Extracellular Enzymes of *Bacillus licheniformis* from Milkfish Gut as Degradation Agent of *Chlorella vulgaris* Cell Wall

Mulyani Putri Dwi[1], Nashrurrokhman Muh[1], Handayani Setya[1], Ardiansyah Syamsul Arif[1] and Purwestri Yekti Asih[1,2]*

1. Biochemistry Laboratory, Tropical Biology Department, Faculty of Biology, Universitas Gadjah Mada Yogyakarta, INDONESIA
2. Research Center for Biotechnology, Universitas Gadjah Mada Yogyakarta, INDONESIA
*yekti@ugm.ac.id

Abstract

Bacillus licheniformis from milkfish gut (Chanos chanos) has been known to have cellulolytic ability and is expected to be used as Chlorella cell walls degradation agent to produce Single-cell proteins. Other abilities and characteristics of extracellular enzymes from Bacillus licheniformis are still unknown. Detection of the extracellular enzymes from Bacillus licheniformis was carried out by observing the presence of clear zones surrounding the colonies on selective mediums and measured using spectrophotometric techniques. The measurement of cellulase and hemicellulase activities was carried out by the DNS method. The optimal pH and temperature of cellulase and hemicellulase activities have also been determined. Chlorella cell wall degradation test was carried out by applying the enzyme on cell and observed under the inverter microscope. We found that the best incubation time for Bacillus licheniformis in producing cellulase and hemicellulase is 48 h and 72 h respectively with enzymes activities of 6.016 U/ml and 2.482 U/ml respectively. The optimum pH and temperature for cellulase and hemicellulase activities were pH 7 and pH 6 and 29 °C and 37 °C. This research is a step towards the production of single cell proteins using alternative enzymes.

Keywords: *Bacillus licheniformis*, Chlorella, Extracellular enzyme, Milkfish, SCP.

Introduction

Chlorella cell walls are known to be very complex and difficult to digest. In fact, Chlorella has great potential in the fields of industry, both food, medicine, cosmetics, and biofuels. Some efforts that have been made to destroy the Chlorella cell wall are physically and chemically. Unfortunately, the degradation of cell walls is chemically costly and expensive, while if done physically, it can reduce the nutrient content in Chlorella. Therefore, enzymatic destruction of cell walls is considered the most appropriate way because it is cheaper, environmentally friendly and can work specifically specific. Generally, the degradation cell wall of Chlorella enzymatically uses commercial enzymes such as cellulase, proteases, lysozyme and pectinase[29]. However, the commercial enzymes are expensive, and this is the barrier in the industry for producing Single-cell proteins (SCP).

Natural alternative enzymes that have the potential to degrade Chlorella cell walls can be a solution for the problem of SCP production. Alternative enzymes derived from selective microorganisms can be used for the protoplast isolation process. Microorganisms are more often chosen as a source of enzymes than animals or plants. This is because microorganisms can grow using inexpensive substrates, short production times, and are easier to control enzyme production through manipulation of the conditions of growth.

Previous research by Lathifah et al (2009) has succeeded in isolating cellulolytic bacteria from the digestive tract of milkfish (*Chanos chanos*) which consumes Chlorella.[17] Bacteria have been identified and have been known as *Bacillus licheniformis*[13]. *B. licheniformis* is known to have great potential in producing extracellular enzymes such as xylanase[3], amylase[14], cellulose[6] and several other enzymes.

Many bacteria undergo adaptation quickly according to their environment. Consequently, if the environment is different, the ability of bacteria to produce enzymes can also vary even if they come from the same species. In order to optimally utilize the extracellular, it is necessary to determine the optimum pH and temperature as well as the ability of these extracellular enzymes to degrade *Chlorella vulgaris* cell wall.

Material and Methods

Materials used in this study included: *B. licheniformis* isolates derived from the milkfish gut (*Chanos chanos*) by Lathifah et al, *Chlorella vulgaris* culture from Biotechnology Laboratory Department of Biology UGM, Zobell 2216 (consisting of 15.0 g Bacto-agar, 5.0 g Bacto-peptone, 1.0 g yeast extract and sterile seawater up to 1 L volume), starch (1 %), lugol's iodine solution 1 %, paper disc, skim milk (1 %), Tween 80, congo red solution, Avicel, Whatmann paper, nutrient agar, sterile aquades, Dinitrosalicylic Acid Reagent (DNS), phosphate buffer, citric acid buffer and universal pH indicator.

Rejuvenation of isolates: Isolates of *B. licheniformis* were inoculated on NA medium and were incubated at 37 °C for 24–72 h.

