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Biofilm Effects of the Soil *Bacillus cereus* Metabolites: Isolation, Characterization and Antimicrobial Activity Against Methicillin-Resistant *Staphylococcus aureus*

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Abstract
The methicillin-resistant *Staphylococcus aureus* (MRSA) causes serious health problems such as community-acquired infections and nosocomial infections, posing a significant public health threat worldwide. The ability of MRSA to adhere to host tissues and medical implants form a mature biofilm that effectively covers host cells in a polymer-based matrix leads to the deduction in the host-immune defences, antimicrobial therapy and difficulty in disease eradication. The present investigation mainly focusing the isolation of the bacteriocin producing microbes from soil and testify their biofilm inhibition ability against MRSA. Bacteriocin-producing bacteria are identified by utilizing nutritious agar media and the agar well-diffusion bioassay in the presence of MRSA as an indicator organism. The 16S rDNA gene sequencing, molecular weight identification and antibiofilm activity showed the four isolates (PU1, PU2, PU3, and PU4) produce bacteriocin and have a wide-ranging antagonistic activity against MRSA. Among them, PU3 exhibits maximum inhibitory activity against MRSA at 100AU/mL concentration using agar well diffusion method. The biochemical assay and 16S rDNA sequencing confirm the isolate PU3 affirmed as *Bacillus cereus* with a molecular weight of ~ 3 kDa. In situ zymogram assay showed a zone of inhibition corresponding to the estimated protein band size and Time-kill study also displayed that *B. cereus* producing metabolites (bacteriocin) comprises a bactericidal effect and 90% reduction in biofilm biomass toward MRSA. This study showed that soil isolated bacteria-based antimicrobial compounds control microbial infections and may instruct future antibacterial research.

Keywords 16S rDNA · Bacteriocin · *Bacillus cereus* · Zymogram · Methicillin-resistant *Staphylococcus aureus*

Introduction

Biofilm-associated infections are linked to a variety of health problems in both animal and human. The biofilm detachment allows the cells to spread the other body parts by blood and body fluids (David et al. 2013). Most biofilm-forming bacterial strains are highly resistant to antibiotics and host-innate defences (Alejandro et al. 2013). In addition, *Klebsiella pneumoniae* (Liu et al. 2020), *Staphylococcus aureus* (Gomes 2019), and *Pseudomonas aeuroginosa* (Eladawy et al. 2019) are among the most common biofilm-producing bacteria. Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA), a causative agent for nosocomial infections anchored within the extracellular hydrated polymeric matrix and promoted increases death rates in patients (Tong et al. 2015). The antibiofilm reasons for antimicrobial substance mostly speak to in two viewpoints, for instance, to repress the improvement of biofilm and dispense with biofilm arrangement and expelling the made biofilm. Fundamentally, the examines reports that antimicrobial substances can suppress the biofilm arrangements since they can successfully execute planktonic microscopic organisms (Pinto et al. 2019; Strempel et al. 2014). Recently, certain bacteriocins consist of antibiofilm capabilities used to deconstruct biofilm development, impede grip, and prevent biofilm formation (Harsh et al. 2018). The newly developed bacteriocin, named Auranofin (AF), is a blended ligand gold compound to treat the antimicrobial and hostile to parasitic action from biofilm formation (She et al. 2019). Bacteriocins are a

