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Isolation, Characterization, and Production of Biofertilizer using *Bacillus megaterium* Strain Isolated from Potato Plants Endemic to Himalayan Regions

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) offer a cost-effective and profitable alternative to pesticides and chemical fertilizers in agriculture. The rhizosphere of potato plants, indigenous to the Himalayas, was examined to isolate PGPR. A total of 65 isolates belonging to the *Bacillus* species were obtained from the rhizosphere. Among these, 7 isolates exhibited positive results for hydrogen cyanide (HCN) production, 5 isolates tested positive for urease production, and 4 isolates showed positive results for nitrogen-fixing potential. Subsequently, the isolates were evaluated for additional plant growth-promoting (PGP) traits, such as ammonia production, nitrate reduction, siderophore production, phosphate solubilization, and antifungal activity. Among all the PGP strains, RA2 followed by DO1 exhibited the highest siderophore units (68% and 61%, respectively) and phosphorus-solubilizing potential (285.22 µg/ml and 249.11 µg/ml, respectively). *In vitro* stress tolerance to saline and alkaline conditions was also assessed for isolates RA2 and DO1. RA2, along with 4 other bacterial isolates, underwent in vivo testing for promoting the growth of Pea (*Pisum sativum*). RA2 demonstrated the most notable plant growth-promoting traits and was identified as *Bacillus megaterium* through biochemical analysis, further confirmed by 16S rRNA sequencing. Moreover, the plastic biodegradation potential of isolate RA2 was examined via scanning electron microscopy (SEM) analysis. Following this, isolate RA2 was successfully encapsulated in sodium alginate beads and talcum powder-based formulations for commercialization, aiming to enhance soil fertility and crop productivity.

Keywords: PGPR, Biofertilizer, Plastic degradation, formulation, P-solubilization, siderophore.

Introduction

Potato cultivation spans across 130 countries worldwide, ranking fourth in production following rice, maize, and wheat. However, in the subsistence agriculture of the Himalayan region, potato crop yields remain low to medium due to inadequate soil fertility and nutrient availability, compounded by fungal diseases such as black scurf and dry rot caused by *Rhizoctonia solani* and *Fusarium solani* (Goswami et al., 2014). Leveraging beneficial microorganisms presents an environmentally sustainable approach to enhancing crop yields and mitigating disease incidence.

Plant growth-promoting rhizobacteria (PGPR) play a pivotal role in augmenting plant growth through various mechanisms, including accelerated nutrient uptake such as phosphorus solubilization, synthesis of growth-stimulating phytohormones like indole-3-acetic acid (IAA), and antagonism against pathogens via the production of inhibitory substances or competition for root colonization (Sharma et al., 2023, Singh et al., 2020). *Bacillus* strains are frequently reported as PGPR, known for their antibacterial or antifungal activities against phytopathogens through the production of secondary metabolites. Certain *Bacillus* products, such as those derived from *B. subtilis*, have been utilized as seed dressings for plant disease biocontrol (Widnyana and Javandira, 2016).

Notably, *Bacillus* strains are capable of forming endospores, imparting them with high stability as bio-fungicides or biofertilizers (Calvo et al., 2010). The Himalayan region's diverse climatic conditions, ranging from tropical foothills to tundra-like environments, present unique challenges and opportunities for agricultural production. Cold-adapted bacteria found in the Himalayan cold-temperature ecosystems possess plant growth-promoting capacities that can be harnessed to improve plant productivity in these challenging environments.

Plant growth-promoting rhizobacteria (PGPR) encompass a diverse array of soil bacteria that, when associated with host plants, stimulate host plant growth through both direct and indirect mechanisms. These mechanisms include nitrogen fixation, phosphate solubilization, siderophore and phytohormone production, and biocontrol against pathogens. Biological nitrogen fixation by PGPR holds particular promise for increasing crop production in agriculture, especially in cold climates like those of the Himalayan hill regions, where mesophilic PGPR struggle to thrive.

Farmers in the Himalayan region encounter numerous challenges, including low soil fertility, small seed size, limited nutrient availability, drought stress, soil erosion, low soil moisture content, and biotic interactions (Kumar et al., 2017). Addressing these challenges through the utilization of psychrotolerant PGPR and other sustainable agricultural practices is essential for enhancing agricultural productivity and promoting food security in the Himalayan region.

