Biological Forum – An International Journal 15(10): 938-944(2023)

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Isolation, Characterization, and Antagonistic Activity of Multifaceted Plant Growth-Promoting Bacteria, Including Metaltolerant *Lysinibacillus macroides*, From Vegetable-Cultivated Soils in Namakkal District, Tamil Nadu, India

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(Received: 12 August 2023; Revised: 13 September 2023; Accepted: 30 September 2023; Published: 15 October 2023)

(Published by Research Trend)

ABSTRACT: The study isolates and characterizes multifaceted bacteria, like *Lysinibacillus macrolides*, with metal tolerance and pathogen control, showcasing their potential in sustainable agriculture and environmental management. The aim of this work is to isolate and characterize multifaceted microorganisms, particularly *Lysinibacillus macrolides*, for sustainable agriculture and environmental management. Soil samples from Namakkal were used to isolate rhizospheric bacteria via serial dilution plating. Isolates were evaluated for multiple plant growth-promoting traits, metal resistance, and antagonistic activity against phytopathogenic fungi. DNA sequencing was employed for isolate identification. The study identified 25 bacterial isolates with multiple plant growth-promoting traits, metal tolerance, and antagonistic activity. *Lysinibacillus macroides* exhibited high lead and chromium tolerance and effective antifungal activity against *Fusarium* sp., with an 84% growth inhibition. Based on the results, it can be concluded that the isolates are the best and can be used to improve soil fertility and this PGP bacterium was hold great promise for sustainable agriculture and environmental improvement.

Keywords: PGPF, Siderophore, Metal resistance, *Lysinibacillus macroides*, Antagonistic activity.

INTRODUCTION

The escalating pace of industrialization and human activities has led to the widespread contamination of soil ecosystems with toxic heavy metals, engendering a complex web of ecological and health challenges. Cadmium (Cd), copper (Cu), zinc (Zn), and nickel (Ni), among other heavy metals, persistently infiltrate soil matrices, disrupting the delicate balance of ecosystems and posing substantial threats to human well-being (Alloway, 2013). Metal pollution poses significant risks to both human health and agricultural productivity. Toxic metals like cadmium, lead, and arsenic can accumulate in crops, entering the food chain and endangering human consumers. This contamination can lead to chronic health issues, developmental problems, and even cancer. In agriculture, metal pollution disrupts soil ecosystems, impairing nutrient cycling and reducing crop yields. A study by Kabata-Pendias and Mukherjee (2007) highlights the adverse effects of metal pollution on soil-plant systems and human health.

The bioaccumulation of heavy metals within the food chain compounds these concerns, further emphasizing the imperative for robust mitigation strategies. The conventional remediation methods, frequently entailing disruptive and resource-intensive practices, underscore the pressing need to investigate alternative and sustainable solutions. As heavy metal pollution continues to pose risks to ecosystems and human health, innovative approaches are essential to ensure long-term environmental integrity and well-being (Hansda *et al*., 2017). In this context, the exploration of eco-friendly and efficient strategies is pivotal to address the challenges posed by heavy metal contamination and to pave the way for a healthier and more sustainable future.

Microorganisms inhabiting metal-contaminated soils have evolved diverse mechanisms to withstand the challenges posed by elevated metal concentrations. These mechanisms include metal efflux pumps, metal-binding proteins, and enzymatic reduction, allowing microorganisms to thrive in these hostile environments (Nies, 1999). Remarkably, certain metal-resistant microorganisms also display antagonistic activity against plant pathogens, signifying their potential to bolster plant health. This dual functionality—metal resistance combined with antagonistic prowess—highlights their potential to serve as versatile agents in agro ecosystems (Zhang *et al*., 2023).

