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RESEARCH ARTICLE OPEN ACCESS

Antimicrobial Potential of Rhizospheric Bacteria *Streptobacillus* sp.

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Abstract

Rhizospheric bacteria exhibiting antagonistic effects are a good source for the production of antibiotics. The antibiotics produced are naturally bactericidal or bacteriostatic in nature. In the present investigation thirty-five rhizospheric bacteria were isolated from different soil samples. Agar well diffusion method, streak agar method, disc diffusion method and biochemical tests were performed to screen the ten antibiotic-producing bacteria. Among them, strain JRR34 selected on the basis of primary antagonistic activity was identified as *Streptobacillus* sp. Media optimisation was done to ensure maximum production of secondary metabolites. *Streptobacillus* sp. JRR34 showed good inhibitory activity against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. The ethyl crude extract of *Streptobacillus* sp. JRR34 rhizobacteria possessing good antagonistic activity against a wide range of pathogenic bacteria can be a vital source of novel antibiotics.

Keywords: Antibiosis, crude extract, pathogens, rhizospheric bacteria, secondary metabolites, *Streptobacillus*

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INTRODUCTION

Bacterial isolates produce organic compounds known as antibiotics, which are toxic towards pathogens and helps the bacterial community to overcome the biotic stress by suppressing the growth of the invading microorganisms.[1] Flocculated microorganisms exhibit high resistance to biocidal compounds.[2] The microorganisms producing antibiotics shows more longevity as compared to the non-producers. The on-going global research for last four decades has increased the commercial production of antibiotics upto two million tons per annum.[3] Recently it was observed that antibiotics show varied effects on other microorganisms in terms of virulence, modification in transcription, biofilm formation and motility.[4] The production of antibiotics occurs maximally during stationary phase and is influenced by several factors like nutrient supply, oxygenation, temperature and pH.[5] Singh et al.[6] reported that *Brevibacillus laterosporus* GI-9 produces bacteriocin which has proteolysis and bactericidal activity. *Burkholderia seminalis* secretes bioactive compound pyrrolo(1,2-a) pyrazine-1,4-dione,hexahydro (PPDH) and pyrrolo(1,2-a)pyrazine-1,4-dione,hexahydro-3(2-methylpropyl) (PPDHMP) which exhibit growth-suppressing activities towards multidrug resistant *Staphylococcus aureus*.[7] The present study was therefore aimed to observe the antibiosis abilities of the rhizospheric bacterial isolates against pathogenic bacteria viz. *Escherichia coli (E. coli), Pseudomonas aeruginosa (P. aeruginosa)* and *Staphylococcus aureus (S. aureus)* through antagonistic activity.

MATERIALS AND METHODS

Sample collection

Rhizospheric soil samples were randomly collected during winter season from different locations of agricultural research farm (ARF), Institute of Agricultural Sciences and Rajiv Gandhi South Campus (RGSC) of Banaras Hindu University (25.3176° N, 82.9739° E), Varanasi, India having a tropical climate. The soil samples were collected in isothermic polybags and stored in refrigerator at 4°C.

Isolation & characterization of bacterial isolates

Each soil samples weighing 0.5g were suspended in 5ml of sterile distilled water amended with sodium chloride (8.5 g/L) followed by shaking using vortex mixer. Further 5-fold serial dilution was done. 20 µl of each dilution (10^{-3}, 10^{-4}, 10^{-5}) was spread evenly on nutrient agar medium (pH 7.2) through spread plating technique and further incubated at 37°C for 48 hours. A total of 35 bacterial colonies developed on NA plate of which ten bacterial colonies showing discrete morphology were picked up and each of them were re-streaked to get a pure culture plate.[8,9] Morphological study of the 10 bacterial isolates was done for the characters viz. colour, dryness, roughness, colony shape, colony size, gram staining and endospore staining. Parameters selected for biochemical characterization of the selected isolates were catalase production, mannitol fermentation and methyl red test.[11]

