Production of antibacterial substance by immobilized cells of *Geobacillus subterraneus* Tm6Sp1 isolate of mount kamojang crater, west java, against pathogenic bacteria

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ABSTRACT

Geobacillus is a thermophilic bacterium known for its ability to produce various metabolites, including thermostable enzymes, chemical compounds, and antimicrobial peptides, particularly bacteriocins. This study aimed to assess the antibacterial potential of *Geobacillus subterraneus* Tm6Sp1, isolated from the crater of Mount Kamojang in Indonesia, as a novel approach for combating pathogenic bacteria. The utilization of cell immobilization techniques provided an environment conducive to antibacterial substance production and facilitated higher cell density for enhanced communication. Antibacterial preparations were carried out by cultivating *G. subterraneus* in Mueller-Hinton Broth supplemented with $CaCl_2$ and $MgSO_4$ for 18 h. The antibacterial activity of free and immobilized cells was evaluated at 55°C, with culture broth collected at 18-h intervals for up to four repeated batch fermentations. The resulting *G. subterraneus* filtrates were then tested for their antibacterial activity against pathogenic bacteria. The findings revealed that the maximum antibacterial activity was observed in the first batch of free cells, exhibiting inhibition zones of 6.90 mm, 10.05 mm, and 16.54 mm against *Staphylococcus aureus*, *Salmonella enteritica*, and *Escherichia coli*, respectively. Conversely, immobilized cells in the fourth batch demonstrated higher inhibition zones of 8.54 mm, 19.75 mm, and 18.04 mm against *S. aureus*, *S. enteritica*, and *E. coli*, respectively. In conclusion, immobilized cells of *G. subterraneus* exhibited optimal antibacterial substance production in the fourth batch of fermentation, showing antagonistic activity against several pathogenic bacteria.

1. INTRODUCTION

Thermophiles have unique metabolic capabilities and enzymes adapted to function in extreme conditions [1]. Therefore, these organisms serve as a valuable source of stable proteins, enabling microorganisms to conserve energy and nutrient resources that would otherwise be expended on protein degradation and synthesis [2]. Subsequently, an extensive exploration of *Geobacillus* and *Parageobacillus* transcriptomes and secretomes has shown many proteins with either proven or potential industrial and medicinal applications [3,4], such as bacteriocins or BLIS [5,6]. Bacteriocins are ribosomally synthesized antimicrobial peptides (AMPs) produced by bacteria to inhibit other bacteria from establishing predominance in environmental microbial communities [7]. AMPs have emerged as highly promising alternatives

to antibiotics due to their potential in treating bacterial infections, including those caused by multidrug-resistant pathogens [8]. They have also been shown to have low or no cytotoxicity on eukaryotic cell lines even when used at very high doses [9].

Numerous AMPs with distinct activity spectra and mechanisms of action have been characterized [8], such as toebicin 218, thermoleovorins, and termosin, which are produced by *Geobacillus toebii* [10], *Geobacillus thermoleovorans* [11], and *Geobacillus stearothermophilus* [12], respectively. In the study by Alkhalili [13], *Geobacillus* sp. has a source of novel antibacterial peptides different from various other types. The AMPs secreted by *Geobacillus thermodenitrificans*, *Geobacillus* ZGt-1, and *Geobacillus* spp. are known to effectively inhibit pathogenic bacteria, *Staphylococcus aureus*, *Salmonella enterica* serovar *Typhimurium*, and *Klebsiella pneumoniae*, respectively [2,14,15].

Currently, immobilized microorganism technology is increasingly used for producing AMP. This technique is a broad term used to describe the physical trapment of viable microbial cells within a

specific, defined area (polymer). This confinement restricts their free movement and imparts hydrodynamic properties that are distinct from those of the surrounding environment, thereby increasing fermentor cell density and stabilizing continuous or repeated cultures [16]. This, in turn, enhances the productivity of various bacteriocins [17,18]. It is also susceptible to the effects of inhibitory compounds, which reduce the risk of cell damage and contamination [19]. Naghmouchi *et al*. [20] investigated the highest production of pediocin PA-1 obtained during repeated-cycle batch cultures using *Pediococcus acidatili* UL5 immobilized in k-carrageenan beads. This repeated-cycle batch culture process was stable and resulted in high pediocin PA-1 production (4096 AU/mL) and volumetric productivity (342 AU/mL/h) at the end of 0.75- and 2-h incubation cycles.