In this context, empirical research has demonstrated the potential of these adaptable microorganisms. The work of Ma *et al*. (2019) has illuminated the isolation of metal-resistant bacteria from contaminated sites and their capacity to enhance plant growth. Additionally, the study by Rana *et al*. (2020) underscores the significance of these isolates in mitigating plant diseases. These studies collectively underscore the value of delving into the multi-dimensional potential of these microorganisms, as they offer a comprehensive solution to the intricate challenges presented by heavy metal pollution and the pursuit of sustainable agriculture. However, the study of this integrative function remains largely unexplored. Microorganisms with these synergistic functions can provide holistic strategies to address the multifaceted challenges of heavy metal pollution and agricultural productivity (Mehmood *et al*., 2023). In the current study, we aim to isolate multifaceted microorganisms by systematically identifying and characterizing metal-resistant bacteria with antagonistic and PGP attributes from soil samples. Through thorough analysis, this research seeks to uncover the potential applications of these microorganisms in remediating heavy metal-contaminated soils and promoting enhanced plant growth.

METHODOLOGY

Soil samples from the Namakkal area, used for cultivating vegetables, were collected and individually placed in plastic bags. These samples were transported to the laboratory for the isolation of rhizospheric bacteria. To isolate these bacteria, 1 gram of soil was subjected to serial dilution plating on Nutrient Agar plates (manufactured by Himedia, India). Subsequently, the plates were incubated at a temperature of 28 ± 2◦C for duration of 24 to 48 hours. Individual colonies were then selected and streaked onto new Nutrient Agar plates for further purification.

Isolation of isolates exhibiting plant growth-promoting factors: All obtained rhizobacterial isolates underwent screening for various plant growth-promoting traits. Each isolate was inoculated onto modified Pikovskaya agar media containing tricalcium phosphate (TCP) and incubated at a constant temperature of 30±0.1°C for 5 to 7 days. The presence of a clearance zone surrounding the colonies was observed, indicating positive phosphate solubilization.

Isolation of IAA producing isolates. The Bric *et al*. (1991) methodology was updated to use a qualitative method for the assessment of IAA (Indole-3-Acetic Acid) synthesis. Bacterial cultures were introduced into nutrient broth supplemented with tryptophan (1 mg/ml) and then incubated at 35 ± 2ºC for 7 days. The development of a pink color was indicative of IAA production after the addition of 2 drops of orthophosphoric acid and 4 ml of Salkowski's reagent (comprising 50 ml 35% perchloric acid and 1 ml 0.5mM $FeCl_3$).

Isolation of Siderophore producing isolates. By inoculating the isolates on solid CAS agar plates and incubating them at 35 2°C for 72 hours, siderophore production by the isolates was evaluated. According to the procedure outlined by Schwyn and Neilands (1987), the appearance of a yellow-orange halo zone around the colonies indicated siderophore formation and release on the agar plate.

Isolation of ammonia producing isolates

To determine ammonia production, isolates were inoculated into peptone water and incubated for 48 hours at 35 ± 2°C. Subsequently, 0.5 ml of Nessler's reagent was added to each tube, and the development of a brown to yellow color indicated a positive test for ammonia production, as per Cappuccino and Sherman (1992).

Isolation of HCN (Hydrogen Cyanide) producing isolates. For assessing HCN (Hydrogen Cyanide) production, bacterial cultures were streaked on nutrient agar medium containing 4.4 gm/l of glycine. Inside the lid of each plate, a Whatman filter paper No. 1 soaked in a 0.5% picric acid solution (in 2% sodium carbonate) was placed. The plates were then sealed with parafilm and incubated at 35 ± 2ºC for 4 days. The presence of an orange to red color development indicated HCN production, as per Ahmad *et al*. (2008).

Isolation of Nitrogen-fixing bacteria. Using yeast extract mannitol selective culture media (YEM) and Bromthimol blue (BTB), nitrogen-fixing bacteria were purportedly recovered from the soil. The Geniaux *et al*. (1993) approach was used to identify colonies that had yellow haloes on the blue medium as nitrogen-fixing bacteria.

Isolation of Protease producing bacteria. Using skim milk agar medium, protease activity of bacterial isolates was assessed. According to Ghosh *et al*. (2008), the isolates were spot-inoculated onto skim milk agar medium, and after two days of incubation at 30°C, proteolytic activity was determined by the existence of a clear zone surrounding the colonies.

