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In vitro antibacterial characterization of *Streptosporangium* sp. (AI-21) a new soil isolate against food borne bacteria

Amita Gaurav Dimri¹, Abhishek Chauhan²^[] M.L Aggarwal¹

¹Deaprtment of microbiology, Shriram Institute for Industrial Research, 19, University Road, Delhi-11007 ²Amity Institute of Environmental Toxicology, Safety and Management, Amity University, Sector-125, Noida, Uttar Pradesh, India [×] <u>akchauhan@amity.edu</u> Received Date: Jan 24, 2020 / Accepted Date: June 22, 2020 / Published Date: June 27, 2020

Abstract

In the present investigation, *Streptosporangium* sp. (AI-21) a new soil borne isolate was characterized against food borne bacteria isolated from thirty-five food samples. Based on the morphological and biochemical characteristics, it was found that all food borne bacteria belong to *Escherichia, Pseudomonas, Salmonella, Staphylococcus* and *Bacillus* group of microorganisms. Colony morphology and biochemical characterization was the base of identification of actinomycete species. The actinomycete isolate was grown at a mass level by inoculum build up in mother flasks. Extraction of antibacterial substance from isolated actinomycetes was done by solvent- extraction method. Ethyl acetate extract exhibited activity against *Staphylococcus* sp. and *Escherichia* sp. The MICs of this extract was recorded in the range of 512 μ g/ml to 2048 μ g/mL. A maximum zone of 23.15mm was observed against *Staphylococcus* sp. The study not only targeted food borne isolates but also suggested how soil could be explored for the extraction of bioactive molecules by actinomycetes. This study could be a platform for new drug discovery.

Keywords: Food borne bacteria, soil actinomycetes, antimicrobial compounds and drug discovery

Introduction

Actinomycetes are Gram-positive, aerobic and thread like bacteria, free-living, saprophytic, and abundant in soil, water, and colonizing nodulating plants. Actinomycetes assume a significant job in reusing squanders in the earth and they are additionally the makers of thousands of metabolic items, which display organic action. For isolation and characterization of antimicrobial compounds numerous factors must be recalled such as source of screening, initial treatment of samples, growth medium both selective and non-selective, culture conditions and identification of colonies on Petri-plates (Nolan and Cross, 1988) using pretreatment of soil, drying and heating, stirred the isolation of rare Actinomycetes (Kim et al., 1994). Isolation procedure must be more selective by adding certain chemicals (Hayakawa et al., 1991). As it is very well known, actinomycetes are most important group of bacteria studied extensively and known to produce a wide range of bioactive compounds with diverse biological activity. Secondary metabolites obtained from various potential strains of actinomycetes are very effective against Gram-positive and Gram-negative bacteria. Many researchers both nationally and internationally isolated this potential group of microorganisms from soil, water, sediment etc. and checked their potential antimicrobial activity (Dimri et al., 2018a,b. Actinomycetes are also explored against various spp. of Candida albicans and against various pathogenic fungi. Bioactive compounds/antimicrobial compounds/antibacterial from agent/molecules discovered this group of microorganisms are used in medicine and agriculture. They are also known to produce branching mycelium of two types first one is substrate and second one is aerial mycelium. They are extensively dispersed in natural and manmade environments and reported by researchers their important role in the degradation of organic matter. The genus

Streptomyces is extensively study for antimicrobial potential and for the production of bioactive molecules (the biggest producer of antibiotics).

Due to the outstanding history of actinomycetes in the production of bioactive molecules for human interest, a large number of efforts have been made on the isolation, characterization and identification from terrestrial sources in the past half-decade. Presently, the rate of exploring novel compounds from soil actinomycetes has fallen down, whereas the amount of isolating commercialized bioactive molecule has inflated. Thus, new strain of actinomycetes must be isolated from unexplored or underexploited habitats for the development of innovated antimicrobial compounds for the benefits of society and humankind.

