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Submission date: 11-May-2020 12:35PM (UTC+0700)
Submission ID: 1321429601
File name: ijpbs-THESS-Antimicrobial_Carpet-no_cover.pdf (606.85K)
Word count: 3277
Character count: 18138

FP-08

THE ANTIMICROBIAL EFFECTIVENESS OF NOVEL NON-RESISTANT THESS FOR CARPET APPLICATION

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ABSTRACT

Antimicrobial additives are used in numbers of products for their availability to prevent product degradation and to provide hygienic properties. These additives are also used for carpet application in controlling bio-contaminants in indoor environments. Bacteria contamination on the carpet has been recognized as one of the most common cause of diseases, such as Norovirus infection, Campylobacteriosis, Kawasaki Syndrome and many others. The aim of this study was to analyze two of the pertinent test methods to analyze the effectiveness of Tetra Hydroxyl Ethyl dibiSulphite-2-Sodium (THESS) for carpet application against *Escherichia coli* and *Staphylococcus aureus*. THESS is a novel class of antibacterial agent with a unique killing mechanism that works from outside of the bacterial cell. The unique feature of THESS as a sulphide chelating agent is the very strong bond formed with the target ligands. This bond is the main key of the bacterial peptidoglycan porosity enlargement which caused lysis and lead to bacterial cell death. The macrodilution technique was conducted for determining the minimal inhibitory concentration (MIC) of THESS. Antimicrobial effectiveness test was conducted by two methods, modified AATCC 174 test I and OECD guideline which was harmonized to ISO 22196. Both are well known standard methods for measuring the qualitative and quantitative inhibition of microbial growth on carpet. The effectiveness of the two methods was then analyzed. MIC of THESS was determined at 0.5% w/v solution against the two tested bacteria. In conclusions, the AATCC 174 test I did not show any inhibition zone in both THESS- carpet and untreated carpet, while the OECD guideline showed the effectiveness of THESS-carpet against *E. coli* and *S. aureus* with the reduction of colony numbers 95.86% and 95.07%, respectively. This result suggested that application of THESS as antimicrobial in carpet is considered effective according to the standard of minimum 90% reduction of bacterial colonies.

KEYWORDS:
Antimicrobial additives, THESS, carpet applications, AATCC 174 test I, OECD guideline

INTRODUCTION:

The term ''antimicrobial'' means the ability of a substance to kill bacteria, viruses, protozoa, algae, fungi, and other pathogens. Pathogens can exist in various surroundings for a long time without triggering any reactions. In the past two decades, much attention has been devoted to polymeric materials with antimicrobial properties (Huang et al., 2016, Alvarez-Paino et al., 2017). It was proven that addition of antimicrobial to polymer, even at low concentrations (from 0.1 to 3%), provides effective protection. In addition, their small amount will only slightly increase the cost of previously manufactured products as well as make it safe and innovative (Varesano et al., 2011).

The increased use of carpets in house, schools, hospitals, and other places demonstrates the need for an additional antimicrobial in carpeting (Tietjen et al., 2003; Rivero et al., 2015). Carpet is a famous textile that is commonly used indoors for comfort, place to sit on the floor, reducing sound from walking, thermal

properties, slip preventer and others. While bringing benefits to our life, carpet also can be a reservoir to many bacteria since fiber used in the carpet can trap dirt which is the nutrient for bacteria to grow (Moody & Needles, 2004). Bacteria contamination on the carpet has been recognized as one of the most common cause of diseases, such as Norovirus infection, Campylobacteriosis, Kawasaki Syndrome and many others (Siegel et al., 2007). If the carpet is not properly cleaned, it may cause a health problem to the one who is incontact with it. Many methods were being used to overcome these problems such vacuuming and shampooing the carpet routinely. However, it does not remove the bacteria that are already attached to the carpet. To control the spread of bacteria and minimize the possibility of people infected by pathogen bacteria attached on the carpet, antimicrobial-carpet are finally traded (Haldar, et al., 2007). Yet, the antimicrobial carpet that have been available in the market contained a lot of chemical additives such as formaldehyde and triclosans which are harmful for human body (Allsopp et al., 2001).

