BIODIVERSITAS
Volume 21, Number 6, June 2020
Pages: 2827-2831

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d210660

Antibacterial activity of CaO from blood cockle shells (*Anadara granosa*) calcination against *Escherichia coli*

AMANDA FIRZA RUSDARYANTI, ULFAH AMALIA♥, SLAMET SUHARTO
Department of Fish Products Technology, Faculty of Fisheries and Marine Science, Universitas Diponegoro. Jl. Prof. Soedarto, SH, Tembalang, Semarang 50275, Central Java, Indonesia. Tel.: +62-24-7474698, Fax.: +62-24-7474698, ♥email: ulfah.amalia@live.undip.ac.id

Manuscript received: 17 March 2020. Revision accepted: 29 May 2020.

Abstract. *Rusdaryanti AF, Amalia U, Suharto S. 2020. Antibacterial activity of CaO from blood cockle shells (*Anadara granosa*) calcination against* Escherichia coli. *Biodiversitas 21: 2827-2831.* Calcium carbonate ($CaCO_3$) in blood clamp shells (BCS) was able to be converted into calcium oxide (CaO) through a calcination process. Some research stated that CaO can be used in the food industries b, one of which is as an antibacterial agent. The purpose of this study was to determine the most optimal size of BCS's powder during calcination as an antibacterial agent and its effect on the activity of *Escherichia coli*. Data were analyzed using analysis of variance and Kruskal-Wallis test. The results showed that 200 mesh size of BCS's powder produced the highest yield of CaO at about 98.68% compared to 120 and 230 mesh size. The best concentration of CaO powder as an antibacterial was 3.5% with a pH of 11.3 ± 0.17. CaO powder had antibacterial activity against *E. coli* with minimum inhibitory concentration's value (MIC) of-0.115; a minimum bactericidal concentration's value (MBC) of 0 CFU/mL; inhibition zone of 3.23 ± 0.2 mm. By the PCR method, DNA degradation has occurred in *E. coli* cells. The differences of CaO powder concentration had significant effect ($P < 0.05$) on the inhibition zone of *E. coli.*

Keywords: Antibacterial, calcination, CaO, shells

INTRODUCTION

Blood cockle shells (BCS) are one of the export commodities that have a high economic value in the fisheries sector for a country's economy. The total export volume of fishery products in 2018 was supported by one of the export commodities of captured fisheries products, which are 3154.8 tons of shells or coral products, with a value of US$ 5169.1 (Ministry of Fisheries 2018).

Blood cockle shells belong to fisheries waste and often cause unpleasant off-odors (Saharudin et al. 2017). Generally, BCS used as raw material for souvenirs and lime betel making (Afranita et al. 2014). Previous research stated that calcium in blood cockle shells could also increase the value of food nutrition (Agustina et al. 2011). Using Atomic Absorption Spectrophotometry analysis, Zikri et. al. (2015) found that calcium carbonate ($CaCO_3$) in BCS was 76.6%.

At the temperature of approximately 700°C, $CaCO_3$ could convert into calcium oxide which has antibacterial activity (Hou et al. 2016); the heat treatment used the calcination method. The process of changing the calcium carbonate component required a container with a low risk of contamination, because in the calcination process, the addition of acid was not carried out and the sample did not need to be monitored. However, the risk still existed when the substances evaporated and condensed during cooling (Herrman 1970). The activity of antibacterial substances could be bactericidal or bacteriostatic, and inhibit the germination of bacterial spores (Sartika et al. 2013). Calcium oxide, including in the form of nanoparticles, having antibacterial activity, requires low costs in the making process, and is easily accessible and biocompatible in its application (Aziz and Yousef 2017).

Li et al. (2014) explained that the content of CaO compounds is universally recognized as an antibacterial agent with a relatively higher pH, i.e. more than 12. These nanoparticles acquire bactericidal properties through the generation of reactive oxygen species that are able to target physical structures, metabolic pathways, and prokaryotic cell DNA synthesis which causes cell death (Gold et al. 2018). Metal-based nanoparticles can interfere with the potential and integrity of cell membranes by electrostatically binding to bacterial cell walls and or releasing metal ions (Beyth et al. 2015). The purpose of this study was to determine the most optimal size of powder BCS in the calcination process as an antibacterial agent, and their effect on the activity of *Escherichia coli*.