Amongst all Actinomycetes strains, Streptomyces remains unique producer of antibiotic. Streptomyces had produced 500 species of this genus, which have been reported to produce antimicrobials (Euzeby, 2008). This may be because as for non-Streptomycetes species requires new and innovative techniques for isolation from soil, sediment and another natural environment. In addition to this, their complex conservation and cultural procedures, which commonly need some precise and scarce conditions. This is the reason these organisms are called rare organisms. Most of the species recovered on plates containing selective media through basic conventional isolation techniques (serial dilution, pour plate, spread plated and pre and postusing enrichment highly selective media). Non-Streptomycetes or rare actinomycetes have observed, whose occurrence is minor than that of strains of Streptomycetes. This had increased physiology, habitat and efficiency of bioactive compounds from rare actinomycetes. Ecologically important potential of actinomycetes expected worth which lead to screen sources to develop into unusual surroundings.

Actinomycetes are explored for diverse group of antimicrobial compounds notably polyenes, polyketides, aminoglycosides glycopeptides, beta-lactams, macrolides, Actinomycins and tetracyclines. Most of the antibiotics (approximately two third of the naturally occurring) are obtained from extracts/fractions of Streptomycetes. The Streptomyces species isolated from various natural environments showed moderate to strong antibacterial and antifungal activities. Streptomyces sp. strain US80 isolated from Tunisian oasis soil, investigated for antifungal and antibacterial substances (Fguira et al., 2005). The isolate was evaluated against various bacteria. The results showed that US80 is the only defined strain, which yields all these three active molecules at once. Similarly, Coringa mangrove forest soils from Andhra Pradesh were studied to isolate actinomycetes for enzyme production and antimicrobial activity (Deepthi et al., 2012). Terrestrial samples from Western Ghat, forest rock, Tamil Nadu, India were analyzed for the isolation, characterization and antimicrobial activity of ERI-3, a Streptomyces spp. (Arasu et al., 2009). Thirtyseven soil samples were investigated for isolation of species of Actinomycetes from agriculture field in Vengodu, District Thiruvannamalai, Tamil Nadu, India. Jeffrey et al (2007) assessed the Malaysian soil for the isolation, characterization and antimicrobial potential enzymatic activities. The investigated report by Gurung et al (2009) has focused on antimicrobial potential of extracts of actinomycetes isolated from soils of Kalapatthar Mount Everest. El-khawaga et al (2012) have reported the antibacterial and insecticidal potential of crude extract of twenty Actinomycetes isolated from sandy soil of Cairo, Egypt. Marine actinomycetes are also a significant source of antimicrobials as the environmental conditions of the sea are completely diverse from the terrestrial conditions (Meiying and Zhic heng, 1998). Many interrogated researches have also obtained novel antibiotics/antimicrobials/crude extracts from the marine environment (Sujatha et al., 2005). Mohanraj et al (2013) explored actinomycetes isolated from marine sediments to explore bioactive molecules to combat the disease caused by pathogenic microorganisms. Five isolates were selected for antimicrobial spectrum of ethyl acetate extract. MIC of active extract was also determined. Study concluded with the statement that naturally occurring actinomycetes have a potential to produce antimicrobial compounds against dermatophytes supporting the discovery of new antibiotics (Deepa et al., 2011). The current study was designed and investigated to study antibacterial activity of isolates of actinomycetes against food borne bacterial isolates.

Method and materials

Media and reagent

The dehydrated microbiological growth media used for the study were of certified brand and procured from Hi Media Laboratories, Mumbai, India. Media was prepared as per the manual instructions. The sterilization of media done at 121°C, 15 Psi for 15 minutes and pH was checked after sterilization. Growth promotion test of each media carried out before evaluation of samples (Chauhan et al., 2013 a, b). Rabbit plasma for serological test was procured from BD Difco Laboratories.

Collection of food samples

Ninety-one food samples (chole-bhatoore, chole-rice, breadpakora, puri-sabzi, kachori-sabzi, chole-kulche and besangola were collected from different locations in Delhi. Gamma radiated sterilized pet jars were used for the sampling maintaining all aseptic conditions. All the food samples were brought to the laboratory maintaining the temperature conditions in an ice pack box.

