

# Freeze-thaw system for thermostable $\beta$ -Galactosidase isolation from Gedong Songo *Geobacillus* sp. isolate

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## Abstract

The effective isolation of intracellular enzymes from thermophilic bacteria is challenging because of their sturdy membrane. On the other hand, the low-cost and nontoxic method is essential for industrial food enzymes. The freeze-thaw cycles using acetone-dry ice as a frozen system was studied for efficient isolation of thermostable  $\beta$ -galactosidase from *Geobacillus* sp. dYTae-14. This enzyme has been known for application in the dairy industry to reduce the lactose content. In this study, the freeze-thaw method was performed with cycle variations 3, 5, and 7 cycles. Acetone-dry ice ( $-78^{\circ}\text{C}$ ) is used as a frozen system and boiling water for thawing. The  $\beta$ -galactosidase activity was assayed using ortho-Nitrophenyl- $\beta$ -galactoside (ONPG) as substrate and protein content determined with the Lowry method. The results show that the most effective freeze-thaw is five cycles. The enzyme's highest specific activity is 3610.13 units/mg proteins at 40–60 % ammonium sulfate saturation, with a purity value of 2.52.

## 1. Introduction

$\beta$ -Galactosidase is one of the enzymes that have been widely applied in industry, which has a function to reduce the content of lactose in milk [1].  $\beta$ -Galactosidase is commonly used in the dairy industry. Milk with a low level of lactose can be consumed by lactose intolerance sufferers [2, 3]. Since thermostable enzymes have been proven to be efficient for industry, the dairy industry's need for thermostable  $\beta$ -Galactosidase has triggered various studies to explore novel thermostable  $\beta$ -Galactosidase. The potential source of these enzymes comes from thermophilic bacteria. The thermostable enzymes from thermophilic bacteria are preferred by the industry because they are more efficient, save time, reduce contamination, improve mass transfer rates, and decrease the solution viscosity. *Geobacillus* sp. dYTae-14 is a thermophilic bacterium isolated from Gedongsongo hot spring [4, 5] and has been reported having the potential of thermostable  $\beta$ -Galactosidase. Based on Ratri *et al.* [5], it was found that there was an

intracellular  $\beta$ -Galactosidase from Gedong Songo *Geobacillus* sp. dYTae-14 isolates.

Effective isolation of high-quality materials is essential in molecular biology, biochemistry, and genetic studies. The  $\beta$ -Galactosidase enzyme is an intracellular enzyme; therefore, the effective bacterial cell lysis is critical to obtain enzymes. Generally, cell lysis can be carried out in simple ways such as adding chemicals; for example, lysozyme, antibiotics, Sodium Dodecyl Sulphate (SDS), Ethylene Diamine Tetra Acetic Acid (EDTA), so forth [6]. However, those methods have several disadvantages: high cost, contaminants, and damage to target enzymes. Large-scale use of antibiotics is almost impossible because antibiotics have a high cost, and not all bacteria can be lysed effectively using antibiotics.

Freeze-thawing is one of the methods used for cell lysis with freeze the cells on ice temperature conditions, usually using acetone-dry ice, chloroform- $\text{N}_2$ , ethanol-dry ice, and others, then melting the cells suspension. This process is repeated several times during the cycles.

Repetition of the cycles is needed to get the efficient lysis process. The advantages of the freeze-thawing method are cheaper than other lysis methods as it does not require special tools and are contaminant free. However, the efficiency of bacterial cell lysis differs in the number of the cycles and freeze-thaw temperature technique for different types of cells. Schneegurt *et al.* [7] conducted four freeze-thawing cycles using dry ice-ethanol and water with a soil bacteria temperature of 80°C. The research showed that 90% of the cells were successfully lysed. Sriwongsitanont and Ueno [8] have performed freeze-thawing using liquid N<sub>2</sub> (-196°C) and water with a temperature of 40°C with the best result obtained in ten cycles. Figueroa-Pizano *et al.* [9] varied the freezing temperature (-4°C, -20°C, and -80°C) and the freeze-thawing cycles (four cycles, five cycles, and six cycles). Yang *et al.* [10] used -196°C as a frozen system and then thawed at 25°C. Chen *et al.* [11] carried out a combination of chemicals and freeze-thaw (liquid nitrogen and 65°C) for the effective isolation of several mesophilic bacteria genomes.

