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Research Article

IN VITRO ANTIMICROBIAL ACTIVITY, OPTIMIZATION OF BIOACTIVE SECONDARY METABOLITES AND MOLECULAR CHARACTERIZATION OF *BACILLUS SUBTILIS* ISOLATED FROM SOILS OF WESTERN GHATS, INDIA

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ABSTRACT

Low molecular mass of secondary metabolites was usually produced from microbes during their late growth phase. They are of huge importance in terms of human health. In this regard, soil samples were collected from Nilgiris district in Western Ghats of Tamil Nadu, India to isolate bioactive potential bacteria. A total of 76 pure bacterial cultures were isolated by serial dilution method and the isolates were initially differentiated based on morphological and staining characteristics. Among these isolates *Bacillus subtilis*, the identity of which was confirmed by 16S rRNA analysis, showed strong antimicrobial activity against the tested pathogens compared with other strains. Optimization studies revealed that the highest antibiotic production was obtained from fermentation broth medium. Parameters of the culture revealed that 5 days of incubation at 30ºC with pH 8.0 of the production medium exhibited maximum yield. The Minimum Inhibitory Concentration (MIC) and minimum bactericidal and fungicidal concentrations (MBC/MFC) were also determined against all tested pathogens.

Keywords: *Bacillus subtilis*, Fermentation broth, Secondary metabolites, Soil, Western Ghats.

INTRODUCTION

The demand for new antibiotics continues to grow due to the rapid emerging of multiple antibiotic resistant pathogens causing life-threatening infection. However, nature still remains the richest and the most versatile source of new antibiotics[1-3]. Soil is considered one of the most suitable environments for microbial growth[4]. Soil bacteria perform important services related to disease suppression. Antibiotics produced by bacteria as secondary metabolites are employed in a wide range of diseases. This antimicrobial metabolite includes a chemically heterogeneous group of small organic molecules of microbial origin[5]. These metabolites have played a key role in the discovery and development of many antibiotics[6]. *Bacillus* species produce a great diversity of secondary metabolites with biological activity. The potential of *Bacillus subtilis* to produce antibiotics has been recognized for 50 years[7]. Some strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* have been known to produce antifungal peptides[8,9]. These strains, in response to nutritional stress, activate a variety of processes including sporulation, synthesis of extracellular degradative enzymes and antibiotic production[10].

Biosynthesis of antibiotics from microorganisms is often regulated by nutritional and environmental factors. El-Banna[11] reported that antimicrobial substances produced by bacterial species were greatly influenced by variation of carbon sources. Several abiotic factors, such as pH and temperature, have been identified as having an influence on antibiotic production from bacteria[12]. Manipulating nutritional and environmental factors can promote the biosynthesis of secondary metabolites and thus facilitate the discovery of new natural products[13]. However, not all microorganisms are equally capable of producing secondary metabolites. In fact, this capability is at the moment restricted to a few groups of microbes. The aim of the present study is to isolate the antibiotics- producing bacteria from forest soil from Western Ghats, identify them at molecular level and to investigate their antimicrobial properties against pathogenic bacteria and fungi. Optimization of culture conditions to improve antimicrobial potential and get high yields of the antimicrobial metabolites.

MATERIALS AND METHODS

Isolation of bacteria from soil samples

The soil samples were collected from Avalanche reserve forest of south zone Nilgiri district (Lat 11^0 08' to 13^0 37' N, Long 77^0 27' to 80^0 4' E) Western Ghats, Tamil Nadu, India. The samples were collected from 5 to 25 cm depth in sterile plastic bags and transported aseptically to the laboratory. Isolation and enumeration of bacteria were performed by serial dilution and spread plate technique[14]. One gram of soil was suspended in 9 mL of sterile double distilled water. The dilution was carried out up to 10^{-5} dilutions. Aliquots (0.1 mL) of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were spread on the Nutrient Agar (NA) medium containing (g/L) Peptone 5.0; NaCl 5.0; Beef extract 1.5; Yeast extract 1.5 and Agar Agar 15.0 and plates were incubated at 37°C for 2 days. Bacterial colonies were purified by repeated streaking. The purified colonies were stored at 4°C for further analysis.