Isolation of cellulase producing bacteria. The isolates were screened for cellulase production by incubating them on plates supplemented with 10 g l^{-1} carboxymethyl cellulose (CMC) for 48 hours at 30°C. Subsequently, the plates were flooded with congo red dye, and the formation of clear halos surrounding the colonies indicated their cellulolytic activity, following the method outlined by Bera *et al*. (2015).

Isolation of amylase producing bacteria. The bacterial isolates were spot-inoculated on starch agar plates (consisting of Beef extract 3.0, peptone 5.0, soluble starch 2.0, Agar 15.0, and Distilled water 1 liter) and incubated at 30°C for 48 hours in order to produce amylase. According to Choubane *et al*. (2016), after the incubation period, iodine solution was flooded onto the plates, and the existence of a clear zone around the colonies was a sign that amylase had been produced. Isolation of metal resistance isolates.

Isolation of metal resistance isolates. The bacteria that were chosen were examined to assess their ability to tolerate lead and chromium. This was done using the agar dilution method, as described by Verma *et al*. (2015). In this method, bacterial cultures that were actively growing were streaked onto agar plates that had been amended with cadmium nitrate at various concentrations ranging from 0.25mM to 2.0mM. The ability of the bacteria to resist cadmium was determined based on their growth after 3 to 4 days of incubation. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of the metal ion that completely inhibited bacterial growth.

Isolation of Antagonistic activity producing PGP isolates. The Using PDA plates, in vitro antagonistic interactions between bacterial isolates and phytopathogenic fungi were studied. *Fusarium* sp of plant pathogen was isolated from infected brinjal leaf samples, which was subjected to antagonistic activity. Two isolates of pathogenic fungal and antagonistic activity producing bacterial isolates were inoculated on the plate at two equal distance sites, 3 cm apart from the colony of fungal pathogen in the center, after placing an agar plug of the growing fungal mycelium in the center of the PDA plate. At 30°C for 7 days, the Petri plates were then incubated. The following formula suggested by Alenezi *et al*. (2016) has measured the percentage of fungal growth inhibition.

% of fungal growth inhibition=1−(a/b)×100%

where a is the distance between fungal growth edge (from the bacterial side) and the

bacterial isolate growth edge (from the fungus side) and b is the distance between the

fungal upper growth edge and the upper edge of the control Petri dish

Isolation of potential isolates. DNA sequence analysis was performed on a biological sample at Barcode Bioscience in Bangalore. The identity of the isolate was verified using the 16S rRNA sequence, a Sanger method for bacterial and archaeal classification. The 16s rRNA genes were amplified using universal primers and the amplicans were sequenced in an automated gene sequencer. After the sequencing process, BLAST analysis was performed with existing 16srRNA bacterial sequence available in the nucleotide databases.

RESULT AND DISCUSSION

Plant growth-promoting (PGP) bacteria have gained significant attention in agriculture and environmental science due to their multifaceted roles in enhancing plant growth and soil health. These beneficial microorganisms exhibit various traits and the ability to suppress phytopathogens. Beyond their PGP capabilities, some of these bacterial isolates also possess remarkable tolerance to heavy metals and antagonistic activity against plant pathogens. This convergence of traits in a single microorganism has profound implications for sustainable agriculture and environmental management, offering novel avenues for improving crop yields, remediating metal-contaminated soils, and reducing the reliance on chemical pesticides.

Previous studies have highlighted the co-occurrence of plant growth-promoting and metal resistance traits in various bacterial isolates (Verma *et al*., 2015; Tirry *et al*., 2018; Mandal *et al*., 2022). These isolates not only promote plant growth and protect crops from diseases but also exhibit resilience in metal-polluted environments. Such dual-functioning microorganisms have the potential to revolutionize agricultural practices in regions affected by heavy metal contamination, where soil quality and plant health are compromised. Furthermore, the antagonistic activity exhibited by some PGP isolates against phytopathogens has gained recognition as a sustainable alternative to chemical fungicides. These isolates offer a natural defense mechanism for crops, reducing the ecological and health risks associated with synthetic pesticides (Alenezi *et al*., 2016). In light of this, a recent study by Sandilya *et al*. (2022) showed how PGPR isolates could effectively lower the blight disease caused by *Alternans* sp. Similar to this, Vanegas *et al*. (2021) reported that they had used PGPR isolates to successfully prevent vascular wilting caused by fungal isolates.

