ISSN: 1410-8917
e-ISSN: 2597-9914

Jurnal Kimia Sains dan Aplikasi 23 (11) (2020): 383–389

Jurnal Kimia Sains dan Aplikasi
Journal of Scientific and Applied Chemistry

Journal homepage: http://ejournal.undip.ac.id/index.php/ksa

Freeze-thaw system for thermostable β-Galactosidase isolation from Gedong Songo *Geobacillus sp.* isolate

Ken Ima Damayanti [a], Nies Suci Mulyani [b,*], Agustina Lulustyaningati Nurul Aminin [b,*]

[a] Chemical Engineering Department, Engineering Faculty, Universitas Serang Raya, Serang, Indonesia
[b] Biochemistry Laboratory, Chemistry Department, Faculty of Sciences and Mathematics, Universitas Diponegoro, Semarang, Indonesia

* Corresponding author: agustina.aminin@live.undip.ac.id

https://doi.org/10.14710/jksa.23.11.383-389

Article Info

Article history:

Received: 18th August 2020
Revised: 18th November 2020
Accepted: 20th November 2020
Online: 30th November 2020

Keywords:
freeze-thawing; acetone-dry ice; β-galactosidase; ONPG; *Geobacillus sp*

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 β-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°C) is used as a frozen system and boiling water for thawing. The β-galactosidase activity was assayed using ortho-Nitrophenyl-β-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

β-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]. β-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 β-Galactosidase has triggered various studies to explore novel thermostable β-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 β-Galactosidase. Based on Ratri *et al.* [5], it was found that there was an intracellular β-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 β-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-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 N2 (-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 β-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 β-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 ½ Luria Berthani Medium for 24 hours. For the bacterial growth curve, a fresh *Geobacillus sp.* dYTae-14 culture was grown aerobically in ½ LB medium at 65°C for 14 hours. The medium turbidity at a wavelength of 600 nm (OD600) was measured every 2 hours. For β-Galactosidase enzyme production, a fresh *Geobacillus sp.* dYTae-14 culture was grown aerobically in ½ 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 H2O, 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 β-Galactosidase enzyme was carried out by adding the ONPG substrate into the crude enzyme with a ratio of 1:1. After that, the β-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% (F1), 20-40 % (F2), 40-60 % (F3), 60-80 % (F4), and 80-100 % (F5) 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 BaCl2, 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 β-Galactosidase activity assay used an ortho-Nitrophenyl-β-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 Na2CO3 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 β-Galactosidase enzyme is important. The intracellular β-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 ½ 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 ½ Luria Berthani medium is shown in Figure 1. (c) and (d).

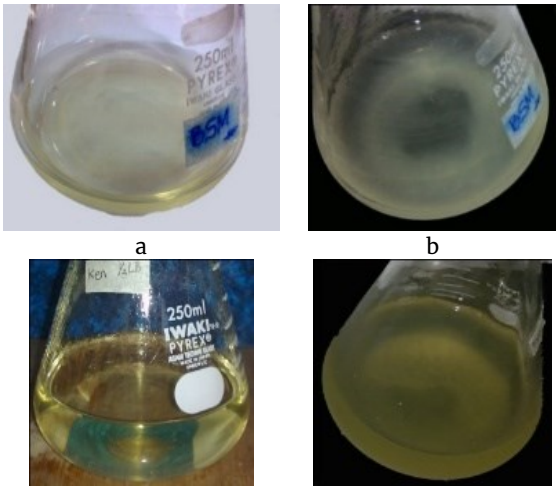

Figure 1. Comparison of bacterial growth on BSM and ½ LB liquid media, (a) negative control on BSM liquid media, (b) culture on BSM liquid media, (c) negative control on ½ LB liquid media, (d) ½ LB culture liquid medium

Physically, it is observed that the ½ 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 ½ 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, ½ LB was then used as a growth medium of *Geobacillus sp.* dYTae-14 to obtain highly thermophilic bacterial cells.

