

# Isolation and Identification of Bacteriocin Producing Microbial Strains from Rhizosphere Soil

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## Vahid *et al.*: Isolation and Identification of Bacteriocin Producing Microbial Strains

Emergence and spread of antibiotics resistance among bacteria poses a serious concern for human health and disease therapy. In addition to this, novel pathogens are also surfacing rapidly. Discovery and development of newer, potent antimicrobial and therapeutic agents is constantly required. Soil is perplexing at different climatic conditions thereby providing variable source for the production of antibiotic producing microorganisms. Bacteriocin is a proteinaceous substance produced by bacteria which inhibits the growth of closely or exactly related bacterial strains. It is natural, safe secondary metabolite and its development and need for better antibiotic spectrum is always in high demand. In the present investigation, the bacteriocin producing microorganisms were isolated from the local rhizospheric soil samples. The isolates were subject to antibacterial susceptibility test against the common pathogenic bacteria i.e., *Pseudomonas*, *Aeromonas*, *Bacillus* and *Escherichia coli* by agar well diffusion method. The prominent zone of inhibition shown by isolate strains was recorded. Out of the 11 isolates, 5 isolated bacterial strains displayed significant inhibitory activity against the pathogenic bacteria. For further identification, biochemical tests were performed for these strains. These bacterial strains were identified with high similarity to *Streptococcus* sp., *Enterococcus* and *Gemella berger*. This investigation indicates that these bacteria have great potential for the production of antimicrobial property and this can be further characterized and utilized to control the growth of microbes in the future.

**Key words:** Antibiotics, rhizosphere, bacteriocin, agar diffusion assay, *Streptococcus*

Humans are constantly exposed to a variety of microbes in the environment and a small portion of these microbes can interact with the host in ways that cause infections and illnesses. To control disease infection, antimicrobial compounds and antibiotics are generally used therapeutically. Development and spread of antibiotic resistance among bacteria has become a crucial issue in disease therapeutics. The prolonged overuse of antibiotics has allowed pathogens to adapt and to develop antibiotic resistance thereby reducing the effectiveness of antibiotic therapy. Additionally, resistant bacterial strains persist, spread as well as transfer resistance genes to other bacteria in environment. Another major component in the expansion of antibiotic resistance is treatment of livestock with antibiotics similar to those employed to treat human infection<sup>[1]</sup>. The emergence of novel pathogens further presents a major hazard to public health<sup>[2]</sup>. To figure out this problem search and development of new therapeutic

antimicrobial agents has become increasingly important.

Bacteriocin is a ribosomally encoded proteinaceous substance produced by bacteria which inhibits the growth of closely or exactly related bacterial strains<sup>[3-5]</sup>. They are usually considered to be narrow spectrum antibiotics produced by both gram negative and gram positive bacteria. Few bacteriocins exhibit a specific antibacterial activity against species that are closely associated to the producers, while others shows broad antibacterial spectrum. Narrow spectrum bacteriocins are of particular interest as targeted therapeutics since they could be expected

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to have minimal impact on resident microbiota. Also, these peptides show great potential to prohibit the growth of certain antibiotic-resistant bacteria. Since the discovery of first bacteriocin, colicin V more diverse bacteriocins have been identified and describe from variety of bacterial species<sup>[6-8]</sup>.

Currently, the bacteriocin nisin reported in lactic acid bacilli *Lactococcus lactis*, is approved as food preservative by the European Union (registered with the E number E234 as a food additive), World Health Organization and Food and Drug Administration<sup>[9,10]</sup>. Also nisin is approved for use in veterinary industry<sup>[10]</sup>. Besides this, several bacteriocins have been shown to be effective against many pathogenic bacteria<sup>[5,11]</sup>. Bacteriocins are also reported to inhibit important pathogens of plant and animals, such as Enterotoxigenic *Escherichia coli* (*E. coli*) (ETEC), Shiga Toxin-Producing *E. coli* (STEC), Methicillin-Resistant *Staphylococcus aureus* (MRSA), Vancomycin-Resistant Enterococci (VRE), *Agrobacterium* and *Brenneria* sp.<sup>[12-14]</sup>.

