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Production, characterization and growth inhibitory potential of metabolites produced by *Pseudomonas* and *Bacillus* species

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ABSTRACT

A variety of metabolites obtained from microbes are indicated to have anti-inflammatory, anticancer and antibiotics activities. This study was therefore aimed at assessing the antibacterial potentials of metabolites produced by four bacterial species against selected pathogens. The study was carried out under batch conditions. A 24 h old culture of each bacteria species was inoculated into 200 mL medium in 250 mL capacity conical flasks and incubated. The metabolites showed antimicrobial activity against the selected typed and clinical isolates. In presence of the respective crude metabolites, growth of *Salmonella typhi* and *Klebsiella pneumoniae* were observed to be inhibited during the period of incubation, except for setup that contained metabolites produced by the *Bacillus cereus.* The metabolite combinations from *P. aeruginosa and P. fuscoginae, P. aeruginosa and B. subtilis* and from *P. aeruginosa and B. cereus* were observed to inhibit most of the isolates. Structural characterization of the metabolites detected the presence of peptide-like moiety, as well as aliphatic hydrocarbons. The antimicrobial potential shown by the metabolites is a promising indication for further studies to be carried out with respect to possible safe use of the purified metabolites as potential antimicrobials in humans and animals.

Introduction

Compounds of microbial origin are vital because they are environmentally friendly, hence do not constitute a main source of pollution and are preferred alternatives to conventional compounds of chemical origins [21]. Microorganisms are veritable sources of biologically active secondary metabolites with vast chemical diversity. They are vital source of natural products and have remarkably contributed to every aspect of animal, human, plant and environment.

A variety of products that are synthesized by microorganisms have showed nutritional, agricultural and healthcare values. Several primary metabolites have been used as food supplements and industrial compounds while a number of secondary

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metabolites have served therapeutic and agricultural values [21]. A variety of metabolites obtained from microbes have led to the production of pharmaceutical products with anti-inflammatory, anticancer and antibiotics functions [20].

Although secondary metabolites are not essential for growth and biosynthesis of microorganisms, they are indicated to show a number of potentials for animal and human health. Although a number of microorganisms, such as bacteria and fungi are reported to produce a vast array of bioactive compounds and a number of studies on microbial secondary metabolite production have been published, it is hypothesized that there is still limitation in the understanding on how to enhance the production of such metabolites [15, 16, 22]

Similarly, although antibiotics have contributed greatly to increased life expectancy of humans, worldwide, the emergence of antibiotic-resistant pathogens in healthcare is widespread and a major healthcare concern. Microbial resistance to antibiotics is partly attributed to the short lifespan of effectiveness of current antibiotics, lack of compliance of patients and indiscriminate use in agriculture. Despite the need for novel antibiotics, it is indicated that there is decrease in antibiotic discovery programs in the pharmaceutical industry [7]. This has necessitated the constant attempt to find new antibiotics [13, 14].

A number of *Bacillus* species have reportedly known to produce a variety of bioactive secondary metabolites, which have been applied as agents for control of plant pathogens, antimicrobial drugs and biosurfactants [9, 11]. In addition, over production of secondary metabolites by Pseudomonas strains has been reported [19]. Despite the fact that several strategies of modifying currently existing new synthetic antibiotics have evolved in more recent years, these have not proven to be effective enough. There is therefore the need to search for other strategies for new antibiotics drugs. One of such strategies is the use of products of bacterial metabolism [3]. This study was therefore aimed at assessing the antibacterial potentials of metabolites produced by four bacterial species against selected pathogens.

Materials and methods

Test bacterial strains metabolite production

The microbial species used for the study were *Bacillus subtilis, Bacillus cereus, Pseudomonas fuscoginae* and *Pseudomonas aeruginosa*. The isolates, which were part of the laboratory stock in the Department of Microbiology, Landmark University, Omu-Aran, Kwara State, Nigeria and were all isolated from soil. Before usage, the cultures were resuscitated separately in nutrient broth. After 24 h incubation at 37 °C, they were sub-cultured on nutrient agar plates, to ascertain their purity. The respective pure colonies were then transferred to nutrient agar slants for maintenance at 4 °C.

Extraction of crude metabolites

To extract the crude metabolites produced by the respective test bacterial species, 120 h old broth cultures of the respective isolates were centrifuged for 15 min at 5000 rpm to obtain cell free supernatants. The respective cell-free supernatants were then decanted into 1 L beakers and acidified by reducing the pH to 2.0, using 1 mL HCl. The acidified solutions were then refrigerated overnight at 4 °C. The precipitated metabolites were then extracted by adding a solvent mixture of ethyl acetate and methanol (3:1 v/v). The solvent was then separated from the broth by decantation and evaporated by placing the beakers in a water bath to obtain the crude metabolites, which were then dried, quantified and kept in the refrigerator until when needed.

Viable count

For viable count, the standard pour plating procedure was employed, using the 10-fold serial dilutions of the broth cultures of the isolates. Following inoculation of the test medium for metabolite production, 1 mL of broth was withdrawn from the respective flasks every 24 h for a 120 h duration for 10 fold serial dilutions and pour plating. Medium used for plating was nutrient agar with incubation temperature being 37 °C ± 2 °C for 24 h. At the end of incubation, colonies were counted after visible growth on the plates and expressed as colony forming units per millilitres (CFU/mL).

