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Pseudomonas aeruginosa SWUC02 Cell-Free Culture as a Potential Antimicrobial Agent Against Household Antibiotics-Resistant *Staphylococcus aureus*

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Abstract

Contamination of household environments with pathogenic bacteria poses a significant risk of foodborne illnesses. This study aimed to investigate the effectiveness of cell-free culture obtained from *Pseudomonas aeruginosa* SWUC02 (CF-SWUC02) against *Staphylococcus aureus*, a common pathogen associated with food poisoning outbreaks. The antagonistic activity of *P. aeruginosa* SWUC02 and CF-SWUC02 against various pathogenic bacteria, particularly *S. aureus*, was assessed. The active antimicrobial compounds produced by *P. aeruginosa* SWUC02 demonstrated resistance to protease enzymes and high temperatures. Optimal culture conditions for inhibiting *S. aureus* were determined as LB media supplemented with 0.01% $CuCl_2$, inoculated with $1x10^5$ $CFU.mL^{-1}$ of *P. aeruginosa* SWUC02, and incubated at 32°C with agitation (100 rpm) for 12 days. Eighty-six *S. aureus* isolates were collected from household environments and tested for antibiotic resistance, with 55 isolates exhibiting resistance to penicillin, and 17 isolates were identified as methicillin-resistant *S. aureus* (MRSA). CF-SWUC02 demonstrated inhibitory effects against all *S. aureus* isolates, including MRSA. In conclusion, CF-SWUC02 displayed antagonistic activity against drug resistant *S. aureus*, suggesting its potential as a valuable resource for combating these pathogens. Furthermore, the presence of heat and protease stable antimicrobial compounds in CF-SWUC02 highlights the need for further investigation to explore their potential applications in the field of antimicrobial research.

Keywords**:** *MRSA; S. aureus; antagonists; phenazine; Pseudomonas aeruginosa*

1. Introduction

Staphylococcus aureus is a species of common pathogenic bacteria that can be found in the noses of both healthy and unsanitary individuals. It can cause various symptoms such as skin and soft tissue infections (SSTIs), abscesses, and purulent abscesses. In addition, consuming foods contaminated with *S. aureus* toxins can lead to food poisoning and diarrhea. This bacterial species can also be transmitted by touching contaminated areas (Frank et al., 2010). In recent years, there have been several reported cases of food poisoning caused by *S. aureus*. From 2000 to 2005, the Department of Medical Sciences in Thailand investigated cases of diarrhea, food poisoning, and SSTIs, and reported that the patients had drug-resistant *S. aureus* that were resistant to methicillin (24-27%), clindamycin (37-69%), erythromycin (94-97%), fosfomycin (7.7-17%), teicoplanin (0.2-1.3%), and vancomycin (0.1-0.8%) (Tishyadhigama et al., 2009). In a survey conducted in Thailand in 2015, it was found that 46% of *S. aureus* isolates were Hospital-Acquired

Methicillin-resistant *S. aureus* (HA-MRSA) and were resistant to ciprofloxacin (99.4%), gentamycin (85.7%), and tetracyclin (84.5%) (Yanti et al., 2015). In 2018, food contamination with *S. aureus* and *Bacillus cereus* was the main cause of a food poisoning outbreak in Yasothon province, Thailand (Wonghirundecha et al., 2019). At the same time in Vietnam, there was a reported case of food poisoning caused by *S. aureus* contamination, with *S. aureus* and staphylococcal toxins being the main cause of a large-scale food poisoning outbreak and resulting in the treatments of 352 patients (Le et al., 2021).

S. aureus was initially well-controlled by penicillin in the mid-20th century, but a strain of penicillin-resistant *S. aureus* was discovered shortly thereafter. This strain produced penicillinase, which could hydrolyze the beta-lactam ring of penicillin, leading to penicillin resistance. To combat penicillin-resistant *S. aureus*, penicillinase-resistant semisynthetic penicillin derivatives, such as methicillin, were developed and used in place of penicillin. Unfortunately, a few years after the use of methicillin, a methicillin-resistant *S. aureus* (MRSA) strain was also discovered. Today, *S. aureus* can be classified as methicillin-sensitive *S. aureus* (MSSA) and MRSA based on their methicillin resistance characteristics. In terms of the source of infection, MRSA can be further divided into hospital-acquired MRSA (HA-MRSA) and community-acquired MRSA (CA-MRSA) (Guo et al., 2020). To address the problem of drug-resistant *S. aureus* contamination, various antibiotics have been used. In 2009, it was recommended to use penicillinase-resistant penicillin or ciprofloxacin to treat catheter-related bloodstream infections with MSSA, while vancomycin was recommended for MRSA in adult patients (Mermel et al., 2009). More recently, patients with *S. aureus* bloodstream infections are recommended to take flucloxacillin or cefazolin for MSSA and vancomycin or daptomycin for MRSA (Kimmig et al., 2021).

