Original Research Article

Antimicrobial Property of Microorganisms Isolated from Soil and Water – Body Samples in Ghana

ABSTRACT

Introduction: Soil and aquatic microorganisms have been the major sources of novel antimicrobial agents over the past years. The continues use of these agents against pathogenic organisms have resulted in multi-drug resistant pathogens, hence, the need to search for new and potent antimicrobial agents.

Methods: In this study, microorganisms were isolated from 24 samples collected from soil, the Kakum River (water and sediments) and the Gulf of Guinea (water and sediments). The microorganisms present in these samples were screened for their antimicrobial producing potentials.

Results: A total of 138 microorganisms were isolated out of which thirty-six (36) showed growth–inhibitory activity against at least one of the test organisms used for the screening. The extract of a selected isolate, $GKSE_1$, showed antibacterial activity against *B. subtilis, S. aureus, S. epidermidis, S. pyogenes, E. faecalis, E. coli, K. pneumonia, P. aeruginosa, S. typhi, S. typhorium and S. dysentriae* with minimum inhibitory concentration ranging from 1.563–6.250 mg/mL. The extract was stable in aqueous solution for more than three months and also had activity after autoclaving at 121 ^{o}C for 15 minutes. TLC analysis of the crude extract revealed 5 spots with 2 regions of inhibition in a bioautography assay.

Conclusion: This study has shown that microorganisms isolated from soil, Kakum River and the sea has the potential to produce antimicrobial agents with the isolate $GKSE_1$, identified as *Enterococcus faecalis* having excellent activity.

Keywords: Antimicrobial metabolites, Kakum River, Drug resistance, MIC, Bioautography

1. INTRODUCTION

Microorganisms are ubiquitous but very dominant in tropical regions due to the conducive climate for their growth. High numbers of living organisms inhabiting most of our natural environment are extensively involved in the biological processes of the ecosystem [1]. The soil environment inhabits more microorganisms than the other environments and this may be due to favorable growth conditions such as pH, temperature, aeration, moisture and organic materials from dead decaying plants and animals [2]. Some of the microorganisms cause various kinds of human, animal and plant diseases and also have been the main sources of secondary metabolites that have industrial, pharmacological and physiological applications [3]. The secondary metabolites include; antibiotics, enzymes, biosurfactant and insecticides [4,5]. Antibiotics are produced by microorganisms to enable them defend themselves against other organisms [6]. Several antimicrobial agents have been isolated from a number of organisms particularly those of the genera actinomycetes following the discovery of penicillin in 1929 [7–9]. Streptomycin and tetracyclines were isolated from Streptomyces species whiles

gentamicin and butremycin were isolated from Micromonospora species. Bacitracin and polymyxin on the other hand were obtained from some Bacillus species [10,11]. These agents have been used in the treatment of many microbial infections and the exposure of microorganisms to these agents has resulted in the development of antimicrobial resistance [7,12]. The increasing prevalence of microbial resistance to the existing antimicrobial agents in the world has necessitated the increased search for new and potent antimicrobial agents to combat resistant pathogens. This current study sought to search for potent antimicrobial producing microorganisms from soil, river (water and sediments) and sea (water and sediments) samples collected in Ghana.

2. MATERIAL AND METHODS

Sample Collection

Twenty four (24) samples consisting of 8 soil samples from Ejisu Zongo (6º 43' 1" N, 1º 28' 34" W and 6º 43' 1" N, 1º 28' 30" W) in the Ashanti Region, and 9 water/sediments samples from River Kakum (at Kakumdo: 5º 8' 49'' N, 1º 17' 15'' W; Kwaprow: 5º 7' 34" N, 1º 18' 10" W; Etre: 5º 5' 53" N, 1º 19' 19" W) and 7 sea water/sediments samples (at Etre: 5º 5' 50'' N, 1º 19' 12'' W; Oyster Bay: 5º 5' 39" N, 1º 19' 51" W) in the Central region were collected. All samples were transported to the Pharmaceutical Microbiology laboratory of the Department of pharmaceutics, Kwame Nkrumah University of Science and Technology, Kumasi.