Qualitative test of extracellular enzymes: Rejuvenated bacteria were grown on several agar mediums containing inducers such as CMC for qualitative cellulase test, xylan for qualitative test of hemicellulase, starch for qualitative amylase test, skim milk for qualitative protease test, and tween 80 for qualitative lipase test. The bacteria were incubated for 24 h at 37 ℃. For the qualitative test of cellulase and hemicellulase, the overgrown medium of colonies was given red congo to see whether or not the clear zone exists around the colony.

Measurement of growth and testing of enzyme activity

a. Measurement of growth: Prior to growth measurement, bacteria were first inoculated on a liquid medium, the culture medium used in this research is CMC medium and NB medium containing 1 % xylan, then incubated at 37 °C. Measurements of bacterial growth and enzyme activity testing were performed at 24 h intervals. Bacterial growth was measured using a 600 nm wavelength spectrophotometer. Growth curves were plotted as absorbance vs time. Enzyme activity was also calculated at 24 h intervals.

b. Enzyme assay: For extracellular enzyme assay, 5 mL of the parent culture was taken. The sample was centrifuged at 10,000 rpm for 10 min until the pellet and supernatant were obtained. The supernatant obtained is a crude enzyme that will be tested for its activity. The product formed in the form of reducing sugar was measured by using 3.5 dinitrosalicylic acid (DNS methode[18] and its concentration was converted to standard glucose and xylose. One unit of relative enzyme activity is the number of enzymes that can produce 1 µmol reducing sugar (glucose and xylose) per minute per mL of substrate (CMC and xylan) solution under test conditions[7].

Enzyme characterization: Enzyme characterization includes determination of pH and optimum temperature and substrate accordingly. The characterization of extracellular enzyme activity was obtained by mixing 1 mL of the crude enzyme with 1 mL of the substrate, then incubating for 10 min and the reaction was stoped by adding DNS and heating on 100 ˚C, then the formed reduction sugar product was measured by spectrophotometer at 540 nm. The determination of the optimum pH was carried out by dissolving the crude enzyme on the substrate under various conditions of pH using citric acid buffer (pH 4, 5 and 6), phosphate buffers (pH 7, 8 and 9), and optimum temperature determination was performed by testing cellulase activity at various temperatures of 29 °C, 37 °C, 50 °C, 60 °C, 70 °C, and 80 °C.

Testing of enzyme activity on cell wall degradation: 10 mL of Chlorella culture was centrifuged in a conical tube with 3,300 rpm for 15 min. Supernatant obtained from centrifugation was removed and then replaced with 1 mL of aquadest. Suspension of pellet and aquadest was centrifuged again with 3,300 rpm for 15 min. Supernatant from the previous centrifugation was removed. 5 mL of enzymes was added to the pellet and then incubated for 2 h to test enzymes activity in degrading Chlorella's cell wall. The degradation of Chlorellah cell wall was observed under a binocular microscope.

Results and Discussion

Qualitative test results of extracellular enzyme activity: The ability of cellulase, hemicellulase, amylase and protease to degrade substrate can be determined based on clear zone around the colony. The clear zone indicates that *B. licheniformis* isolate can hydrolyze polysaccharide such as cellulose and xylan, starch and also skim milk as a carbon source or produced hydrolytic enzymes which were secreted to the growth medium. These enzymes degrade β-1, 4-glycosidic bond in CMC and xylan. Nevertheless, clear zones were qualitatively reproducible with the plate overlay technique. The clear zone became clearer by coloring with congo red and iodine which strongly interacted with β-1, 4-glycosidic bound.[23,25,28]

This result suggests that *B. licheniformis* from milkfish gut has the ability to produce extracellular enzymes such as amylase, protease, cellulase and hemicellulase, and appropriate with other studies that *B. licheniformis* can produce cellulase[6], hemicellulase[24], amylase[14], proteinase[15]. In another study *B. licheniformis* can produce lipase[21], but in this study the result showed that there is no lipase activity that formed clear zone around the colony. This can be caused due to differences in strain and environment. As a result, bacteria can have different abilities in producing enzymes even in the same species.

Quantitative test results and extracellular enzyme characterization: Quantitative test was conducted by measuring the activity of crude enzyme from *B. licheniformis* bacteria using spectrophotometric technique. The quantitative tests are focused to determine the activities of cellulase and hemicellulase. Wijosono[27] reported that the cell wall of Chlorella composed of 15.4 % cellulose and 31 % hemicellulose which is the thickest part among the other cell wall components. Because of this, we consider the importance of measuring the optimum conditions for producing cellulase and hemicellulase from *B. licheniformis* before being tested on the Chlorella cell wall. Some previous studies have also used these two enzymes to degrade Chlorella cell walls including for ethanol production[19] and enhanced lipid recovery from *Nannochloropsis* microalgae[31].

