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Research Article

ISOLATION AND CHARACTERIZATION OF MULTI-TRAIT *ENTEROBACTER HORMAECHEI*

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Abstract: The multi-trait *Enterobacter hormaechei* strain was isolated from Jeevamrut (an organic manure prepared from cow dung, cow urine, jaggery and gram flour) prepared at Indore Biotech Inputs and Research Pvt. Ltd. The strain was identified using 16s RNA sequencing and was found to have the ability to solubilize minerals such as phosphate, potassium and zinc, calcite solublization, and oxidize sulphur *in vitro*. The isolate also produces Indole Acetic Acid, and siderophores. Furthermore, the *Enterobacter* strain reported here can retard the growth of *Fusarium oxysporium* significantly *in vitro*. All these properties make this strain a strong candidate for use as a bio inoculant.

Keywords: *Phosphate solubilizing bacteria, Potassium solubilizing bacteria, Zinc solubilizing bacteria, siderophore production, Jeevamrut, Calcite solubilization*

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Introduction

The microbial biodiversity of soil is one of the most important indicators of its fertility. The micro-ecosystem developed by the micro-flora significantly affects not only the nutrient availability, texture, and organic matter content of the soil; but also affects the nutrient uptake, resistance to biotic stress and overall growth of the plants. Synthetic fertilizers and pesticides may have deleterious effect on this micro-ecosystem. Moreover, their negative impact on environment and human health has also become evident from many studies.

Phosphate and Potassium are two important macronutrients required for the healthy growth of plants. Phosphorus is involved in several key functions, such as energy transfer, movement of nutrients, photosynthesis, conversion of sugars and starches and is also an integral component of genetic material. Its deficiency leads to stunted growth, delay maturity, lesser yield, and reduced resistance to diseases [1]. Similarly, potassium is also involved in many vital functions and acts as activator of enzymes involved in synthesis of proteins, starch, and ATP.

It also helps in maintaining the turgor, while reducing water loss and wilting in leaves, helps in root growth and drought resistance, and improves resistance to diseases [2]. When these nutrients are added to the soil in form of chemicals, almost half remain unused and form complexes inside the soil. Formation of these unavailable forms slowly renders the soil infertile.

Furthermore, nutrient leaching of Phosphorus, Potassium and Nitrogen, applied as fertilizers, has been reported to cause groundwater and waterways contamination [3]. Hence the exploration of alternative and competitive strategies for improving plant growth has drawn interest from farming as well as scientific communities. Recently, the use of plant growth promoting bacteria (PGPB) as bio-inoculants has proven to be a promising practice for sustainable agricultural production while bringing down the requirement of chemical fertilizers significantly [4]. Some of the plant beneficial activities shown by PGPB include mineral solubilization; asymbiotic N_2-fixation; production of plant growth promoting hormones such as indole-acetic acid (IAA), cytokinins, Gibberellins (GA); and antagonistic activities against soil borne plant pathogens [5-8].

Enterobacter hormaechei that belongs to the family Enterobacteriaceae has been reported to have several plant growths promoting activities. *E. hormaechei* (MF957335) was found to show phosphate, potassium, and calcium solubilizing activity, was able to fix atmospheric nitrogen, produce auxin, confer disease resistance to the plant, and increase tomato yield both under saline and non-saline soil conditions [9]. In another study, where 18 strains of *E. hormaechei* were studied for their soil friendly properties, all the strains were found to exhibit Nitrogen fixation, IAA production and siderophore production. In addition, two strains were able to produce gibberellic acid, while one was found to produce exopolysaccharide. Okra seeds bio-primed with these strains showed significant improvement in plant growth and increased P and K uptake as compared to control seeds [10]. The ability of various *Enterobacter* strains to fix atmospheric nitrogen was found to be at par with that by Azotobacter species in a study performed on paddy [11]. The exopolysaccharides secreted by some *Enterobacter* species help the microcolonies of the bacteria in developing at the root tips and assist other microbes to survive in the rhizosphere [11].

