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Report On Isolation Of Plant Growth Promoting Bacteria (PGPB) From The Gut Of Lesser Horseshoe Bat Collected From The North-Eastern Part Of India.

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Abstract

Current trend in agricultural practices are focused on searching for an alternative sustainable and environmentally friendly fertilizers. One of these alternatives is the use of microbial inoculants known as plant growth promoting bacteria (PGPB) in conventional farming. The aim of the study is to evaluate the effect of bacteria isolated from the gut of Lesser Horseshoe bat and characterizing them as a potential PGPB. Qualitative screening assays like Phosphate solubilization, production of Hydrogen cyanide, Ammonia, Indole acetic acid, and Siderophores were performed to determine for PGPB traits of the isolates. Hydrolytic enzymes assay like cellulase, protease, and amylase which are known to play a vital role in plant growth were also performed. Another important criterion of PGPB is to exhibit antifungal activity against common soil pathogen (*Fusarium oxysporum*). Of the 35 samples isolated from the gut of lesser horseshoe bat which was tested for their PGPB property, CSL (*Pseudomonas aeruginosa* data under publication) showed promising results in all the assays performed. Hence, seed germination test was further carried out only for CSL isolate alone. We conclude that bacterial isolate (CSL) when used as inoculants (PGPB), can contribute to alternative environmental friendly fertilizers thereby minimizing environmental pollutants.

Keywords: *Plant growth promoting bacteria, Microbial communities, Lesser Horseshoe Bat, Agriculture, and Germination*.

1. Introduction

With an increasing population which is predicted to escalate up to 8 billion by 2025 and 9 billion by 2050 (Vejan et al., 2016), there is a necessity to regulate the increasing demand for food security. However, to cope up with an increase in food demand, the use of large-scale chemical fertilizers and pesticides has become an essential component in modern agricultural practice (Adesemoye et al., 2009). This overuse of chemical fertilizers and pesticides can in-turn cause unanticipated environmental pollutions with potential risk to human health. Therefore, moving towards a sustainable agricultural vision, it is imperative that we find an alternative to minimize the use of chemical fertilizers and pesticides as to positively affect the overall soil fertility, disease resistant and ultimately be used efficiently in conventional farming. Throughout evolution, plants have developed adaptive mechanisms related to interactions with microorganisms (Rosenberg Z and Rosenberg E., 2008) Knowledge of microbiota composition in wildlife is limited especially in bats. Being the second most diverse group of mammals in the world, bats not only play a major role in various ecological services such as pollination, seed dispersion, and insect-pest control but are

known carriers of various zoonoses (Calisher et al., 2006). To date, there are only a handful of records on microbiota from bats with originating from fecal matter or different parts of the gastrointestinal tract, namely the stomach, intestine, and rectum (Herd et al., 1997, Anand and Sripathi, 2004, Whitakker et al., 2004, Gloriana 2006, Muhldorfer 2010, Apun at al., 2011). Thus, establishing a link between microbial ecological and gut microbiota is necessary for determining the role of this microbiota in agricultural practice.

Microbial communities associated with crops growing under diverse biotic and abiotic stress condition have received much attention in recent years. It is widely known that plant-roots react to different environmental conditions through secretion of a wide range of compounds that can interfere with the plant-bacteria interaction. Literature survey suggested that these microbial communities promote agricultural production via the synthesis or production of plant hormones such as indole-3-acetic acid (IAA), gibberellic acid, cytokines, ethylene and auxins which directly impact the plant growth by stimulating uptake of nutrients (Jha and Saraf, 2012, Vessey 2003, Costacurta and Vanderleyden, 1995). They also promote associative nitrogen fixation (Richardson et al., 2009), production of siderophores (Lemanceau et al., 2009), hydrolytic enzymes such as glucanases, chitinases, proteases, cellulases, amylases (Bashan and de-Bashan, 2005) and hydrocyanic acid (HCN) (Voisard et al., 1989). All these features classify them as plant growth-promoting bacteria (PGPBs). These PGPBs are known to accelerate seed germination, improve seedling emergence, protect plants from disease and promote root growth (Lugtenberg and Kamilova, 2009). PGPB can also act as a bio-control of pathogenic fungi through the production of antibiotics, competition for nutrients or by the induction of systemic resistance (Glick, 2012). Moreover, microbial isolate can exploit, translocate, mineralize and mobilize soil phosphate, potassium, Fe reserves, increase organic matter or fix Nitrogen from the atmosphere (Owen at al., 2015, Leifheit et al., 2014, Ahemad and Kibret, 2014) thereby improving soil fertility.