Determination of optimum incubation time: The effect of incubation time on enzyme production was studied from 24–72 h using CMC and xylan as substrate respectively. The production increased with increase in fermentation period. From fig. 1, it can be seen that *B. licheniformis* has an optimum incubation time to produce cellulase within 48 h (6.016 U/mL). It takes longer to obtain optimum incubation time on producing hemicellulase after incubation for 72 h (2.482 U/mL). This is in accordance with the research which

states that the maximum growth of the *B. licheniformis* was obtained within 24 h to 84 h of cultivation whereas the activity of the α-amylase reached a maximum within 36 h after inoculation[4].

In another research, *B. licheniformis* can produce maximum xylanase after 72 h of fermentation period in solid state fermentation[12,16], 48 h for producing amilase[20] and 60 h for producing cellulase[2].

This difference of incubation time shows the potential of bacteria in producing enzymes. Based on these results, it is known that *B. licheniformis* from milkfish gut has a better potential for producing cellulase than hemicellulase. In another research, it was found that *B. licheniformis* SVD1 predominantly produced hemicellulase (xylanase), and showed minimal production of mannanase, CMCase and avicelase[9]. The difference of enzyme activity might be due to the culture conditions[10,22], and also the origin of environmental conditions of *B. licheniformis* with various nutrients available in them. This causes *B. licheniformis* to adapt to survive through the production of enzymes that can digest available nutrients.

Determination of optimum temperature: Temperature has an important role for enzymatic reaction. The effect of temperature on cellulase and hemicellulase activity of the crude enzyme was determined over a temperatures range of 29 °C to 80 °C at pH 7.0 (fig. 2). At all temperature points focused, cellulase activity was higher than hemicellulase. The optimum temperature for *B. licheniformis* cellulase activity was 29 °C at which activity was 6.632 ± 0.045 U/mL. The activity decreased linearly with the increasing of temperature until it reaches up to 60 °C and inclined thereafter. This result is supported by data from Seo et al[24] finding that the range of optimum temperatures of cellulace activity from *B. licheniformis* was at 20 °C to 40°C.

From fig. 2, it can be seen that hemicellulase activity from *B. licheniformis* was highest at 37 °C (2.48 ± 0.345 U/mL). But, the hemicellulase activity of *B. licheniformis* showed almost the same value and is not much different at temperatures of 29 °C to 80 °C. This shows that the optimum temperature range for bacterial hemicellulase activity is very wide. This is supported by literature. We found that optimum temperature for hemicellulase activity of *B. licheniformis* varied. Bajaj and Manhas[5] reported *B. licheniformis* P11 (C) efficiently utilizes agricultural residues as a source of carbon and nitrogen to produce large amounts of xylanase which shows activity and stability over a higher temperature range of 40 °C to 100°C. In another research, Irfan et al[16] reported that *Bacillus* sp. xylanase activity was optimum at 25 °C to 50°C.

Determination of optimum pH: Based on data, it is known that the optimum pH for cellulase and hemicellulase from *B. licheniformis* is sequentially at pH 7 and pH 6. Most microorganisms grow optimally within a wide pH range. *B. licheniformis* in this research is isolated from milkfish gut which has pH that tends to be acidic to netral, so it is estimated that this bacterium will also be optimum under acidic to netral conditions.

This is evidenced by the optimum activity of hemicellulase at pH 6 and cellulase activity at pH 7. At alkaline range of pH, both enzymes production started to decrease significantly. Acharya and Chaudary[2] reported that cellulase production from *B. licheniformis* with CMC medium was best at pH 6.5. Another study report, that the optimum pH of xylanase is 5 with the maximum activity in range 4 – 6[24].