Pseudomonas spp. are well-known for producing a variety of secondary metabolites. Genome mining has revealed that *Pseudomonas* spp. produce antimicrobial compounds such as 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide (HCN), pyrrolnitrin, phenazine, alkylresorcinol, and putative lankacidin, as well as siderophore compounds such as pyridine-2,6-thiocarboxylic acid (PDTC), pyoverdine, and achromobactin. In addition, *Pseudomonas* spp. produce a range of antimicrobial compounds, including acyl-homoserine lactones (AHL) and phenazines, which are produced more strongly during biofilm formation compared to planktonic stage (Rieusset et al., 2020). Navarro et al. (2020) demonstrated that *P. aeruginosa* LV had antibacterial activity against both Gram-positive and Gram-negative pathogenic bacteria, such as *S. aureus*, *S. saprophyticus*, and *Enterococcus faecalis*. *P. aeruginosa* LV could produce pyocyanin, and inhibit biofilm production by *S. saprophyticus*, *Acinetobacter baumannii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Escherichia coli* (Feghali & Nawas, 2018). In a previous study, *P. aeruginosa* SWUC02 was isolated from the surface of lime fruit from chemically treated crops, and it showed no toxicity to lime seedlings. *P. aeruginosa* SWUC02 and its cell-free culture (CF-SWUC02) had strong antagonistic activity against the citrus canker pathogen *Xanthomonas citri* pv. *citri* (*Xcc*) (Sudyoung et al., 2020). In this study, we aimed to investigate the efficacy of CF-SWUC02 in inhibiting drug-resistant *S. aureus*, and to characterize the antagonistic traits of CF-SWUC02. We also optimized the culturing conditions of *P. aeruginosa* SWUC02 to increase the potential of CF-SWUC02 for inhibiting *S. aureus* and examined the prevalence of drug-resistant *S. aureus* in household wash basins.

2. Objectives

The objectives of this study were to examine the effectiveness of CF-SWUC02 against drug-resistant *S. aureus* and to optimize culture conditions for CF-SWUC02 production.

3. Materials and methods

3.1 Evaluation of the antagonistic activity of *P. aeruginosa* SWUC02 and CF-SWUC02

P. aeruginosa SWUC02 was isolated from lime fruit (Sudyoung et al., 2020). The antagonistic activity of *P. aeruginosa* SWUC02 was performed by the dual culture method. Bacterial pathogens were spread on tryptic soya agar (TSA) containing 0.01% $CuCl_2$. Wells were then created with a cork borer, and 100 µL of *P. aeruginosa* SWUC02 suspension (10^8 $CFU.mL^{-1}$) was pipetted into each well. After incubation at room temperature (30 ± 2 °C) for three days, inhibition zones were measured. The inhibition zone was calculated by subtracting the diameter of the clear zone by the colony size of *P. aeruginosa* SWUC02. The pathogens used in this study were *A.*

baumannii, E. coli, Enterobacter sp., *Klebsiella* sp., *Salmonella* sp., *Shigella* sp., *S. aureus*, and *B. cereus*, all of which were obtained from Department of Microbiology, Faculty of Science, Srinakharinwirot University. The experiments were independently repeated three times.

To prepare CF-SWUC02, *P. aeruginosa* SWUC02 was cultured in tryptic soya broth (TSB) containing 0.01% $CuCl_2$ at room temperature with agitation (100 rpm) for six days. The culture was centrifuged at 8,000 xg, 4°C for 10 minutes, and then, filtered through a PES membrane filter whose pore size was 0.45 µm. The CF-SWUC02 was investigated for antagonistic activity by agar well diffusion assay. The bacterial pathogens were spread on tryptic soya agar (TSA) containing 0.01% $CuCl_2$, and 100 µL of the CF-SWUC02 was pipetted into each of the wells created by a cork borer. The inhibition zone was calculated as described above. The experiments were independently repeated three times.

3.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of CF-SWUC02 against *S. aureus* and *B. cereus*

To prepare the diluted CF-SWUC02, it was mixed with 2X-TSB to obtain a 50% CF-SWUC02 concentration in TSB. Then, 50 µL of this mixture was combined with 50 µL of TSB and serially diluted two-fold in TSB. Next, 50 µL of *S. aureus* or *B. cereus* was pipetted into the wells containing the diluted CF-SWUC02 finally ranging from 12.50 to 0.049% CF-SWUC02, in a 96-well plate. The final concentration of *S. aureus* and *B. cereus* in each well was $1x10^5$ $CFU.mL^{-1}$. After incubation at room temperature for 24 hours, the growth of the pathogens was observed by turbidity measurement at a wavelength of 600 nm using a microplate spectrophotometer. The MIC was determined as the minimum concentration of CF-SWUC02 that prevented visible growth, resulting in a clear medium. To confirm the absence of growth, all clear inoculated TSB was dropped onto TSA and incubated at room temperature for 24 hours. The MBC was determined as the minimum concentration of CF-SWUC02 where no growth of pathogens was observed on the TSA medium.

3.3 Antimicrobial characters of *P. aeruginosa* SWUC02

Genes involving in antimicrobial compound production of *P. aeruginosa* SWUC02 were investigated. The genome of *P. aeruginosa* SWUC02, extracted by the AccuPrep® Genomic DNA extraction kit, was used as a DNA template for PCR. The genes involving in phenazine, pyoluteorin, pyrrolnitrin, and DAPG production were examined by PCR using specific primer pairs shown in Table 1. PCR cycles were carried out according the those described in the references (Table 1). The PCR products were analyzed by agarose gel electrophoresis.

P. aeruginosa SWUC02 was investigated for the ability to produce ammonia, HCN, lipase, protease, β-1,3 Glucanase, and chitinase. *P. aeruginosa* SWUC02 was cultured in 4% peptone broth for three days, and the culture broth was tested with the Nessler's reagent to observe the ammonia production. TSA with 1% Tween 80, minimal salt medium with 1% laminarin, skim milk agar, and Colloidal chitin agar were used for determining the enzymatic activities of lipase, β-1,3 Glucanase, protease, and chitinase, respectively. The HCN production was assayed following Castric (1975) method.

