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Characterization of phosphate solubilizing plant growth promoting rhizobacterium *Lysinibacillus pakistanensis* strain PCPSMR15 isolated from *Oryza sativa*

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ABSTRACT

The rhizosphere soil is a source for a diversity of microorganisms which play a vital role in the enhancement of plant health through the mechanism of symbiotic interaction thereby influencing the plant growth. The present study aimed at isolating potential phosphate solubilizing rhizobacteria from rice (*Oryza sativa*) crop for which, four different rhizosphere soil samples were collected from different locations of Tiruvallur district, India. Isolates were cultured on nutrient agar medium followed by serial dilutions and different colonies with morphological variations were isolated from each dilution. A total of 52 bacteria were isolated and maintained as pure cultures. Out of the 52 isolates, 16 strains showed phosphate solubilizing ability and amongst them, 4 were highly potential which were subjected to morphological and biochemical characterization. Phosphate solubilizing bacterial strains when assessed for their possible effect of their inoculation on the growth and development of mung bean seeds significantly enhanced the growth of the plants. Furthermore, the potential bacteria were analysed for Indole Acetic Acid (IAA) production, which was found to be directly proportional to the plant growth promotion. Upon the comparative analysis of the four potential isolates, PCPSMR15 exhibited remarkable plant growth promoting traits. A detailed biochemical and molecular analysis identified the promising strain PCPSMR15 as *Lysinibacillus pakistanensis*. The present study, thus signifies the strain, PCPSMR15 for exploration as an inoculant for improving soil fertility, enhancing phosphorus availability to plants and improved crop production and sustainability.

Abbreviations

IAA	Indole acetic acid
PCR	Polymerase chain reaction
PGPR	Plant growth promoting rhizobacteria
PSB	Phosphate solubilizing bacteria
PVK	Pikovskaya's agar medium

1. Introduction

Phosphorus (P) makes up about 0.2% - 0.8% of the plant dry weight, is the second major common limiting macronutrient after nitrogen that is required for plant growth and development as it is involved in the basic biological functions of the plant. The plants absorb phosphorous as phosphate anions from the soil which are extremely reactive and can be immobilized through precipitation with cations such as Fe^{3+}, Mg^{2+}, Al^{3+} and Ca^{2+} and this small proportional supply can cause deficiency of phosphorous (Schachtman et al., 1998; Kochian et al., 2004; Balemi and Negisho, 2012; Kalayu, 2019).

Over the years, several scientists have discovered the ability of certain microorganisms that aid in phosphate solubilization. The microorganisms in the rhizosphere mainly bacteria, have the ability to compose complex substances based on the plant exudates (Glick, 2012; Brahmaprakash et al., 2017). The bacteria colonizing the soil and those that are associated with the plant rhizosphere are called rhizobacteria. Most bacterial species affect the chemical properties of the soil and also have a beneficial effect upon the plant growth, hence are termed as plant growth promoting rhizobacteria (PGPR). Their influence can also be neutral or deleterious (Schroth and Hancock, 1981, 1982; Davison, 1988; Beneduzi et al., 2012).

Phosphate solubilizing bacteria (PSB) such as *Bacillus, Pseudomonas, Azospirillum, Rhizobium, Azotobacter, Agrobacterium* etc., convert

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insoluble inorganic phosphate compounds into soluble forms. Phosphate solubilization takes place through the process of acidification, chelation and exchange reactions leading to the production of organic and inorganic acids which in turn lower the pH of the soil causing increase in solubility and release of phosphorous (Khan et al., 2009; Bhattacharyya and Jha, 2012; Seshachala and Tallapragada, 2012; Alori et al., 2017).

Occurrence of PSB in the soil are ubiquitous in different forms and an elevated PSB population are seen in agricultural soils (Khan et al., 2009; Kalayu, 2019). Studies have reported that bacterial strains isolated from rice fields have the remarkable ability to solubilize phosphorus from insoluble phosphorus through production of organic acids and phytohormones that increases phosphate uptake and improves the growth of rice plants (Othman and Panhwar, 2014).

The present study was designed and conducted with the objectives to isolate, identify, characterize and analyse the diversity of culturable bacteria from the rice rhizosphere. Also, to establish the ability of the bacteria for phosphate solubilization, IAA production and for its plant growth promoting capabilities on mung bean.

