Antibacterial Activity of Biosecur® Citrus Extract Surface Cleaner Against *Vibrio Vulnificus*

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Abstract: This study evaluated the antibacterial activity of Biosecur® citrus extract surface cleaner against *Vibrio vulnificus* using plate count method. Two concentrations, 0.5% and 2% of Biosecur® surface cleaner were plated on *Vibrio vulnificus* Agar (VVA) and tested for reduction of *Vibrio vulnificus*. In order to investigate the lasting residual activity of Biosecur®, antibacterial activity tests were also performed at time intervals up to 2.5 h after Biosecur® was plated on VVA. Biosecur® showed 6-log reduction of *Vibrio vulnificus* at 2%, and 3-log reduction of *Vibrio vulnificus* at 0.5%. The antibacterial activity of 2% Biosecur® against *Vibrio vulnificus* was shown to be equivalent to that of tetracycline. The residual activity of 2% Biosecur® was shown to maintain for at least 2.5 h after application. This study confirmed the high activity and long lasting residual effect of a safe, non-toxic organic food grade surface cleaner.

Keywords: Antibacterial activity, citrus extract, residual activity, surface cleaner, *Vibrio vulnificus*.

INTRODUCTION

Vibrio vulnificus is a leading cause of seafood-associated illness and death in the United States [1, 2]. Since 2000, the incidence of *Vibrio* infections due to eating raw or under-cooked oysters has shown a sustained increase [3]. *Vibrio vulnificus* could be introduced through discharges of feces or post-harvest processing of aquaculture products, and it could also occur from contaminated drinking water, by skin contact, or by wound infection. *Vibrio vulnificus* is a salt-requiring bacterium that is widespread in marine environments. This organism is most frequently of clinical significance as an agent of wound infections associated with exposure to seawater or primary septicemias resulting from the consumption of raw oysters [4]. In addition, other clinical presentations have been described, including pneumonia and sepsis in a drowning victim and a corneal ulcer [5]. It could also cause gastrointestinal disease, associated with ingestion of raw bivalves and crabs [6].

A high level of sanitation is needed for food-processing equipment especially food contact surfaces to ensure safety. In January 2001, the United States Food and Drug Administration issued a rule requiring food manufacturing process in the US to achieve a 5 log reduction of pathogens [7]. Commercial cleaners are usually applied to food contact surfaces in aid for decontamination, however, they are not specifically formulated for the purpose of killing pathogens, but rather removing mineral and soil deposits. Commercial cleaners usually contain acid-anionic surfactant, known to have optimal bactericidal activity at a pH range of 1.5-3.0 [8]. Unfortunately, they were shown to have relatively low inhibition against pathogens, resulting in only 2~3 log reduction [9].

Natural products have been a particularly rich source of anti-infective agents, of which flavonoids have become the subject of medical research. Flavonoids are ubiquitous in photosynthesizing cells and are commonly found in fruit, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis and honey. For centuries, preparations containing these compounds as the principal physiologically active constituents have been used to treat human diseases. Increasingly, this class of natural products is becoming the subject of anti-infective research, and many groups have isolated and identified the structures of flavonoids [10-13]. Flavonoids can function as direct antioxidants and free radical scavengers, and have the capacity to modulate enzymatic activities and inhibit cell proliferation [14]. In plants, they appear to play a defensive role against invading pathogens, including bacteria, fungi and viruses [15]. The peel of citrus fruits is a rich source of flavonoids. Citrus flavonoids have a large spectrum of biological activity and have been documented to possess antibacterial activity against a wide range of Gram-negative bacteria [16-18].

Biosecur® is a non-toxic, alcohol-free surface cleaner containing citrus bioflavonoids extracted from the *flavedo* and *albedo* layers of various citrus fruits and dissolved in food grade glycerin. It offers effective antibacterial surface cleaning with long-lasting residual benefits and was certified organic and GRAS (generally recognized as safe). Biosecur® corresponds to the natural and organic trend in food process-

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ing industry, and it provides additional protection to food processing plants.

In order to assess the antibacterial activity of Biosecur® and compare it to a standard antibiotic used for *Vibrio vulnificus* treatment, tetracycline was selected as a positive control and basis for comparison. Tetracycline was recommended as the standard clinical therapeutics for *V. vulnificus* [6, 19-22], and it was recently found to be the most effective therapy in mice experimentally infected with *V. vulnificus* [23].

In this study, the potential of Biosecur® to be used as antibacterial surface cleaner was assessed. This report presents a study on the antibacterial activity of Biosecur® against *Vibrio vulnificus* and its equivalence to tetracycline.