Numerous reports of potential bacteriocin producing rhizospheric and plant-associated bacteria have been identified exhibiting wide range antimicrobial activity toward economically important plant pathogens<sup>[15]</sup>. To date, approximately 500 bacteriocins have been identified and characterized of which majority are produced by rhizosphere bacteria<sup>[4]</sup>. In this study, bacterial species from local rhizospheric soil samples were isolated and screened for their ability to produce bacteriocins. The cell free culture media of soil isolates was screened for antimicrobial activities against pathogenic bacteria. Following antimicrobial assays, the isolates were subjected to identification using biochemical assays and biochemical test result analysis by ABIS online software.

## MATERIALS AND METHODS

### Isolation of rhizospheric bacteria:

A total of 4 soil samples were collected from different location in local area of Noida, Uttar Pradesh, India. The samples were collected in a sterile plastic container and transferred to laboratory Helix biogenesis, Noida, India for further analysis.

For the isolation of bacterial strains from rhizospheric soil samples, serial dilution method was utilized. Briefly, 10 g each of soil samples were serially diluted in 100 ml of sterile distilled water and homogenized. Each of soil solution samples was further diluted to obtain dilution factor of 10<sup>-6</sup>. 100 ml of appropriate

serial dilution were plated with nutrient agar plates and incubated at 30° for 48 h. After incubation, plates were observed for bacterial growth and distinct colonies were randomly selected. Selected colonies that were picked out were repeatedly streaked on nutrient agar plates until pure cultures were obtained. The pure cultures were maintained on nutrient agar plate at 5° and sub-cultured at 2 w intervals. Also glycerol stocks of the cultures were prepared and stored at -20°.

### Preparation of a cell free culture supernatant:

All the isolated strains were grown by propagating in Lysogeny Broth (LB) for 72 h at 30°. Following this growth, the broth culture was centrifuged at 5000 rpm for 15 min. The supernatant was transferred into a new Eppendorf tube followed by another round of centrifugation until a pure supernatant was obtained. The indicator bacterial strains, *Aeromonas*, *E. coli*, *Pseudomonas* and *Bacillus* were obtained from the culture collection centre (IMTECH, Chandigarh, India). The strains were maintained on nutrient agar slants at 5° and transferred at 2 w intervals.

### Agar well diffusion method:

Antibiotic sensitivity testing using agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts<sup>[16]</sup>. The different strains isolated from soil sample were sub-cultured and tested for antimicrobial property by agar well diffusion method. Standard bacterial pathogens stains *Pseudomonas*, *E. coli*, *Aeromonas* and *Bacillus* were cultivated in 10 ml LB and kept overnight in shaking incubator at 37°. A total of 4 petri plates were prepared each containing 30 ml of Luria agar media. Solidified media plates were spread with 200 µl of bacterial suspension of standard strains respectively. The plates were left for 15 min and wells were punched in the inoculated media plates. Freshly prepared culture supernatant (50 µl) of rhizospheric isolates was loaded in the wells. The plates were incubated at 37° for 24-48 h. Inhibition of pathogenic bacteria growth was recorded as the diameter of the inhibition zone (cm).

### Identification of isolated microbes by morphological and biochemical characterization:

The colonies obtained after incubation of each isolated rhizospheric strain on nutrient agar were examined for the type of growth, size, shape, elevation, consistency and pigmentation. Selected

strains C1-C11 were examined by gram staining and biochemical characterization using the following biochemical test assays viz., aerobic growth, anaerobic growth, growth at 10° and 45°, growth in 6.5 % Sodium chloride (NaCl) medium, Voges-Proskauer test, esculin hydrolysis, glucose test, sorbitol test, lactose test, maltose test, mannitol test, sucrose test and starch test. The results of biochemical assay results were recorded and entered for analysis in ABIS ([https://www.tgw1916.net/bacteria\\_logare\\_desktop.html](https://www.tgw1916.net/bacteria_logare_desktop.html)) software for confirmation of bacterial species.