Materials and Methods

Microbial Cultures (Isolation and Preliminary Screening)

Rhizospheric soil samples were collected from potato plants in five distinct regions (Harsil, Jhala, Raithal, Uttarkashi City, and Matli) of the Himalayas within Uttarkashi district,

Uttarakhand. Samples were collected from a depth of 0-10 cm, air-dried, sieved (2 mm), and stored at 4°C. Predominant *Bacillus* strains were isolated from these soil samples using the heat treatment method outlined by Walker et al., (1998). Isolated cultures were maintained on nutrient agar plates at 30 ± 1 ºC for 48 hours. Identification was confirmed via Gram staining, noting colony morphology, and preparing glycerol stocks preserved at -80°C.

Selection of PGPR Traits

Isolated *Bacillus* strains underwent screening for plant growth-promoting traits, including:

1. **Qualitative Screening for HCN and Urease Production:** Rhizospheric *Bacillus* isolates were assessed for HCN and urease production. HCN production was determined using the picrate assay by Castric (1974), with changes in filter paper colour indicating HCN presence. Urease production was assessed using urea broth tubes containing phenol red, observing colour changes.
2. **Ammonia Production and Nitrogen-Fixing Potential/Nitrate Reduction Test:** Ammonia production was determined by growing *Bacillus* strains in peptone broth and detecting ammonia with Nessler's reagent. Nitrogen fixation ability was tested on Jensen medium, and nitrate reduction was assessed in peptone nitrate broth (Marakana, T. *et al.*, 2018).
3. **Determination of Antifungal Activity/Mycoparasitism:** Dual culture plate assays were conducted to evaluate antagonistic activity against Aspergillus niger and Fusarium oxysporum phytopathogens. Observations were made for microbial growth and pathogen parasitization over 7 days at 30 ± 1°C (Dennis and Webster, 1971).

Qualitative and Quantitative Estimation of Siderophore

Siderophore production was qualitatively assessed using Chrome Azurol S (CAS) agar medium following the method outlined by Schwyn and Neiland (1987). The formation of a yellow-orange zone around colonies indicated positive siderophore production. Additionally, $FeCl_3$ testing was conducted for qualitative siderophore detection. Quantitative estimation was performed through CAS-shuttle assay (Kumar *et al.*, 2017),(Singh et al., 2020).

Qualitative and Quantitative Estimation of Phosphate Solubilization

Qualitative assessment of phosphate solubilization was conducted by spot inoculating isolated bacterial strains onto Pikovskaya agar plates amended with tricalcium phosphate. Halo zone formation indicated positive solubilization. Quantitative analysis involved inoculating active cultures into mineral salt medium broth containing zinc phosphate, followed by spectrophotometric measurement of phosphate levels (Pikovaskya, 1948).

***In Vitro* Screening of Potential *Bacillus* Isolates Under Stress Conditions**

Selected bacterial isolates were subjected to pH and saline stress tolerance tests. Tolerance to varying pH levels (2-12) and NaCl concentrations (2.0-10.0% w/v) was assessed spectrophotometrically over 5 days (Gupta and Pandey, 2019).

Assessment of Plant Growth-Promoting Traits (Seed Germination Test) and *In Vivo* Pot Trials

Selected *Bacillus* isolates were evaluated for their plant growth-promoting abilities using a seed vigour assay on peas (*Pisum sativum*) and pot trials. Surface-sterilized seeds were inoculated with overnight bacterial cultures, and germination was monitored for 10 days (Singh *et al.,* 2013). Pot trials involved inoculating germinated plantlets with bacterial suspension and measuring vegetative parameters after 6-8 days.

Biochemical Characterization

Biochemical characterization of the potent strain RA2 was conducted using the HiCarbohydrate kit by HIMEDIA, comprising 35 tests for carbohydrate utilization and enzyme production. Metabolic changes were indicated by colour changes in the media.

Strain Characterization

The potential isolate RA2 was characterized via PCR amplification and sequencing of the partial 16S rRNA gene. Phylogenetic analysis was performed using BLASTn at NCBI.

Evaluation of Plastic Biodegradation

Screened *Bacillus* isolates were assessed for plastic degradation potential using Yeast Mannitol Broth (YMB) medium supplemented with glucose for easy adjustment of the bacteria to the medium and low-density polyethylene (LDPE) strips. Percentage degradation was calculated based on weight loss after 2 months of incubation, which is further confirmed by scanning electron micrograph (SEM) analysis (Munir et al., 2018).