Preceding this, the identification of plant growth-promoting isolates was carried out using soil from vegetable cultivation, and the current study focuses on evaluating their combined metal resistance and antagonistic characteristics on the PGP isolates. A total of 30 bacterial isolates from eight distinct genera were found in this study. The highest occurrence was *Bacillus* genus, which includes *Bacillus subtilis*, *Bacillus cereus*, *Bacillus amyloliquefaciens*, and an unnamed *Bacillus* species and followed by *Pseudomonas* sp. It's interesting to note that out of the seven genera seen, 26.6% of the isolates belonged to the gram-positive group, while 73.3% were gram negative (Fig.1). This finding is consistent with a prior investigation carried out in the Namakkal region in 2018 that also discovered a bacterial consortium of isolates that promote plant development (PGPF) (Sundaram and Murali 2018). Additionally, from soil samples from vegetable-cultivated fields, Singh *et al*. (2017) at Banaras Hindu University discovered different bacterial populations.

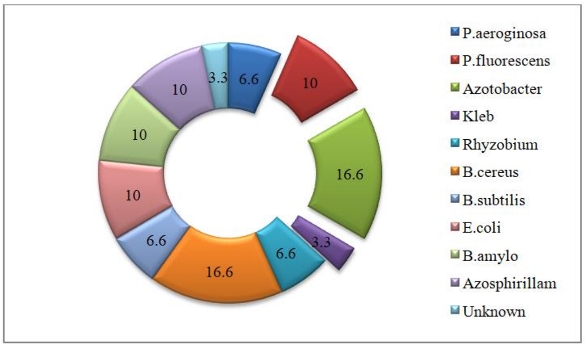

Fig. 1. Occurrence of bacterial isolates from vegetables cultivate soil samples.

In the current study, all isolates underwent screening for plant growth-promoting factors (PGPF), with each isolate exhibiting specific plant growth-promoting traits. Notably, 56.6% of the bacterial isolates were found to produce IAA, and all isolates of *P.fluorescens*, Klebsiella sp, *B.amyloliquefaciens* and unknown were positive for IAA production. Moreover, other essential PGPR characteristics, such as 40% of the isolates exhibited ammonia-producing capabilities, and 33.3% demonstrated phosphate solubilization, with 16.6% of soil isolates contributing to both ammonia production and phosphate solubilization. A similar tendency was also noted in India by Ponmurugan *et al.* (2012) and more recently, a study by Sagar *et al.* (2022) identified the distinct plant growth traits connected to *Azotobacter* sp.

Siderophores play a central role in various ecological processes, including promotion of plant growth and biological control activities. They are synthesized by a variety of microorganisms to facilitate the extraction of iron (III) from iron hydroxide complexes. In our current study, 13.3% of the isolates showed positive siderophore production. Of the seven genera examined, single isolate of unknown *Bacillus* sp and *Rhyzobium* spp. were shown to be major siderophore producers, with a significant number of *Azotobacters* also displaying siderophore production capabilities. These results are consistent with those of Jenifer *et al*. (2013) who reported siderophore production by free-living rhizosphere *Azotobacter* isolates (16.22%) as well as by *Pseudomonas* spp. Observed. and *Bacillus* spp.

HCN is a chemical compound that has gained recognition for its potential as a plant growth promoter. It has been proposed that HCN can also serve as a biocontrol agent against plant pathogens. According to earlier studies, the synthesis of HCN by rhizospheric isolates can shield a variety of plants from fungi that cause soil-borne root illnesses (Zahir *et al*., 2004). In our current study, approximately 10% of the isolates tested positive for HCN production, including *Azotobacter*s sp., *Rhizobium* sp., and unknown isolate. Ponmurugan *et al*.'s earlier investigation (2012) also noted *Azotobacter*s sp. producing HCN in the Namakkal region. Additionally, in 2017, *Lysinibacillus* sp. that produces HCN was found in soil samples from maize fields by Naureen *et al*. (2017).

