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Original Research Article

Isolation and molecular identification of soil bacteria exhibiting a broad-spectrum of antibacterial activity against multi-drug resistant strains

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Abstract

Due to the increase in drug-resistant in pathogens, the search for new antibiotics from an unexplored natural environment is gaining momentum to combat Multidrug-resistant (MDR) bacteria. The genus bacillus has been found as a prolific source of antibacterial metabolites synthesis. The current study was intended to isolate antibacterial metabolites producing *Bacillus spp*. From the unexplored region of Pakistan. Initially, 49 *Bacillus spp*. Were isolated from 9 soil samples and evaluated for antibacterial activity against a set of American Type Culture Collection (ATCC) and MDR clinical bacterial strains. The isolate MK-12.1 antagonized the growth of ATCC, as well as MDR pathogenic bacterial strains, was selected for further study. The antibacterial metabolites were characterized and confirmed to be protein in nature, showed no hemolysis on human blood agarand molecular weight was >20 kDa. The antibacterial metabolite producing bacterial isolate was identified as *Bacillus safensis* strain MK-12.1 on the basis 16S rRNA gene sequencing analysis supported with biochemical testing. Various culturing parameters such as temperature, pH, incubation time and growth medium were optimized for growth and antibacterial metabolites synthesis. The optimum antibacterial activity and biomass was achieved at pH 8, 30 ^{0}C after 48 hours in the shaking incubator when cultured in modified OPT medium. The present study revealed that soil of Kohat, Pakistan is rich in terms of antibacterial metabolites producing bacteria and could be a good source for antibacterial which leads to fight against MDR bacteria in the future.

Keywords: *Bacillus spp*. Antibacterial metabolites, Multi-drug resistant bacteria, Optimization

INTRODUCTION

The exploration of new antibacterial agents from natural habitat is becoming more critical due to the fast-growing resistant in humen pathogens. In a natural habitat, soil contains Streptomyces and Bacillus spp. Which are a prolific source of natural bio-active compounds (Kemung et al. 2018; Sumi et al., 2015). The natural bioactive compounds produced by bacteria are playing an important role to treat infection and seem to be a good source in the future (Kumar and Kumar 2016). The standard research strategies conducted in the past decade, from genome mining via high-throughput screening, did not get any new therapeutic agent. Even new improved natural compounds library for bioavailability did not reveal accurate physicochemical properties of a therapeutic agent. Moreover, the natural bioactive compounds, augmented for ant-bacterial

Table 1. Test ATCC and local clinical Multi drug resistant (MDR)strains used in the current study.

Test strains	Description
Staphylococus aureus	ATCC (29213)
Staphylococcus epidermidis	ATCC (12228)
Streptococcus pneumonia	ATCC (6305)
Salmonella typhimurium	ATCC (14028)
Shigella flexneri	ATCC (12022)
Klebsiella pneumoniae	ATCC (13889)
Vibrio cholerae	ATCC (9459)
Pseudomonas aeruginosa	ATCC (27853)
Echerichia coli	ATCC (25922)
Acinetobacter baumannii	Local clinical MDR
Staphylococcus aureus	Local clinical MDR
Escherichia coli	Local clinical MDR
Pseudomonas aeruginosa	Local clinical MDR

activity, not evaluated for human or animal applications may be associated with love activity and high cytotoxicity. The main drawback in natural bioactive compounds research is, it appears like a plain sailing cakewalk which is considered as an easy option. Often, already reported bio-active compounds or bioactive compounds producing bacteria had been re-isolated. Collectively with the circumstances the cost of new drug near to zero, that's why facing drug development cost of approximately 1.103 USD. Subsequently, only small margin expected for novel antibiotics that could be used against MDR pathogenic bacteria. The new antibiotic will reserve option and will outcome in rather low sale statistics. Therefore, most of the pharmaceutical industries and research groups avoid working in this field. Currently, a total of about 500 investigators belongs to 50 research groups are active across the globe in the field of antibiotic discovery (Stern et al. 2017). The chance of success of a bio-prospecting research project aims to identify novel antibiotic can be increased if it is based on a good rational. The natural environment is a reservoir of too many useful resources and may contain many potential antimicrobial metabolites producing microbes that can be employed for the synthesis of novel antibiotics. Dumps soil remains the most significant target for bio-prospect screening research as its inhabitant harbor widespread distribution of antimicrobial compounds. These compounds regulate the microflora of the specific biological niche and use particularcompounds to communicate andsurvive in a consortium(Ghanmi et al. 2016; Raaijmakers and Mazzola 2012).Many important antibiotics such as vancomycin and kanamycin produced by Streptomyces orientalis and Saccharopolyspora erythraea respectively was isolated from soil sample (Butler 2008; Iv 1981). The bacterial classbacilli described as a bio-resource with high potential for bioactive compounds synthesis after actinomycetes (Chaudhary et al. 2013; Sumi et al. 2015).