Characterization of isolates

Bacterial isolates were initially identified based on color, culture morphology, and Gram's staining test according to the Bergey's manual[15].

Gram Staining

Smear was prepared by spreading the broth culture on a glass slide followed by heat drying. The smear was covered with crystal violet for 30-60 seconds and washed with water. The smear was covered with Gram's iodine for 30-60 seconds, decolorized with alcohol, and washed with water. Finally the smear was stained with safranin counter stain for 2 min. After washing and drying, the slides were viewed at 100× under phase contrast microscope. The gram positive bacteria retain the crystal violet dye and gram negative bacteria retain the safranin dye[16,17].

Morphological characterization

The bacterial colonies were observed under a high power magnifying lens and colony morphology was noted with respect to color, margin, consistency and appearance.

Secondary metabolites from isolated bacteria

The bacterial isolates were cultured in 100 ml of Nutrient broth medium (NB) containing (g/l): Peptone 5.0; NaCl 5.0; Beef extract 1.5; Yeast extract 1.5; and DH_2O 1L in 250 ml Erlenmeyer flasks. After 3 days of incubation at 130 rpm at 37°C in rotary shaker, cultures were centrifuged at 10000 rpm for 15 minutes and the supernatant was used for extraction process. The crude metabolite was extracted using equal volume of ethyl acetate with supernatant (1:1). The solvent layer thus collected was evaporated in a rotary evaporator under vacuum to obtain the crude residue which was dissolved in dimethylsulfoxide (DMSO) at a concentration of 2 mg/ml and employed for antimicrobial activity.

Microbial organisms

The following Gram-positive and Gram-negative bacteria as well as a fungus were used for the experiment. Gram-positive: *Enterococcus faecalis* (ATCC 29212). Gram-negative: *Enterobacter aerogenes* (MTCC 111), *Alcaligenes faecalis* (MTCC 3104). Fungi: *Candida albicans* (ATCC 10231). The reference bacterial cultures were obtained from the Institute of Microbial Technology, Chandigarh, India.

Antimicrobial Assay

The antimicrobial activity of the crude ethyl acetate extract was assayed using the standard Kirby-Bauer disc diffusion method[18]. The 0.5 Mc Farland Standard was used to adjust the turbidity of the tested pathogens. The turbidity standard may contain approximately 1.5 x 10^8 CFU/ml of the cell. Petri plates were prepared with 20 ml of sterile Muller Hinton agar (MHA, Himedia) for bacteria and Sabouraud Dextrose Agar (SDA Himedia) for fungal pathogens. The test cultures were swabbed on the top of the solidified media. The crude extract loaded discs were placed on the surface of the medium. A negative control was prepared using the respective solvent [dimethylsulfoxide (DMSO)]. Streptomycin (10 mg/disc) for bacteria and ketoconazole (10 mg/ disc) for fungi were used as positive controls. The plates were incubated overnight at 37°C and zones of inhibition were recorded.

Molecular Characterization

Isolation of genomic DNA

The genomic DNA of active strain was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel), according to manufacturer instructions. The pure bacterial culture was taken in a micro-centrifuge tube. 25 µl of proteinase K and 180 µl of T1 buffer were added with the bacterial culture and incubated in a water bath at 56°C. After incubation, 5 µl of RNase A solution added with the suspension and incubated for five minutes at room temperature. After that, 200 µl of B3 buffer added with the suspension and incubated for ten minutes at 70°C. 210µl of ethanol (100%) was added in the suspension and mixed uniformly using vortex mixture. The mixture was pipette into Nucleo Spin Tissue column and centrifuged at 12,000 rpm for one minute at room temperature and washed with 500 µl of BW buffer followed by 600 µl of B5 buffer. After that, Nucleo Spin Tissue column was placed in a clean 2 ml tube and bacterial DNA was eluted out using 50 µl of BE buffer. Finally the DNA was eluted by centrifuging at 11,000 rpm for one minute.

Analysis of 16S rRNA

The primers 16S-RS-Forward- CAGGCCTAACACATGCA AGTC and 16S-RS- Reverse- GGGCGGWGTGTACAAGGC (5' → 3') were used to amplify 16S ribosomal sequences. Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Bio systems) using the Big Dye Terminator v3.1 Cycle sequencing Kit (Applied Bio systems, USA). The quality of the sequence was tested using Sequence Scanner Software v1. The alignment of the sequence and necessary editing of the sequence was done by Geneious Pro v5.6.

