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Bioactive Compound from *Streptomyces* sp. KB1 TISTR2504 as Effective Disinfectant against Microorganisms

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Abstract: A host environment cleaning using disinfectants is the most effective way to prevent the pathogen's transmission. This study compared the activity among of commercial liquid disinfectants [3% hydrogen peroxide (HP), and 70% ethyl alcohol (EA)] and bioactive-compounds (BCs) from *Streptomyces* sp. KB1 on indicating microorganisms: *Bacillus subtilis* ATCC 6633 as representative of spores; *Staphylococcus aureus* TISTR 517 and clinical methicillin-resistant *S. aureus* as representative of gram-positive bacteria, *Escherichia coli* TISTR 887, *Pseudomonas aeruginosa* TISTR 1467, and extended spectrum beta-lactamase (ESBL)-*Klebsiella pneumoniae* 342 as representative of gram-negative bacteria; *Candida albicans* TISTR 5779 as representative of yeast. Results showed that *B. subtilis* spores are resistant to 70% EA and BCs from strain KB1—which are biocidal to TISTR strains and clinical isolate MRSA, ESBL—in the free floating microorganism (suspension test). On the other hand, this resistance was not observed with 3% HP. Thus, 3% HP showed more promise for disinfecting microorganisms than that 70% EA and BCs. However, BCs did show the antimicrobial activities equal as 70% EA. The results might be implied that the using of BCs from selected bacterial strain as effective disinfectant against microorganism, especially vegetative cell, is a sustainable application in nearly future, to reduce using the chemical substances and zero wastes.

Key words: Bioactive compounds, disinfectant, *Streptomyces* sp.

1. Introduction

Microorganisms are either normal flora or environmental isolate that can make their way into respiratory organs from the air or on the ground. In addition, they can form biofilm along the surface of various medical devices, i.e., endotracheal tube, develop into infection involving the respiratory airway. Consequently, they can cause serious illnesses in immunocompromised patients [1, 2] such as HIV patients [3], diabetic patients [4], and patient with cancer [5]. Unfortunately, the most, there is as yet not no vaccine against those microorganisms. Regular cleaning of host environment using disinfectants that can interrupt microorganisms transmission is so far the best way to prevent the disease induced by the pathogenic microorganisms [6].

Disinfectants can be classified into five agents: alkylating, sulfhydryl combining, oxidizing, dehydrating, and permeable [7]. Interestingly, bioactive compounds (BCs) are the groups of alternative biological substances for interrupting the transmission of microorganisms, used as bio-disinfectant. In this study, two of the mentioned chemical agents are considered "challenged disinfectants". The other one is bio-disinfectant, BCs produced from *Streptomyces* sp. KB1 TISTR2304. Hydrogen peroxide, the representative oxidizing agent, functions as an oxidant by producing hydroxyl free radicals that attack essential cell components including lipid, protein, and DNA. Furthermore, it degrades rapidly into the innocuous products, water

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and oxygen; therefore, it is a relatively environmentally friendly disinfectant [8, 9]. Alcohol, a dehydrating agent, causes the cell membrane damage, rapid denaturation of proteins with subsequent metabolism interference and cell lysis [10, 11]. It shows rapid broad-spectrum antimicrobial activity with regard to vegetative bacteria, viruses, and fungi, but does not manifest sporicidal ability [9]. In addition, BCs from strain KB1 cause the cell membrane disruption with subsequent cell lysis [12].

The objective of this study is to determine the most effective disinfectant to prevent indicating microorganisms. In order to state that, two readily available disinfectants were utilized: 3% hydrogen peroxide (3% HP), 70% ethyl alcohol (70% EA) and one of bio-disinfectant, BCs, from strain KB1. Indicating microorganisms used were: *Bacillus subtilis* ATCC 6633 as representative of spores; *Staphylococcus aureus* TISTR 517 and clinical methicillin-resistant *S. aureus* (MRSA) as representative of gram-positive bacteria, *Escherichia coli* TISTR 887, *Pseudomonas aeruginosa* TISTR 1467, and extended spectrum beta-lactamase (ESBL)-*Klebsiella pneumoniae* 342 as representative of gram-negative bacteria; *Candida albicans* TISTR 5779 as representative of yeast.