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bacterial source, heat-stable peptides with low sub-atomic weight and sensitivity to proteolytic agents (Karthikeyan and Santhosh 2009; O'sullivan et al. 2003). Bacteriocin ordinarily shows its antimicrobial activity by meddling with the cell divider or film of target living beings, either restraining the cell divider blend or premise pore arrangement, resulting in death (Lopez and Belloso 2008). Bacteriocins are proteins or protein mixes with bactericidal movement coordinated against common species, especially with maker microorganisms. They are heterogeneous and exacerbate that shift in atomic weight, biochemical properties, movement spectra, and activity instrument (Klaenhammer 1988). The activity component of lantibiotic nisin and pediocin-like bacteriocins relies on the cell film protein, layer potential, pH, lipid arrangement, and explicit amino acids and essential spaces (Montvillle and Chen 1998). The bactericidal activities of bacteriocin were examined Vidhya Prakash et al., which *L. fermentum* and *L. casei* 335 framed demonstrated compelling against anti-toxin safe *E.coli* and tranquillize safe *Salmonella typhi* microorganisms (Prakash et al. 2018). A notable, microscopic organism of the *Bacillus* sort has solid flexibility to various conditions and that few species produce profoundly safe spores; they have been segregated from fish (Aly et al. 2008; Ghosh et al. 2002; Sugita et al. 1998), delicate corals and wipes (Ivanova et al. 1999) just as other marine spineless creatures (Khandeparker and Anil 2003; Miyanishi et al. 2003). One benefit of utilizing *Bacillus* spp. as probiotics in aquaculture is that they will probably not use qualities from Gram-negative microscopic organisms (e.g., Vibrio) that may present anti-microbial opposition (Rabinowitz and Roberts 1986). *Bacillus cereus* is around 60 delegates of the broadly fluctuated *Bacillus* variety. It incorporates the supposed Bacillus cereus gathering from the start with fundamentally the same as species *B. mycoides, B. thuringiensis* and *B. anthracis*. Bacillus is a large and ubiquitous bacteria family that is frequently isolated from soil and waste environment. There are tiny differences between these four species. *B. cereus* is commonly found as a saprophyte in soil, water, plants, and air, from which it is efficiently transported to nourishment, either from novel crude material or during nourishment preparations. At that point, they are associations with the *Bacillus* bunch are all around considered the great producer of antimicrobial substances, including peptide and lipopeptide anti-toxins, and bacteriocins (Stein 2005). Therefore, the current research mainly focuses on developing an antibiofilm agent from *Bacillus cereus* to eradicate MRSA disease (Jiang et al. 2020).

In the present study, we explore bacteriocin delivering *Bacillus cereus* microorganisms separated from many regions in Salem. We attempt to identify and characterize bacteriocin producing bacteria from food waste soil. We assessed the antibacterial capacity and molecular mass of a bacteriocin from isolated bacteria. Those bacteria are growing an alternative source of the bacteriocin to control many important antibiotic-resistant pathogenic bacteria in the future.

Methodology

Sample Collection

A soil sample collected in the kitchen waste disposal area, K.R. Thoppur, Salem District, Tamil Nadu, India (11°40′46.9″N 78°00′28.9″E). 5 g of soil sample was collected by using a clean and dry sterile spatula in a clean polythene bag.

Spread Plate Method

1 g of soil was dissolved in 1 ml of 0.8% NaCl to make soil suspension. Serial dilution was carried out. To get a concentration range from 10^{-1} to 10^{-3}. A volume of 0.1 ml of each dilution was transferred aseptically to nutrient agar plates. The sample was spread uniformly. The plates were incubated overnight at 37 °C (Prashad 2014).

Screening of Bacteriocin Producing Bacteria

The single colonies obtained from each sample were screened for their antimicrobial activity against the methicillin-resistant *Staphylococcus aureus* (Rosado and Seldin 1993). Nutrient agar plates are preparing to overlay with 20 ml of soft agar (0.75% agar) inoculated with MRSA Concentration of 10^6 CFU/ml. To patch the single colonies and plates were incubated at 37 °C for 16–18 h and the appearance of clear zones showing the antagonistic activity was observed.

Genomic DNA Extraction of Bacteria

The genomic DNA was extracted from the isolated bacteria colonies were by using the slightly modified protocol described (Govindarajan et al. 2016). The 12 h cultures of bacterial isolates were taken in the microcentrifuge tube. The tube was centrifuged at 10000 rpm for 10 min. The pellet was collected and 90 µl of 10% SDS was added. The tubes were incubated at 37 °C for 1 h(Govindarajan et al. 2016). After incubation, an additional 150 µl of 5 M NaCl was added before the addition of 100 µl of 10% Cetyl trimethyl ammonium bromide (CTAB). The sample was mixed thoroughly and incubated at 65 °C kept in a water bath for 30 min, after incubation, add phenol, chloroform and Isoamylalcohol in the ration of 25:24:1 (Vol/Vol/Vol) (Govindarajan et al. 2016). The tube was centrifuged at 13000 rpm for 15 min and the aqueous layer was separated into a fresh

