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Bacterial Colloids Silver from Slurry of Silver Craft Industry and Its Activity as an Antibacteria

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Abstract: Colloidal silver is a mineral with a very small size (1-100 nm), accumulated in the form of mucus material on liquid waste from silver craft industry. Several species of bacteria from slurry of silver craft industry waste were tolerant and able to grow on the form of colloids. Bacterial colloids silver was formed concomitant with bacterial growth specific characters of bacterial resistance upon Ag^+ ion. Colloidal silver is the most potential antiseptic and antimicrobial agent against viruses, bacteria, fungi and pathogenic microorganisms. The purpose of the study was to obtain resistant bacteria which synthesize colloids nano silver; and to elucidate their antibacterial activity against bacterial pathogen. The research was commenced with selection of silver tolerant bacteria from slurry of silver craft industry waste based on their growth ability on different $AgNO_3$ concentration. Formation of bacterial colloids silver was observed through growth experiments using selected isolate grown on *Triptone Yeast Extract* (TYE) broth containing 0.1 mg/L $AgNO_3$. The bacterial colloids silver formed was detected using laser. Results revealed that only one strain (BAgAK 6) grew on medium containing 5 mg/L $AgNO_3$, and formed colloid mass. These colloids demonstrated as red line after exposed to laser beam. The bacterial colloids silver of BAgAK 6 inhibiting the growth of *Escherichia coli* FNCC 0091 and *Staphylococcus aureus* FNCC 0049 with inhibition index of 7.1 and 2.4, respectively. Conclusion of the research were strain BAgAK 6 resembled to *Bacillus* sp., resistant to a high concentration of Ag^+ was able to form bacterial colloids silver, as antibacterial agent against to both gram negative and gram positive bacteria.

Keywords: resistant, silver, bacterial colloids silver, antibacterial, *Bacillus* sp.,

Introduction

Generally, liquid waste from the silver craft industry contained a number of heavy metals (Ag, Cu, Zn and Cr) (Giyatmi *et al*., 2008), and also microbes growing floating on the surface area produced mucus layer, called colloids. Colloidal silver is a mineral form of microbial growth in industry liquid waste silver, especially bacteria (Hidayati, 2015; Xiu *et al*., 2014). The Colloids was character of microbial colonies resistant to ion Ag. The colloids was assumed as colloidal silver (Soetarto *et al*., 2012). Colloidal silver is a complex material consist of inorganic materials or minerals (Ag, Cu and Cr) with very small size (1-100 nm) and organic materials, including bacteria (Jeevan *et al*., 2012). These anorganic materials or minerals, especially ion Ag, accumulated as mucus from results of bacterial activity in the silver craft industry liquid waste. The mucus shows typical resistance to silver ions (Ag^+). In general, the levels of Ag in the silver craft industry (from silver plating) approximately 0.052 mg/L (Giyatmi *et al*., 2008, Soetarto *et al*., 2012), still below for maximum threshold value in the environment (0.1 mg/L) (Governor Roles of the Yogyakarta Special Region No. 281/KPTS/1998).

Most of the liquid waste from the silver craft industry has not been treated before released directly into the environment or dumped into a reservoir wells (Soetarto *et al*., 2012). These liquid waste could causes environmental pollution (Soetarto, 1989; Giyatmi *et al*., 2008). The various efforts to reduce the toxicity of pollutants and environmental pollution were carried out through physically, chemically and biologically treatment. It has been known, silver possesses antibacterial activity and has been used clinically for treatment of ulcers and skin wound (Shrivastava *et al*., 2007). Recently, interest in silver motivates to emerge nano silver to overcome the increasing prevalence of bacterial infections. Generally silver use has been limited to

humans caused silver ions toxicity; however, nanotechnology has facilitated the production of finer silver particles with large surface area-to-volume ratios, greater efficacy against pathogenic bacteria (Guzman *et al*., 2009); the most important is to lower toxicity to humans (Rai & Bai, 2011). The purpose of this study was to obtain bacteria resistant to ion Ag and grow that shape the slime as colloidal silver, as well as analyze the antibacterial activity against several types of pathogenic bacteria.

Materials And Methods

Bacterial isolates media and selection

Three pure cultures of bacterial isolates (strain BAgAK6; BAgSK1-2 and BAgBK1-1) were silver tolerant bacteria, obtained from previous research (Hidayanti, 2014; Soetarto *et al*., 2012); cultivated on TYEG (tryptone 1 g/L, yeast extract 3 g/L and glucose 1 g/L; pH 7.0) agar medium at 37°C for 24 h. Colonies grown separately was picked up and transferred on TYE agar slant medium to be use as pure cultures for further investigation. The cell morphology, colony appearance, spore formation and pigmentation were recorded. Pure culture of *Escherichia coli* FNCC 0091 and *Staphylococcus aureus* FNCC 0049 used as testing bacterial models.