Primary screening for Antibiosis

Antibiosis test is helpful in identifying microbes producing diffusible low molecular weight compounds possessing antifungal/ antibacterial activity. The ten bacterial isolates were therefore screened on the basis of their inhibitory activity against the pathogenic *bacteria (E. coli, P. aeruginosa* and *S. aureus)*. For primary antibiosis test, broth cultures of the isolated bacteria were used to check its inhibitory activity against the selected pathogens by agar well diffusion method. 20µl inoculum of each pathogen was spread on solidified nutrient agar media plates. Sterilized 1000 µl micro tip (diameter 8cm) were used for making the wells on the agar plate. 50µl of the bacterial broths were pipetted in the wells and incubated at 37°C for 24 h.[10] Bacterial strain JRR34 manifesting significant inhibitory activity during primary screening was optimized for growth conditions and subjected to secondary screening.

Optimization of growth conditions

Growth conditions were optimized for the strain JRR34 to get maximum secondary metabolite production. The liquid fermentation nutrient broth containing carbon sources (conc. 1%): glucose, sucrose, lactose and mannitol; nitrogen sources (conc. 1.3%): yeast extract, beef extract, peptone and ammonium chloride; metal ions (conc. 0.1%): ferrous sulphate, lead nitrate and copper sulphate was used in the present study. Furthermore, pH range: 5, 7, 9, 11 and temperature: 4 °C, 25 °C, 37 °C, 50 °C were also

Table 1. Morphological & biochemical characters of bacterial isolates

No.	Site of collection	Bacterial isolates	Colony morpholgy staining (G+/G-)	Gram staining	Endospore	Catalase	Mannitol test	MR test
1	RGSC	JRR2	Punctiform, moist, convex, white, entire	-	-	+	+	-
2	RGSC	JRR14	Punctiform, moist, convex, white, entire	+	-	+	+	-
3	RGSC	JRR19	Round, slimy, convex, white, entire	-	-	++	+	+
4	RGSC	JRR20	Irregular, moist, raised, orange, lobate	-	+	++	+	+
5	RGSC	JRR21	Punctiform, translucent, convex, white, entire	-	-	+	+	-
6	RGSC	JRR23	Irregular, slimy, convex, milky, entire	+	-	+	+	-
7	ARF	JRR34	Punctiform, moist, convex, white, entire	-	+	+++	+	+
8	ARF	JRR35	Irregular, slimy, convex, white, undulate	-	-	+	+	+
9	ARF	JRR36	Irregular, slimy, convex, white, undulate	-	+	++	+	+
10	ARF	JRR39	Irregular, slimy, convex, white, undulate	-	+	+	+	+

(ARF: Agricultural Research Farm; RGSC: Rajiv Gandhi South Campus).

optimized. Each media was sterilized at 121 °C for 15 min. The optimized media in which the isolate JRR34 showed maximum growth as measured through optical density was used for further studies.

Secondary screening for Antibiosis

Secondary screening was done to evaluate the inhibitory activity of secondary metabolites extracted in different organic solvents. Production of antimicrobial component was done by submerged fermentation technique.[12] JRR34 isolate (100µl) was grown in 100 ml of optimized media broth and incubated at 37°C for 7 days at 120 rpm rotation under sterile conditions.[13] Thereafter, centrifugation of the media was done at 10,000 rpm for 10 min for the removal of cellular debris. Resultant fermented broth was equally divided and amended with equal volume of 70% ethanol, 80% methanol respectively. Rotatory shaker was used for shaking the samples vigorously. The solvent phase was collected and evaporated using a desiccator. Later the desiccated residues were redissolved in dimethyl sulfoxide (DMSO), lyophilized and stored for further studies.[14,15] Antagonistic effect of the crude extracellular extracts was examined by agar disc diffusion method. Lawns of pathogen were made on solidified nutrient agar media and discs dissolved in 50 µl of each crude extract were placed in the plate followed by incubation at 37 °C for 24 h to measure the zone of inhibition. Antibiosis test confirmed the presence of antimicrobial compounds in the organic crude extracts of microbes. The inhibitory zones were measured in three replicates.

