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Antimicrobial Activities of Bacteriocin-Like Extracellular Metabolites Produced by Soil Bacteria

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Abstract

In an attempt to competitively inhibit other organisms within the same environment, microbial cells produce a wide range of bioactive bacteriocins and bacteriocin-like extracellular metabolites (BLEM). Bacterial species (*Bacillus* and *Pseudomonas*) were isolated from soil samples, screened for BLEM production and the crude activity of cell free metabolites determined by agar diffusion assay. Partial purification of metabolites was achieved by protein precipitation with ammonium sulfate and BLEM sensitivities to temperature, pH, surfactant and ultraviolet radiation along with the mode of action of partially purified BLEM on indicator strains and other pathogens were characterized. *Bacillus subtilis* SF8 and *Pseudomonas aeruginosa* SF4 produced BLEM inhibitory to closely related strains with partially purified activity units of 768 and 2048AU/mL respectively. BLEMs retained large percentages of their inhibitory activities over a wide range of temperature and pH, 71%, 92% and 80% activity was retained by BLEM from *B. subtilis* SF8 while 91%, 81% and 89% activity was retained by BLEM from *P. aeruginosa* SF4 after 60 minutes of BLEM pre-incubation at 90^0C, pH 4 and 10, respectively. Ultraviolet radiation enhanced BLEM production in *P. aeruginosa* SF4 but not in *B. subtilis* SF8. Fourier Transformed Infrared (FTIR) spectroscopy revealed that BLEMs from both *B. subtilis* SF8 and *P. aeruginosa* SF4 interfered with cell membrane components of *B. macerans* SF2 and *P. fluorescence* BS2 respectively. The BLEM also had a broad inhibitory range on test bacterial and fungal pathogens.

1 Introduction

Microbial bacteriocin-like peptides are unique groups of extracellular antimicrobial metabolites produced by living microbial cells within a particular environment and also considered to be part of the host innate immunity[1]. Bacteriocins and bacteriocin-like metabolites are usually produced as excretory metabolites of microbial cells usually against closely related and at times distant species to the producing organism[1,2]. The bacteriocin family includes diverse microbial excretory proteins in terms of size, mode of action, transportation, release, microbial target and immunity mechanisms and can be broadly divided into those produced by Gram-negative and Gram-positive bacteria[3].

Bacteriocins from Gram positive bacteria are often shorter chain, low molecular weight peptides and generally divided into classes based on their morphology, size, physical, and chemical properties[4]. The genus *Bacillus* contains a number of bacteriocinogenic species, which produce bacteriocins such as subtilin, subtilosin, coagulin and megacin as well as bacteriocin-like metabolites[2, 5]. Several species of *Pseudomonas* are excellent bacteriocin and bacteriocin-like metabolite producers. *Pseudomonas aeruginosa* presents an example of such excellence, and many of its strains produce bacteriocins such as the phage tail pyocins, pore formers and several other bacteriocin-like inhibitory metabolites as well as an uncharacterized array of bacteriocins[6,7].

Some limitations ascribed to the use of prescribed therapeutic antibiotics include possible initiation of allergic reactions in individuals, elimination of normal flora when administered, and ineffectiveness against several antibiotic-resistant resistant microorganisms. The search for antimicrobial bacteriocins, some of which are already proven to kill bacteria in animal models[8], is imperative due to their activity against antibiotic resistant isolates, safety, as well as reduced allergic reactions in individuals when compared to regular microbial antibiotics as regards their clinical utilization and in food and feed production[9].

The intent of this research work was therefore to screen for, partially purify and characterize bacteriocin-like extracellular antimicrobial metabolites from *Pseudomonas* and *Bacillus* species isolated from soil and study their inhibitory effects on closely related species, other representative pathogenic bacteria and fungi.

2 Materials and Methods

2.1Sample collection, microbial isolation and identification

Soil samples were taken from several locations within Ibadan, Oyo state and used in the isolation of bacteria. Ten grams (10g) of each soil sample were placed in 90 mL distilled water and vortexed vigorously. Serially diluted soil samples were thereafter plated on nutrient agar, tryptone soy agar and *Pseudomonas* isolation agar using the pour plate method and incubated at 30^{o}C. The isolated bacteria were identified morphologically and biochemically[10] as *Bacillus* and *Pseudomonas* strains.