2. Materials and methods

2.1. Collection of soil samples

Rhizosphere soil samples around the roots of rice (*Oryza sativa*) were collected from different rice fields in Minjur and the neighbouring places of Tiruvallur district, India. The collected samples were placed into sterile zip lock plastic bags individually, placed in a cool box and transported to the laboratory for further analysis.

2.2. Isolation of phosphate solubilizers

Bacterial colonies were isolated by serial dilution and spread plate methods. Approximately 2 g of the soil sample was dispersed in 9 mL of sterile distilled water and vortexed thoroughly. Subsequent serial dilutions of this stock were carried out to obtain dilutions of 10^{-8} and a 100 μL of each dilution was pour plated on to the nutrient agar medium. To isolate the phosphate solubilizing bacteria, the individual colonies were picked and inoculated on to Pikovskaya's agar medium (PVK) (Pikovskaya, 1948; Goenadi et al., 2000) containing insoluble tricalcium phosphate and incubated at 28 °C for 7 days for the bacterial growth. The presence of clear zone around the bacterial colony after incubation for 2 days was used as an indicator for positive phosphate solubilization (Husen, 2003). Pure cultures of PSB were spot inoculated on to PVK medium and incubated at 28 °C for 48–72 h for the detection of their phosphate solubilizing efficacy (Sharma et al., 2011). The phosphate solubilization index (SI) was calculated for all the potential phosphate solubilizers and the pure cultures were analysed for their colony morphology and following evaluations were carried out.

2.3. Characterization of phosphate solubilizing bacteria

The PSB isolates showing maximum phosphate solubilization were subjected to morphological and biochemical characterization according to Bergey's Manual of Systematic Bacteriology (Whitman et al., 2012).

2.4. Indole acetic acid (IAA) production

The production of IAA was evaluated by the Gordon and Weber (1951) colorimetric method with minor modifications. A 100 μL of phosphate solubilizing bacterial cultures were inoculated into tubes containing nutrient broth supplemented with tryptophan 5 mg/mL and incubated at 37 °C for 72 h. After incubation, the culture broth was centrifuged at 10,000 rpm at 10 °C for 10 min. The IAA production was determined by mixing 1 mL of supernatant and 2 mL of Salkowski reagent ($FeCl_3 \bullet 6H_2O$ - 2% and $HClO_4$ - 37%) and incubated in the dark for 30 min for the development of reddish pink coloured complex. The presence of IAA was visually observed by the reddish pink colour and quantified by measuring the absorbence at 530 nm in a UV-Spectrophotometer (Singh and Prasad, 2014; Rahman et al., 2010). All experiments were performed in triplicates and plain nutrient broth with tryptophan was used as control.

2.5. Pot experiment to evaluate growth promotion of Vigna radiata by PSB

Seeds of mung beans (*Vigna radiata*) were surface sterilized using 95% ethanol and 2.5% sodium hypochlorite followed by sterile distilled water wash for 3 times. The procedure of Shahab et al. (2009) was followed with minor modification for seed bacterization by soaking healthy seeds of similar size in bacterial culture (liquid culture) for 30 min. The seeds were then placed into Petri plates lined with soaked filter paper and incubated at 37 °C for 4 h. Post incubation period, 10 seeds were sown per cup containing 200 g of sterile soil at an equal depth of $1cm^2$. Equal volumes of sterile distilled water were used to water the treated plants daily whereas control (untreated seeds) was watered with distilled water and tap water. The root, shoot and leaf length were measured after 20 days using a centimetre scale.

Statistical analysis were performed using SPSS software (version 20). The results were considered to be significant at $P < 0.05$. Correlation coefficient was calculated for the data wherever necessary.