Table 1 Primer sets used in this study

Primers	Sequences	Specific to	References
phzEf	GAAGGCGCCAACTTCGTYATCAA	Phenazine gene	Schneemann et al. (2011)
phzEr	GCCYTCGATGAAGTACTCGGTGTG		
PRND1	GGGGCGGGCCGTGGTGATGGA	Pyoluteorin gene	de Souza et al. (2003)
PRND2	YCCCGCSGCCTGYCTGGTCTG		
PLTC1	AACAGATCGCCCCGGTACAGAACG	Pyrrolnitrin gene	de Souza et al. (2003)
PLTC2	AGGCCCGGACACTCAAGAAACTCG		
Phl2a	GAGGACGTCGAAGACCACCA	DAPG gene	Raaijmakers et al. (1997)
Phl2b	ACCGCAGCATCGTGTATGAG		

3.4 Optimization of *P. aeruginosa* SWUC02 culturing conditions for antimicrobial compound production

In this study, we used one-factor-at-a-time (OFAT) method to optimize the culturing conditions for *P. aeruginosa* SWUC02. OFAT involves studying each factor separately in order to determine the optimal conditions for each factor. The experiments were conducted with 25 mL cultures in 200 mL flasks. The Primary conditions were as follows: TSB with 0.01% $CuCl_2$ inoculated with $1x10^3$ CFU.mL^{-1} of *P. aeruginosa* SWUC02 incubated at room temperature with agitation (100 rpm) for 6 days. The effects of inoculum sizes ($1x10^3$, $1x10^4$, and $1x10^5$ CFU.mL^{-1}), $CuCl_2$ concentrations (0.005%, 0.01%, 0.015%, and 0.02% (w/v), agitation rate (0, 50, 100, 150, and 200 rpm), temperature (26, 28, 30, 32, and 34 °C), incubation period (3, 6, 9, 12, and 15 days), and culture media (TSB, nutrient broth (NB), King's B, and Luria-Bertani broth (LB) were investigated, respectively. The antimicrobial activity of *P. aeruginosa* SWUC02 against *S. aureus* ATCC 25923 was evaluated using agar well diffusion assay. We chose *S. aureus* ATCC 25923 as a control in this experiment because this strain is MSSA, which is likely to represent the majority of the *S. aureus* population found in household environments. The experiments were repeated three times for each factor, and the data were analyzed using Kruskal-Wallis method. Multiple comparisons between treatments were performed using Mann-Whitney U method.

3.5 Heat and enzyme tolerance characteristics of CF-SWUC02

P. aeruginosa SWUC02 was cultured under the optimized culturing conditions, and CF-SWUC02 was then prepared. CF-SWUC02 was assessed for heat tolerance by incubation at 35°C, 40°C, 45°C, 50°C, 60°C, 70°C, 80°C, 90°C, 100°C and 121°C for 30 minutes, followed by antagonistic activity assay against *S. aureus* ATCC 25923 using agar-well diffusion method. In addition, to evaluate its proteolytic enzyme tolerance, CF-SWUC02 was mixed with 1000 μg.mL^{-1} of proteinase K and pepsin, and incubated at 37°C for two hours. The treated CF-SWUC02 was tested for antagonistic activity against *S. aureus* ATCC 25923 using agar-well diffusion method. The experiment was repeated three times, and the data were analyzed using Kruskal-Wallis and Mann-Whitney U methods for multiple comparison between treatments.

3.6 Determination of *S. aureus* prevalence in household wash basins

The prevalence of *S. aureus* in household wash basins was determined by collecting samples from 108 wash basins in 36 households located in Bangkok, Thailand, between June and November 2022. Each household provided three wash basins for sampling. A 100 cm^2 surface area of each wash basin was swabbed using a sterile cotton bud moistened with sterile phosphate buffered saline (PBS). The cotton bud was then placed in a tube containing 1 mL of sterile PBS and stored in a cool box during transportation.

In the laboratory, the tubes were vortexed for approximately one minute to ensure proper mixing. From each tube, 0.1 mL of the resulting cell suspension was aseptically spread onto Baird-Parker Agar (BPA) plates. The BPA plates were incubated at 37 °C for 24 hours. Following incubation, the small black colonies with a halo zone, indicative of *S. aureus*, were counted. The number of colonies was multiplied by 10 to account for the 0.1 mL sample volume used from the 1 mL cell suspension. The results were then reported as CFU of *S. aureus* per 100 cm^2 of each wash basin surface.

3.7 Antibiotic resistance characterization of *S. aureus* isolates from household wash basins and evaluation of CF-SWUC02 antimicrobial activity

All selected isolates were confirmed as *S. aureus* using the coagulase test. To evaluate the antibiotic resistance of the *S. aureus* isolates, one isolate was selected from each wash basin, and their resistance to penicillin, cefoxitin, and vancomycin was determined using the Clinical and Laboratory Standards Institute (CLSI) method (2021). The minimum inhibitory concentration (MIC) breakpoints recommended by CLSI were employed to determine the antibiotic-resistant profiles.

The distinction between MRSA and MSSA isolates was determined through cefoxitin MIC testing, following the guidelines provided by CLSI (2021). Isolates with penicillin, cefoxitin, and vancomycin MICs below 0.12, 4, and 2 μg.mL^{-1}, respectively, were classified as susceptible. Conversely, isolates with penicillin, cefoxitin, and vancomycin MICs exceeding 0.25, 8, and 16 μg.mL^{-1},

respectively, were considered resistant. For vancomycin, isolates with MICs between 4 and 8 $\mu g.mL^{-1}$ were classified as vancomycin intermediate.