The thermophilic bacterial isolate, *Geobacillus subterraneus* Tm6Sp1, from Mount Kamojang Crater, West Java, Indonesia, seems to be a promising source of AMPs, which was confirmed by the coding gene's availability [21]. The focus of this study is on *G. subterraneus* and its potential for producing antibacterial substances against various pathogenic bacteria utilizing an immobilized cell system.

2. MATERIALS AND METHODS

2.1. Microorganisms and Maintenance of Culture

G. subterraneus Tm6Sp1 isolate was obtained from Mount Kamojang Crater, West Java, Indonesia. In a previous study, Tm6Sp1 was identified and found to have more than 99% similarity to *G. subterraneus* (accession no. CP051162.1) [21]. Furthermore, it was maintained from glycerol at 4°C to Mueller-Hinton Agar (MHA) at 55°C for 24-48 h. The test indicator organisms were *Streptococcus mutans* ATCC 700610, *S. aureus* ATCC 29213, *S. enterica* serovar Typhimurium ATCC 49416, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *K. pneumoniae* ATCC 700603. They were grown on Tryptic Soya Agar at 37°C for 24 h.

2.2. Inoculum Preparation and Cell Count

G. subterraneus Tm6Sp1 culture was grown in Luria Bertani (LB) broth at 55°C for 24–48 h in an incubator shaker at 150 rpm [22]. The growth phase of bacteria was determined with two replications by measuring the number of cells in McFarland turbidity value converted to Colony form unit (CFU)/mL (this conversion referred to Dalynn Biological [23]). This observation of the cell number of bacteria was carried out every 2 h for 36 h. Furthermore, the supernatant was taken after reaching the late logarithmic phase as the initial inoculum for producing bacteriocins [15].

2.3. Repeated Batch Production of Antibacterial Substances by Free Cells (F)

The inoculum from a fresh culture of *G. subterraneus* Tm6Sp1 was prepared by adjusting to the 0,5 McFarland turbidity standard (equivalent to 1×10^8 CFU/mL) and then diluting it 1/10 (v/v). The resulting suspension was used as the starter inoculum, which was transferred to 40 mL of modified Mueller-Hinton Broth (MHB) containing additional salts such as $MgSO_4$ and $CaCl_2$ as the production medium [16]. According to the growth curves, this suspension was incubated with batch fermentation in an incubator shaker at 55°C and 150 rpm until it reached the late logarithmic phase, which occurred at the 18th h. The culture broth was then centrifuged at $10000 \times g$ for 20 min at 4°C (Eppendorf centrifuge 5424 R). This centrifugation step separated the cells in the pellet for further treatment.

For the free cells (F) treatment, the pellet was resuspended in 75 mL of fresh MHB medium and subjected to repeated fermentation. As for the immobilized cell treatment, the pellet was used to prepare immobilized cells as described in Section 2.5. The supernatant obtained from the centrifugation step was filtered using 0.22 µm syringe filters to remove bacterial cells. The fourth batch of fermentation was performed to obtain data at four different observation times.

F1: First batch filtrate of *G. subterraneus* Tm6Sp1 free cells
F2: Second batch filtrate of *G. subterraneus* Tm6Sp1 free cells
F3: Third batch filtrate of *G. subterraneus* Tm6Sp1 free cells
F4: Fourth batch filtrate of *G. subterraneus* Tm6Sp1 free cells

All filtrates were tested for antibacterial activity by disk diffusion method.

2.4. Repeated Batch Production of Antibacterial Substances by Immobilized Cells (I) In Agar Beads

The method of cell immobilization used in this study was based on the procedure described by Alkhalili *et al*. [16], with some modifications. The cell pellet obtained from centrifugation was resuspended in 5 mL of sterile MHB and mixed with 25 mL of 3,5% (w/v) sterile molten agar solution that had been pre-warmed to 60°C. This mixture was dropped into cold mineral oil using a syringe needle to form agar beads with an average diameter of 5 mm. Furthermore, the agar beads were washed thoroughly with sterile phosphate buffer saline and distilled water to remove the oil before being transferring to 75 mL of fresh, sterile MHB. The bead suspension was incubated at 55°C and 150 rpm for 18 h. After incubation, the culture broth was carefully separated from the beads by pouring it into sterile bottles. The culture broth was then centrifuged at $10,000 \times g$ for 20 min at 4°C. The resulting supernatant was filtered using a 0.2 µm syringe filter. Finally, the filtered supernatant was stored for further analysis.

The beads were transferred back to the cultivation flask with 75 mL of fresh, sterile MHB, and another cultivation cycle was conducted. The beads were further recycled up to the fourth batch to obtain four observation times.