Thus, the aim of this study is to isolate bacterial communities from the gut of lesser horseshoe bat and evaluate them as potential PGPB, followed by screening for antifungal activity against *Fusarium oxysporum*.

2. Materials and Methods

2.1 Chemical

All bacteriological media used in this study were purchased from Hi-media laboratories and chemicals of analytical grade were obtained from Sigma-Aldrich. Co., St.Louis, USA, Himedia laboratories and Sisco Research Laboratory, Mumbai, India.

2.2 Sample site and collection

The Arwah Cave (25.2717'N, 91°7308'E) is located at Cherrapunji, Meghalaya and was a habitat of many insects and bat species. Geologically, Khasi Hills is located at the northeastern extension of the Indian Peninsular Shield (Ghosh et al., 2005). Sampling sites were chosen on the basis of available information of bat roosts and foraging sites. The bats were captured using mist nets, set up at various locations of their flying routes within the cave. The trapped bats were untangled from the nets and kept in cages for easy transportation to the laboratory.

2.3 Enumeration of bacteria

Gut dissection is performed as per (Nordgard et al., 2005) with few modifications. Briefly, 1 gm of the sample from each individual was finely ground in a sterile motor and pestle using isotonic saline (0.9%) followed by suspending the aliquot in 1 ml of sterile distilled water and mixed thoroughly followed by serial dilution of the samples up to 10^9 and plated onto different bacteriological media (Himedia) and incubated overnight at 37˚C for 24 to 48 hours. Colonies with different morphology were sub-cultured into the pure culture by inoculating in freshly prepared nutrient agar plates.

2.3 Production of Indole acetic acid (IAA)

IAA was performed as per Hariprasad et al., 2014. Bacterial isolates were grown at 37°C for 72 hours in Luria Bertani broth (LB) with (100 µg ml-1, L tryptophan supplemented) and centrifuged at 3000 RPM for 30 min. 1 ml of the supernatant was mixed with 4 ml of the Salkowski reagent (50 ml of 35% of Perchloric acid plus 1 ml of 0.5 M FeCl3). The intensity of the color was measured at 530 nm and a standard curve was plotted using standard IAA.

2.4 Phosphate solubilization

The screening method for the detection of phosphate solubilization was done as per Singh et

al., 2017 with few modifications. 35 isolates were tested for their tri-calcium phosphate (TCP) solubilizing activity on Pikovskaya Agar (PVK) plates. The isolates were spot inoculated onto the agar plate aseptically and were incubated at 30±2°C for 5-days. A clear zone around the colony indicates the phosphate solubilization activity.

2.5 Hydrogen cyanide (HCN) production

Screening of bacterial isolates for HCN production was done as per Castric et al., 1975 with some modification. Bacterial cultures were streaked onto nutrient agar medium containing 4.4 g/L of glycine. A Whatman filter paper No.1 was soaked in 0.5% picric acid solution mixed with 2% sodium carbonate and placed inside the lid of a plate. Plates were then sealed with parafilm and incubated at 30±0.1°C for 4 days. Development of light brown to dark brown color indicates HCN production.

2.6 Ammonia production

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated at 30°C for 72 hours. Nessler's reagent (0.5 ml) was added in each tube. Development of yellow to brown color is tested positive for ammonia production (Cappuccino and Sherman, 1992).

2.7 Siderophore production

Siderophores are important for the survival and growth of bacteria in the soil and aqueous environment. Siderophore production was tested qualitatively using chrome azurole S (CAS) agar medium. An orange halo around the colonies after 48 hours incubation indicates the production of siderophore. The CAS assay (Schwyn and Neilands, 1987) is a universal chemical assay for siderophore detection and it is based on siderophores high affinity for the ferric ion. When siderophore is present the following reaction releases the free dye which is orange in color.