Various antimicrobial treatments are currently used in the carpet industry to impart antimicrobial properties to the manufactured carpet. The antimicrobial that is seeded in the carpet should have a broad spectrum of activity against numerous bacteria and also possess very low toxicity (Moody & Needles, 2004). Since Tetra Hydroxyl Ethyl dibiSulphite-2-Sodium (abbreviated as THESS) has been proven to have very low toxicity and capable to fight against numerous bacteria, THESS-carpet is now introduced for carpet application. In the manufacturing process, THESS is seeded on the carpet surface and it binds permanently to the pore of the carpet fibres (Wardoyo, unpublished data).

THESS is a novel antimicrobial agent with a unique killing mechanism that works from outside of the bacterial cell by binding with peptidoglycan cell wall. Before THESS-carpet enter the market, several tests including controls and parameters have to be performed. Methods used to substantiate claims of THESS for carpet application effectiveness include: quantitative assay by measuring zone of inhibition tests using a sample piece and qualitative assay by direct inoculation of the surface under evaluation. AATCC 174 test I and OECD guideline which harmonized to ISO 22196 were chosen for substantiate claims since both are well known for standard method for measuring the qualitative and quantitative inhibition of microbial growth on carpet (AATCC, 2008; OECD, 2012).

MATERIAL AND METHODS:

Reagent and Microorganism

The materials used in this research were 0.75% THESS-carpet based polystyrene butadiene latex and conventional carpet, distilled water, ethanol 70%, Mueller Hinton Broth (HIMEDIA, India), agar bacteriological (OXOID, England), $BaCl_2$ (Merck, Germany), H_2SO_4 (Merck, Germany), Tetra Hydroxyl Ethyl di Sulphate (THESS) liquid and powder (Novis Natura Navita, Indonesia). *Escherichia coli* and *Staphylococcus aureus* acquired from Chemistry and Microbiology Laboratory of Pusat Penelitian Kimia, LIPI (Study Center of Chemistry, Indonesian Institution of Science).

Culture bacteria

The effectiveness of THESS for carpet application was assessed against *E. coli* and *S. aureus*. Bacteria was inoculated by taking 2 colonies from bacteria stock using sterilized loop and transferred into the sterilized nutrient broth. The bacterial suspension was put in the 36ºC ± 1ºC incubator. Overnight cultures were kept for 16 hours and bacterial suspension was diluted with sterilized nutrient broth to a density of $6x10^5$ CFU/ml.

MIC determination

Inoculum was prepared as described previously, however, bacterial suspension was diluted with sterilized nutrient broth to a density of $1x10^6$ CFU/ml (turbidity = 0.5 McFarland standard with 100 times dilution). Further 1:2 serial dilutions were performed by addition of culture broth to reach concentrations ranging from 0.25% to 8% w/v of THESS solution. 1 ml of each dilution were distributed in test tubes and was inoculated

with 1 ml of bacterial suspension (10^6 CFU/ml), thus, forming a total volume of 2 ml. The positive control was made by replacing the sample with nutrient broth whereas the negative control was made by putting 2 ml of nutrient broth in the test tube. All experiments were performed in triplicate and the macrodilution tubes were incubated at 35 ± 1^{0}C for 24 hours. The bacterial growth was detected by the absorbance of each solution at 600nm after the incubation period was over. MIC values were defined as the lowest concentration of THESS to completely inhibited or reduced microbial growth.

Qualitative assay for effectiveness of antimicrobial for carpet application

The method was based on AATCC 174 test I with modification (AATCC, 2008). Solutions consist of 0.1, 0.5 and 1% of THESS were prepared in 5 ml distilled water. 6 mm filter paper discs were impregnated with each of different concentration. Paper discs impregnated with streptomycin were used as positive control. The carpet sample was cut and shaped to resemble the 6 mm filter paper. All paper discs and carpet samples were placed onto the agar surface that was impregnated with the bacterial culture. The approximation of cell density of bacterial inoculum should be around 1 x 10^8 CFU/ml in accordance to 0.5 McFarland standard. Test was performed in triplicate. Results were recorded after overnight incubation at 37°C.

