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Production and Charecterization of Lenticin Against Selected Human Pathogenic Bacteria

Rekha Gupta[1], Kiran Vati[2]

[1]Department of Microbiology, Allahabad Agriculture Institute Deemed to be University, U. P.

[2]Department of Biochemistry, University of Lucknow

Abstract: ***This study focuses on investigating the antibiosis activity of soil bacteria isolated from 10 different places in the rhizosphere and various agriculture areas in India. The bacteria were obtained by the use of conventional serial dilution plate procedures. The isolate was morphologically characterized using Gram's staining, revealing that all of them were gramme positive. The isolated bacteria were subjected to testing against Salmonella typhi. The most promising isolates of RS1R01 and RS7R05 were selected based on the screening results. Both isolates exhibited favorable outcomes when tested against S. typhi. The highest level of antagonistic activity, with zone of inhibition, was observed at a concentration of 160 µl for RS1R01. The process of biochemical test was conducted for isolates RS1R01 (due to high zone of inhibition) indicated a similarity with Bacillus lentus***

Keywords: Pathogenic bacteria, Bacillus lentus, Antibiosis activity, Zone of inhibition.

1. Introduction

The phenomenon of antibiotic resistance has evolved gradually, progressing from resistance to certain categories of antibiotics to the more complex forms of multi - drug resistance and extreme drug resistance [1, 2]. Previously, discussions around antibiotics and antibiotic resistance focused on their use as therapies for infections and the prevention of effective treatment, respectively. The mechanisms by which antibiotics work and the mechanisms by which bacteria develop resistance to antibiotics have primarily been investigated in harmful bacteria [3]. In recent years, antibiotic resistance research has shifted its focus towards the environment where antibiotics were first derived, specifically soil microorganisms and the soil ecosystem. Due to a diminishing availability of new antibiotics and a growing resistance problem, researchers have shifted their attention towards studying the natural antibiotic resistome [4]. Their goal is to comprehend the ecology and evolution of antibiotic resistance in non - clinical settings, with the aim of identifying sources of both known and new antibiotic resistance mechanisms [5].

Between the 2000s and the early 2023s, there was a significant decrease of 90% in the approval rate of new antibiotics. Several businesses have abandoned medication research in favour of more viable therapeutic areas due to scientific, regulatory, and commercial challenges that have made antibiotic development less appealing [6]. The microbial resistance is persistently increasing, the availability of antibiotics is decreasing, and the bulk of the population remains oblivious to this crucial predicament. Presently, infectious diseases are responsible for approximately 13 million deaths globally each year. This number is steadily increasing, facilitated by the presence of resistance genes [7].

Due to their unique structure, natural products continue to be a significant and potential source of secondary metabolites [8]. Additionally, they have demonstrated antibacterial efficacy against pathogenic microorganisms. Microorganisms remain the most promising source of future antibiotics, as they yield natural compounds with great potential [9]. Soil is regarded as a highly favourable habitat for microbial development among several undiscovered environments. The microorganisms obtained from soil are the primary source of antibiotic discovery. The soil and plant - associated habitats include a large number of bacteria that create antibiotic compounds. These compounds have either specialized or broad - spectrum actions against other microorganisms that are present [10 - 12].

The effectiveness of these antibiotics depends on factors such as pH, nutrient availability, and humus content. The abundance and variety of soil organisms are controlled by a system of non - living and living variables [13]. The primary abiotic elements encompass climate variables such as temperature, precipitation, soil texture, soil structure, salt, and pH. The physiology of soil organisms is influenced by climatic circumstances, which vary globally and also within the same locations over different seasons. Although the soil contains abundant bacteria that have the ability to produce antibiotics, it is not well understood how often this synthesis occurs at quantities that are ecologically meaningful [14]. Despite the fact that the traditional method of random screening has been used for the past 50 years to discover novel antibiotics that are beneficial to humans [15]. With this in mind, the objective of the current work was to isolate and characterize bacteria that produce antibiotics from the rhizosphere soil of various places in, Allahabad.

2. Methodology

Study area:

The specimens were gathered from ten different places of research field of Allahabad Agriculture institute deemed to be university, Allahabad. The soil in this region primarily comprises sediments such as alluvium, laterites, and brown sands. Hydromorphic saline soils are present in the regions where mostly rocky soil types are prevalent.