Fe3+ - dye (blue) + Siderophore → Fe3+ - Siderophore + dye (orange).

2.8 Antifungal activity by dual culture method

Antagonistic effect of isolates was evaluated by a dual-culture assay using the relative growth of *F. oxysporum* (Khan et al., 2001). A 5 mm mycelial mat of soil-borne fungi, *F. oxysporum* was placed on one side of a potato dextrose agar (PDA, Himedia), and each bacterial isolate was streaked on the other side of the medium. The PDA plate was then cultured at 28°C for 7 to 14 days. Other plates were inoculated with same size plug of *F. oxysporum* colony in the absence of test strain as the control. All treatments were performed in triplicates and the plates were incubated at 30°C for 5 days. During the cultivation, antagonistic effects of the bacterial isolates against the fungal isolates were confirmed by inhibition zones formed between the bacterial isolates and fungal isolate. The formula for calculating the zone of inhibition in dual culture assay was followed as per Skidmore and Dickinson, 1976 .

Inhibition of mycelial growth % = R1-R2/R1×100

R1 = Radial growth of fungus in control.

R2 = Radial growth of fungus in dual culture.

2.9 Screening of hydrolytic enzymes from bacterial isolates

2.9.1 Amylase activity

The bacterial isolates were subject to screening for amylase activity as per Collins et al., 1995. The isolates were spot inoculated onto starch agar medium and incubated at 30°C for 48 hours. The plates were then flooded with iodine solution and kept for a minute which was later poured off the plates. We then observe for the appearance of zones around the colonies.

2.9.2 Cellulase activity

Cellulase production was determined using the method as per Wood et al., 1988 with some modifications. 35 isolates were inoculated into the medium containing carboxymethylcellulose (CMC) and incubated for 2 days at 37°C. After cell growth, the presence of extracellular cellulase was detected by flooding the plates with 0.3% Congo red solution for 15 min and plates were stained with 0.1% NaCl for 15 min. The plates were then visualized for halo zone for positive cellulase production.

2.9.3 Protease activity

Protease activity was determined as per Strauss et al., 2001. Culture medium composed of 1% skim milk, 5 g/L peptone, 3 g/L yeast extract, 10 g/L glucose, 20 g/L agar and pH is adjusted to 5.0. After 3 days of growth at 30°C, isolates producing a transparent halo around the colonies indicates positive protease activity.

2.9.4 Seed germination

Seeds of Peas (*Pisum sativum*) were surface sterilized with 1% sodium hypochlorite for 2 minutes followed by repeated washing with sterile distilled water. The experiment was conducted in four groups. a) seeds soaked in CSL broth culture along with CMC, b) seeds soaked in CSL culture, c) seeds soaked in only CMC as control and d) seeds without any treatment were kept as control. Three seeds of each treatment were kept equidistance in sterilized petri plates containing moist filter paper and the petri plates were incubated at 30°C. Seed germination and present seedling emergence were calculated using the following formula (Mia et al., 2012).

$$\% \text{ Emergence} = \frac{\text{Number of seedlings}}{\text{Number of seed sown}} \times 100$$

2.9.5 Data analysis

Data are expressed as mean standard deviation (SD) for all the parameter. All data were analyzed with SPSS for Windows statistical package (version 20.0, SPSS Institute Inc., Cary, North Carolina. Statistical significance between the different groups was determined by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests and the significance level was fixed at $p<0.05$.

3. Result and Discussion

Microbial communities play a pivotal role in the functioning of plants by influencing their physiology and development. PGPB occupy the rhizosphere of many plant species and have beneficial effects on the host plant. They may influence the plant in a direct or indirect manner. A direct mechanism would be via increasing plant growth by supplying the plant with nutrients and hormones. Indirect mechanisms, on the other hand, include reduced susceptibility to diseases and activating a form of defense referred to as induced systematic resistance. The use of PGPB occupies a small but growing niche in organic agriculture and it is reasonable to expect increased use of PGPB. PGPB based inoculation method can be considered an important strategy for sustainable management and for reducing environmental problems by decreasing the use of chemical fertilizers (Hungria et al., 2010, Hungria et al., 2013). Of all the 35 isolates, CSL was found to have its effect on both direct and indirect mechanism, signifying a potent PGPB property. Hence, seed germination test is further performed to justify that CSL being a non-pathogenic strain can be used as PGPB. The findings are briefly summarized in Table 1 and further discuss below.