MATERIALS AND METHODS

Bacterial Strains and Growth Media

Vibrio vulnificus (ATCC 29306) was grown at 33°C for 18 h in tryptic soy broth (Becton, Dickinson and Company). The NaCl content of the broth was adjusted to 2% for optimal *Vibrio* growth. To maintain *Vibrio* cells in the stationary phase, fresh *Vibrio* culture was grown every day. The 18 h culture from the previous day was removed from incubator and preserved at room temperature, until inoculated into fresh tryptic soy broth for the next day.

Bacterial Culture Enumeration

Serial dilutions of *Vibrio vulnificus* ($10^{-1} \sim 10^{-5}$) were made daily with phosphate saline buffer. A hundred microliters of each dilution was plated on *Vibrio vulnificus* Agar (VVA) in duplicates. *Vibrio vulnificus* Agar contained 20 g peptone, 30 g NaCl, 25 g agar, 10 ml 100×dye stock solution per liter of medium; the 100×dye stock solution contained 0.6 g Bromothymol blue per 100 ml of 70% ethanol [24, 25]. All plates were incubated at 33°C for 18 h. The dilution displaying between 20 to 200 colonies was selected as basis for enumeration.

Antibacterial Activity Testing

Biosecur® H730D concentrate (Biosecur Lab Inc., Mont St-Hilaire, Quebec, Canada) was diluted with filter-sterilized distilled water to 0.5% and 2% (v/v). Since Biosecur® surface cleaner used glycerin as a carrier, we also diluted glycerin with sterile water to 0.5% and 2% (v/v) for antibacterial activity test. Tetracycline hydrochloride was dissolved in distilled water at 10 mg ml^{-1} and then filter-sterilized. The sterile tetracycline solution was then diluted with sterile water to 1 mg ml^{-1}, 100 µg ml^{-1}, 10 µg ml^{-1}, 1 µg ml^{-1} and 0.1 µg ml^{-1}. As a standard treatment for *Vibrio*, tetracycline served as a positive control and basis for comparison regarding antibacterial activity.

The antibacterial activity test was performed according to the procedure of Pati and Kurade [26]. Briefly, *Vibrio vulnificus* dilutions ($10^{-4} \sim 10^{0}$) were spread plated on VVA at 100 µl per plate, after which 100 µl of Biosecur® (0.5% and 2%); glycerin (0.5% and 2%); tetracycline (1 mg ml^{-1}, 100 µg ml^{-1}, 10 µg ml^{-1}, 1 µg ml^{-1} and 0.1 µg ml^{-1}) were plated on VVA as different treatments. All the treatments were tested in du-

plicates. *Vibrio vulnificus* dilutions 10^{-5}, 10^{-4} and 10^{-3} were spread plated on VVA at 100 µl per plate as untreated control. This study chose spread plate technique instead of agar overlay technique to avoid heat sensitivity and insolubility of Biosecur®.

Equivalent CFU Determination

All the VVA plates were examined for colony formation after overnight (18 h) incubation. The number of colony forming units (CFU) of diluted *Vibrio vulnificus* was multiplied by the dilution factor to give the equivalent CFU of undiluted *Vibrio vulnificus*. Equivalent CFU of each treatment was compared to that of untreated control, and the antibacterial activity was determined based on the difference between equivalent CFU of treatment and control.

Residual Activity Test

Biosecur® (2%) and tetracycline (100 µg ml^{-1}) were tested in the residual activity test. A hundred microliters of each substrate was plated on VVA plates and left in the biosafety cabinet to dry, for 0 s, 30 s, 1 min, 2 min, 5 min, 20 min, 30 min, 40 min, 60 min, 80 min, 90 min, 105 min, and 120 min. A hundred microliters of undiluted *Vibrio vulnificus* was then plated on the treated VVA. All the tests were performed in duplicates. Residual activity test was further tested with 2% Biosecur® alone. Biosecur® (2%) was plated at 100 µl on VVA plates and left in the biosafety cabinet to dry, for 30 min, 60 min, 90 min, 120 min, 150 min, before the undiluted *Vibrio vulnificus* was plated. All the plates were examined for colony formation after overnight (18 h) incubation. Number of colonies on each plate was counted, and plates of different time intervals were compared for differences in CFU.

Statistical Analysis

Analysis of Variance (ANOVA) with statistical software JMP was applied to analyze the significance of difference in equivalent CFU between treatments and control.