Collection of soil, sediment and water samples

A number of fifty-seven soil samples were collected from various depths at different places of Dehradun, Hardwar district, Uttarakhand, India. Samples were dried in room temperature for 24 hrs. Then ground and sterilized as recorded by Saadoun et al (1999). Fifteen sediments and fifteen lakes water samples were collected from different lakes and then added in 90 ml of autoclaved distilled water. With the help of serial dilution technique, each dilution was spreaded onto Actinomycetes isolation agar (AIA) medium. Colony morphology and biochemical characterization was the base of identification of Actinomycetes from soil and sediments.

Morphological and biochemical characteristics of actinomycetes isolates

The Actinomycetes isolates screened and named AI-2, AI-4, AI-15, AI-18, AI-19, AI-21 and AI-24. Gram staining and biochemical tests were performed for identification of the isolates. Biochemical tests included- methyl red, voges-proskauer, urease, citrate utilization, indole production, lysine, malonate, nitrate reduction, arginine, hydrogen-sulphide production, phenylalanine-deamination test, ONPG (ortho- nitrophenyl –beta-D- galactopyranose) test, glucose, sucrose inositol, xylose, mannitol, sorbitol, rhammose, adonitol, arabinose and raffinose.

Mass culturing of actinomycetes isolates

The actinomycete isolates were grown at a mass level by inoculum build up in mother flasks (nutrient broth). Stock culture of isolated actinomycetes was inoculated in 40ml of mother flask. The mother flasks incubated at 28°C on rotatory shaker for 48 hrs. After incubation, 5ml of aliquot of inoculum from respective mother flasks was transferred to 50 ml of fermentation medium (Arginine Glycerol Medium). The fermentation medium flasks incubated at 28°C on rotatory shaker for 96 hrs.

Extraction procedure

Extraction of antibacterial substance from isolated actinomycetes was done by solvent- extraction method. A weight 10 g of dried biomass of actinomycete isolates was

taken in centrifuge tube followed by 25mL of organic solvents- methanol, Ethyl acetate and hexane. The centrifuge tube was shaked and vortexed for 30 minutes for proper mixing of extract with the solvent. Supernatant was collected and dried in nitrogen gas at a temperature of 50°C. After drying the crude extract was make up with DMSO (100 mg/mL) and then used within twenty-four hours for the assessment of antibacterial potential. Antibacterial activity of the mycelia was also checked.

Food borne isolates and inoculum preparation

All food borne isolates (FBIs) were maintained and preserved in Nutrient Agar slants. For experiment, all isolates were set a concentration of 0.5 Mc Farland (10^8 cfu/ml) in normal saline (0.85%) with the help of densitometer. Validation of inoculum size was done by serial dilution pour plate method.

Bacterial Strains as control

Positive bacterial cultures were used as to compare and identify the isolates from food samples. Positive strains of bacteria were procured from NCIM (National Collection of Industrial Microorganisms), Pune (Maharashtra). The cultures procured named- Escherichia coli (ATCC- 8739), Pseudomonas aeruginosa (ATCC- 9027), Salmonella typhimurium (ATCC- 23564), Staphylococcus aureus (ATCC- 6538) and Bacillus cereus (ATCC- 11778), Staphylococcus epidermidis (ATCC- 12228), Shigella flexineri (ATCC- 29508), Clostridium perfringens (ATCC-13124), Acinetobacter junii (ATCC- 17908), Proteus vulgaris (ATCC- 6380), Bacillus subtilis (ATCC- 6633), Enterobacter aerogenes (ATCC- 13048) and Klebsiella pneumoniae (ATCC- 10031). All the isolates and the positive cultures once identified, marked properly were preserved at 4°C in refrigerator in Nutrient Agar slants. The sub culturing was done as per ATCC, as to maintain the viability of the cultures. For the experimental purpose, cultures were subcultured onto the fresh Nutrient agar slants. The temperature of the refrigerator was also monitored and recorded for the better storage of cultures.