Statistical analysis

Three sets of experiment were carried out in randomized design and one-way analysis of variance (ANOVA) was done. Least significant difference (LSD) test was used to compare the statistical significance between the treatments at $P \leq 0.05$ probability level using SPSS Version 16 statistical analysis software (SPSS Inc., Chicago, IL).

RESULTS

Morphological and biochemical characterisation

Thirty-five bacteria were isolated from different rhizospheric soil samples through serial dilution technique. Morphological and

biochemical characteristics of the isolates showing discrete colony are listed in Table 1.

The selected isolate JRR34 was identified as *Streptobacillus* sp. on the basis of morphological and biochemical studies as described in Bergey's Manual of Determinative Bacteriology[16] and Manual for the identification of Medical Bacteria.[17] JRR34 was gram negative, rod-shaped bacteria, growing in long chains and showing slightly acidic growth condition and optimum growth temperature 37°C.

Test for Antibiosis

Bacterial strains isolated from rhizospheric soil showed significant antibiosis activity as measured through the inhibition zone (Fig. 1 a & b). The pathogens used were *E. coli, P. aeruginosa* and *S. aureus*. The bacterial strain JRR34 showed maximum antimicrobial activity during the primary screening against the pathogens. The well diffusion plates are shown in Fig. 1 (a).

Optimization of growth conditions

Optimization of culture conditions was done to provide ideal conditions for growth to the isolate JRR34 and for better production of secondary metabolite under in vitro conditions. The growth of JRR34 was optimized in 100 ml nutrient broth media and the growth was measured in terms of optical density. Maximum growth of JRR34 was observed on day 5 (1.18) and day 6 (1.24). Day 7 (1.22) onwards a decrease in optical density depicting death phase initiation was observed. Fig. 2 depicts the growth of the selected isolate graphically representing exponential and stationary phase of the isolate. Also, maximum growth (0.48) was observed in pH 5 whereas minimum growth (0.06) was observed at

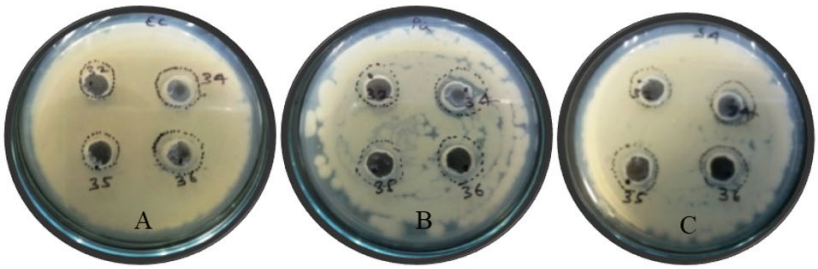

Fig. 1(a). Screening bacterial isolates for antibiosis activity by well diffusion method against (A) *E. coli* (B) *P. aeruginosa* (C) *S. aureus.*

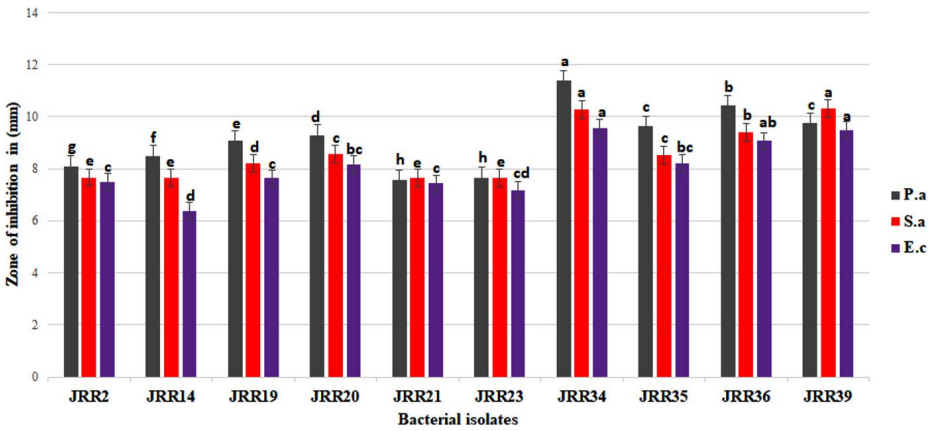

Fig. 1(b). Primary screening of bacterial isolates for antibiosis activity by well diffusion method against pathogenic bacteria *P. aeruginosa, S. aureus* and *E. coli.*

(Results are expressed as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among bacterial isolate activity taken for the same bacterium according to Duncan's multiple range test at P ≤ 0.05)

pH 11. Fig. 3 represents the effect of pH on growth of JRR34. Further it was observed that growth of JRR34 was best at 37 °C when compared for the temperature conditions. The streak plates showing the growth of JRR34 under various temperature conditions is shown in Fig. 4 .