Sample collection:
Rhizosphere soil samples were obtained from 10 distinct locations in various crop fields in Lucknow, India. The detritus from soil samples was eliminated before to collection. The site was excavated to a depth of 5–15 cm, and roughly 10 g of the soil surrounding the roots was collected in a sterile tube. The tube was then brought to the laboratory and stored at a temperature of 4 °C. [16].

Isolation of bacteria:
The usual serial dilution plate approach was used to isolate soil microorganisms. A quantity of 1 gramme of each soil sample was measured and immersed in 10 millilitres of sterile physiological saline. The samples were subsequently diluted in a sequential manner. From each of the four dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) of each sample, 100 µl was taken to prepare nutritional agar spread plates. The plates were cultured for a maximum of 7 days to identify colonies of hostile bacteria. The colonies that exhibited antagonism were individually selected and streaked onto nutrient agar plates to achieve pure isolated colonies. The pure culture was preserved at a temperature of 4 °C for further investigations [17].

Preparation, isolation, and characterization of Bacillus species using phenotypic analysis:
The culture obtained from the enrichment broth was transferred to mannitol egg yolk polymyxin agar plates and then incubated at a temperature of 37°C for a duration of 24 hours. Biochemical characterization, including Gramme staining, motility, haemolysis, Voges - Prokauer, oxidase, methyl red, nitrate reduction, citrate utilisation, indole, coagulase, and urease tests, was used to identify typical colonies of *Bacillus species*, following the recommendations of Talaiekhozani. [18]. The isolates were additionally verified using a Microgen® Bacillus ID identification kit (Microgen Bioproducts, U. K.).

Antimicrobial susceptibility test:
The antibiotic susceptibility pattern was assessed using the Kirby - Bauer - NCCLS modified single disc diffusion technique on Mueller - Hinton agar [19].

Media optimization:
The effect of different parameters on the enhancement in growth of isolate was standardized in respect of temperature, pH, carbon source, and nitrogen source. The optimization of media is depended on one factor at a time method [20, 21].

3. Results

Microbial isolates isolation and maintenance
70% of Bacillus spp. Were isolated from the collected soil sample out of which 2.5% spp. belongs to *Bacillus lentus.*

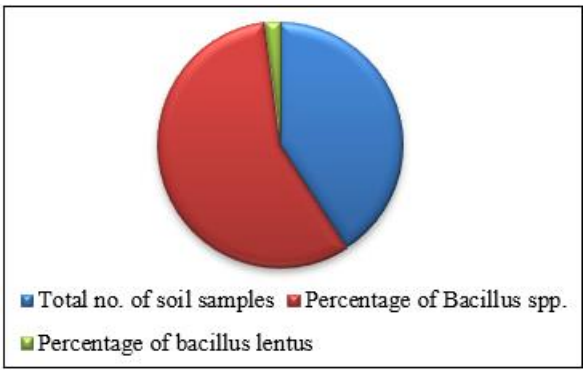

Figure 1: Isolate percent obtained from various soil samples

The colony forming units (CFU) of all soil samples exhibited a wide range of diversity. The appropriate dilution was chosen based on the plate containing a quantifiable number of colonies. The soil sample (R06) had the highest number of colony - forming units (CFU), while the soil sample (S2) had the lowest amount of CFU. Among the 10 soil samples that were examined, Five soil specimens (S1, S2, S4, S7, and S10) exhibited antagonistic characteristics at dilutions of either 10^{-2} or 10^{-3}. S7 had three colonies that exhibited hostile behavior. Subsequently, S1 was accompanied by two colonies. Sample S2, S4, and S10 each displayed a solitary colony with antagonistic behavior (Table 2). Therefore, these 8 antagonistic bacteria (S1R01, S1R02, S2R03, S4R04, S7R05, S7R06, S7R07, S10R08) were chosen for additional screening.