In the present study we report a multi-trait *Enterobacter hormaechei* strain that was isolated from Jeevamrut (an organic manure prepared from cow dung, cow urine, jaggery and gram flour) prepared at Indore Biotech Inputs and Research Pvt. Ltd. This exercise was conducted to isolate and identify phosphate solubilizing bacteria using Pikovskaya's medium as a selective medium. The Isolated strain was identified using 16s RNA sequencing and was found to have the ability to solubilize minerals such as phosphate, silica, potassium, calcite and zinc, and oxidized Sulphur *in vitro*. The isolate also produces Indole Acetic Acid, and siderophores. Furthermore, the *Enterobacter* strain reported here is able to retard the growth of *Fusarium oxysporium* significantly *in vitro*.

Materials and Methods

Sample preparation and isolation

For preparation of Jeevamrut, 2.5kg of local cow dung and 2.5 litres of cow urine were mixed with 50 litres of water in a barrel. To this, 0.5 kg of jaggery, 0.5 kg of gram flour and handfuls of soil was added. The mixture was stirred well, covered with muslin cloth and kept for 48 hours in shadow at room temperature. The mixture was stirred a couple of times for a minimum of 10 minutes and left for fermenting. After 48 hours a 100 ml sample was drawn from the mixture and serial dilutions were made using this sample.

The samples were spread on sterilized PVK-BPB (Pikovskaya's-Bromophenol Blue), where 0.1% Bromophenol blue was added to Pikovskaya agar medium (Himedia). Negative control consisted of uninoculated PVK-BPB agar medium. Positive control consisted of a known PSB strain of Bacillus megaterium inoculated on PVK-BPB agar medium. The plates were incubated at 37°C for three days. Based on the appearance of the yellow zone, strains were visually assayed. All the assays in this experiment as well as ensuing experiments were set up in triplicates.

Potassium and Zinc solubilization assays

100 ul of liquid culture of each selected Phosphate solubilizing bacteria was spread on modified Aleksandrov Agar (Himedia) (ALK-BTB) with 0.01% bromothymol blue (BTB) to observe Potassium solubilization; and for zinc solubilization, media consisting (g/l); Glucose, 10.0g; K_2HPO_4, 0.1g; $(NH_4)_2SO_4$, 1g; KCl, 0.2g; $MgSO_4$, 0.2g, ZnO, 1gm, Agar, 2%, pH 7.1 ± 2 bromothymol blue (BTB) as indicator (Zn-BTB) was used. A known Potassium and Zinc solubilizing bacterial reference strain (*Paenibacillus polymyxa*, MTCC122) was used as positive control for both the assays. The plates were incubated at 37°C for a period of 3 days. The bacterial strain showing both potassium and zinc solubilization was selected for further characterization and sent for 16S RNA sequencing.

Production of Indole Acetic Acid (IAA)

Selected bacterial strain was grown at 30ºC in nutrient broth containing tryptophan (100 mg/l) for a period of 96 hours [12] in the dark and was centrifuged. IAA production was determined in the supernatant by Salkowski's method and OD was measured at 530 nm. Standard curve was made using various concentrations of Loba Chemie's Indole-3 Acetic acid (0ug-100ug at an interval of 10ug).

Siderophore production

Siderophore production was detected by using Chrome azurol-S (CAS) agar medium [13]. The plate of Chrome azurol-S (CAS) agar medium was inoculated with a loop of active cultures of a positive control (Pseudomonas fluorescens), a negative control which was uninoculated and a loopful inoculation with *Enterobacter hormaechei*. The plates were incubated at 28 ± 2°C for 2 days. Siderophore production was indicated by the formation of yellowish orange color around the bacterial growth against the blue background of the medium.

Sulphur oxidation

10 ml of thiosulphate broth [14] consisting of (g/l); $Na_2S_2O_3$, 5g; K_2HPO_4, 0.1g; $NaHCO_3$, 0.2g; NH_4Cl, 0.1g; Dextrose, 5 g; pH 8.0 ± 0.1, bromocresol purple 0.1%, was inoculated with loopful of the selected strain (*Enterobacter hormaechei*). The culture was incubated at room temperature for 7 days with continuous shaking at 120 RPM. Controls consisted of uninoculated broth; positive control consisted of Sulphur oxidizing bacteria inoculated with a loopful of *Thiobacillus* sps.