Maximum growth of the selected isolate JRR34 was observed to be in the optimized media containing Yeast extract (1.25%), $FeSO_4$ (0.1g/l), K_2HPO_4 (0.5g/l), Lactose (1.0%), NaCl (0.2g) having pH 5 with incubation temperature of 37°C for 5 days.

Secondary screening

Assessment was done for the antimicrobial properties of *Streptobacillus* sp. extracellular components purified by solvent extraction method. Ethyl extract showed higher inhibition of the pathogens as compared to methyl extract. Tetracycline and DMSO were used as positive and negative controls respectively. Plates with zone of inhibition are shown in Fig. 5 .

A comparative analysis showed that *Streptobacillus* sp. had a significant difference in antimicrobial activity (Fig. 6) in different solvents. Although it exhibited inhibiting activity in both methyl and ethyl extracts the activity was higher in ethyl extract. It conferred 2% increase in zone of inhibition against *E. coli*, 4% increase against *P. aeruginosa* and 13% increase against *S. aureus* in ethyl extract as compared to methyl extract.

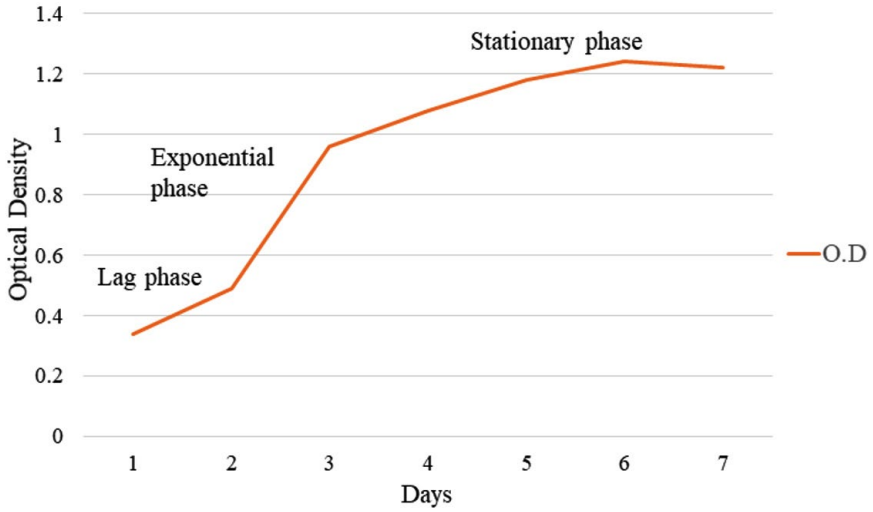

Fig. 2. Growth curve of JRR34

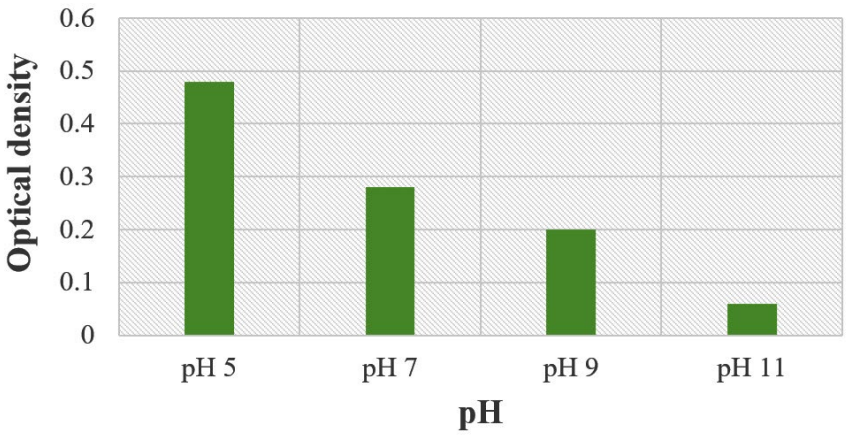

Fig.3. Effect of pH on growth of JRR34

Streptobacillus sp. exhibited more inhibitory action against *S. aureus* (22.2 mm) as compared to *E. coli* (18.2 mm) and *P. aeruginosa* (19.5 mm) in ethyl extract. Tetracycline taken as positive control showed maximum inhibition zone of 21.5mm, 24.6mm, 23.8mm against *E. coli*, *P. aeruginosa* and *S. aureus* respectively.

DISCUSSION

Thirty-five bacterial isolates were obtained from rhizospheric soil of which ten isolates which were morphologically discrete were used for primary screening purpose. The bacterial isolate JRR34, showing significant inhibitory activity against bacterial pathogens *E. coli*, *P. aeruginosa* and *S. aureus*, was identified as *Streptobacillus* sp. using Bergey's manual.