MATERIALS AND METHODS

Sample preparation

The shells used in the making of CaO powder are blood cockle shells (BCS) of *Anadara granosa* which were obtained from Rejomulyo traditional market in Semarang, Central Java. The cockle shells were washed and cleaned using a brush to remove dirt attached to the surface of the shell, then soaked in distilled water for 1 h and dried using oven at 100°C for 1 h (Mohamed et al. 2014). The shells were crushed and sieved to obtain 120 mesh, 200 mesh, and 230 mesh powder sizes, and finally were processed in a furnace at 900°C for 2 h.

Characterization of CaO powder

The chemical composition of CaO powder from calcination was analyzed using X-ray Fluorescence Spectrometry. The results of qualitative and quantitative analysis provided information on the elemental content expressed in units of counts per second (cps) and converted to units of weight percentages or parts per million (ppm) (Masrukan et al. 2007).

pH of CaO powder

The hydrogen potential of the CaO powder solution was measured using a pH meter. One gram of CaO powder was added to 10 mL of phosphate buffer solution (pH 5.8-7.4) (Chen et al. 2015).

Antibacterial activity

The antibacterial activity test was performed using different CaO powder concentrations, 0.5%; 1.25%; 2%; 2.75%; and 3.5%. The antibacterial activity of CaO powder from BCS against *Escherichia coli (E. coli)* was measured based on Minimum Inhibitory Concentration (MIC), as follows: several colonies were picked from a fresh (18 to 24 h) non-selective agar plate to broth containing 0.9% NaCl. In this study, *E. coli* used as positive control, followed by the other samples were *E. coli* added with some concentrations of CaO powder as stated above, respectively. After inoculation, the final step was incubation for 24 h at 37°C using shaker incubator. Qualitative analysis of antibacterial activity was determined by observing changes in the conditions of the samples which were interpreted based on the rating scale (1+ = very light haze, 2+ = light haze, 3+ to 4+ = heavy turbidity), whereas, the quantitative analysis was determined by measuring the absorbance value of the sample using a spectrophotometer with a wavelength (λ) of 600 nm. For Minimum Bactericidal Concentration (MBC), it used a ready-made medium, added with 1 mL of the MIC's test results. It was flattened using a spreader and incubation was done for 24 h at 37°C. Furthermore, the number of colonies and disk diffusion was analyzed using disc paper. The results were derived from the formation of clear zones around the disk paper which were then measured using a caliper (Henry 2007).

Polymerase chain reaction method to detect bacteria

Polymerase Chain Reaction (PCR) method in this study refers to Fariba et.al. (2016), and it was used to detect and analyze the degradation of bacterial DNA. The bacterial DNA extraction was carried out by transferring a 400 µL sample solution of each treatment into a 1.5 mL microtube using Chelex method. Amplification was carried out using 16rRNA bacterial universal primers (27F and 1492R) with the MyTaq Red Mix protocol, followed by electrophoresis.

Statistical analysis

Statistical analysis of variations between concentration samples was compared using one-way ANOVA, followed by Tukey's post hoc test. Significance of differences was defined at $p < 0.05$. Statistical analyses were performed using SPSS 24 software with three replications.

RESULTS AND DISCUSSION

Characteristics of blood cockle shell powder

It was obtained that the CaO content of blood cockle shells (BCS) powder with different particle sizes was 98.61% to 98.68%, and the optimal particle size of CaO powder was 200 mesh (Table 1). It was suggested that antibacterial activity increases with the increase of particle size and antibacterial content. This result was in line with previous research done by Narayanan et. al. (2012) showing that the antibacterial activity against human pathogen (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa*) increased with increasing concentration of the zinc oxide nanoparticles.

pH of CaO Powder

The pH values of CaO powders with different concentrations are presented in (Figure 1).

The degree of acidity (pH) of CaO powder solution increased by 11.43 ± 0.05 to 11.73 ± 0.05 at 0 h, and then decreased at 4 h and 8 h until it became stable at 11.3 ± 0.03 at 12 h for different CaO concentrations (Figure 1). Calcination at high temperatures was used to decompose the chalk. These products include basic alkaline products with a pH> 12 and are usually in the form of powder or granules (Suleiman et al. 2013).

The reaction mechanism that occurred in CaO powder with the addition of water was: $CaO + H_2O = Ca(OH)_2 = Ca^{2+} + 2OH^-$. CaO hydration caused an alkaline effect which resulted in the mechanism of antibacterial activity in a calcined powder solution. Reactive oxygen produced from CaO powder can destroy bacterial cells. The nature of CaO powder with a high affinity for water that reacts with humidity can form $Ca(OH)_2$ which changes the biological properties of bacterial cell walls, deactivates membrane transport, and damages the bacterial cell nucleus. According to Sawai (2011), the alkaline effect caused by the hydration of CaO is considered as one of the main mechanisms of bactericidal action.