2.6. Biochemical and molecular characterization of PCPSMR15

Detailed biochemical analyses were carried out for the most potential isolate PCPSMR15 following Bergey's Manual of Systematic Bacteriology (Whitman et al., 2012). Genomic DNA was isolated from the bacteria using NucleoSpin® Tissue Kit as per manufacturer's instructions. The 16S rRNA was amplified using polymerase chain reaction (PCR) with the universal primers, forward primer 5′- AGAGTTTGATCCTGGCTCAG −3′ and reverse primer 5′- GGTTACCTTGTTACGACT −3′ (Weisburg et al., 1991). PCR reactions contained 10 mM of dNTPs, 5 pmol of each primer, 2 mM $MgCl_2$, 1X PCR buffer, 1.25 U of Taq DNA polymerase (Fermentas®) and 40 ng of genomic DNA (Thomas et al., 2018). Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) as per manufacturer's protocol. The 16S rRNA sequence was deposited in GenBank and the sequencing result obtained was analysed for nucleotide similarity and comparison with the sequences placed in NCBI database using the BLAST tool (Seshachala and Tallapragada, 2012). The phylogenetic tree was built using MEGA X (htpp://www.megasoftwere.net). The 16S rRNA gene sequence of strain PCPSMR15 was compared with the 16S rRNA gene sequences of closely associated species with validly published names. Phylogenetic tree was constructed using the neighbour-joining algorithm, and tree topology was evaluated by bootstrap analyses.

3. Results

3.1. Isolation of PSB and detection of phosphate solubilization index (SI)

A clear zone around the colony indicates phosphate solubilization after the incubation period at 28 °C for 48–72 h. These clear zones were formed as the PVK medium acts as a specific isolation medium for PSB due to the presence of calcium triphosphate which is known to be the factor for the halo zone formation. A total number of 52 isolates were obtained from the four different rhizosphere samples. Upon screening these 52 isolates, 16 isolates were possessing phosphate solubilizing potency of which, 4 strains were revealing remarkable phosphate solubilizing activity with the high colony-halo zone ratio, the SI (Table 1), of which isolate PCPSMR15 was the most promising at 24 mm which correspond to an SI of 4 (Fig. 1).

Table 1
Phosphate solubilizing efficacy of the four potential isolates.

Isolates	Zone of clearance (mm)	Colony diameter (mm)	Solubilization Index (SI)
PCPSKR37	10	6	2.7
PCPSMR7	11	9	2.2
PCPSSR53	18	12	2.5
PCPSMR15	24	8	4.0

Value shows the average of triplicates mean ± SD.

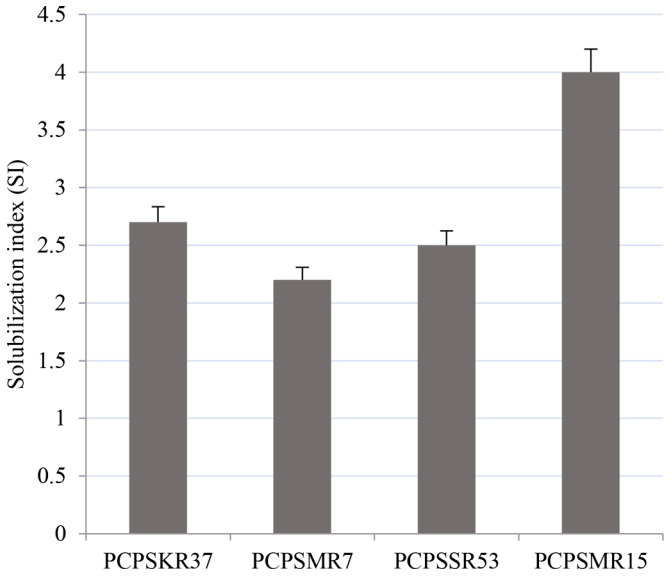

Fig. 1. Solubilization Index (SI) of the four isolates exhibiting remarkable phosphate solubilizing ability.

3.2. Characterization of the phosphate solubilizing isolates

The colony phenotypes of the four strains exhibiting phosphate solubilizing activity revealed clear variations (Table 2). Amongst these four isolates, three were Gram positive and one isolate was Gram negative bacteria. All the four isolates were producing cellulase and hydrogen cyanide, on contrast, only three were producing protease and gelatinase and two were producing catalase with only one strain producing amylase. All the four bacteria were exhibiting varied lipase activity based on the sources used and the overall results are tabulated in table 3.

3.3. Screening for IAA

The potential four isolates proved to have the ability to produce IAA. The concentration of IAA produced by the isolates ranged between 5.069 μg/ml - 10.571 μg/ml. It was found that isolate PCPSMR15 produced the highest concentration of IAA (10.571 μg/ml), followed by PCPSSR53 (8.899 μg/ml), PCPSMR7 (7.486 μg/ml) and PCPSKR37 (5.069 μg/ml) (Table 4).