I1: First batch filtrate of *G. subterraneus* Tm6Sp1 immobilized cells
I2: Second batch filtrate of *G. subterraneus* Tm6Sp1 immobilized cells
I3: Third batch filtrate of *G. subterraneus* Tm6Sp1 immobilized cells
I4: Fourth batch filtrate of *G. subterraneus* Tm6Sp1 immobilized cells

All filtrates were tested for antibacterial activity by the disk diffusion method.

2.5. Antibacterial Activity Test

The filtrates (F1-F4 and I1-I4) were checked for pH, which must be neutral before use, and adjusted with 5 M NaOH [24]. The filtrate was put in a vial containing sterile blank disks of 6 mm diameter, and the disks were soaked for 1 h at room temperature. After soaking, the disks were placed on the MHA plate surface, inoculated with the test bacteria, and incubated at 37°C for 24 h. The production of antibacterial substances using free and immobilized cells was compared based on their antibacterial activity in the diameter (mm) of the inhibition zone. This was interpreted as follows: diameters of the inhibition zone >20 mm, 10–20 mm, 5–10 mm, and 0–5 mm represent very strong, strong, moderate, and no inhibitory activity or weak, respectively [25].

2.6. Statistical Analysis

The data obtained from this research regarding antibacterial activity were subjected to statistical analysis using the non-parametric

Kruskal-Wallis test in the Statistical Package for the Social Sciences 25th version software. A significance level of 0.05 was employed for the analysis. All analyses were done with three replications.

3. RESULTS

3.1. Growth of *G. subterraneus* Tm6sp1

Bacterial growth was observed every 2 h during 36 h of incubation in an MHB medium, and CFU/mL determined the number of bacteria. A graph was made between the number of bacteria and the incubation time to identify the logarithmic phase, as shown in Figure 1.

Based on the analysis of growth curve parameters, it was determined that the generation time of Tm6Sp1 was relatively rapid compared to other *Geobacillus* strains, with an approximate value of 36.78 min. This finding is consistent with the observations made by Marchant *et al*. [26], who reported a doubling time of around 30 min for several *Geobacillus* strains isolated from soil samples in nutrient-rich media. For the production of the first batch of free cell filtrate (F1), the bacterial culture was harvested during the late logarithmic phase after 18 h of incubation.

3.2. Physical Properties of The Agar Beads With Embedded *G. Subterraneus* Tm6Sp1

Spherical particles immediately formed and hardened into beads when an agar solution containing embedded Tm6Sp1 was dropped into cold mineral oil. The final particles were pellucid white spheres of approximately 3–4 mm diameter, as shown in Figure 2.

3.3. Antibacterial Activity by Free Cells (F)

The inhibition zones can show antibacterial activity by free cells, as graphically presented in Figure 3.

The results showed that the production of antibacterial activity by free cells was at its maximum, with strong inhibition observed in the initial batch (F1) or during the final logarithmic phase before being repeated. However, upon reusing the cells, the antibacterial activity experienced a sharp decline and was not detectable in the subsequent batches (third to fourth). Overall, the inhibition zone size ranged from 8.63 mm to 16.43 mm against *E. coli* [Figure 4] under optimal conditions.

3.4. Antibacterial Activity by Immobilized Cells (I)

Antibacterial activity by immobilized cells can be seen by the inhibition zones formed, as shown in Figure 4.

Antibacterial production using immobilized cells exhibited maximum activity at the fourth batch (I4), inhibiting 18.04 mm against *E. coli* [Figure 4]. In the second batch (I2), a faint inhibition was observed against *E. coli*, *S. mutans,* and *S. aureus*, with inhibition zones measuring 7.05 mm, 7.23 mm, and 8.21 mm, respectively. Therefore, the production using immobilized cells increased along with the culture batch period until the fourth batch without declining [Figure 5].