The property of isolates as silver tolerant bacteria was proved by growing them on different concentrations (0, 1, 2, 3, 4, and 5 mg/L) of $AgNO_3$. All cultures were selectively expanded by subculturing twice as enrichment cultures. Each time transferring at 1% (v/v) cell cultures to the same fresh medium including the 0.1 mg/L $AgNO_3$, were incubated under the same conditions. The resulting potential silver nitrate resistant enriched culture was detected by producing colloids and then exposed by laser beam. The red line produced through colloids in bacterial isolates culture indicated colloidal silver formed. The red line in isolate culture was selected. The selected isolates were purified using single cell colony technique on TYE agar medium containing 0.1 mg/L $AgNO_3$.

Experiments of formation of bacterial filament on medium containing silver

Formation of bacterial filament was carried out through bacterial growth experiments with three replicates and one control, at 100 mL TYE liquid medium containing 0.1 mg/L $AgNO_3$. All cultures were incubated at 37°C for 24 h or until mucus substances appeared on surface of medium.

Synthesis of bacterial colloids silver was detected using laser beam (Harsojo, 2015, personal communication) and the colloidal silver was determined spectrophotometrically UV-VIS ($A_{200\text{-}600\ nm}$) (Shrivastava *et al*., 2007). The high peak showed by the bacterial culture of about $A_{400\ nm}$ was determined as colloidal nano silver formed by the isolates, and would be used for further experiments.

Determination of antibacterial activity test of bacterial colloids silver

Antibacterial activity was measured with disc diffusion method Kirby Bauwer (Guzmán *et al*., 2009) using filter paper Whatman 42 (paperdisk). Firstly, selected bacterial isolates were grown on TYE liquid medium containing 0.1 mg/L $AgNO_3$, incubated at 37°C for 24 h or until growth occurs (at $A_{600\ nm}$ ~ 0.8 or equivalent with biomass ~ 10^7 cfu/mL) as antibacterial agent. Two pure cultures of testing bacteria (*E. coli* FNCC 0091 and *S. aureus* FNCC 0049) were grown on Muller-Hinton agar medium (Jevan *et al*., 2012) with a pour plate method, sterile paperdisk dipped in the bacterial colloids silver culture was put on the middle of those bacterial culture surface, incubated at 37°C for 24 h. In order to determined strength of antibacterial activity, the nanofiber material containing 5% and 10% $AgNO_3$ and three antibiotic solution (ampicillin, chloramphenicol, and penicillin-streptomycin) were used as a positive control. The antibacterial activity was demonstrated by clear zone produced surround paperdisk.

Bacterial identification methods

The potentially silver resistant bacteria isolate was cultivated on TYEG (tryptone 1 g/l, yeast extract 3 g/l and glucose 1 g/l; pH 7.0) agar medium at 37°C for 24 h. The colony appearance, cell morphology, spore formation and pigmentation were recorded.

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Catalase, hydrolysis of casein, hydrolysis of gelatin, hydrolysis of starch, MR-VP, indole test, nitrate reduction, Simmon citrate test, hydrogen sulfide production, and utilisation of carbohydrates and oxidation or fermentation of glucose were performed as described by Holtz (1994).

Results And Discussion

Bacterial isolates ability to grow on liquid medium

Three isolates strains (BAgAK6; BAgSK1-2 and BAgBK1-1) tolerant to Ag^+ revealed varies ability to grow on the liquid medium. Strain of BAgSK1-2 and BAgBK1-1 grew well on medium containing 0 to 2 mg/L $AgNO_3$ concentrations, only strain BAgAK6 grew on medium containing a maximum concentration of 5 mg/L $AgNO_3$ (Table 1).

Table 1. The capability grow of isolates strains on TYE liquid medium with various concentration of $AgNO_3$ (mg/L)

No.	Isolates strains	Growth of isolates strains on TYE liquid medium with various concentration of $AgNO_3$ (mg/L)					
		0	1	2	3	4	5
1.	BAgAK6	++	++	++	+	+	+
2.	BAgSK1-2	++	+	+	-	-	-
3.	BAgBK1-1	++	+	+	-	-	-

Note:
+ = the isolates strains capable to grow
++ = the isolates strains capable to grow well
- = no grow of the isolates strains

Table 1 showed that three strains of isolates were silver tolerant bacteria, while strain BAgAK6 was silver resistant bacterium capable grown up to a concentration of 5 mg/L $AgNO_3$.. Silver as a toxic metal inhibited the growth of bacteria. This metal reacts with protein having thiol group, that lead to an activation of protein (Lehninger *et al.*, 1993). the interaction of Ag^+ with thiol group makes bacteria inactive (Liau *et al.*, 1997).