tube. Then it was precipitated with 70% ethanol and centrifuged at 7000 rpm for 5 min. Pellets were suspended in 30 µl of TE buffer(Govindarajan et al. 2016). The DNA sample was separated according to their molecular weights under the electrophoresis system. Finally, the DNA band was visualized under a gel documentation system (Lark, Germany). The DNA concentration was determined by measuring the absorbance at the ratio 260/280 nm and the DNA suspension was stored at − 20 °C is used for further analysis.

PCR Amplification of 16S rDNA Gene

A reaction mixture containing approximately 50 ng of template DNA, l0X PCR buffer, a 20 pmol concentration of each PCR primer (27F AGA GTT TGATCMTGG CTC AG and 1492 R TAC GGY TAC CTT GTT ACG ACTT), a 2.5 mM concentration of dNTPs and 2.5U of Taq DNA polymerase in a total volume of 50 µl was prepared. After a 10 min denaturation at 94 °C, the reaction mixture was run through 35 cycles of denaturation for 20 s at 94 °C, annealing at 58 °C, and extension for 1 min at 72 °C, followed by a final extension for 10 min at 72 °C. 10 µl of PCR product was electrophoresed on 1% agarose gel to determine the size of the product. Both negative and reagent controls were included in PCR amplification (Lu et al. 2000).

Phylogenetic Analysis

The 16S rDNA amplicon of these four isolates was used to decide the sequence of the first 500 nucleotides. A blast search assumed to find out the similarity of these two sequences with the most similar 16S rDNA sequences of the GenBank database. The sequence of 16S rDNA was aligned with related sequences retrieved from the database to construct a neighbour-joining tree showing the nearest phylogenetic tree (Chowdhury et al. 2004).

Production of Bacteriocin

The isolated strain was grown in Luria broth at 37 °C for 12 h. After incubation, the broth was centrifuged at 6000 rpm for 10 min and the cells were separated. To collect the supernatant and pellet, then they are used to determine the antimicrobial activity (salasiah kormin et al. 2001).

Determination of Inhibitory Spectrum

An agar well diffusion method was used to access the production of antimicrobial compounds by the selected isolates from bacteria against MRSA. Duplicate plates were used for each target organism (Anita et al. 2014). 100 µl of bacterial culture supernatant be added to the wells in the plates. The finding of clear inhibition zones around the wells on the inoculated plates is an indication of antimicrobial activities (Anita et al. 2014).

SDS-PAGE and Zymogram Analysis

SDS-PAGE was performed according to the method of (Laemmli 1970) using Proviga (India) electrophoretic apparatus. 15% separating and 4% stacking gel were used to separate the proteins. Per well, 50 µl samples were loaded. SDS-PAGE was run until the bromophenol blue front reached the end of the gel (30 V for stacking gel and 50 V for separating gel was applied) (Selvam et al. 2016). While one half of the gel was stained with Coomassie brilliant blue R250, the other half was washed 3times with sterile ddH_2O, overlaid with cultures of MRSA and incubated at 37 °C for 24 h.

Time Kill Assay

In time kill studies, an inoculum of MRSA was prepared from test organisms subcultured until the exponential phase and Cultured in Luria Britani broth were transferred in a separate flask, and crude bacteriocin extract was added from an MRSA culture flask. A growth control flask with tested strain without bacteriocin was included in the experiment. The cultures were incubated at 37 °C, and samples were collected obtained at every 3 h interval. Every sample to make a duplicate. Growth rates and bacterial concentrations were determined by measuring OD at 600 nm at different time points (Selvam et al. 2016).