The β-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 β-Galactosidase enzyme from Geobacillus stearothermophilus were found to be pH 7.0 and 70°C. Jensen et al. [21] reported that the thermostable β-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 ½ 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.

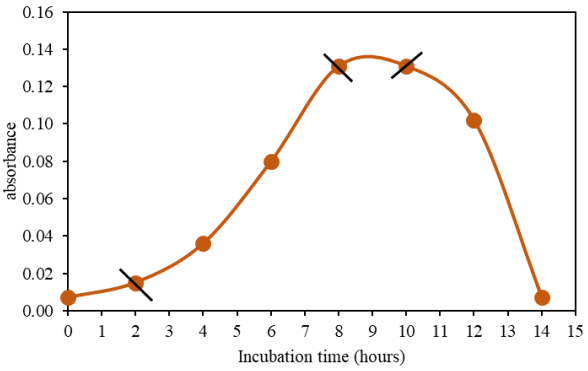

Figure 2. The growth rate of *Geobacillus sp.* dYTae-14 in ½ LB liquid medium

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 β-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 β-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

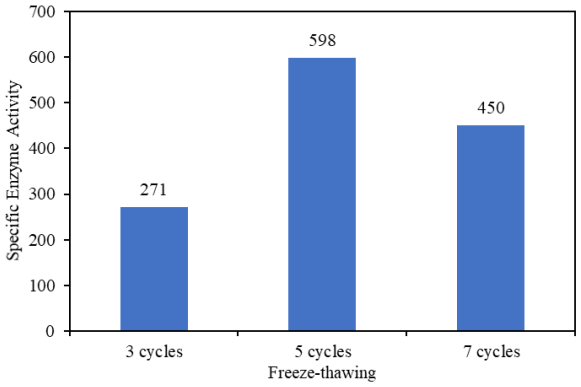

Figure 3. The specific activity of β-Galactosidase at different freeze-thawing cycles

The specific activity of the β-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°C) and boiling water (100°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°C [4], so that boiled water (100°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°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 β-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 β-Galactosidase enzyme. The purification of β-Galactosidase was performed with ammonium sulfate precipitation and dialysis [34]. The determination of the specific activity of the β-Galactosidase enzyme was achieved by analyzing the enzyme activity and protein content. The specific activity of the β-Galactosidase enzymes is defined as one unit β-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

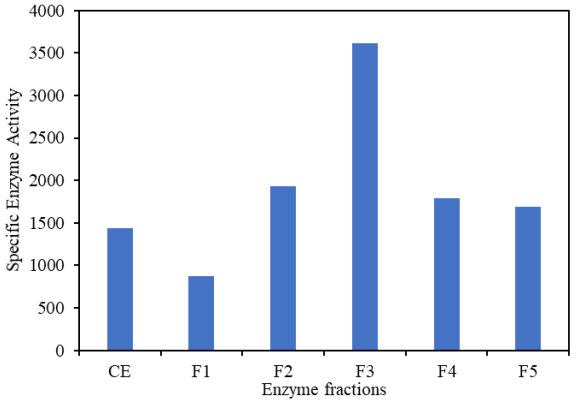

Figure. 4. Specific activity of crude extract enzyme and precipitated fractions; CE: Crude Extract Enzyme; F1: 0-20% fraction (purity 0.61); F2: 20-40% fraction (purity 1.35); F3: 40-60% fraction (purity 2.52); F4: 60-80% fraction (purity 1.25); F5: 80-100% fraction (purity 1.18)

The results show that the addition of 1% lactose could generate β-Galactosidase because the lac operon is induced. One unit of β-Galactosidase enzyme activity is defined as the amount of ONP (µg) produced as a result of β-Galactosidase enzyme activity per mL of enzymes under optimal conditions [36]. Gheytanchi et al. [37] also reported that β-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 β-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 β-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.

Acknowledgment

This research was partially supported by the Inter-University Research Cooperation Program (PEKERTI), Ministry of Research and Technology, Republic of Indonesia.