3.5. Antibacterial Spectrum Against Pathogenic Bacteria

Based on the optimization of batch results using both free and immobilized cells, the first and fourth batches demonstrated the most
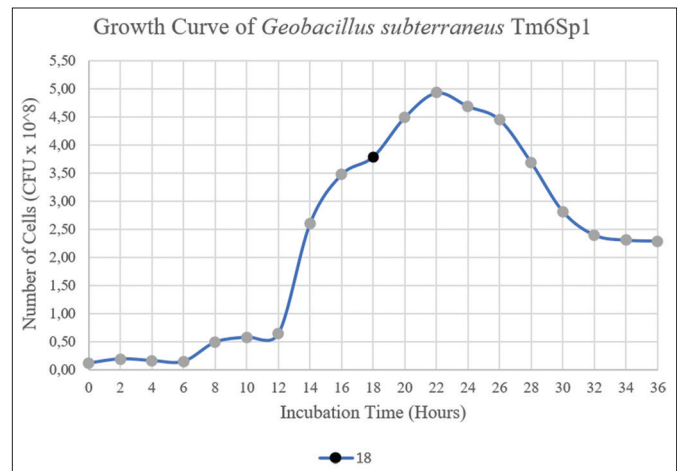
Figure 1: Growth curves of *G. subterraneus* Tm6Sp1 at their optimal growth condition for 36 h. The black dot (●) represent the time (h) of culture sampling for the first batch of free cells and the initial inoculum of immobilized cells. culture sampling for the first batch of free cells and the initial inoculum of immobilized cells.


Figure 2: Agar beads embedded with *G. subterraneus* Tm6Sp1 formed with 3% w/v agar bacteriological and cold mineral oil.
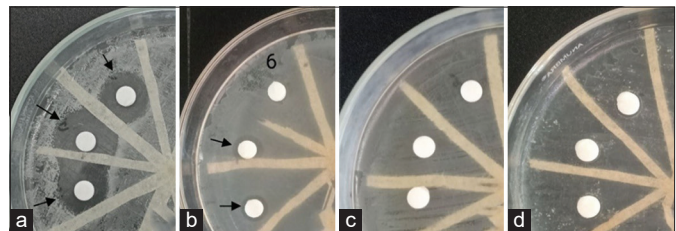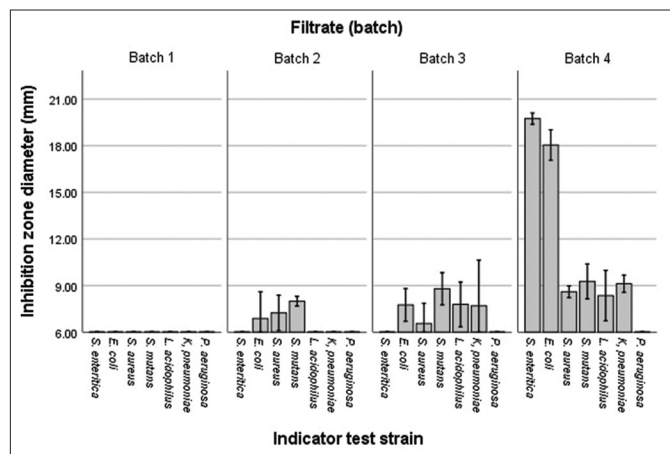

Figure 3: Antibacterial activity by *G. subterraneus* Tm6Sp1 free cells against *E. coli* at (a) first, (b) second, (c) third, and (d) fourth batch.

favorable conditions in terms of antimicrobial activity. Additionally, the spectrum activity was also examined against several pathogenic bacteria, as shown in Table 1.
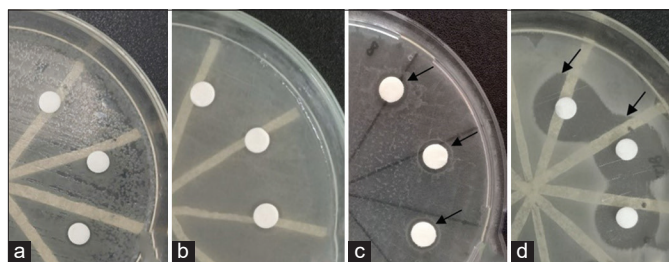
Figure 4: Antibacterial activity by *G. subterraneus* Tm6Sp1 immobilized cells against *E. coli* at (a) first, (b) second, (c) third, and (d) fourth batch.

Figure 5: Antibacterial activity by immobilized cells of Tm6Sp1 for four batches. (Very strong: >20 mm, strong: 10–20 mm, moderate: 6–10 mm, weak or not detected: <6 mm).
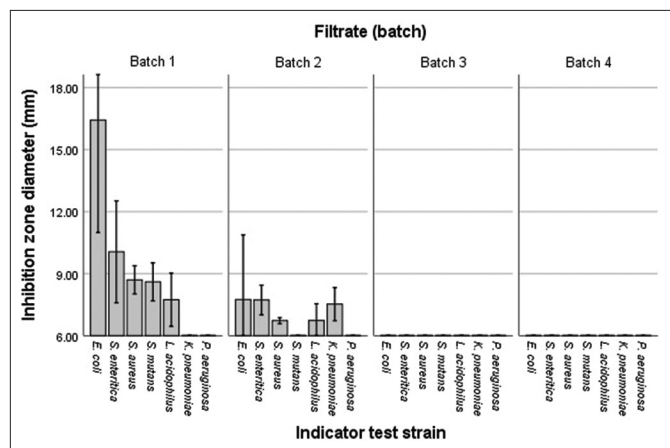
Figure 6: Antibacterial activity by free cells of Tm6Sp1 for four batches. (Very strong: >20 mm, strong: 10–20 mm, moderate: 6–10 mm, weak or not detected: <6 mm).