Antibacterial potential and growth inhibition studies of the metabolites

Both typed and clinical bacterial isolates were used for antibacterial susceptibility testing in agar medium. The typed isolates used were *Escherichia coli* (ATCC 25,922), *Salmonella typhi* (ATCC 20,971), *Klebsiella pneumoniae* and *Staphylococcus aureus* (ATCC 6538), while the clinical isolates used were *Salmonella* sp. (STYAA0639), *E. coli* (ECO0503352), *Salmonella* sp. (STYAAA0026), *E. coli* (ECO050102) and *Salmonella* sp. (SALP5049). The clinical isolates were obtained from the University College Hospital, Ibadan, Oyo State, Nigeria. All the isolates were first sub-cultured in nutrient broth and streaked in nutrient agar to ascertain their purity before usage.

For the antibacterial testing in agar medium, the crude metabolites solution was used singly and in combination at a ratio of 1:1. The antimicrobial activity of the crude metabolites was tested using the agar well diffusion method.

For the test, nutrient agar was prepared in 100 mL conical flasks and allowed to cool. A portion of broth cultures of each bacterial isolate were added to their respective flasks. The flasks were then swirled to achieve homogenization. Approximately, 20 mL of the agar was then poured into well-labelled plates and allowed to solidify. Using a sterile cork borer, four

holes each were bored into the agar plates containing the microorganisms to accommodate the known concentration of the metabolite solution. To the bored holes, few drops of the metabolites were added using a micropipette. The plates were left for the metabolites to diffuse into the agar after which they were incubated at 37 °C for 24 h. The zones of inhibition were measured in millimetres.

For growth inhibition in liquid medium, two bacterial species (*Klebsiella pneumoniae* and *S. typhi*) were used. The medium that was used for cultivation was peptone water. To a 90 mL quantity of sterile medium in a 100 mL capacity conical flask, 1 mL of test bacterial species in 24 h old nutrient broth culture was inoculated. Following inoculation, 5 mL of 200 mg/mL of a crude metabolite was added and incubated in a rotary shaker at 30 °C ± 2 °C for 120 h at a shaking speed of 100 rpm. Just before incubation, and every 1 h for a 10 h duration, 5 mL sample was withdrawn from the respective flasks for absorbance reading, using a UV–visible spectrophotometer, at wavelength of 750 nm. All absorbance readings were in duplicates.

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) of the respective crude metabolites tested were determined against the following clinical and typed bacterial isolates: *Salmonella sp*. (SALPS049), *E. coli* (ECO050102), *Salmonella* sp. (STYAAA0639), *Salmonella* sp. (STYAAA0026), and *E. coli* (ECO050335) while the typed isolates were; *E. coli* (ATCC 25,922), *Staphylococcus aureus* (ATCC 6538), *S. typhi* (ATCC 20,971), and *Klebsiella pneumoniae.*

Eleven different concentrations ranging from 20 mg/mL to 200 mg/L of the respective crude metabolites were used for the determination of the MIC, using the microbroth dilution method. To sterile microtitre plates, 25 µL of broth sterile nutrient broth was dispensed into respective wells in a microtitre plate. This was followed by the addition of 10 µL each of 24 h old broth culture of the respective test isolates. Ten µL of the serially diluted concentrations of the respective metabolites were then added incubated at 37 °C for 24 h. A control well that contained only broth and isolate was also setup for each category. After 24 h incubation period, 10 µL iodonitrotetrazolium dye was added into each well to detect presence or absence of growth of the respective bacteria across the different concentrations. Growth was detected by development of pinkish red colouration in the well. The minimum concentration of a metabolite at which growth was inhibited was taken as the MIC.

Characterization of crude metabolite

The crude metabolites were characterized using Fourier Transform Infrared (FTIR) and Gas Chromatogram-Mass Spectrometry (GC–MS).

The FT-IR spectrometer used for the analysis was the Infrared spectrometer Varian 660 MidIR Dual MCT/DTGS Bundle with ATR. This was used to confirm the chemical structure of all samples. Prior to the analysis, the samples were first dried in an auto- desiccator for 24 h. During analysis, the samples were directly applied to a diamante crystal of ATR, with the resulting spectra recorded in transmittance mode (4000 to 500/400 cm^{-1}) at a resolution of 4 cm. All resulting spectra during analysis were corrected for background air absorbance.

GC–MS analysis of the samples was carried out using a Varian 3800 gas chromatograph equipped with an Agilent fused silica capillary CP-Sil 5 CB column (30 $m \times 0.25$ mm i.d.) connected to a Varian 4000 mass spectrometer. ITROGE was used as the carrier gas at a flow rate of 1.0 ml/min and a split ratio of 1 :10. The operating temperatures were 60 °C for 1 min; rising at 3.0 °C/min to 240 °C and held for 1 min while the injector and the detector were held at 240° C. The samples were diluted with chloroform at 1:10 (v/v), with 0.2 µl of the mixtures always injected automatically in the splitless mode. The mass spectra were obtained by electron ionization at 70 eV, using a spectral range of *m/z* 30–1000. The quantification and detection of the components of the metabolites as detected by the mass spectrometer while identification of the constituents was through comparison of their retention times and fragmentation pattern of mass with those of published data or with those of the Wiley 9 and NIST 08 mass spectral libraries.