Antibiofilm Activity

The culture of MRSA was incubated into the 96 well flat-bottomed tissue culture plates and it's incubated at 37 °C for 2 days. After 2 days, the different concentration (10–40%) of bacteriocin was added and incubated at 37 °C for 24 h using the blank group as a control. After incubation, the spent media was aspirated gently and the wells were washed with 200 µl of PBS and then air-dried. 200 µl of 99% ethanol was added and incubated for 15 min for fixation, and aspirated and allowed to dry later (Selvam et al. 2016). The wells were stained with 200 µl of 0.1% crystal violet for 5 min. The excess stain was gently rinsed with distilled water and the plate was air-dried (Mataraci and Dosler 2012). The stain was resolubilized in 200 µl of 33% acetic acid and the cell concentration was measured at OD 595 nm and check the morphological changes in antibiofilm activity (Rukmanikrishnan et al. 2020).

Result

Screening of Bacterial Isolates for Bacteriocin Production

As described in the materials and methods section, Four bacterial isolates were obtained from soil samples and tested for the bacteriocin activity toward MRSA as an indicator organism. Among them, one bacterial (PU3) isolates showed the antagonistic activity against MRSA. The antagonistic bacterial strain reported to be effective in supressing selected as found to the zone of inhibiting the growth of MRSA (Fig. 1). In our bacteriocin efficacy was compared with three isolates, PU3 produced maximum activity against MRSA.

Antibacterial Activity

The agar well diffusion method was used to determine the bacteriocin minimum inhibitory activity. The findings revealed that the bacterial strain PU3 produced a high level of bacteriocin (100 AU/ml) when compared to other strains (Fig. 2).

To assess their morphological and biochemical characterization, the isolated strain PU3 was subjected to the microscopic examination, the findings revealed that the PU3 strain exhibited rod in shape, motile, spore formed and can cultivate at 37 °C. In addition, the various biochemical and physiological tests showed that PU3 is catalase, urease, citrate, gelatin, oxidase and lipase positive; however, starch hydrolysis, methyl red, Voges-Proskauer and indole negative, as depicted in Table 1. Based on the characterization and the PU3 strain was phenotypically identified as *Bacillus* sp.

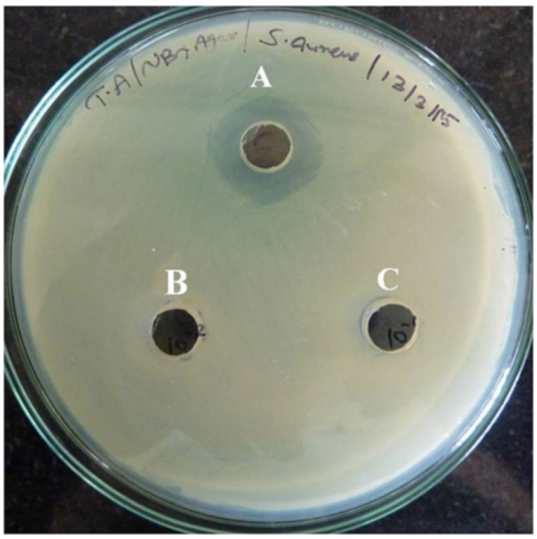

Fig. 2 Antibacterial activity of culture supernatant of PU3. **A** Cured; **B** 10^{-1}; **C** 10^{-2}

Isolation of Genomic DNA From Bacteria

The genomic DNA isolation from soil bacteria by alkaline lysis method. The isolation DNA was l 1% agarose gel, after the run finish of gel, the genomic DNA band was observed under the UV eliminator.

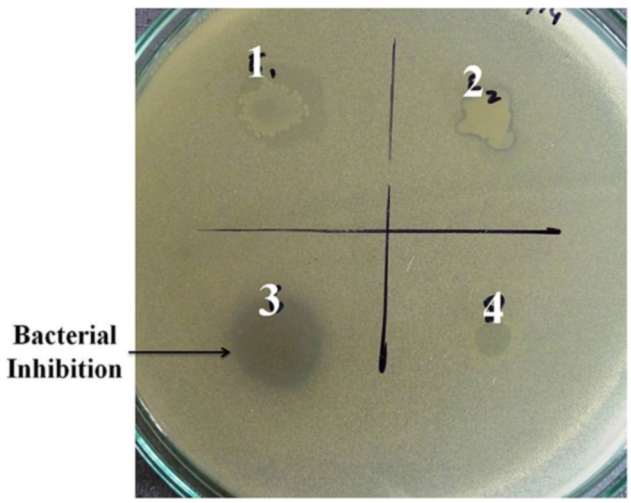

Fig. 1 Screening of bacterial isolates for bacteriocin production. **1** PU1; **2** PU2; **3** PU3; **4** PU4