Sample Preparation, Cultivation and Isolation of Microorganism

For each of the soil and sediment samples, 1 g was suspended in 10 mL sterile normal saline and allowed to settle after which 1 mL of the supernatant was withdrawn and serially diluted twice in 9 mL of sterile normal saline. Using the pour plate method [13], 1 mL quantity of the diluted supernatant was then inoculated into 30 mL sterile Humic Acid Vitamin (HAV) agar in a sterile petri dish [14,15]. The water samples (fresh and sea) were also separately inoculated undiluted (1 mL) into 30 mL of the sterile HAV agar. All the plates were incubated inverted at 34 ^{o}C for 14 days with daily observation. Portions of the prepared samples were also similarly cultivated in sterile Glycerol Asparagine (GA) agar plates [16,17]. After the 14 days of incubation, morphologically different colonies were separately isolated into different test tubes containing 10 mL Nutrient Broth (Oxoid Limited, Basingstoke, UK) and incubated at 34 ^{o}C for 24 hours.

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Test Organisms Used

The microorganisms used to evaluate the antimicrobial potential of the microbial isolates were *Bacillus subtilis* NTCC 10073, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 4853, and clinical strains of *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Salmonella typhorium*, *Shigella dysentriae* and *Candida albicans*. Prior to screening, these test organisms were diluted in normal saline to 0.5 McFarland standard [18].

Screening of Isolates for Antibiotic Production

The agar well diffusion method [19, 20] was used to screen the isolates for antibiotic production. Each isolate was cultivated in Nutrient Broth (Oxoid Limited, Basingstoke, UK) for 7 days and then centrifuged at 1400 rpm for 15 minutes. A cork borer of diameter 12 mm was used to create wells in nutrient agar plates which were previously seeded with the test microorganisms. The wells were then filled with 100 µL of the supernatant. The plates were incubated at 37 ^{o}C for 24 hours after which they were observed for zones of growth inhibition. The screening was done in triplicate and the results obtained were recorded (Table 1). Based on the screening results obtained, an isolate coded $GKSE_1$, isolated from the sediment of the Kakum River at Etre (5º 5' 53" N, 1º 19' 19" W) was selected for further investigation.

Fermentation and extraction of metabolite of $GKSE_1$

Four bottles each containing 500 mL of sterile Nutrient broth were each inoculated with 20 mL of 24 hours broth cultures of isolate $GKSE_1$. The bottles were then incubated at 34 ^{o}C with 2 minutes of shaking every 8 hours for 7 days [20], after which the culture was filtered through Whatman No.1 filter paper. The filtrate was then extracted four times with equal portions of ethyl acetate in a separating funnel. The ethyl acetate portions were pooled together and concentrated to a syrupy mass at 45 ^{o}C using a rotary evaporator (Buchi, Swaziland) which was further dried in vacuo at room temperature. The extract was kept at 4 ^{o}C until needed.

Antimicrobial activities of $GKSE_1$ extract

Agar diffusion method

Two – fold dilutions ranging between 12.5 – 200 mg/mL of $GKSE_1$ extract were prepared and their antimicrobial activities were assessed using the agar well diffusion method as described above on the test microorganisms listed excluding *Candida albicans* [19]. Ciprofloxacin was used as a positive control. The test was conducted in triplicates and zones of growth inhibition were recorded (table 2).

Minimum inhibitory and bactericidal concentrations determination

The minimum inhibitory and bactericidal concentrations (MIC & MBC) were also determined using the microbroth dilution method in a 96-well microtiter plates [21]. Briefly, Two-fold dilutions of the extract ranging from 0.05 – 50 mg/mL were prepared and 70 µL portions were dispensed into wells previously filled with 100 µL of sterile double strength Nutrient broth (Oxoid Limited, Basingstoke, UK). A 30 µL of the test microorganisms prepared from 18 hours broth cultures (diluted to 0.5 McFarland standard) were dispensed into the wells of the plates and incubated at 37 ^{o}C for 24 hours. Bacterial growth in the wells were assessed after addition of 20 µL of 0.25 mg/mL MTT (Applichem Chemical Synthesis Services, Germany).