Bioformulation Preparation

Biofertilizer formulations of isolate RA2 were prepared using talcum powder and sodium alginate as carrier materials. Talcum powder formulations were sterilized and mixed with bacterial inoculum, while alginate beads were prepared by mixing bacterial culture with sodium alginate solution and dropping it into chilled calcium chloride (Trivedi *et al.,* 2005, Trivedi *et al.,* 2008).

Statistical Analysis

Experiments were conducted in triplicates using a completely randomized design (CRD). Data were analyzed using two-way ANOVA in SPSS/STPR2, with statistical significance set at $p < 0.05$. Results were reported in terms of critical difference (CD) and standard error of mean (SEm).

Result and Discussion

A total of 65 morphologically distinct *Bacillus* isolates were obtained from *Solanum tuberosum* samples plated on nutrient agar from various regions. Specifically, 15 isolates were collected from the Harsil region, 7 from the Jhala region, 13 from Raithal, 17 from Uttarkashi city, and 13 from the Matli region. These isolates underwent morphological screening via Gram staining, and their colony morphologies were documented. Additionally, qualitative screening for plant growth-promoting rhizobacteria (PGPR) traits was performed.

HCN production and Urease production test.

Among the 65 isolates examined, assessment for hydrogen cyanide (HCN) and urease production unveiled that only two isolates, specifically DO1and RA2, displayed notable HCN production. Isolates lacking HCN production capability failed to elicit a discernible colour change in Whatman filter paper no. 1 immersed in a solution comprising 0.05% picric acid and 2% Na_2CO_3, thus maintaining their original appearance. Conversely, RA2, UKI-1, and DO-1 triggered a conspicuous shift in the filter paper's colour, transitioning from a faint yellow hue to orange-brown, indicative of substantial HCN production. RA1 and MAT2 exhibited a marginally lower level of HCN gas production (Figure 1).

Furthermore, five *Bacillus* isolates (DO1, DO4, UKI1, RA2, and MAT2) from the pool of 65 isolates demonstrated positive results for the urease production test (Figure 1). The presence of these test cultures on nine plates resulted in a distinct alteration in the colour of the medium, transitioning from yellow to pink, as evidenced by phenol red. In contrast, no such colour change was observed in the control plates.

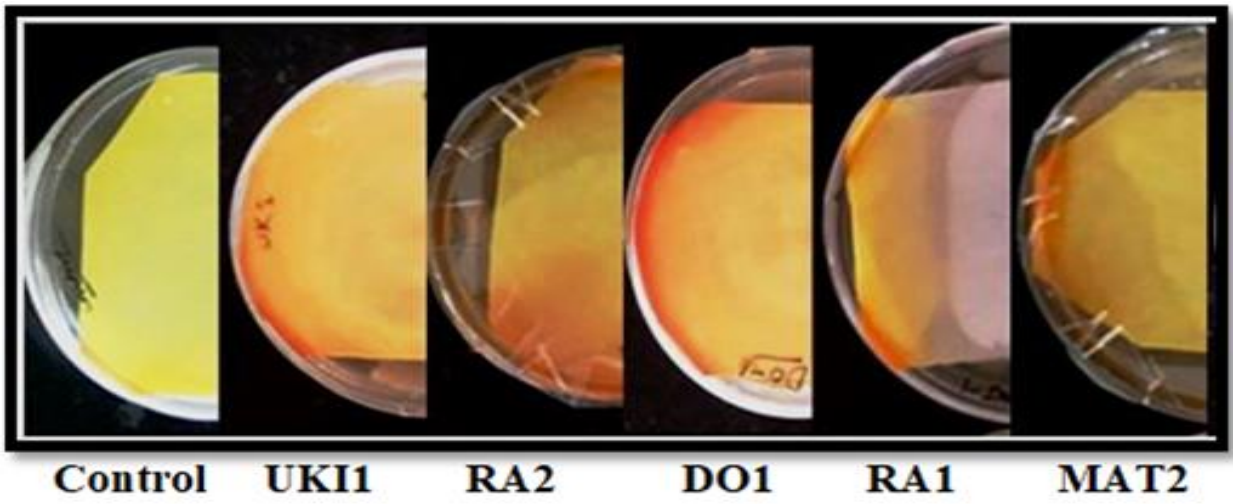


Figure 1. Qualitative detection of HCN through picrate assay test by isolated bacterial isolates