2.2 Screening for bacteriocin-like metabolite production by Bacillus and Pseudomonas species

Primary screening was done by growing pure cultures of bacterial isolates in 10ml of Nutrient broth at 37° C for 24 hours. Aliquots, (2µl), of the cultures were spotted onto Nutrient agar plates which after 18 hrs were overlaid with 5ml of 0.7% soft agar inoculated with the cell suspension of the test indicator strain. The plates were then incubated at 30°C for 24-72 hours. Inhibition against indicator organism was scored positive if the zone formed was wider than or equal to 2mm in diameter under each test condition[11]. The antimicrobial activity of metabolites from the selected species of *Pseudomonas* and *Bacillus* against other closely-related bacterial species isolated from the same ecological niche (secondary screening) was determined by the agar-well diffusion assay method, using the cell-free metabolites of the producer strains[12].

2.3 Bacteriocin titre determination

The titre of inhibitory metabolites produced was quantified by a two-fold serial dilution of the metabolites in saline solution[13]. The unit of antimicrobial activity of the metabolite was then defined as the reciprocal of the highest dilution showing at least 2mm diameter inhibition of the indicator lawn and expressed in Activity units per ml (AU/ml).

2.4 Partial purification of bacteriocin-like metabolite

Partial purification of cell free bacteriocin sample was done by protein precipitation with ammonium sulfate from 0% - 70% saturation followed by 70% - 90% saturation and centrifuged after storing overnight at 4^{o}C[14]. The centrifuged precipitates were pooled, re-suspended in 10mM phosphate buffered saline (pH 7) to the initial volume of culture filtrate; then dialyzed against the same buffer in a tubular cellulose membrane (spectra por number four dialysis tube) for 18 hours at 4°C for further purification and elimination of salt.

2.5 Characterization of partially purified metabolite

2.5.1 Sensitivity to temperature and pH

To determine the effect of temperature and pH on the residual activity of partially purified bacteriocin-like metabolites produced by selected *Pseudomonas* and *Bacillus* species, aliquots (500µl) of their partially purified metabolite contained in screw-capped tubes was incubated at various temperatures (30, 40, 50, 60, 70, 80, 90 and 100 °C) for 15, 30 and 60 minutes to determine their sensitivity to temperature. Incubation of equal volumes of BLEM (500µl) was done with appropriate buffers at pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10 to determine the effect of pH on the activity of these partially purified metabolites[15]. The inhibitory activity of untreated metabolite, in each case of percentage residual activity determination (assayed at room temperature and pH 7), was measured as control at 100% inhibition.

2.5.2 Effect of ultra violet radiation on BLEM production

Fresh four hour old cultures of each of *Bacillus* and *Pseudomonas* species, (500µL) were exposed to ultraviolet irradiation within time ranges of 2 to 10 minutes at 25^{o}C[16]; at a distance of 30 cm from an 8W (254 nm) UV transilluminator. To compare the yield of their inhibitory metabolites, nutrient broth cultures of these organisms grown in the absence of ultraviolet irradiation were used as controls. The amount of bacteriocin-like inhibitory metabolite expressed by these cultures was then determined using the agar well diffusion method and compared.

2.5.3 Hemolysis and hemagglutination tests

For hemolysis test, 5% mice erythrocytes in Nutrient agar plate were inoculated with purified BLEM, incubated for 24hours and observed for clearing zones (positive) while 50% (v/v) BLEM was mixed with 5% mice erythrocytes for 15minutes and observed for settling (negative) or agglutination (positive).

2.5.4 Mode of action of partially purified bacteriocin-like metabolite

The effect of the partially purified metabolite activity on closely related indicator organisms was analyzed by FTIR spectroscopy

after subjecting to the treatment described below. The purified *Bacillus* BLEM was added at 10% (v/v) to 0.5 McFarland cell suspensions of the indicator organism, *B. macerans* SF2, while the *Pseudomonas* BLEM was added to *P. fluorescence* BS2. After incubation for 60 minutes, both treated and untreated (control) cells of the indicator organisms were washed 3 times with sterile distilled water and 20µL of each bacterial sample (treated and control indicator cells) was evenly applied onto the spectroscope's ZnSe optical plate, dried for approximately 15 minutes and then analyzed to generate curves based on the detection and abundance of cellular molecules through bond vibration frequency. The effect of each BLEM on indicator cells was determined through the differences observed in the orientation and peaks of generated curves with both experimental and control cultures[17].