Quantitative assay for effectiveness of antimicrobial for carpet application

The assay was adapted from OECD guideline harmonized with ISO 22196 with modification (OECD, 2012). The carpet sample was cut up to a size 1 cm x 1 cm that is fit for the vials used during the the inoculation, incubation, and neutralization process and placed inside each vials. Each samples were inoculated with 200 µl of cell suspensions. Later, incubation process at 37^{0}C for 24 hours was performed. After 24 hours incubation, the carpet sample that has been inoculated then transferred to another vials for neutralization. An aliquot 10ml of distilled water validated for the active substances employed in the treated material was added to each vials that contains the carpet sample. An aliquot from this vials then mixed with agar medium by pour method. Same as ISO 22196, untreated carpet were required as negative control. The amounts of bacterial growth on THESS-treated and untreated carpet was determined by colony count method and the obtained results were compared.

Data Analysis

Results were expressed as mean value ± standard error of the mean. Statistical differences between colonies found on treated and untreated carpet, and the MIC values obtained from 2 different bacteria were observed by T-test. Replication in all experiment was analyzed by Analysis of Variance (ANOVA) single factor. P values lower than 0.05 ($p < 0.05$) were considered significant. The data graphic was built using Microsoft Excel 2013. The inhibition percentage of concentrated solution in macrodilution method was analyzed using following formula:

$$\text{Inhibition percentage (\%)} = \frac{\text{Absorbancy of control} - \text{Absorbancy of sample}}{\text{Absorbancy of control}} \times 100$$

RESULTS AND DISCUSSION:

Based on the result shown in Figure 1, THESS solution was indicated strongly reduce the growth of all isolates at concentration 0.5% w/v. At this point, only approximate MIC value could be determined. In order to find more precise MIC value, the same procedure should be repeated in a smaller THESS concentration range. Also, an aliquot of solutions from the macrodilution testing tube that show inhibition (at and above the MIC) should be diluted 1:1000 in saline or broth and 0.1 ml of the final dilution should subcultured to an agar medium so that the number of colonies that grow on subculture can be compared with the actual number of organisms inoculated into the MIC tubes. If the number of colonies found on a subculture plate less than 0.1% indicating 99.9% of the initial inoculum has been killed, a bactericidal effect has been achieved (Mahon, et al., 2014).

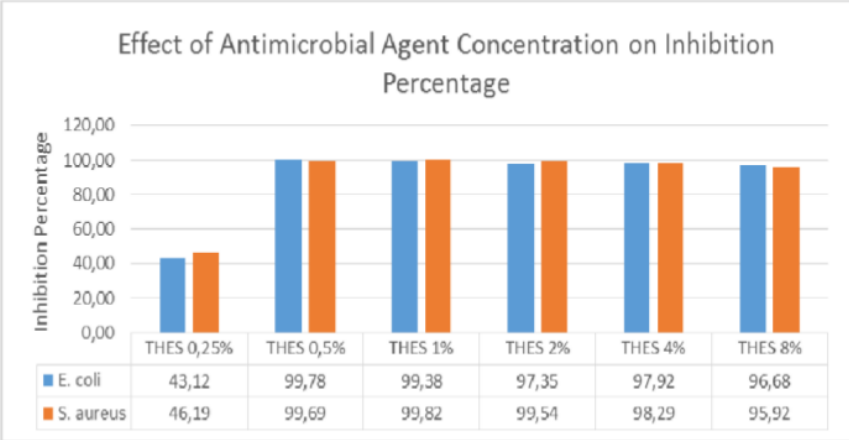

Figure 1
Effect of Antimicrobial Agent Concentration on Inhibition Percentage

In this study, 0.5 to 8% w/v solution of THESS were successfully reduced the growth of *Staphylococcus aureus* and *Escherichia coli* by about 96-99%, respectively, after 24 hours of treatment. At concentration 0.5%, THESS has attained its MIC and concentrations above 0.5% might cause diverse reaction. At first, when solutes dissolved, their particles were interacted with the solvent. Consequently, forming an unsaturated solution. However, when excessive amount of solutes were added into a solution, it became saturated. This described as a condition where the rate at which solute particles leave the surface of the solid equals the rate at which they return to the surface of the solid (National Science Foundation, 2016). At some environmental conditions, a saturated solution can be transformed into a supersaturated solution. Thereby, THESS solution at concentration above 0.5% was predicted to present similar or lower results to the 0.5% THESS solution due to the formation of saturated solution.