Database searching and phylogenetic analysis

The sequences were compared for similarity with the reference species of bacteria contained in genomic database, using the NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree was constructed using the neighbor-joining DNA distance algorithm using software MEGA (version 4.0).

Optimization for maximum metabolite production

The parameters studied include Suitable medium, pH, temperature and incubation period. Four different broth media used for the secondary metabolite production. Such as,

- Nutrient Broth Medium (NB) containing (g/l): Peptone 5.0; NaCl 5.0; Beef extract 1.5; Yeast extract 1.5; and DH2O 1L.
- Lactose Broth (LB) containing (g/l): Peptone 5.0; Lactose 5.0; Beef extract 3.0 and Yeast extract 1.5.
- Fermentation Broth (FB) containing (g/l): glucose 10.0; NH_4NO_3 5.0; yeast extract 5.0; NaCl 5.0; K_2HPO_4 1.0; KH_2PO_4 1.0 and $MgSO_4$, $7H_2O$ 1.0.
- Luria Bertani Broth (LBB) containing (g/l): Tryptone 10.0; NaCl 5.0; Yeast extract 5.0.

The fermentation conditions were optimized for selected bacterial strain to enhance the bioactivity. The conditions include pH (5, 6, 7, 8 and 9), temperature (20^0C, 25^0C, 30^0C, 35^0C and 40^0C) and incubation period (1, 3, 5, 7 and 9 days). The pH of medium was adjusted with 1M HCl and 1M NaOH by using pH meter. The optimization process was done by altering one factor at a time and maintaining other factors as constant.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was determined by the macro broth dilution method[19]. The overnight culture of tested pathogens was adjusted with 0.5 Mcfarland standards. The 200 µg of crude metabolite was dissolved in 1 ml of DMSO and diluted into 100 µg, 50 µg, 25 µg, 12.5 µg and 6.25 µg in sterile NB and SBD for bacteria and fungi respectively. 100 µl of test micro organisms was disposed in each tube. MIC was considered the lowest antibiotic concentration that showed no visible growth and no increase in the optical density (OD600) read at the UV visible spectrophotometer. The experiments were carried out in triplicate. The bacteria containing tubes were incubated at 37°C for 24 h. The fungi containing tubes were incubated at 30°C for 3 to 5 days. The percentage cell inhibition was calculated using the following formula

$$\text{Percentage of inhibition}(\%) = ([\text{Control OD} - \text{Test OD}] / (\text{Control OD}) \times 100$$

Determination of minimum bactericidal and fungicidal concentrations (MBC/MFC)

For the determination of the MBC and MFC, fresh nutrient agar and sabouraud dextrose agar plates were inoculated with one loop ful of culture taken from each of the first three broth cultures that showed no growth in the MIC tubes. While MBC assay plates were incubated for 24 h, MFC assay plates were incubated for 3 to 5 days. After the incubation periods, the lowest concentration of the crude extract that did not produce any bacterial or fungal growth on the solid medium was regarded as MBC and MFC values for this extract[20].

Statistical analysis

All tests were conducted in triplicate. Data are reported as mean± standard deviation (SD). Results were analyzed statistically using Graph pad prism software.

RESULTS AND DISCUSSION

Collections of soil samples and isolation of bacteria

Totally 76 bacterial isolates were obtained from the soil samples collected from Avalanche reserve forest of south zone Nilgiris district, Western Ghats, Tamil Nadu, India. These isolates were initially differentiated into eight strains based on the colour, colony morphology and Gram staining characteristics following Bergey's Manual of Determinative Bacteriology[15] (Figure 1 and Table 1).