Results

Growth of the isolates during incubation

During the period of incubation, viable count of the respective isolates did not follow any consistent pattern of increase or decrease with time. Highest counts of 1.0×10^8 CFU/mL and 8.50×10^7 CFU/mL were observed at 96 h of incubation for *B. cereus and P. aeruginosa*, respectively. For *B. subtilis* and *P. fuscoginae*, highest viable counts of 5.5×10^7 CFU/mL and 8.5×10^7 CFU/mL were recorded after 96 and 48 h of incubation, respectively (Fig. 1).

Antibacterial activity in solid medium

Generally, all the crude metabolites showed inhibition against the test clinical and typed isolates investigated. For the metabolites produced by *Pseudomonas aeruginosa,* highest zone of inhibition of 30 mm was observed against the *Klebsiella pneumoniae.* For that produced by the *P. fuscoginae, B. subtilis* and *B. cereus,* highest zones of inhibition were recorded against *E. coli* (ECO0503352), *Salmonella* sp (STYAAA0026) and *Klebsiella pneumoniae,* respectively (Table 1).

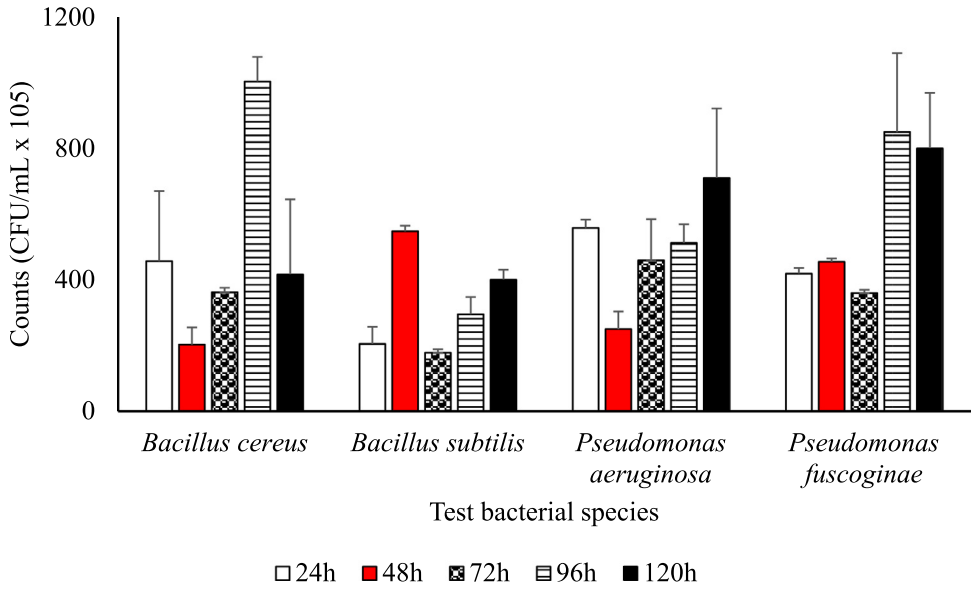

Fig. 1.. Viable count of the isolates during the period of incubation.

Table 1.
Inhibitory effect of the metabolites on selected typed and clinical bacterial isolates.

	Metabolites			
	A	**B**	**C**	**D**
Zones of inhibition (mm)				
Typed isolates				
E. coli (ATCC 25,922)	+ (24)	+ (21)	+(22)	+(20)
S. typhi (ATCC 20,971)	+ (20)	+ (19)	+(18)	+(14)
Klebsiella pneumoniae	+ (30)	+ (22)	+ (23)	+ (24)
Staphylococcus aureus (ATCC 6538)	+ (23)	+ (14)	+ (20)	+ (12)
Clinical isolates				
Salmonella sp. (STYAA0639)	+ (14)	+ (13)	+ (11)	+ (15)
E. coli (ECO0503352)	+ (19)	+ (23)	+ (20)	+ (21)
Salmonella sp. (STYAAA0026)	+ (17)	+ (15)	+ (30)	+ (20)
E. coli (ECO0050102)	+ (24)	+ (21)	+ (19)	+ (13)
Salmonella sp. (SALP5049)	+ (23)	+ (14)	+ (19)	+ (15)

+ indicate sensitive. Values in parenthesis indicate the zone of inhibition of the metabolites on the isolates. 'A', 'B', 'C' and 'D' represent metabolite produced by *P. aeruginosa, P. fuscoginae, B. subtilis* and *B. cereus,* respectively.