The thermophilic are single-celled organisms that do not have a nucleus but have a unique, rigid outer cell wall. The freeze-thawing system for thermophile cell lysis has minimal information. Yang *et al.* [12] reported using a pretreatment method combining freezing/thawing with *Geobacillus sp.* for WAS hydrolysis and subsequent short-chain fatty acids (SCFAs) production. Freezing at -18°C for 72 hours, and then 16 thawings for another 3 hours at 29°C was performed. The long hours for the freeze-thaw system in this study was carried out because the temperature was not extreme. Based on the previous studies, an alternative freeze-thaw system to thermophilic bacteria cell lysis was studied. The *Geobacillus sp.* dYTae-14, as a local thermophilic bacterium isolated from Gedong Songo hot spring, was used as a model to isolate the thermostable  $\beta$ -Galactosidase enzyme. The freeze-thawing method generally uses acetone-dry ice as a freezing system and thawing at 37°C. In this study, boiling water (100°C) was used as a thawing system. According to the previous studies, different freeze-thawing cycles were performed and effective at different cycles such as 3, 5, or 7. The effectiveness of the cell lysis was assayed based on the activity of the  $\beta$ -Galactosidase enzyme.

## 2. Methodology

### 2.1. Media and Culture

*Geobacillus sp.* dYTae-14 of Gedong Songo isolates (Aminin culture collections) was grown aerobically at 55°C in Basal Saline Medium (BSM) at pH 6 for 28 hours, supplemented with Yeast-Tryptone (YT) 0.05 % sterile and at 65°C in 1/2 Luria Berthani Medium for 24 hours. For the bacterial growth curve, a fresh *Geobacillus sp.* dYTae-14 culture was grown aerobically in 1/2 LB medium at 65°C for 14 hours. The medium turbidity at a wavelength of 600 nm (OD<sub>600</sub>) was measured every 2 hours. For  $\beta$ -Galactosidase enzyme production, a fresh *Geobacillus sp.* dYTae-14 culture was grown aerobically in 1/2 LB medium at 65°C. After 2 hours from the start,

sterile 1% lactose was added and continued incubation for 7.5 hours.

### 2.2. Freeze-Thawing Modification Methods

Fresh *Geobacillus sp.* dYTae-14 culture was centrifuged at 5600 RPM cold conditions for 30 minutes. The pellets were washed with sterile H<sub>2</sub>O, then centrifuge again. The cell pellets were suspended in a 0.08 M phosphate buffer pH 6.0. The bacterial cell lysis process used a modified freeze-thawing method to obtain crude enzymes. The freezing period used acetone-dry ice (-78°C) for 1 minute, while the thawing period used boiling water (100°C) for 2 minutes. The transition period between the freezing and thawing phases of the cycle should not exceed 5 seconds. Variations of freeze-thawing cycle lysis have been carried out with variations of three lysis cycles, five lysis cycles, and seven lysis cycles. The cell lysis mixture was then centrifuged at 5600 RPM for 30 minutes, and the filtrate, a crude enzyme, was taken. The qualitative assay for the existence of the  $\beta$ -Galactosidase enzyme was carried out by adding the ONPG substrate into the crude enzyme with a ratio of 1:1. After that, the  $\beta$ -Galactosidase enzyme-specific activity test was carried out to determine the effectiveness of the freeze-thawing method lysis cycle. The most efficient lysis cycle results were used in the subsequent process, such as the initial purification of the enzyme with ammonium sulfate precipitation and dialysis. The supernatant was stored at 0–2°C.

### 2.3. Ammonium Sulphate Precipitation and Dialysis

Proteins were sequentially precipitated from the crude enzyme by the gradual addition of solid ammonium sulfate under stirring at a certain degree of saturation, followed by incubation on an ice water bath (0–4°C) for at least 2 hours and centrifugation at 5600 RPM. The pellet obtained after centrifugation was resuspended in a 0.08 M phosphate buffer pH 6.0. The steps above were carried out for 0–20% (F<sub>1</sub>), 20–40 % (F<sub>2</sub>), 40–60 % (F<sub>3</sub>), 60–80 % (F<sub>4</sub>), and 80–100 % (F<sub>5</sub>) of ammonium sulphate saturation, respectively.