Figure 2. Qualitative estimation of urea by isolated bacterial cultures

Ammonia Production

The evaluation of ammonia production among the *Bacillus* strains was conducted by culturing the isolates in peptone broth for duration of 4-5 days. Out of the 65 *Bacillus* PGPR (Plant Growth-Promoting Rhizobacteria) strains isolated from *Solanum tuberosum*, 10 strains exhibited significant ammonia production (Figure 3). This was determined by a distinct colour change in the broth from its original state to a yellow/brown hue, as compared to the control samples which exhibited no such change. This finding indicates the presence of ammonia in the culture medium, produced by the metabolic activities of these specific *Bacillus* strains.

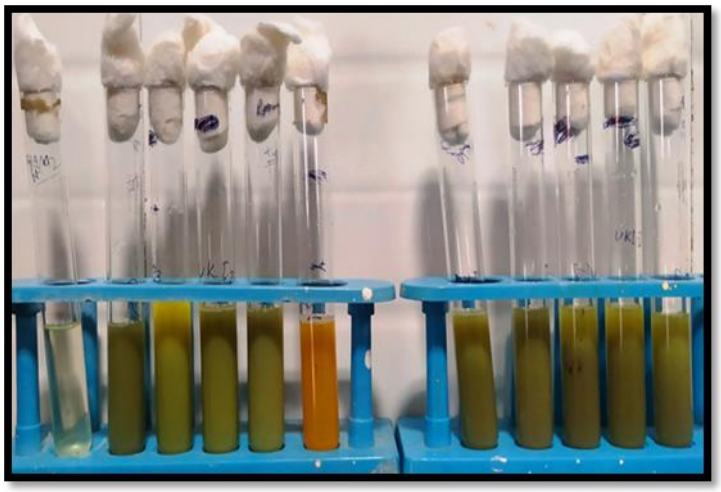


Figure 3. Qualitative estimation of ammonia production test by isolated *Bacillus* cultures.

Nitrate Reduction Test

The nitrate reduction capabilities of the *Bacillus* isolates were assessed using the nitrate reduction test. Among the 65 isolates, only 5 demonstrated the ability to reduce nitrate to nitrite. This was visually confirmed by a red colouration in the test tubes, which indicates the presence of nitrite as a reduction product of nitrate (Figure 4). This biochemical trait is significant as it highlights the potential of these isolates to participate in the nitrogen cycle by converting nitrate to more usable forms for plant uptake.

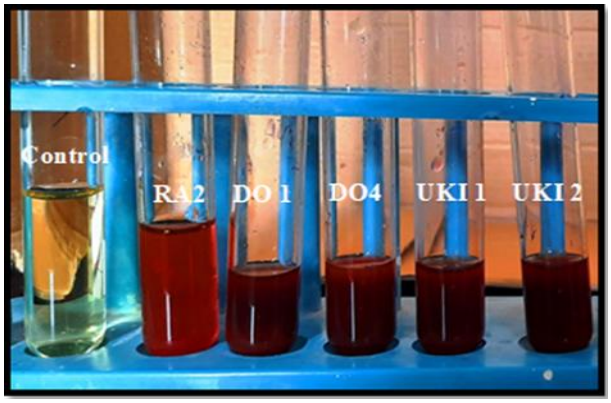


Figure 4. Qualitative estimation of nitrate reduction test

Nitrogen Fixation Potential

The nitrogen fixation potential of the *Bacillus* isolates was examined using Jensen agar media, a nitrogen-free growth medium. Of the 65 isolates tested, 4 displayed positive results for nitrogen fixation. These isolates were able to grow on the Jensen agar, indicating their capability to fix atmospheric nitrogen and convert it into a form that can be utilized by plants. This trait is particularly valuable for enhancing soil fertility and promoting sustainable agricultural practices (Figure 5).

The comprehensive screening of the *Bacillus* isolates for ammonia production, nitrate reduction, and nitrogen fixation revealed significant variability in PGPR traits among the strains. Specifically, 10 isolates were proficient in ammonia production, 5 were capable of nitrate reduction, and 4 demonstrated nitrogen fixation abilities. These findings underscore the potential application of these *Bacillus* isolates in improving soil fertility and promoting plant growth through various mechanisms of nitrogen metabolism. The identified strains with these beneficial traits could be further studied and potentially developed as biofertilizers to enhance agricultural productivity.