## RESULTS AND DISCUSSION

A total of 12 bacterial colonies were picked from bacterial isolation plates from the 4 soil samples collected in this study. We were able to grow pure culture of 11 isolates and stored the pure cultures as glycerol stocks. Sub-culturing was done from glycerol stock of pure culture for all experimental procedures. In order to identify potential bacteriocin producing strains, all the soil isolates were screened

by *in vitro* antimicrobial activity assay against 4 major pathogenic bacteria-*Pseudomonas*, *E. coli*, *Aeromonas* and *Bacillus*. All 11 isolates were also subjected to gram staining and biochemical characterization for identification.

Antimicrobial Susceptibility Testing (AST) is a measurement of the susceptibility of bacteria to antimicrobial agents. Bacteria are becoming increasingly resistant to conventional antibiotics; hence, has become an important requirement to find out newer and better antimicrobial agents. Agar well diffusion method is a generally used technique to evaluate the antimicrobial activity of plants or microbial extracts<sup>[16]</sup>. In this study, the different bacterial strains isolated from soil sample were sub-cultured and culture supernatant was tested for antimicrobial property by agar well diffusion method. The strains showing the distinct inhibition of growth of standard pathogenic bacteria were recorded and diameter of zone of inhibition was measured. The results are shown in fig. 1 and Table 1.

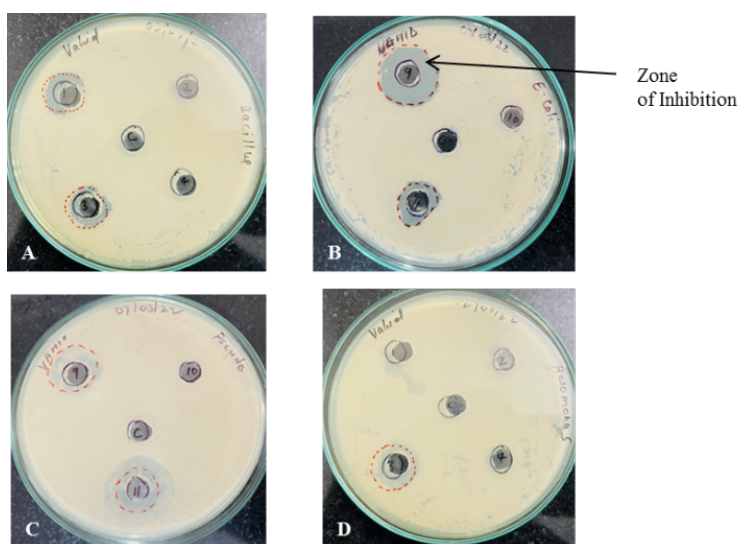


Fig. 1: Zone of inhibition against different bacteria, (A): *Bacillus*; (B): *E. coli*; (C): *Pseudomonas* and (D): *Aeromonas*

TABLE 1: DETAILS OF ZONE OF INHIBITION OBSERVED IN ASSAY FOR ANTIMICROBIAL PROPERTY BY AGAR WELL DIFFUSION METHOD

Isolate name	Diameter of inhibition zone (cm)			
	<i>E. coli</i>	<i>Aeromonas</i>	<i>Pseudomonas</i>	<i>Bacillus</i>
C1	1.5 cm	-	-	1.7 cm
C2	-	-	-	-
C3	1.5 cm	1.65 cm	-	1.4 cm
C4	-	-	-	-
C5	-	-	-	-
C6	1.6 cm	-	-	-
C7	-	-	-	-