The dialysis process using a cellophane membrane was conducted in a beaker glass containing 0.0008 M phosphate buffer pH 6.0. The dialysis process was stopped when there was no white precipitate in the 0.0008 M phosphate buffer after the addition of BaCl<sub>2</sub>, 1:1. The aliquot sludge fraction was analyzed for its enzyme activity and protein concentration assay. As a comparison, part of the crude enzyme was also directly subjected to the same analysis.

### 2.4. Enzyme Activity Assay

The  $\beta$ -Galactosidase activity assay used an ortho-Nitrophenyl- $\beta$ -Galactoside (ONPG) 5.0 mM as a substrate in 0.08 M phosphate buffer pH 6.0, which was incubated at 65°C for 10 minutes. The activity was determined by incubating the samples for 30 minutes at 65°C, and stopping it by adding 1.5 mL Na<sub>2</sub>CO<sub>3</sub> 2M, then letting it cool before measuring the absorbance (420 nm). The inactive enzyme was used as a control.

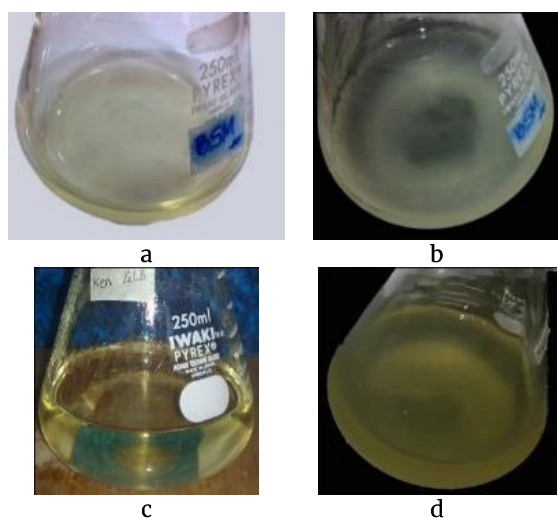
### 2.5. Protein Concentration Assay

The protein content in the samples during purification was determined by the Lowry method. The Bovine Serum Albumin 1 mg/mL in 0.08 M phosphate buffer pH 6.0 was used as a protein standard. The assay was determined by incubating the samples for 30 minutes after adding a 0.5 mL Lowry D solution and then measuring the absorbance (750 nm).

Note: The samples of enzyme solutions used for enzyme activity and protein concentration assay were crude enzymes and precipitated fractions; 0–20% (F1), 20–40% (F2), 40–60% (F3), 60–80% (F4), and 80–100% (F5) which has undergone dialysis.

### 3. Results and Discussion

Research on the isolation of thermophilic  $\beta$ -Galactosidase enzyme is important. The intracellular  $\beta$ -Galactosidase enzyme is very precious because of its high concentration but not as the extracellular enzyme. *Geobacillus* sp. dYTae-14 isolates were grown on a starter medium to get fresh and productive bacteria to obtain optimal enzyme activity. The isolates were inoculated into Basal Saline Medium and  $\frac{1}{2}$  Luria Berthani medium to compare the effective medium for the growth of *Geobacillus* sp. dYTae-14. Comparison of *Geobacillus* sp. dYTae-14 growth in Basal Saline Medium is shown in Figure 1. (a) and (b) while the growth of the isolates in  $\frac{1}{2}$  Luria Berthani medium is shown in Figure 1. (c) and (d).



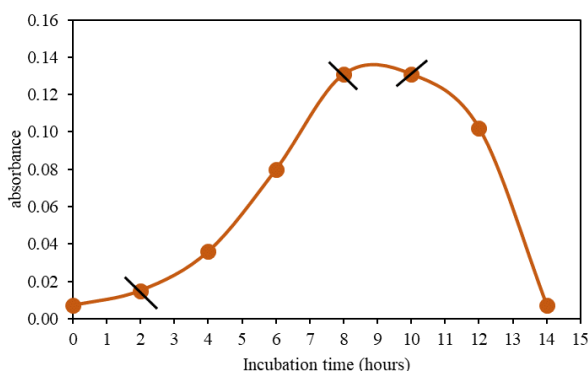
**Figure 1.** Comparison of bacterial growth on BSM and  $\frac{1}{2}$  LB liquid media, (a) negative control on BSM liquid media, (b) culture on BSM liquid media, (c) negative control on  $\frac{1}{2}$  LB liquid media, (d)  $\frac{1}{2}$  LB culture liquid medium