In the present study, unexplored sampling sites of northern Pakistan were studied. Several antibacterial metabolites producing bacterial isolates were isolated and one isolate MK-12.1 exhibited broad-spectrum antibacterial activity against ATCC as well as MDR clinical strains.

MATERIALS AND METHODS

Soil samples collection and pre-treatment

The soil samples (n=9) were obtained from diverse localities in Kohat region(33.5638^{o} N, 71.4656^{o} E), Pakistan. These localities include mill effluent soil, riverbank soil, rose garden rhizosphere soil, university lawn soil, dry mountain soil, cultivated land soil, stream bank soil, forest soil and waste dump soil. The soil samples were pre-treated for the rational isolation of bacillus spp.using physic-chemical method as previously described (Hayakawa et al., 1991).Afterward, the samples were serially diluted and 200 µl from each dilution was plated on tryptic soy agar (TSA) plates and culture was purified by repeated suc-culturing.

Screening for antibacterial metabolites synthesis

Preliminarily all isolated bacterial isolates were screened for antibacterial activity against test bacteria using a modified cross streaking method (Kamat and Velho-Pereira 2012). Later on the cell-free supernatant from antibacterial metabolites producing isolateculture was evaluated for antibacterial activity against 9 ATCC and 4 Clinical MDR strains (Table 1). The zone of inhibition was measured around the wells in mm.

Identification of antibacterial metabolites producing isolates

The antibacterial metabolites producing bacterial isolate was initially characterized based on colony morphology,

cell morphology and biochemical testing. Subsequently, the isolate was molecularly identified by 16S rRNA gene sequencing analysis. The genomic DNA from soil isolate was extracted using the phenol/chloroform method as described previously (Wright, Adelskov, and Greene 2017). The 16S rRNA gene was amplified using 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTTGTTACGA-3'). the amplification and visulizeiation of of amplified 16S rRNA gene product was performed as described by (Nasfi et al. 2018). The purified PCR product was sequenced by di-deoxy chain termination method using the commercial services of Macroge Korea. The 16S rRNA gene sequence was aligned with other related sequences freely available at NCBI by ClustalW and refined using bioedit sequence editor. A phylogenetic tree was constructed based neighbor-joining method using MEGA-X program (Kumar et al. 2018).To infer the evolutionary history of the soil isolate MK-12.1, bootstrap values were calculated on 1000 replicates (Efron, Halloran, and Holmes 1996).

Optimization of growth and antibacterial metabolites production

Culturing parameters such as incubation time, temperature, pH and medium was optimized for growth as well as antibacterial metabolites production. To evaluate the effect of temperature and pH, an Erlenmeyer flask containing 100 ml medium was inoculated with 1ml standardize inoculum (equal to 1 McFarland) of soil bacterial MK-12.1.The pH of medium and incubation temperature was set to 6, 7, 8, 9 and 25, 30, 37 and 42 respectively. For optimization of culturing medium a standardize inoculum of 1ml was poured in various media such as Luria Bertani (LB) broth, Tryptic soy broth (TSB), Nutrient broth (NB), Brain heart infusion (BHI) broth (BHI), OPT broth (Akpa et al., 2001) and OPT broth with little modification and maintained at 30 ^{0}C for 48 hours.The effect of incubation time and aeration was examined by culturing the antibacterial producing isolate in shaking/static incubator and activity and growth was determined at different time intervals i-e 24, 48, and 72 hours. The antibacterial activity of CFS from MK-12.1was measured in mm and growth as O.D at 600 nm.