Formation of bacterial filament on medium containing silver

Biomass grown produced mucus substances seemed like colloids (Fig. 1). The colloids was assumed as colloidal particles silver because this colloids showed red line after exposed to laser beam and the highest peak spectrofotometrycally of A390 nm (Fig. 2). The colloidal particles silver was also synthesized by bacteria culture on liquid medium containing 0.1 mg/L AgNO3 (Jevan et al., 2012; Nair and Panda, 2012). Based on red line appeared on liquid culture, strain BAgAK6 was determined as a silver resistant bacterium.

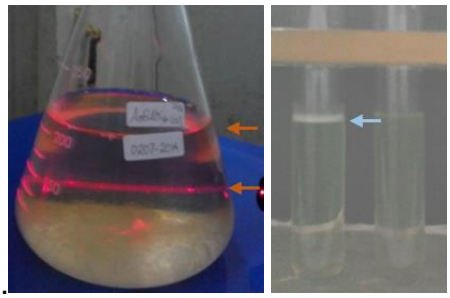

Figure 1. Strain BAgAK6 culture grown on TYE liquid medium after 24 h incubation. Red line appeared (←) after exposed laser beam as colloidal particles silver (a), mucus layer formed on the surface of liquid medium (←) (b).

The appearances of red line was caused Tyndall effect (Harsojo, 2015, private communication). The Tyndall effect used to determined colloids form from a liquid culture bacteria. The red line caused by scattering light of colloidal particle after exposed to laser beam.

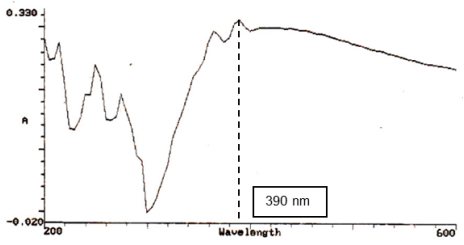

Figure 2. Absorption spectrum of colloidal silver from isolate strain BAgAK 6 culture

The size, morphology and interaction between strain BAgAK6 and colloidal silver are illustrated in the Trasmission Electron Microphotographs (TEM) (Figure 3).

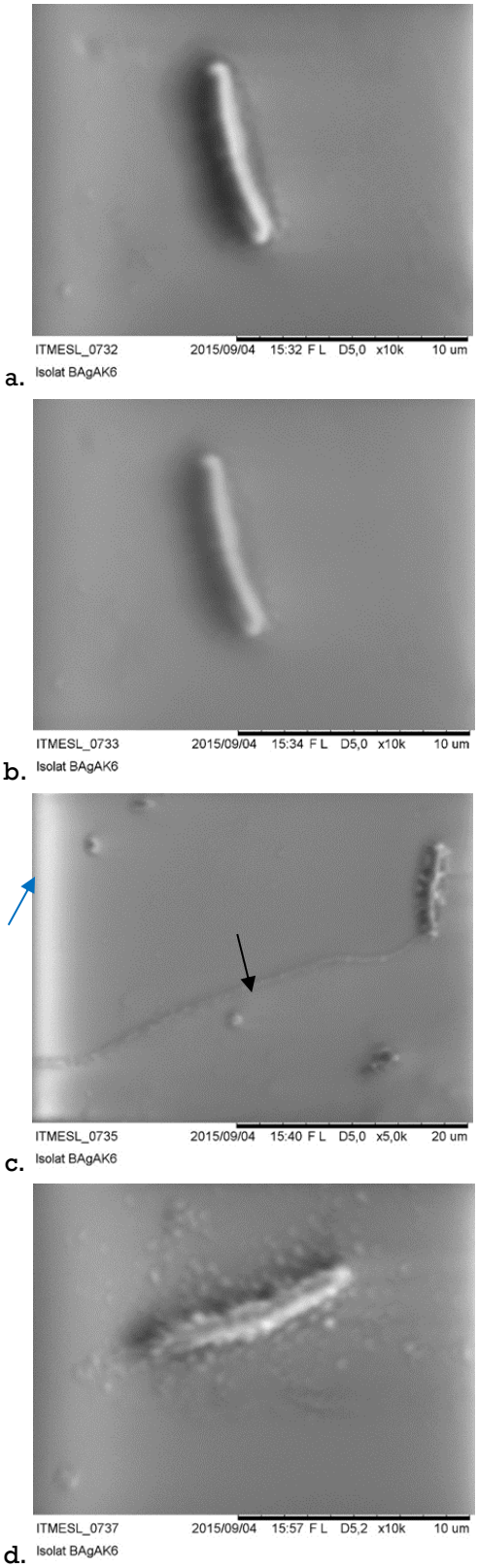

Figure 3. TEM microphotographs of strain BAgAK6 cell: cell morphology from BAgAK6 culture witout $AgNO_3$ (a,b) cell with flagel and surrouded particle silver (c) cell accumulated particle $AgNO_3$ (d)