4. DISCUSSION

The growth phase of the *G. subterraneus* Tm6Sp1 isolate was determined by observing the growth curve. The results showed that the exponential phase of this strain occurred between 12 and 18 h of cultivation, as depicted in Figure 1. To obtain the first batch of free cell filtrate (F1), the culture was taken during the late logarithmic phase, specifically after 18 h of incubation. During this phase, the supernatant was filtered, resulting in the filtrate containing Tm6Sp1-free cells from

Table 1: Antibacterial spectrum against pathogenic bacteria from an optimal batch of *G. subterraneus* Tm6Sp1.

Test pathogen strain	Free cells at first batch (F1)	Immobilized cells at fourth batch (I4)
Gram-positive		
Streptococcus mutans	-	+
Staphylococcus aureus	+	+
Lactobacillus acidophilus	+	+
Gram-negative		
Pseudomonas aeruginosa	-	-
Klebsiella pneumoniae	-	+
Salmonella enteritica	++	++
Escherichia coli	++	++

Classification of antibacterial activity, +++ (very strong): >20 mm, ++ (strong): 10–20 mm, + (moderate): 6–10 mm, - (weak or not detected): <6 mm

Table 2: Antibacterial activity in every batch against *E. coli*.

Cells system preparation	Antibacterial activity in every batch against *Escherichia coli*			
	1	2	3	4
Free cells	++	+	-	-
Immobilized cells	-	+	+	++

the first batch. On the other hand, the centrifuged pellets were utilized in two different treatments, followed by repeated batch fermentation using the first batch as the initial inoculum to manufacture beads into immobilized cells.

The late logarithmic phase has been identified as the optimal time for the synthesis of antibacterial compounds in many bacteriocins produced by Bacilliaceae. Several studies reported that maximum production occurs during the mid-log phase in certain strains such as *B. lentus*, *B. mycoides,* and *L. brevis* [27]. Similarly, the production of thermosin 10, thuricin 439, and cerein 7 from *G. stearothermophilus* 32A [11], *B. thuringiensis* B439 [28], and *B. cereus* Bc7 [28], respectively, has been found to be most effective during the late log phase or early stationary phase. Ozdemir *et al*. [10] also discovered that bacteriocin-like inhibitory substances (BLIS) produced by *G. toebii* were maximally produced at the late logarithmic phase. Based on the promising results obtained in shake flasks, repeated batch fermentations were conducted using free and immobilized cells of *G. subterraneus* Tm6Sp1 to optimize the culture conditions, as presented in Table 2.

The maximum antibacterial activity observed with free cells was 16.43 mm and 10.05 mm, respectively, that were detected in the first batch of filtrate against *E. coli* and *S. enteritica*, respectively. This finding is related to the previous study [29] on bacteriocin production. During batch fermentation, substances accumulate during the growth phase, when bacteria experience rapid growth and reach their peak. However, it was observed that the antibacterial activity sharply decreased to 8.63 mm against *E. coli* after the first batch of free cell culture. Furthermore, no antibacterial activity was detected in the third to fourth batches, as shown in Table 2. The statistical analysis revealed significant differences ($P < 0.05$) in the diameter of the zone of inhibition within every batch of fermentation [Table 3]. Additionally, the different batches of fermentation in each cell system preparation resulted in significant variations in the antibacterial activity, as

Table 3: Kruskal-Wallis test assessing the impact of batch fermentation on the antimicrobial activity exhibited.

Variable	Cell system preparation	Batch 1	Batch 2	Batch 3	Batch 4	Kruskal-Wallis H	df	*P*-value
Diameter	Free cells	62.07	51.93	28.00	28.00	43.789	3	0.000
	Immobilized cells	22.50	34.31	46.79	66.40	43.564	3	0.000

The *P*-value represents the significance level of the Kruskal-Wallis, where $P \leq 0.05$ indicate statistical significance differences

evidenced by the formation of clear zones against the selected bacterial pathogens.

