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Optimized Novel Antibacterial Production from *Geobacillus kaustophilus* Tm6T2 (a) as Treatment for *Salmonella typhimurium*

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ABSTRACT

Geobacillus sp. is recognized for its potential to produce bacteriocins, antibacterial substances that hold promise in addressing gastrointestinal illnesses. This study aimed to optimize the medium and pH conditions for producing antibacterial substances by *Geobacillus kaustophilus* Tm6T2(a). The research employed a descriptive and experimental methodology. Growth studies were conducted in Mueller Hinton Broth with $CaCl_2$ and $MgSO_4$ and Nutrient Broth with KCl and $MgCl_2$ across 6, 7, and 8 pH values. Subsequently, antibacterial substance production was achieved at the late logarithmic phase and was assessed against the gastrointestinal pathogen *Salmonella typhimurium*. Interestingly, findings indicated that antibacterial substance production might not solely correlate with bacterial cell count. Despite a lower bacterial cell count, the highest inhibition zone against *S.typhimurium* was observed at 13.11 mm in NB salt at pH 8. Analytical results show that the variation of pH and both mediums significantly affects the presence of the inhibition zone ($p < 0.10$). This finding suggests the complexity of factors influencing antibacterial activity. Overall, the optimum condition for antibacterial production in *G.kaustophilus* Tm6T2(a) was identified at pH 8 using NB salt. These findings have potential implications for developing antibacterial solutions targeting gastrointestinal pathogens.

Keywords: Antibacterial activity, bacterial cell count, environmental condition,*Geobacillus kaustophilus* Tm6T2 (a), KCl, $MgCl_2$

INTRODUCTION

Many studies have investigated the potential of *Geobacillus* bacteria as a producer of antibacterial substances with activity against Gram-positive and Gram-negative bacteria.[1-3] Alkhalili *et al*. reported that the thermophilic bacteria *Geobacillu ss*p. ZGt-1 produces an antibacterial substance in the form of lantibiotic bacteriocins synthesized from lantipeptides, classified as class-I lantipeptide.[4]

Environmental factors, such as nutrient composition and pH, can influence the production of antibacterial substances, particularly peptides. The production of these substances can be directly proportional to the existing biomass, indicating that conditions rich in nutrients are necessary for their generation.[5] Therefore, it is essential to investigate the optimum conditions for producing antibacterial peptides.

Bacteriocin can be a potential drug for some gastrointestinal pathogens. The infection is primarily contracted through the consumption of contaminated food. Based on the Nutritional Status Survey in 2020, Indonesia experienced a prevalence rate of 9.8% for diarrheal occurrences.[6] This infection is commonly caused by bacterial pathogens such as *E.coli, Staphylococcus*, and *Salmonella*.

This research aimed to determine the optimum medium and pH conditions for *G.kaustophilus* Tm6T2 (a) 's production of antibacterial substances.

METHODS

Thermophilic bacteria *G.kaustophilus* Tm6T2 (a) was isolated from Kamojang Crater, West Java, Indonesia. Pathogen bacteria *Salmonella typhimurium* ATCC 49416 were obtained from the microbe collection from the Microbiology Laboratory of the Faculty of Medicine located at the Center for Academic, Innovation, Technology, and Research (PAMITRAN) West Java, Indonesia.

The media used for antibacterial production of *G.kaustophilus* Tm6T2 (a) are Mueller Hinton Broth (MHB) supplemented with $CaCl_2$ and $MgSO_4$ salt and