Fig. 3 illustrated the interaction between strain BAgAK6 and silver particle on liquid medium after 72 h. In normal condition, the bacterium culture without $AgNO_3$ perfomed as basillus rod-shaped with a smoth appearances (Fig. 3a,3b), while those bacterium culture with $AgNO_3$ perfomed of cell changed with the a long flagel ( ) and surrounded by silver particle ( ) (Fig. 3c). After more than 72 h incubation, cell was able to accumulated silver particle on the cells surface (Fig. 3d). This interaction were found to anchor to the bacteria cell wall. The interaction of silver particle was found to be much less with the gram-positive bacteria (Shrivastava *et al*., 2007). Silver particle was more interated with gram-negative bacteria. This might be composed of contents of the gram-positive bacteria cell wall and possibly represent a defence mechanism to locally deplete silve concetration. The cumulative affect of these factors would lead to retardation in bacteria growth but not complete annihilation.

Colloidal silver antibacterial activity of bacterial strains BAgAK6

Colloidal silver strain BAgAK6 shows the growth inhibitory activity with a minimum concentration (MIC) against two testing bacteria models (*E. coli* FNCC 0091 and *S. aureus* FNCC 0049), a clear zone was formed around paperdisk with inhibition zone about 10 mm and 3 mm, respectively (Fig. 4) and inhibition index (IP) about 7.1 and 2.4.

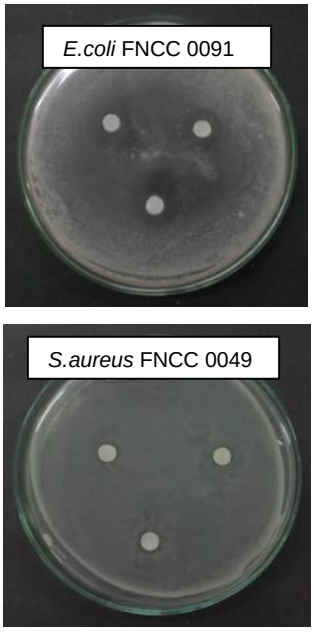

Figure 4. Antibacterial activity of colloidal silver from isolate strain BAgAK6 culture against *E. coli* FNCC 0091 (a) and *S. aureus* FNCC 0049 (b).

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The differences between gram-positive and gram-negative bacteria essentially rest in the structure of their respective cell wall. The gram-negative bacteria have a layer of lipopolysaccharide at the exterior, followed underneath by a thin layer of peptidoglycan (Madigan *et al*., 2012). Although the lipopolysaccharides are composed of covalently linked lipids and polysaccharides, they lack strength and rigidity. Negative charges on the lipopolysaccharides are attracted towards weak positive charges available on silver particles. On the other hand, the cell wall in gram-positive bacteria is principally composed of a thick layer of peptidoglycan (Baron, 1996). The rigidity and extended cross-linking not only endow the cell walls with fewer anchoring sites for the silver nanoparticles but also make them difficult to penetrate.

The extent of inhibition of bacterial growth reported in this study was dependent on the concentration of particles silver on liquid medium. Interaction between particles silver and the cell wall of bacteria would be facilitated by the relative abundance of negative charges on the gram-negative bacteria, which was congenial to the fact that growth of gram-negative bacteria was more profoundly affected by the silver particles silver than that of the gram-positive organisms. As demonstrated by MIC test, the inhibitory activity of colloidal silver in gram-negative bacteria is better than gram positive, while the MIC value is smaller than controls (nanofiber silver and antibiotics). In fact, colloidal silver bacterial are promising as a potential antibacterial agent and more active than the synthetic material (Jeevan *et al*., 2012; Nair and Panda, 2012).

Identification of selected silver tolerant bacterial isolates

Strain BAgAK6 are irregular shaped colony morphology with cream colour, bacillus rod-shaped, spore forming, gram-positive bacteria with specific biochemistry properties (Table 2). Strain BAgAK6 resembled to Bacillus sp.Table 2. Biochemistry test of isolate strain BAgAK6

Test	BAgAK6
Catalase	-
Casein hydrolysis	-
Gelatin hydrolysis	-
S*tarch* hydrolysis	+
MR-VP test	-
Indole production	-
Nitrate reduction	+
Simmon citrate test	-
H_2S production	-
Utilization of	
Glucose	+
Fructose	-
Sucrose	+
Lactose	-
Maltose	-
Manitol	-
Oxidation-Reduction of $AgNO_3$	-

Conclusion

Bacterial strain BAgAK 6 is resistant to Ag ion, able to forming colloidal nano silver and has antibacterial power against, both gram positive and gram negative pathogenic bacteria. Bacterial strain BAgAK 6 has similar characteristics to Bacillus sp.

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