When the crude metabolites were combined, combinations from *P. aeruginosa and P. fuscoginae, P. aeruginosa and B. subtilis* and *P. aeruginosa and B. cereus* inhibited almost all the typed and clinical isolates used for investigation, except *Salmonella* sp. (STYAAA0026) that was only inhibited by combination of metabolites from *P. aeruginosa and B. cereus*. Metabolite combinations from *P. fuscoginae and B. subtilis, P. fuscoginae and B. cereus* and *B. subtilis and B. cereus* were only effective against the growth of *Klebsiella pneumoniae* (Table 2).

Generally, concentration of 200 mg/mL was observed to be the minimum inhibitory concentration (MIC) of the respective metabolites against the majority of the test isolates. This observation was irrespective of the metabolites used for investigation. Concentration of 180 mg/mL was however observed for be the MIC for surfactant from *P. aeruginosa* against *Salmonella sp*. (SALPS049), *Salmonella* sp. (STYAAA0639), *Salmonella* sp. (STYAAA0026) and *E. coli* (ATCC 25,922). Against *E. coli* (ECO050335), MIC of 160 mg/mL was observed for all the respective metabolites (Tables SM1 and SM2).

Growth inhibition in liquid medium

In the presence of the respective crude metabolites, growth of *S. typhi* was observed to be inhibited throughout the 10 h incubation period, except for medium that contained metabolites produced by the *Bacillus cereus.* From initial absorbance values of 0.087, 0.283, 0.032 and 0.0795, values of 0.198, 0.437, 0.323 and 0.811 were recorded after the 10 h incubation period in medium containing metabolites produced by the *P. aeruginosa, P. fuscoginae, B. subtilis and B. cereus,* respectively.

Table 2.
Inhibitory effects of combinations of the metabolites on selected typed and clinical bacterial isolates.

	Metabolite Combinations					
	A	**B**	**C**	**D**	**E**	**F**
Typed isolates						
Zones of inhibition (mm)						
E. coli (ATCC 25,922)	+ (20)	+ (20)	+ (15)	–	–	–
Staphylococcus aureus (ATCC 6538)	+ (14)	+ (19)	+ (21)	–	–	–
S. typhi (ATCC 20,971)	+ (19)	+ (22)	+ (15)	–	–	–
Klebsiella pneumoniae	+ (19)	+ (13)	+ (25)	+ (12)	+ (11)	+ (14)
Clinical isolates						
Salmonella sp.(STYAA0639)	+ (21)	+ (21)	+ (25)	+ (16)	–	–
E. coli (ECO0050335)	+ (17)	+ (21)	+ (21)	-	–	–
Salmonella sp. (STYAAA0026)	–	–	+ (21)	-	–	–
E. coli (ECO0050102)	+ (15)	+ (16)	+ (15)	-	–	–
Salmonella sp. (SALP5049)	+ (20)	+ (18)	+ (25)	+ (11)	+ (10)	–

'+' and '-'indicate sensitive and resistant respectively. Values in parenthesis indicate the zones of inhibition. A, B, C, D, E and F connote combination of metabolite from *P. aeruginosa and P. fuscoginae,* from *P. aeruginosa and B. subtilis,* from *P. aeruginosa and B. cereus,* from *P. fuscoginae and B. subtilis,* from *P. fuscoginae and B. cereus,* and from *B. subtilis and B. cereus,* respectively.

In the control medium that was without the surfactant, absorbance reading increased from 0.002 to 0.893 after the period of incubation (Fig. 2).

The growth of *Klebsiella pneumoniae* was observed to be inhibited throughout the incubation period of 10 h in the presence of metabolites produced by *P. aeruginosa, P. fuscoginae, B. subtilis*, with the exception of medium containing metabolite produced by *B. cereus.* From initial absorbance values of 0.0165, 0.021, 0.028, 0.0245 and 0.0135 recorded, values of 0.2335, 0.109, 0.2675, 0.9115 and 0.9495 were recorded after the 10 h incubation period in medium containing (Fig. 2).

Fourier transform infra-red (FTIR)

The FTIR spectra of the metabolite produced by the *P. aeruginosa,* revealed the presence of 133 peaks. Some of these peaks corresponded to O–H stretching vibration, C–H symmetric stretching vibration of the connection of the CH_2 group, C–H stretching vibration, of aliphatic akane, C≡N stretching vibrations, C = O stretching vibration of a carbonyl bond, C–O-C asymmetric stretching vibration, C–O–C and C–C–O Stretching vibration due to the accumulation of polyester compounds (poly-3-hydroxybutyrate and probably phospholipids, C–C Stretching vibration of amide group and C–Cl C-Br, I Stretching vibrations (Table 3).

A total of 15 peaks were identified from the metabolite produced from the *P. fuscoginae.* These peaks corresponded to O–H stretching vibration, C–H symmetric stretching vibration of the connection of the in CH_2 group, C–H stretching vibration, of aliphatic Alkane, C≡N stretching vibrations, C = O stretching of aldehydes and ketones, N = C = S stretching vibration, C = C–C aromatic ring stretch, CH_3 symmetric bending vibrations, C–O–C and C–C–O stretching vibrations due to the accumulation of polyester compounds (poly-3-hydroxybutyrate and probably phospholipids, C–C stretching vibration of amide group and C–Cl C-Br, I Stretching vibrations (Table 3).