Table 1 Morphological characteristics and biochemical tests for isolated strain

Biochemical tests	Result
Media used for inoculums	LB medium
Colony character	Rod
Grams staining	Positive
Biochemical tests	
Indole tests	–
Methyl red test	–
VP tests	+
Citrate test	+
Oxidase test	–
Catalase test	+
Gelatin	–
Urease	+
Lipase	+
Starch hydrolysis	–

Identification of Bacteriocin Producing Bacteria Gene Sequence

The ribosomal RNA gene sequences are relatively conserved among the bacteria. Therefore, the 16S rDNA sequence was analyzed to confirm the species level identity of the bacterial isolate. The 16S rDNA region (1500 bp) was amplified from the genomic DNA of the bacterial isolate (Fig. 3). The sequences were aligned (1419 bp) and searched for sequence homology in Ribosomal Database Project II (RDP). The results revealed that the sequence had a high score bit of homology with the 16S rDNA sequence of *Bacillus cereus.* The selected bacterial isolate (PU3) was conformed as *Bacillus cereus* and the nucleotide sequence of the 16S rDNA of the PU3 strain was deposited in GenBank under the accession number KX548263.

Phylogenetic Tree Analysis

Based on BLAST and phylogenetic results, strain PU3 was identified as *Bacillus cereus.* The 16S rDNA sequence of isolates determined direct analysis of the genetic distance. The phylogenetic tree and related type strain were used to assemble the phylogram using the neighbor-joining method and displayed in tree view (Fig. 4).

SDS-PAGE and Zymogram Analysis

To estimate the molecular weight of the bacteriocin, the protein in the active fraction was resolved on 15% SDS-PAGE. Based on the electrophoretic mobility, the molecular weight of the single protein band that exhibited antimicrobial activity was estimated to be ~ 3 kDa as indicated by the complete clear zone of inhibition for MRSA, respectively (Fig. 5).

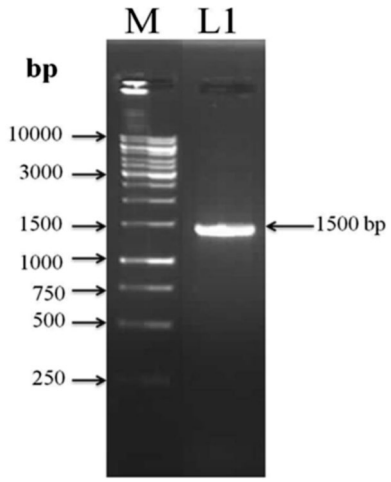

Fig. 3 PCR amplification of 16S rDNA of bacterial stain. M—1 kb DNA Ladder, L1—PCR amplification of *Bacillus cereus* PU3

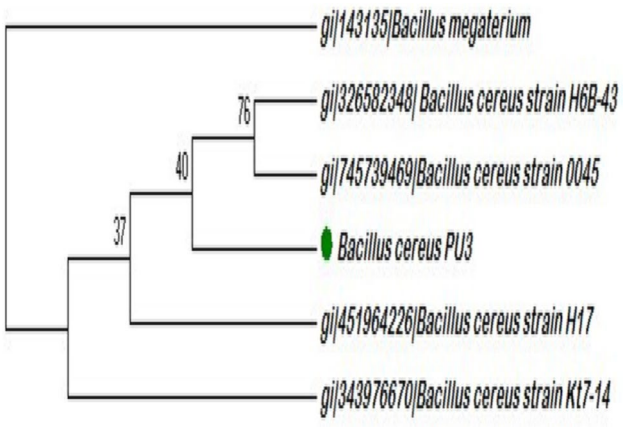

Fig. 4 Phylogenetic analysis of bacteria *Bacillus cereus* PU3

Time Kill Assay

The bactericidal activity was assessed by time-kill assay in the result showed that the *Bacillus cereus* producing bacteriocin had good antibacterial activity against MRSA at different time duration (Fig. 6).