Figure 5. Determination of N- fixing potential of isolated bacterial isolates on Jensen's agar media.

Antifungal Activity

The antagonistic activity of the bacterial isolates was assessed by measuring the inhibition zone diameter, which serves as an indicator of the reduction in growth of two root rot/wilt-causing pathogenic fungi: *Aspergillus niger* and *Fusarium oxysporum*. Isolate RA2 exhibited significant antifungal activity against both *Aspergillus niger* and *Fusarium oxysporum*. Additionally, the *Bacillus* isolates D01 and UKI1 demonstrated positive antifungal activity specifically against *Fusarium* spp. (Figure 6).

Figure 6. Determination of antifungal activity by isolate RA2 against (A) *Fusarium oxysporum* (B) *Aspergillus niger*

Siderophore Production

Qualitative assessment of siderophore production was initially conducted using the Chrome azurol S (CAS) agar plate assay on active cultures of all 65 isolates. Results revealed that 6 out of 65 isolates demonstrated the ability to produce siderophores extracellularly, as evidenced by the formation of orange zones surrounding the colonies (Figure 7). The transition of the CAS dye from blue to yellow indicated the effective chelation of ferric ions by the siderophores, corroborating their strong chelating potential (Schwyn and Neiland, 1987). Further confirmation of siderophore production was obtained through the ferric chloride ($FeCl_3$) test, wherein all six microbial cultures exhibited a reddish-brown coloration, indicating a positive reaction and confirming their siderophore-producing potential (Pahari et al., 2016).

Quantitative estimation of siderophore production was performed spectrophotometrically by measuring absorbance at 630 nm using the CAS assay. Siderophore quantification was conducted using culture supernatants, and the percent siderophore unit was calculated according to established protocols (Nithapriya *et al*., 2021). The siderophore units (% w/v) ranged from 32% to 61% across different culture filtrates. Notably, the highest siderophore production was observed in bacterial isolate DO1 (61%) after 120 hours of incubation, followed by RA2 (51.6%) under similar incubation conditions (Figure 8). These findings are consistent with previous studies, indicating the reproducibility and reliability of the observed results (Figure 9).

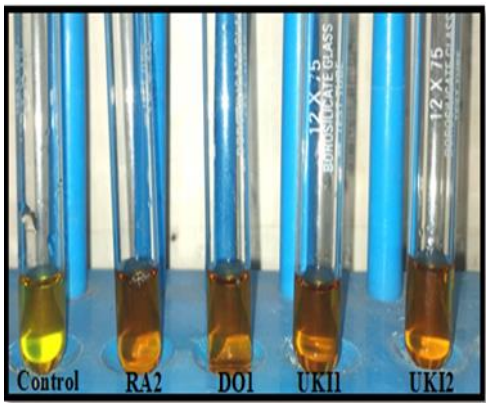


Figure 7. Qualitative detection for siderophore production by test cultures during FeCl3 test.

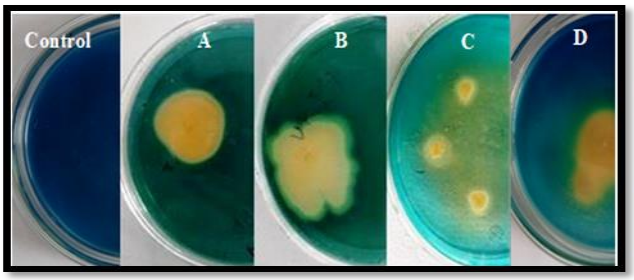


Figure 8. Qualitative detection for siderophore production on CAS agar plates by selected bacterial isolates.