Table 2
Morphological characterization of the isolates.

Morphological traits	PCPSKR37	PCPSMR7	PCPSSR53	PCPSMR15
Colour	Transparent white	Pale yellow	White	Pale yellow
Texture	Shiny	Shiny	Rough	Glossy
Margin	Entire	Undulate	Lobate	Undulate
Shape	Bacilli	Bacilli	Bacilli	Bacilli
Elevation	Convex	Convex	Convex	Convex
Size in diameter	0.5cm	0.3cm	0.5cm	0.3cm

Table 3
Biochemical characterization of the isolates.

Characterization Tests	PCPSKR37	PCPSMR7	PCPSSR53	PCPSMR15
Gram staining	+	+	–	+
Actinomycetes	+	–	–	–
Amylase	–	–	+	+
Catalase	–	–	–	+
Cellulase	+	+	+	+
Gelatinase	+	–	+	+
Hydrogen cyanide	+	+	+	+
Lipase-coconut	+	+	+	+
Lipase-groundnut	+	+	–	+
Lipase-sesame	+	–	–	–
Pectinase	+	+	+	+
Protease	+	+	+	+

+ indicates presence or positive; - indicates absence or negative.

Table 4
IAA production of the isolates.

Isolate code	IAA production (μg/ml)
PCPSKR37	5.069
PCPSMR7	7.486
PCPSSR53	8.899
PCPSMR15	10.571

3.4. Plant growth promotion of Vigna radiata by PSB

Enhancement of the growth of mung beans (*Vigna radiata*), in terms of length of root, shoot and leaf was observed when the seeds were treated with the four isolates viz. PCPSKR37, PCPSMR7, PCPSSR53 and PCPSMR15. Of these four isolates, PCPSMR15 induced the remarkable overall plant growth (Table 5). With regard to the length of root, shoot and leaf, plants inoculated with the strain PCPSMR15 ranked the highest and it was much significant than that of the uninoculated plants (control) (Fig. 2, 3).

3.5. Characterization of PCPSMR15

The most potential isolate PCPSMR15, when subjected to detailed biochemical analysis revealed that the isolate is a gram negative, facultatively anaerobic, rod shaped, motile bacterium and has a pale yellow colony colour. Interestingly, the isolate was able to grow in the presence of 10% (w/v) NaCl indicating it to be a NaCl tolerant strain. The bacterium was also growing between temperatures and pH ranging from 20 to 45 °C and 6–9. The PCPSMR15 exhibited amylase, catalase, cellulase, protease and urease activities with no evidence for citrate utilization and glucose fermentation (Table 6).

3.6. Molecular characterization of PCPSMR15

Nucleotide sequencing of PCR-amplified 16S rRNA gene (accession no: MK215192) when compared with the sequences available in the GenBank using the BLAST algorithm allowed the identification of the nearest neighbour sequence. Based on a sequence identity of 97%, the isolate was found to be affiliated to the genus *Lysinibacillus*. Thus, preliminary phylogenetic placements based on the results of the BLAST analysis revealed that the isolate clustered together to member of well

Table 5
Efficiency of PCPSMR15 on the growth of *Vigna radiata* seedlings.

Measurements (Length in cm)	Control (DH_2O)	Control (Tap water)	PCPSMR15 (Sterile water)
Root	1.3	2.8	3.1
Stem	7.1	13.7	20.8
Leaf	1.6	2.1	2.9

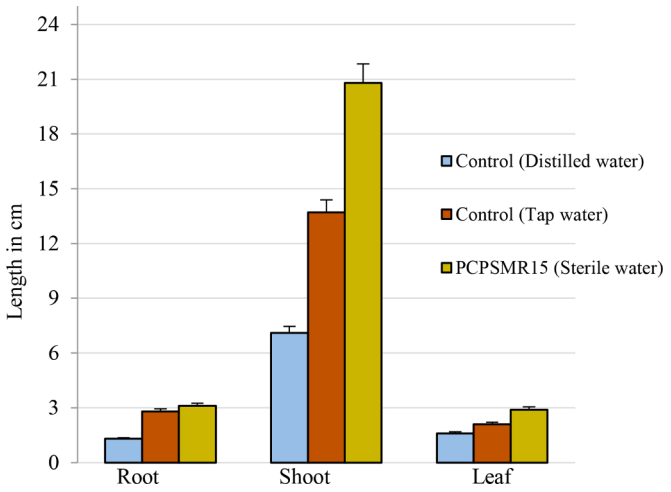

Fig. 2. Efficiency of PCPSMR15 treated and control conditions on the growth of mung beans.