In other to determine the MBC of the extract, 100 µL of the content of the wells that showed inhibitory activity were inoculated onto the surface of sterile nutrient agar plates, incubated at 37 ^{o}C for 48 hours and observed for signs of growth. The least concentration that did not show growth was recorded as the MBC.

Effect of temperature on the antimicrobial activity of $GKSE_1$ extract

To assess the stability of the bioactive components of the extract at various degree of temperature, eight screw capped test tubes were filled with 10 mL of 200 mg/mL concentration of $GKSE_1$ extract. Six test tubes were respectively heated at temperatures of 60, 70, 80, 90 100 and 110 ^{o}C for one hour whereas one was autoclaved 121 ^{o}C for 15 minutes. The remaining tube was kept at room temperature as a control. After cooling to room temperature, the heat-treated extracts including the control were tested for antimicrobial activity against *B. subtilis* and *K. pneumoniae* using the agar well diffusion method [19]. The activity was carried out in triplicates and the zones of inhibitions measured were recorded.

Stability of $GKSE_1$ extract in water

The extract (10 mL of 200 mg/mL) was freshly prepared with sterile distilled water and its activity was tested against *B. subtilis* and *K. pneumoniae* using the agar well diffusion method. The remaining extract solution was dispensed in 4.5 mL quantities into two screw capped test tubes. One of the tubes was kept in a refrigerator at 4 ^{o}C whiles the other was kept at room temperature and their antimicrobial activities were determined weekly for 12 weeks.

Thin layer chromatography (TLC)and Bioautography assay of $GKSE_1$ Extract

A 100 mg/mL solution of the extract was spotted on 6 different thin layer chromatography plates of dimension 1.5 × 6 cm (TLC silica gel 60 F254, Merck, Germany) with the help of a capillary tube. The plates were developed in chloroform–ethanol (85:15) solvent system. The Rf values of the separated fractions of the extract were determined after observing the chromatograms under UV light (254 nm and 365 nm) as well as after treating two of the plates with p–anisyladehyde spray. Two untreated plates were then overlaid [22] with 18 hours broth culture of *B. subtilis* (0.5 Mcfarland standard) and incubated at 37 ^{o}C for 24 hours. Areas of growth inhibition were identified after spraying the plates with MTT dye. The experiment was repeated using *K. pneumoniae*.

Characterization and identification of isolate $GKSE_1$

The isolate was characterized using a number of morphological and biochemical assays such as gram staining, oxidase, catalase, H_2S and indole productions, MR–VP, nitrate reduction, and citrate utilization assays following the methods described in Cowan and Steel's manual for the identification of medical bacteria [23]. The isolate was further identified using Matrix-Assisted Laser Desorption Ionization–Time of Flight (MALDI-TOF) device (Bruker Daltonik GmbH, Karlsruhe, Germany) as described by Elabbasy et al., [24].

3. RESULTS AND DISCUSSION

Isolation and screening of isolates

The 'microbial world' has served as a useful reservoir of chemical compounds that continues to be exploited for the development of potent antibiotics. Most of the antimicrobial metabolites are produced by microorganisms as a means of defense against other organisms they compete with in their habitat [19]. In this study, a total of 138 microorganisms were isolated from the samples obtained (soil, water and sediment). Thirty–six (36) of these isolates (26%) exhibited antimicrobial activity against at least one of the nine test microorganisms that were used in the screening of the isolates using the agar well diffusion method (table 1). This finding agrees with other reports and suggest that the microorganisms isolated produced secondary metabolites with inhibitory activities. Tawiah *et al*. [20] reported 27 antimicrobial metabolite producing microbial isolates out of 119 microorganisms recovered from water body samples collected in Ghana. Kumar *et al*. [4] also reported that 45 out of 78 marine *Actinobacteria* isolated, showed some level of antimicrobial activity. Another study by Gebreyohannes *et al*. [25] in Ethiopia showed that out of 31 actinomycetes isolated from water and sediments samples of Lake Tana, 13 displayed antibacterial activity against at least one test microorganism. Many of the reported antimicrobial metabolites of microorganism are molecules of proteins, lipopeptides, Glycopeptides, and lipoglycopeptide [26].