It has been similarly reported by Awais *et al*. [30] that during free cell synthesis of peptide antibiotics, maximum activity (14 mm) was seen at the first cycle of 24–48 h of incubation. Furthermore, the activity abruptly declined at 72 h due to the Tm6Sp1 cells entering the death phase. During batch fermentation experiments, Callewaert *et al*. [29] observed a significant decrease in amylovorin L471 titer after reaching peak activity during the active growth phase. This decrease in activity was accompanied by a decrease in the growth of *Lactobacillus amylovorus* DCE 471 in colony-forming units (CFU), indicating cell death. Moreover, it could be argued that the conditions for maximal bacteriocin production increase linearly with the cell density of the producer strain as a primary metabolite [12]. These results also showed that the free cell system was not suitable for repeated cycle culture.

In contrast, antibacterial peptides synthesis with an immobilized cell system were produced in the second batch (7.05 mm) and kept increasing during the third and fourth fermentation cycles. The highest antibacterial activity was obtained in the fourth batch, with strong inhibition against *S. enterica* and *E. coli* at 19.67 mm and 18.04 mm, respectively, as seen in Figures 5 and 6. The result showed that immobilized cell system preparation was advantageous for long-term bacteriocin production, and the encapsulated cells could be reused more than two times after filtration and resuspension in a new medium. Similarly, Hasan *et al*. [31] stated that the production of an antibacterial substance by immobilized *B. pumilus* SAF1 is higher than that of free cells. The results on the reusability of the entrapped cells by repeated batch fermentations showed that increased production was observed up to four cycles. This may be due to the proper adaptation in this microenvironment and the proper growth of cells in the beads. In the study by Alkhalili *et al*. [2], immobilized cells could be recovered and recycled for sequential batch fermentation with increasing antibacterial activity up to the fourteenth batch. Compared to free cells, entrapment inside the gel beads provides a protective environment for the cells that are more active at producing certain metabolites than free cells [32]. However, free and immobilized cells could produce AMPs and had strong inhibition against several bacteria.

The antibacterial compound produced by *G. subterraneus* Tm6Sp1 worked antagonistically against six test pathogens, with strong inhibition against *S. enterica* and *E. coli* and moderate inhibition against *S. aureus*, *S. mutans*, *L. acidophilus*, and *K. pneumoniae*. Furthermore, there was strong inhibition reported against Gram-negative bacteria. This spectrum is similar to that of the other bacteriocins, such as Geobacilin I from *G. thermodenitrificans* OH2-1 and *Geobacillus* sp. M10EXG, which could inhibit *S. aureus* [14], while Geobacillin I from *Geobacillus* ZGt-1 inhibits *S. aureus* and *S. enterica* [16]. Moreover, thermoleovorin-S2 and thermoleovorin-N9 from *G. thermoleovorans* inhibit the pathogenic bacteria *S. enterica* [11]. Antibacterial activity also occurs against *K. pneumoniae* by bacteriocins produced by *Geobacillus* spp. [16]. BLIS from *G. toebii* HBB-247 can inhibit the growth of several strains, including *G. stearothermophilus*, *Listeria* sp., *Enterococcus*

faecalis, *Enterococcus avium* AS-3, *Anoxybacillus* sp. HBB-134, *Geobacillus* sp. HBB-269, *Clostridium pasteurianum*, and *Cellulomonas fimi* [10]. Therefore, the spectrum of bacteriocins produced by *Geobacillus* had a broader antibacterial activity to inhibit pathogenic bacteria, a suitable prospect for application in medicine.

5. CONCLUSION

The antibacterial substance of *G. subterraneus* Tm6Sp1 can be produced using free and immobilized cells in their optimum condition. Strong inhibition against pathogenic bacteria was obtained at 10.88 mm to 19.75 mm by immobilized cells in the fourth batch. The production of this substance was initiated in the second batch and increased with the incubation period cycle (repeated batch) up to the fourth batch without a decrease. In contrast, the antibacterial activity detected in the free cells system between 10.05 mm and 16.43 mm was only detected in the first batch, during the late logarithmic phase of fermentation. The result concluded that the free cell system was unsuitable for reuse in repeated batch fermentation and that the antibacterial spectrum effectively inhibited gram-negative bacteria, *E. coli*, and *S. enteritica*.

7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All data relating to the manuscript has been provided in script.

12. PUBLISHER'S NOTE

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