Antibiofilm Activity

The different concentrations of bacteriocin were incubated with MRSA for 4 h at 37 °C for adherence to the wells of microtiter plates; it inhibited biofilm attachment

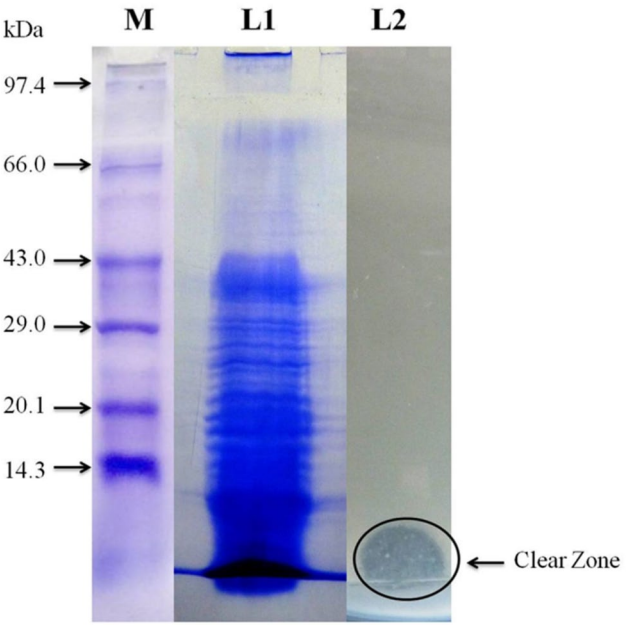

Fig. 5 SDS—PAGE analysis of the bacteriocin of *Bacillus cereus* PU3. M—Medium range protein marker, L1—*Bacillus cereus* supernatant, L2—Zymogram assay zone of inhibition

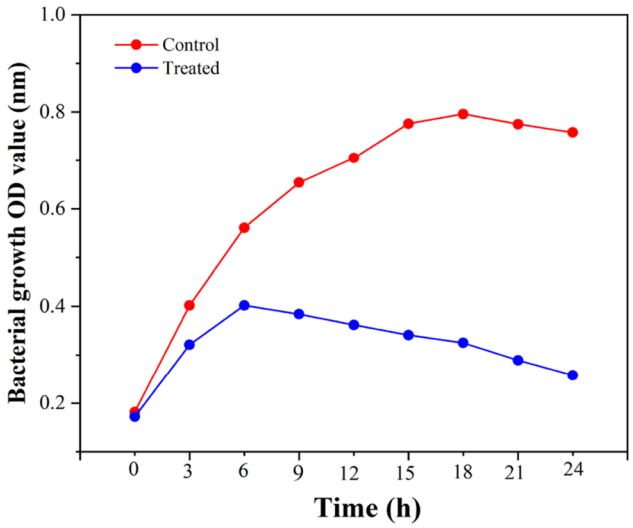

Fig. 6 Time kill assay of MRSA by bacteriocin

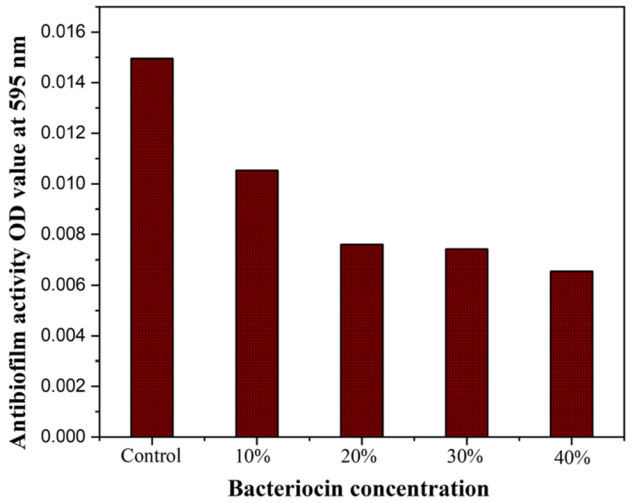

Fig. 7 The graphical representation of MRSA biofilm inhibition with bacteriocin (Control- *S. aureus*; 10–40 bacteriocin concentration)

in a concentration-dependent manner (Fig. 7). About 0.020 ± 0.001 attachment of biofilm was observed in the presence of 30–40% of bacteriocin and also examined morphological changes of bacteriocin treated with biofilm. Bacteriocin showed significant inhibitory activity against MRSA biofilm formation at 24 h, about its concentration (Fig. 8).