Table 1: Studies on the antagonism properties of isolates

Soil sample	Number of colonies	Dilution	Number of CFU/ ml	Antagonism
RS1	35	10^{-3}	3.5×10^{6}	R01, R02
RS2	85	10^{-3}	8.5×10^{5}	R03
RS3	61	10^{-3}	6.1×10^{6}	-
RS4	94	10^{-3}	9.4×10^{6}	R04
RS5	32	10^{-3}	3.2×10^{5}	-
RS6	200	10^{-2}	20.0×10^{6}	-
RS7	60	10^{-3}	6.0×10^{6}	R05, R06, R07
RS8	30	10^{-2}	3.0×10^{6}	-
RS9	230	10^{-3}	23.0×10^{6}	-
RS10	180	10^{-3}	18.0×10^{6}	R08

Table 2: Shape of selected strain

Strain no.	Remark
R01	Gram positive, *Bacillus*
R02	Gram positive, *Bacillus*
R03	Gram positive, *Bacillus*
R04	Gram positive, *Coccus*
R05	Gram positive, *Bacillus*
R06	Gram positive, *Coccus*
R07	Gram positive, *Bacillus*
R08	Gram positive, *Bacillus*

Antimicrobial screening:
Following the AST through well diffusion methods, the isolates RS1R01 and RS7R03 showed the highest potential with strong antagonistic activity against four human pathogens: *S. typhi* (Table 2). Both isolates exhibited positive results for *Enterococcus sp.* and *S. aureus.* The isolate RS1R01 exhibited a zone of inhibition measuring 22.65±1.29 mm and 30.12±0.52 mm against *S. typhi* at volumes of 80 µl and 160 µl, respectively. The antibiotic disc

Norfloxacin 30 (Amoxyclav) exhibited a zone of inhibition measuring 29.53±2.24 mm against *S. typhi a*s shown in figure 2 and table 3. Further the identification of the selected isolate RS1R01 strain by analyzing its biochemical characteristics and colony shape as shown in table 4.

Table 3: Zone of inhibition produced by RS1R01 and RS7R05 against *S. typhi.*

Isolates	80 µl	160 µl	Norfloxacin 10µg/ml
RS1R01	22.65±1.29 mm	30.12±0.52 mm	29.53±2.24 mm
RS7R05	21.67±0.21 mm	27.23±1.23 mm	29.53±2.24 mm

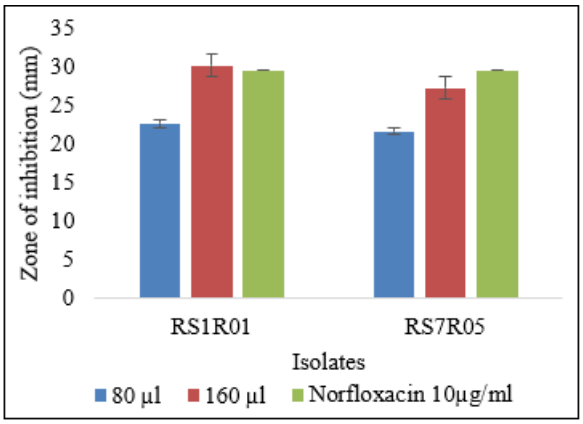

Table 2: Zone of inhibition produced by RS1R01 and RS7R05 against *S. typhi.*

Table 4: Identifying the RS1R01 strain by analyzing its biochemical characteristics and colony shape.

S. no.	Test	Results
1	Colony appearance	Smooth and Shiny
2	Colony colure	Cream
3	Surface	Glossy
4	Shape	Rod
5	Margine	Entire
6	Gram's reaction	+ve
7	Temperature for growth	30°C
8	Cell length (µm)	3/3/3/3
9	Colony shape	Circular, convex
101	Arrangement	Chain
11	Oxidase test	+ve
12	Catalase test	+ve
13	Indole test	+ve
14	Citrate utilization	+ve
15	Vogues Proskaur test	+ve
16	Motility test	+ve
17	Reduction test	+ve
181	Starch hydrolysis test	+ve
19	H_2S production test	+ve
20	Urease test	+ve
21	Sugar fermentation	+ve

The biochemical and colony morphological characteristics suggest that the chosen bacterial strain is likely *Bacillus lentus.*