Antibacterial activity

The antibacterial activity of CaO powder with different concentrations against *Escherichia coli* is presented in Table 2 (qualitative data's) and Table 3 (quantitative data's).

Table 1. Chemical composition of blood cockle shell powder

Element	Content (%) in various sizes		
	120 mesh	200 mesh	230 mesh
CaO	98.63 %	98.68%	98.61%
SrO	0.30%	0.29%	0.28%
K_2O	0.29%	0.30%	0.24%
Cl	0.25%	0.23%	0.24%
P_2O_5	0.23%	0.16%	0.25%
SO_3	0.20%	0.24%	0.27%
Fe_2O_3	0.03%	0.04%	0.05%
SnO_2	0.02%	0.01%	0.02%
ZnO	-	-	0.01%
Ta_2O_5	-	0.01%	-

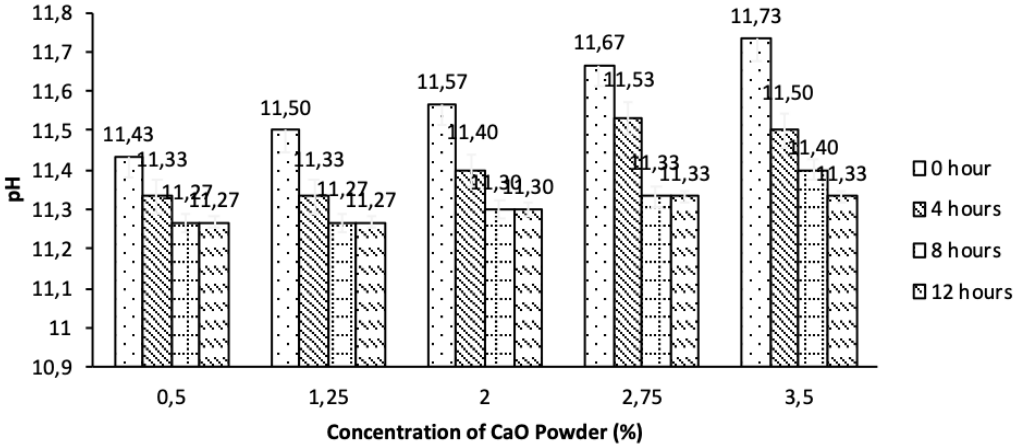

Figure 1. pH value of CaO powder solution with different concentration and time

Table 2. Qualitative Minimum Inhibitory Concentration (MIC) of CaO powder

Indicator of bacteria presence	*E. coli* with CaO powder in different concentration (%)					*E. coli* as a positive control
	0.5	1.25	2	2.75	3.5	
Very light haze			+	++	++	
Light haze		+++				
Heavy turbidity	+++					++++

Note: + and ++: Inhibition occurred, +++ and ++++: No inhibition

Table 3. Quantitative Minimum Inhibitory Concentration absorbance value of CaO powder

Concentration of CaO powder (%)	Before incubation	After incubation	ΔOD
0 (*Escherichia coli*)	0.23	0.16	0.12
0.5	0.15	0.03	0.073
1.25	0.20	0.16	0.071
2	0.27	0.36	-0.029
2.75	0.13	0.25	-0.091
3.5	0.11	0.19	-0.115

Table 4. Minimum Bactericidal Concentration of CaO powder

Concentration of CaO powder (%)	Number of colonies (CFU/mL)
0.5	254 ± 59.74
1.25	67 ± 2.00
2	1.66 ± 0.44
2.75	2.3 ± 0.58
3.5	0 ± 0.00

Note: Data on antibacterial activity are interpreted by an average ± standard deviation of 3 replications

The results of qualitative MIC showed that there was no inhibition on 0.5 and 1.25% of CaO powder against the growth of *Escherichia coli*. Based on Table 2, it could be seen that culture media of *E. coli* with 2% of CaO powder resulted in very light haze, which means no turbidity. The situation of no turbidity indicated that there was no bacterial growth. The same condition also applied to culture media of *E. coli* with 2.75 and 3.5% of CaO powder. This study is in line with Suparno et. al. (2018) who stated that the lowest concentration of a substance that has the power to inhibit the growth of microorganisms is indicated by the absence of turbidity in the test tube that has been incubated for 24 h at 37°C.