Standard antibiotic

Antibiotic (Ciprofloxacin) used in the study was procured from Indian Pharmacopeia commission. On the basis of claim (potency 99.6%), weighed accurately 50.2 mg powder of ciprofloxacin in to 50 mL of volumetric flask. Dissolved and sonicated for five minutes and then make the volume to the mark with distilled water. Took 1 ml and diluted it to 100 ml. This dilution contained 10 μ g/mL. Prepared double dilution to obtain 5 μ g/mL.

Agar well diffusion assay

In this assay, 100 μ L of each bacterial culture were mixed into 100 ml of sterile, molten, cool Mueller Hinton Agar, mixed well and poured into sterile Petri-plates. After solidification two wells of 6mm diameter per plate were made with the help of cork borer. The plates were marked properly with respect to the extract, Media used, Date and test food borne isolates. A 100 μ l of respective extracted solvent was put into the wells (used as controls). Petri-plates were incubated at 37°C for overnight. Zone of inhibitions (in mm) measured with the help of vernier calipers (Goyal et al., 2008; Gupta et al., 2009; Chauhan et al., 2010; Kaushik et al., 2010)

Determination of minimum inhibitory concentration (MIC)

For determining the MIC, tube dilution method was used. The active extract of Actinomycetes was diluted in the range of 1-2048 μ g/mL as per NCCLS guidelines. Adjusted bacterial culture were added in the MHB tubes having diluted extracts. After incubation at 37° C, MIC values for all extracts recorded.

Results and discussion

The food borne bacterial isolates isolated from thirty-five collected food samples were named as Staphylococcus spp. (FBI-11-17) and Bacillus spp. (FBI-28-35). Gram-negative bacteria food borne isolates of Escherichia spp. (FBI-1-10), Salmonella spp. (FBI-18-21)and Pseudomonas spp. (FBI-22-27). In the case of Streptosporangium AI-21 only ethyl acetate extract exhibited activity against Staphylococcus spp. and Escherichia spp. as compared to Pseudomonas, Bacillus and Salmonella isolates in which no activity was observed. Amongst Escherichia isolates, the maximum inhibition zone was recorded against FBI-5 (19.11 mm) followed by FBI-7 (16.11 mm) FBI-3 (15.20 mm) FBI-6 (15.02 mm) FBI-8 (14.22 mm) FBI-2 (14.11 mm) FBI-1 (13.02 mm FBI-4 (10.11 mm). However little zone of inhibition observed against FBI-9 (8.11 mm) and FBI-10 (9.11mm). Similarly, all isolates belong to Staphylococcus were found susceptible when tested with this extract. the maximum inhibition zone was recorded against FBI-16 (23.15 mm) followed by FBI-15 and 12 (23.10 mm) FBI-11 (21.20 mm) FBI-17 (21.10 mm) FBI-14 (20.19 mm) FBI-13 (19.15 mm. MIC of the ethyl acetate extract determined against Escherichia and Staphylococcus isolates only. Amongst Escherichia isolates,

the maximum inhibition zone was recorded against FBI-5 (19.11 mm) followed by FBI-7 (16.11 mm) FBI-3 (15.20 mm) FBI-6 (15.02 mm) FBI-8 (14.22 mm) FBI-2 (14.11 mm) FBI-1 (13.02 mm FBI-4 (10.11 mm). However little zone of inhibition observed against FBI-9 (8.11 mm) and FBI-10 (9.11mm). Similarly, all isolates belong to *Staphylococcus* were found susceptible when tested with this extract. The maximum inhibition zone was recorded against FBI-16 (23.15 mm) followed by FBI-15 and 12 (23.10 mm) FBI-11 (21.20 mm) FBI-17 (21.10 mm) FBI-14 (20.19 mm) FBI-13 (19.15 mm)

Table 1 Morphological and biochemical characteristics of *Strpetosprangium* sp.