For this experiment, cefoxitin sodium salt and penicillin G sodium salt from Glentham Life Sciences Co. Ltd. (U.K.), and vancomycin from Lek Pharmaceuticals d.d. (Slovenia) were used. The inhibitory effect of CF-SWUC02 against the *S. aureus* isolates was evaluated using an agar well diffusion assay.

3.8 Statistical analysis

The data were checked for normal distribution using a normal Q-Q plot. For parametric data, statistical differences were determined using one-way ANOVA with the Fisher's LSD test for multiple comparisons between treatments. For non-parametric data, Kruskal-Wallis method and Mann-Whitney U test were applied.

4. Results and Discussion

4.1 Antagonistic activity of *P. aeruginosa* SWUC02 and CF-SWUC02 against common pathogenic bacteria

P. aeruginosa SWUC02 showed antagonistic activity against various common pathogenic bacteria, including *A. baumannii*, *E. coli*, *Enterobacter* sp., *Klebsiella* sp., *Salmonella* sp., *Shigella* sp., *S. aureus*, and especially *B. cereus* (Table 2). Transparent zones against all pathogenic bacteria were observed. CF-SWUC02 showed outstanding antagonistic activity against Gram-positive bacteria, including *S. aureus* and *B. cereus* (Figure 1). The different antagonistic activity against the pathogenic bacteria between *P. aeruginosa* SWUC02 and CF-SWUC02 might imply that growth of the pathogens was suppressed by a different mechanism. It should be noted that the antagonistic activity of CF-SWUC02 came from 6-day culture of *P. aeruginosa* SWUC02, while that of *P. aeruginosa* SWUC02 was carried out by dual culture method with three days incubation time. A longer incubation period of CF-SWUC02 can increase the production of bioactive compounds against *S. aureus*, which may be secondary metabolites.

We then determined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of CF-SWUC02 against *S. aureus* and *B. cereus*. The results showed that 0.195% and 0.781% of CF-SWUC02 were the MIC and MBC, respectively, against *S. aureus*, while 0.781% was the MIC and MBC against *B. cereus* (Table 2). These results strongly suggest that CF-SWUC02 is highly effective at inhibiting *S. aureus.*

Table 2 Antagonistic activity of *P. aeruginosa* SWUC02 against various pathogenic bacteria

Pathogenic bacteria	Antimicrobial activity		MIC	MBC
	***P. aeruginosa* SWUC02**	**CF- SWUC02**		
Gram-negative bacteria				
A. baumannii	2.48 ± 0.32^{c}	-	ND	ND
E. coli	4.74 ± 0.58^{bc}	-	ND	ND
Enterobacter sp.	-	-	ND	ND
Klebsiella sp.	10.77 ± 2.16^{ab}	-	ND	ND
Salmonella sp.	6.01 ± 0.49^{b}	-	ND	ND
Shigella sp.	3.67 ± 0.86^{c}	-	ND	ND
Gram-positive bacteria				
S. aureus	8.13 ± 1.03^{ab}	$11.70 \pm 1.50^{*a}$	0.195%	0.781%
B. cereus	11.67 ± 0.31^{a}	$3.33 \pm 0.58^{*b}$	0.781%	0.781%

Data are shown as mean ± standard deviation of halo zone and clear zone (*) (mm). Different superscript letters above the antimicrobial activity value indicate that there are significant differences ($p<0.05$) in the column. The data were validated for normal distribution using a Q-Q plot and analyzed using the Kruskal-Wallis method. The Mann-Whitney U test was employed for multiple comparisons between treatments. "ND" means no data.

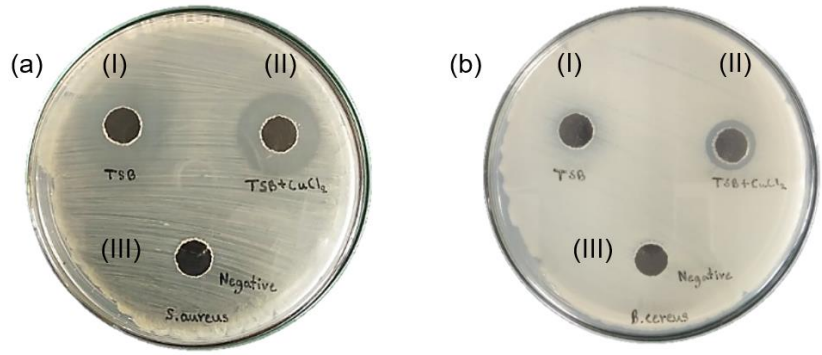

Figure 1 Antimicrobial activity of CF-SWUC02 against *S. aureus* (a) and *B. cereus* (b). By agar well diffusion assay, (I) and (II) indicate wells filled with cell-free culture obtained by culturing *P. aeruginosa* SWUC02 in TSB and TSB with 0.01% $CuCl_2$, respectively. (III) indicates well filled with TSB medium as a control.

There are several studies that have reported the effectiveness of biosurfactants produced by *P. aeruginosa*. El-Sheshtawy & Doheim (2014) found that *P. aeruginosa* ATCC-10145 showed a greater reduction in surface tension compared to *B. subtilis* NCTC-1040. Similarly, Bharali & Konwar (2011) discovered that biosurfactants from *P. aeruginosa* strain OBP1 were more effective against Gram-positive bacteria than Gram-negative bacteria. Among Gram-positive bacteria, greater inhibition was observed against *S. aureus* compared to *B. subtilis, B. circulan, S. epidermidis*. This can be attributed to the difference of cell walls between Gram-positive and Gram-negative bacteria. Sharma et al. (2021) found that the properties of surfactants and the structure of the bacterial cell wall influence their effectiveness in killing bacteria. These findings support the observed differences in the activity of CF-SWUC02 against *S. aureus, B. cereus* and Gram-negative bacteria.