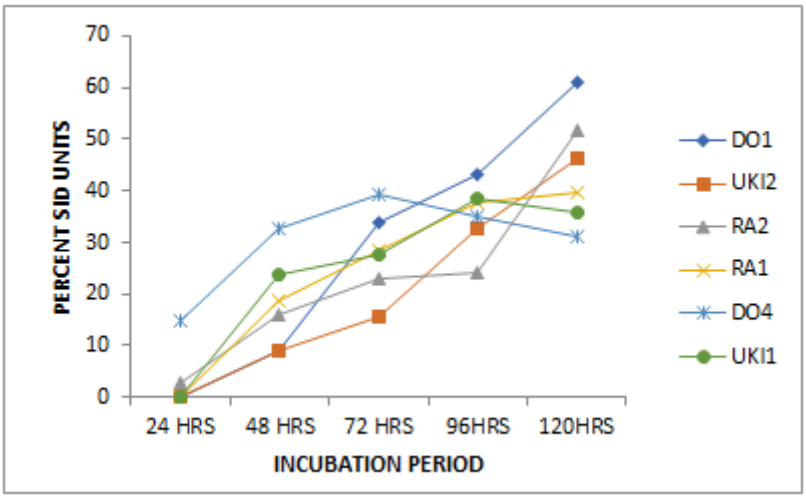


Figure 9. Graphical representation of siderophore production by the bacterial cultures in iron-free media.

Phosphate Solubilization

Phosphorus is a crucial macronutrient essential for plant growth and development (Duangpaenga *et al*., 2013; Zheng BX *et al*., 2018). Certain plant growth-promoting rhizobacteria (PGPRs) have the ability to solubilize insoluble phosphate forms, thereby enhancing its availability for plant uptake (Zheng et al., 2018).

In this study, 20 out of 65 *Bacillus* isolates exhibited the capacity to solubilize phosphorus in plate-based assays, forming clear halo zones exceeding 2.8 mm around the colonies. Subsequently, based on qualitative screening results, 6 *Bacillus* isolates underwent quantitative estimation of phosphate (Table 1).

All cultures exhibited robust growth in the medium, utilizing zinc phosphate as a phosphorus source. Notably, isolate RA2 demonstrated the highest phosphorus solubilization potential, with a concentration of 285.22 µg/ml observed after 72 hours, followed by DO1 with 249.11 µg/ml. Despite a slight decrease in pH values from 7.1 to 5.28 (**Table 1**), the process of P-solubilization was not attributed to acid production; instead, it may be attributed to the secretion of extracellular alkaline phosphatase enzymes. Numerous strains of *Bacillus* sp. have been identified as proficient phosphate solubilizers in previous studies (Ahmad et al., 2018), supporting the findings of this investigation.

Table 1. Phosphate concentration in culture filtrates of selected *Bacillus* cultures strains released upon solubilization of zinc phosphate

S. No	Bacterial cultures	Phosphate conc. (µg/mL) at different time intervals (h)				
		0	24	48	72	96
1	DO1	17.03	132.02	155.01	249.11	143.97
2	DO4	18.55	129.67	147.45	248.15	133.21
3	RA1	15.18	134.10	172.43	245.20	135.39

4	**RA2**	20.21	126.78	144.67	285.22	155.45
5	**UKI1**	13.55	110.09	131.21	227.09	202.77
6	**UKI2**	15.52	129.33	187.20	241.89	176.10
SEm±CD (5%)						

In vitro Stress Tolerance in Response to Saline and Alkaline Stress

The potential of all *Bacillus* strains to tolerate high salt concentrations (2-10%) and pH stress conditions ranging from pH 2 to pH 12 was evaluated. Regarding salinity stress, isolate RA2 and DO1 exhibited notable tolerance, with RA2 showing growth in 8% NaCl and DO1 in 10% NaCl after 72 hours of incubation. However, the maximum growth rate for RA2 was observed at 0% NaCl, followed by 2% NaCl concentration, while DO1 exhibited optimal growth at 0% and 2% NaCl concentrations (Figure 10 (1) & (2)).

Additionally, the tolerance of *Bacillus* isolates RA2 and DO1 to pH stress was assessed. RA2 demonstrated resilience to pH stress up to pH 11, while DO1 exhibited similar tolerance up to pH 11 as well. Notably, maximum growth rates were observed at pH 7 for both isolates. However, deviations from neutral pH resulted in a decline in growth rates, indicating sensitivity to extreme pH conditions (Figure 11 (a) & (b)).