2. Materials and Methods

2.1 Microorganisms, Media and Culture Conditions

The *Streptomyces* sp. KB1 TISTR2304 was collected from air at Aonang, Krabi province, southern Thailand, used as source of BCs, bio-disinfectant. It was cultured in half formula of Luria-Bertani (LB/2) [12].

The BCs have been produced after culturing the strain KB1 for 2 days at 30 ℃, shaking at 200 rpm. The BCs in culture broth, cell free supernatant, were collected by centrifugation at 4 ℃, 10,000 rpm for 30 min and filtered pass through the membrane (Millipore), pore size 0.45 micrometer. The protein concentration in BCs was measured for quantitation. It was kept at -20 ℃ until use, used as bio-disinfectant for investigation of the efficacy disinfectant against indicating (tested) microorganisms.

The indicating microorganisms named as TISTR used in this study were obtained from Thailand Institute of Scientific and Technological Research, Bangkok, Thailand. The *B. subtilis* ATCC 6633 is kindly provided as gift from Assoc. Prof. Chanpen Wiwat, Mahidol University, Thailand. The ESBL-*K. pneumoniae* 342 is a clinical isolate, obtained from Maharaj hospital, Nakhon Si Thammarat, Thailand. Indicating microorganisms were incubated at 37 °C and activated by incubating for a period of 24 h in a Luria-Bertani (LB) medium (Sharlau) or sabouraud dextrose (SD) medium (Pronadisa).

All strains were maintained in the LB/2, LB or SD broth medium containing 15% glycerol at -80 °C. For routine work, they were kept as LB/2 or LB or SD agar plates.

2.2 Preparation of Cell-Free Culture Broth

Two liters of supernatant from culture broth of strain KB1 was filtered through a 0.2 μm pore-size of cellulose acetate filter (Sartorius, Germany) to remove residual bacterial cells by vacuum pump (GAST, U.S.A.), named as cell-free culture broth and kept at 4 ℃. This process was again performed until obtaining the 12 L of cell-free culture broth.

2.3 Extraction of BC(s)

Twelve liters of cell-free culture broth from strain KB1 was extracted by 3-time with ethyl acetate (EA) at ratio of 1:1 (*v*/*v*), harvested and pooled the solvent layer. The pooled EA extracts were evaporated by using rotary evaporator at 45 ℃ under reduced pressure and harvested the slag. The EA extract slag was weighed, dissolved in the least amount of 100% acetone and named as crude EA extract.

2.4 Disinfectants

Two commercial chemical disinfectants were selected for testing. They were 30% hydrogen peroxide (Merck)

and 99% ethyl alcohol (Carlo Erba).

In the suspension test, each disinfectant, at original concentration was diluted with microorganisms suspensions or 0.85% sodium chloride (0.85 NaCl) in order to come up with the final disinfectant concentrations (3% HP and 70% EA). All disinfectants were prepared and utilized on the same day.

2.5 Disinfectant Activity against Free-Floating Microorganisms

After preparing the disinfectant solutions, inoculums of 1.5×10^8 colony forming units/mL (CFU/mL), made of a 0.5 McFarland standard, were prepared for the indicating microorganisms. A 500 µL of the prepared inoculums were then separately added to 500 µL of each disinfectant solution and incubated at 37 °C for 10 min. A 100 µL were then pipetted and plated onto LB or SD agar plates and incubated at 37 °C overnight. Colonies were then counted up to 100 then multiplied by the dilution factor of 20 and result was documented. Mixed solution with 0.8% NaCl and microorganisms without disinfectants was employed as a positive control, and 0.85% NaCl without microorganisms was utilized as a negative control. All tested were repeated three times.

