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RESEARCH ARTICLE

THE INFLUENCE OF pH ON THE ANTIBACTERIAL ACTIVITY OF THERMOPHILIC BACTERIA ISOLATED FROM A MOUNTAIN CRATER IN WEST JAVA AGAINST *Streptococcus mutans*

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Abstract

Thermophilic bacteria that live in hot springs are a potential source of valuable biochemical capable of producing antibacterial regarding its ability to produce antimicrobial peptide (AMP). The study aims to investigate how pH affects the antibacterial activity of a bacterium isolated from a mountain crater in West Java against *Streptococcus mutans*, the predominant bacterium in the creation of oral biofilms. According to Buchanan and Gibbons, samples collected from the water of Mount TangkubanPerahu's crater were identified. The ideal pH and incubation duration were ascertained using a disc diffusion antibacterial test against *S. mutans*ATCC 25175, considering the diameter of the inhibitory zone. The sample was determined to be a Gram-positive bacterium that, at its ideal pH, forms the highest inhibitory zone, indicating antibacterial activity against *S. mutans*. At an optimum pH of 6–8, thermophilic bacteria isolated from a mountain crater in West Java have antibacterial activity against *S. mutans*.

Introduction:-

Discovering microorganisms that can produce antimicrobials in sufficient quantities to replace the available antibiotics that lost their effectiveness due to the multidrug resistance phenomenon is necessary because multidrug-resistant pathogenic microorganisms are becoming a problem and a threat to human health. It is well-recognized that extreme environments support a unique biodiversity of microorganisms, many of which have the potential to produce bioactive chemicals[1]. One sourceofantibacterialcurrentlyofconcerntoresearchersisthermophilicbacteriathat livein hotsprings as a potentialsourceofvaluablebiochemicalscapableofproducingthermostableenzymes, antibiotics, anti-fungi, and anti-cancer. This bacterium is extensively used to discover alternative antibiotics for resistant bacteria caused by the use or excessive use of antibiotics[2].There are two types of thermophilic bacteria: facultative and obligatory thermophilic bacteria. Facultative thermophilic bacteria can thrive at temperatures below 38 °C or in the range of 50 to 66 °C, while obligatory thermophilic bacteria can only survive at temperatures over 50 °C, and several obligatory thermophilic bacteria can proliferate at 77 °C. The compost that breaks down in the soil and the high temperatures it receives allow thermophilic bacteria to flourish under these conditions.[3].

Thermophilicbacteriahavethepotentialforvariousapplications, particularly in theindustrialsector.Becausetheycanproducesecondarymetabolitesthat are morestableathightemperatures, thermophilicbacteriacansurviveathightemperatures (thermostable).These days, the usage of this metabolite is crucial

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for the pharmaceutical and biotechnology industries[3].Thesecondarymetabolitesthey produce are in theformof AMP or bacteriocin, whichisstableathightemperaturesandiseffectiveagainstGram-positiveandGram-negativebacteria[4], sotheycanbeusedtolookforalternativeantibiotics, especiallyforbacteriathat are resistanttoantibioticsduetoabuseoroveruse[5].

Previousresearch has proventhat AMP caninhibitplanktonicbacteriaandbiofilmswhich are closelyrelatedto oral health[6]. Oral biofilm formationbeginswhen a layer ofsalivaryglycoproteincoatsthetoothsurfaceandisthenfollowedbybacterialcolonizationinitiatedby Gram-positivebacteriaof S. mutans[7]. These bacteria produce extracellular polymeric substances (EPS), which facilitate the attachment of other microorganisms and acid-producing bacteria. This raises the virulence of the dental biofilm and leads to tooth caries. Therefore, inhibitingthegrowthandvirulenceof*S. mutans* in biofilmsisvery. Importanttoreduceitscariogenicproperties[6]. The purpose of this study is to evaluate how pH affects the potency of AMP produced by thermophilic bacteria were isolated from Mount in the West Javan region to combat *S. mutans*.

Materials and Methods:-

Samples isolation/collection.

Samples were collected from the 45°C to 76°C hot springs in the mountain crater in West Java, with a pH of 1 to 2. It was incubated at 55°C for 5 to 7 days after being put in a test tube containing 100 mL of liquid Thermus medium. Additionally, the material was continuously purified using the same subculturing procedure on MHA solid medium.

Identification of bacterial morphology

The identification of bacterial morphology was determined according to Buchanan and Gibbons[8]. Pure colonies were isolated, then observed macroscopically by looking at the size, pigmentation, shape, margins, and elevation of the bacteria. Gram stain was used for the microscopic observation of colonies.

The Growth Curve

Preparation of liquid inoculum

The isolates on MHA were cultivated in 50 mL of LB liquid medium, produced in a falcon tube, up to 0.5 McFarland suspension, or 1.5 x 10^8 CFU/ml. For 24 h, bacteria were cultured in a shaker incubator set to 150 rpm and 55°C.