The metabolite produced by *B. subtilis* revealed the presence of 8 peaks, which were O–H stretching vibration, C–H symmetric stretching vibration, CH_3 symmetric bending vibrations, C–O-C asymmetric stretching vibration, C–O Stretching vibration, C–C–O stretching vibration, C–C Stretching vibration of amide group and C–Cl C-Br, I Stretching vibrations (Table 4).

The metabolite produced by the *B. cereus* showed the presence of 16 peaks. The peaks were O–H stretching vibration, strong N–H stretching vibration of H-bonding (due to water) and possible metal binding of amide groups in proteins, C–H stretching vibration, of aliphatic Alkane, C–H stretching vibration, of aliphatic Alkane, CH_2 symmetric bending vibration, C–O-C asymmetric stretching vibration, RCH=N = N stretching vibration, C≡N stretching vibrations, C–O–C and C–C–O Stretching vibration due to the accumulation of polyester compounds (poly-3-hydroxybutyrate and probably phospholipids, C–C Stretching vibration of amide group, Rocking CH_2 mode due to the accumulation of polyesters and C–Cl C-Br, I Stretching vibrations (Table 4).

Gas chromatogram (GC–MS) of the crude metabolites

From the GC–MS of the metabolite produced by the *P. aeruginosa,* the following compounds were identified to be present: Hexanoic acid, 2-methyl-Benzoic acid, 2-amino-5–chloro-, methyl ester, Ethyl 2,4-, dihydroxy-6-methylnicotinate, Benzyl methyl ketone, Benzoic acid, 1-methylethyl ester, 5,7-Dodecadiyn-1,12-diol, dl-Citrulline Gluconic acid, 2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl- Glucose n-Hexadecanoic acid, L-Aspartic acid, N-glycyl-Heptadecanoic acid, Pentadecanoic acid, 13-methyl-, methyl ester, 1-Hexadecanol, 3,7,11,15-tetramethyl-, Maltose, Stigmasterol, Cystine, 9-Octadecenoic acid, (E)-Sucrose, Heptadecanoic acid, 16-methyl-, methyl ester, Monolinolenin, Glycerol 1-palmitate and α-TocospiroB (Figure SM1).

For the crude metabolite produced by the *P. fuscoginae,* Hexanoic acid, 2-methyl-Benzoic acid, 2-amino-5–chloro-, methyl ester, Ethyl 2,4-dihydroxy-6-methylnicotinate, Benzoic acid, 1-methylethyl ester Benzyl methyl ketone, 5,7-Dodecadiyn-1,12-

Table 3.
Assigned peaks from the FTIR spectra of the metabolite from the *Pseudomonas* species.

Run	Peak	Trans	Abs	Assignment
P. aeruginosa				
1	3792.46	77.41	0.1121	O-H stretching vibration
2	3133.28	63.03	0.2005	C–H symmetric stretching vibration of the connection of the CH_2 group
3	2472.55	68.32	0.1655	C–H stretching vibration, of aliphatic Alkane
4	2410.63	60.00	0.2218	O-H stretching vibration
5	2286.51	69.76	0.1564	C≡N stretching vibrations
6	1796.53	76.65	0.1155	C = O stretching vibration of a carbonyl bond
7	1228.34	64.08	0.1933	C–O–C asymmetric stretching vibration
8	1121.30	65.73	0.1822	C–O–C and C–C–O Stretching vibrations due to the accumulation of polyester compounds (poly-3-hydroxybutyrate and probably phospholipids
9	1031.05	72.41	0.1402	C–O–C and C–C–O Stretching vibrations due to the accumulation of polyester compounds (poly-3-hydroxybutyrate and probably phospholipids
10	864.60	70.84	0.1497	C-C Stretching vibration of amide group
11	799.48	70.80	0.1500	C–H Stretching vibration
12	700.41	73.15	0.1358	Rocking CH_2 mode due to the accumulation of polyesters
13	621.44	72.56	0.1393	C-Cl C-Br, I Stretching vibrations
P. fuscoginae				
1	3792.46	86.11	0.0650	O–H stretching vibration
2	3192.43	72.34	0.1406	C–H symmetric stretching vibration of the connection of the in CH_2 group
3	2592.85	37.16	0.4299	C-H stretching vibration, of aliphatic Alkane
4	2410.63	18.74	0.7272	O–H stretching vibration
5	2286.51	24.80	0.6055	C≡N stretching vibrations
6	2148.33	65.53	0.1836	C = O stretching of aldehydes and ketones
7	1998.71	66.00	0.1085	N = C = S stretching vibration
8	1820.73	82.41	0.0840	C = C–C aromatic ring stretch
9	1326.54	20.00	0.6990	CH_3 symmetric bending vibrations
10	1192.37	28.61	0.5435	C–O–C and C–C–O Stretching vibrations due to the accumulation of polyester compounds (poly-3-hydroxybutyrate and probably phospholipids
11	1031.05	38.27	0.4171	C–O–C and C–C–O Stretching vibrations due to the accumulation of polyester compounds (poly-3-hydroxybutyrate and probably phospholipids (PLs))
12	864.60	74.82	0.1260	C-C Stretching vibration of amide group
13	700.41	60.00	0.2219	Rocking CH_2 mode due to the accumulation of polyesters
14	657.18	53.56	0.2712	C–O Stretching vibration
15	551.03	76.17	0.1184	C-Cl C-Br, I Stretching vibrations

Trans and Abs represent transmittance (%) and Absorbance, respectively. Peak is cm^{-1}.