Partial purification of antibacterial metabolites

The antibacterial metabolites producing bacteria *B.safensis* MK-12 was cultured in optimized condition. Subsequently, the culture was centrifuged at 6000 rpm for 20 minutes. To remove cell debris and other impurities the CFS was passed through 0.45 pore size membrane. The antibacterial metabolites were extracted from CFS using ethyl acetate (1:1 ethyl acetate and CFS) and solvent was evaporatedusing rotary evaporator.

Determination of minimum inhibitory concentration (MIC)

The MIC of extracted metabolites was determined against 11 tested bacterial strains using the serial dilution method (European Society of Clinical Microbiology and Infectious Diseases 2003). The minimum concentration of metabolites that completely restric the growth of tested bacteria was called MIC.

Characterization of antibacterial metabolites

The molecular weight of antibacterial metabolites was determined using the dialysis membrane with pore size of 20 < kDa (Muhammad et al. 2015). To determine the nature of active metabolites the extracted antibacterial metabolites were mix with proteinase K and kept for 30 minutes at room temperature. Temperature sensitivity was assessed by heating the antibacterial metabolites from 20 to 121 ^{0}C for 15 minutes and later on antibacterial activity was examined. Hemolytic activity of antibacterial metabolites was studied on 5% human blood agar plates.

RESULTS

Plate count and isolation of antibacterial metabolites producing bacteria

Plate count for each soil sample was calculated on TSA plates, ranged from 4 $\times 10^5$ to 7 $\times 10^5$ colony-forming unit (CFU) per gram soil. The lowest CFU/g 4 $\times 10^5$ was determined for soils sample obtained from mill affluent area. A total of 47 *Bacillus spp*. purified and 12 were found to be producing antibacterial metabolites. Out of 12 bacterial isolates, 3 exhibited antibacterial activity only against gram-negative strains while 9 showed antagonistic activity against at least one gram-negative and one gram-positive bacterial strain. One isolate MK-12.1 revealed promising antibacterial activity against all tested bacterial strains as shown in Figure 1. The extracted antibacterial metabolites showed maximum antibacterial activity against *Salmonella typhimurium* on the other hand *Klebsiella pneumoniae*presented resistant towards antibacterial metabolites produced by *Bacillus safensis* MK-12.1 (Figure 2).

Identification of antibacterial metabolites producing bacteria

The antibacterial metabolites producing bacteria *B.safensis* MK-12.1was characterized in TSA medium. The cell morphology reveals that it is grampositive, spore-forming rodshaped bacteria. Colony morphology

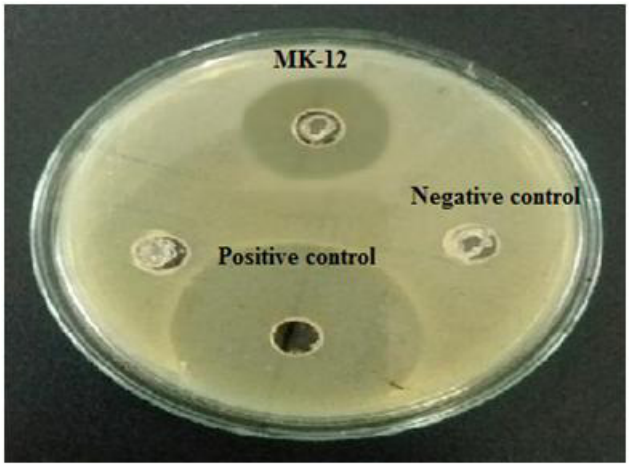

Figure 1. Showing antibacterial activity of partially purified antibacterial metabolites produced by *B.safensis*MK-12.1 against *S.aureus*.