Nutrient Broth (NB) supplemented with KCl and $MgCl_2$ salt.[4,7] Subsequently, variations of pH 6, 7, and 8 were obtained by adding sterile HCl or NaOH. *G.kaustophilus* Tm6T2 (a) cultures were incubated in a shaker incubator at 55^{0}C and 150 rpm for 36 hours. The growth phase of the bacteria was determined by measuring the McFarland value on a McFarland densitometer (DEN-1B 89402-910), which was later converted to CFU/mL. Observations were made every 2 hours after that, up to 36 hours.[8] Growth curves were generated using Microsoft Excel software to determine the correlation between biomass and the activity of antibacterial substances. The following formulas can calculate the generation time and bacterial growth rate constant values.[9]

$$Generation\ time\ (g) = \frac{0.301}{slope}$$

$$Growth\ rate\ constant\ (k) = \frac{0.693}{g}$$

Antibacterial activity was tested using disc diffusion. Each sample was repeated in triplicate. Ten micrograms of ampicillin discs (OXOID) were used as a positive control, while discs that had been soaked with aquadest were used as a negative control. The test bacteria and discs were incubated at 37^{0}C for 24 hours, and an inhibition zone was observed.[10] Data obtained from optical density observation and zone of inhibition will be processed and analyzed using Microsoft Excel and Statistical Products and Services (SPSS).

RESULTS AND DISCUSSIONS

The growth phase of *G.kaustophilus* Tm6T2 (a) can be divided into lag, logarithmic/exponential, stationary, and death phases, as shown in Figure 1. The various phases started at different times for each pH and other medium. For both the cultures in MHB salt pH 7 and NB salt pH 6, the lag phase ended at the earliest, at 8 hours of incubation. The highest bacterial cell count was observed in MHB salt at pH 7, with approximately 5.07 x 10^8 CFU/mL achieved during the 8-hour logarithmic phase. Conversely, the lowest bacterial cell count was obtained in NB salt at pH 8, with approximately 1.21 x 10^8 CFU/mL achieved during the 6-hour logarithmic phase.

Genus *Geobacillus* produces some antibacterial substances that are classified as several bacteriocins, antibiotic pigments, Bacteriocin-Like Inhibitory Substances (BLIS), and volatile organic substances.[3] Bacteriocin, a peptide that exhibits antibacterial activity against pathogens, is synthesized ribosomally during the primary growth phase and is maximized at the late logarithmic/exponential phase.[11] This substance is most likely bacteriocin or BLIS. The result is consistent with Pranckutė *et al.*, where *Geobacillus* species (*G.stearothermophilus* NUB36187, *G.uzenensis* VKM B-2229, *G.subterraneus* VKM B-226, *G.thermocatenulatus* VKM B-1259, *G.thermoleovorans* DSM 5366, *G.thermodenitrificans* DSM 466, and *G.lituanicus* N-3) from oil wells produced bacteriocin in exponential phase. The maximum production occurs in the late logarithmic/exponential phase.[1] Throughout the growth of thermophilic bacteria, various substances are produced, as mentioned above. Therefore, to ensure that the zone of inhibition observed was not due to a substance other than bacteriocin or BLIS, the effects were neutralized using NaOH and HCl.