Physically, it is observed that the  $\frac{1}{2}$  LB medium is preferred by bacteria than the BSM medium since it is more cloudy. Quantitative analysis of bacterial turbidity measured by turbidimeter proved that the cell turbidity in BSM is 200 NTU, while in  $\frac{1}{2}$  LB medium is 633 NTU. The turbidity value reflects the number of bacterial populations in the medium. Nephelometric Turbidity Unit (NTU) is a measurement unit for turbidity. Based on this result,  $\frac{1}{2}$  LB was then used as a growth medium of

*Geobacillus* sp. dYTae-14 to obtain highly thermophilic bacterial cells.

The  $\beta$ -Galactosidase enzyme produced by *Geobacillus* sp. dYTae-14 is thermostable for use in high-temperature production. High-temperature production has several advantages over conventional production using mesophilic enzymes. The metabolic rate is usually higher at high temperatures because thermophiles are strong and temperature-tolerant enzymes. Thermophilic organisms generally have low cell growth yields; hence, more substrate carbon is directed towards the product. Thermophilic fermentation is less susceptible to mesophilic contamination. Growth at high temperatures facilitates the recovery of volatile products, such as ethanol. Fermentation at high temperatures reduces the need for refrigeration [13, 14, 15]. Examples of thermophiles metabolic engineering have long been limited but have recently begun to emerge [16, 17, 18, 19]. In the study by Chen et al. [20], the optimal conditions for the  $\beta$ -Galactosidase enzyme from *Geobacillus stearothermophilus* were found to be pH 7.0 and 70°C. Jensen et al. [21] reported that the thermostable  $\beta$ -Galactosidase enzyme from *Geobacillus stearothermophilus* had optimal aerobic conditions for the enzyme activity at 70°C and pH 6.4. The natural condition of Gedong Songo hot springs where *Geobacillus* sp. dYTae-14 was isolated was 55–65°C [4]. In this study, 65°C is used for growing the isolate.

Enzyme isolation from bacteria was performed at the logarithmic phase. To investigate the period of this phase, the growth rate of bacteria in a particular medium needs to be carried out. The growth rate study was achieved by measuring culture turbidity every 2 hours in wavelength of 600 nm [22, 23, 24] using spectrophotometer UV-Vis. The culture turbidity represents the population or biomass bacterial cells in the medium, in which the  $\frac{1}{2}$  LB liquid medium was used as a negative control. Lactose was added to the culture after 2 hours as an enzyme inducer. The *Geobacillus* sp. dYTae-14 growth rate is shown in Figure 2.



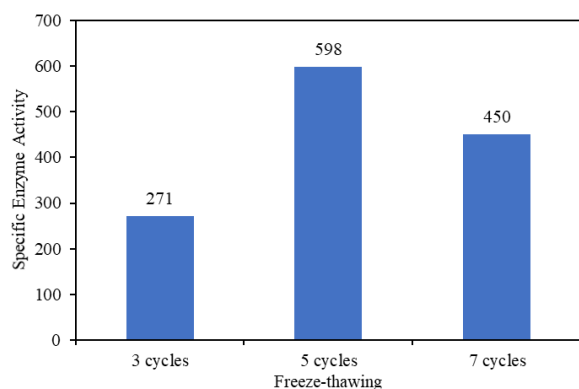
**Figure 2.** The growth rate of *Geobacillus* sp. dYTae-14 in  $\frac{1}{2}$  LB liquid medium

Based on the growth rate in Figure 2., the logarithmic phase is seen at an incubation time of 4 to 7.5 hours. For an optimal result of the enzyme isolation, the cell biomass of *Geobacillus* sp. is harvested at the end

of the logarithmic phase. The enzyme is gained through effective cell lysis. Cell lysis with the freeze-thawing modification method aims to get a significant cycle in lysis of the cell walls of *Geobacillus* sp. dYTae-14. The effectiveness of the freeze-thawing method was evaluated through the quantitative assay of the  $\beta$ -Galactosidase enzyme specific activity. It is essential to determine the quality changes that occur during multiple freeze-thawing treatments [25]. The choice of the cell lysis method is very significant, limiting only the cell wall without destroying the intracellular  $\beta$ -Galactosidase enzyme. The enzyme activity and protein content based on the different freeze-thawing cycles are shown in Table 1, and the enzyme-specific activity in Figure 3.