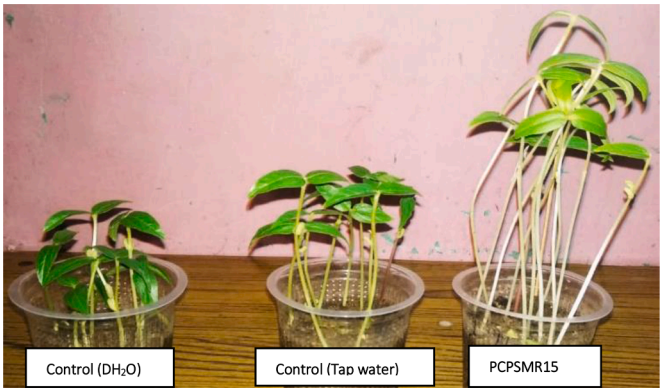

Fig. 3. Efficiency of PCPSMR15 on the growth of mung beans.

Table 6
Biochemical characterization of the isolate PCPSMR15.

S. No.	Biochemical test	PCPSMR15
1.	Growth condition	FA
2.	Indole production	–
3.	Methyl red	–
4.	Voges-Proskauer	–
5.	Oxidase	–
6.	Catalase	+
7.	Starch hydrolysis	–
8.	Protease	+
9.	Citrate utilization	–
10.	Urease	+
11.	TSI: H_2S	–
	TSI: Gas	–
	TSI: K/K	A/A
12.	Glucose Fermentation	–
13.	Sucrose Fermentation	–
14.	Growth temperature	20–45 °C
15.	Growth pH	6–9
16.	Growth NaCl	0–10%
17.	Gram staining	+ve

FA: Facultative anaerobic; TSI: Triple Sugar Iron; K/K: Alkaline/Alkaline; (+) indicates presence or positive; (-) indicates absence or negative.

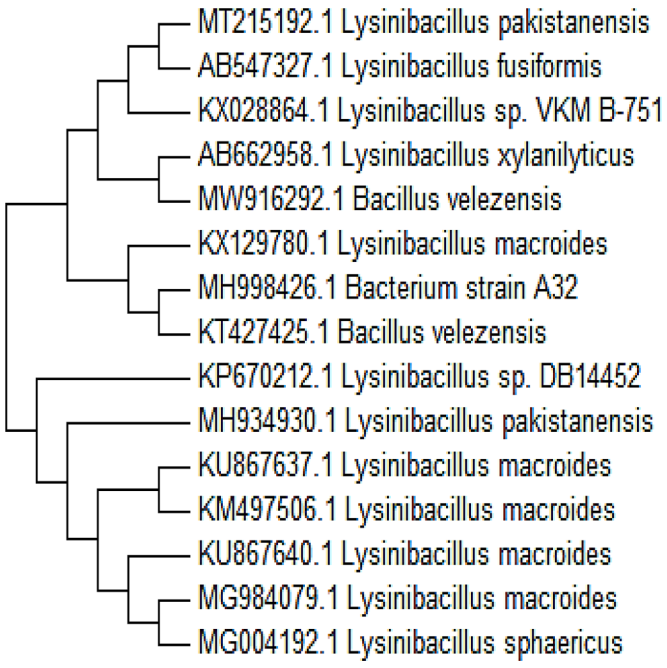

Fig. 4. Phylogenetic tree of the isolate PCPSMR15. Phylogenetic tree construction by neighbour joining method revealing the evolutionary relationship of *L. pakistanensis* PCPSMR15 (MT215192) with closely related species.

characterized and previously named bacterial species. Hence, the isolate PCPSMR15 was identified as *Lysinibacillus pakistanensis* (Fig. 4).