In order to discover nitrogen-fixing bacteria within soil samples, attempts were made to recognize the critical significance of nitrogen as a major nutrient in activities such as chlorophyll synthesis, amino acid creation, and protein building. The nitrogen-fixing test findings showed that 23.3% of the isolates had the ability to fix nitrogen. Notably, nitrogen-fixing activity was present in 50% isolates of *Rhyzobium* and 40% *Azotobacter* and *B.cereus*. Additionally; nitrogen fixation was discovered in unknown isolate, *Azosphirillam* sp also. These results highlight the importance of these microbes for preserving soil nitrogen levels, promoting healthy plant growth, improving soil fertility, and lowering dependency on synthetic nitrogen fertilizers. In 2022, Patel *et al*; Negi *et al*. (2022) were observed the various type of bacterial isolates that capability of nitrogen fixing from agriculture soil samples. In addition, enzymes can aid in the breakdown of pollutants and waste materials, which can improve the health of the soil and plant growth (Liu *et al*., 2018). Protease, cellulose, and amylase production rates for isolates in the current investigation were 50%, 73.3%, and 40%, respectively (Fig. 2). The two genera with the highest incidence of enzymes among the seven obtained were *Pseudomonas* and *Bacillus* sp. Apart from cellulose, several plant development boosting elements were detected in this isolate, which according to the overall characterization, harbors the majority of the plant growth promoting components.

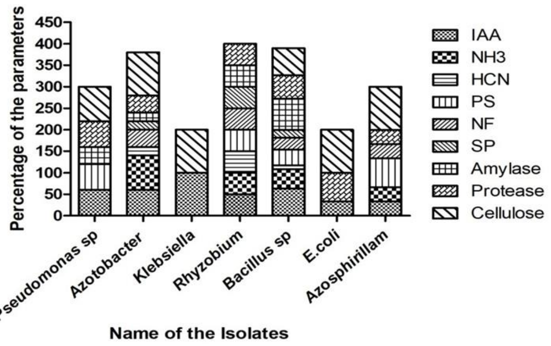

Fig. 2. Isolation of *plant growth promoting* traits.

Due to their low tolerance for heavy metal concentrations, the effectiveness of using PGP bacteria is restricted in areas contaminated with heavy metals. The resistance of bacteria found in areas contaminated with heavy metals is widely acknowledged to be greater than that of bacteria isolated from soil that is free from contamination (Efe, 2020). Keeping this concept in mind, soil was collected from the agricultural area without metal contamination and tested for the presence of factors favorable to the growth of the plant growth and it was tested for tolerance against metals such as chromium and lead. Totally 30 isolates were subjected to preliminary screening of metal tolerance assay, among them, 5 of only were grown on 0.25mM concentration of chromium and lead containing media, leading to further testing of these isolates for metal tolerance.

According to tolerance assay, 2 *Bacillus* sp was tolerate up to 1.5mM of chromium and single isolate of unidentified bacteria was tolerate up to 2mM. Simultaneously, same *Bacillus* sp, *Pseudomonas* sp and unidentified bacteria were tolerated up to 5.5mM of lead metal. Previous studies show that *B. cereus* has a significant heavy metal (Pb, As, Co, Cd, Ni, Cr, Hg and Zn) tolerance which supports the results of the current study (Efe, 2020). Recently, PGPR strains such as *Bacillus sp*., *Cupriavidus sp*., *Cupriavidus sp*., *Fulvimonas choli* and *Novosphingobium* sp., isolated from rhizosphere soil, were tolerant to heavy metal concentrations (Syed *et al*., 2023). Based on present study, unidentified bacteria exhibited significant tolerance to both metals, with higher tolerance to lead compared to chromium.

The potential isolate of unidentified bacteria was subjected to identification process through sequence. This isolate was put through a 16srRNA sequence in order to identify the exact species since it may be an efficient strain of bacteria that promotes plant growth. Universal primers were used to amplify the 16s rRNA genes, and an automated gene sequencer was used to sequence the amplicons. Following the sequencing procedure, BLAST analysis was carried out using the 16srRNA bacterial sequence that was already present in the nucleotide databases. The homology of isolate was discovered by examination. Sequencing and BLAST results showed that the isolate had 100% sequence similarity to *Lysinibacillus macroides*.