**Table 1.** The enzyme activity based on the different cycles of freeze-thawing

Enzyme	The absorbance of Enzyme Activity	Enzyme Activity (unit/mL)	The absorbance of Protein Content	Protein Content (mg/mL)
3 cycles	0.039	1012	0.161	3.74
5 cycles	0.078	2806	0.185	4.69
7 cycles	0.052	1610	0.157	3.58



**Figure 3.** The specific activity of  $\beta$ -Galactosidase at different freeze-thawing cycles

The specific activity of the  $\beta$ -Galactosidase enzyme shows that five cycles are the most effective cell lysis. The three cycles produce the lowest specific activity; perhaps this system cannot destroy the whole cells. On the other hand, several enzymes have been denatured in seven cycles due to a drastic temperature change over a more prolonged period. The highest rate of water loss was observed when the number of freezing and thawing increased. It is emphasized that ice crystals formed as a result of repeated thawing. The process damages the cell membrane and organelle [25]. The temperature range and freeze-thawing time cause irreversible changes toward the protein texture. The enzyme is part of bacterial cell membrane proteins that can trigger denaturation due to freeze-thawing temperature changes. Freeze-thawing is often accompanied by product damage and loss of product [26]. Quality deterioration is seen during freezing and thawing storage due to the osmotic removal of water, denaturation of protein, and mechanical damage [27]. Applying inaccurate freezing, storage, and thawing

processes generally causes microbiological, chemical, and physical deterioration. The changes induced by the freezing-thawing cycle are mainly due to three phenomena that are often closely related; mechanical damage, denaturation of proteins, and loss or water-holding capacity [28]. Freezing and thawing a biopharmaceutical product can change the chemical and physical properties of the product solution. In turn, it can suppress proteins and can permanently change the structural properties of complex macromolecules and change their stability [29]. However, Castejon et al. [30] reported that neither change in the reactivity characteristic of anti-treponemal antibodies in the weak reactive serum nor the absence of expressive effect on the results of the analyzed samples had occurred.

Freeze-thawing modification using acetone-dry ice ( $-78^{\circ}\text{C}$ ) and boiling water ( $100^{\circ}\text{C}$ ) has never been done before. The acetone-dry ice used as the frozen system and the boiling water used as the melting system were chosen because they have extreme temperature changes that cause a heat shock effect on the cell wall of thermophilic bacteria. Acetone-dry ice is easy to find and relatively cheaper than others. Gedong Songo *Geobacillus* sp. dYTae-14 isolates is a thermophilic bacterium that can grow at  $65^{\circ}\text{C}$  [4], so that boiled water ( $100^{\circ}\text{C}$ ) is used as a melting system to break down the bacterial cell wall in a shorter time. When a drastic temperature change occurs during the freeze-thawing process, the cells will not have time to adapt, so the cell will likely cause lysis due to the cell membrane damage/rupture. According to Gazali and Tambing [31], cold shock due to a sudden drop in temperature below  $0^{\circ}\text{C}$  will decrease cell viability. This is related to the transition from the membrane lipids that leads to a separation stage and degradation of selective permeability properties of the living cell biological membrane. The cooling rate and temperature interval influence cell sensitivity level toward cold shock. The main effects of cold shock are permeability, changes in the membrane lipid components accompanied by enzyme secretion, movement of ions through the membrane, and decreased content of fats such as phospholipids and cholesterol, which play a vital role in maintaining the structural integrity of the plasma membrane. The freeze-thawing method causes cells to become distended during freezing and shrink during thawing until they finally burst. Repetition of cycle times is required for efficient lysis. Freeze-thawing demonstrates the effective release of recombinant proteins present in the cytoplasm of bacteria [32]. The freeze-thaw method developed for disintegrating *E. coli* cells producing T7 lysozyme used in the pBAD expression system is straightforward and rapid. Its efficiency for small volume samples is proportional to the sonication efficiency [33]. Based on the results, cell lysis with five cycles was used for the rest of the work to obtain  $\beta$ -Galactosidase intracellular enzymes from Gedong Songo *Geobacillus* sp. dYTae-14 isolate.