Development of growth curves

A total of 2.22 ml of inoculum was put in 20 ml of liquid MM with variations of pH 6, and pH 8 in different falcon tubes[9].Bacteria were incubated in a shaker incubator at 150 rpm with a temperature of 55°C for 24 hours[10]. The McFarland value on the McFarland densitometer can be used to determine the growth phase of bacteria by measuring it and converting it to CFU/ml. The McFarland value was monitored starting at the zero hour and continued every 2 hours to 24 hours. A growth curve was created using Microsoft Excel software based on the pH.

Optimization of pH for antibacterial activity

A total of 10% (v/v) inoculum of 0.5 McFarland in LB medium was added to 50 mL of MHB+salt liquid medium at pH of 6 and pH of 8 in glass bottles and incubated in a shaker incubator at 150 rpm with a temperature of 55°C until the stationary phase (10 hours). The culture thenwas centrifuged at 10,000 x g for 20 min at 4°C then the supernatant was discarded.5 mL of MHB+ salt was used to suspend the pellet, which had pH variations between 6 and 8. Furthermore, it was kept in a shaker incubator for 4 days at 150 rpm and 55°C, with daily observations conducted.Cultures were obtained every 24 hours and put into microtubes for 20 minutes at 4°C of 10,000g centrifugation. After that, a 0.22 µm filter was used to filter the supernatant, and a disc diffusion antibacterial test against *S. mutans* ATCC25175was performed to determine the optimal pH and incubation period based on the inhibitory zone's largest diameter[11].

Results:-

The bacterialisolateshavedifferentcellandcolonymorphologies. The colonies were foundtobewhitetocream in color, withnosignsofpigmentation. Under a microscope, eachwasseen as a singleorgrouped in shortor long chainsofrods. Gram-variablecultures are thosethat were seentobelightpurple. The bacteria were observedtobeaerobic in culturetubes.

Antimicrobial activity

The results of the thermophilic antibacterial test against *S. mutans*are shown in the **Table 1.** below.

Tabe l1:- InhibitionZoneMeasurementResults (mm).

Isolates	Optimum pH	Inhibitionszonesagainst*S. mutan*(mm)						
		Replicationoffinal log			Replicationoffinal stationary			Average
		1	2	3	1	2	3	
SP1	6	7.44	9.1	6.77	8.92	7.56	0	7.96
SP2		0	7.51	6.96	6.57	6.73	7.02	6.96
SP3		6.63	0	0	0	0	6.38	6.5
SP4		7.88	7.82	7.53	8.14	7.89	7.26	7.75
SP5	8	0	0	0	6.49	0	0	6.49
SP6		0	7.35	0	7	8.4	7.74	7.08

Statistic Analysis

Final Log andFinal StationaryEffectivenessTest

General linear model repeated measure ANOVA was used to compare the effect size between these two treatments to test the effectiveness of final log and final stationary on the inhibition zone. The results test of the effectiveness between Final Log and Final Stationary on the S. mutans bacteria inhibition zone is shown in **Table 2**.

Table 2:- Effectiveness Test Results.

Descrtiptive	Inhibition zones				p value	EffectSize
	I	II	III	Average		
Final Log						
Mean	3.66	5.30	3.54	4.17	0.464	0.112
SD	4.03	4.15	3.89	3.87		
Final Stationary						
Mean	6.19	5.10	4.73	5.65	0.576	0.067
SD	3.18	3.99	3.69	3.16		

Based on the test results, it can be seen that there is no significant difference between the final log inhibition zone for *S. mutans* bacteria in replication I to replication III. This can be seen from the probability value (p-value) of 0.464, where this value is higher than 0.05. Then the final log effect size on the inhibition zone of *S. mutans* bacteria was 11.2%.

The final stationary test results showed that there was no significant difference in the inhibition zone of *S. mutans* bacteria from replication I to replication III. This can be seen from the probability value (p-value) of 0.576, where this value is higher than 0.05. Then the final stationary effect size on the inhibition zone of *S. mutans* bacteria is 6.7%. From these results, it can be concluded that the final log is more effective in the inhibition zone of S. mutans bacteria than in the final stationery.

pH Optimization

ThisanalysisisusedtoseethepHoptimizationoftheinhibitionzoneof*S. mutans*bacteria in the final logs and stationary. The following are theresultsofeffectiveness testing betweenthe final log and final stationaryontheinhibitionzone of *S. mutans*bacteriabasedon optimum pH (**Table 3.**)

Table 3:- Effectiveness Test Results based on Optimum pH.

