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Use of Bioluminescent Indicator *Acinetobacter* Bacterium for Screening and Characterization of Active Antimicrobial Agents

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Abstract Because of the need for new antimicrobial substances with novel mechanisms of action, we report here the use of an *Acinetobacter* reporter system for high-throughput screening of active antimicrobial agents. The bioreporter *Acinetobacter* strain DF4/PUTK2 carrying luciferase genes *lux*CDABE was chosen because of its ecological importance and it is widespread in nature. This bioreporter is genetically engineered to emit light constitutively that can be measured in real time by luminometry. Hence, this reporter system was employed to determine the bacteriostatic actions of spent-culture supernatants derived from twelve bacterial isolates. Out of the results, the strongest bioluminescence inhibitory effect of the supernatants was recorded with *Bacillus cereus* strain BAC (S5). Subsequently, ethyl acetate extracts of extracellular products of strain BAC (S5) were separated by a thin-layer chromatography (TLC). Based on the bioluminescence inhibitory assay, three fractions were found to have antimicrobial activity. One fraction (C) having the strongest antimicrobial activity was further purified using TLC and characterized by IR, ^{1}H NMR, mass spectrometry, SDS-PAGE, and amino acid composition analysis. The results predicted the presence of 2-pyrrolidone-S-carboxylic acid (PCA) and the octadeconic-acid-like fatty acid. Fraction C also demonstrated a broad inhibitory activity on several Gram-negative and Gram-positive bacteria. In conclusion, the *Acinetobacter* reporter system shows great potential to be a reliable, sensitive, and real-time indicator of the bacteriostatic actions of the antimicrobial agents.

Key words: Bacteriocin, antimicrobial activities, *Bacillus cereus*, bioreporter, bioluminescence, bioassay

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Many bacteria of different taxonomic branches and residing in various habitats produce antimicrobial substances that are active against other bacteria [20]. Both Gram-negative and Gram-positive bacteria produce bacteriocins, which constitute a heterologous subgroup of ribosomally synthesized antimicrobial peptides. In general, these substances are cationic peptides that display hydrophobic or amphiphilic properties, and the bacterial membrane is usually the target for their activity [26].

Universally used techniques for testing antimicrobial agents are the plating techniques of the agar well diffusion assay and agar spot test [16, 33, 26]. In those methods, the indicator strain is inoculated into/onto appropriate agar and the culture supernatant to be studied is placed into agar-cut wells (agar well diffusion assay) or a small inoculum of bacteria is spotted on the agar (agar spot test). The inhibitory activity of substances produced by the tested bacteria is then read after an incubation period by determining any inhibitory zone around the sample.

Despite that these traditional methods are relatively simple, several factors such as pH of medium, concentration of substances, and concentration of the strain used can cause them to be less sensitive. Moreover, they are also time-consuming and laborious. To address these shortcomings, more sensitive and rapid screening methods have been developed. There are methods based on, for example, turbidity, flow cytometry, measurement of ATP, and bioluminescence [19, 8, 4].

Recently, genetically engineered bioluminescent bacteria were applied to test antimicrobial activities as whole-cell biosensors [21, 29, 34]. In the present study, an easy, fast, and sensitive method for detection of antimicrobial substances was demonstrated. The method uses bioluminescent

indicator *Acinetobacter* strain DF4/PUTK2 [5, 1] for the detection of antimicrobial activities of bacterial spent culture supernatants. This bioreporter required no addition of substrate for light production because it was transformed with *luxAB* genes together with *luxCDE* genes responsible for regeneration of long-chain fatty aldehyde.

MATERIALS AND METHODS

Bioluminescent Bioreporter and its Growth Conditions

The bioluminescent assays were carried out using *Acinetobacter* bioreporter strain DF4/PUTK2 that had been genetically modified by conjugation [5, 1] to contain the plasmid PUTK2 [9], with the Tn4431 lux transposon downstream of a putative plasmid maintenance promoter to produce continual visible light, so-called bioluminescence. The culture medium for stock cultures of the bioreporter DF4/PUTK2 was LB broth (Merck, Darmstadt, Germany). The working culture was prepared by transferring each bacterium from stock culture to 50-ml conical glass flasks containing 20 ml of medium. Culture flasks were aerobically incubated on a rotary shaker at 30°C for overnight.