2.6 Determination of the Minimum Inhibitory Concentration (MIC)

Vancomycin hydrochloride and oxacillin sodium salt monohydrate were purchased from the Sigma-Aldrich, USA. They were prepared as a stock solution at a concentration of 200 µg/mL by sterile double-distilled water. Meanwhile, the BC was prepared as a stock solution at a concentration of 1 mg/mL by Dimethyl sulfoxide (DMSO). The MIC was determined by using broth microdilution assay according to published protocol [13]. Initially, all isolates of MRSA were grown to mid-log phase in LB broth medium at 37 ℃. Each isolate was correspondingly adjusted, approximately to a 0.5 McFarland standard, then a 10-fold serial dilution was made to dilute out the cells, approximately residue of 1.5×10^6 cells/mL. Two-fold dilutions of BC, vancomycin and oxacillin were freshly prepared in 96 wells plate (Nunc, USA); the wells containing only 100 µL of DMSO or double-distilled water were designed as a control. One hundred microliter of 1.5×10^6 cells/mL of MRSA suspension was added to each well to a final volume of 200 µL. The plates were incubated in a moist chamber at 37 ℃ for 24 hrs. After the incubation, the optical density (OD) was measured at 690 nm with a microplate spectrophotometer (Thermo Scientific, USA). The MIC value was interpreted from the highest dilution showing no growth of MRSA. The MIC determinations were repeated independently three times and each time it was performed in triplicates, always with *S. aureus* TISTR 517 as a control.

2.7 Determination of the Minimum Bactericidal Concentration (MBC)

The MBC value was determined by using subculture technique [13]. One hundred microliter of each MRSA strain of well that showed MIC value and 3 wells with greater than the MIC value was spread on fresh MH agar medium without antibiotic and incubated at 37 ℃ for 24 hrs. After incubation, if the growth of MRSA was observed lower than 5 colonies, it indicated that a concentration of BC, vancomycin or oxacillin of these wells was the MBC value.

2.8 Statistical Analysis

The statistical analysis was carried out by using Statistical Package for the Social Sciences (SPSS) software version 17. The obtained data from assay which was performed in triplicate and repeated independently three times were analyzed by One-way ANOVA and the p-value < 0.05 was assigned as significance. The post-hoc turkey analysis was investigated the differences between groups. Meanwhile, the obtained data from assay which

performed in triplicate were reported as mean ± SD and analyzed by Kruskal-Wallis test. If they were different, Mann-Whitney U was used to compare the mean between groups. Difference was considered as statistical significance when p-value < 0.05.

3. Results and Discussion

After LB medium plate which was used to collect microorganisms from the air sample at Ao-Nang was incubated at 30 ℃ in static incubator for 7 days, the colony that was shown the fungal-like formation and embed in the agar medium was isolated as pure culture by using aseptic technique for Gram-staining. The isolated colony which showed the Gram positive branching formation was known as actinomycetes and named as KB1. Actinomycetes are the largest number of microorganisms which can grow in various environments such as air, soil, water, mangrove sediment, etc. From extensive studies in the past three decades, actinomycetes have been proven themselves as reliable sources of novel BCs and the richest source of secondary metabolites.

3.1 Antimicrobial Activity

Each microorganism suspension was separately incubated with each disinfectant solution for 10 min at 37 ℃ and then plated on the surface of LB plates for bacteria or of SD plates for yeast, by means of spread plate technique. The plates were incubated for a day, and the number of colonies was counted in order to find the reduction of each microorganism. The 70% EA and BCs were active against the free floating of TISTR- and ATCC-derived bacteria and yeast: *S. aureus* (gram-positive bacteria); *E. coli*, *P. aeruginosa*, ESBL-*K. pneumoniae* (gram-negative bacteria); and *Candida albicans* (yeast), no colonies appeared, exceptionally *B. subtilis* (spores). Those results almost similarity occurred with 3% HP. Based on these results, *B. subtilis* spores manifested stronger disinfectant resistant than other indicating microorganisms. However, the strong resistance of *B. subtilis* spores could be penetrated by using 3% HP (Table 1).

The mode of action of disinfectants can be classified into five agents: alkylating, sulfhydryl combining, oxidizing, dehydrating, and permeable [7]. By the ways, bio-disinfectants may also show difference from those of mode of actions, especially antioxidant activity from phenolic compounds that indicated the antimicrobial activity [12]. However, sellers of BCs often attribute health benefits to these compounds, but there is still insufficient research into the effectiveness and safety of these substances, either in long-term use or in quantities that exceed normal consumption levels. In addition, as BCs are not essential, advice on daily intake or usefulness is often unregulated [14, 15]. Interestingly, brine shrimp lethality bioassay has been ever used to preliminarily assess the cytotoxicity of the active molecules from BCs produced from strain KB1. It did not show cytotoxicity against brine shrimp [12]. Such, it might be implied that the using of BCs from selected bacterial strain as effective disinfectant against microorganism, especially vegetative cell, is a sustainable application in near future, to reduce using the chemical substances.