Table 1: Screening of isolates using the agar well diffusion method

	Test organisms/Mean zones of inhibition (mm)								
Isolates	*B. sub*	*S. typ*	*E. fae*	*S. aur*	*S. pyo*	*K. pne*	*E. col*	*P. aer*	*C. alb*
$GSSA_4$	-	18.3±0.3	-	-	-	-	-	-	-
$GSSC_1$	-	16.0±0.0	-	-	-	-	-	-	17.7±0.3
$GSSC_{14}$	-	17.7±1.8	-	-	-	-	-	17.3±0.3	-
$HSSA_1$	-	-	-	-	-	-	18.3±0.3	-	-
$HSSA_{2a}$	16.3±0.3	-	-	-	-	-	-	-	-
$HSSA_{3a}$	-	28.0±0.6	-	-	-	-	-	35.0±0.6	-
$HSSA_5$	15.0±0.6	-	-	-	-	-	-	-	-
$HSSA_6$	-	-	-	21.3±0.3	-	-	-	-	-
$HSSC_2$	-	18.3±0.7	-	-	-	-	-	-	-
$GFWC_{2b}$	-	16.3±0.3	-	-	-	-	-	17.3±0.3	-
$GFWK_{1a}$	16.7±0.3	-	-	-	-	-	19.7±0.3	-	-
$GKSC_3$	24.0±1.2	-	-	-	-	-	-	-	-
$GKSE_1$	33.3±1.5	24.0±0.0	-	-	-	29.7±0.3	-	35.3±0.3	-
$HKSC_{10}$	15.3±0.9	-	-	-	-	-	-	-	-
$HKSC_{2b}$	16.0±0.0	16.0±0.6	-	15.7±0.3	-	-	-	-	-
$HKSC_{2c}$	16.7±0.7	-	-	-	-	-	-	-	-
$HKSC_{3a}$	-	-	-	19.7±0.3	-	-	-	24.3±0.3	-
$HKSE_{10}$	17.3±0.7	-	-	-	-	-	15.3±0.3	-	-
$HKSE_4$	-	-	-	-	-	-	-	30.0±0.0	-
$HKSK_2$	-	20.7±0.9	-	-	-	-	-	-	-
$HKSK_{3b}$	-	-	-	31.0±0.6	-	-	-	-	-
$HKSK_{4b}$	-	-	-	-	-	-	-	20.3±0.3	
$HKSK_{7c}$	-	24.3±0.3	-	-	-	-	26.3±0.3	-	-
$HKSK_{8a}$	-	-	-	16.0±0.0	-	-	-	-	-
$GSWE_{1b}$	-	18.3±0.3	-	-	-	-	16.7±0.3	-	-
$GSWE_{2a}$	-	16.0±0.6	-	-	-	-	-	17.0±0.0	-
$HSWO_{1a}$	17.0±1.0	-	-	-	-	-	-	-	-
HSWO3	-	-	-	-	-	-	16.3±0.3	-	-
$GSSO_{11}$	15.3±0.3	-	-	25.0±0.6	-	-	-	-	
$GSSO_4$	-	-	-	15.7±0.7	-	-	-	19.7±0.3	-
$HSSE_1$	-	-	-	16.3±0.3	-	-	18.0±0.0	-	-
$HSSE_{2a}$	17.7±0.7	-	-	-	-	-	16.3±0.3	16.7±0.3	-
$HSSE_{3a}$	-	16.3±0.3	-	27.0±0.6	-	-	16.0±0.0	-	-

$HSSO_2$	-	17.7±0.7	-	-	-	-	17.7±0.3	19.3±0.3	-
$HSSO_{4c}$	-	-	-	16.7±0.3	-	-	-	-	-
$HSSO_{5a}$	-	-	-	16.3±0.3	-	-	17.0±0.0	-	-

B. sub=*B. subtilis*, E. fae=*E. faecalis*, S. typ=*S. typhi*, S. aur=*S. aureus*, S. pyo=*S. pyogenes*, K. pne=*K. pneumoniae*, E. col=*E. coli*, P. aer=*P. aeruginosa* and C. alb=*C. albicans*. Diameter of cork-borer=12 mm.