Preliminary screening of antimicrobial of activity

All the eight bacterial strains were evaluated for their antimicrobial potential against test pathogens. Among these three (37.5 %) isolates showed antimicrobial activity against *Enterococcus faecalis*, four (50 %) isolates showed activity against *Enterobacter aerogenes*, one (12.5) isolate showed activity against *Alcaligenes faecalis* and three (37.5 %) isolates showed activity against *Candida albicans*. One promising isolate, exhibited strong antimicrobial activity against the all tested pathogens with the zone of inhibition *Enterococcus faecalis* 14.75 ± 0.21, *Enterobacter aerogenes* 14.10 ± 0.14, *Alcaligenes faecalis* 13.85 ± 0.07 and *Candida albicans* 15.30 ± 0.42, So the isolate was selected for the further study (Table 2 and Figure 2). Similarly, Ganesan *et al.*[21] isolated 106 soil actinomycetes from Western Ghats in Tamil Nadu and screened against pathogenic microbes. They reported that one isolate, FMS-20, was highly active against all tested pathogens. Arasu, *et al.*[22] reported several *Streptomyces sp.* designated as ERI-26, ERI-3, ERI-04 etc, isolated from southern Western Ghats regions mainly from Nilgiri hills. *Streptomyces* sp. ERI-26 exhibited resistance to streptomycin and showed activity against several bacteria and fungi such as *Staphylococcus aureus, Staphylococcus epidermidis, Xanthomonas* sp. and *Candida albicans* at 0.25 mg/ml concentration. Saravanan *et al.*[23] isolated *Pseudomonas* sp. from forest soil of Western Ghats with potent antibacterial activity against a wide range of Gram positive and Gram negative bacteria. Islam *et al.*[24] reported several bacterial isolates from Western Ghats soil samples, which produce antifungal compounds. The present study is in accordance with all the previous studies.

Molecular characterization of efficient isolate

The active strain was further identified by 16s rRNA sequence analysis and Blast analysis, which confirmed that the isolate belonged to the *Bacillus subtilis* species. The obtained nucleotide sequence of the bacterial strain was deposited in the NCBI Genbank with the accession number MH198042. The quality of the DNA isolated was checked using agarose gel electrophoresis. The UV trans illumination used for the visualization of gels and the image was taken using Gel documentation system under the UV light. The 2-log DNA ladder was used as a molecular standard (Figure 3). A phylogenetic tree (Figure 4) based on 16S rRNA gene sequences of members of the genus *Bacillus* was constructed according to the bootstrap test of neighbor-joining algorithm method[25].

Optimization of culture conditions

The production of antimicrobial metabolites by *Bacillus subtilis* was determined at various culture media and physical conditions by antimicrobial disc diffusion assay method measuring the zone of inhibition against tested pathogens.

Effect of suitable medium

The composition of a fermentation medium is a significant factor that influences the level of antimicrobial production by a microorganism[26]. Among the four different broths medium Fermentation Broth (FB) produces maximum antimicrobial metabolite against tested pathogens (Figure 5). Therefore, designing an appropriate medium and conditions for cultivation has prime importance in improving the antibiotic yield[27].

Effect of pH

The pH of a culture medium is usually not constant throughout fermentation and the changes that occur are highly affecting the metabolite synthesis. To determine the optimal pH for antimicrobial metabolite production by different pH values (5, 6, 7, 8 and 9). The highest level of antimicrobial metabolites production showed in pH 8 (Figure 6). Dezfully and Ramanayaka[28] reported the *in-vitro* antimicrobial compound production by *Streptomyces flavogriseus* could be obtained in MISP-2 medium having a pH 8. Similar findings were found in *Bacillus* sp. FAS1 where the antimicrobial compound was produced at alkaline pH[29]. Das *et al.*[30] also reported the optimum pH for antibacterial metabolite production by *Micrococcus* sp. AM7 is highly varied and the production was optimal at pH 8.0. Our present study is in agreement with this. However, pH 8 was used for further optimization experiments.