Disinfectants are not expected to kill all bacterial spores and are used to decontaminate devices that ordinarily do not penetrate tissues or that touch only intact skin [16-18]. The appropriate use of effective disinfectants is very important in preventing transmission of the pathogenic microorganisms easily discovered in the air or on the ground as environmental isolate, not safe for the elderly, neonates and immunocompromised patients. Moreover, there is still no vaccine available against all environmental isolate pathogenic microorganisms, using appropriate disinfectants is the most effective way to prevent pathogenic infections [1-6].

First, several chemical disinfectants that are easily available and belonging to different classifications were used: 3% HP (the oxidizing agent), and 70% EA

Table 1 Disinfectant activity of 3% hydrogen peroxide (HP), 70% ethyl alcohol (EA) and bioactive compounds (BCs) towards six free-floating microorganisms.

Microorganisms	CFUs			
	Solutions			
	0.85% NaCl (control)	3% HP	70% EA	BCs
E. coli	> 2,000, > 2,000, > 2,000	0, 0, 0	0, 0, 0	0, 1, 0
S. aureus	> 2,000, > 2,000, > 2,000	0, 0, 0	1, 0, 0	0, 0, 0
MRSA	> 2,000, > 2,000, > 2,000	0, 0, 0	1, 0, 0	0, 0, 0
P. aeruginosa	> 2,000, > 2,000, > 2,000	0, 0, 0	0, 0, 0	0, 45, 0
B. subtilis	> 2,000, > 2,000, > 2,000	0, 0, 0	26, 0, 0	40, > 300, > 300
ESBL-*K. pneumoniae*	> 2,000, > 2,000, > 2,000	0, 0, 0	0, 45, 0	400, 0, 0
C. albicans	> 2,000, > 2,000, > 2,000	0, 0, 0	0, 0, 0	0, 10, 1

After making the mixed solution with both disinfectant and microorganism that counted 1.5 × 10^7 CFUs/mL. They were incubated for 10 min at 37 °C, plated onto appropriate medium plates, incubated overnight, and the number of colonies was counted. All tests were repeated three times. CFUs = colony forming units; ESBL = extended spectrum beta lactamase; *E. coli* = *Escherichia coli*; *S. aureus* = *Staphylococcus aureus*; MRSA = methicillin-resistant *S.* aureus; *P. aeruginosa* = *Pseudomonas aeruginosa*; *B. subtilis* = *Bacillus subtilis*; *K. pneumoniae* = *Klebsiella pneumoniae*; *C. albicans*= *Candida albicans*.

(the dehydrating agent). Interestingly, biodisinfectant, BC from strain KB1 (the permeable disruption of cell membrane) [12], is one of the alternate biological disinfectants to control the environmental microorganisms. However, the most effective chemical disinfectant against indicating microorganisms, 3% HP, is not expensive and is therefore not an economical barrier in eliminating pathogenic microorganisms. Second, various microorganisms were utilized; *B. subtilis*as representative of spores; *S. aureus* as representative of gram-positive bacteria, MRSA, as representative of drug resistant bacteria, *E. coli*, *Ps. aeruginosa*, and ESBL-*K. pneumoniae* as representative of gram-negative bacteria; *C. albicans* TISTR 5779 as representative of yeast. Among the microorganisms tested, *B. subtilis* spores showed the strongest disinfectant resistant to 70% EA and BCs, but 3% HP is its most effective disinfectant that confirms similar findings from Jang et al. [19].

Bacterial resistant originated from either a natural property of an organism [9, 20]. Thus, it can be inferred that *B. subtilis* spores in this study could have also been this property. Spores are highly resistant to environmental stresses such as high temperature (some endospores can be boiled for several hours and retain their viability), irradiation, strong acids, disinfectants, etc. [9, 21].