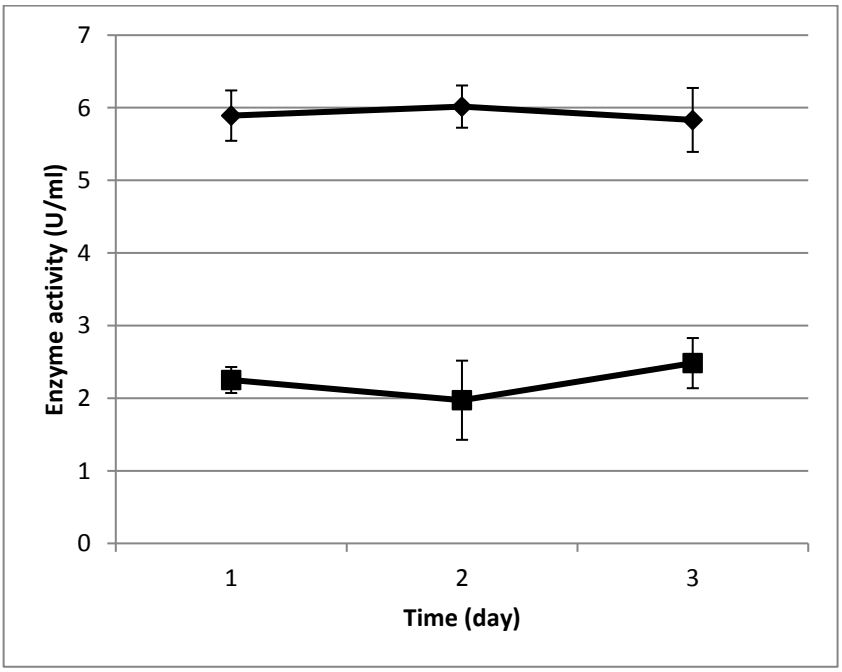


Figure 1: Determination of optimum incubation time of *B. licheniformis* for cellulase (♦) and hemicellulase (■) activity from *B. licheniformis*

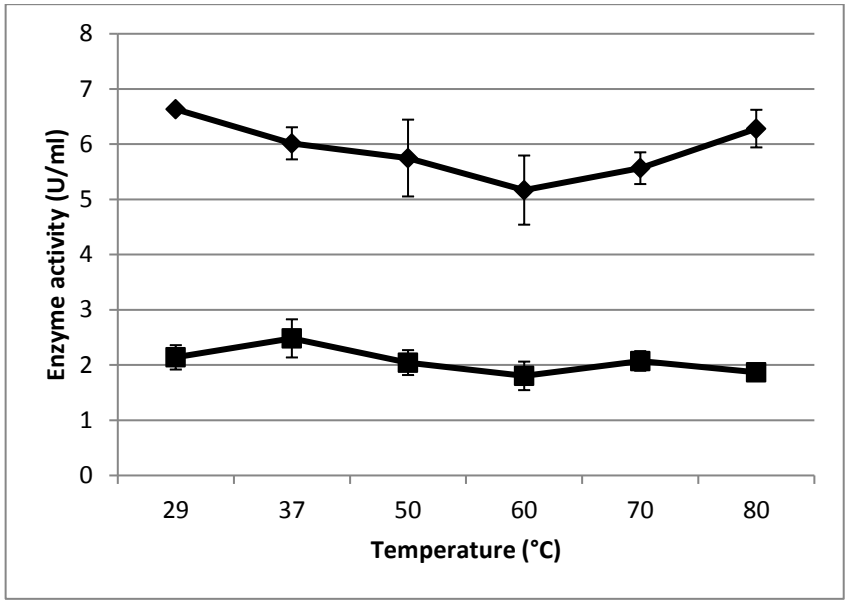


Figure 2: Determination of optimum temperature of cellulase (♦) and hemicellulase (■) activity from *B. licheniformis*