Table 4.
Assigned peaks from the FTIR spectra of the metabolite from the *Bacillus* species.

Run	Peak	Trans	Abs	Assignment
B. subtilis				
1	3792.46	87.52	0.0579	O–H stretching vibration
2	3133.28	72.10	0.1421	C–H symmetric stretching vibration of the connection of the in CH_2 group
3	1326.54	80.00	0.0969	CH_3 symmetric bending vibrations
4	1216.31	89.74	0.0470	C-O-C asymmetric stretching vibration
5	998.50	88.56	0.0528	C–O Stretching vibration
6	946.27	85.50	0.0531	C–C–O stretching vibration
7	850.31	88.76	0.0518	C–C Stretching vibration of amide group
8	621.44	32.64	0.4862	C-Cl C-Br, I Stretching vibrations
B. cereus				
1	3998.26	58.21	0.0545	O–H stretching vibration
2	3308.41	32.74	0.4849	strong N–H stretching vibration of H-bonding (due to water) and possible metal binding of amide groups in proteins
3	3199.74	23.92	0.6212	C-H stretching vibration, of aliphatic Alkane
4	3021.58	49.11	0.3088	C–H symmetric stretching vibration of the connection of the in CH_2 group
5	2999.58	52.76	0.2777	C–H symmetric stretching vibration of the connection of the in CH_2 group
6	2982.55	58.91	0.2298	C–H stretching vibration
7	2843.62	68.00	0.1675	RCH=$N = N$ stretching vibration
8	2228.71	80.00	0.0969	C≡N stretching vibrations
9	1400.97	41.36	0.3834	CH_2 symmetric bending vibration
10	1200.04	55.02	0.2595	C-O-C asymmetric stretching vibration
11	1026.48	52.00	0.2840	C–O–C and C–C–O Stretching vibration due to the accumulation of polyester compounds (poly-3-hydroxybutyrate and probably phospholipids
12	898.27	81.52	0.0887	C-C Stretching vibration of amide group
13	800.00	73.41	0.1342	CH bending vibration of alkenes
14	747.35	74.26	0.1292	Rocking CH_2 mode due to the accumulation of polyesters
15	632.07	72.50	0.1400	C–O Stretching vibration
16	530.73	56.22	0.2501	C-Cl C-Br, I Stretching vibrations

Trans and Abs represent transmittance (%) and Absorbance, respectively. Peak is cm^{-1}.