There is much research being currently underway regarding the bacterial resistance of environmental isolates against disinfectants. Most reports have only analyzed the effect of disinfectants against reference strains from ATCC, but susceptibility of environmental or clinical isolates to disinfectants is now attracting special attention. ATCC strains that are laboratory adapted may not be good predictor for the susceptibility of strains extracted from patients. They concluded that disinfectant efficacy should be confirmed with recently isolated organisms [22]. It is interesting that ESBL-*K. pneumoniae* as an clinical isolate in this study also showed susceptibility similarly from TISTR-strains. On the basis of these results, for the future to decide the efficacy of disinfectants, it is more accurate to use isolates extracted directly from the environment or patient than to utilize ATCC- [19, 22, 23] or TISTR strains.

3.2 Anti-MRSA Activity of BC(s)

3.2.1 The MIC and MBC Value

This method, individual MRSA was grown in 96 wells plate containing different concentrations of BC or antibiotic. The wells become clear when BC or antibiotic is able to inhibit the growth of MRSA,

Table 2 The MIC and MBC values of bioactive compound (BC), vancomycin and oxacillin against MRSA and MSSA.

Microorganisms	MIC* (μg/mL)			MBC* (μg/mL)		
	Bioactive compound	Vancomycin	oxacillin	Bioactive compound	vancomycin	oxacillin
MRSA clinical isolate No.						
106	31.25	1.56	> 100	31.25	1.56	> 100
142	31.25	1.56	> 100	31.25	1.56	> 100
189	31.25	1.56	> 100	31.25	1.56	> 100
1424	31.25	1.56	> 100	31.25	1.56	> 100
7181	15.63	1.56	> 100	15.63	1.56	> 100
1195	15.63	1.56	> 100	31.25	1.56	> 100
1801	15.63	1.56	> 100	31.25	1.56	> 100
2468	15.63	1.56	> 100	15.63	1.56	> 100
7234	31.25	3.12	> 100	31.25	3.12	> 100
8176	62.50	3.12	> 100	62.50	3.12	> 100
MSSA TISTR No.						
517	31.25	1.56	1.56	31.25	1.56	1.56

* The data were expressed as the mode value which obtained from triplicate and three-time independence.

otherwise show turbid due to presence of viable MRSA. The minimum concentration of BC or antibiotic which is able to inhibit the growth of MRSA is referred as MIC value. The wells that have shown the MIC value and 3 wells with greater than the MIC value are continuously sub-cultured for determining the MBC value. In this study, the MIC and MBC values of BC, vancomycin and oxacillin were shown in Table 2. Statistical analysis of one-way ANOVA and post-hoc Turkey test revealed that the MIC and MBC value of BC was higher than of vancomycin, but lower than of oxacillin (p-value < 0.05). Vancomycin and oxacillin were often used when other antibiotics have failed. As a result, antibiotic-resistant strains of staphylococci have appeared and caused a major clinical problem in hospitals [24]. The MIC values of both oxacillin and vancomycin against MSSA TISTR 517 were in the range 0.78-1.56 μg/mL. Those MIC values were within the currently accepted range of susceptibility (≤ 2 μg/mL) by CLSI because MSSA TISTR 517 (*S. aureus* TISTR 517) is laboratory strain which used within laboratory only. Shockingly, the MIC values of vancomycin against clinical isolates of MRSA varied from 1.56-3.13 μg/mL. This result implied that the vancomycin MIC of some isolate was higher than the susceptibility cutoff value (≤ 2 μg/mL). This is evidence suggesting a tendency toward higher vancomycin MIC in these clinical isolates, also mentioned to as "MIC creep" [25]. For BC which do not appear the report of anti-MRSA activity, it showed the MIC value against the clinical isolates of MRSA significantly higher than vancomycin about 20-folds. But, from MBC value found that exhibited the bactericidal activity which was considered from the MBC/MIC value lower than or equal 4 (≤ 4).

4. Conclusions

In conclusion, to prevent the *B. subtilis* spores, it is more effective to use 3% HP as an oxidizing agent rather than using 70% EA and BCs. BCs did show the antimicrobial activities equal as 70% EA as tabulated in Table 1. Interestingly, BCs did show higher anti-MRSA activity than oxacillin (Table 2).

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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