Calcite-Solubilizing activity

The isolated *E. hormaechei* was grown on selective calcite agar medium by inoculating a loopful of bacteria in the centre of the media plate [Medium composition (in g/100 ml): dextrose-1.0; $CaCO_3$- 0.5, $(NH_4)_2SO_4$- 0.05; KCl- 0.02; $MgSO_4.7H_2O$-0.1; yeast extract- 0.5; agar- 1.5 and pH- 7.0] and incubated at 280 for 7 days [15]. Un-inoculated plate was considered a negative control while *Paenibacillus polymyxa* was inoculated as a positive control.

Antagonistic activity against *Fusarium oxysporum*

The antifungal activity of the reported strain against *Fusarium oxysporum* was determined by a modified dual-culture plate antagonism method. Spores of *Fusarium oxysporum* were plated on PDA media by pour plate method. After solidification, 0.1 ml of *Enterobacter hormaechei* grown in nutrient broth (cfu $5.3X10^7$) was spread on the surface. Negative controls consisted of one PDA plate without any inoculation and nutrient broth spread on the surface, and one PDA plate with 0.1 ml of *Enterobacter hormaechei* grown in nutrient broth (cfu $5.3X10^7$) spread on the surface. One positive control consisting of *Fusarium oxysporum* spores plated on PDA media by pour plate method with 0.1 ml nutrient broth spread on the surface. All the plates were incubated at 26°C for 7 days.

Results

Jeevamrut is an excellent natural plant growth promoter and protectant. It is very rich in plant beneficial microorganisms. We carried out isolation of Phosphate solubilizing bacteria from Jeevamrut prepared in our lab. For this serially diluted samples of Jeevamrut were spread over selective Pikovskaya's-Bromophenol Blue (PVK-BPB) media. Four morphologically different bacterial colonies were found to grow on PVK-BPB media. Two colonies were found to give zones of solubilization in the form of yellow halos. The colony giving greater zone of solubilization was selected for further characterization. Morphologically, the isolate produced slimy, rounded, and semi-transparent colonies comprising of cells that were gram negative short-rod when observed under microscope.

Multiple traits exhibited *in-vitro*

The selected strain produced yellow halos by de-colorization of blue colour of PVK-BPB, ALK-BTB and Zn- BTB, agar media and the decolorization of PVK-BPB, ALK- BTB and Zn- BTB media and formation of yellow halo was like the positive control used in each experiment, indicating that the selected strain is efficient in mineral solubilization [Table-1]. The strain was subjected to 16 S RNA sequencing to confirm its identity and was confirmed as *Enterobacter hormaechei*. Several other plant beneficial activities were also confirmed using respective assays. The bacteria are an excellent producer of Indole Acetic Acid and was found to produce 60ug/ml +2 of IAA under the conditions mentioned earlier, calculated using standard curve method. The CAS/HDTMA agar plates showed change in colour from blue to orange around the bacterial colony of *E. hormaechei* due to siderophore production that acts as a strong iron chelator which removes iron from the dye complex, bringing about the change in colour [Fig-1]. Also, the selected strain brings about reduction in pH of the thiosulphate-based media from 8.0 to 4.5 by formation of acid and is indicated by the change in colour from blue to yellow. Similar change was seen in the broth inoculated with *Thiobacillus* sps. while the uninoculated controls did not show any change in colour [Fig-2]. The selective calcite agar plates showed clear-halos around the *E. hormaechei* colonies indicating calcite-solubilizing activity. The negative control showed no clear halo zone. While the positive control plate showed a much smaller halo zone compared to *E. hormaechei* [Fig-3 and Table-1].

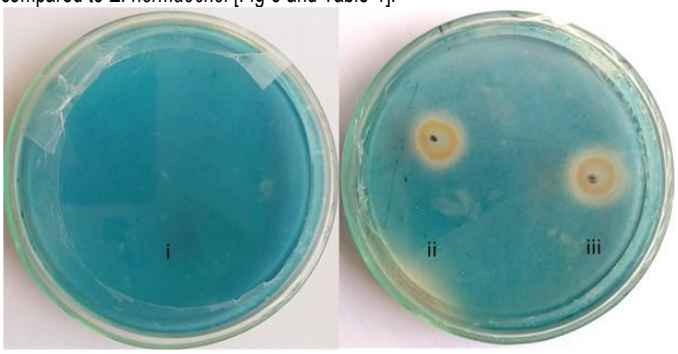

Fig-1 *In-vitro* siderophore production by the reference strain and *Enterobacter hormaechei* as observed on CAS/HDTMA agar
i. uninoculated control, ii. *Pseudomonas fluorescens*, iii. *Enterobacter hormaechei*