C8	-	-	-	-
C9	2.3 cm	1.9 cm	1.7 cm	-
C10	-	-	-	-
C11	1.5 cm	-	2.5 cm	-

Note: (-): No zone of inhibition

Of all 11 isolated bacterial strains, 5 strains (C1, C3, C6, C9 and C11) showed distinct zone of inhibition against at least one pathogen with inhibition zone diameter ranging from 1.4-2.5 cm (Table 1). All the 5 isolates inhibited the bacterium *E. coli* and isolate C9 exhibited the highest zone of inhibition (2.3 cm). Inhibition against *Aeromonas* was shown by isolates C3 (zone of inhibition=1.65 cm) and C9 (zone of inhibition=1.9 cm) only. *Pseudomonas* was inhibited by isolates C9 (zone of inhibition=1.7 cm) and C11 (zone of inhibition=2.5 cm) while, *Bacillus* growth was inhibited by isolates C1 and C3.

All 11 isolates were subjected to biochemical tests and morphological test which included gram staining. Gram staining results showed that all 11 pure cultures isolated from total 4 soil samples, were positive. Fig. 2 shows the results of biochemical tests performed on the rhizospheric soil isolates. Table 2 summarizes the observation for all tests. Most bacteria isolated were aerobic in nature and two of them are anaerobic too. Many sugars like lactose, sorbitol, sucrose and glucose were fermented by these bacteria and resulted in the production of acid. Some bacteria were positive for the starch hydrolysis test and show the capacity to hydrolyze the starch present in the medium and most bacteria were halotolerant as they can grow in high salt concentrations (6.5 % NaCl).

All the microscopic and biochemical test result were analyzed by ABIS online software. Table 3 shows the results of analysis. Based on biochemical test results, the strain of bacteria isolated from the soil sample which was identified with high similarity (81.4 %-91.9 %) to *Streptococcus* sp. (8 cultures), *Enterococcus* sp. (2 cultures) and *Gemella berger* (*G. berger*) (1 culture). The 5 isolates that were found to exhibit antimicrobial activity in agar well diffusion assay were identified as follows, isolate C1 was identified as *G. berger* (81.4 % similarity) and isolate C3 as *Streptococcus agalactiae* (*S. agalactiae*) with 90 % similarity. Isolate C6 showed high similarity (86 %) to *Streptococcus thoralensis* (*S. thoralensis*), isolate C9 showed high similarity (90.6 %) to *Streptococcus equinus* (*S. equinus*) and isolate C11 was highly similar (90.6 % similarity) to *Streptococcus parasuis* (*S. parasuis*).

Several bacteria produce bacteriocins which are biologically active against other gram-positive and gram-negative bacteria and inhibit growth selectively<sup>[17]</sup>. Lactic acid bacteria represent a group of gram-positive bacteria, that produce lactic acids, organic acids, secondary metabolites, inhibitory enzymes and bacteriocins as well<sup>[18,19]</sup>. Notably, bacteriocins production has been reported in several species of the lactic acid bacteria genus *Streptococcus* such as Bovicin HJ50 from *Streptococcus bovis*<sup>[20]</sup>; Mutacins B-Ny266, II, 1140/III from *Streptococcus mutans*<sup>[20,21]</sup>; *Streptococcins* A-FF22, A-M49 and A-M57, streptin, salivaricin A, SpbMN, Blp1, and Blp2 from *Streptococcus pyogenes*<sup>[22]</sup>; Salivaricin-A, salivaricin A3, salivaricin 9 from *Streptococcus salivarius*<sup>[21,23]</sup>; thermophilin 347 from *Streptococcus thermophilus*<sup>[21]</sup> and ubericin A, and uberolysin from *Streptococcus uberis*<sup>[21,24,25]</sup>. In present study 4 out of 5 isolates that exhibited antimicrobial activity belonged to *Streptococcus* genus.