4.2 Antimicrobial characters of *P. aeruginosa* SWUC02

We studied the antagonistic traits of *P. aeruginosa* SWUC02 and investigated the genes involved in the production of bioactive compounds. *P. aeruginosa* SWUC02 contains a gene responsible for the production of phenazine, as confirmed by PCR using phenazine-specific primers (Schneemann et al., 2011), which generated a 450 bp product. However, we did not find genes involved in the production of pyoluteorin, pyrrolnitrin, or 2,4-diacetylphloroglucinol in this study. Additionally, we examined several antimicrobial metabolites produced by *P. aeruginosa* SWUC02. Although we did not detect the presence of ammonia, lipase, or chitinase enzyme from *P. aeruginosa* SWUC02 *in vitro*, the results showed that *P. aeruginosa* SWUC02 produced HCN, proteases, and β-1,3-glucanase (Table 3). Importantly, β-1,3-Glucanase is an enzymatic molecule that specifically cleaves glucan chains, which are essential components of fungal cell walls in varies fungal species (Mouyna et al., 2013). The demonstrated antifungal activity of CF-SWUC02 holds significant value for further investigations in this field.

It has been reported that *P. aeruginosa* produces various antimicrobial compounds, such as 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin, and phenazine (Raaijmakers & Mazzola, 2012). It is possible that *P. aeruginosa* SWUC02 also produces antimicrobial secondary metabolites that are produced during a long incubation period in a culture medium containing 0.01% $CuCl_2$. It has been reported that *P. aeruginosa* LV, when cultured in NB medium containing 0.01% $CuCl_2$ for 10 days, produced phenazine and antimicrobial organo-metallic compounds (de Oliveira et al., 2016). The antimicrobial compounds produced by *P. aeruginosa* LV caused cell lysis of the bacterial pathogen *Xcc*. Additionally, the metalloantibiotic compound fluopsin C, produced by *P. aeruginosa* LV, showed strong antibiotic activity due to its ability to disrupt cell membranes (Navarro et al., 2019). The cell-free culture of *P. aeruginosa* LV was also found to inhibit drug-resistant *K. pneumoniae and S. aureus,* and phenazine-1-carboxylic acid produced by *P. aeruginosa* LV exhibited antagonistic activity against phyto-pathogenic fungi, *Botrytis cinerea* (Cardozo et al., 2013; Kerbauy et al., 2016).

Table 3 Antimicrobial characteristics of *P. aeruginosa* SWUC02

Characteristics		Results
Gene involving the production of	phenazine	+*
	pyoluteorin	-
	pyrrolnitrin	-
	2,4-diacetylphloroglucinol	-
Antimicrobial metabolites	Ammonia	-
	HCN	+
	Lipase	-
	Protease	+
	β-1,3 Glucanase	+
	chitinase	-

*"+" and "-" are an indicator whether a particular characteristic was detected or not. * The presence of the phenazine-related-producing genes in P. aeruginosa SWUC02 were confirmed by whole genome sequencing (Srisangchun et al., 2023).*

Table 4 Antagonistic activity against *S. aureus* ATCC 25923 of CF-SWUC02 obtained from *P. aeruginosa* SWUC02 cultured under optimized conditions

Culturing conditions	Factors	Antimicrobial activity (Clear zone; mm)	Obtained Condition
Inoculum size ($CFU.mL^{-1}$)	$1x10^3$	12.85 ± 0.68^b	TSB with 0.01% $CuCl_2$ inoculated with $1x10^5$ $CFU.mL^{-1}$ of *P. aeruginosa* SWUC02 incubated at room temperature with agitation (100 rpm) for 6 days.
	$1x10^4$	13.66 ± 0.44^a	
	$1x10^{5*}$	14.30 ± 0.66^a	
$CuCl_2$ concentration (w/v)	0.000%	4.65 ± 0.89^b	TSB with 0.01% $CuCl_2$ inoculated with $1x10^5$ $CFU.mL^{-1}$ of *P. aeruginosa* SWUC02 incubated at room temperature with agitation (100 rpm) for 6 days.
	0.005%	13.80 ± 0.35^b	
	0.010% *	14.86 ± 0.34^a	
	0.015%	15.42 ± 0.23^a	
	0.020%	14.83 ± 0.54^a	
Shaking rate (rpm)	50	3.13 ± 0.65^c	TSB with 0.01% $CuCl_2$ inoculated with $1x10^5$ $CFU.mL^{-1}$ of *P. aeruginosa* SWUC02 incubated at room temperature with agitation (100 rpm) for 6 days.
	100*	15.26 ± 0.48^a	
	150	14.85 ± 0.58^{ab}	
	200	14.50 ± 0.43^b	
Temperature (°C)	26	3.51 ± 0.52^c	TSB with 0.01% $CuCl_2$ inoculated with $1x10^5$ $CFU.mL^{-1}$ of *P. aeruginosa* SWUC02 incubated at 32°C with agitation (100 rpm) for 6 days.
	28	14.16 ± 0.62^b	
	30	14.63 ± 0.50^{ab}	
	32*	15.46 ± 0.58^a	
	34	14.76 ± 0.66^{ab}	
Incubation time (days)	3	9.54 ± 0.38^c	TSB with 0.01% $CuCl_2$ inoculated with $1x10^5$ $CFU.mL^{-1}$ of *P. aeruginosa* SWUC02 incubated at 32°C with agitation (100 rpm) for 12 days.
	6	14.90 ± 0.88^b	
	9	15.39 ± 0.64^b	
	12*	17.07 ± 0.47^a	
	15	17.16 ± 0.36^a	
Culture medium	TSB	18.34 ± 0.85^a	LB or TSB with 0.01% $CuCl_2$ inoculated with $1x10^5$ $CFU.mL^{-1}$ of *P. aeruginosa* SWUC02 incubated at 32°C with agitation (100 rpm) for 12 days.
	NB	15.50 ± 0.85^b	
	King' s B	16.72 ± 0.88^b	
	LB*	18.07 ± 0.98^a	