In-vitro* antagonistic activity against *Fusarium oxysporum

Antagonistic activity against *Fusarium oxysporum* was determined by a modified dual-culture plate antagonism method as mentioned earlier. It was observed that the negative control with only nutrient broth spread on the surface showed no growth while the negative control with *Enterobacter hormaechei* spread on the surface showed only the growth of *Enterobacter hormaechei*. The positive control consisting of only *Fusarium oxysporum* pour plated on a PDA plate showed luxuriant growth of the fungus with yellow spore formation at the end of 7 days. Similar result was observed on the plate where *Fusarium oxysporum* was pour plated and nutrient broth was spread on the top.

Table-1 *Solubilization of Phosphate, Potassium, Zinc and calcite by reference strains and Enterobacter hormaechei seen by zone of clearance on the selective media plates*

Name of Bacteria	Average diameter of halo (in cm) on various media			
	PVK-BPB	ALK-BTB	Zn-TrypB	Calcite Agar
Control	0	0	0	0
Paenibacillus polymyxa	NA	2.5±0.1	1.3±0.1	1.5±0.1
Bacillus megaterium	2.4±0.1	NA	NA	NA
Enterobacter hormaechei	2.6±0.2	2.5±0.2	2.2±0.2	3±0.1

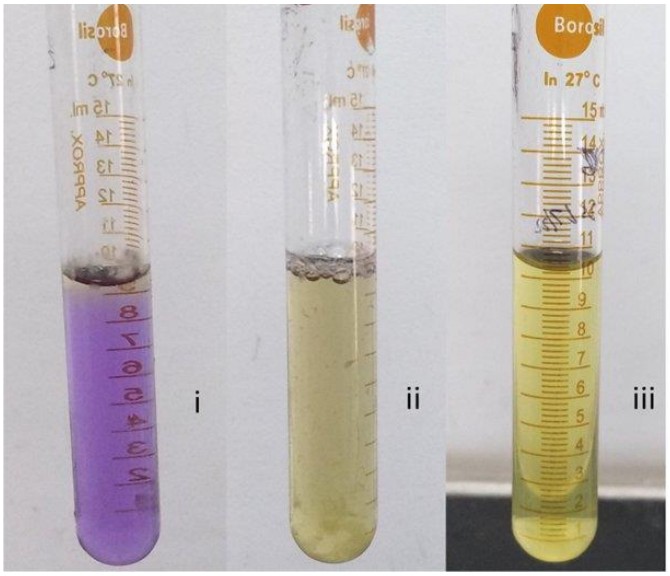

Fig-2 *In-vitro* sulphur oxidation by the reference strain and *Enterobacter hormaechei* as seen in thiosulphate broth
i. Uninoculated control, ii. *Thiobacillus* spp, iii. *Enterobacter hormaechei*

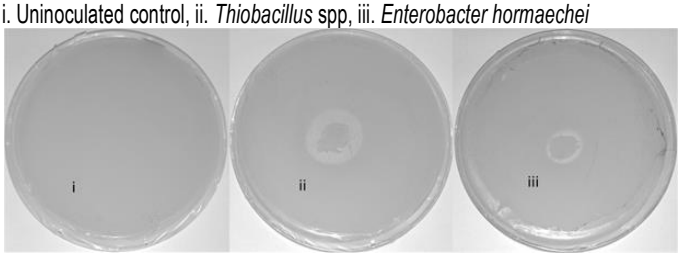

Fig-3 *In-vitro* calcite solubilization by the reference strain and *Enterobacter hormaechei* as observed on calcite agar medium
i. Uninoculated control, ii. *Paenibacillus polymyxa*, iii. *Enterobacter hormaechei*

Fig-4 *In-vitro* antagonistic effect against *Fusarium Oxysporum* shown by *Enterobacter hormaechei* as observed on potato dextrose agar
i. Uninoculated control, ii. *E. hormaechei* control, iii. *F. oxysporum* control,
iv. Antagonism shown by *E. hormaechei* against *F. oxysporum*

However, highly retarded mycelial growth was found in the plate consisting of *Fusarium oxysporum* and *Enterobacter hormaechei* spread on the top. These results indicate that the *E. hormaechei* strain reported efficiently retards the growth of the pathogen *F. oxysporum* and is also able to inhibit its sporulation [Fig-4].