2.5.5 Antimicrobial spectrum of bacteriocin-like metabolites

Antifungal properties of the bacteriocin-like extracellular metabolites of the producer bacteria (*B. subtilis* and *P. aeruginosa*) on post-harvest spoilage and pathogenic fungal species was carried out by introducing 50µl of BLEM into a 6 mm well bored in a potato dextrose agar plate which had been surface seeded with 0.5 ml haemotocytometer-count of 5 x 10^6 indicator fungal cells per ml[18]. The plates were incubated at 28±2^{o}C and observed daily over 5 days[19].

Inhibitory activity of BLEM on methicillin-resistant *Staphylococcus aureus, Enterococcus faecalis*, *P. aeruginosa* ATCC 29853, *Salmonella typhi* 33458, *E. coli* 35218 and *E. coli* 23218 was performed using the agar well diffusion procedure[12]. 50µl each of BLEM was introduced into 6mm agar wells in nutrient agar plates seeded with 0.5 McFarland cell suspensions of the bacteria cells and incubated at 37^{o}C for 24 hours. In all tests, a clear zone (≥ 2mm) around the well was taken as inhibition against each test pathogen, while the cell suspension solution (saline solution or buffer) without the cells was used as control for each case of BLEM activity determination.

3 Results and Discussion

3.1 Microbial isolation and screening for BLEM production

Eleven of the 46 isolate screened were found positive for the spot and/or agar overlay inhibitory metabolite production test(s) and thereafter subjected to further screening based on the characteristics of their inhibitory metabolites. Cell-free extracellular metabolite of *Bacillus subtilis* SF8 (pH 6.8) had higher activities and inhibited the growth of closely related strains; *B. cereus, B. subtilis* SC1 and *B. macerans* SF2 while the metabolite from *Pseudomonas aeruginosa* SF4 (pH 7.3) inhibited the growth of *P. aeruginosa*ATCC29853 and *P. fluorescence* BS2 (Table 1). In contrast to traditional antibiotics, bacteriocins usually target organisms closely related to the producer species (although some exceptions exist)[24].

As a result, *Bacillus macerans*SF2 and *Pseudomonas fluorescence* BS2 were selected as indicator organisms for subsequent tests with the BLEMs produced by *Bacillus subtilis*SF8 and *Pseudomonas aeruginosa*SF4 respectively. It was also observed that BLEMs were not inhibitory to the producer-strain in each case.

3.2 Partial purification of bacteriocin-like metabolite

As similarly reported for partial purification of bacteriocins from *Lactobacillus* species[14], inhibitory activity was detected only in the salt precipitate and not in the supernatant of both strains of *Bacillus* and *Pseudomonas* species. The activity unit of inhibitory metabolite of *B. subtilis* SF8 against its indicator strain (*B. macerans* SF2) increased from 640 to 768 AU/ml after partial purification (Table 2) while a higher increment was observed in the partially purified excretory metabolite of *P. aeruginosa* SF4 (1536 AU/ml to 2048 AU/ml).

3.3 Characterization of partially purified metabolites

One fascinating property of bacteriocins and BLEM is their ability to retain large percentages of their inhibitory activities over a wide range of temperature[25]. Analysis of the effect of temperature on the stability of the inhibitory activities of BLEMs from *B. subtilis* SF8 *and P. aeruginosa* SF4 showed that these metabolites were relatively thermostable, more than 70% residual activity was retained by the partially purified inhibitory metabolites of both *B. subtilis* SF8 *and P. aeruginosa* SF4 at 30^{0}C to 90^{0}C pre-incubation temperature range for 60minutes (Figures1a and 1b). At 100^{0}C, the bacteriocin-like excretory metabolite of *B. subtilis* SF8 exhibited 83.3, 54.3 and 41% residual activities after pre-incubation periods of 15, 30 and 60min, respectively. Likewise, the bacteriocin-like extracellular metabolite of *P. aeruginosa* SF4 retained more than 90% inhibitory activity at 90^{0}C even after 60 minutes pre-incubation time. Residual activities of 92.1, 92.1 and 85.3% were retained at 100^{0}C after 15, 30 and 60 minutes pre-incubation periods respectively. All activity was lost in both BLEMs after autoclaving the metabolites for 15 minutes.