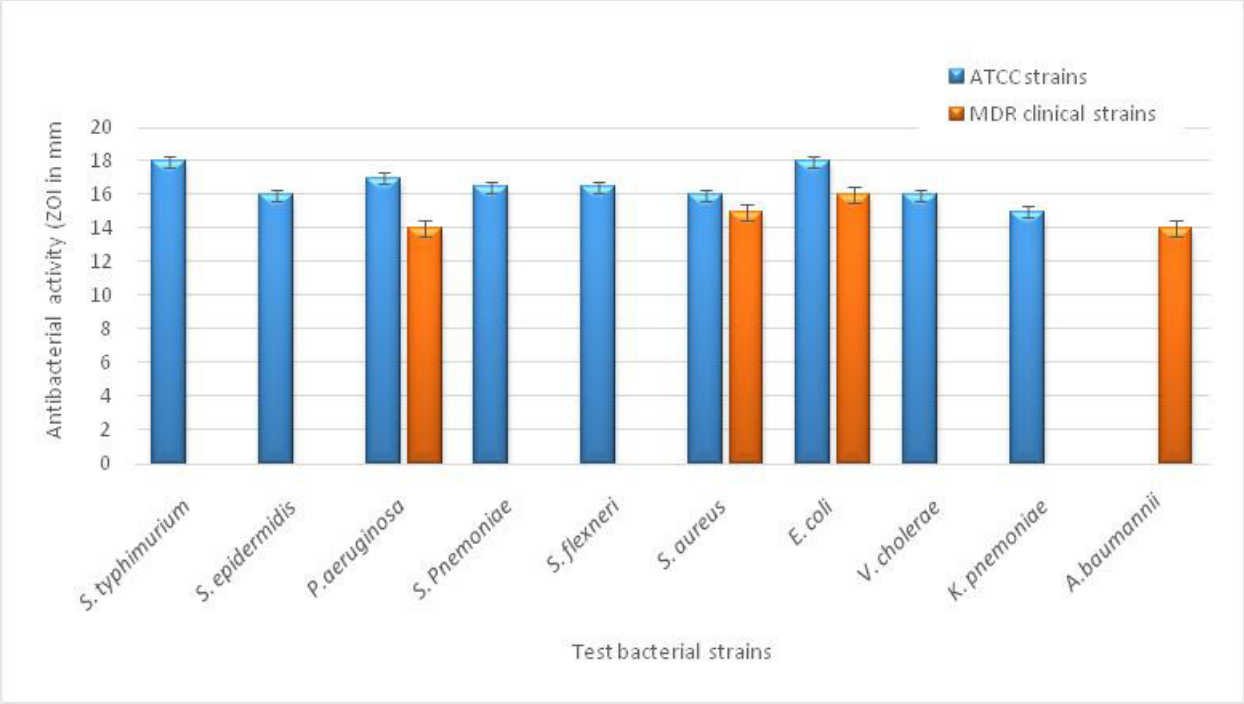

Figure 2. The antibacterial activity of *B.safensis* MK-12.1 against test ATCC and MDR clinical bacterial strains.

was round, undulant with irregular margin, non-luminescent and dull-white in color. Biochemical testing showed that MK-12.1 is positive for oxidase, catalase, Voges Proskauer test and negative for methyl red, indole, DNase, amylase, H_2 S and ureas production. To infer diversity and phylogeny of MK-12.1, the 16S rRNA gene sequence was compared with publically available sequences deposited in NCBI database. This revealed that soil isolate belongs to *Bacillus spp.*and identified as *bacillus safensis*strain MK-12.1. It seems that *Bacillus* is abundantly found in soil and play critical role in the biogeochemical process by producing various type of bioactive metabolites. The abundance of *Bacillus spp*. in soil is probably due to their ability to form a highly resistant endospore that helps them to survive under extreme environment and our pre-treatment of samples clearly favors spore-forming bacteria.The nucleotide sequencing data of MK-12.1was deposited to Gene bank database under the accession no. MN519460. The BLAST results of 16S r RNA sequence showed high level of similarity (99.73) with *Bacillus safensis* strain NBRC.