Discussion

Most of the microbial inoculant products available in the market are single microbes with single trait or consortia consisting of single trait microbes. As compared to these, multi-trait bio inoculants may prove to be more economical to the consumers and beneficial for the soil. Consortia comprising of such multi-trait microbes can also be expected to have better chances of survival in the soil. We isolated a multi-trait *Enterobacter hormaechei* from Jeevamrut which can solubilize phosphate, potassium, and zinc *in vitro*.

These are macro nutrients which are essential in abundant quantity for normal growth and development of a plant. Furthermore, the strain is a prolific producer of Indole Acetic Acid (IAA) whose role has been implicated in several developmental processes of plants [16]. It has also been reported that indole-3-acetic acid is a signalling molecule and affects gene expression in some microorganisms and could also be involved in plant-microbe interactions [17]. Siderophore production was also observed in this strain. Siderophores are low molecular mass molecules that are produced by microbes, especially under Fe-limiting conditions [18]. Iron is an essential component of electron chains and is a cofactor of many enzymes. Its deficiency may cause suboptimal growth and chlorosis in plants [19]. Siderophores produced by soil microbes promote iron dissolution through various mechanisms [20].

The *E. hormaechei* reported here also exhibits heterotrophic sulphur oxidizing ability *in-vitro*. Sulphur is an essential secondary plant nutrient as it is essential for plant proteins as it is a part of certain amino acids. It is also an essential constituent of some important biological molecules such as vitamins, acetyl coenzyme A, ferredoxin *etc*. Sulphur is absorbed by the plant's roots from the soil in the form of sulphates. It is released from amino acids that accumulate in the soil as a result of degradation of plant and animal proteins. This sulphur is then oxidized to sulphates by the microorganisms present in the soil [21]. The carbon dioxide emitted in the environment is partially absorbed by certain soil bacteria and is converted into insoluble calcium carbonate ($CaCO_3$) or calcite. Increased amount of calcite results in stiffening of the soil and hinders the growth of roots and eventually the whole plant. Solubilization of this calcite is carried out by bacteria that produce organic acid, thus maintaining the balance between soluble and insoluble calcium content of the soil [22]. Furthermore, significant retardation of *Fusarium oxysporum in-vitro* mycelial growth and spore formation was also observed in the antagonistic assays using the reported strain of *E. hormaechei*. Some strains of *F. oxysporum* are pathogenic to various plant species. They cause wilt disease and are responsible for severe damage on many economically important plants [23]. The disease management frequently involves fumigation of soil with broad spectrum chemical biocides which are deleterious to the environment [24]. Use of bio inoculants that inhibit or retard the growth of pathogens is more desirable.

Conclusion

The *E. hormaechei* strain reported here is a multi-trait bacterium that exhibits several soil and plant friendly activities and has the potential to act as a plant protector. This strain of *E. hormaechei* could prove to be an excellent bio inoculant for helping in plant's growth, development, and protection against biotic stresses.

Application of research: Study of bio inoculant for helping in plant's growth, development, and protection against biotic stresses

Research Category: Agriculture Microbiology

Abbreviations: sp.: species; PGPB: plant growth promoting bacteria; IAA: Indole-Acetic Acid; GA: Gibberellins; P: Phosphorus; K: Potassium; RNA: Ribonucleic Acid; PVK-BPB: Pikovskaya's-Bromophenol Blue; Zn-BTB: Zinc containing media with Bromothymol blue; ALK: Aleksandrov Agar; BTB: bromothymol blue; CAS: Chrome azurol-S; RPM: Revolutions per minute; PDA: Potato Dextrose Agar; PVP-BPB: Pikovskaya's-Bromophenol Blue; HDTMA: Hexadecyltrimethylammonium

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Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: Indore Biotech Inputs & Research Pvt. Ltd., Indore, 452001

Strain name: *Enterobacter hormaechei*

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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