[18] These pathogens selected cause human diseases like urinary tract infection, pneumonia and cellulitis and therefore the present study was focussed on the antibiosity of the crude extract of *Streptobacillus* sp. against these opportunistic pathogens. Rhizospheric bacteria often use the antibiotics produced as a competitive tool to survive in the soil environment.[19] Bacteria with antimicrobial potential help improve plant health.[20]and the bacterial interspecific interactions activates production of antibiotics.[21,22] Optimization of media is important to maximize the production of secondary metabolites. Temperature and pH are significant factors affecting production of secondary metabolite. The selected isolate, *Streptobacillus* sp, grew profoundly in pH 5 and at incubation temperature of 37 °C. In a similar study it was reported that *Streptobacillus* sp. and *Corynebacterium* sp. showed optimum growth in pH7 and at a temperature of 32 °C.[23] Similarly, *Streptomyces albidoflavus* produced maximum antimicrobial compound at pH 7[24,25] while optimum incubation temperature noted was 28 °C.[26,27] A combination of lactose, yeast and $FeSO_4$, K_2HPO_4 showed optimum growth of *Streptobacillus* sp. in the present study. Kanmani et al.[28] reported that production of bacteriocin which is responsible for antimicrobial property, in MRS media containing peptone, lactose and malt extract was maximum. Further it was observed

Fig. 4. Effect of temperature on growth of JRR34 (A) Streak plate at 4 °C (B) Streak plate at 25 °C (C) Streak plate at 37 °C

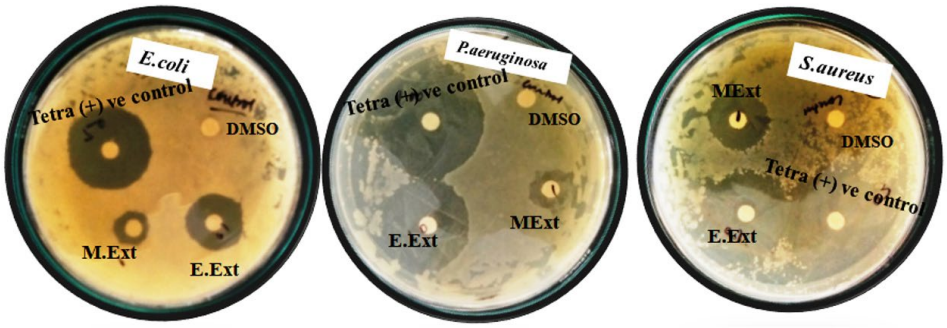

Fig. 5. Determination of antimicrobial activity of *Streptobacillus* sp. JRR34 crude extract obtained in different solvents against *E. coli, P. aeruginosa* and *S. aureus* by disc diffusion method.