Partially purified BLEMs of both *B. subtilis* SF8 *and P. aeruginosa* SF4 retained more than 80% of their inhibitory activities over the pH range of 4 to 8 but had no activity at pH 2 (Fig. 2).The activity of antimicrobial metabolite from *B. subtilis*SF8 increased gradually from pH 3 and reached its maximum inhibitory activity at neutral pH (pH 6 and 7), above which there was a sharp decline in inhibition activity. That of *P. aeruginosa* SF4 recorded its maximum activity between pH 7 and 8 and still recorded 86.1% activity at pH 10. Bacteriocins and bacteriocin-like extracellular metabolites have been broadly documented to retain a significant fraction of their inhibitory properties over a wide pH range[26, 27, 28]. The bacteriocin-like metabolite of *P. aeruginosa* SF4 and *B. subtilis* SF8 retained

more than 80% of its inhibitory activity within the pH range of 4-8 with optimum activity recorded at pH 6 to neutral pH.

The addition of surfactants to the partially purified metabolite of *B. subtilis* SF8 recorded an increment in percentage inhibition varying between 0 and 9% with the addition of 30% SDS and EDTA, and 4.5% increment with triton X-100 (Fig. 3a). The range of increment in percentage inhibition of 38.9 - 58.3% was measured with the addition of different concentrations of SDS to the bacteriocin-like inhibitory metabolite of *P. aeruginosa* SF4 while the range of increment in activity of 55.5 – 58.3% was measured when different concentrations of triton X-100 was added to this metabolite (Fig. 3b).

Table 1: Activity of crude and partially purified BLEMs of *B. subtilis* SF8 *and P. aeruginosa* SF4 on indicator strains

BLEM Producer strains	Activity Unit (AU/mL)		Indicator strain/Zone of inhibition (mm)					
	Crude	Partially purified	*P. fluorescence* BS2	*P. putida*	*P. aeruginosa ATCC9853*	*B. macerans* SF2	*B. cereus* SF4	*B. subtilis* SC1
B. subtilis SF8	640±42.4	768±36.77	0±0.00	0±0.00	7±0.00	12±2.83	11±0.00	11±2.83
*P. aeruginosa*SF4	1536±25.46	2048±0.00	18±2.83	12±4.24	8±1.41	0±0.00	0±0.00	0±0.00

* results are average of triplicate values

Table 2: Antimicrobial activities of BLEM from *B. subtilis* SF8 and *P. aeruginosa* SF4 on plant pathogenic fungi and clinical pathogenic bacteria

Indicator organisms	BLEM producer strain/Diameter of inhibition (mm)	
	B. subtilis SF8	*P. aeruginosa* SF4
Rhizopus nigricans ART98	4	0
Penicillium oxalicum ILE1-OB	5	0
Penicillium chrysogenum TZPB10	4	6
Fusarium oxysporum RA1486	0	0
Fusarium compacticum	0	0
Aspergillus niger OK1482	0	0
Methicillin Resistant *S.aureus*	0	13
Enterococcus faecalis	0	0
P. aeruginosa ATCC 29853	7	8
Salmonella typhi 33458	0	0
Escherichia coli 35218	0	0
Escherichia coli 23218	0	6

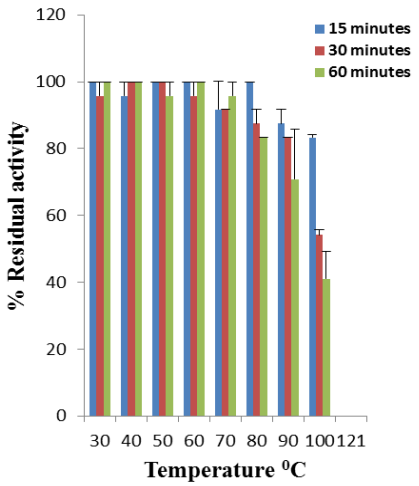

Fig 1a: Effect of temperature on the stability of BLEM produced by *B. subtilis* SF8