Data are shown as mean ± standard deviation of clear zone (mm). Different superscript letters above the antimicrobial activity values indicate that there are significant differences ($p<0.05$). Asterisks indicate the optimal factors. The data were validated for normal distribution using a Q-Q plot and analyzed using the Kruskal-Wallis method. The Mann-Whitney U test was employed for multiple comparisons between treatments.

4.3 Optimization of culture conditions for enhancement of antimicrobial compound production in *P. aeruginosa* SWUC02

Primarily, *P. aeruginosa* SWUC02 was grown in TSB with 0.01% $CuCl_2$ with the inoculum size of $1x10^3$ CFU.mL^{-1}, and incubated at room temperature with agitation (100 rpm) for 6 days. The ambient temperature was 28-35 (°C) and the pH of TSB without adjustment is around 6-7. We improved the production of antimicrobial compounds in *P. aeruginosa* SWUC02 through the optimization of culture conditions. Using OFAT optimization method, the inoculum size was first studied, followed by $CuCl_2$ concentration, agitation rate, temperature, incubation time, and culture medium, respectively. Consequently, to achieve strong antagonistic activity against *S. aureus* ATCC 25923, *P. aeruginosa* SWUC02 was cultured in LB or TSB medium containing 0.01% $CuCl_2$ (w/v) at pH 7, with $1x10^5$ CFU.mL^{-1} inoculum size, and incubated at 32°C with agitation at 100 rpm for 12 days, as shown in Table 4.

We compared the antimicrobial activity of CF-SWUC02 obtained from the optimized culture conditions against those obtained from culture conditions reported by previous studies. The study of Sudyoung et al. (2020) was the use of *P. aeruginosa* SWUC02 and its cell-free culture against *Xcc*. Lopes et al. (2012) and de Oliveira et al. (2016) cultured *P. aeruginosa* LV to extract bioactive compounds against *X. axonopodis* and *Xcc*, respectively. As shown in Table 5, *P. aeruginosa* SWUC02 cultured under optimized conditions in this study showed stronger antimicrobial activity than CF-SWUC02 obtained from *P. aeruginosa* SWUC02 cultured under Lopes et al. (2012), de Oliveira et al. (2016), and Sudyoung et al. (2020) conditions. The different culture conditions included culture media, inoculum sizes, temperatures, and incubation times, while pH, $CuCl_2$ concentration, and agitation rate were held constant across the four methods. Therefore, we used these optimized culture conditions to obtain CF-SWUC02 for further study.

4.4 Heat and enzymes tolerant characteristics of CF-SWUC02

CF-SWUC02 obtained from optimized culture conditions retained antagonistic activity against *S. aureus* even after treatments with proteinase K and pepsin. Incubated at 100°C for 30 minutes did not reduce its antagonistic activity against *S. aureus*, indicating the heat-tolerant characteristics (compared to the control incubated at 32 °C shown in Table 6). Furthermore, autoclave conditions (121°C for 15 minutes) did not abolish the antagonistic activity of CF-SWUC02. These results suggest that the bioactive compounds produced by CF-SWUC02 responsible for inhibiting *S. aureus* are less likely to be proteinaceous in nature; however, the results do not rule out the possibility of being a bacteriocin entirely.

Table 5 Comparison of antimicrobial activity against *S. aureus* ATCC 25923 of CF-SWUC02 obtained from optimized and reference culturing conditions.

Methods	Current study	Sudyoung et al. (2020)	de Oliveira et al. (2016)	Lopes et al. (2012)
Culture conditions				
Inoculation seed	$1x10^5$ CFU.mL^{-1}	$1x10^4$ CFU.mL^{-1}	$1x10^4$ CFU.mL^{-1}	$1x10^4$ CFU.mL^{-1}
Medium	LB	TSB	NB	NB
$CuCl_2$	100 mg/L $CuCl_2$	100 mg/L $CuCl_2$	100 mg/L $CuCl_2$	100 mg/L $CuCl_2$
pH	pH 7	pH 7	pH 7	pH 7
Temperature	32°C	28°C	28°C	28°C
Agitation	100 rpm	100 rpm	100 rpm	100 rpm
Incubation period	12 days	3 days	10 days	15 days
Antimicrobial activity (Clear zone; mm)*	18.08 ± 0.34^a	10.17 ± 0.67^d	13.64 ± 0.50^c	15.09 ± 0.67^b

**Clear zone in millimeters are shown as the mean ± standard deviation. Different superscript letters indicate significant differences ($p<0.05$). The data were validated for normal distribution using a Q-Q plot and analyzed using the ANOVA method. The Fisher's LSD test was employed for multiple comparisons between treatments.*