3.1 Qualitative and quantitative production of Indole acetic acid (IAA)

IAA is a plant growth hormone produced by a large number of microbial species and also serves as a signaling molecule in several plant-microorganism interactions (Lin et al., 2012). Most microorganisms need tryptophan to produce IAA, given that four of the five routes for the synthesis of auxin are tryptophan-dependent (Hassan and Bano 2015). Qualitative analysis (Table 2) showed that out of 35 isolates tested for the production of IAA, 33 isolates showed changes in color on the addition of Salkowski's reagent which correlates to about 94.28% IAA production (Table 1). Quantitative analysis also showed no IAA production only in 2 isolates, whereas 33 isolates showed results ranging from 0.8µg/ml to 89.19µg/ml with CSL showing maximum concentration plotted using standard IAA curve $Y = 0.0093x + 0.0992$, $R2= 0.9797$.

3.2 Phosphate solubilization

The function of phosphate in a cell cannot be performed by any other nutrient and adequate supply of phosphate is required for survival and reproduction of plants. Plants can absorb phosphate only in a soluble form and transformation of insoluble phosphate into soluble form is needed to be carried out by a number of microbes present in the soil (Musin et al., 2014). 35 isolates were tested for phosphate solubilization on PVK agar plate containing insoluble tricalcium phosphate (TCP). Only CSL showed a clear halo around the colony as depicted in Fig 1 & Table 2. The ability of CSL to solubilize phosphate clearly indicates its ability to produce phosphate required for plants survival.

3.3 Hydrogen cyanide (HCN) production

HCN also promote plant growth and some Pseudomonas spp. colonize competitor niches produce iron-chelating, antibiotic compounds and excrete volatiles which induces plant systemic resistance (Santoyo et al., 2012). However, (Pal et al., 2000) reported that HCN was an unlikely a biocontrol agent and that bacterial product, like pigments and antibiotics, were much more effective against fungal pathogens. Later, Rijavec and Lapanje., 2016 reported that HCN is involved in sequestration of metals and the consequential indirect increase of nutrient availability, which is beneficial for the rhizobacteria and their plant

hosts. In both cases the production of HCN is essential and our result also showed positive HCN production for 2 bacterial isolates (CSL and BA1) depicted in Table 2.

3.4 Ammonia production

Another important PGPB mechanism that indirectly influences plant growth is ammonia production. 14 isolates showed positive results shown in Table 2. Similar results were observed in a study conducted by Cherif-Silini et al., 2016 where ammonia production was observed in strains isolated from the wheat rhizosphere.

3.5 Assay of siderophore production

Iron (Fe) is an essential micronutrient for plants and microorganisms, as it is involved in various important biological processes such as photosynthesis, respiration and chlorophyll biosynthesis (Kobayashi and Nishizawa, 2012). Microorganisms have developed active strategies for Fe uptake. Bacteria can overcome the nutritional Fe limitation by using chelator agents called siderophores (Desouza et al., 2015). Siderophore production has been reported to be beneficial in growth promotion and disease suppression in plants. A total of 35 isolates from the gut of bat were screened on CAS agar plates (qualitative estimation) for siderophore production. Only CSL and BA1 (Table 2) of the 35 isolates showed a clear light orange zone surrounding the colony and the diameter of the zone depicted the amount of siderophore excreted by the isolates (Fig. 2).

3.6 Dual culture

A dual culture test is extensively used as a reliable In-vitro test for preliminary screening of biological control agents (Desai et al., 2012). According to the previous reports, dual culture tests have shown that Bacillus isolates from livestock manure and cotton-waste composts have antagonistic effects against the isolates of soil-borne fungi, *F. oxysporum*, *P. capsici*, *R. solani* and *S. sclerotiorum* (Kim et al., 2008). Similarly, CSL, when tested for antagonistic effects against a soil pathogenic fungi (*F. oxysporum*), showed the inhibition zone of 59.69%, (Fig. 3) displaying its ability to reduced susceptibility against common phytopathogens.