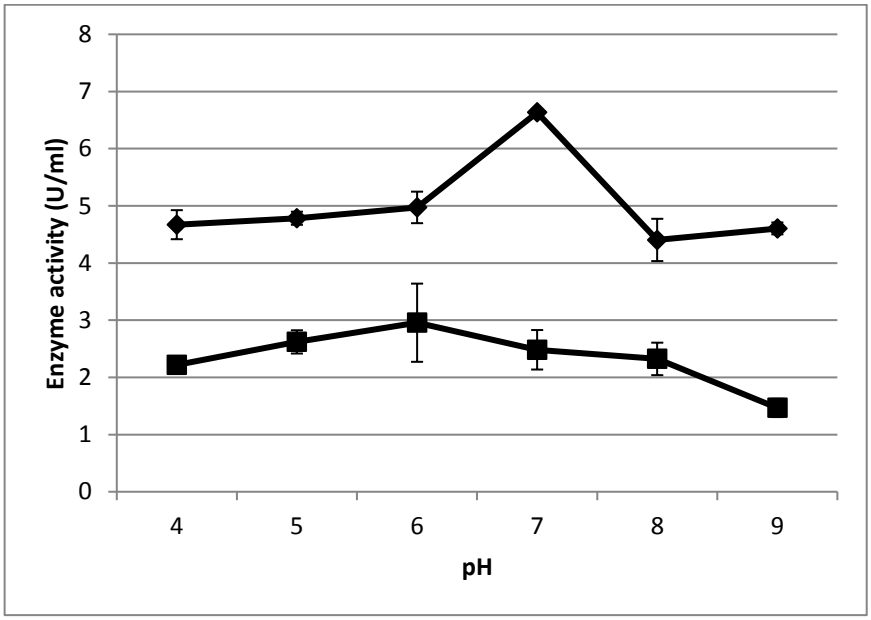


Figure 3: Determination of optimum pH of cellulase (♦) and hemicellulase (■) activity from *B. licheniformis*

Chlorella cell wall degradation test by cellulase and hemicellulase *B. licheniformis*: In this study, cellulase and hemicellulase from *Bacillus licheniformis* were used which had known pH conditions and optimum temperature activity. Some of research which used enzyme to degradate Chlorella cell wall had been conducted such as lysozyme combined with several enzyme using frourescence DNA staining and electron microscopy. This research used safranin and light microscopy to analyze Chlorella cell wall after giving crude cellulase and hemicellulase treatment.

Based on table 2, it can be seen that Chlorella cells with cellulase and hemicellulase treatments have darker color than control. Safranin staining will enter to the cell and gives darker color when Chlorella cells are degradated by enzyme. The treatment with cellulase gives a darker color than hemicellulase. This proves that cellulase is more effective to degrade Chlorella cell wall than hemicellulase due to thicker cellulase content than hemicellulase on the Chlorella cell wall.

This is in accordance with Jannatunaim et al[17] who reported that cellulases from digestive tract bacteria of milkfish (*Chanos chanos*) successfully hydrolyzed the cell walls of *C. zofingiensis*.

Xi et al[30] also proved that the main component of the cell wall of Chlorella protothecoides cell is cellulose. It is estimated that giving cellulase and hemicellulase together will provide more optimal results in degrading Chlorella cell walls. As with Braun and Aach[8] studies, it successfully degrades of algae cell walls using a mixture of enzymes containing cellulase activity, hemicellulase, and pectinase.

Conclusion

The conclusions obtained based on the research are:

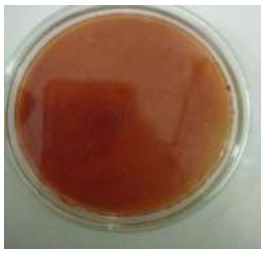
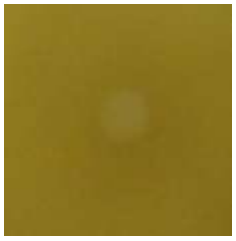
a. The types of enzyme capable of being produced by *Bacillus licheniformis* from the milkfish gut are cellulase, hemiselulase, proteinase, and amylase.

b. *Bacillus licheniformis* optimum produces cellulase after incubation for 48 h, and hemicellulase after incubation for 72 h.

c. Cellulase and hemicellulase bacterial isolates of *Bacillus licheniformis* from the milkfish gut are optimum at a temperature of 29 °C and 37 °C and at pH 7 and 6 respectively.

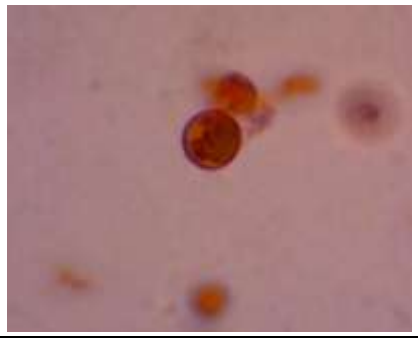
d. Cellulase and hemicellulase *Bacillus licheniformis* from the milkfish gut are capable of degrading Chlorella cell walls and are potentially used for protoplast isolation in the production of SCP.

Table 1
Qualitative test results of extracellular enzyme activity *B. licheniformis*

Enzymes treatment	**Results**	
	+/-	**Clear zone formation**
Protease	+	
Cellulase	+	
Amylase	+	
Hemicellulase.	+	
Lipase	-	

Note: the sign (+) indicates the presence of enzymatic potency and the presence of clear zones around the colony

Table 2
Extracellular enzyme activity test results of *B. licheniformis* in degrading Chlorella cell wall

Enzymes Treatment	Results	
	Chlorella cell wall degradation test results	Degradation of cell wall
Control	-	
With Cellulase	+	
With hemicellulase	+	

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