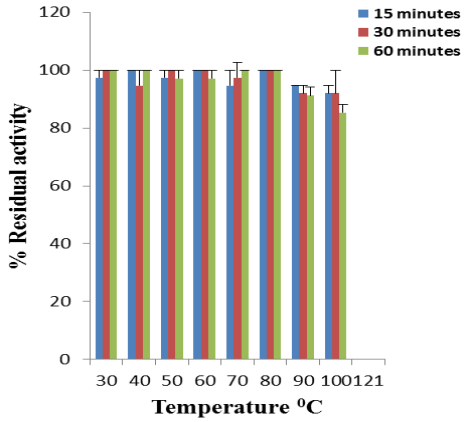

Fig 1b: Effect of temperature on the stability of BLEM produced by *P. aeruginosa* SF4

However, the activity of this partially purified metabolite was not significantly affected with the addition of EDTA. The peptidoglycan layer in Gram negative bacteria, compared to that

of Gram-positive bacteria, is generally thinner; though the outer membrane in the former group protects cells from many external agents. It is, however, possible to weaken the protective abilities of this outer membrane by various membrane-disorganizing permeabilizers like EDTA and surfactants, which could result in an increased permeability of such membrane to toxic agents[29].

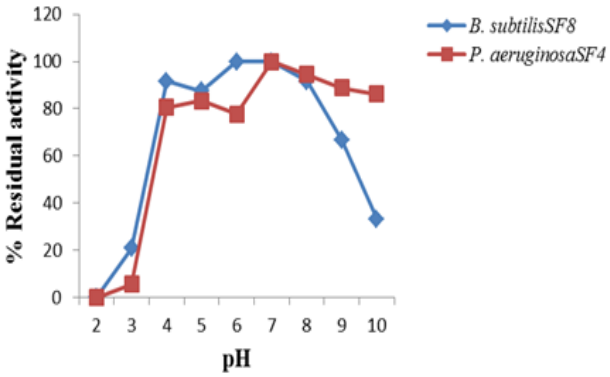

Fig 2: Effect of pH on the activity of BLEMs produced by *B. subtilis* SF8 and *P.aeruginosa* SF4

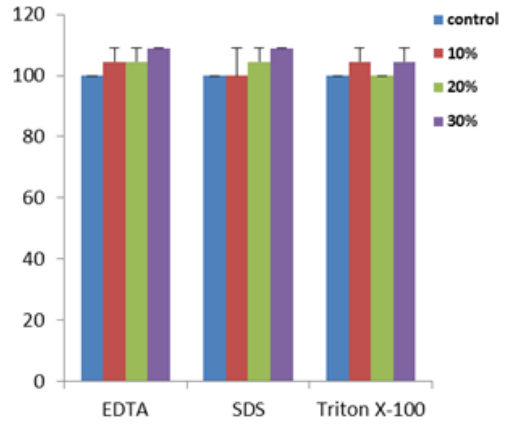

Fig 3a: Effect of surfactants on the activity of BLEM produced by *B. subtilis*SF8

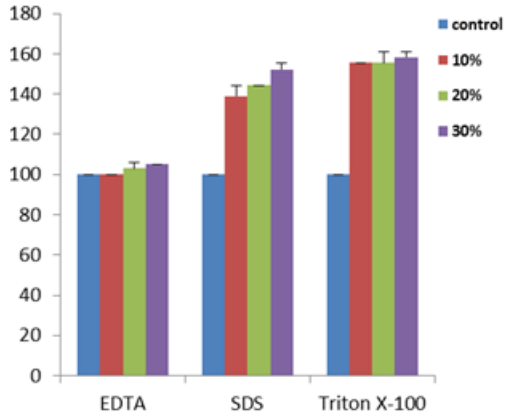

Fig 3b: Effect of surfactants on the activity of BLEM produced by *P. aeruginosa*SF4

3.4 Effect of ultra violet radiation on BLEM production

The activity of bacteriocin-like metabolite produced by *B. subtilis* SF8 was reduced by 12.5 and 50% after the exposure of young cultures of this strain to ultraviolet radiation for 8 and 10 minutes respectively. Increment in antimicrobial activity of 28 and 11% was observed for bacteriocin-like metabolite from *P. aeruginosa* SF4 after 6 and 8 minutes UV irradiation of its young cultures respectively. An abrupt reduction in the inhibitory activity was observed for the culture irradiated for 10 minutes (Fig. 4). It was reported that the biosynthesis of Gram-negative bacteriocins is regulated by host's SOS gene cluster system, which could be induced under stress conditions such as UV exposure and DNA damage[30].