In conclusion, the addition of 0.5-8% w/v THESS solution had successfully reduce the growth of *Staphylococcus aureus* and *Escherichia coli.* Based on the obtained results, antimicrobial macrodilution method appear to be more reproducible since it provides sensitivity and MIC value even though macrodilution may produce in exact MIC data due to the performance of doubling dilutions (Patel, 2012). This study revealed that THESS is greatly effective as an antibacterial agent.
After the antibacterial propeties of THESS pure substance has been successfully confirmed, then test was perfomed for THESS-carpet. Methods used to substantiate claims of THESS for carpet application effectiveness include: Quantitative assay by measuring zone of inhibition tests using a test piece and qualitative assay by direct inoculation of the surface under evaluation. AATCC 174 test I and OECD guideline which harmonized to ISO 22196 were chosen for substantiate claims since both are well known for standard method for measuring the qualitative and quantitative inhibition of microbial growth on carpet (AATCC, 2008; OECD 2012).

In qualitative as which is conducted with AATCC 174 test I method, THESS carpet was cut into a specific size and placed on the surface of agar plate that has been inoculated with a bacterial suspension of *S. aureus* and *E. coli.* After overnight incubation, the result shown that THESS carpet has no zone of inhibition against these two types of inoculum since the carpet sample only contain 0.75% of THESS. The zone of inhibition must be a minimum of 2 mm for Gram positive bacteria and a minimum of 1 mm for Gram negative bacteria (AATCC, 2008). This means, the higher the concentration, the wider zone of inhibition will be detected.

Taken altogether, it should be highlighted that the effectiveness of antibacterial agent is not only determined from the antibacterial agent or antibiotics producing the widest zone of inhibition. Careful consideration

should be taken in terms of the culture medium, diffusion rate, concentrations, sensitivity, and the interaction between drug and the medium (Poliak & Tsvetkova, 2007).

Based on OECD guideline, the bacterial colonies reduction on THESS-treated and untreated carpet was determined by colony count method and the results were compared. Figure 2 shown below expressed a mean value ± standard error of the mean of colony forming unit found on THESS-treated and untreated carpet after overnight incubation. By looking through the results, the ability of THESS-treated carpet to inhibit the growth of microorganisms can be verified.

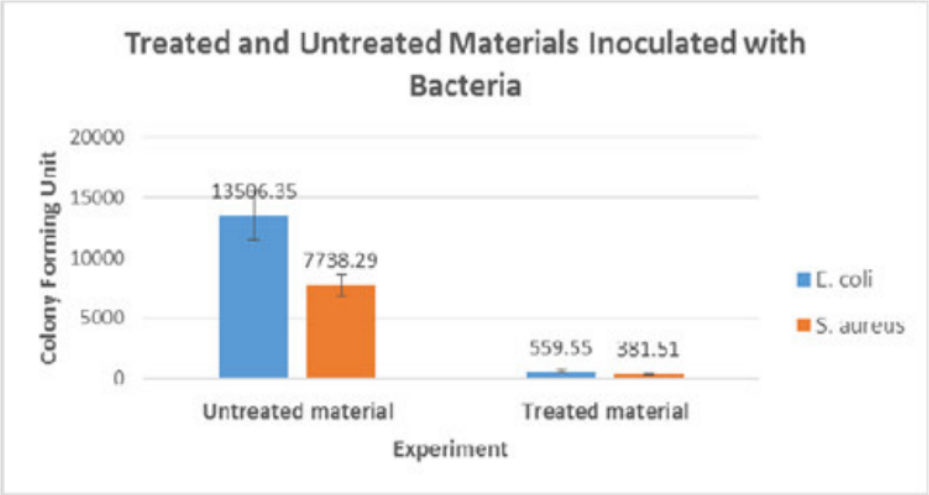

Figure 2
Colonies found on treated and untreated materials inoculated with bacteria

The result shown that the number of colonies found on untreated carpet is higher than THESS-treated carpet. It can be deduced that 0.75% THESS in carpet gives the opportunity to reduce the bacterial growth. A minimum of 90% reduction against each bacterium is required to be considered effective its application in the carpet product. Based on the figure above, THESS for carpet application reduced the number of *E. coli* and *S. aureus* colonies, 95.86% and 95.07%, respectively. Its mean, THESS for carpet application is considered effective since it can reduce more than standard of minimum bacterial colonies reduction which is 90% (AATCC, 2008). Figure 3 below respresented the colonies visible on agar plate for each test.

