The potency of B-G31 isolate associating with valanganigricornis as a probiotic candidate to digest cellulose

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Abstract. Cellulose is a polymer that is abundant in the environment, but they are unable to digest by the human digestive system. This study aims to determine the anti-pathogenic ability and measure the cellulase activity of B-G31 isolate. Auto-aggregation and co-aggregation methods were used to analyze the anti-pathogenic role of B-G31 against biofilm formed by Escherichia coli and Staphylococcus aureus. To assess glucose concentration obtained from cellulose degradation, B-G31 supernatant was reacted in different CMC concentrations (0.5%, 1%, 1.5%, 2%, and 2.5%) and was measured their absorbance (OD540) using ELISA spectrophotometer. The study has revealed that the percentage of B-G31 auto-aggregation is 26% and they can explicitly inhibit colonization of E. coli and S. aureus biofilm accounted for 20.21% and 21.20%, respectively, the bacteria also exhibited antagonistic activity towards two bacterial pathogens. Furthermore, enzyme activity was relatively high in the presence of 2% CMC with 0.913 U/mL to yield average glucose of 411.75 ppm and significantly different from that in the control group (p < 0.05). However, the enzyme value in 0.5% CMC concentration was about 0.345 U/mL and not significantly different from control group (p > 0.05). Our results indicated that B-G31 isolated from Valanganigricornis can form aggregates against bacterial-tests biofilm and increase degradation of cellulose, thus, the isolate could probably be used as probiotics to digest cellulose.

1. Introduction

In 2012, the Food and Agriculture Organization (FAO) reported that food consumption needs will increase in 2050 as many as 230 million tons [1]. Increased population and the need for food are not comparable anymore since food availability shrunk out annually [2]. We offer a notion to solve the food problem with an advanced solution to use several new types of materials as a source, especially cellulose. The polymers compose of glucose molecules by β-1,4 D glycosides bonds and they are abundant in nature [3] [4]. Nevertheless, a human can not digest the cellulose due to the absence of cellulase -an enzyme that can digest cellulose- in the digestive system [5]. Thus, there should be an agent for humans in their digestive system to produce cellulase to be able to process cellulose.

Recently, there are types of microorganisms, probiotics, which can associate to give benefit to the digestive process both in humans or animals [6]. Several pieces of evidence are studied that probiotics would improve intestinal health, enhance the immune system, reduce serum cholesterol, and even prevent cancer. Generally, types of microorganisms implemented in the market are species from Lactobacillus and Bifidobacterium genera since they easily live in an acid condition. However, lactic acid bacteria (LAB) have gained their interest as new probiotic properties and needed to be further studied.
Although many insects are a nuisance to plants, several of the benefits to humanity. Based on the study showed by Abdullah et al. (2018), bacterial candidates who were able to secrete cellulase has been isolated from insect intestine and could be used as a probiotic candidate to digest cellulose [7]. Ferbiyanto et al (2015) also isolated microorganisms potentially secreting cellulase from other species of the insect [8]. In this study, we exploit Valanganigricorns, an insect animal, that has been observed to have a mutualistic symbiosis with microorganisms synthesizing essential compounds, cellulase, to increase the efficiency of the insect’s digestive system through absorption and digestion [9].

Previously, we have studied that-G31 (Bacteria of Grasshopper gut no. 31 which isolated from V. nigricornis) did not exhibit any pathogenic activity to human blood in the hemolysis tests and tolerate to live in a low level of pH [7]. Since B-G31 was obtained from insect consuming materials containing cellulose, we hypothesize that the bacteria could digest cellulose. To test our hypothesis, the study aims to (1) determine the anti-pathogenic activity and (2) measure cellulase activity of B-G31 isolate.

2. Material and methods

2.1. Preparation of inoculum

Bacterial culture was isolated from Valanganigricornis intestine using the method mentioned by (Abdullah et al., 2018). The inoculum was inoculated into the MRS Broth medium and incubated for 48 hours at 37°C [10]. Then, the bacterial stock was stored at -800C.

2.2. Auto-aggregation test

The auto-aggregation ability of B-G31 bacteria was analyzed using methods explained by [11]. B-G31 was incubated for 18 hours and compared the turbidity, with 0.5 MacFarland. The 18-hours culture was centrifuged at 5000 rpm for 15 minutes and the pellets were washed twice with phosphate-buffered saline (PBS) before they were deposited in the same buffer. 4 ml of B-G31 cell suspension was observed for its absorbance value with a wavelength of 600 nm by ELISA spectrophotometer after pellets were incubated for approximately five hours. The value of % auto-aggregation was obtained from a formula as follows,

\[
\text{Autoaggregation(\%)} = \left[1 - \left(\frac{A_t}{A_0}\right)\right] \times 100\%
\]

At is the amount of absorbance at the time \(t = 5\) hours of pellet incubation and \(A_0\) is the absorbance at \((t) = 0\).