Effect of temperature

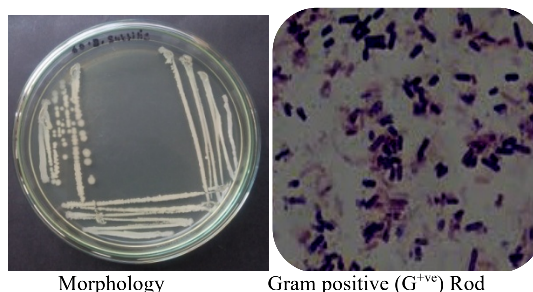
A wide range of temperatures (20, 25, 30, 35 and 40°C) was used in this study. The incubation temperature 30°C was found for maximum bioactive metabolite production by *Bacillus subtilis* (Figure 7). Islam *et al.*[24] reported the optimum temperature for production of antibiotic substances from isolate *Bacillus subtilis* (C9) was found at 30°C, which was similar to the present findings. This is in agreement with the report of Oskay[31] on *Streptomyces* sp. KGG32, wherein maximum biomass and antimicrobial production was observed at 30˚C. Hassan *et al.*[32] also has observed maximum antibiotic production by *Streptomyces violatus* at 30˚C. Also, Adinarayana *et al.*[33] found that, maximum neomycin production by *Streptomyces marinensis* was obtained at 30°C. However, El- Mehalawy *et al.*[34] had observed that maximum antifungal production by *Streptomyces lydicus* was at 24˚C whereas by *Streptomyces erumpens*. (*Streptomyces antimycoticus* removed from the text). The antimicrobial activity was reduced at higher temperature. These results accord with the fact, that extreme pH and temperature are unfavorable for antibiotic production[35].

Effect of incubation period

Fermentation time determination is of high importance for maximum harvesting of the metabolite because the incubation period required for growth and metabolite production vary significantly with different bacteria. The highest level was obtained on 5th day of incubation and then production was declined gradually (Figure 8). Kathiresan *et al.*[36] reported that 5 days of incubation gave the broad activity of secondary metabolites. The *Streptomyces albidoflavus* reached to maximum levels of cell growth and antibiotic production after 4 days[37].

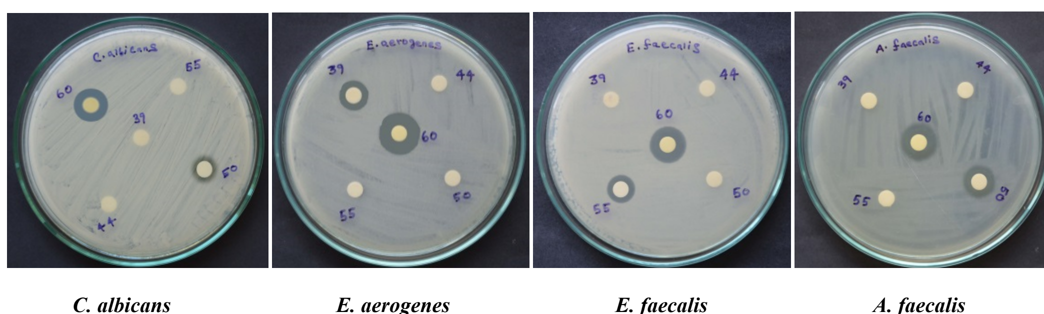
Minimum inhibitory concentration (MIC) and Minimum bactericidal and fungicidal concentrations (MBC/MFC) of crude extract of *Bacillus subtilis*

The minimum inhibition concentration (MIC) of crude extract was determined by NCCLS Macro broth dilution method. Different concentrations (200 µg,100 µg, 50 µg, 25 µg, 12.5 µg, and 6.25µg) of the crude extract were tested against the pathogens. The results observed in the MIC against the tested pathogens ranged between 50 to 100µg/ml. The Minimum bactericidal and fungicidal concentrations determined that no growth on the plates represented approximately 99 to 99.5 % killing the tested pathogens. The MBC and MFC of the crude extract against tested bacteria and fungi were found at 50 to 200 µg/ml (Table 3). So, it can be concluded that the crude extract of *Bacillus subtilis* was able to inhibit the growth of tested pathogens in the *in-vitro* studies. Arasu *et al.*[22] *Streptomyces* sp. isolated from the rock soil samples collected from Western Ghats, showed strong antimicrobial activity against *Staphylococcus epidermidis, Xanthomonas* sp., *Staphylococcus aureus* and *Candida albicans* with the MIC value of 0.25 mg/ml. A quantity of biologically active compounds can saturate more target sites and cause rapid bactericidal action against the bacteria[38].