The highest decrease in absorbance value, which was-0,115, was seen in 3.5% CaO powder concentration indicating a low turbidity level. The absorbance value will be higher if turbidity is also increasing because the turbidity indicates the growth of *E. coli* in the growing media (Table 3). This study is in line with Warokka et al. 2016) who stated that the decrease of the absorbance value can inhibit bacterial growth. Higher concentration of the extract lowers the bacterial growth activity because of the greater antibacterial content of the extract.

The growth of bacterial colonies was not found in CaO powder with a concentration of 3.5% and maximum control, but in CaO powder with concentration of 2% and 2.75%, there was a growth of bacterial colonies of 1.66 CFU/mL and 2.3 CFU/mL after incubation process. This shows that the concentration of 2% CaO powder is the lowest concentration that can inhibit the growth of *Escherichia coli* bacteria (Table 4). This study is in line with (Balouiri et al. 2016) who stated that the bactericide endpoint has been subjectively defined as the lowest concentration, where 99.9% of the final inoculum is killed. This is confirmed by (Julianti et al. 2017)] who stated the Minimum Bactericidal Concentration is obtained after

determining the MBC. The MBC is the smallest amount of drug needed to kill 99.9% of bacteria. The MBC of the test sample is determined by inoculating a sample from a plate that does not show bacterial growth.

It can be seen that the inhibition zone formed on the CaO antibacterial powder with different concentrations of *Escherichia coli* bacteria is 0.56 mm to 3.23 mm as shown in Figure 2. Greater use of CaO powder concentration results in wider diameter of the inhibitory zone, because the amount of active ingredient will also be higher (Table 5). This study is in line with (Dizaj et al. 2014) who stated that the antimicrobial activity of metal nanoparticles and metal oxides shows that particle size and concentration are important parameters that determine the antimicrobial effectiveness of metal nanoparticles. According to Susanto (2012), the broad categorization of inhibition zones consists of very strong, strong, moderate, and weak. The inhibition zone area of 6-10 mm is categorized as moderate, while inhibition zone smaller or equal to 5 mm is categorized as weak.

Detection of *Escherichia coli* by addition of CaO powder using the *Polymerase Chain Reaction* method

The purity of *E. coli* 's DNA in this study presented in Table 6. DNA concentrations of *E. coli* after purification had the values about 5.9 µg/µl to 52.4 µg/µl. These results were influenced by the presence of CaO powder in this study's treatments, as it was a material that can affect or contaminate the results. On the other hand, as we saw in Table 6, the purity of *E.* coli's DNA was 1.80 to 1.86, and it was good indicator of DNA extraction process. This study was in line with Mostafa (2015), who stated that the presence of other substances affects the results of the concentration of isolated DNA, whether in the form of proteins or other materials. According to Desjardins and Conklin (2010), the success of DNA isolation is demonstrated by the concentration and purity of obtained DNA. Pure nucleic acids produce a ratio of 260/280 ~ 1.8 and ~ 2.0 for DNA and RNA.

Overall, visualization of samples treated with CaO powder by PCR resulted in clear band. The visualization of line 3 to 6 showed different locations of band compared to line 1 (*E. coli* as a positive control). This phenomenon was caused by the presence of CaO powder which probably damaged *E. coli* 's cells. In Figure 3, it can be seen that the band appeared in line 2 to line 5 tended to be similar and very much like to line 1, and it meant that the concentration of CaO powder from 0.5 to 2.75% could inhibit *E. coli* 's activities. This study is in line with Alizadeh et. al. (2018) who stated that silver nanoparticles not only have an inhibitory effect on bacteria by increasing the concentration of antibacterial activity, but they also affect the bacterial genome DNA sequence and change them into other bands.

Table 5. CaO Powder Activity in Inhibiting the Growth of *E. coli* through Disk Diffusion Method

Concentration of CaO powder (%)	Inhibition zone diameter (mm)
0.5	0.56±0.30 [a]
1.25	1.36±0.32 [ab]
2	2.1±0.3 [bc]
2.75	2.5±0.36 [cd]
3.5	3.23±0.20 [d]
Without CaO	10.60±0.65[e]

Note: Data on antibacterial activity are interpreted by an average ± standard deviation of 3 replications; Different superscript letters show significantly different results based on Tukey's post hoc test (P <0.05).