Extraction and antimicrobial activities of isolate $GKSE_1$ extract

Ethyl acetate extraction of the secondary metabolites from $GKSE_1$ fermentation yielded 2.39 g brown extract. The extract displayed a concentration–dependent inhibitory activity against both gram-positive and gram-negative bacteria using the agar well diffusion method [19] with the least concentration tested (12.5 mg/mL) having activity against staphylococcus epidermidis only (Table 2). In the MIC determination, the least concentration to inhibit the growth of the test pathogens was 1.56 mg/mL whereas the highest concentration was found to be 6.25. The assessment of the MBC revealed that a concentration of 3.25 mg/mL had the potential of killing a test pathogen and the highest concentration shown to be bactericidal was found to be 12.5 mg/mL (Tables 3). The activity of Ciprofloxacin was comparatively better and this is because of it being a pure compound.

Table 2: Antimicrobial activity of the crude extract and Ciprofloxacin.

Test organisms	Zone of Inhibition of $GKSE_1$ Extract (mm)					Ciprofloxacin
	12.5 mg/mL	25 mg/mL	50 mg/mL	100 mg/mL	200 mg/mL	1 µg/mL
B. subtilis	0.0	10.00±0.0	12.00±0.58	13.00±0.58	17.33±0.33	40.00±0.0
S. aureus	0.0	10.33±0.33	10.67±0.33	13.67±0.67	16.67±0.33	45.33±0.67
S. epidermidis	10.0±0.0	13.00±0.58	13.67±0.67	19.67±0.67	24.33±1.33	46.67±1.20
S. pyogenes	0.0	10.67±0.33	12.00±0.57	16.33±0.33	20.67±0.33	28.33±0.67
E. faecalis	0.0	10.00±0.0	16.00±0.0	25.33±0.88	29.67±0.33	33.67±0.67
E. coli	0.0	10.00±0.0	12.67±0.33	17.67±1.20	28.33±1.67	43.33±0.88
K. pneumoniae	0.0	12.33±0.33	11.00±0.0	12.67±0.33	16.00±0.58	33.00±0.0
P. aeruginosa	0.0	10.00±0.0	11.00±0.0	13.00±0.58	16.33±0.33	32.33±0.33
S. dysentriae	0.0	10.00±0.0	11.33±0.33	12.67±0.33	15.33±0.33	48.67±0.67
S. typhi	0.0	11.33±0.33	12.67±0.33	16.67±0.67	19.67±0.33	48.67±0.67
S. typhorium	0.0	10.33±0.33	13.33±0.33	15.33±0.88	17.33±0.67	34.33±0.33

Diameter of cork-borer: 8 mm

Table 3: The minimum inhibitory and bactericidal concentrations of $GKSE_1$ extract and ciprofloxacin.

Test organisms	$GKSE_1$ Extract (mg/mL)		Ciprofloxacin (µg/mL)	
	MIC	MBC	MIC	MBC
B. subtilis	6.25	12.5	5.0	10.0
S. aureus	1.56	6.25	1.25	2.5
S. epidermidis	3.13	3.13	2.5	10.0
S. pyogenes	3.13	6.25	1.25	2.5
E. faecalis	3.13	3.13	2.5	1.25
E. coli	3.13	3.13	1.25	1.25
K. pneumonia	6.25	6.25	2.5	10.0
P. aeruginosa	3.13	3.13	2.5	5.0
S. dysentriae	3.13	3.13	10.0	20.0
S. typhi	1.56	3.13	1.25	2.5
S. typhorium	6.25	12.5	2.5	10.0