Morphology   Gram positive (G^{+ve}) Rod

Figure 1: Morphology and Gram staining of the *Bacillus subtilis*



C. albicans ***E. aerogenes*** ***E. faecalis*** ***A. faecalis***

Figure 2: Antimicrobial activity of crude bacterial extract

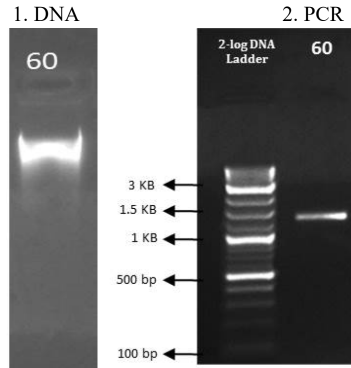


Figure 3: *Bacillus subtilis* DNA and PCR amplification

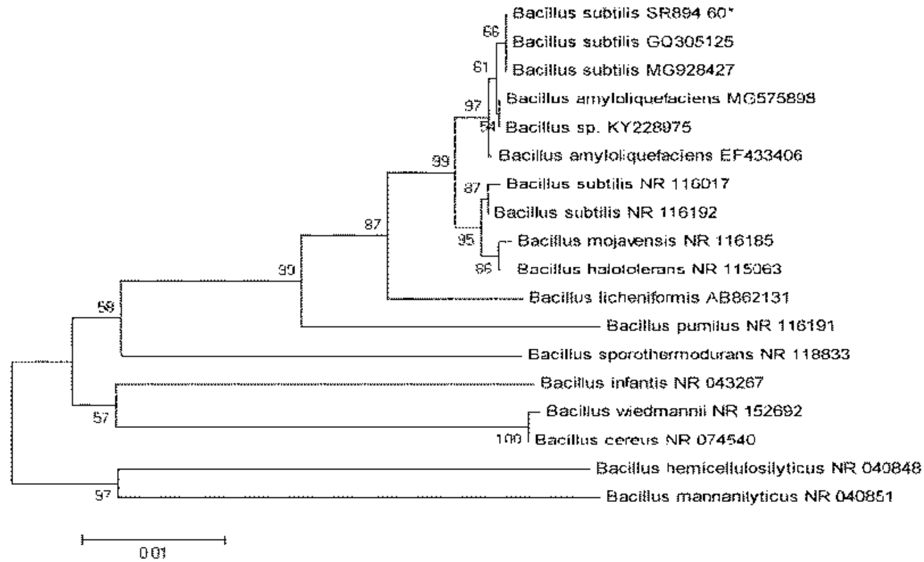


Figure 4: Phylogenetic tree of the *Bacillus subtilis*

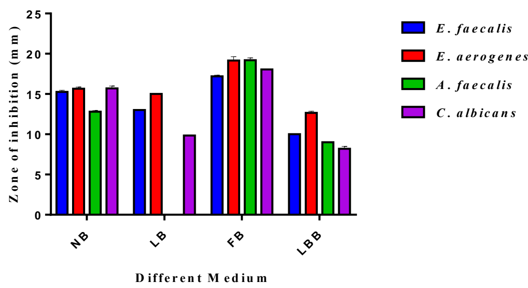


Figure 5: Effect of suitable media

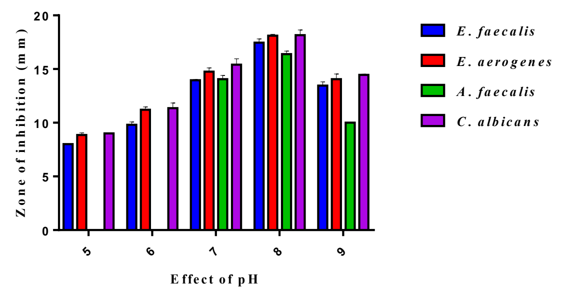


Figure 6: Effect of pH

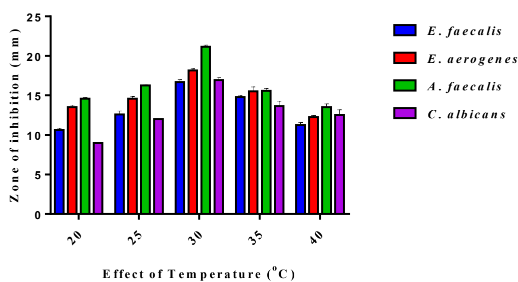


Figure 7: Effect of temperature

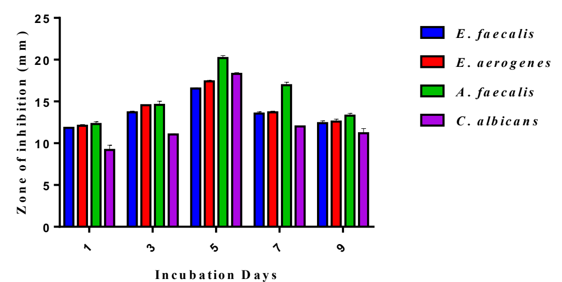


Figure 8: Effect of incubation period

Table 1: Morphological characteristics of the selected bacterial isolate

Characters	Isolate (*Bacillus subtilis*)
Color	White
Margin	Undulate
Appearance	Smooth
Consistency	Dry
Gram Staining	G+
Shape	Rod

Table 2: Antimicrobial activity of crude extracts of soil bacteria

Bacterial isolate No	*E. faecalis*	*E. aerogenes*	*A. faecalis*	*C. albicans*
	Zone of inhibition (mm)			
60	14.75±0.21	14.10±0.14	13.85±0.07	15.30±0.42
66	08.90±0.14	09.25±0.35	-	11.95±0.49
69	-	-	-	-
79	10.35±0.21	09.00±0.00	-	-
99	-	-	-	-
107	-	-	-	-
123	-	09.50±0.14	-	-
127	-	-	-	10.00±0.00
Streptomycin	**13.00±0.07**	**14.00±0.00**	**13.00±0.00**	**-**
Ketoconazole	-	-	-	**15.00±0.00**

Table 3: MIC, MBC and MFC values of bacterial crude extracts against the tested pathogens

	Pathogens		**Concentration of the crude extract (µg/ml)**					
			200µg	**100µg**	**50µg**	**25µg**	**12.5µg**	**6.25µg**
MIC	*A. faecalis*		-	-	+	+	+	+
	E. aerogenes		-	-	+	+	+	+
	E. faecalis		-	-	+	+	+	+
	C. albicans		-	-	-	+	+	+