Screening for Antimicrobial Agents

Briefly, cell-free supernatants were prepared as follows: twelve bacterial isolates, described elsewhere [2], were grown in tryptone soya broth (TSB, Oxoid Co. Hampshire, U.K.) for overnight at 30°C with aeration. Subsequently, bacterial cells were removed by centrifugation and the resulting supernatants were filter-sterilized through a 0.22-μm-pore-size filter (Millipore, Corp.). The cell-free supernatants were either used immediately or stored frozen at -20°C. The bioluminescent bioreporter strain DF4/PUTK2 was grown for overnight at 30°C in LB medium. Afterward, cultures were diluted 1:10 in LB medium and incubated with shaking at 30°C to an optical density of 0.6 at 600 nm. The culture was washed two times in LB medium and resuspended in an equal volume of LB. Two ml of the culture was then added to 20-ml scintillation vials containing 2.0 ml minimal salts medium supplemented with 10% cell-free supernatant [3]. Aliquots of 200 μl were removed from each scintillation vial and transferred to black 96-well microtiter plates (Nunc, Roskilde, Denmark) to produce 3 replications of each dilution. Wells were covered with transparent plate sealer and placed in a microtiter plate reader luminometer (Lumistar Galaxy, BMG Lab. Technologies GmbH, Offenburg, Germany) for luminescence detection at room temperature. A control, which was free of any chemical induction, was run with each experiment. In all measures, the percent of bioluminescence inhibition (BI %) was determined by comparing the response given by a control (no addition) to that corresponding with the sample [12].

Extraction and Isolation of Bactericidal Compounds

To obtain metabolites with antimicrobial activity, isolate BAC (S5) was inoculated into 1-l cultures of M-9 minimal liquid medium [13] supplemented with 2% glutamic acid as a carbon source and cultured in a bottle for 2 days at 30°C. Cultures were centrifuged at 10,000 rpm for 20 min and the supernatant was sterilized by 0.2 mm Millipore filtration. Supernatant was extracted with 1 l of analytical grade ethyl acetate (Merck, Darmstadt, Germany) in separating funnels at room temperature (20°C). The ethyl acetate soluble fraction was concentrated using a rotary evaporator and dewatered using Na_2SO_4. The crude extract was separated into its constitutive fractions by thin-layer chromatography (TLC). A 9:1 mixture of ethyl acetate and petroleum ether was found to be the best eluent in preliminary trials. Three fractions A, B, and C appeared, and subsequently, several TLC runs were made to purify these fractions and calculate their rf values. The fractions were visualized under UV light.

Bacteriostatic Activity of Metabolite Fractions

Bioluminescence assays using the bioreporter strain DF4/PUTK2 of the three fractions (A-C) were performed using the same procedure as described above. However, the analysis was performed using various concentrations ranging from 1–45% of each fraction. Out of the bioluminescence inhibitory assay, fraction C showed the strongest activity. Subsequently, it was subjected to further bioassay of effects of this metabolite on different Gram-positive and Gram-negative bacterial indicators. The antimicrobial activity of fraction C against all indicator strains (listed in Table 2) was detected by agar disc diffusion assay [25].

Purification and Characterization of Fraction C

The C fraction was of particular interest because of its broad spectrum of antibacterial activity. After initial separation from the crude metabolite extract, this fraction was purified twice by TLC using the eluents listed above. The purified C fraction was then subjected to characterization studies using IR (Bruker Optics Billerica, MA, U.S.A.), NMR (^{1}H NMR spectrum was recorded on a Bruker DRX300 [300 MHz] spectrometer with DMSO as a solvent), and MS analysis (recorded on a Jeol SX-102 double-focusing spectrometer, Tokyo, Japan). To determine amino acids composition, the TLC-purified C fraction was first hydrolyzed in 6 M HCl under vacuum at 110°C for 24 h. The amino acids were then derived with *ortho*-phthalaldehyde and analyzed with the EzChrom amino acid analyzer (EzChrom Scientific, San Ramon, CA, U.S.A.).