References

[1] Sophie Drouault, Jamila Anba, Gérard Corthier, *Streptococcus thermophilus* is Able To Produce a β-Galactosidase Active during Its Transit in the Digestive Tract of Germ-Free Mice, *Applied and Environmental Microbiology*, 68, 2, (2002), 938 https://dx.doi.org/10.1128/AEM.68.2.938-941.2002

[2] Ayşegül Ayyildiz, Characterization of Catalytic Phenotype of beta-Galactosidase From LacI Mutant, E. Coli CSH-36, as a Tool For The Management of Lactose Intolerance, *Turkish Journal of Medical Sciences*, 29, 5, (1999), 521-528

[3] Aziz Tanriseven, Şenay Doğan, A novel method for the immobilization of β-galactosidase, *Process Biochemistry*, 38, 1, (2002), 27-30 https://doi.org/10.1016/S0032-9592(02)00049-3

[4] Agustina Lulustyaningati Nurul Aminin, Fida Madayanti Warganegara, Pingkan Aditiawati, Akhmaloka, Simple enrichment and independent cultures to expand bacterial community analysis from Gedong Songo hot spring, *Journal of Bioscience and Bioengineering*, 106, 2, (2008), 211-214 https://doi.org/10.1263/jbb.106.211

[5] Maulida Indriyaning Ratri, Purbowatiningrum Ria Sarjono, Agustina L. N. Aminin, Studi Filogeni dan Uji Potensi Bioremediasi serta Enzim Termostabil Ekstraseluler Isolat Geobacillus sp. dari Sumber Air Panas Gedong Songo, *Jurnal Kimia Sains dan Aplikasi*, 12, 2, (2009), 31-39 https://doi.org/10.14710/jksa.12.2.31-39

[6] Mohammed Shehadul Islam, Aditya Aryasomayajula, Ponnambalam R. Selvaganapathy, A Review on Macroscale and Microscale Cell Lysis Methods, *Micromachines*, 8, 3, (2017), https://doi.org/10.3390/mi8030083

[7] Mark A. Schneegurt, Sophia Y. Dore, Charles F. Kulpa Jr, Direct extraction of DNA from soils for studies in microbial ecology, *Current issues in molecular biology*, 5, 1, (2003), 1-8 https://doi.org/10.21775/cimb.005.001

[8] Supaporn Sriwongsitanont, Masaharu Ueno, Effect of freeze-thawing process on the size and lamellarity of peg-lipid liposomes, *The Open Colloid Science Journal*, 4, 1, (2010), 1-6 http://dx.doi.org/10.2174/1876530001104010001

[9] María Dolores Figueroa-Pizano, Itziar Vélaz, María Elisa Martínez-Barbosa, A Freeze-Thawing Method to Prepare Chitosan-Poly(vinyl alcohol) Hydrogels Without Crosslinking Agents and Diflunisal Release Studies, *Journal of Visualized Experiments*, 155, (2020), 1-9 https://dx.doi.org/10.3791/59636

[10] Runqiang Yang, Qianru Hui, Xiaoyun Feng, Li Feng, Zhenxin Gu, Pei Wang, The mechanism of freeze-thawing induced accumulation of γ-aminobutyric acid in germinated soybean, *Journal of the Science of Food and Agriculture*, 100, 3, (2020), 1099-1105 https://doi.org/10.1002/jsfa.10118

[11] Feifei Chen, Jianren Ye, Chonlong Chio, Wanhui Liu, Jiyuan Shi, Wensheng Qin, A simplified quick microbial genomic DNA extraction via freeze–thawing cycles, *Molecular Biology Reports*, 47, 1, (2020), 703-709 https://doi.org/10.1007/s11033-019-05176-w