Table 6. Purity of *E. coli* 's DNA in samples treated

Sample	Abs 260	Abs 280	Abs 260/ Abs 280	DNA conc. (µg/µl)
E. coli	0.42	0.225	1.86	21
E. coli + 0.5% of CaO powder	0.908	0.501	1.81	45.4
E. coli + 1.25% of CaO powder	0.801	0.443	1.80	40
E. coli + 2% of CaO powder	1.049	0.579	1.81	52.4
E. coli + 2.75% of CaO powder	0.13	0.07	1.85	5.9
E. coli + 3.5% of CaO powder	0.434	0.241	1.80	19.2

Figure 2. The inhibition zone formed on the CaO antibacterial powder with different concentrations of *Escherichia coli* bacteria

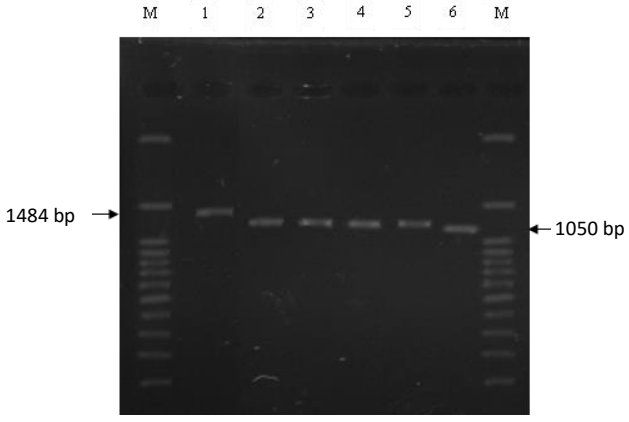

Figure 3. Visualization of *E. coli's* DNA with CaO's powder at different concentrations. Line M: Marker; Line 1: *E. coli* (positive control) at 1484 bp; Line 2-6: samples of *E. coli*'s culture media with CaO powder at different concentration: 0.5, 1.25, 2, 2.75, and 3.5%, respectively.

In conclusion, blood cockle shells (BCS) can be utilized as an antibacterial agent through calcination process. This study concludes that 200 mesh sizes of BCS resulted in the highest yield of calcium oxide (CaO) by about 98.68%. The different concentration of CaO powder as an antibacterial agent has a different effect on the activity of *Escherichia coli*. CaO with 3.5% concentration has the following features: pH of 11.3 ± 0.17 which can inhibit *E. coli* with a Minimum Inhibitory Concentration (MIC) absorbance value of -0.115; Minimum Bactericidal Concentration (MBC) value of 0 CFU/mL; and inhibition zones of 3.23 ± 0.2 mm. E. coli 's DNA or cell degradation can be detected by using Polymerase Chain Reaction.

ACKNOWLEDGEMENTS

We would like to extend our appreciation and thanks to Sumardianto and Romadhon, for their contributions in the form of suggestions and criticisms for this article.

REFERENCES

Afranita G, Anita S, Hanifah TA. 2014. Potential of ash shells (*Anadara Granosa*) as white tin ion adsorbent. J Online Mahasiswa 1 (1): 1-5. [Indonesian]

Agustina TW, Fahmi AS, Widowati I, Sarwono A. 2011. Utilization of scallop shell waste (*Amusium pleuronctes*) in making calcium rich cookies. J Indon Fish Prod Process 24 (1): 8-13.

Alizadeh V, Eimani BG, Amjady F. 2018. Genomic effect of silver nanoparticles in *Staphylococcus aureus* bacteria. J Water Environ Nanotechnol 3 (1): 51-57.

Aziz DMAEl, Yousef NMH. 2017. Antimicrobial effects of calcium oxide nanoparticles and some spices in minced meat. ARC J Anim Vet Sci 3 (2): 38 45.

Balouiri M, Sadiki M, Ibnsouda SK. 2016. Methods for in vitro evaluating antimicrobial activity: a review. J Pharm Anal 6: 71-79.

Beyth N, Haddad YH, Domb A, Khan W, Hazan R. 2015. Alternative antimicrobial approach: nano-antimicrobial materials. Evid-Based Compl Altern Med 2015:1-6.

Chen YC, Lin CL, Li CT, Hwang DF. 2015. Structural transformation of oyster, hard clam, and sea urchin shells after calcination and their antibacterial activity against foodborne microorganisms. Fish Sci 1-8.

Desjardins P, Conklin D. 2010. Nanodrop microvolume quantitation of nucleic acids. J Visual Exp 45: 1-4.

Dizaj SM, Lotfipour F, Jalali MB, Zarrintan MH, Adibkia K. 2014. Antimicrobial activity of the metals and metal oxide nanoparticles. Mater Sci Eng C 44: 278-284.