Discussion

Bacteriocins are promising antimicrobial operators, ribosomally integrated peptides and delivered by Gram-positive and Gram-negative microbes for the evident motivation behind wrecking their rivals (Cotter et al. 2005). The quantity of MRSA disease has been expanding and turns into a difficult issue in general well-being worldwide. Noval antimicrobial operators are quickly expected to battle this safe medication issue. The utilization of bacteriocin as an elective specialist to beat the issue is promising (Papagianni and Anastasiadou 2009). In this way, the microorganisms enduring such a domain are relied upon to deliver a wide range of antimicrobial movement. In my investigation, a few bacterial strains separated from the dirt examples indicated antimicrobial movement against MRSA. The inhibitory range of these strains was assessed and found that they showed antimicrobial action against Gram-positive microscopic organisms MRSA. The bacteriocins show a tight range towards the related strains (Kim et al. 2001). The 16S rDNA sequencing of these strain was completed to learn the species-level recognizable proof locale was exceptionally preserved, for assurance that the species were distinguished as *Bacillus cereus*. The phylogenetic tree was the manufacture and microscopic organisms are ordered. They are now detailed for this investigation 16S rDNA quality sequencing uncovered that all the strains have a place with *Bacillus licheniformis* yet neglected to show huge variety in the arrangement of these strains. By and large, the 16S rDNA arrangement is utilized in *Bacillus* order as a structure for animal varieties portrayal (Stackebrandt and Swiderski 2002). The utilization of 16S rDNA quality sequencing is used for the ID of bacterial soil test disconnects, which showed vague biochemical profiles (Woo et al. 2000, 2001). A few specialists have likewise revealed the *B. licheniformis* is known to deliver bioactive proteins with various atomic loads from 2.0 to 150 kDa that was purged from the way of life supernatant. Cerein 7, an antibacterial peptide delivered by *B. cereus* (Oscariz and Pisabarro 2001), bacillocin 490 (Martirani et al. 2002) and lichenin (Cladera-Olivera et al. 2004) are hydrophobic proteins with low atomic mass (< 2 kDa). In my present examination, the bacteriocin movement protein band was complied with in SDS PAGE investigation and afterwards, Zymogram action indicated an away from the restraint of ~ 3 kDa. The comparative aftereffect of some report that the innovation of type I, II thermostable bacteriocins and phenol-dissolvable modulins (PSMδ; MW 3 kDa) by *S. epidermidis* can specifically eliminate bacterial pathogens, for example, *S. aureus* (Cogen et al. 2010; Claudel et al. 2019). A period slaughter test was performed with bacteriocin to affirm the bactericidal impact of *B. cereus*. Development hindrance and adequacy of the bacteriocin were time-subordinate, creating particular time-kill profiles for the tried microscopic organisms. The unrefined concentrate of bacteriocin licheniocin 50.2 demonstrated noteworthy bactericidal actions against *L. monocytogenes* beginning with the primary hour of utilization (Beric et al. 2013). The advancement of biofilm is a serious issue in the clinical environment and nourishment