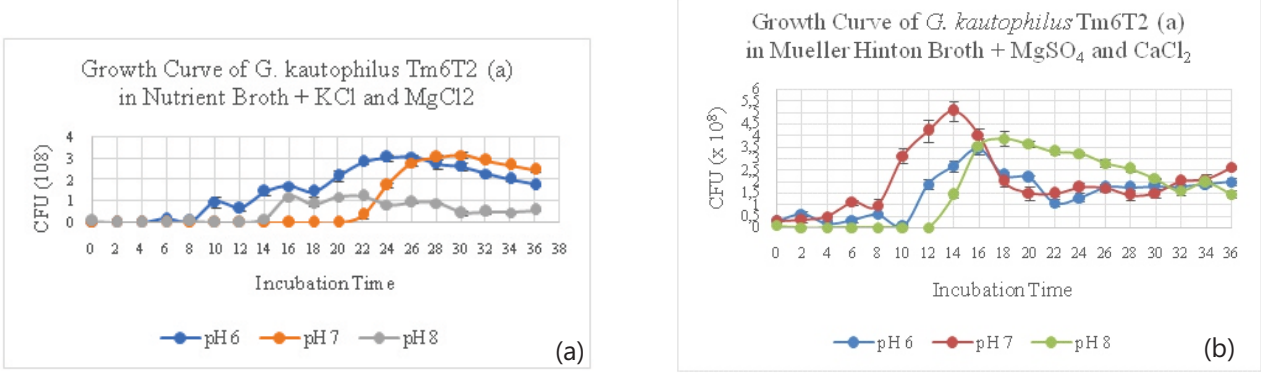

Figure 1. Growth curve kinetics of *G.kaustophilus* Tm6T2 (a) in (a) NB salt. (b) MHB salt. The color blue indicates pH 6, orange indicates pH 7, and grey indicates pH 8. All experiments were repeated in triplicate

The highest specific growth rate constant (k) of hours^{-1} was achieved in NB salt at pH 8 (Table 1). This parameter describes the rate of cell division per unit time. The growth rate constant can be used as a benchmark to determine the carrying capacity of the medium for cell growth and division. Under these conditions, bacteria have a generation time (g) of 0.320, requiring 0.320 hours (19.2 minutes) to double the number of cells. Meanwhile, in the MHB salt medium at pH 7, which has the highest bacterial cell count, the specific growth rate constant (k) was 1.178 hours^{-1}, with the generation time of 0.588 hours (35.2 minutes).

Production of this antibacterial substance can be biomass-dependent, indicating that it could be a primary metabolite.[12] However, high bacterial cell count only sometimes leads to high activity of antibacterial substances. Despite a bacterial cell count of no more than 5.07 x 10^8 CFU/mL in this research, the culture grown in NB salt at pH 8 exhibited the most effective antibacterial activity. It is worth noting that some class II bacteriocins where production was regulated by quorum sensing, can be induced by environmental factors and are not solely dependent on bacterial cell count.[13] Growth curve kinetics was then used to harvest the antibacterial substance at the end of the log/early stationary phase.

Antibacterial substances from *G.kaustophilus* Tm6T2 (a) exhibited different zones of inhibition against *S.typhimurium* in various production media. Bacteria grown on NB salt showed the largest inhibition zone of 13.11 mm. This was followed by MHB salt, exhibiting an inhibition zone of 10.58 mm. Both were classified as having vigorous inhibitory activity, as shown in Figure 2.

The results showed that pH also influences the activity of antibacterial substances. *G.kaustophilus* Tm6T2 (a) was grown on NB salt at pH 8, indicating the largest inhibition zone of 13.11 mm, classified as vigorous inhibitory activity. Meanwhile, bacteria grown in the same medium with different pH showed no zone of inhibition, as presented in Figure 2. Statistical analysis demonstrated that the variation of pH and MHB salt medium significantly affects the presence of the inhibition zone ($p < 0.10$), meaning

Table 1. Generation time, growth rate constant, and maximum cell count of *G.kaustophilus* Tm6T2 (a) in different media and pH*

Media	pH	Generation Time (g)	Growth Rate Constant (k)	Maximum Cell Count (CFU/mL)
NB+KCl and $MgCl_2$	6	3.077	0.255	3.07 x 10^8±0.11
	7	0.588	1.178	3.15 x 10^8±0.15
	8	0 320	2.161	1.21 x 10^8±0.02
MHB+$MgSO_4$ and $CaCl_2$	6	0.534	1.295	3.42 x 10^8±0.21
	7	1.137	0.609	5.07 x 10^8±0.43
	8	0.653	1.059	3.87 x 10^8±0.31