Fariba A, Bahram GE, Farrokh K, Tabrizy S, Pooneh SS. 2016. An investigation of the effect of copper oxide and silver nanoparticles on *E. coli* genome by RAPD molecular markers. Adv Biotech Micro 1 (2): 1-6.

Gold K, Slay B, Knackstedt M, Gaharwar AK. 2018. Antimicrobial activity of metal and metal-oxide based nanoparticles. Adv Therap 1: 1-15.

Henry DI. 2007. Clinical Microbiology Procedures Handbook. American Society of Microbiology. Washington DC:

Herrman R. 1970. The Application of Flame Photometry in Biology and Medicine. Analytical Flame Spectroscopy. Macmillan Education, UK.

Hou Y, Shavandi A, Carne A, Bekhit AA, Ng TB, Cheung RCF, Bekhit AEA. 2016. Marine shells: potential opportunities for extraction of functional and health-promoting materials. Crit Rev Environ Sci Technol 46 (11-12): 1047-1116.

Julianti E, Rajah KK, Fidrianny I. 2017. Antibacterial activity of ethanolic extract of cinnamon bark, honey, and their combination effects against acne-causing bacteria. Sci Pharm 85 (19): 1-8.

Li M, Yao ZT, Chena T, Lou ZH, Xia M. 2014. The antibacterial activity and mechanism of mussel shell waste-derived material. Powder Technol 264: 577-582.

Masrukan, Rosika, Aggraini D, Kisworo J. 2007. Comparative analysis of AlMgSI1 alloys composition using *X*-ray Fluorescence (XRF) and Emission Spectroscopy. Prosiding PPI-PDIPTN 2007 Pustek Akselerator dan Proses Bahan-BATAN Yogyakarta, 10 Juli 2007. [Indonesian]

Ministry of Fisheries. 2018. East Java Fishery Capture Statistics. Ministry of Fisheries, Jakarta. [Indonesian]

Mohamed M, Yusup S, Maitra S. 2012. Decomposition study of calcium carbonate in cockle shell. J Eng Sci Technol 7 (1): 1-10

Mostafa AA. 2015. Antibacterial activity of zinc oxide nanoparticles against toxigenic *Bacillus cereus* and *Staphylococcus aureus* isolated from some Egyptian food. Intl J Microbiol Res 6 (2): 145-154.

Narayanan PM, Wilson WS, Abraham AT, Sevanan M. 2012. Synthesis, characterization, and antimicrobial activity of zinc oxide nanoparticles against human pathogens. Bio Nano Sci 2: 329-335.

Saharudin SH, Shariffuddin JH, Nordin NIAA. 2017. Biocomposites from (*Anadara granosa*) shells waste for bone material applications. IOP Conf Ser Mater Sci Eng 257: 012061. DOI: 10.1088/1757-899X/257/1/012061.

Sartika R, Melki, Purwiyanto AIS. 2013. Antibacterial activity of *Eucheuma cottoni* seaweed extract against bacteria *Escherichia coli, Staphylococcus aureus*, *Vibrio cholera*, and *Salmonella typhosa*. J Maspari 5 (2): 98-103.

Sawai J. 2011. Antimicrobial characteristics of heated scallop shell powder and its application. Biocontrol Sci 16 (3): 95-102.

Suleiman IE, Abubakar A, Otuoze HS, Suleiman MA, Momoh RO, Aliyu SN. 2013. Effects of particle size distribution on the burn ability of limestone. Leonardo Electr J Pract Technol 23: 115-130.

Suparno O, Panandita T, Afifah A, Marimin, Purnawati R. 2018. Antibacterial activities of leave extracts as bactericides for soaking of skin or hide. IOP Conf Ser Earth Environ Sci 141: 012028. DOI: 10.1088/1755-1315/141/1/012028.

Susanto, Sudrajat, Ruga. 2012. Study of the content of active ingredients of plants red devices (*Shorea leprosula* Miq.) as a source of antibacterial compounds. J Health 11 (2): 1-15.

Warokka KE, Wuisan J, Juliatri. 2016. Minimum Inhibitory Concentration Test (MIC) of binahong leaf extract (*Anredera cordifolia Steenis*) as an Antibacterial against *Streptococcus mutants* growth. J e-GiGi 4 (2): 155-159.

Zikri A, Zultiniar-Amri A, Yelmida. 2015. Synthesis of Precipitated Calcium Carbonate (PCC) from blood shells (*Anadara granosa*) with variation in acid type and carbonation time. J Eng 2 (2): 1-6.