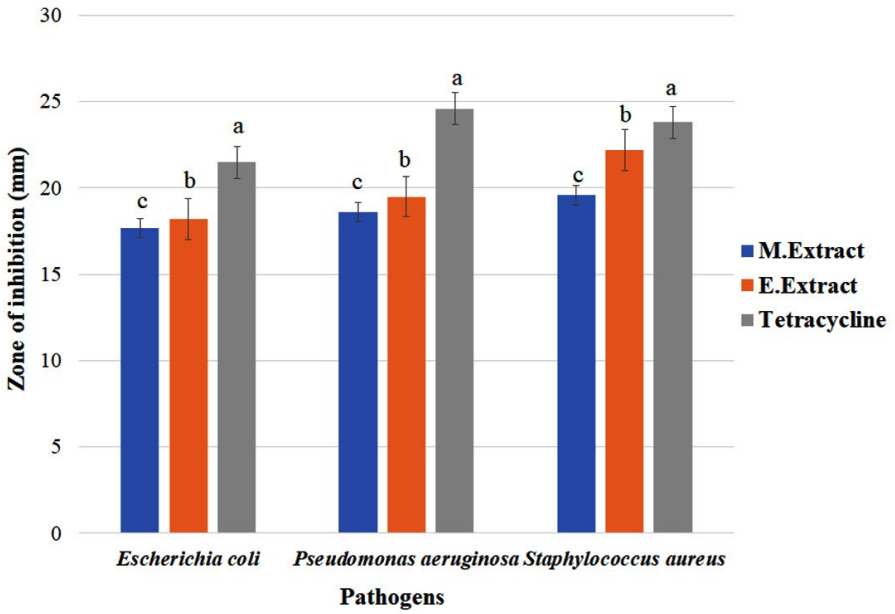

Fig. 6. Comparative analysis of zone of inhibition by *Streptobacillus* sp. crude extract obtained in different solvents against pathogens.
(Results are expressed as means of three replicates and vertical bars indicate standard deviations of the means. Different letters indicate significant differences among the results according to Duncan's multiple range test at $P \leq 0.05$)

that K_2HPO_4 plays an important role in antibiotic production in *Streptomyces fradiae*.[29] During the secondary screening process it was observed that methyl and ethyl extract of *Streptobacillus* sp. showed good antimicrobial property against *E. coli*, *P. aeruginosa* and *S. aureus*. The inhibitory effect was observed largely in ethyl extract. Similarly, Abeysinghe[30] reported that ethanol extracts exhibited higher inhibition property than other aqueous solvents. Moreover, in the present study it was found that *Streptobacillus* sp. is susceptible to tetracycline which aligned with the results of Habib et al.[23] which proved that *Streptobacillus* was resistant to trimethoprim-sulfamethoxazole and amoxicillin whereas it was susceptible to tetracycline and phenoxymethyl-penicilline, though it showed intermediate resistance towards Ciprofloxacin. *Streptobacillus* sp. exhibited more inhibitory action against *S. aureus* than *E. coli* and *P. aeruginosa*. However, it was observed that the susceptibility of gram negative bacteria was more as compared to gram positive bacteria, as the antimicrobial property was more noticeable against gram negative bacteria. This may indicate that the antibiotics produced cannot lyse the thick wall of peptidoglycan present in Gram positive bacteria. This study may contribute in understanding the biocontrol activity of *Streptobacillus* sp. and can be used in antibiotic therapy. As the present investigation is limited to few samples, it does not reveal the bigger picture but defined and precise result can be obtained if it is carried out widely.

CONCLUSION

The selected bacterial isolate JRR34 identified as *Streptobacillus* showed alterations in growth rate in response to modified growth media, pH and temperature. The inhibitory effects of *Streptobacillus* sp. ethyl crude extract against the pathogens *E. coli*, *P. aeruginosa* and *S. aureus* was significant as measured through antibiosis activity. The ethyl crude extract of *Streptobacillus* can be a vital source of novel antibiotics and the study also opens scope for extensive exploration against the multiple drug resistant microbes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approve it for publication.

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DATA AVAILABILITY

All datasets obtained or studied during this study are incorporated in the manuscript.

ETHICS STATEMENT

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

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