		Positive control	**200µg**	**100µg**	**50µg**	**25µg**	**12.5µg**	**6.25µg**
OD value at 600nm	*A. faecalis*	0.984±0.002	0.018±0.003	0.020±0.001	0.024±0.002	0.232±0.001	0.513±0.001	0.993±0.001
	E. aerogenes	1.154±0.004	0.020±0.002	0.024±0.003	0.214±0.002	0.564±0.004	0.720±0.001	1.010±0.003
	E. faecalis	0.846±0.002	0.018±0.001	0.023±0.004	0.189±0.003	0.395±0.004	0.538±0.002	0.794±0.001
	C. albicans	1.324±0.004	0.016±0.004	0.019±0.006	0.024±0.001	0.418±0.002	0.813±0.001	1.214±0.002
			200µg	**100µg**	**50µg**	**25µg**	**12.5µg**	**6.25µg**
Percentage (%) of inhibition	*A. faecalis*		98.17	97.96	97.56	76.42	47.86	07.21
	E. aerogenes		98.26	97.92	81.45	51.12	37.60	12.47
	E. faecalis		97.87	97.28	77.65	53.30	36.40	06.14
	C. albicans		98.79	98.56	98.18	68.42	38.59	08.30
			200µg	**100µg**	**50µg**	**25µg**	**12.5µg**	**6.25µg**
MBC	*A. faecalis*		-	-	+	+	+	+
	E. aerogenes		-	-	+	+	+	+
	E. faecalis		-	+	+	+	+	+
MFC	*C. albicans*		-	-	-	+	+	+

(+) = Presence of growth, (-) = Absence of growth
Positive control: Without addition of crude extract in pathogenic cultures

CONCLUSION

This study indicates that bacteria isolated from soil environment produce many pharmaceutically important bioactive compounds with antimicrobial potential under optimum conditions. The isolate *Bacillus subtilis* showed good antimicrobial activity against test pathogens. Soil bacteria revealed their potential to yield bioactive metabolite which may play an important role of providing bio molecules for drug designing in pharmaceuticals industry. Hence, further studies have to be taken to carry out purification, characterization and identification of bioactive metabolites of *Bacillus subtilis* for drug discovery and exploitation.

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