*S. equinus* is an amyolytic lactic acid bacterium commonly associated with ruminal acidosis in cattle and gastrointestinal infections in humans<sup>[26]</sup>. *In silico* genome analysis of *S. equinus* has revealed presence of large spectrum of bacteriocin encoding gene clusters such as bovine HC5, bovicin HJ50 bovicin 255, wo-component system nsuRK and the immunity genes nsuFEG, SB15, which have further validated with *in vitro* protein characterization and assay studies<sup>[20,27,28]</sup>. *S. parasuis*, a potential opportunistic zoonotic pathogen is also close relative of *Streptococcus suis* (*S. suis*), initially classified as *S. suis* serotypes 20, 22 and 26<sup>[29,30]</sup>. *S. suis* is reported to produce suicin 3908 and suicin 90-1330<sup>[31,32]</sup>.

*S. agalactiae* is a Group B Beta-hemolytic *Streptococcus* (GBS). Genome analysis identified production of the bacteriocin Agalactacin by the *S. agalactiae* type strain ATCC 13813<sup>[22]</sup>. Agalactacin has shown high similarity to nisin, and possessed antimicrobial activity against several gram-positive species. Another bacteriocin nisin P, identified in *S. agalactiae* strain DPC7040, which restricted several gram-positive species, especially *Lactobacilli* and *Staphylococcus*<sup>[6]</sup>. In another study, genome assessments for secondary metabolites



and antimicrobial peptides detected the presence of bacteriocin zoocin A in *S. agalactiae* strains<sup>[33]</sup>. Furthermore, the production of bacteriocin-like substances in *S. agalactiae* strain isolated from vaginitis in Iraq was also reported.

*S. thoralensis* is known to cause zoonotic disease and recently identified as human pathogen that generally isolated in oral flora of humans<sup>[34,35]</sup>. Genomic analysis has revealed presence of nicin like genes<sup>[36]</sup>. *Gemella* is a genus of gram-positive facultatively anaerobic hemolytic bacteria, i.e., indigenous in the oral cavity. Bacterial culture supernatants of *Gemella haemolysans* have been reported to inhibit the growth of *Porphyromonas gingivitis* a periodontal pathogens<sup>[37]</sup>. *G. bergeri* is catalase-negative, facultatively anaerobic cocci

reported from clinical sources<sup>[38,39]</sup>.

Considering the growing potential of many new bacteriocins being discovered in *Streptococcus*, it is pivotal to follow up such findings with molecular and functional analysis studies. Additional confirmation of the stain identities is required to be done using molecular identification tools such as 16S ribosomal Deoxyribonucleic Acid (16S rDNA) amplification, sequencing and phylogenetic analysis for complete identification of the isolates. Also future work is required for characterization of the antimicrobial substance/bacteriocin produced by these organisms using gene amplification, sequencing and isolation and characterization of antimicrobial protein from culture supernatant.

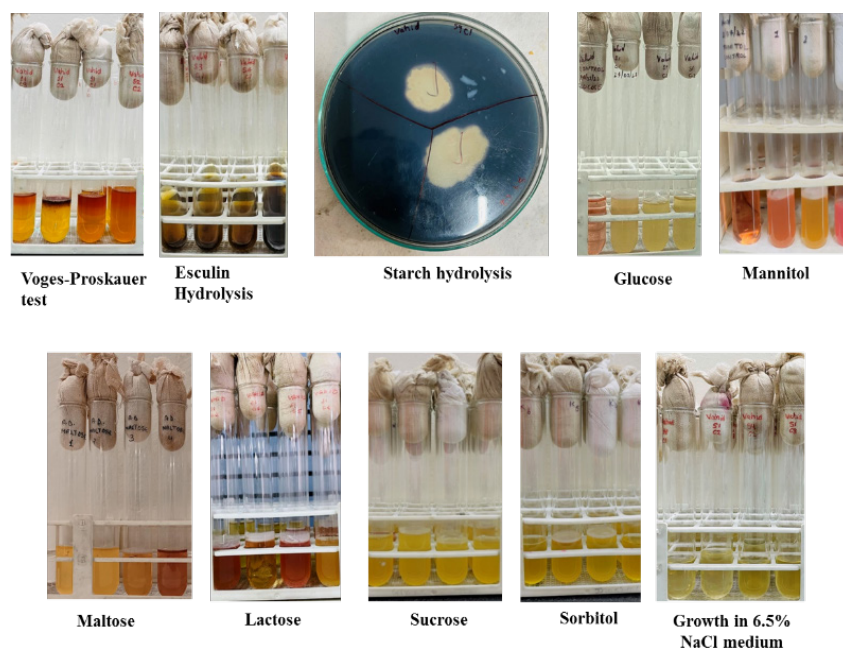


Fig. 2: Biochemical tests results for identification of bacterial strains isolated from rhizosphere