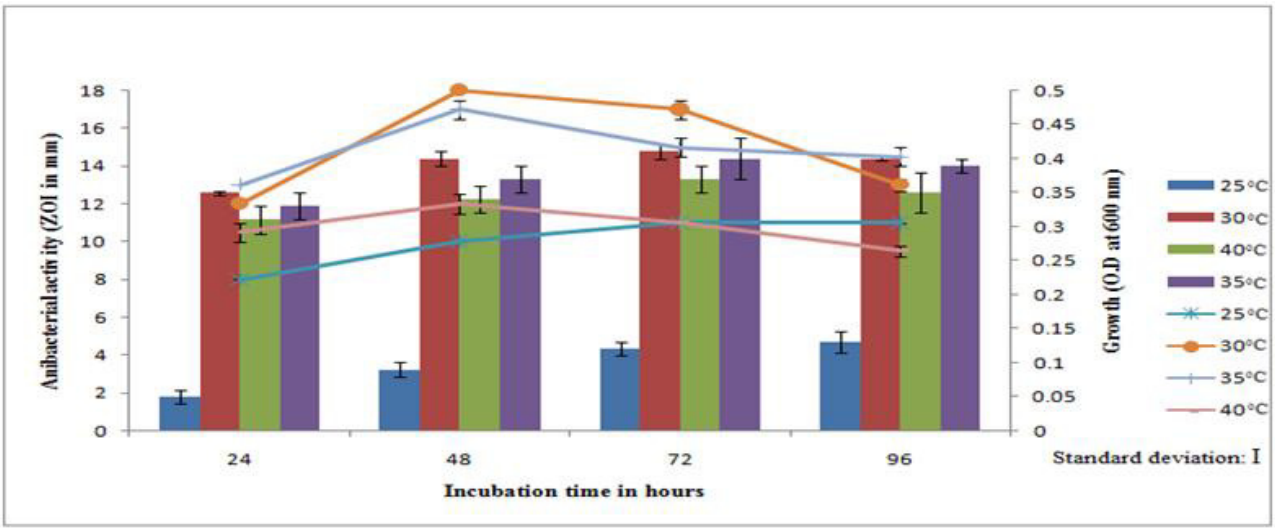

Figure 3. Effect of temperature and incubation time on growth and antibacterial activity of *B.safensis*MK-12.1 against *S.aureus*