Binita *et al*. (2018) investigated metal resistance and plant growth promoting properties in *Lysinibacillus* sp. Later, Ahsan and Shimizu (2021) reported similar characteristics in *Lysinibacillus* sp. However, to the best of my knowledge, this study represents the first documented evidence of resistance to lead and chromium in *Lysinibacillus* sp. additionally; it is noteworthy that the resistance exhibited by these isolates to lead was significantly higher which was consistent with the findings reported by Kamaruzzaman *et al*. (2020). The findings of this study thus imply that native bacteria in lead-chromium deposit soils have advantageous characteristics for the restoration of polluted soils.

In an attempt to assess *Lysinibacillus macrolides* antagonistic activity, the inhibition zone's diameter was measured as an indicator of its ability to reduce pathogen growth. This isolate exhibited notable antifungal activity against *Fusarium sp*. The first likely interaction between the isolate and the pathogen occurred 2-3 days after inoculation. This interaction resulted in a substantial suppression of *Fusarium sp* growth, with a percentage inhibition rate of 84%. Conversely, the PDA control plates showed rapid mycelial growth, while the treatment plates demonstrated a significant reduction in fungal growth.

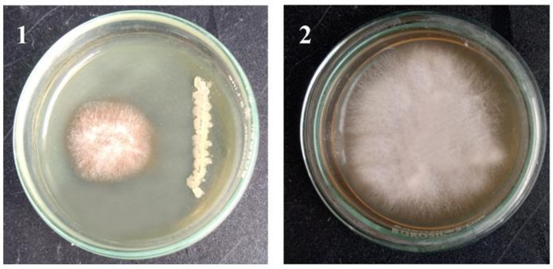

Fig. 3. 1: *Fusarium sp* treated with *Lysinibacillus macrolides,* **2:** Control without treated.

The bacterial isolate can suppress plant pathogens by a number of mechanisms, including competition for resources and space, production of bacteriocins, lytic enzymes, antibiotics, and siderophores. Using volatile organic molecules such alcohols, aldehydes, acids, ketones, unsaturated hydrocarbons, and aromatic hydrocarbons, these antagonistic bacteria rapidly breakdown pathogen cells (Che *et al*., 2017). Several prior studies against *Colletotrichum acutatum, Aspergillus niger, Fusarium moniliforme, Fusarium oxysporum*, and *Fusarium solani* looked at the antifungal potential of *Lysinibacillus* sp (Kamaruzzaman *et al*., 2020; Shabanamol *et al*., 2021; Passera *et al*., 2021). However, there is limited knowledge regarding the mechanisms by which the plant growth-promoting bacterium *Lysinibacillus macrolides* inhibits plant pathogenic fungus isolates.

CONCLUSIONS

PGP bacteria like *Lysinibacillus macrolides* offer a comprehensive solution for sustainable agriculture and environmental management. Their unique combination of PGP traits, metal tolerance, and pathogen control reduces reliance on chemicals, enhances crop health, and improves soil fertility. This study broadens our understanding of diverse PGP isolates with specific traits, including nitrogen fixation and enzyme production. Notably, the discovery of lead tolerance in *Lysinibacillus macrolides* has implications for polluted soil remediation. Additionally, their potent antifungal activity against Fusarium sp. underscores their role as a natural crop defense mechanism, reducing the need for synthetic pesticides. This PGP bacterium was hold great promise for sustainable agriculture and environmental improvement.

FUTURE SCOPE

Future research will concentrate on field inoculation development and formulation as well as bio safety analyses. The strains' responses to disease suppression in less-infested soils will be assessed in the greenhouse.

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How to cite this article: R.C. Senthamil Selvi and N. Thangaraj (2023). Isolation, Characterization, and Antagonistic Activity of Multifaceted Plant Growth-Promoting Bacteria, Including Metaltolerant *Lysinibacillus macroides,* From Vegetable-Cultivated Soils in Namakkal District, Tamil Nadu, India. *Biological Forum – An International Journal, 15*(10): 938-944.