TABLE 2: DETAILS OF BIOCHEMICAL TESTS RESULTS FOR IDENTIFICATION OF BACTERIAL STRAINS ISOLATED FROM RHIZOSPHERE

S. no	Test	Bacterial cultures										
		C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11
1	Glucose	+	+	+	+	+	+	+	+	+	+	+
2	Starch	+	-	-	+	-	+	-	-	-	+	+
3	Lactose	-	+	-	+	-	+	+	+	+	+	+
4	Mannitol	-	+	-	+	-	+	+	+	-	+	+
5	Sucrose	+	+	+	+	+	+	+	+	+	+	+
6	Sorbitol	+	+	+	+	+	+	+	+	+	+	+
7	Maltose	+	+	+	-	+	-	+	+	+	+	+

8	Aerobic growth	+	+	+	+	+	+	+	+	+	+	+
9	Anaerobic growth	+	+	-	-	-	-	-	-	-	+	-
10	Growth at 10°	-	-	-	-	-	-	-	-	-	-	-
11	Growth at 45°	+	+	+	+	+	+	+	+	+	+	+
12	Growth in 6.5 % NaCl medium	+	+	+	+	+	+	+	+	+	+	+
13	Esculin hydrolysis	-	+	-	-	-	-	+	-	+	+	-
14	VP test	-	+	+	+	+	+	+	+	+	+	-

Note: (+): Production and (-): No production

**TABLE 3: IDENTIFICATION OF BACTERIA BY ANALYSIS OF BIOCHEMICAL TEST RESULTS WITH ABIS SOFTWARE**

S. no	Bacterium	Similarity %	Organism
1	C1	81.4	<i>G. bergerie</i>
2	C2	91.9	<i>Streptococcus salivarius</i> subsp. <i>salivarius</i>
3	C3	90	<i>S. agalactiae</i> ( <i>S. diffcilis</i> )
4	C4	82.9	<i>Streptococcus ferus</i>
5	C5	81.5	<i>Streptococcus salivarius</i> subsp. <i>thermophilus</i>
6	C6	85	<i>S. thoralensis</i>
7	C7	85	<i>Enterococcus thailandicus</i>
8	C8	85	<i>Enterococcus thailandicus</i>
9	C9	90.6	<i>S. equinus</i>
10	C10	84.4	<i>Streptococcus alactolyticus</i>
11	C11	87.9	<i>S. parasuis</i>

Note: Isolates C1, C3, C6, C9 and C11 exhibiting positive antimicrobial activity

In this study, strains of bacteria were isolated from the soil sample and analyzed for antimicrobial activity and biochemical test analysis for strain identification. Bacteria isolated are aerobic in nature and two of them are anaerobic too. The isolated bacteria give a clear zone of inhibition against pathogenic test organisms. From the above-performed experiments, and biochemical test result analysis by ABIS online software, the isolated strain of bacteria from the soil sample which shows antimicrobial activity were identified with high similarity to *Streptococcus* sp. (4 cultures), *Enterococcus* and 1 culture of *G. berger*. Confirmation of the stain identities is required to be done using molecular identification tools such as 16S rDNA amplification, sequencing and phylogenetic analysis for complete identification of the isolates. Also future work is required for characterization of the antimicrobial substance/bacteriocin produced by these organisms using gene amplification, sequencing and isolation and characterization of antimicrobial protein from culture supernatant.

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#### Conflict of interests:

The authors declared no conflict of interests.

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