Media optimization for production of antimicrobial compounds

Various carbon sources were tested for the generation of antimicrobial compounds. The largest zone of inhibition, measuring 30±2.12 mm, was observed in the presence of starch. When alternative carbon sources such as maltose and sucrose were present, zones of inhibition measurements of 21±2.5 mm and 19±1.25 mm were obtained, respectively. No zone of inhibition was identified in the presence of lactose. Multiple nitrogen sources were evaluated for the production of antibacterial substances. The biggest zone of inhibition, measuring 32±2.78 mm, was detected when Peptone was present. Zones of inhibition readings of 19±0.52 mm and 22±1.36 mm were found when alternate carbon sources like Yeast extract and Ammonium nitrate were used. No zone of inhibition was observed when Beef extracts were present given in figure 3 and table 5.

Various incubation temperatures were tested for the synthesis of antibacterial compounds. The largest zone of inhibition, measuring 35±1.33 mm, was observed at 30 °C. Zones of inhibition measured 22±0.85 mm at 40 °C and 20±0.11 mm at 50 °C. There was no zone of inhibition detected at 60 °C. Multiple incubation pH levels were explored for the production of antibacterial substances. The biggest zone of inhibition, measuring 40.12±1.83 mm, was seen at pH 9. Zones of inhibition were 19±0.35 mm at pH 8 and 10±0.08 mm at pH 10. No zone of inhibition was seen at pH 11. As shown in figure 3 and table 5.

Table 5: Effect of media components, temperature and pH antibiotic activity against *S. typhi.*

Factors	Components	Zone of Inhibition in mm
Carbon source	Starch	30±2.12
	Maltose	21±2.5
	Sucrose	19±1.25
	Lactose	0
Nitrogen source	Yeast extract	19±0.52
	Beef extracts	0
	Peptone	32±2.78
	Ammonium nitrate	22±1.36
Incubation Temperature (°C)	30	35±1.33
	40	22±0.85
	50	20±0.11
	60	0
pH	8	19±0.35
	9	40.12±1.83
	10	10±0.08
	11	0

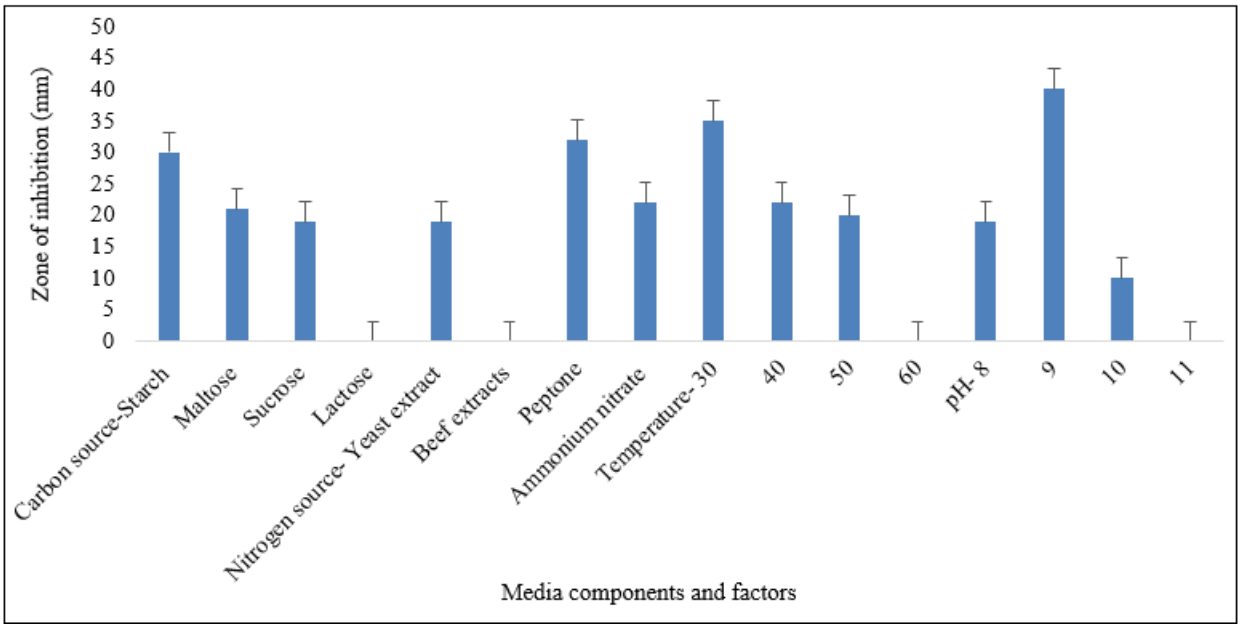

Figure 3: Effect of media components for activity enhancement as antibiotic activity against *S. typhi*