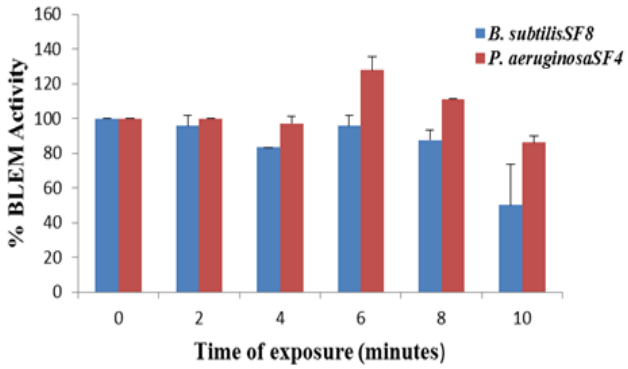

Fig 4: Effect of ultraviolet radiation on the production of BLEMs by *B. subtilis* SF8 and *P. aeruginosa* SF4

3.5 Hemolysis and hemagglutination tests

Negative hemolysis and hemagglutination results were recorded for the BLEM from *B. subtilis* SF8, however, tests on BLEM of *P. aeruginosa* SF4 showed that this metabolite exhibited both hemolysis and hemagglutination properties. Hemolysin, lectin and other extracellular metabolites with phospholipase and agglutination properties produced by *P. aeruginosa* could be precipitated (as proteins) along bacteriocin-like excretory metabolites of this organism[31, 32, 33] which could be responsible for the hemolysis and agglutination observed in its partially purified metabolite. However, such hemolysin and lectin within the metabolites of *P. aeruginosa* SF4 appeared to exist alongside the BLEM produced by this strain due to the inhibitory characteristics of this metabolite, including the inhibition of very closely related strains and strains from similar ecological niche, as well as its characteristic mode of action revealed by FTIR.

3.6 Mode of action of partially purified BLEM

Orientations of curves generated from the FTIR spectroscopic investigation of the mode of action of partially purified BLEM produced by *B. subtilis* SF8 on *B. macerans* SF2 (Figures 5a and 5b) revealed that the percentage proportion of the detected cellular components had significant peak differences at 2093.00 cm^{-1} (19%), 2097.14 cm^{-1}(46%) and 1645.90 cm^{-1}(2%), 1644.09 cm^{-1} (36%) of untreated and treated cell suspensions respectively. This corresponded to membrane fatty acid regions

(2093.00 and 2097.14 cm^{-1}), NH_2 bending, C=O stretching and amides I and II (1645.90 and 1644.09 cm^{-1})[34]. While FTIR spectroscopy also revealed a large difference in the peaks of curves generated as a result of the inhibitory effects of *P. aeruginosa* SF4 metabolite on *P. fluorescence* BS2 at 2092.21 cm^{-1} (13.5%), 2092.26 cm^{-1} (17.5%) and 1259.06 cm^{-1} (10%), 1269.91 cm^{-1} (13.5%) of untreated, treated cell suspensions; with assignments of P=O antisymmetric stretching and C-H aliphatic stretching respectively (Figures 6a and 6b).

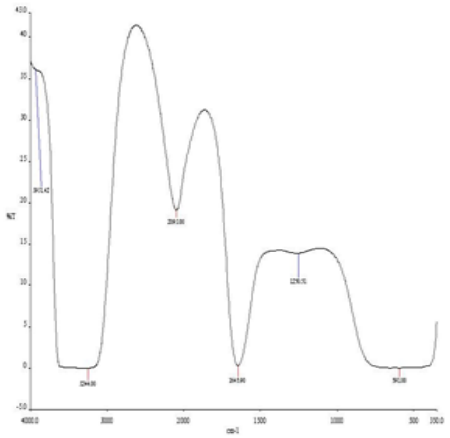

Fig 5a: FTIR analysis of *B. macerans* SF2 cell suspension

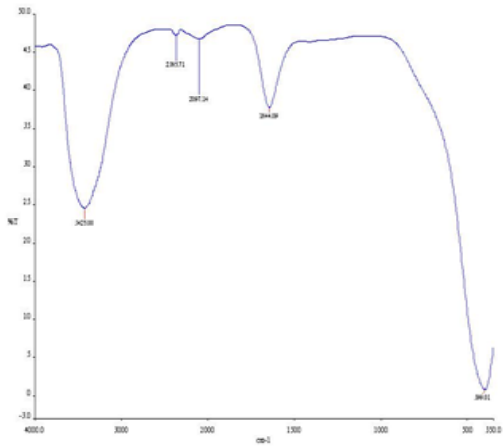

Fig 5b: Effect of partially purified BLEM of *B. subtilis* SF8 on *B. macerans* SF2