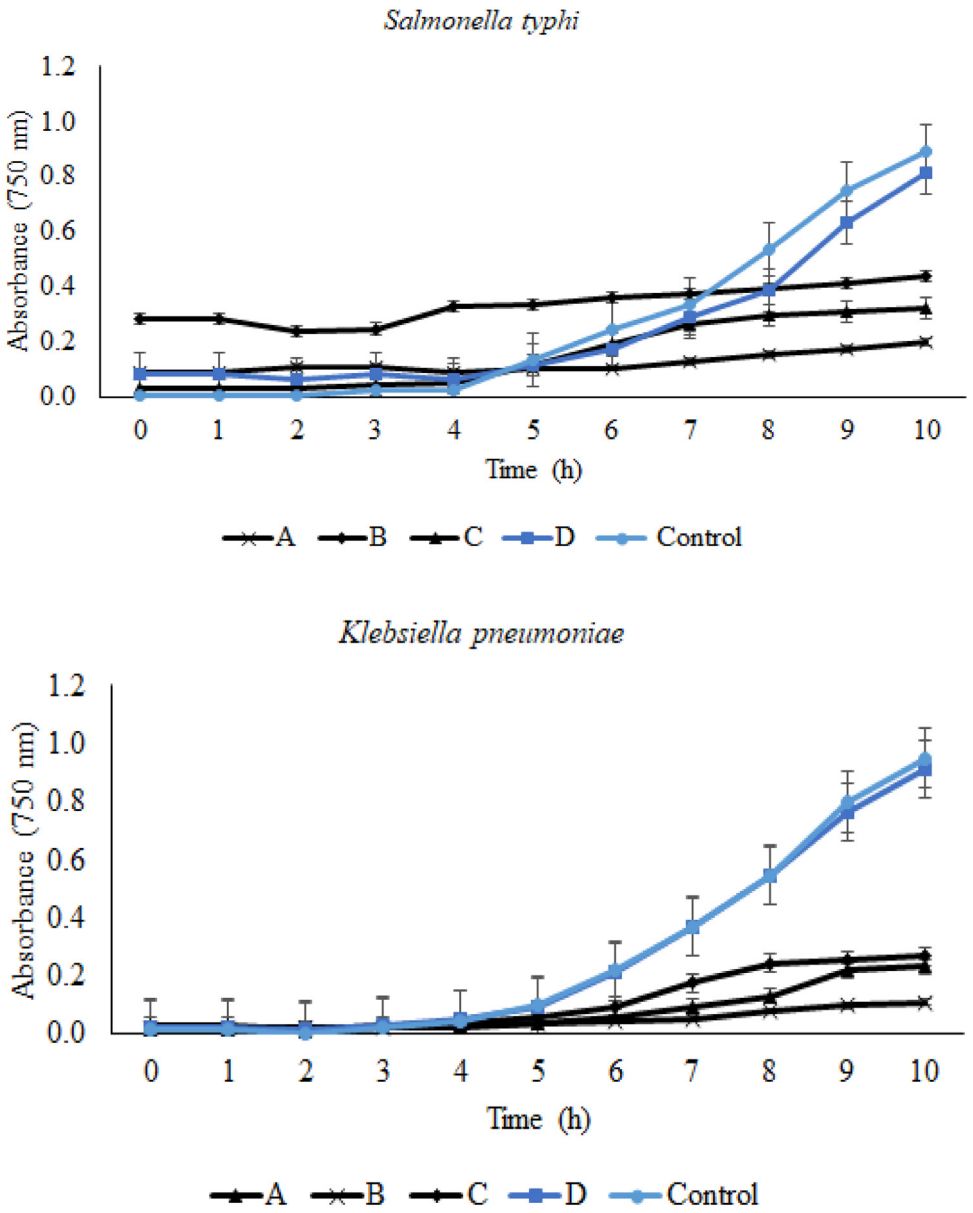

Fig. 2.. Growth of the *Salmonella typhi* and *Klebsiella pneumoniae* in the respective broth media. A, B, C and D represent medium with metabolites produced by *P. aeruginosa, P. fuscoginae, B. subtilis and B. cereus,* respectively. Control indicates medium with no metabolite.

diol, dl-Citrulline Gluconic acid, 2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, Glucose, n-Hexadecanoic acid, L-Aspartic acid, N-glycyl-Heptadecanoic acid, Pentadecanoic acid, 13-methyl-, methyl ester 1-Hexadecanol, 3,7,11,15-tetramethyl-, Maltose, Stigmasterol, Cystine, 9-Octadecenoic acid, (E)-Phytol, Monolinolenin,Heptadecanoic acid, 16-methyl- methyl ester, Glycerol 1-palmitate α-TocospiroB and Sucrose were identified to be present (Figure SM1).

The following compounds were identified to be present in the crude metabolite produced by the *B. subtilis*: Benzoic acid, 2-amino-5–chloro-, methyl ester, Ethyl 2,4-dihydroxy-6-methylnicotinate Benzoic acid, 1-methylethyl ester, Benzyl methyl ketone, 5,7-Dodecadiyn-1,12-diol, dl-Citrulline, Pentadecanoic acid, 13-methyl-, methyl ester, Glucose, L-Aspartic acid, N-glycyl-, Gluconic acid, Sucrose, n-Hexadecanoic acid, Heptadecanoic acid, 1-Hexadecanol, 3,7,11,15-tetramethyl-, Maltose, Stigmasterol, Cystine, 9-Octadecenoic acid, (E)-, Heptadecanoic acid, 16-methyl-, methyl ester, Monolinolenin, Glycerol 1-palmitate and α-TocospiroB (Figure SM2).

In the case of the surfactant produced by the *B. cereus,* compound that were identified to be present were Hexanoic acid, 2-methyl-, Benzoic acid, 2-amino-5–chloro-methyl ester, Ethyl 2,4-dihydroxy-6-methylnicotinate Benzoic acid, 1-methylethyl ester, Benzyl methyl ketone, 9-Octadecenoic acid, (E)-, Pentadecanoic acid, 13-methyl-, methyl ester, dl-Citrulline, Glucose,

L-Aspartic acid, N-glycyl-, Gluconic acid, 5,7-Dodecadiyn-1,12-diol, n-Hexadecanoic acid, Heptadecanoic acid, 1-Hexadecanol, 3,7,11,15-tetramethyl-, Maltose, Sucrose, Cystine, Heptadecanoic acid, 16-methyl-, methyl ester, α-TocospiroB (Figure SM2).

Discussion

In this study, the crude metabolite produced showed inhibition against all the test clinical and typed isolates investigated using the agar diffusion test. The results are in corroboration with existing reports of antibiotic effect exhibited by rhamnolipid metabolite [25]. In a study by Gomaa [8], metabolites produced by *Bacillus licheniformis* were investigated for potential antimicrobial activity by using the disk-diffusion method against several Gram-positive and Gram-negative bacteria. In addition to *Candida albicans,* the study reported extremely distinct antibacterial activity of the metabolite against the bacteria and yeast species. Some other investigators [23], have indicated that *Bacillus* strains were able to produce a large number of antimicrobial peptides with different chemical structures, such as bacteriocins, iturin A and surfactin. In another study carried out by Bharali et al., [5] the antimicrobial activity of the metabolite produced by *Pseudomonas aeruginosa* was estimated against four pathogenic strains of bacteria (*E. coli, Staphylococcus aureus, Klebsiella pneumoniae,* and *Bacillus subtilis*) and two pathogenic strains of fungi (*Aspergillus flavus* and *Aspergillus niger*), and the findings revealed antibacterial activity against these organisms except, *Staphylococcus aureus.*