Effect of temperature on the antimicrobial activity of the extract

The structure of most compounds is destroyed when they are subjected to high temperatures hence rendering them biologically inactive. In this current study, temperature was not found to have effect on the bioactive metabolite produced by isolate $GKSE_1$ when the extract was subjected to temperatures up to 110 ^oC for 1 hour and also after autoclaving at 121 ^oC for 15 minutes and then testing against *K. pneumonia* and *B. subtilis* (Figure 1). This finding agrees with that reported by Xiao–Yan *et al*., [27] where Trichokonins isolated from *Trichoderma koningii* SMF2 was found to be heat resistant. In contrast with this current study, Bharti *et al*., [28] observed a reduction in the antimicrobial activity of an extract obtained from *Burkholderia gladioli* when the extract was exposed to temperatures of 70 ^oC and above.

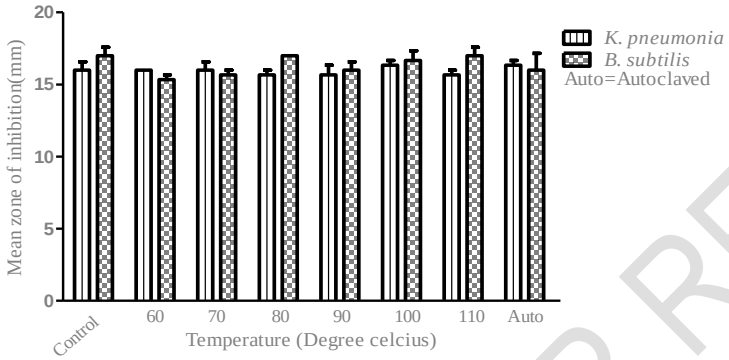

Figure 1.0. Effect of temperature on the antimicrobial activity of the extract. There was no significant difference in activity between the control group and the other heat treated groups.

Stability of the extract in aqueous solution

The bioactive metabolite of the extract was found to be stable and active against *K. pneumonia* and *B. subtilis* when kept in aqueous solution and tested weakly for a period of 12 weeks. Nevertheless, the activity of the extract was not dependent on the temperature at which it was kept (4 ^oC and room temperature) during the study period (Figures 2a and b). This may be due to the antimicrobial agent's ability to withstand degradation in aqueous solution for the entire period of the study.

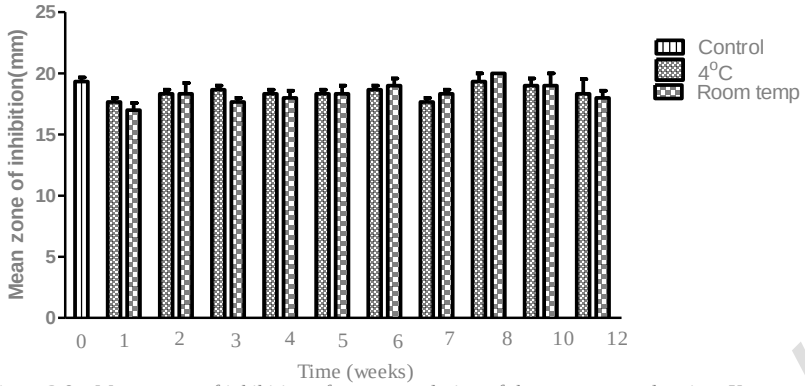

Figure 2.0a: Mean zones of inhibition of aqueous solution of the extract tested against *K. pneumonia* for a period of twelve weeks. There was no significant difference between the mean zone of inhibition observed on day 0 and the other weeks.

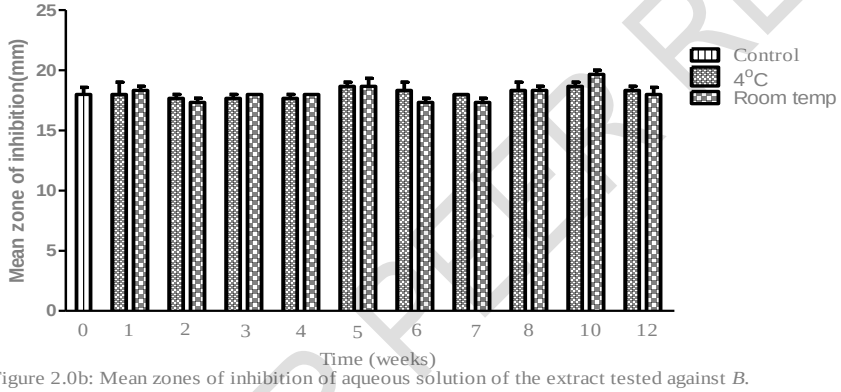

Figure 2.0b: Mean zones of inhibition of aqueous solution of the extract tested against *B. subtilis* over a period of twelve weeks. There was no significant difference between the mean zone o inhibition obtained on day 0 and the other weeks