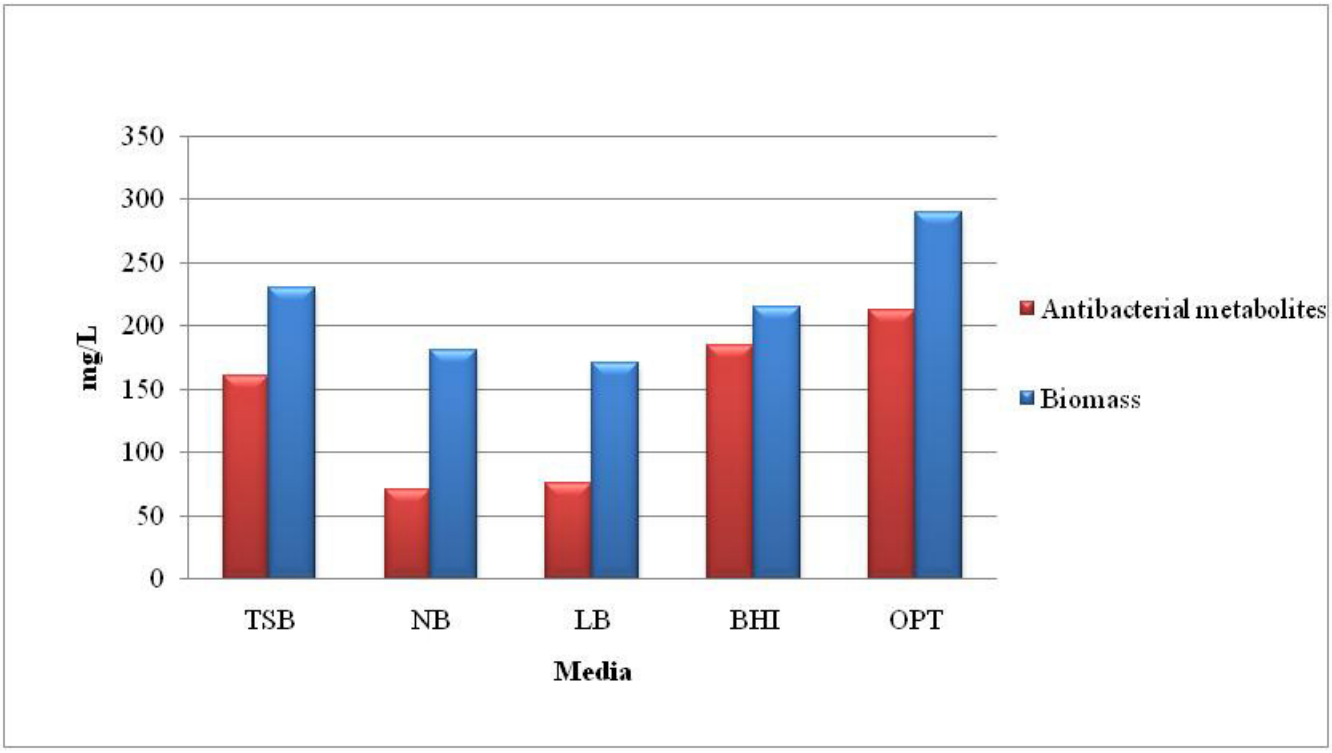

Figure 4. Antibacterial metabolites production by *B.safensis*MK-12.1 grown on various culture media. Trypticase soya broth (TSB), Nutrients broth (NB), Luria bretani (LB), Brain heart infusion (BHI) and Optimized (Opt) broth medium.

Optimization for Growth and antibacterial metabolites production

The CFS obtained from *B.safensis* MK-12.1 cultured at 30 C exhibited maximum activity i-e 18 mm zone of inhibition followed by 35 ^{0}C with 17 mm ZOI as shown in figure 3. On the other hand minimum, antibacterial activity was noted when MK-12.1 was grown at 45 ^{0}C and 25 ^{0}C indicate that its mesophile in nature and prefer to grow in moderate condition. The present study result revealed that nitrogen-rich media such as modified OPT, TSB and BHI boost antibacterial metabolites production as compared to other media (Figure 4). Maximum ZOI and biomass was obtained when MK-12.1 was grown in a modified OPT medium (Table 2). MK-12.1 exhibited higher activity at pH 8 as compared to pH 5, 6, 7 and 9.

Table 2. Composition ofOptimized (Opt) medium (component/ L)

Component	Quantity
Peptone	35 gm
Sucrose	20 gm
Yeast extract	8 gm
Potassium di-hydrogen phosphate	2 gm
Magnesium sulphate	0.045 gm
Trace elements	9 mL

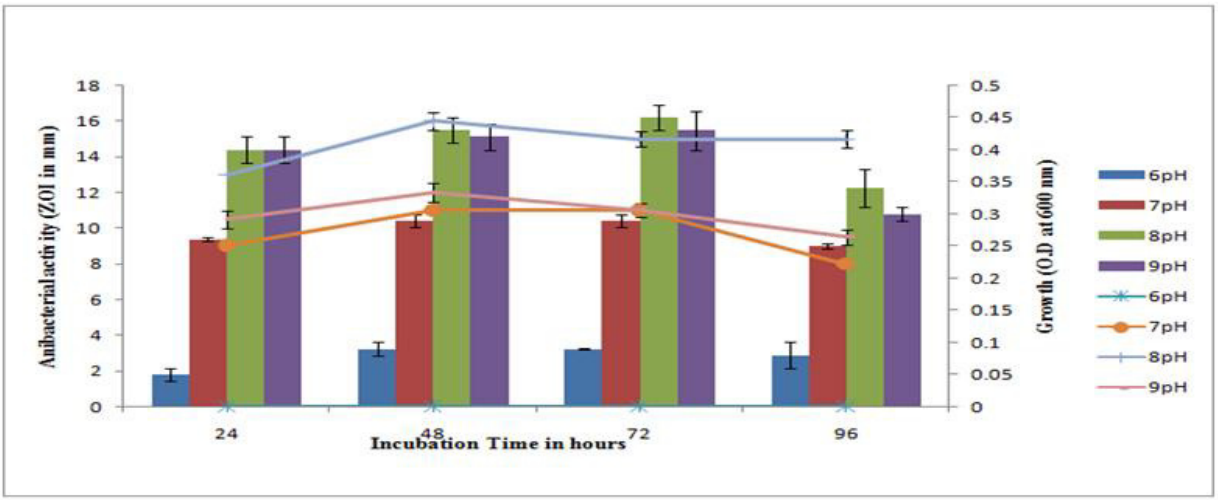

Figure 5. Effect of pH and Incubation time on growth and antibacterial activity of *B.safensis*MK-12.1 against *S.aureus.*