RESULTS

Antibacterial Activity Testing

Glycerin at 0.5% and 2% had no apparent antibacterial activity against *Vibrio vulnificus* (Fig. **1**). The plates of *Vibrio vulnificus* 10^{-5} dilution treated with 0.5% and 2% glycerin had 0.3-0.8 log CFU and the plates of *Vibrio vulnificus* 10^{-4} dilution treated with 0.5% and 2% glycerin had 1.9-2.3 log CFU, similar to that of untreated *Vibrio vulnificus* dilutions. The plates of undiluted *Vibrio vulnificus* treated with 0.5% and 2% glycerin had massive colonies (>1000).

Tetracycline was shown to have a minimum inhibitory concentration of 100 µg ml^{-1}. Lower concentration of tetracycline showed no significant antibacterial activity, 0.1 µg ml^{-1}, 1 µg ml^{-1} and 10 µg ml^{-1} tetracycline all reduced the CFU of *Vibrio vulnificus* dilutions by only 0.4-0.8 log and resulted in massive colonies (>1000) on undiluted *Vibrio vulnificus* culture. There were no apparent differences in antibacterial activity between these concentrations. Tetracycline (100 µg ml^{-1}) reduced the undiluted *Vibrio vulnificus*

culture (5.5 ± 0.3 log CFU) to 0 CFU during repeated trials. Plates of *Vibrio vulnificus* treated with 100 µg ml^{-1} tetracycline exhibited the same appearance as fresh VVA. The blue color of the agar was maintained and no cellobiose decomposition was observed (Fig. **2A**).

Biosecur® (0.5%) showed little inhibition against *Vibrio vulnificus*. When tested on *Vibrio vulnificus* dilutions, the results varied among trials. Biosecur® (0.5%) had less than 3 log reduction and resulted in massive colonies (>1000) on undiluted *Vibrio vulnificus* culture.

Biosecur® (2%) reduced the undiluted *Vibrio vulnificus* culture (5.5 ± 0.3 log CFU) to 0 CFU during repeated trials. Plates of *Vibrio vulnificus* treated with 2% Biosecur® exhibited color change of yellow, possibly due to pH change induced by the citrus extract (Fig. **2B**).

Statistical Analysis

Despite apparent differences in equivalent CFU (Fig. **1**), both concentrations of glycerin failed to reduce the level of *Vibrio vulnificus*. Tetracycline only significantly reduced *Vibrio vulnificus* at concentrations exceeding 100 µg ml^{-1}. Tetracycline (100 µg ml^{-1}), 0.5% and 2% Biosecur® exhibited significant inhibitory effect, as their equivalent CFU were significant lower than that of untreated control (Table **1**).
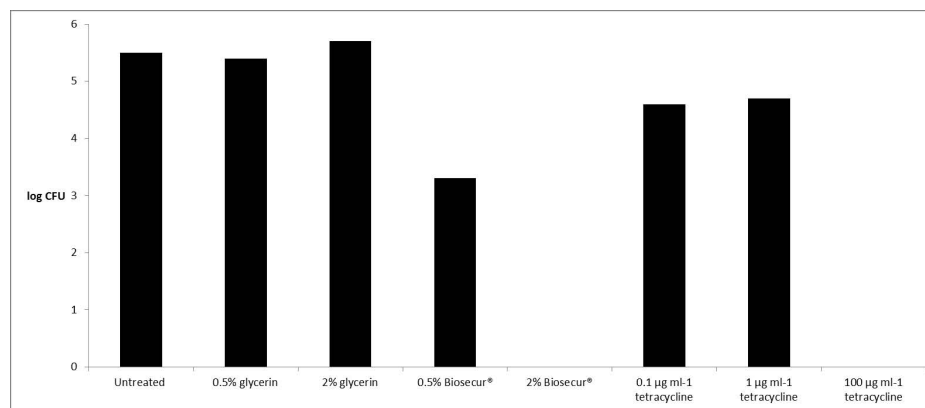
Residual Activity Test

The residual activity of Biosecur® was shown to last at least 2.5 h. The time interval was gradually increased from 5 min to 2.5 h after the observation that 2% Biosecur® maintained 6-log reduction against *Vibrio vulnificus* after 5 min. Even after 2.5 h of application, VVA plates with 100 µl 2% Biosecur® still reduced the undiluted *Vibrio vulnificus* culture (5.5 ± 0.3 log CFU) to 0 CFU. Tetracycline (100 µg ml^{-1}) exhibited the same activity, reducing undiluted *Vibrio vulnificus* culture to 0 CFU after 2 h of application.