The objective of the study was to extract antibiotic - producing bacteria from the rhizosphere zones of several are in Gomtinagar, Lucknow. The region of rhizosphere soil has been chosen for sampling due to its higher abundance of microbial communities compared to other environments. The rhizosphere microorganisms have a significant degree of hostile activity [22].

Soil is a preferred choice among scientists for isolating new antibiotics due to its abundance of antibiotic - producing bacteria, particularly Actinomycetes as noted by researcher. The study [20] also demonstrates that the biodiversity of soil microbes is greatly influenced by the variability of soil, which creates an assortment of ecological niches. This result was associated with our applied methodology for sample collection, which involved collecting samples from various places and diverse cultivations. The present investigation utilised the usual approach to sample collection known as random sampling, according to by Williams and Vickers [23].

The morphological characterisation was conducted using the Gramme staining method, a widely used conventional technique for characterization as reported by several scientists. All of the bacterial isolates were found to be gramme positive based on the Gram's staining. The results are consistent with those of Baker & Satish [24], who found that the majority of soil isolates were gramme positive. The isolates underwent for screening using the agar well diffusion method to determine their ability to produce antibiotics. This study aligns with earlier publications that employed identical approaches to screen isolates.

The isolates RS1R01 and RS7R05 were subjected to secondary screening against *S. typhi.* Both isolates, RS1R01 and RS7R05, exhibited the largest zone of inhibition against S. typhi. The findings of this study supported the outcomes of Manandhar et al., [25], who indicated that the soil isolates exhibited the highest level of inhibition against S. aureus. Similarly, Gandra et al. [26] observed that the isolate exhibited the largest zone of inhibition towards S. typhi. Contrary to the previous findings, the highest level of inhibition zone was observed at a concentration of 12.5 µg/ml (Minimum Inhibitory Concentration) for *K. pneumonia,* and then *E. coli [*27].

Bacterial identification was conducted by a series of biochemical tests. An important benefit of this procedure is that it allows for the identification of bacterial isolate within a week. The isolates were confirmed to be *Bacillus lentus* using biochemical characterization investigation of RS1R01. This outcome offers robust validation to previous research that has already established Bacillus species as the primary bacteria found in soil.

This also reinforces the findings of prior research that have demonstrated *Bacillus lentus* to be a highly effective source of antibiotics. The study conducted by Abdulkadir, & Waliyu, [28] has demonstrated that *Bacillus lentus* is capable of producing a wide range of antimicrobial peptides and secondary metabolites, which aligns with our current findings. The findings are consistent with that *Bacillus lentus* produces the highest levels of antimicrobial proteins, particularly against gram - positive *S. aureus*. Gram - positive bacteria are more vulnerable to antibiotics due to their sole outer peptidoglycan layer, which is not a highly efficient barrier. The rationale for both RS1R01 and RS7R05 to create bigger zones of inhibition against *S. typhi*, a gramme positive bacterium, may be attributed to this.

4. Conclusion

Bacillus lentus has demonstrated significant efficacy as a source of antibiotics against plant infections. It is primarily found in the rhizosphere of plants, which further strengthens the relevance of our investigation. In addition, lenticin which is produced by *Bacillus lentus*, has been shown to be effective against human infections like *S. typhi*. In our study, we observed that *Bacillus lentus* exhibited the highest level of inhibition against *S. typhi.* Furthermore, *Bacillus lentus* is capable to producing lenticin. *Bacillus lentus* has been employed as an antibacterial agent to combat pathogenic bacteria in dairy and veterinary animals. All of these

discoveries offer robust evidence and form the basis of our conclusion that *Bacillus lentus* is a powerful reservoir of antibiotics.

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