*Each experiment was repeated in triplicate

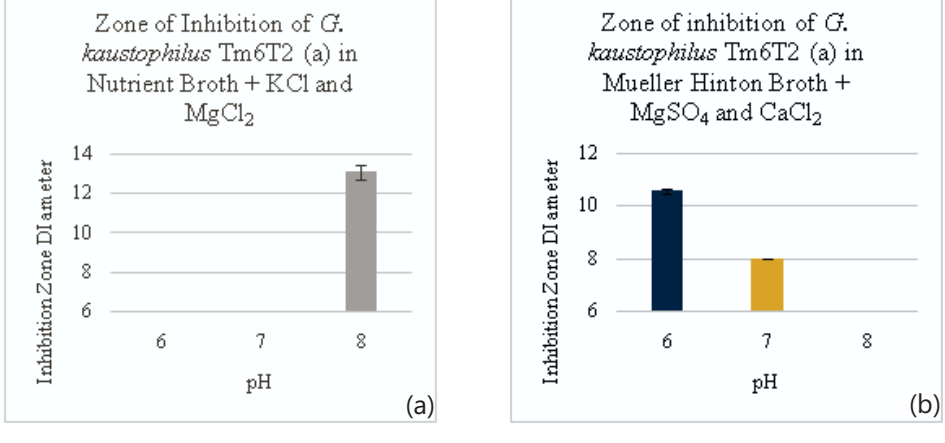

Figure 2. Activity of antibacterial substance from *G.kaustophilus* Tm6T2 (a) free cell culture against *S.typhimuriumin* (a) NB salt. (b) MHB salt. The color black indicates pH 6, orange indicates pH 7, and grey indicates pH 8. All experiments were repeated in triplicate

that different pH will lead to a different production of an antibacterial substance that was projected by the inhibition zone. Meanwhile, in the NB salt medium, the pH and NB salt medium variation did not significantly affect the presence of the inhibition zone ($p > 0.10$).

Media composition for antibacterial substance production should consist of carbohydrates, proteins, amino acids, organic nitrogen sources, minerals, and vitamins. Based on the results, culture in NB supplemented with salt exhibited the best antibacterial activity. This medium contains peptone, beef extract, yeast extract, and sodium chloride, which fulfill all the needs for bacteriocin production. Peptone and beef extract serve as sources of carbon, nitrogen, vitamins, and minerals for bacterial growth. Yeast extract provided vitamins of the B-group. Meanwhile, sodium chloride is used to maintain the osmotic pressure of the medium.[14] Addition of magnesium chloride could be correlated with the use of magnesium cation for protein synthesis, as its deficiency might interfere with enzyme synthesis and activity, as well as potassium chloride.[15] Since both belong in the same class, it is possible that the salt had a similar effect on *Geobacillus.* This finding is consistent with Kaunietis *et al*., which evaluated the combination of salt and achieved the best bacteriocin activity produced by *Geobacillu ssp*. when using potassium chloride and magnesium chloride salt. Finally, the activity increases from 40 AU/mL (without the addition of salt) up to 320 AU/mL.[7]

The pH value plays a crucial role in the production of antibacterial substances, as it can affect the enzymes of bacterial cells. In this experiment, NB salt, pH 8 medium exhibited the widest inhibition zone. This result is in line with Avci *et al*., which recorded the best bacteriocin production at pH 8 by *Bacillus sp.*ZBP4 bacteria.[16] Furthermore, the ionic interactions between H and OH ions will make the enzyme more stable to bind to its substrate. It is worth noting that the pH value of the media can also affect the stability of the antibacterial substances secreted out of cells. Finally, any change in pH can modify the charge on the amino acids that make up antibacterial substances, altering the substrate's activity.[17]

CONCLUSIONS AND SUGGESTIONS

The optimization of medium and pH proved pivotal for antibacterial production by G.kaustophilus Tm6T2 (a). Optimal conditions at pH 8, using NB with KCl and $MgCl_2$ resulted in a substantial 13.11 mm inhibition zone against *S.typhimurium*. This study highlights the bacterium's potential as a source of potent antibacterial agents. Further research should focus on characterizing and isolating the antibacterial substance to uncover its attributes and mechanisms. This advancement holds promise for biotechnological applications and combating gastrointestinal pathogens.

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