2.3. Co-aggregation assay

A Co-aggregation test was carried out to determine the ability of B-G31 probiotic against colonization of pathogen biofilm. Two strains of pathogenic bacteria, Staphylococcus aureus (ATCC 25923) and Escherichia coli (ATCC 35218), were used as partners in the co-aggregation of tested bacteria. The cell suspension is prepared as the same methods as the auto-aggregation test. Then, 2 mL of each suspension was combined in pairs between pathogens and candidate probiotic bacteria using vortex for 10 seconds [11]. The tube that contains 4 mL of bacterial suspension was incubated for 5 hours at room temperature. The value of co-aggregation is measured at OD600. The % co-aggregation value is obtained from the following formula,

\[
\text{Coaggregation(\%)} = \left[\left(\frac{A_{\text{Pathogen}}}{A_{\text{probiotic}}}\right) - \left(\frac{2 \times A_{\text{mix}}}{A_{\text{pathogen}} + A_{\text{probiotic}}}\right)\right] \times 100\%
\]

2.4. Cellulase activity test

To test cellulase activity, B-G31 Isolate was cultivated into 100 mL of 1% CMC broth medium at 120 rpm at 45°C for 24 hours and centrifuged at 4000 rpm for 10 minutes at 40C to obtain culture supernatant (Manhar et al., 2016). 1 mL of supernatant was combined with 1 mL different concentration of CMC (0.5; 1; 1.5; 2; 2.5%), 1 mL of DNS reagent and filled with distilled water to reach a
maximum volume of 10 mL in total. The mix was incubated for 5 minutes at 45°C (digestive temperature) [5] and stopped reaction with boiling the mix at 100°C for 5 minutes. The amount of reducing sugar (glucose) of the sample was determined using 540 nm of ELISA spectrophotometer [12] and the cellulase activity was assessed using the following formula,

\[
\text{Enzyme activity (U/mL)} = \frac{GC}{\text{GMW} \times t} \times \frac{H}{E}
\]

GC is glucose concentration, GMW is molecule weight of glucose, t is a time of incubation, H is the total volume of enzyme-substrate, and E is the volume of the enzyme.

3. Results and discussion

3.1. Auto-aggregation, co-aggregation and antagonistic assay

The Auto-aggregation ability of B-G31 isolate was investigated after five hours of sedimentation periods. The result showed that absorbance was 2.711 and yielded 26% of the auto-aggregation value, interestingly, it was significantly different from the control group (p < 0.05). Moreover, the average absorbance and co-aggregation of cultures mixed between E. coli and B-G31 were 1.822 and 20.21%, respectively. The mix of S. aureus and B-G31 produced co-aggregation for about 21.2% with 2.247 absorbances and significantly different from the control group (p < 0.05). The detail can be seen in table 1.

| Isolate  | Auto-aggregation (%) | Absorbance | Co-aggregation (%) | Antagonistic activity (mm) |
|----------|----------------------|------------|--------------------|---------------------------|
|          | E. coli              | S. aureus  |                    |                           |
| B-G31    | 26                   | 0.0035     | ± 1,822 ± 0.0040   | 20.2                      | 21.2                     | 22           | 26           |

The Auto-aggregation rate for biofilm formation of B-G31 was quite good and following another group. For example, Ramos et al. (2013) revealed that auto-aggregation is strain-specific among L. Plantarum, L. fermentum, as well as L. brevis and the rate of auto-aggregation, ranged from 12 to 61% after five hours of incubation [13]. Todorov et al. (2011) reported that L. Plantarum (isolate CH3 and CH41) and L. Brevis (isolatesFFC199 and SAU105) exhibited auto-aggregation no more than 21% after five hours of sedimentation [14]. However, some reports were not identical as the current study, based on a study conducted by Ng et al. (2015), Lactobacillus Brevis and L. Plantarum in their study were found to have auto-aggregation features to form biofilm for about >40% after 20 hours of sedimentation in 37°C [15]. The % auto-aggregation value of a study conducted by Sirichokkatchawan et al. (2018) showed that the auto-aggregation of L. plantarum 22F and 25F for four hours of incubation yielded 43.01% and 42.63%, respectively, the value also increased almost doubled depended on the incubation time of the pellets just in the range of one-hour incubation [16].

Our results are classified as a good property of auto-aggregation for a probiotic candidate since the level is more than 20%. Wang et al. (2010) presented that the level of auto-aggregation of 40% is enough to refer to as good auto-aggregation but must be higher than 10% [17]. However, there is no standard classification because another researcher claimed that, to be assessed to contain good property, the level of auto-aggregation should be more than 70% [18]. That kind of pattern is crucial for probiotic candidates because it would simply relate to the quantity of surface-layer protein for
bacterial adhesion onto the host’s intestinal wall [19] [20] [21]. In case if the layer is removed or reduced, the auto-aggregation ability would shrink out and they would not protect the host from any pathogens. B-G31 may be considered as a potential candidate of probiotics.