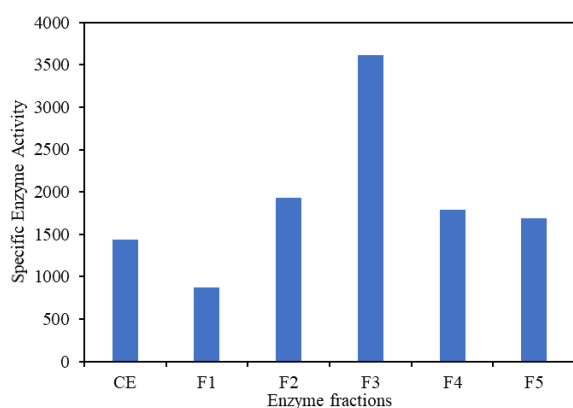
The results of cell lysis are crude extract; therefore, a purification process is needed to obtain pure  $\beta$ -Galactosidase enzyme. The purification of  $\beta$ -Galactosidase was performed with ammonium sulfate



precipitation and dialysis [34]. The determination of the specific activity of the  $\beta$ -Galactosidase enzyme was achieved by analyzing the enzyme activity and protein content. The specific activity of the  $\beta$ -Galactosidase enzymes is defined as one unit  $\beta$ -Galactosidase activity per mg protein [35]. Enzyme activity and protein content of crude enzyme and enzyme fractions can be seen in Table 2. The specific activity enzymes are shown in Figure 4.

**Table 2.** Data of crude and fractions enzyme

Enzyme	The absorbance of Enzyme Activity	Enzyme Activity (unit/mL)	The absorbance of Protein Content	Protein Content (mg/mL)
CE	0.046	1334	0.301	0.93
F1	0.039	1012	0.36	1.16
F2	0.051	1564	0.272	0.81
F3	0.079	2852	0.266	0.79
F4	0.045	1288	0.25	0.72
F5	0.064	2162	0.39	1.28



**Figure 4.** Specific activity of crude extract enzyme and precipitated fractions; CE: Crude Extract Enzyme; F1: 0–20% fraction (purity 0.61); F2: 20–40% fraction (purity 1.35); F3: 40–60% fraction (purity 2.52); F4: 60–80% fraction (purity 1.25); F5: 80–100% fraction (purity 1.18)

The results show that the addition of 1% lactose could generate  $\beta$ -Galactosidase because the lac operon is induced. One unit of  $\beta$ -Galactosidase enzyme activity is defined as the amount of ONP ( $\mu$ g) produced as a result of  $\beta$ -Galactosidase enzyme activity per mL of enzymes under optimal conditions [36]. Gheytauchi et al. [37] also reported that  $\beta$ -Galactosidase activity was identified to increase through the addition of 1 % lactose. However, the enzyme activity decreased when 1% of glucose was added as a sole carbon source. The ONPG test is recommended for biochemical tests because it is fast, cheap, and simple in all laboratories. The specific activity enzymes can be described as the purity of enzyme fractions. The highest specific activity of enzyme fraction is found in 40–60% salt saturation (F3) with 3610.13 unit/mg. This means that most of the 40–60% fraction is  $\beta$ -Galactosidase enzymes. Ammonium sulfate saturation level increased significantly ( $p < 0.05$ ), increasing the specific activity even to the saturation level of 65% [34].

#### 4. Conclusion

Based on this study, it can be concluded that the most efficient freeze-thawing cycles to lyse the local thermophilic *Geobacillus* sp. dYTae-14 is five cycles using acetone-dry ice as a freezing system and boiling water as a thawing system. The  $\beta$ -Galactosidase enzymes from *Geobacillus* sp. of Gedong Songo has the highest purity level at 40–60% ammonium sulfate saturation with purity value 2.52 and specific activity 3610.13 unit/mg proteins.

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