DISCUSSION

The antibacterial activity of Biosecur® was observed during multiple tests. Two concentrations, 0.5% and 2% were recommended by Biosecur Lab Inc., however, only 2% Biosecur® exhibited a necessary 6-log reduction against *Vibrio vulnificus*, while 0.5% Biosecur® showed varied antibacterial activity among different trials, and it did not possess the ability to completely inhibit *Vibrio* growth. The lack of consistency suggested that 0.5% Biosecur® was not efficient as an antibacterial agent. Analysis of Variance (ANOVA) suggested that both concentrations significantly reduce the number of *Vibrio vulnificus*, but the activity of 0.5% Biosecur® was significantly lower than 2% Biosecur® (P=0.01).

In order to confirm the antibacterial activity of Biosecur®

Fig. (1). Comparison of antimicrobial activity of all treatments, as indicated by differences in each treatment's log equivalent CFU.

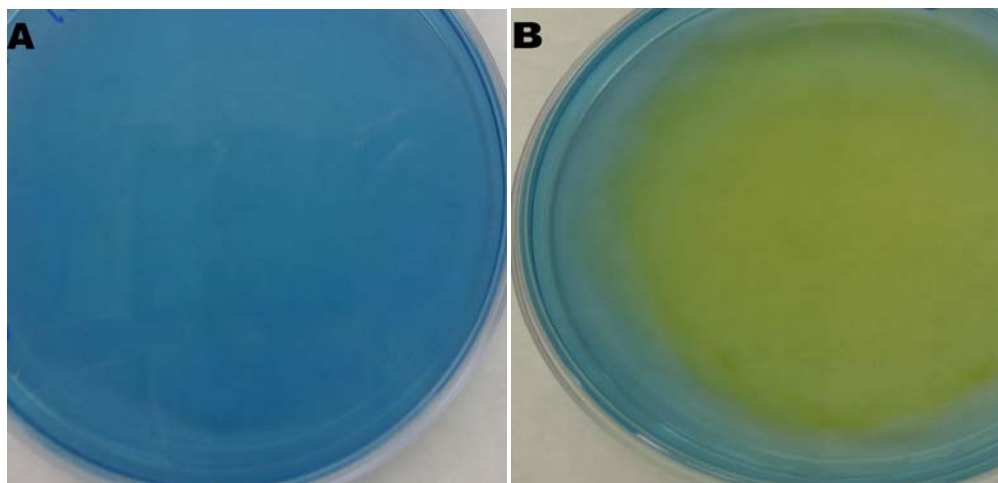**Fig. (2).** Appearance of *Vibrio vulnificus* on VVA under different treatments. (**A**) 100 µl *Vibrio vulnificus* culture and 100 µl 100 µg ml^{-1} tetracycline chloride on *Vibrio vulnificus* Agar; (**B**) 100 µl *Vibrio vulnificus* culture and 100 µl 2% Biosecur® on *Vibrio vulnificus* Agar.

Table 1. **Statistical Analysis of Antimicrobial Activity of Different Substances Against *Vibrio vulnificus***

Condition	Replicate	Log CFU	ANOVA Prob>F
Untreated	12	5.5 ± 0.3	1
0.5% glycerin	7	5.4 ± 0.6	0.8289
2% glycerin	8	5.7 ± 0.4	0.2108
0.5% Biosecur®	5	3.3 ± 0.6	0.0091
2% Biosecur®	15	ND	<0.0001
0.1 µg ml^{-1} tetracycline	2	4.6 ± 0.2	0.1164
1 µg ml^{-1} tetracycline	2	4.7 ± 0.3	0.1273
100 µg ml^{-1} tetracycline	12	ND	<0.0001

Analysis of Variance (ANOVA) was used for mean comparison between every treatment and the untreated control. Value of Prob>F less than 0.05 indicates a significant difference at α level of 0.05.

was from the citrus fruit and not from its carrier, glycerin, testes were also performed on 0.5% and 2% glycerin. ANOVA indicated that glycerin did not possess significant antibacterial activity against *Vibrio vulnificus*. Therefore it was concluded that the observed antibacterial activity of Biosecur® was due to the citrus extract alone.

To standardize the activity of 2% Biosecur®, tetracycline was used as a reference. As the most commonly used antibiotic against *Vibrio vulnificus* [6, 20, 23], tetracycline exhibited 6-log reduction of *Vibrio vulnificus* with a minimum inhibitory concentration of 100 µg ml^{-1}. Biosecur® (2%) was proven to have an inhibitory effect on *Vibrio Vulnificus* equivalent to 100 µg ml^{-1} tetracycline (P=1.0). Therefore, 2% Biosecur®'s complete inhibition of *Vibrio vulnificus* proved equivalence to tetracycline.

In conclusion, Biosecur® could be used as a food grade surface cleaner with strong inhibitory effect against *Vibrio vulnificus*.

CONFLICT OF INTEREST

The authors have no conflicts of interest to report.

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