Parameters	Strpetosprangium sp.							
	AI-21							
Aerial mycelium	Pinkish White							
Substrate mycelium	Brownish white							
Gram stain	+							
Spore chain	Straight Chain							
Methyl Red	+							
Voges-Proskauer test								
Malonate test	-							
H ₂ S production test	-							
Colony margin	Filaments							
Citrate test	-							
Urease test	-							
Nitrate test	+							
Phenylalanine- deamination test	-							
Indole	-							
Lysine test	-							
Arginine test	-							
ONPG test	-							
Mannitol	+							
Rhammnose	-							
Lactose	+							
Glucose	+							
Xylose	-							

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Sucrose	-	Sorbitol	-
Arabinose	+	Adonitol	-
Inositol	+	Raffinose	-

Table 2 Antimicrobial potential of *Streptosporangium sp.* AI-21 against food borne gram-positive and gram-negative bacteria

ood Borne Isolates	Antibacterial Spectrum (Zone of Inhibition in mm)									
Bacteria and	Actinomycetes Extracts/Fraction									
Isolate No.		Organic So	Mycelium	Ciprofloxacin						
	Methanol	Hexane	Ethyl acetate							
Escherichia sp. (FBI-1) 0.0		0.0	13.02±.22	0.0	29.11±.22					
Escherichia sp. (FBI-2)	0.0	0.0	14.11±.13	0.0	23.10±.30					
Escherichia sp. (FBI-3)	0.0	0.0	15.20±.03	0.0	22.10±.10					
Escherichia sp. (FBI-4)	0.0	0.0	10.11±.9	0.0	26.10±.23					
Escherichia sp. (FBI-5)	0.0	0.0	19.11±.37	0.0	23.10±.21					
Escherichia sp. (FBI-6)	0.0	0.0	15.02±.20	0.0	26.10±.12					
Escherichia sp. (FBI-7)	0.0	0.0	16.11±.21	0.0	27.10±.14					
Escherichia sp. (FBI-8)	0.0	0.0	14.22±.5	0.0	25.10±.33					
Escherichia sp. (FBI-9)	0.0	0.0	08.11±.8	0.0	28.10±.30					
Escherichia sp. (FBI-10)	0.0	0.0	09.11±.19	0.0	22.10±.10					
Staphylococcus sp. (FBI-11)	0.0	0.0	21.20±.10	0.0	26.10±.10					
Staphylococcus sp. (FBI-12)	0.0	0.0	23.10±.11	0.0	23.27±.10					
Staphylococcus sp. (FBI-13)	0.0	0.0	19.15±.11	0.0	26.10±.19					
Staphylococcus sp. (FBI-14)	0.0	0.0	20.19±.14	0.0	24.30±.61					
Staphylococcus sp. (FBI-15)	0.0	0.0	23.10±.13	0.0	25.10±.17					
Staphylococcus sp. (FBI-16)	0.0	0.0	23.15±.18	0.0	24.23±.17					
Staphylococcus sp. (FBI-17)	0.0	0.0	21.10±.30	0.0	24.10±.26					
Salmonella sp. (FBI-18)	0.0	0.0	0.0	0.0	25.12±.14					
Salmonella sp. (FBI-19)	0.0	0.0	0.0	0.0	28.14±.10					
Salmonella sp. (FBI-20)	0.0	0.0	0.0	0.0	26.12±.62					
Salmonella sp. (FBI-21)	0.0	0.0	0.0	0.0	24.20±.12					
Pseudomonas sp. (FBI-22)	0.0	0.0	0.0	0.0	23.12±.02					

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Pseudomonas sp. (FBI-23)	0.0	0.0	0.0	0.0	28.23±.12
Pseudomonas sp. (FBI-24)	0.0	0.0	0.0	0.0	26.10±.24
Pseudomonas sp. (FBI-25)	0.0	0.0	0.0	0.0	25.21±.01
Pseudomonas sp. (FBI-26)	0.0	0.0	0.0	0.0	24.13±.02
Pseudomonas sp. (FBI-27)	0.0	0.0	0.0	0.0	23.10±.1.8
Bacillus sp. (FBI-28)	0.0	0.0	0.0	0.0	25.20±0.4
Bacillus sp. (FBI-29)	0.0	0.0	0.0	0.0	22.12±.01
Bacillus sp. (FBI-30)	0.0	0.0	0.0	0.0	24.13±.13
Bacillus sp. (FBI-31)	0.0	0.0	0.0	0.0	28.12±.17
Bacillus sp. (FBI-32)	0.0	0.0	0.0	0.0	23.12±.13
Bacillus sp. (FBI-33)	0.0	0.0	0.0	0.0	25.02±.21
Bacillus sp. (FBI-34)	0.0	0.0	0.0	0.0	25.02±.07
Bacillus sp. (FBI-35)	0.0	0.0	0.0	0.0	27.14±.31