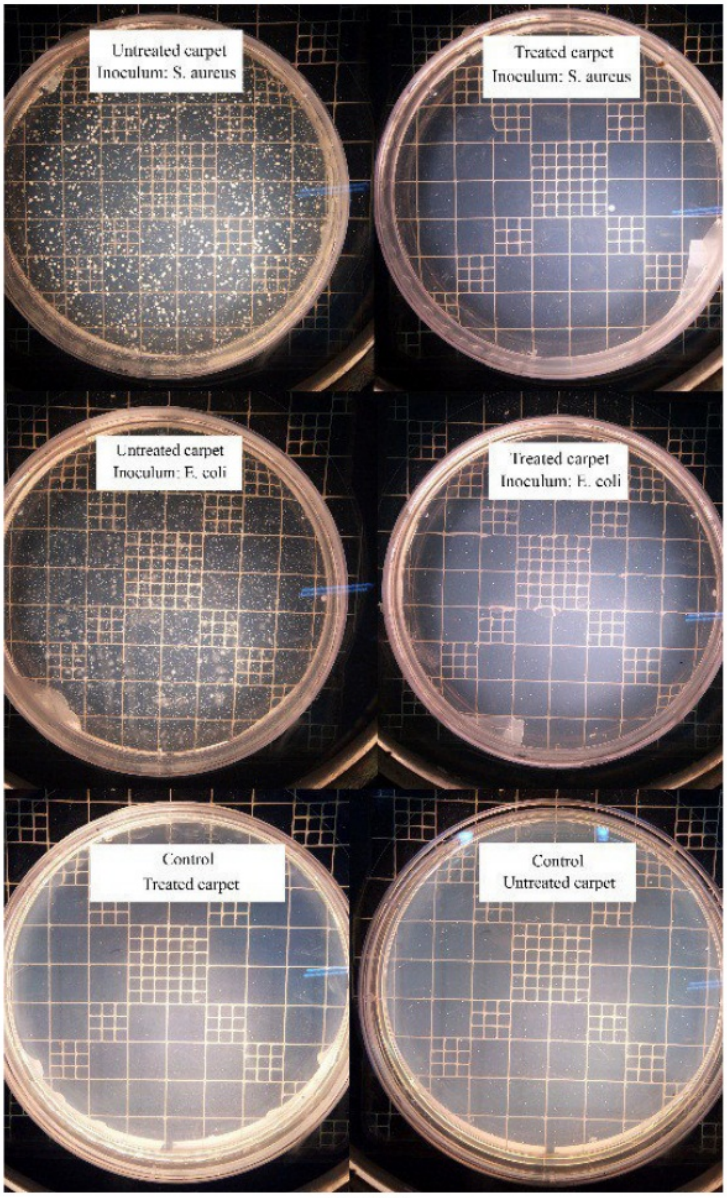

Figure 3.
Colonies found on agar plates

From this experiment, quantitative method established by OECD guideline were found to be excellent for evaluating the antimicrobial activity of treated materials and proving the quality of product THESS- treated and untreated carpet.

CONCLUSIONS:

This study suggested that THESS is potential as a novel non-resistant antimicrobial agent. The minimal inhibitory concentration of THESS in the macrodilution method found at 0.5% w/v solution (5mg/ml). Based on qualitative assay result which was conducted with AATCC 174 test I method shown that THESS carpet has no zone of inhibition against *E. coli* and *S. aureus*, while the OECD guideline showed the effectiveness of THESS-carpet against *E. coli* and *S. aureus* with the reduction of colony numbers 95.86%

and 95.07%, respectively. This result suggested that application of THESS as antimicrobial in carpet is considered effective according to the standard of minimum 90% reduction of bacterial colonies.

ACKNOWLEDGMENT:

The authors would like to express their gratitude to PT. N3 (Novis Natura Navita) who had provided research funding. The authors also wished to express their gratitude to Haryanto Wardoyo, Bobby Hapraja and the late Dr. Doddy Kustaryono (STKIP Surya) and all those who had assisted the authors' research activities either directly or indirectly.

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