3.7 Qualitative analysis of hydrolytic enzymes

Some Actinomycetes are known to produce hydrolytic enzymes such as protease, cellulase, and amylase (Stanford et al., 2002, Mukherjee and Sen et al., 2004, Ramesh and Mathivanan 2009). These enzymes help in preventing crops from phytopathogens and deleterious microbes by degrading their cell walls. These microorganisms play an important role in the promotion of plant growth by decomposing organic matter and nutrient mineralization. Out of the 35 isolates screened for hydrolytic enzymes Table 3. 7 isolates showed positive with the highest zone diameter of 2.76±0.25 attained by CSL for cellulase enzyme. In addition, CSL again showed the highest zone diameter of 1.76±0.25 of the 5 isolates tested for amylase activity. For protease activity, a total of 11 isolates were positive with the highest zone diameter of 2.866±0.15 depicted by BA1.

From the above result, it can be concluded that CSL, identified as *Pseudomonas aeruginosa* has a creditable trait as PGPB when compared to other 35 isolates. Keeping this into account we have selected CSL as a potential PGPB and further tested for trials on seed germination of *Pisum sativum*.

3.8 Seed germination

Using CSL as PGPB our result showed that there is a significant increase in seedling length when compared to Control and CMC + Control. In line with these finding, it is obvious that CSL treated group showed better growth in seedling length. However, we could also observe a significant increase in seedling length in CSL+ CMC when compared to CSL treated alone. This can be justified as CMC could have increased the adhesive property of CSL to the seeds, furthermore enhancing the PGPB property of CSL isolate. Result for seed germination test is illustrated in Fig. 4.

4. Conclusion

This study focussed on isolating the bacterial communities from the gut of Lesser Horseshoe bat which dwells inside the caves of Meghalaya. It is known that guano harbor beneficial microbes and have been efficiently used as a bio-fertilizer for plant growth in many studies. In line with this, we suggest that the bat gut may also harbor these beneficial microbes. Hence, in this study, we focussed on screening bacterial isolates and understanding the animal-plant-microbe interactions towards developing efficient PGPB

inoculants. Thus, we can conclude that the bacterial isolate (CSL), when used as inoculant, showed credible PGPB properties and this can contribute to alternative environmental friendly biofertilizers thereby minimizing environmental pollutants.

Conflict Of interests

The author declares no conflict of interest.

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Ethics statement

Due to ethical considerations and permit limitations, three individuals were collected. Ethical clearance was obtained from the Chief Conservator of Forest of the state of Meghalaya States, (No: 23(4)/ (MEMO.NO.FWC/G/173/PT-III/2270/74). In total, 3 intestinal biopsy samples were used for this study and it was conducted under proper biological safety conditions.

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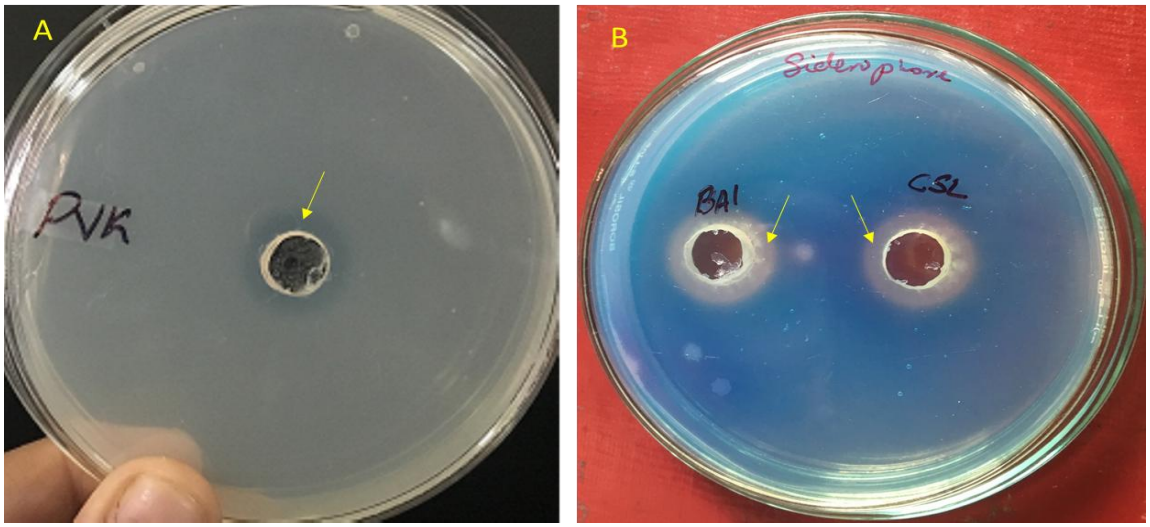
Figures and Tables

Figure 1: Phosphate solubilisation and Siderophores production by CSL isolate.