[12] Chunxue Yang, Wenzong Liu, Zhangwei He, Sangeetha Thangavel, Ling Wang, Aijuan Zhou, Aijie Wang, Freezing/thawing pretreatment coupled with biological process of thermophilic Geobacillus sp. G1: Acceleration on waste activated sludge hydrolysis and acidification, *Bioresource Technology*, 175, (2015), 509-516 https://doi.org/10.1016/j.biortech.2014.10.154

[13] Ramesh Chander Kuhad, Ajay Singh, Lignocellulose Biotechnology: Current and Future Prospects, *Critical Reviews in Biotechnology*, 13, 2, (1993), 151-172 https://doi.org/10.3109/07388559309040630

[14] Mark A. Payton, Production of ethanol by thermophilic bacteria, *Trends in Biotechnology*, 2, 6, (1984), 153-158 https://doi.org/10.1016/0167-7799(84)90032-5

[15] Juergen Wiegel, Lars G. Ljungdahl, Arnold L. Demain, The Importance of Thermophilic Bacteria in Biotechnology, *Critical Reviews in Biotechnology*, 3, 1, (1985), 39-108 https://doi.org/10.3109/07388558509150780

[16] Ashwini Bhandiwad, A. Joe Shaw, Adam Guss, Anna Guseva, Hubert Bahl, Lee R. Lynd, Metabolic engineering of Thermoanaerobacterium saccharolyticum for n-butanol production, *Metabolic Engineering*, 21, (2014), 17-25 https://doi.org/10.1016/j.ymben.2013.10.012

[17] R. E. Cripps, K. Eley, D. J. Leak, B. Rudd, M. Taylor, M. Todd, S. Boakes, S. Martin, T. Atkinson, Metabolic engineering of Geobacillus thermoglucosidasius for high yield ethanol production, *Metabolic Engineering*, 11, 6, (2009), 398-408 https://doi.org/10.1016/j.ymben.2009.08.005

[18] A. Joe Shaw, Kara K. Podkaminer, Sunil G. Desai, John S. Bardsley, Stephen R. Rogers, Philip G. Thorne, David A. Hogsett, Lee R. Lynd, Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield, *Proceedings of the National Academy of Sciences*, 105, 37, (2008), 13769 https://doi.org/10.1073/pnas.0801266105

[19] Douwe van der Veen, Jonathan Lo, Steven D. Brown, Courtney M. Johnson, Timothy J. Tschaplinski, Madhavi Martin, Nancy L. Engle, Robert A. van den Berg, Aaron D. Argyros, Nicky C. Caiazza, Adam M. Guss, Lee R. Lynd, Characterization of Clostridium thermocellum strains with disrupted fermentation end-product pathways, *Journal of Industrial Microbiology & Biotechnology*, 40, 7, (2013), 725-734 https://doi.org/10.1007/s10295-013-1275-5

[20] W. Chen, H. Chen, Y. Xia, J. Zhao, F. Tian, H. Zhang, Production, Purification, and Characterization of a Potential Thermostable Galactosidase for Milk Lactose Hydrolysis from Bacillus stearothermophilus, *Journal of Dairy Science*, 91, 5, (2008), 1751-1758 https://doi.org/10.3168/jds.2007-617

[21] Torbjørn Ølshøj Jensen, Ivan Pogrebnyakov, Kristoffer Bach Falkenberg, Stephanie Redl, Alex Toftgaard Nielsen, Application of the thermostable β-galactosidase, BgaB, from Geobacillus stearothermophilus as a versatile reporter under anaerobic and aerobic conditions, *AMB Express*, 7, 1, (2017), 169 https://doi.org/10.1186/s13568-017-0469-z

[22] T. A. Brown, *Gene Cloning and DNA Analysis: An Introduction*, 6th ed., John Wiley & Sons, 2016

[23] Ana O. Tiroli, Carlos H. I. Ramos, Biochemical and biophysical characterization of small heat shock proteins from sugarcane: Involvement of a specific region located at the N-terminus with substrate specificity, *The International Journal of Biochemistry & Cell Biology*, 39, 4, (2007), 818-831 https://doi.org/10.1016/j.biocel.2007.01.014