The Co-aggregation percentage of B-G31 agreed with other studies that had been conducted with both E. coli and S. aureus. For instance, Sirichokchatchawan et al. (2018) investigated the co-aggregation value of L. Plantarum with E. coli (ranged from 15-27%) and S. aureus (ranged from 22-29%) after four hours of co-incubation [16]. Ramos et al. (2013) presented that co-aggregation for five hours of L. fermentumCH58, L. Plantarum SAU96 and L. Brevis with E. coli was for about 21%, 21%, and 1%, respectively [13]. However, another paper showed different results and tended to have a higher level of co-aggregation than this present study. Based on a study from Gómez et al. (2016), five genera of Lactobacillus elucidated a high co-aggregation for about 38-75% with E. coli and showed a tendency to be strain–pathogen combination-dependent manner [22]. Co-aggregation indicates probiotics’ ability to stick to pathogens reducing pathogen’s adherence to intestinal epithelia, therefore, the host would be protected from any colonization by pathogens [23] [21]. The patterns are desirable to apply as potential probiotics.

Moreover, B-G31 showed an outstanding antibacterial activity against E. coli and S. aureus for about 22 mm and 26 mm, respectively (Table 1). The present result agreed with the previous studies. For example, MGB32 isolates produced an inhibitory zone against E. coli and P. aeruginosa for about 29 mm and 30 mm, respectively [24]. However, another study did not present an identical result, based on a study conducted by Ramos et al. (2013), it revealed that two strains of L. plantarum showed no antagonistic activity against S. aureus, the highest inhibition zone was only about 4 mm [13]. The activity is expected as a result of the existence of bacteriocin substances synthesized by bacteria[25] and it probably triggers by a aggregation activity that is excreted the inhibitory substances [26].

3.2. Cellulase Activity Assay

Cellulase activity was tested by measuring the absorbance value at 540 nm. The results have proved that the cellulase activity of B-G31 depends on the available substrate concentration. The activity of cellulase was0.913 U/mL when the presence of the substrate was 2% and it is significantly different from the control group (p < 0.05). Enzyme activity at 1.5% and 2.5% of CMC were about 0.419 U/mL and 0.631 U/mL, respectively.Nevertheless, there are no significant different of cellulase activity between 0.5% (0.345 U/mL) and 1% (0.366 U/mL)of CMC concentration. The details can be seen in figure 1.

![Figure 1](image-url)  
**Figure 1.** Cellulase activity of B-G31 isolates in different concentrations of CMC. Superscript of a indicates p>0.05 and superscript of b indicates p<0.05 compared with the control group.

Production of cellulase in B-G31 isolate was much higher than other studies that have been conducted by other groups in both bacteria isolated in nature and insect guts. For example,
Bacillus subtilis CK-2 obtained from compost soil [27] and Bacillus megaterium isolated from facultative soil anaerobe [28] were reported producing CMCase for about 0.26 U/mL and 0.102 U/mL in 1% concentration of CMC, respectively. Deka et al. (2011) revealed that bacillus subtilis AS3 expressed 0.43 U/mL of cellulase when 2% CMC was available [29]. Rastogi et al. (2009) also showed that cell-free culture supernatant of Geobacillus sp. isolated from the goldmine field exhibited CMCase and cellulase activity of 0.058 and 0.043 U/mL, respectively [30]. For bacteria gained from insects, Hatefi et al. (2017) had studied that DNH5437 isolate isolated from Osprhanteriacoerulescens gut exhibited the highest activity of cellulase enzyme for about 0.62 ± 0.04 U/mL in 1% CMC [31]. Also, Bhuyan et al. (2018) indicated that MGB05 isolate acquired from mature larvae of Antheraea sp. exhibited the highest CMCase activity (0.262 U/mL) at 72 h of incubation under submerged condition [32].

The differences might be caused by several reasons, firstly, carbon source [33][34][29] and duration of incubation [24][35] could affect enzyme production. Secondly, the effects of pH and incubation temperature [36][37][38] as well as metal ions [39][40][41] were found to be crucial since those parameters would influence the stability of the enzyme. Thirdly, the substrate of the enzyme could reduce the amount of oxygen in the media, therefore, that will affect the production of cellulase. Finally, enzyme-substrate complexes are commonly formed after the optimum phase of enzyme activity is passed where there is no active site of enzyme left even though the substrate increases, thus the condition would also decrease enzyme activity [42][43].

4. Conclusion

Bacteria isolate of B-G31 isolated from the gut of Valanganigricornis has antagonistic activity against staphylococcus aureus and Escherichia coli, intriguingly, the bacteria showed a relatively high cellulase activity. Moreover, the bacteria can be applied as a cellulose probiotic candidate since it could form auto-aggregation and co-aggregation with common bacterial pathogens.

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