RESULTS AND DISCUSSION

Numerous methods for screening new antimicrobial substances now exist; however, these are often laborious techniques,

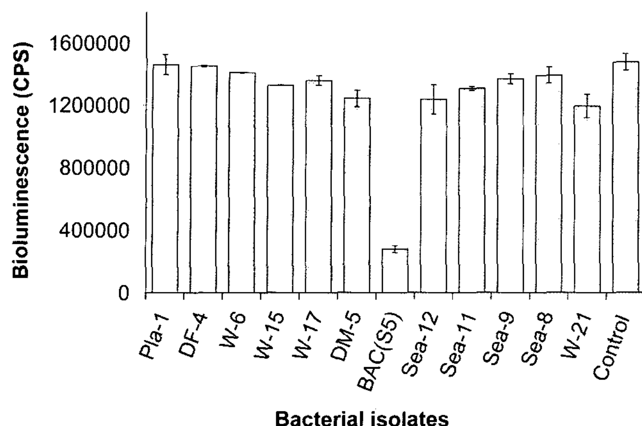

Fig. 1. Screening for antimicrobial activity in twelve bacterial isolates using bioluminescent indicator *Acinetobacter* bacterium strain DF4/PUTK2.

The values of the assay are means of three replications. CPS, luminescence counts per second. Error bars represent standard error of the mean (n=3).

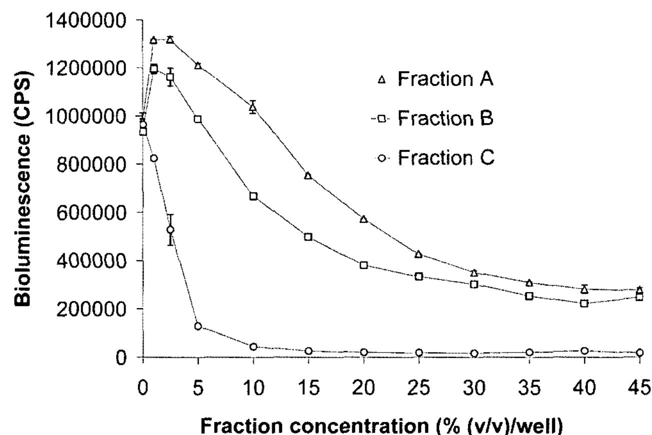

Fig. 2. Bacteriostatic activity of metabolite fractions (A, B, and C) using bioluminescent bioreporter strain DF4/PUTK2.

The values of the assay are means of three replications. CPS, luminescence counts per second. Error bars represent standard error of the mean (n=3).

time-consuming, complex and/or require expensive equipment [16, 19]. Through this study, an easy, fast, and sensitive method for screening for antimicrobial agents was demonstrated. The method uses a bioluminescent indicator *Acinetobacter* bacterium strain to detect antimicrobial activities. The effect of the spent culture supernatants of twelve tested bacterial isolates on the bioluminescence of strain DF4/PUTK2 was conducted at 5 min as the selected shortest time exposure for the bioassay. Obviously, in Fig. 1, bioluminescence was significantly decreased (*t* test, $P=0.05$) with the spent culture supernatant of *Bacillus cereus* strain BAC(S5). However, no considerable bioluminescence response was observed with the rest of the examined isolates ($P>0.6$, *t*-test; data not shown).

Because of the above results, *Bacillus cereus* strain BAC(S5) was chosen for further analysis. It is established that *Bacillus* is an interesting genus to investigate for antimicrobial activity, as the *Bacillus* species produce many peptide antibiotics representing several different basic chemical structures [36]. Members of the so-called *Bacillus cereus* group (consisting of the five closely related species *B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. mycoides* and the psychrotolerant *B. weihenstephanensis*) are capable of producing so-called bacteriocin-like inhibitory substances [26, 37, 7]. At all bioluminescence assays, we did not test the cell viability of strain DF4/PUTK2, as the bioluminescent level changes of the control wells can explain the cell viability. Previously, Virta *et al.* [35] have shown that the changes in bacterial luminescence closely correlate with the number of CFU, when bacteria are killed by antimicrobial agents. They also concluded that the luminometric method offers an alternative to flow cytometry for measuring cell viability.