A: Yellow arrow represent halo zone indicating the ability of the isolate to solubilise phosphate & **B**: Yellow arrow represent a light orange halo around the colony indicating the ability of the isolate to produce siderophores.

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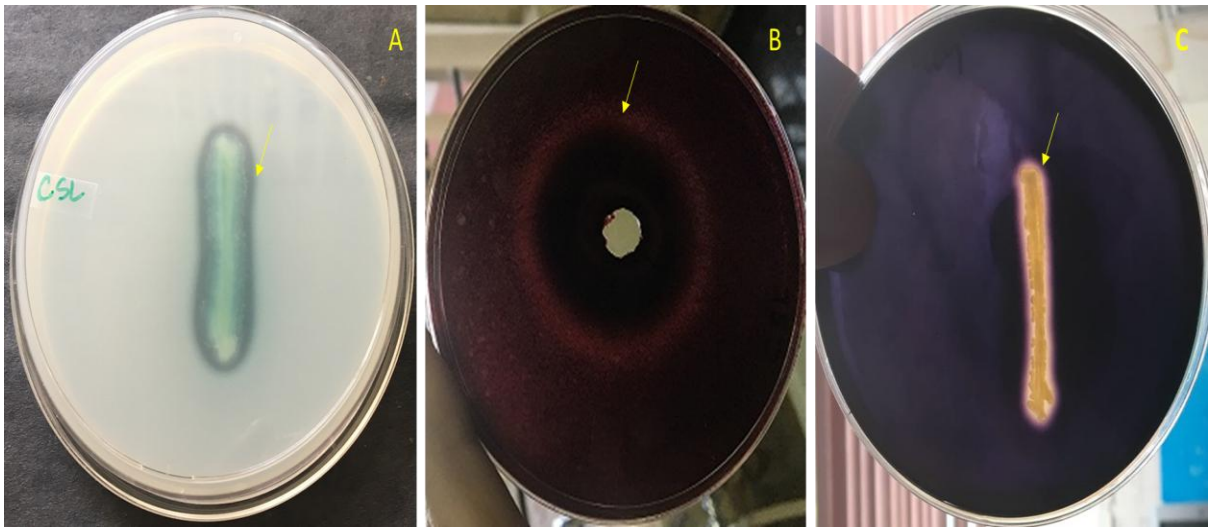
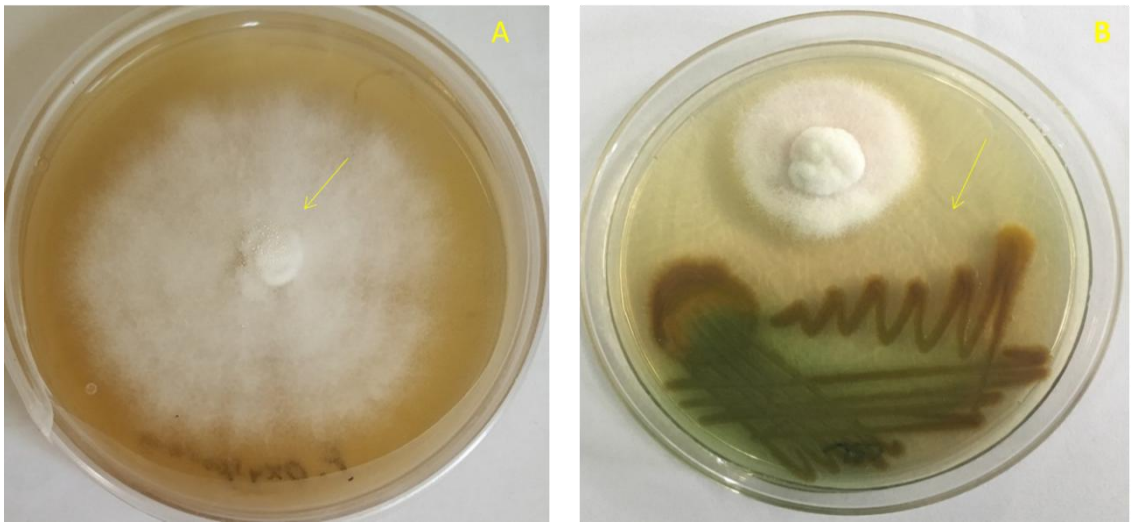
Figure 2: Hydrolytic enzymes production by CSL isolate.