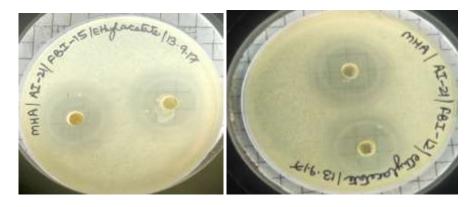


Fig. 1: Zone of inhibition against food borne isolate *Staphylococcus* sp. (FBI-12 and 15): Muller Hinton agar plate, well size: 6mm, well: 1 and 2: 100 µL of ethyl acetate extract (100 mg/mL) of AI-21 isolate; zone size in mm recorded after 24 hrs of incubation at 37°C

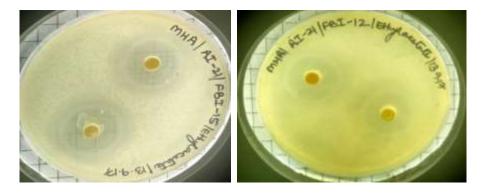


Fig. 2: Zone of inhibition against food borne isolate *Staphylococcus* sp. (FBI-15 and 12): Muller Hinton agar plate, well size: 6mm, well: 1 and 2: 100 μ L of ethyl acetate extract (100 mg/mL) of AI-21 isolate; zone size in mm recorded after 24 hrs of incubation at 37°C

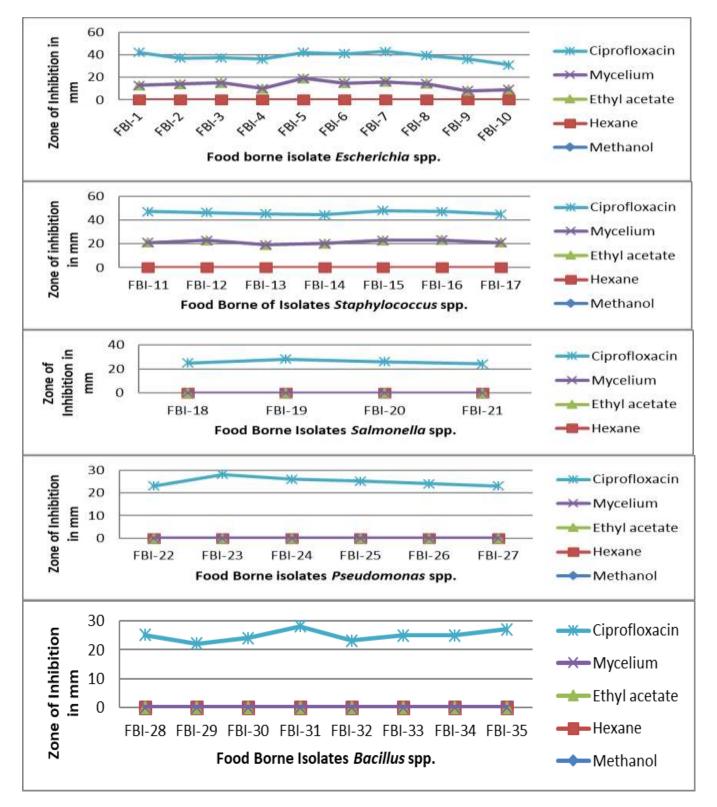


Fig. 3: Antimicrobial spectrum of different extracts of AI-21 Actinomycete isolate against food borne isolates; A: *Escherichia* spp., B: *Staphylococcus* spp.; C: *Pseudomonas* spp. D: *Salmonella* sp. E: *Bacillus* spp.