These regions correspond to membrane fatty acids of bacteria[17]. Several factors may be responsible for the wide disparity observed in the generated curves, these could include the possible production of multiple bacteriocin-like extracellular metabolites (by *B. subtilis* SF8) targeting different sites on the membrane of indicator strain, as well as the release of intracellular components.

3.7 Antimicrobial spectrum of bacteriocin-like metabolites

Bacteriocin-like metabolite of *B. subtilis* SF8 inhibited the growth of *Rhizopus nigricans* ART98*, Penicillium oxalicum* ILE1-OB and *P. chrysogenum* TZPB10 while the metabolite produced by P. *aeruginosa* SF4 inhibited the growth of *Penicillium chrysogenum* TZPB10. Antifungal activities of bacteriocins and bacteriocin-like excretory metabolites of bacteria, especially from *Bacillus* and *Pseudomonas* species, have been reported[19]. BLEM from P. *aeruginosa* SF4 was inhibitory to *P. aeruginosa* ATCC 29853, methicillin resistant *Staphylococcus aureus,* and *E. coli* 23218, while that from *B. subtilis*SF8 also inhibited *P. aeruginosa* ATCC 29853.

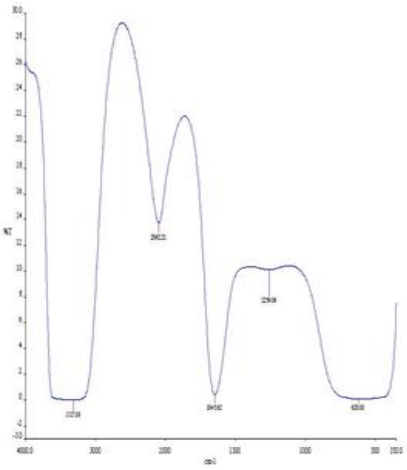

Fig 6a: FTIR analysis of *P. fluorescence* BS2 cell suspension

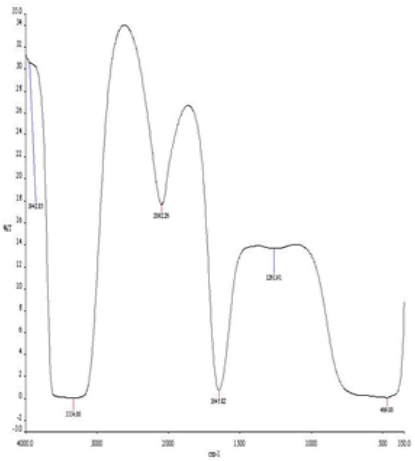

Fig 6b: Effect of partially purified BLEM of *P. aeruginosa* SF4 on *P. fluorescence* BS2

Researchers have reported and documented the antimicrobial activities of bacteriocins against closely related and distant species to the producing bacterium[24].

4 Conclusion

In conclusion, *B. subtilis* SF8 *and P. aeruginosa* SF4 produced bacteriocin-like extracellular metabolites, which retained large percentages of their inhibitory activities over a wide range of characterizing conditions. Investigating the mode of action of these partially purified metabolites by Fourier transformed infrared spectroscopy (FTIR) revealed that both metabolites from *B. subtilis* SF8 *and P. aeruginosa* SF4 interfered with the cell membranes of the indicator species. The activity spectrum

exhibited against clinical and economically significant pathogenic and spoilage organisms is a fascinating property with prospective agriculture and medically relevant potentials. These metabolites could therefore be further investigated and in translation, applied (in place of conventional antibiotics) in various fields.

5 Conflict of interest

The authors declare that there are no conflicts of interest.

6 Author's contributions

AOM carried out the laboratory assay of bacteriocins and manuscript writing. OOA evaluated the work, gave research instructions and edited the manuscript. Both authors read and approved the final manuscript.

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