All the metabolites produced by the test bacterial species were observed to show antibacterial activity when used singly. When used in combination, antibacterial activities of the metabolites were not observed to be enhanced, rather activity was either reduced or lost. In a similar study on the antimicrobial activity of a mixture of a metabolite from *Bacillus subtilis* and an alkaline lipase from *Fusarium oxysporum*, result revealed activity against *S. aureus, Salmonella choleraesuis, P. aeruginosa, E. coli, B. cereus*. B. subtilis and L. innocua. The metabolite was not observed to be active against *E. coli, Pseudomonas aeruginosa*, yeast and C. albicans [18]. Antimicrobial activity of metabolite from *Pseudomonas aeruginosa* RTE4 have been reported to have activity against *Corticum invisium, Fusarium solani* and *Xanthomonas campestris* [6].

In all the metabolites, FTIR analysis showed that O–H stretching vibration and C–H symmetric stretching vibration of the connection of the CH_2 group were evident. The C–O–C and C–C–O stretching vibration due to the accumulation of polyester compounds (poly-3-hydroxybutyrate and probably phospholipids was also observed to be common among the respective metabolites. Furthermore, C–C stretching vibration of amide group and C–Cl C-Br, I stretching vibrations were also evident among all the respective metabolites. A similar study of the FTIR of metabolite produced by *P. aeruginosa* reported 5 peaks which corresponded to hydroxyl group (-OH) free stretch, C–H stretching vibrations of hydrocarbon chain of alkyl (CH_2-CH_3) groups, characteristic carbonyl stretching band denoting the presence of ester compounds, COO− group stretching vibration, characteristics of the glycosidic bond (C–O-C). From this study, it was deduced that the chemical structure of this metabolite is identical to those of previously reported rhamnolipid which comprises of rhamnose ring attached with long hydrocarbon chains [17].

In a study reported by Al-Dhabi et al., [1], FTIR of metabolite from *B. subtilis* strain Al-Dhabi-130 showed various strongly absorbing bands characterized by C = O stretching mode, C–N stretching mode combined with N–H band, C–H stretching mode of the aliphatic alkane, lactone carbonyl absorption which corroborates investigations carried out by de Faria et al., (2011) on characterization of surfactin produced by *B. subtilis* isolate LSFM-05, which revealed the presence of CH_3 and CH_2, as well as N–H and C–H vibration. It can be deduced from the result of this study that metabolite produced by *Pseudomonas aeruginosa, P. fuscoginae, Bacillus subtilis* and *B. cereus* contains peptide-like moiety as well as aliphatic hydrocarbons.

In this study, GC–MS of metabolites produced by the test bacterial species identified 2-amino-5–chloro-, methyl ester, Ethyl 2,4-, dihydroxy-6-methylnicotinate, Benzyl methyl ketone, Benzoic acid and 1-methylethyl ester. Some other compounds also identified among all metabolites produced were pentadecanoic acid, L- Aspartic acid, sucrose and α TocospiroB. Alyousif *et al*., [2] reported GC–MS chromatogram of the metabolite produced by *P. aeruginosa* showing major peaks indicating the presence of different compounds including 2-Octenoic acid, trans-2-Decenoic acid, 3-Hydroxydecanoic acid, trans-2-Dodecenoic acid, Palmitoleic acid, n-Hexadecanoic acid. It was deduced from this study that the results of GC–MS analysis indicated the presence and structure of the major rhamnolipid components. Rhamnolipids are produced by various *Pseudomonas* species especially *P. aeruginosa* [24].

In another study Anitha et al., [4] reported that GC–MS analysis carried out on compound produced by *Bacillus* species was a lipopeptide derivative. The compounds identified in the extract were phenol, 2,4-bis (1–1 dimethyl), 1,2-Benzenedicarboxylic acid and butyl 2-ethylhexyl ester, Hexadecanoic acid, methyl ester, 9- Octadecenoic acid (Z)-methyl ester and Trioxoclane-2- octanoic acid 5 octyl, methyl ester, with the hydrophobic moiety predicted to be an octadecanoic acid methyl ester. The result is in corroboration with the findings of earlier workers [10, 12].

Conclusion

The findings from this study revealed that all the four organisms screened were found to produce metabolites. When used separately, all the metabolites showed antibacterial activity against the pathogens investigated.

Also, due to the antimicrobial potential shown by the metabolites, further studies could be carried out with respect to possible safe use of purified metabolite product as a potential antimicrobial drug candidate in humans and animals, as opposed to presently employed antibiotics that have been constantly abused and resulting in rise of life-threatening diseases due to drug resistance among pathogenic organisms.

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Declaration of Competing Interest

The authors have no competing interests

Supplementary materials

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