Extraction and isolation of the inhibitory compounds revealed the presence of three active fractions (A, B, and C) in the spent culture supernatant of strain BAC(S5). The effect of different concentrations (1–45% v/v) of purified fractions on bioluminescence of the bioreporter DF4/PUTK2 is presented in Fig. 2 and Table 1. Generally, the obtained results showed that bioluminescence inhibition could be correlated with the fraction concentration up to 10% with fraction C (BI%=97–98%) and 45% with fractions A and B (BI%=70–76%), respectively. Obviously, bioluminescence was significantly decreased (*t* test, $P=0.05$) at concentration 2.5% (v/v) of fraction C (Fig. 2) exhibiting BI% value >50% (Table 1). In contrast, both fractions A and B exhibited low bioluminescence inhibition values at concentrations 10 and 5%, respectively. The most significant (*t* testm $P=005$) decrease in the bioluminescence (>50% inhibition) was observed at concentrations up to 25% and 20%, respectively.

Previously, luminescent *Staphylococcus aureus*, *Streptococcus*, *Escherichia coli*, and *Salmonella* have been

Table 1. Bioluminescence inhibition percentages (BI %) of strain DF4/PUTK2 in response to fractions A, B and C and their concentrations (1 to 45%).

Concentration (%)	Bioluminescence inhibition (%)		
	Fraction A	Fraction B	Fraction C
1	-34.4304	-27.9922	14.42419
2.5	-34.6813	-24.2427	50.13185
5	-23.6682	-5.81694	86.45319
10	-5.95795	28.4211	97.17849
15	22.95943	46.61006	97.29269
20	41.52188	59.23534	97.88726
25	56.33028	64.37104	98.12641
30	64.25075	67.68785	98.19974
35	68.47772	72.82356	98.87528
40	71.30384	76.14037	98.21951
45	71.64373	75.35853	98.13289

Table 2. Antibacterial activity spectrum of the extracted fraction C.

Indicator strains	Source*	Inhibition zone (mm)**
Bacillus sp. strain BAC(S-5)	Abd-El-Haleem *et al.* (2002)	25
Bacillus cereus	ATCC 14579	27
Staphylococcus aureus	ATCC 9144	25
Microbacterium sp. strain Pla-1	Abd-El-Haleem *et al.* (2002)	23
Escherichia coli (E1)	Abd-El-Haleem *et al.* (2003)	15
Klebsiella pneumoniae strain DM5	Abd-El-Haleem *et al.* (2002)	12
Pseudomonas aeruginosa (P1)	Abd-El-Haleem *et al.* (2003)	13
Salmonella typhi (S1)	Abd-El-Haleem *et al.* (2003)	15

*ATCC: American Type Culture Collections.
**Diameter of the inhibition zone in millimeter (mm) around the disc.

used successfully for the measurement of antibacterial activity [36, 21, 34]. However, most of these bioreporters are bacterial pathogens that were genetically modified to measure in a narrow range the antimicrobial activity and the mode of action of certain antibiotics (*e.g.*, penicillin, chloroexidine, tetracycline, and moxifoxacin). Here, therefore, the light-emitting *Acinetobacter* DF4/PUTK2 cells were employed as a bioreporter to discover novel antibacterial agents. It is well known that many members of the genus *Acinetobacter* are widespread in nature, and can be obtained from water, soil, living organisms, and even from human skin [1]. In addition, some *Acinetobacter* species have become resistant to almost all antimicrobial agents that are currently available, including the aminoglycosides, quinolones, and broad-spectrum β-lactams [22]. Most *Acinetobacter* strains are resistant to cephalosporins, and resistance to carbapenems is being increasingly reported [27, 23].

Out of these findings, the greatest bioluminescence inhibition was obtained with fraction C. Therefore, it was of particular interest to observe the occurrence of bacteriostatic effects of fraction C on some Gram-negative and Gram-positive bacterial indicators. According to the data presented in Table 2, the inhibitory spectrum of fraction C was particularly more active with the four examined Gram-positive strains, *Bacillus cereus* strain BAC(S-5), *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 9144, and *Microbacterium* sp. strain Pla-1. However, it also included the four tested Gram-negative pathogenic strains, *Escherichia coli* (E1), *Klebsiella pneumoniae* strain DM5, *Pseudomonas aeruginosa* (P1), and *Salmonella typhi* (S1). These data suggested that fraction C might be a bacteriocin that is effective against both Gram-positive and Gram-negative bacteria, and therefore, it was subjected to further characterization study.