TLC and Bioautography analysis of the extract

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TLC analysis of the crude extract yielded five spots with R_f values 0.095, 0.405, 0.524, 0.810 and 0.976 respectively in Chloroform – ethanol solvent system (85:15). This is an indication that the organism produced more than one compound as a secondary metabolite [4]. In the bioautography assay, two regions in the chromatogram were observed to have displayed zones of inhibitions (indicated by clear areas as against a purple background) suggesting that the organism might have produced two major kinds of antimicrobial agents probably as a defense mechanism [6]. This result is in contrast with that reported by Tawiah *et al*., [20] where all seven spots observed in the TLC analysis inhibited the growth of the test organisms in the bioautography assay.

Characterization and identification of isolate $GKSE_1$

The isolate was subjected to series of morphological and biochemical assays as a way of characterizing it (Table 4). MALDI-TOF, a more advanced technique, was used to identify the isolate as an *Enterococcus feacalis*. In recent years, MALDI-TOF has had its application in the identification of both bacteria and fungi to at least the species level [29].

Table 4: Characteristics of isolate $GKSE_1$.

TEST	OBSEVATION	TEST	OBSEVATION
Gram reaction	+	Indole production	-
Shape	Cocci	MR	+
Growth at 6.5% NaCl	+	VP	-
Growth at 45 ^{o}C	+	Nitrate reduction	-
Growth on		Citrate utilization	-
Cetrimide agar	-	Acid produced from	
Bismuth sulphite agar	-	Glucose	+
Mannitol salt agar	-	Lactose	+
MacConkey agar	-	Gas produced from	
Catalase	-	Glucose	-
Oxidase	-	Lactose	-
H_2S production	-		

Comment [ML3]: Pls check it.

Comment [ML4]: Pls check it.

4. CONCLUSION

This study has revealed the presence of antimicrobial producing microorganisms in the soil, Kakum River and the Gulf of Guinea in Ghana. The crude extract of secondary metabolites produced by a selected isolate, $GKSE_1$, showed inhibitory activity against all the test organisms used. The extract was found to be heat stable even after autoclaving at 121 ^{O}C for 15 minutes and also stable in aqueous solution for 3 months. The bioautography analysis of the crude extract of the selected isolate revealed two regions of inhibitions on a developed TLC plates indicating the possibility of production of more than one bioactive metabolite. The isolate was finally identified as an *Enterococcus faecalis* being the first strain of *Enterococcus* species reported to possess an antimicrobial producing potential. Work is ongoing to isolate and identify the compounds responsible for the antimicrobial activities.

COMPETING INTERESTS DISCLAIMER:

AUTHORS HAVE DECLARED THAT NO COMPETING INTERESTS EXIST. THE PRODUCTS USED FOR THIS RESEARCH ARE COMMONLY AND PREDOMINANTLY USE PRODUCTS IN OUR AREA OF RESEARCH AND COUNTRY. THERE IS ABSOLUTELY NO CONFLICT OF INTEREST BETWEEN THE AUTHORS AND PRODUCERS OF THE PRODUCTS BECAUSE WE DO NOT INTEND TO USE THESE PRODUCTS AS AN AVENUE FOR ANY LITIGATION BUT FOR THE ADVANCEMENT OF KNOWLEDGE. ALSO, THE RESEARCH WAS NOT FUNDED BY THE PRODUCING COMPANY RATHER IT WAS FUNDED BY PERSONAL EFFORTS OF THE AUTHORS.

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