[24] Adam L. Mark, Development of a method to generate a soluble substrate for lytic transglycosylases, Department of Molecular and Cellular Biology, The University of Guelph, 2011

[25] T. Baygar, Y. Alparslan, Şükran Çakli, Effects of multiple freezing and refrigerator thawing cycles on the quality changes of sea bass (Dicentrarchus labrax), *Iranian Journal of Fisheries Sciences*, 12, 2, (2013), 289-300

[26] Oliver Spadiut, Thomas Gundinger, Birgit Pittermann, Christoph Slouka, Spatially Resolved Effects of Protein Freeze-Thawing in a Small-Scale Model Using Monoclonal Antibodies, *Pharmaceutics*, 12, 382, (2020), 1-15 https://doi.org/10.3390/pharmaceutics12040382

[27] Kari Thyholt, Tomas Isaksson, Differentiation of Frozen and Unfrozen Beef Using Near-Infrared Spectroscopy, *Journal of the Science of Food and Agriculture*, 73, 4, (1997), 525-532 https://doi.org/10.1002/(SICI)1097-0010(199704)73:4<525::AID-JSFA767>3.0.CO;2-C

[28] Arnaud Hallier, Sylvie Chevallier, Thierry Serot, Carole Prost, Freezing-thawing effects on the colour and texture of European catfish flesh, *International Journal of Food Science & Technology*, 43, 7, (2008), 1253-1262 https://doi.org/10.1111/j.1365-2621.2007.01601.x

[29] Manasi Puri, Sorina Morar-Mitrica, George Crotts, Douglas Nesta, Evaluating Freeze–Thaw Processes in Biopharmaceutical Development, *BioProcess International*, 13, 1, (2015), 34-45

[30] Marcia Jorge Castejon, Rosemeire Yamashiro, Camila C. Oliveira, Elaine L. Oliveira, Edilene P. R. Silveira, Carmem Aparecida F. Oliveira, Effect of multiple freeze-thaw cycles on the stability of positive anti-treponemal serum samples, *Jornal Brasileiro de Patologia e Medicina Laboratorial*, 53, 4, (2017), 246-251 http://dx.doi.org/10.5935/1676-2444.20170038

[31] Muhamad Gazali, Surya Natal Tambing, Kriopreservasi Sel Spermatozoa, *Hayati Journal of Biosciences*, 9, (2002), 27-32

[32] Brian H. Johnson, Michael H. Hecht, Recombinant Proteins Can Be Isolated from E. coli Cells by Repeated Cycles of Freezing and Thawing, *Bio/Technology*, 12, 12, (1994), 1357-1360 https://doi.org/10.1038/nbt1294-1357

[33] Marta Wanarska, Piotr Hildebrandt, Józef Kur, A freeze-thaw method for disintegration of Escherichia coli cells producing T7 lysozyme used in

pBAD expression systems, *Acta Biochimica Polonica*, 54, 3, (2007), 671-672 https://doi.org/10.18388/abp.2007_3241

[34] Krishna Prasad Nooralabettu, Optimisation of ammonium sulfate precipitation method to achieve high throughput concentration of crude alkaline phosphatase from Brown shrimp (Metapenaeus monoceros) hepatopancreas, *International Journal of Analytical Bio-Science*, 2, 1, (2014), 7-16

[35] David L. Nelson, Albert L. Lehninger, Michael M. Cox, *Lehninger Principles of Biochemistry*, W. H. Freeman, 2005

[36] F.G. Winarno, *Enzim pangan*, P.T. Gramedia, 1983

[37] Elmira Gheytanchi, Fariba Heshmati, Bahareh Kordestani Shargh, Jamileh Nowroozi, Farahnaz Movahedzadeh, Study on b-galactosidase enzyme produced by isolated lactobacilli from milk and cheese, *African Journal of Microbiology Research*, 4, 6, (2010), 454-458 https://doi.org/10.5897/AJMR.9000674