Table 6 Heat and enzyme tolerance characteristics of CF-SWUC02

Condition	Antimicrobial activity (Clear zone; mm)
Enzyme [ns]	
Untreated	18.46 ± 0.50
Treated with Proteinase K	17.48 ± 0.45
Treated with Pepsin	17.89 ± 0.80
Temperature (°C)	
32 (30 minutes)	19.57 ± 0.71[a]
40 (30 minutes)	19.63 ± 0.86[a]
60 (30 minutes)	19.72 ± 0.71[a]
80 (30 minutes)	19.50 ± 0.55[a]
100 (30 minutes)	19.07 ± 0.85[a]
121 (15 minutes)	16.96 ± 0.40[b]

Data are shown as the mean ± standard deviation. Different superscript letters in each row indicate significant differences ($p<0.05$). The data were validated for normal distribution using a Q-Q plot and analyzed using the Kruskal-Wallis method. The Mann-Whitney U test was employed for multiple comparisons between treatments. No significant differences are denoted by "ns".

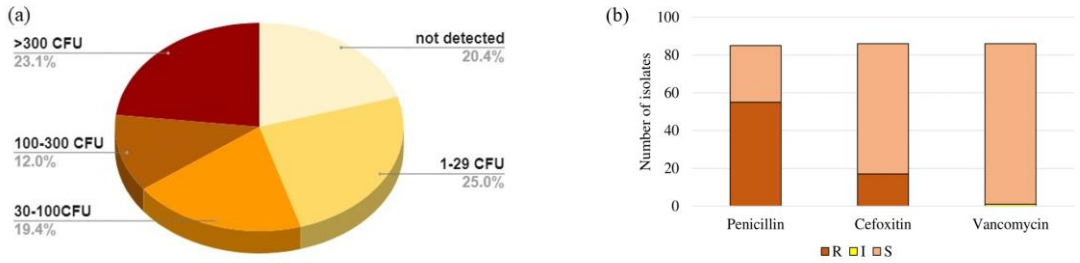

Figure 2 Prevalence of *S. aureus* in household wash basin. (a) Pie chart of the numbers of *S. aureus* (CFU) found per wash basin (100 cm^2), (b) antibiotic resistance characteristic of *S. aureus* isolates. R, I, and S represent resistant, intermediate, and susceptible, respectively.

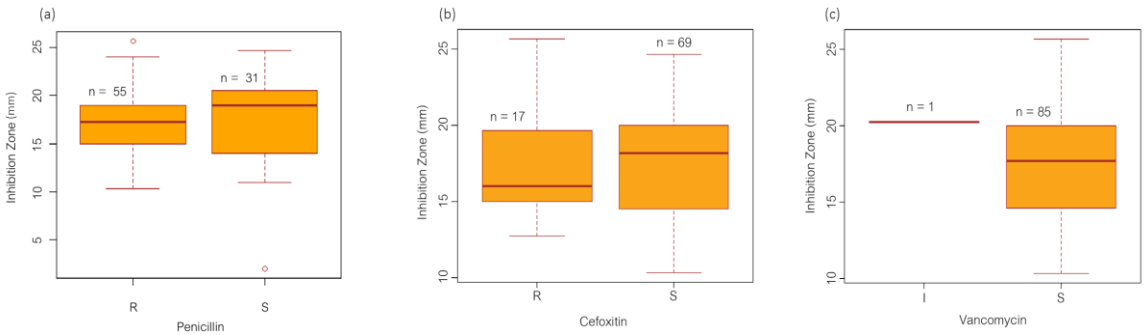

Figure 3 Inhibitory effect of CF-SWUC02 against the *S. aureus* isolates divided by their antibiotic resistance characteristics of (a) penicillin (b) cefoxitin (c) vancomycin. Data are shown as the boxplot of clear zones (mm) of isolates. n represents the number of *S. aureus* isolates. R, I, and S represent resistant, intermediate, and susceptible, respectively.

4.5 Inhibitory effect of CF-SWUC02 against drug resistance household *S. aureus* isolates

First, the prevalence and antibiotic resistance of *S. aureus* in household were assessed. Sampling data from 108 wash basins in 36 households showed that 20% of wash basin samples were not contaminated with *S. aureus*. However, 23% of the total wash basin samples were heavily contaminated

with *S. aureus* (more than 300 CFU/ 100 cm^2) and posed a health risk, while 44% had moderate contamination (less than 100 CFU/ 100 cm^2) (Figure 2a). The chosen *S. aureus* isolates were investigated for their susceptibility to penicillin and cefoxitin. Penicillin resistant isolates were 55 out of 86 isolates, and 20% (17 out of 86 isolates) were also resistant to cefoxitin. According to the CLSI (2021) standard, they were MRSA (Figure 2b and 3b). Fortunately, only one isolate was intermediate to vancomycin (Figure 2b and 3c), and it was not MRSA.

CF-SWUC02 was tested for its ability to inhibit the growth of *S. aureus* isolates, including those that are resistant to antibiotics. As shown in Figure 3, CF-SWUC02 had a significant inhibitory effect on the growth of MRSA, MSSA, and vancomycin susceptible *S. aureus* (VSSA). The inhibition zones ranged from 10-22 mm.