A, B & C: Yellow arrow represent a clear halo zone indicating the ability of CSL to produce hydrolytic enzymes (Protease, Amylase & Cellulase).

Figure 3: Dual culture test using CSL against *F. oxysporum*.



A: Yellow arrow represent control growth of *F. oxysporum* & **B**: Dual culture showing ability of CSL to inhibit the growth of *F. oxysporum***.**

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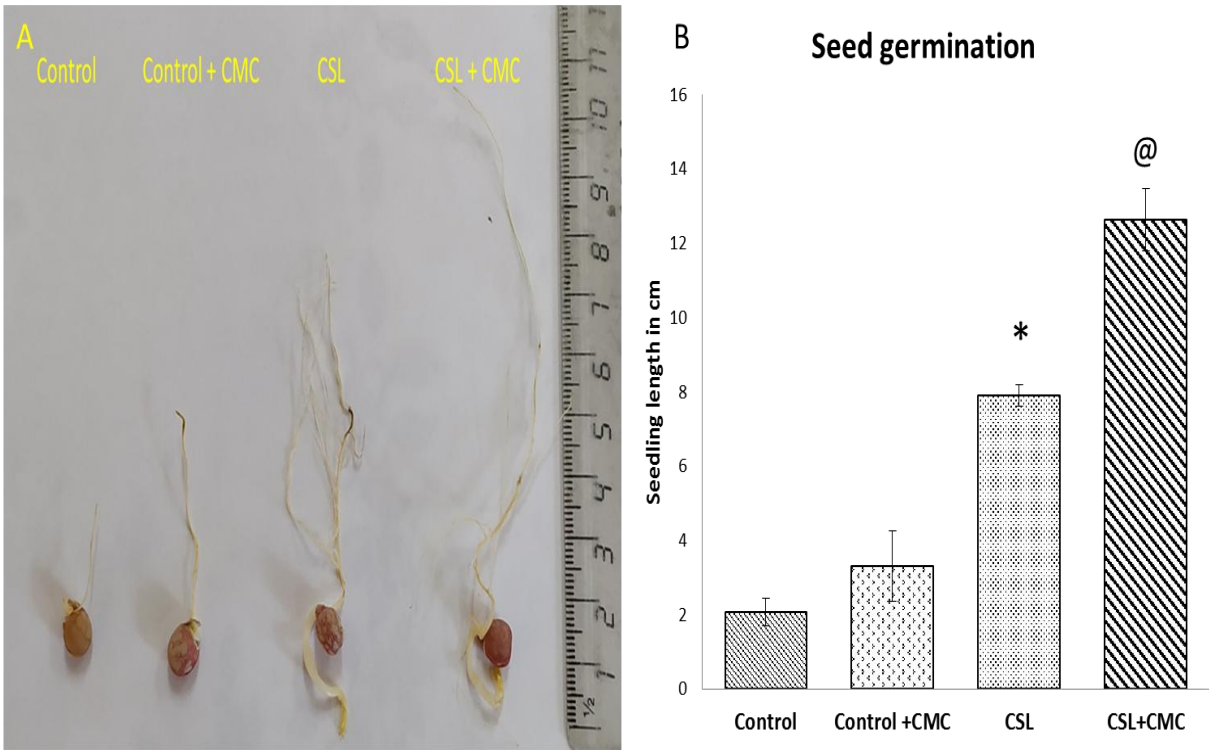
Figure 4: Seed germination using CSL as Plant growth promoting bacteria (PGPB).