The IR absorption spectrum of fraction C (Fig. 3) shows the presence of -OH functions (wide band between 3,500 and 3,400 cm^{-1}), aliphatic chains and $-CH_3$ (peaks at 2,920, and 2,860 cm^{-1}), and an amide moiety (peaks at 1,654 and 1,565 cm^{-1}). The bands of 1,275–1,200 and 1,075–1,020 cm^{-1} indicate -C-O-C functions. The proton NMR spectrum of fraction C is shown in Fig. 4. The following features were observed: methyl protons at 0.88 ppm, methine protons at 1.21 ppm, and $-CH_2$ groups at 1.27 ppm. -C-OH groups could be identified at 3.23, 3.50, and 3.62 ppm. As shown in Fig. 5B, the first mass spectrum profile of the fraction C showed several signals: the molecular ion at *m/z* 129, a base ion at *m/z* 84, and an ion at m/z 56. Out of these peaks, it may be possible to conclude that fraction C

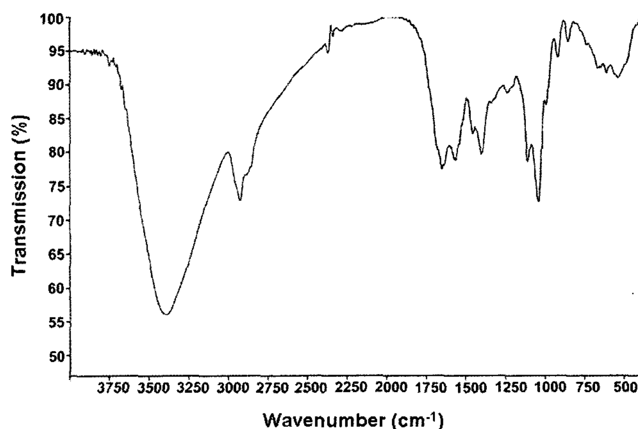

Fig. 3. Infrared spectrum of active fraction C.

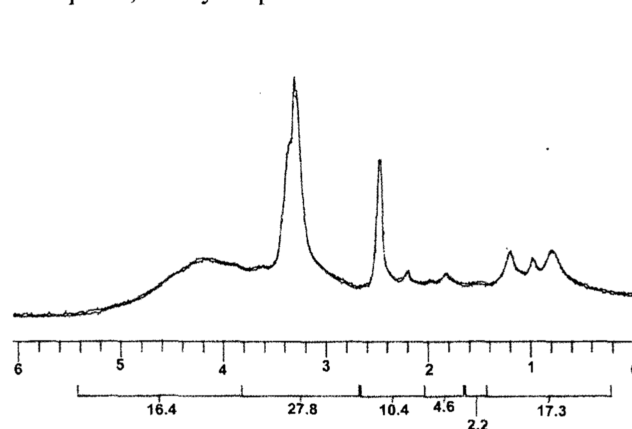

Fig. 4. ^{1}H NMR spectrum of active fraction C.

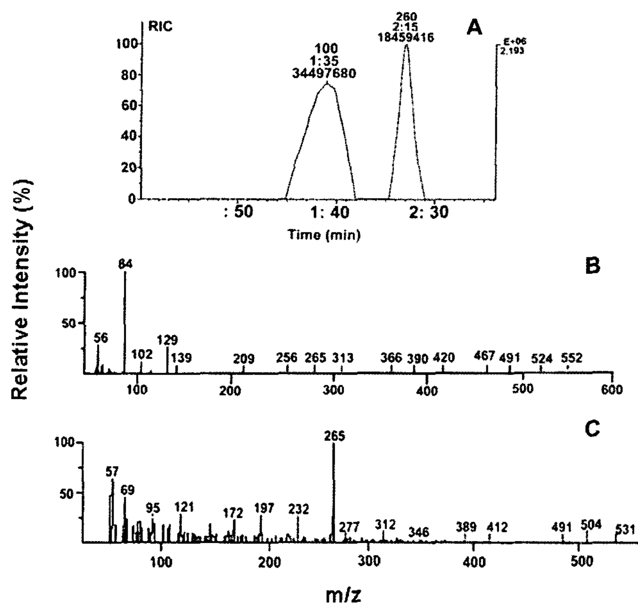

Fig. 5. EI mass spectrum of the purified C fraction; **A**. Total Ion Chromatogram (TIC); **B** and **C** are mass spectrometry profiles of the peaks in the TIC shown in **A**.