Descrtiptive	Inhibitions zones				p value	EffectSize
	I	II	III	Average		
pH 6						
Final Log						
Mean	5.49	6.11	5.32	5.64	0.818	0.021
SD	3.69	4.13	3.56	3.46		
Final Stationary						
Mean	5.91	5.55	5.17	5.54	0.845	0.015

SD	4.06	3.73	3.46	3.41		
pH 8						
Final Log						
Mean	0.00	3.68	0.00	1.23	0.500	0.500
SD	0.00	5.20	0.00	3.00		
Final Stationary						
Mean	6.75	4.20	3.87	4.94	0.603	0.341
SD	0.36	5.94	5.47	3.88		

Based on the test results, it can be seen that at pH 6, there is no significant difference in the final log inhibition zone against *S. mutans* bacteria in replication I to replication III. This can be seen from the probability value (p-value) of 0.818, where this value is higher than 0.05. Then the final log effect size on the inhibition zone of*S. mutans* bacteria is 2.1%. Meanwhile, the final stationery has an effect size of 1.5%.

At pH 8, there was no significant difference in the final log inhibition zone against *S. mutans* bacteria from replication I to replication III. This can be seen from the probability value (p-value) of 0.500, where this value is higher than 0.05. Then the final log effect size on the inhibition zone of *S. mutans* bacteria is 50%. Meanwhile, the final stationary data has an effect size of 34.1%. So, the optimum pH greatly influences the effect size of the inhibition zone on *S. mutans* bacteria.

Discusion:-

Thermophilicbacterialspecies are capableofproducingantimicrobialpeptidesor bacteriocin, whichweighbetween 6 - 7.5 kDaandhavegoodantimicrobialactivityatpH 4-10 against Gram-positive*Geobacillussp*, *Staphylococcushaemolyticus* P903, and Gram-negativepathogens. Bacteriocinsproducedby. Thermophilicbacteriahavedifferentcharacteristicsbasedontheirantibacterialspectrum, sensitivitytoenzymes, pH, andmolecularweight[12].AMP is a moleculewithuniqueandverydiverseproperties, producedbyalllivingorganisms, andfunctions as animmunefactorsothattheseorganismscancontinuetheirlife. Thispeptide has goodantimicrobialactivityagainstviruses, bacteria, protozoa, and fungi, whichcanreactquickly. Anotheradvantageof AMP isthatitisnaturallyabletoact as anantimicrobialforbacteriaresistanttoantibioticssuch as methicillinandvancomycin[13], [14].

The researchconductedbySethy&Beheraresulted in the making ofisolatesofthermophilicbacteriafromcoalminingareas. Accordingtotheirfindings, thermophilicbacteria are capableofinhibitingthegrowthof Gram-positive*Staphylococcusaureus*and Gram-negative*Escherichiacoli*. For *E. coli*, themaximalzoneofinhibitionformsbetween 40°C to 50°C atpH 5–7, whilefor*S. aureus*, it formsat 40°C duringthe 24th incubationhour.*E. coli*exhibits a maximalinhibitionzoneat 40°C to 50°C atpH 5–7, and*S. aureus* has a similarzoneat 40°C duringthe 24th incubationhour. The findingsofthis study demonstratethatthermophilicbacteriacanproduceantimicrobialcompoundswithantibacterialactivityagainstboth Gram-positiveand Gram-negativebacteriaandthesecompoundscanwithstandtemperatures as high as 60°C[15].

Severalstudieshavefoundthat AMP productionishighlydependentonpHbecausetheexpressionofbiosyntheticgenesisregulatedbypH. Additionally, pHisknowntoinfluencecationicpeptidestoassociatewiththecellmembraneofproducerstrains.AMP activity will increase continuously during the exponential growth phase, and the highest activity is achieved at the end of this phase. AMP will be produced maximally in the middle or end of the exponential growth phase or the beginning of the stationary phase, but during the stationary phase, production will decrease. Therefore, it is necessary to know the correlation between pH, OD, and activity[16].

In this present study, the antimicrobial test was carried out based on the growth curve to determine the growth phase of bacteria. It consists of log, exponential, stationary, and death phases of bacteria. Growth curves were also used to establish the effect of pH on bacterial growth based on incubation time. The result showed a significant effect of pH on the number of bacteria. This clearly shows that the maximal growth of bacteria is highly dependent on the medium pH.Based on the bacterial growth curve, the isolates used in this study's antibacterial activity test were tested at pH 6 - 8, where there were a lot of bacteria, and the intervals were 8 and 10 hours. This was because those were the beginning and end of the stationary phase, in which bacteria will produce their maximum amount of

antibacterial activity[17]. In thisresearch, AMPsproduced were stronglyinfluencedbypH, especiallyatpH6 - 8, resulting in fastcellgrowth, whichaffectsthehighestbacteriocinproduction.

Conclusion:-

The antibacterial activity of thermophilic bacteria isolated from a mountain crater in West Java against *S. mutans* is influenced by pH, at an optimum pH of 6-8.

Conflicts of interest

No financialorotherconflictsofinterest in this study.

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