Values are expressed as Mean ± SD, N=3. '*' CSL compared with control '@'CSL+CMC compared with; CSL Significance at $p < 0.05$. CMC: Carboxymethyl cellulose.

Table 1: Number and % of isolate producing Plant Growth Promoting Bacteria (PGPB).

Source Bat Gut	Total	IAA	Phosphate	Ammonia	HCN	Siderophore	Cellulase	Amylase	Protease
No. of bacterial isolates	35	33	1	14	2	2	7	5	11
Percentage obtained	%	94.28	2.85	40	5.71	5.71	20	14.28	31.42

Table 2: Qualitative analysis of Plant growth promoting factors from gut microbial isolates.

Sl.no	Indole acetic acid production	Ammonia production	Phosphate solubilisation	Siderophore production	Hydrogen cyanide production
MGSL01	+	-	-	-	-
MGSL02	+	-	-	-	-
MGSL03	+	-	-	-	-
MGSL04	+	-	-	-	-
MGSL05	+	-	-	-	-
MGSL06	+	-	-	-	-
MGSL08	+	+	-	-	+
MGSL11	+	-	-	-	-
BA1	+	+	-	+	-

BA4	+	+	-	-	-
BA5	+	+	-	-	-
BA6	+	+	-	-	-
BA7	+	+	-	-	-
BA8	+	+	-	-	-
JS23	+	+	-	-	-
M5	+	+	-	-	-
SS1	+	-	-	-	-
SS3	-	-	-	-	-
SS5	+	-	-	-	-
SS6	+	+	-	-	-
SS7	+	-	-	-	-
SS9	-	+	-	-	-
SS10	+	-	-	-	-
SS13	+	-	-	-	-
SS15	+	-	-	-	-
SG03	+	+	-	-	-
SG02	+	+	-	-	-
SG06	+	-	-	-	-
SG09	+	-	-	-	-
SG11	+	-	-	-	-
SG14	+	-	-	-	-
OR	+	-	-	-	-
UK2	+	-	-	-	-
SL	+	-	-	-	-
CSL	+	+	+	+	+

Table 3: Hydrolytic enzyme assays of gut microbial isolates.

Isolates	Protease activity (cm)	Amylase activity (cm)	Cellulase activity (cm)
MGSL01	-	0.7±0.173	-
MGSL02	-	-	-
MGSL03	2.7±0.360	-	-
MGSL04	-	-	-
MGSL05	-	-	-
MGSL06			-
MGSL08	-	1.533±0.152	-
MGSL11	-	-	-
BA1	2.866±0.15	-	-
BA4	-	-	-
BA5	-	-	-
BA6	-	-	-
BA7	-	-	-
BA8	-	-	-
SS1	-	-	2.43±0.208
SS2	-	-	2.2±0.2
SS3	-	-	-
SS5	-	-	2.26±0.11
SS6	-	-	2.23±0.1
SS7	-	-	1.33±0.115
SS9	-	-	-
SS10	1.56±0.11	-	-
SS13	-	-	-

SS15	1.2±0.2	-	-
SG3	1.533±0.15	-	-
SG2	2.2±0.2	-	-
SG6	-	-	-
SG9	1.6±0.173	0.66±0.152	-
SG13	1.23±0.25	-	-
SG14	-	-	1.23±0.115
SG11	-	-	-
UK2	2.23±0.25	-	-
OR	0.56±0.115	0.633±0.23	-
SL	-	-	-
CSL	2.7±0.17	1.76±0.25	2.76±0.25
M5	-	-	-
JS27	-	-	-

Table 4: Seed germination of *Pisum sativum* using CSL as Plant Growth Promoting Bacteria.

Treatment group	Seedling length (cm)	Germination percentage (%)	Vigor index
Control	2.066±0.378	100.0	206.6
Control + CMC	3.300±0.953	100.0	330.0
CSL	7.900±0.300*	100.0	790.0
CSL+CMC	12.633±0.850@	100.0	1263.3

Values are expressed as Mean ± SD, N=3. '*' - CSL compared with control '@' - CSL+CMC compared with; CSL Significance at $p < 0.05$.
CMC: Carboxymethyl cellulose