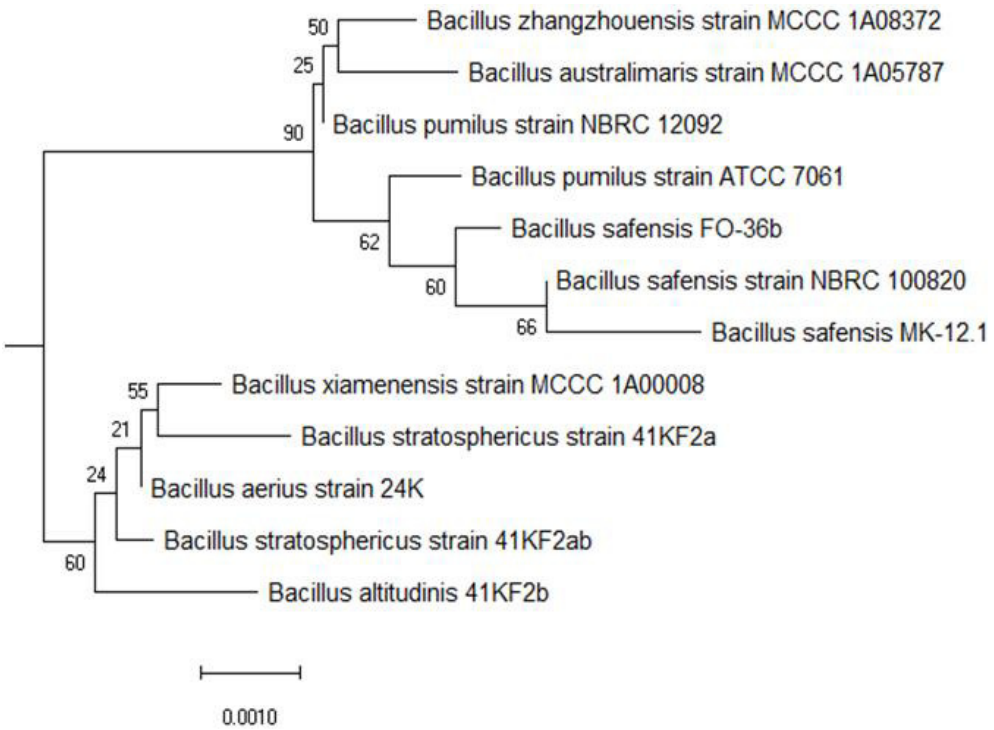

Figure 6. Phylogenetic tree constructed by neighbor-joining method indicating the evolutionary relationship of Bacillus safensis MK-12.1with related bacillus species.

Table 3. MIC of antibacterial metabolites produced by *B.safensis* MK-12.1 against various test bacteria (mg/mL).

S.no	Test bacteria	Strain no.	MIC	
			Mk-12 extract (mg/ml)	Streptomyces (+ive control) µg/ml
1.	*Salmonella typhimurium*	ATCC 29213	3.12	50
2.	*Staphylococcus epidermidis*	ATCC 12228	3.12	25
3.	*Pseudomonas aeruginosa*	ATCC 6305	4.68	25
4.	*Streptococcus pneumonia*	ATCC 14028	2.34	25
5.	*Shigella flexneri*	ATCC 12022	3.12	25
6.	*Staphylococus aureus*	ATCC 13889	3.12	50
7.	*Echerichia coli*	ATCC 9459	2.34	25
8.	*Vibrio cholerae*	ATCC 27853	3.12	50
9.	*Klebsiella pneumoniae*	ATCC 25922	4.68	50
10.	*Acinetobacter baumannii*	Local clinical MDR	6.25	50
11.	*Staphylococcus aureus*	Local clinical MDR	3.12	25
12.	*Escherichia coli*	Local clinical MDR	3.12	25
13.	*Pseudomonas aeruginosa*	Local clinical MDR	3.12	50

This is probably due to the optimum pH that assists the antibacterial metabolites synthesis. Moreover, maximum growth and antibacterial metabolites production were noted in shaking incubator after 48 hours of incubation that after a decline in ZOI and growth was observed (Figure 5).