4. Discussion

There is a continuous requirement to establish an eco-friendly sustainable agriculture which is possible by the symbiotic interactions of plants and PGPR. A greater concentration of bacteria is found around the plant roots due to the nutrients from the plant root exudates (Glick, 2012). On an average, when constituting a portion of the plant dry weight, the Indian soils contain much lesser phosphate due to which PSB are required to solubilise insoluble phosphates and make it available to the plants (Seshachala and Tallapragada, 2012). Strains from the genera *Pseudomonas, Bacillus* and *Rhizobium* are amongst the most powerful phosphate solubilizers (Rodríguez and Fraga, 1999). In the present study, four potential rice rhizobacterial isolates exhibited phosphate solubilizing efficacy. There were variations in SI of the isolates and isolate PCPSMR15 was revealing the highest phosphate solubilizing ability by showing maximum zone of clearance of 24 mm with a phosphate solubilization index of 4. In addition to phosphate solubilizing activity, all the four isolates produced considerable amount of IAA, a plant growth inducing trait.

Phosphate solubilizing bacteria when used as inoculants simultaneously increases P uptake by the plant and crop yield. All the four isolates when studied for their growth promoting ability on *Vigna radiata*, all of them enhanced root and shoot length with remarkable increase in the size of the leaves when compared to the control. PCPSMR15 was the most promising amongst the four with significant enhancement of the plant growth promotion ability and IAA production. Previous reports show the treatment of *Vigna radiata* with phosphate solubilizing bacteria such as *Acinetobacter, Bacillus, Burkholderia, Lysinibacillus, Pseudomonas, Pantoea*, in increasing the yield and quality parameters of the seeds/plant (Walpola and Yoon, 2013; Hassan et al., 2017; Kumar et al., 2017; Tomer et al., 2017; Kumari et al., 2018; Thomas et al., 2018). Physiological characterization of the isolate in terms of pH and temperature indicated its environmental adaptability and the ability of the isolate to grow even in 10% (w/v) NaCl confirms

the halo tolerant nature of the bacterium. In addition to phosphate solubilizing ability and IAA production, PCPSMR15 was also possessing protease, urease, catalase, cellulase, amylase, gelatinase and lipase enzymes activities. Protease, one amongst the cell wall degrading enzyme is considered as a potential antifungal agent (Ji et al., 2020). There are many reports on the abovesaid enzymes which are known to play copious roles in pharmaceutical, food, textile, chemical industries, alcoholic beverages and also in waste water treatment (Hankin and Anagnostakis, 1975; Andro et al., 1984; Mahajan and Badgujar, 2010; Zhang et al., 2010; Balan et al., 2012; Sujoy and Aparna, 2013). Hence, this isolate is a prospective candidate for exploration for further quantitative research on these enzymes for its biotechnological applications.

The 16SrRNA sequence of the isolate PCPSMR15 was deposited in GenBank sequence data library under the accession number MK215192 (Samundeeshwari et al., 2020). Molecular characterization and phylogenetic analysis revealed the identity of PCPSMR15 as *Lysinibacillus pakistanensis* sharing 97% sequence similarity to *L. pakistanensis* (Hayat et al., 2013; Ahmed et al., 2014).

This is the first report to show the phosphate solubilizing efficacy of L. *pakistanensis* isolated from the rice rhizosphere of Tiruvallur district, India. The current study demonstrated that the strain PCPSMR15 has a great prospect to be developed as a biofertilizer. Hence, this strain can be used as a potential inoculant to improve soil fertility and plant growth promotion after assessing its ability in field trails.

5. Conclusion

This is the first report to reveal the potency of L. *pakistanensis* PCPSMR15, a rice rhizobacterium for phosphate solubilization and plant growth promotion. In general, PGPR are known for its significant role in improving plant growth, health and crop yield. Hence, we suggest the usage of this PGPR in the preparation of biofertilizers which can be considered as a replacement to chemical fertilizers. PCPSMR15 exhibiting its halo tolerant trait and the ability to grow in broad range of temperatures and pH indicates its environment adaptability. The isolate possessing various enzyme activities, which are known for its biotechnological and industrial applications recommends this promising isolate for subsequent research. We strongly conclude that this potential bacterium, L. *pakistanensis*, has eventual commercial applications after further research.

Author contribution

Srilakshmi Lelapalli and Samundeeshwari Baskar have equal contribution in carrying out this research work

Sharon Maria Jacob contribution was towards the writing and editing the manuscript

Sripriya Paranthaman is responsible for Conceptualization, Investigation, Project administration and Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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