contains 2-pyrrolidone-S-carboxylic acid (PCA). However, the second mass spectrum profile, presented in Fig. 5C, revealed the presence of octadeconic-acid-like fatty acid, as predicted by the mass spectrometer software tools attached to the instrument. Previously, Engelhardt and coworkers [11] had identified PCA in coffee by mass spectroscopy. They reported that the spectra of the trimethylsilylated sample showed a very weak molecular ion at *m/z* 273, a base ion at m/z 156, and two small ions at *m/z* 258 and *m/z* 230, respectively. The molecular ion peak and base ion peak correspond to those (*m/z* 129 and *m/z* 84) present in our mass spectra obtained in this study.

PCA is a natural constituent of foods of plant origin, including vegetables and fruits [6], and fermented soybean and cereal products [24, 32]. Among lactic acid bacteria strains, *S. bovis* has previously been shown to produce PCA by conversion of glutamine [10]. In addition, Huttunen *et al.* [15] reported that purified PCA [separated from *Lactobacillus casei* ssp. casei LC-I (*LCC*) and *L. casei* ssp. pseudoplantarum LB1931 (*LCP*)] showed inhibition toward several spoilage bacteria, such as *Bacillus subtilis* 1205, *Bacillus subtilis* MCM-I, *Enterobacter cloacae* 1575, and *Pseudomonas putida* 1560-2. It is also known that under certain conditions, some microorganisms possessing lipolytic activities may produce significant amounts of fatty acids [30, 28]. The antimicrobial activity of fatty acids has been recognized for many years. The unsaturated fatty acids are active against Gram-positive bacteria, and the antifungal activity of fatty acids is dependent on chain length, concentration, and pH of the medium [14]. The antimicrobial action of fatty acids has been thought

Table 3. Amino acid composition of the fraction C.

Amino acids	Concentration of residues (μg/ml)	Concentration of residues (%)
Serine	1.84	4.53
Glutamic	17.4	42.82
Glycine	2.45	6.01
Alanine	3.28	8.07
Phenylalanine	0.18	0.45
Cysteine	5.76	14.17
Lysine	2.20	5.41
Arginine	1.74	4.28
Valine	1.04	2.56
Histidine	4.75	11.69

to be due to disassociated molecules, not the anion, since pH had profound effects on their activity, with a more rapid killing effect at lower pH [17].

The amino acid composition of fraction C analyzed by mass spectrometry is shown in Table 3. The composition was particularly high in glutamic acid residues (42.8%). Because of the higher ratio of cysteine (14.17%) and to confirm the presence of disulfide bond, fraction C was subjected to further analysis by reduced and nonreduced SDS-PAGE, as described elsewhere [18]. As shown in Fig. 6, the molecular mass of the bacteriocin was about 30 kDa with 2-ME and about 55 kDa without, which confirmed the cysteine formed disulfide bridge. It is known that disulfide bonds can make considerable contributions

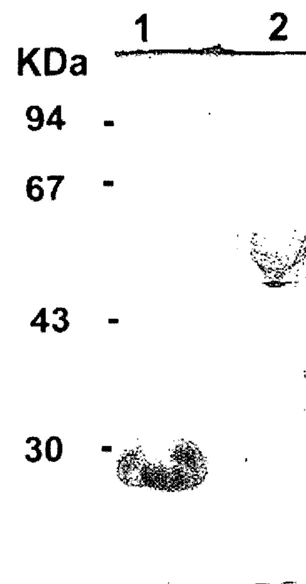

Fig. 6. Reduced and nonreduced SDS-PAGE to confirm the presence of disulfide bond in fraction C.

Lanes 1 and 2 are TLC-purified fraction C with and without 1% 2-mercaptoethanol (2-ME) in the SDS loading buffer as a reducing agent, respectively.

to stability, an effect mainly attributed to the decreased entropy of the denatured protein [18]. In conclusion, the present work has shown that it is possible to use the bioluminescence inhibition assay of the bioreporter DF4/PUTK2 as a simple, fast, and reliable tool for screening of novel antibacterial substrates. More analyses of fraction C including its sensitivity to enzymes, pH, detergents, heat treatment, and molecular cloning of its gene(s) to identify the pathways of synthesis in *Bacillus cereus* strain BAC(S5) are currently underway.

Acknowledgments

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