Partial purification and characterization of antibacterial metabolites

The antibacterial metabolites extracted from *B.safenis* MK-12.1 showed enhance activity against tested bacterial strains as compared to CFS. The antibacterial metabolites lost their antagonistic activity when treated with Proteinase K and high temperature. The susceptibility of metabolites to high temperature and proteinase K results indicates that it is protein in nature. The molecular weight of bioactive metabolites was estimated to be > 20 KDa. Additionally, no hemolytic activity was observed when an antibacterial metabolite was evaluated for hemolysis against human blood.

Determination of MIC

The MIC of extracted antibacterial metabolites was determined and range from 25 - 75 µg/ml against ATCC strains. While against clinical MDR strains MIC ranged from 50 - 75 µg/ml as shown in table 3.

DISCUSSION

The emergence of multi-drug resistant in human pathogen led the scientist to search the novel sources or novel compounds from the existing sources for therapeutic agents to combat the resistant pathogen. Microorganism is a rich source of antimicrobial metabolites and approximately 90% of the current antibiotics in the market are primarily derived from the microorganism (Katz and Baltz 2016).Previously most of the antibiotics are isolated from soil bacteria via antibacterial activity-based screening approaches (Rolain et al. 2016).Therefore, it can also be expected that soil microorganisms will be the most prominent source of new antibiotics in future (Elbendary et al. 2018).Streptomyces and bacilli are the proven proliferative antimicrobial metabolites producing species (Hug et al. 2018).In spite of the fact that many bioactive metabolites have already been isolated from these soil bacteria but still, they contain many more unexplored genes for the synthesis of bioactive compounds (Pidot et al., 2014). Only few studies have been conducted on bacterial potential for bioactive compounds synthesis on waste dump soil where microorganisms should be capable to deal with relatively diverse and extreme environments. Therefore, the chance for isolation of novel bacteria having highly diverse metabolic capabilities (Trabelsi et al. 2016). In the current study, B.safensis MK-12.1 was isolated from unexplored waste dump soil Kohat, Pakistan that showed promising antibacterial activity against tested strains. This is in agreement with the fact that Bacillus is predominant soil bacteria to form endospore and antibacterial metabolites which facilitate them to colonize in such a harsh environment (Yadav et al., 2011).

The evolutionary history of MK-12.1 was inferred based on 16S rRNA gene sequence analysis.The soil isolate bacillus safensis MK-12.1 lies in a clade with B.safensis NBRC 100820. Previous studies demonstrated that 16S rRNA is an improved approach to identify soil bacteria as compared to conventional phenotypic and biochemical methods (Srinivasan et al. 2015). The strainBacillus safensis MK-12.1 showed fascinating bioactivity against all tested ATCC as well as MDR strains. The ATCC strains Salmonella typhimurium,

Shigella flexneri, Staphylococus aureus, Vibrio cholera and Staphylococcus epidermidis was inhibited more efficiently (MICs range from 25 to 50 µg/ml) as compared to other ATCC strains such as Pseudomonas aeruginosa and Klebsiella pneumonia (MICs range from 50 to 75 µg/ml). These results can be related toanother study where antibacterial metabolites from actinomycetes were purified and MIC against various strains was determined (Sharma, Kalita, and Thakur 2016). The study to evaluate antibacterial metabolites production usually involved optimum culture medium and condition. Modified opt medium, pH 8 and 30 0C was found to be the optimum medium and condition for growth and antibacterial metabolites production. Previous studies revealed that pH, temperature, carbon and nitrogen in the medium directly influence the growth and antibacterial metabolites production. These results are in accordance with another report wherelactobacillus sp. produce maximum amount of bacitracin at 25-30 0C (Malheiros et al. 2015). The partially purified antibacterial metabolites from B.safensis MK-12.1 culture loss the antagonistic activity at an elevated temperature and did not show any hemolysis on human blood agar indicate its suitability for further pharmacological study.

Competing interest

None declare

Authors' contribution

SI, MQ, designed the experimental work. SI and FB performed experimental work and drafted the manuscript. HR configured the bioinformatics analysis, MQ and NU edited and critically reviewed the manuscript.

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