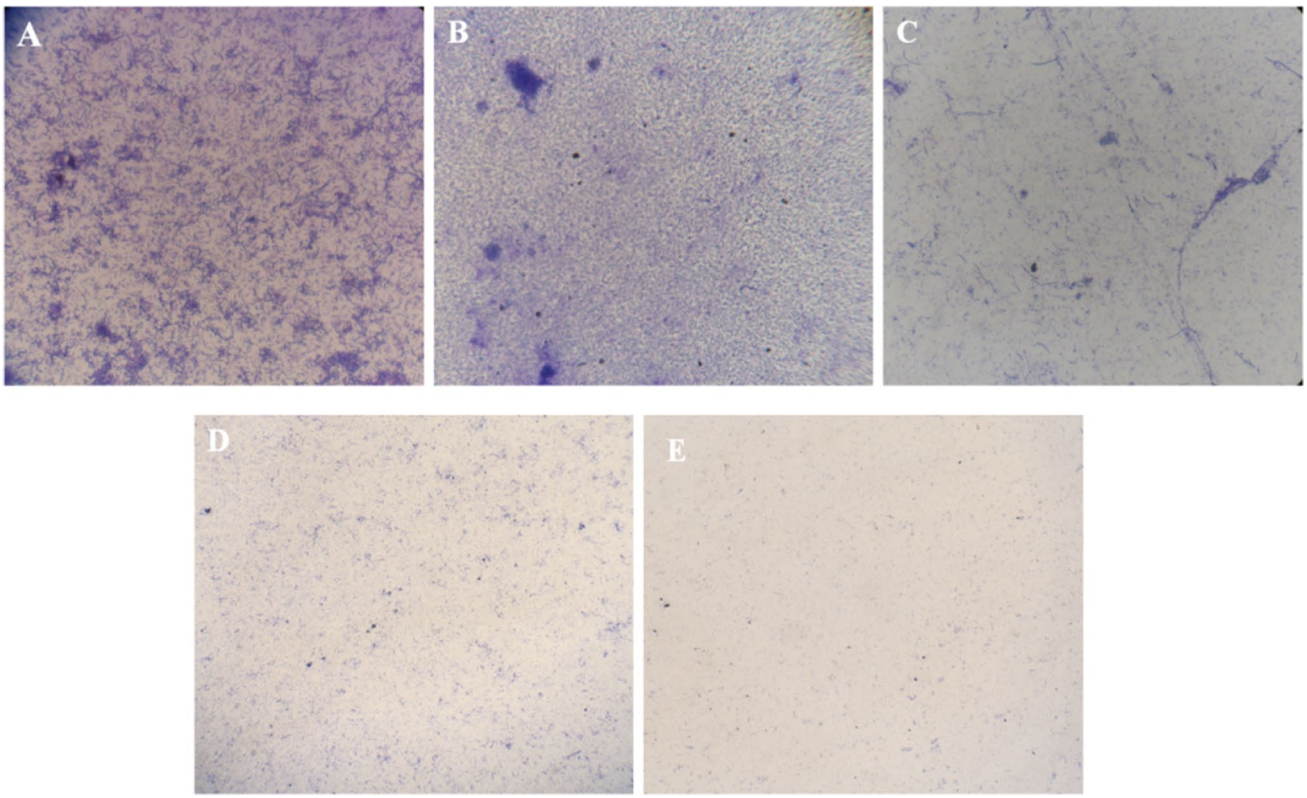

Fig. 8 Microcopy (1000× magnification) analysis of MRSA Biofilm treated with bacteriocin. **A** Control MRSA biofilm; **B** 10% bacteriocin concentration; **C** 20% bacteriocin concentration; **D** 30% bacteriocin concentration; **E** 40% bacteriocin concentration

ventures. In specific reports of biofilm, the cloned and overcommunicated bacteriocin enterocin-B displayed antibiofilm movement against *L. monocytogenes, A. baumanii* and *S. aureus*. This anti-biofilm action was expanded essentially with a blend of enterocin A (Ankaiaha et al. 2018). In my outcome, a significant decrease in biofilm improvement ability to MRSA with bacteriocin was seen at various fixations.

Conclusion

Bacteriocins are produced by many bacteria that have a lot of antagonistic activity. They can suppress MRSA, which has become a significant concern in hospitals around the world. These non-pathogenic bacterial strains are found in nature. In this work, the isolated PU3 bacterial strains from soil samples have an antagonistic impact against MRSA. In the future, they could exploit the bacteriocin produced by these microorganisms as a source of peptide antibiotics against harmful bacteria and for the control of drug-resistant MRSA infection. While resistance to these peptides in target strains has yet to be observed under laboratory circumstances, resistance mechanisms are common in producer strains. To avoid the spread of resistance and loss of efficacy, caution should be given while using bacteriocins and related compounds in clinical settings.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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