Food Borne Isolates	rne Isolates Ethyl Extracts concentration (µg/ml)										MIC		
Bacteria and	2048	1024	512	256	128	64	32	16	8	4	2	1	(µg/ml)
Isolate No.													
Escherichia sp. (FBI-1)	-	+	+	+	+	+	+	+	+	+	+	+	2048
Escherichia sp. (FBI-2)	+	+	+	+	+	+	+	+	+	+	+	+	>2048
Escherichia sp. (FBI-3)	-	-	+	+	+	+	+	+	+	+	+	+	1024
Escherichia sp. (FBI-4)	-	-	+	+	+	+	+	+	+	+	+	+	1024
Escherichia sp. (FBI-5)	+	+	+	+	+	+	+	+	+	+	+	+	>2048
Escherichia sp. (FBI-6)	+	+	+	+	+	+	+	+	+	+	+	+	>2048
Escherichia sp. (FBI-7)	-	-	+	+	+	+	+	+	+	+	+	+	1024
Escherichia sp. (FBI-8)	+	+	+	+	+	+	+	+	+	+	+	+	>2048
Staphylococcus sp. (FBI-11)	-	-	-	+	+	+	+	+	+	+	+	+	512
Staphylococcus sp. (FBI-12)	-	-	+	+	+	+	+	+	+	+	+	+	1024
Staphylococcus sp. (FBI-13)	-	-	+	+	+	+	+	+	+	+	+	+	1024
Staphylococcus sp. (FBI-14)	-	+	+	+	+	+	+	+	+	+	+	+	2048
Staphylococcus sp. (FBI-15)	-	+	+	+	+	+	+	+	+	+	+	+	2048
Staphylococcus sp. (FBI-16)	-	-	-	+	+	+	+	+	+	+	+	+	512
Staphylococcus sp. (FBI- 17)	-	-	+	+	+	+	+	+	+	+	+	+	1024

Table 3: Minimum inhibitory concentration of ethyl acetate extracts of *Streptosporangium sp.* AI-21 against food borne isolates

MIC values of standard antibiotic ciprofloxacin were recorded <1 µg/mL

MIC of the ethyl acetate extract determined against Escherichia and Staphylococcus isolates only.The MIC values were observed in the range of 512 µg/mL to 2048 µg/mL. The lowest MIC value (512 µg/mL) was seen against *Staphylococcus* isolates (FBI-11 and FBI-16) whereas it was 1024 µg/mL against isolate no. FBI-3, FBI-4, FBI-7, FBI-12, FBI-13, 2048 µg/mL against FBI-1, FBI-14, FBI-15 and >2048 µg/mL observed against FBI-2, FBI-5, FBI-6 and FBI-8. In comparison with the standard ciprofloxacin, all the extracts exhibited low to moderate antibacterial activity.

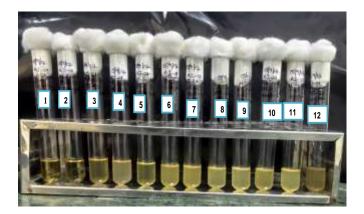


Fig. 4: Minimum inhibitory concentration of ethyl acetate extract of AI- 21 against *Escherichia* sp. (FBI-3)



Fig. 5: Minimum inhibitory concentration of ethyl acetate extract of AI-21 against *Staphylococcus* sp. (FBI-11)

Conclusion

Further investigations are needed to identify the food borne bacterial isolates and actinomycetes isolates at molecular level. The active extracts actinomycetes isolates should be proceed further for identification using various techniques like GC-MS, LC-MS, IR and NMR, the efficacy of potential molecules may also be evaluated using animal model. Thus, more intensive screening of actinomycetes from extreme to be done as to overcome chemically synthesized drugs. The study not only targeted food borne isolates but also suggested how soil could be explored for the production of antimicrobial compounds by actinomycetes. This study could be a platform for new drug discovery.

Conflict of Interest

Authors hereby declare no conflict of interest.

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