The prevalence of MRSA varies depending on the sampling environments. For example, in a study conducted in Nepal in 2019, 71% of 133 clinical specimens were MRSA while 29% were MSSA, and all were susceptible to vancomycin (Sapkota et al., 2019). In contrast, only 4% of *S. aureus* isolated from dairy farms in Ethiopia during the period from July 2020 to January 2021 were found to be MRSA, and 94% of *S. aureus* isolates were resistant to penicillin (Tibebu et al., 2021). In this study, 20% of *S. aureus* isolated from household wash basins were classified as MRSA. It is therefore not surprising that a slightly higher percentage of *S. aureus* was found in this sampling environment compared to that found in the farms. *S. aureus* is a common pathogen usually found on human skins, so it is expected to be present in household wash basins. According to the Regulation of the Ministry of Public Health No. 416 (B.E. 2563 (2020)) of Thailand, *S. aureus* should not be present in ready-to-eat food at levels above 100 CFU/g (Ministry of Public Health (Thailand), 2020). As shown in the results, around 35% of the household wash basins were contaminated with *S. aureus* at levels above 100 CFU/100 cm^2, and 64% of the *S. aureus* isolates were penicillin-resistant, with 31% of them (17 out of 55 isolates) were MRSA. This highlights the importance of proper antibiotic usage to prevent the increase of antibiotic-resistant bacteria and the need for continued research on novel antibiotics.

In this study, the drug resistance characteristics of *S. aureus* isolates were tested by various antibiotic groups including beta-lactam (penicillin and cefoxitin) and glycopeptide (vancomycin). We found that a number of *S. aureus* isolates were resistant to beta-lactam antibiotics. Multiple studies have consistently demonstrated the presence of penicillin resistance in both MSSA and MRSA isolates. Gurung et al. (2020) specifically found complete resistance to penicillin among MSSA isolates. Furthermore, MRSA isolates showed resistance to a broader range of antibiotics compared to MSSA isolates. Similarly, Pai et al. (2010) reported that all MSSA strains exhibited penicillin resistance. These findings collectively indicate a high level of penicillin resistance among both MRSA and MSSA strains. The heightened resistance of MRSA to multiple drugs highlights its substantial threat as a life-threatening infection, particularly in hospital environments.

CF-SWUC02 exhibited inhibitory effects against the tested isolates regardless of their drug resistance or susceptibility. The antimicrobial compounds present in CF-SWUC02 may have different inhibitory mechanisms compared to traditional antibiotics. Notably, CF-SWUC02 exhibited heat stability, which could confer certain advantages in specific applications. Traub & Leonhard (1995) conducted a study on the heat stability of antibiotics using a microtiter broth dilution MIC test. They observed that vancomycin displayed heat stability, with minimal changes in MIC values after autoclaving. In contrast, penicillin G and cefoxitin exhibited partial heat stability and heat lability, respectively, as their MIC values increased following autoclaving. These findings highlight the potential advantage of CF-SWUC02 over cefoxitin in terms of heat stability. This characteristic could make CF-SWUC02 a more reliable option in applications where heat exposure is involved, ensuring sustained antimicrobial activity.

Furthermore, heat tolerance is a valuable characteristic that holds relevance across numerous applications. *Pseudomonas* sp. are known for their ability to produce diverse metabolites with stability. For example, Gupta & Khare (2007) demonstrated that a purified lipolytic enzyme from *P. reinekei* displayed activity across a wide pH range (5.0-9.0) and remained stable for 24 hours at 40°C. This enzyme's properties make it suitable for biodiesel production, detergent formulations, and oil biodegradation. Similarly, alkaline protease from *P. aeruginosa* MTCC 7926 remained active in a pH range of 6-11 and at temperatures ranging from 25 to 65°C, making it a potential alternative for detergent formulations (Patil & Chaudhari, 2009).

Some *Pseudomonas* species are known to produce phenazine-1-carboxylic acid, which

exhibits antimicrobial properties against bacterial pathogens. Zhang et al. (2017) demonstrated the ability of *P. aeruginosa* PA31x to inhibit *Vibrio anguillarum* C312 through the production of phenazine-1-carboxylic acid, resulting in complete lysis of the bacteria. Similarly, Huang et al. (2022) reported that phenazine-1-carboxamide, produced by *P. aeruginosa*, suppressed the growth of clinical strains of *A. baumannii*. Although *P. aeruginosa* SWUC02 contains genes associated with phenazine-1-carboxylic acid production, its presence in our strain was not confirmed by our current study. Nevertheless, it is worth considering the potential involvement of other antimicrobial compounds in CF-SWUC02. The heat-tolerant enzymes and biosurfactants commonly found in *Pseudomonas* species should not be overlooked. A follow-up study will be conducted to identify the major bioactive compounds responsible for inhibiting multi-drug-resistant *S. aureus* and investigate their mode of action.

5. Conclusion

In this study, *P. aeruginosa* SWUC02 was found to have antagonistic activity against various common pathogenic bacteria, and CF-SWUC02 demonstrated particularly strong antagonistic activity against *S. aureus*. The active antimicrobial compounds produced by this strain were resistant to protease enzymes and high temperatures. The optimal culture conditions for inhibiting *S. aureus* were LB media supplemented with 0.01% $CuCl_2$, inoculated with $1x10^5$ $CFU.mL^{-1}$ of *P. aeruginosa* SWUC02, and incubated at 32°C with agitation (100 rpm) for 12 days. *S. aureus* contamination was also observed on household wash basins, with 20% of the isolates being MRSA. However, CF-SWUC02 showed inhibitory effects against all of these isolates, including MRSA. The mechanism for pathogen inhibition of CF-SWUC02 may warrant further study.

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