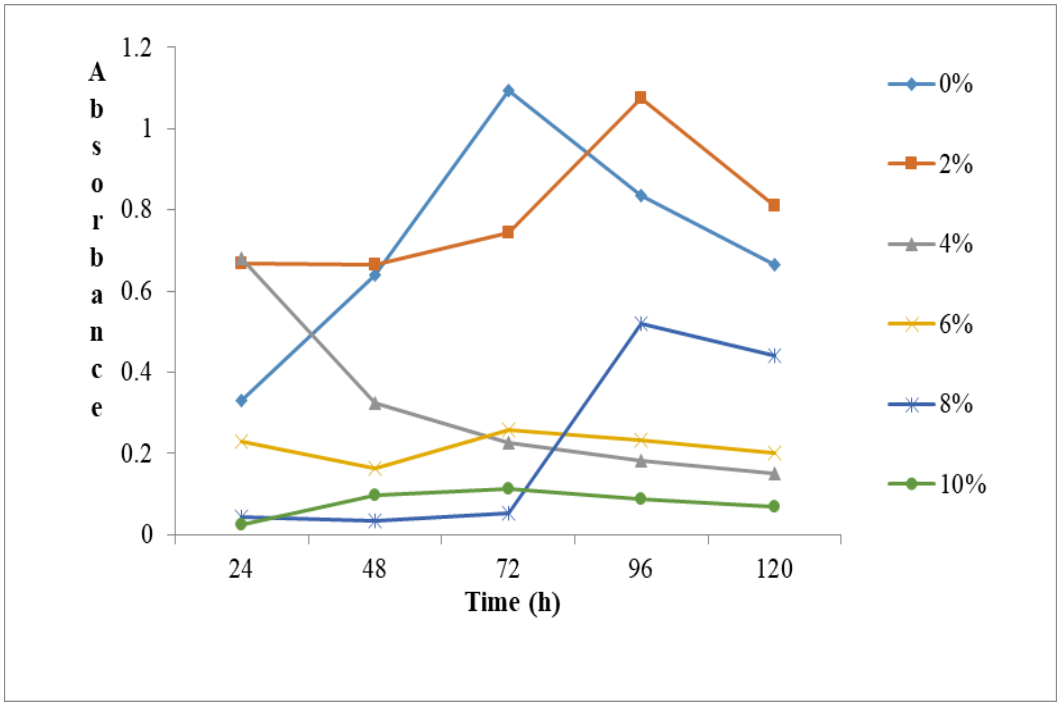


Figure10 (a) Growth curve of DO1 at different salt concentrations

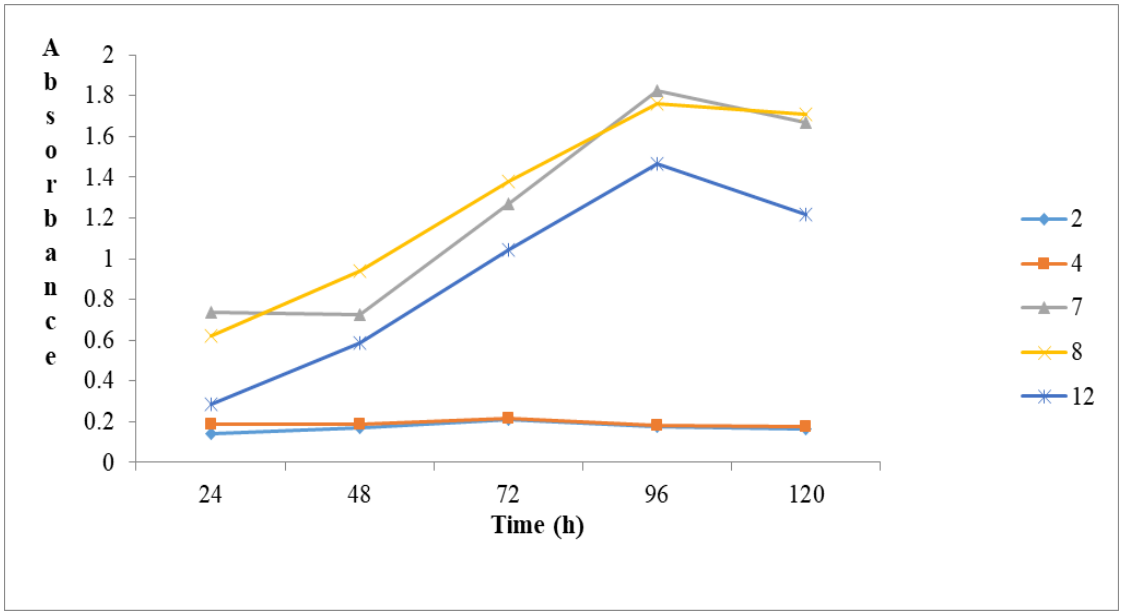


Figure10 (b) Growth curve of DO1 at different pH

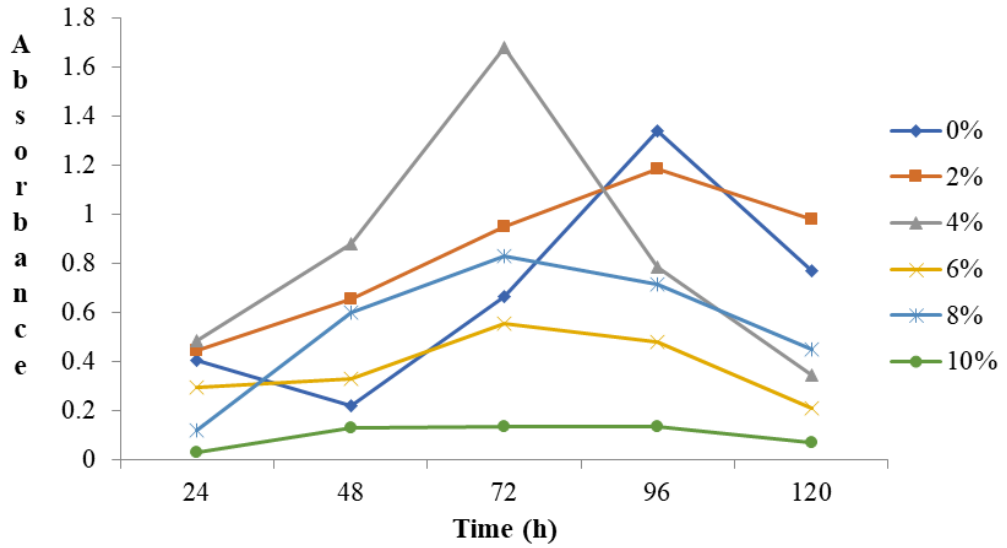


Figure11 (a) Growth curve of RA2 at different salt concentrations

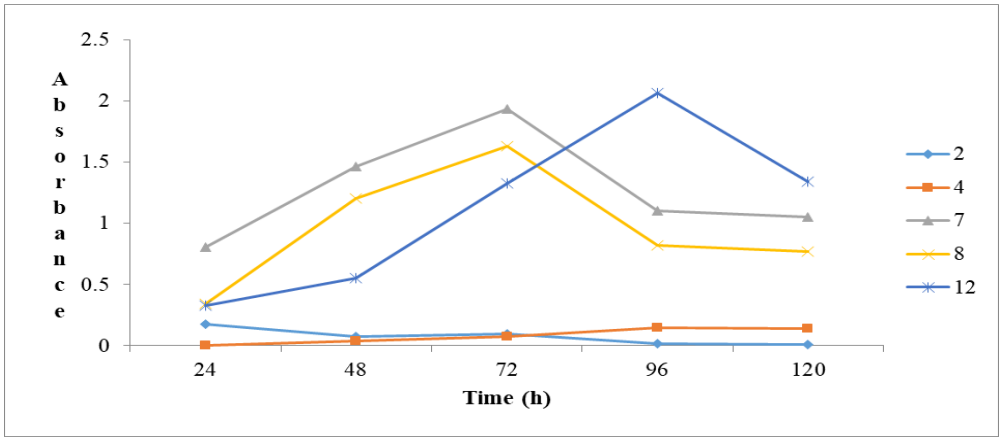


Figure11 (b) Growth curve of RA2 at different pH

Plant Growth Promotion Assessment (Seed Vigour Test) & In vivo Pot Trials

The plant growth promotion potential of selected bacterial isolates was initially evaluated through a seed vigour assay conducted on sweet peas (*Pisum sativum*) using water agar (WA) medium. Following the assessment of plant growth-promoting rhizobacteria (PGPR) traits, the five most promising isolates, namely DO1, RA2, UKI-2, UKI-1, and RA1, along with their consortium, were selected for the seed vigour assay.

The seed vigour assay involved the incubation of bacterial-inoculated sweet pea seeds for duration of 10 days. Readings were taken on the 3rd and 10th days of incubation. Remarkably, the highest germination percentage after 3 days of treatment was observed in seeds inoculated with the consortium of DO1, RA2, and UKI-2, followed by seeds inoculated with RA2 alone. Conversely, the uninoculated control exhibited the lowest growth (Figure 12). These trends persisted on the 10th day of inoculation, reaffirming the efficacy of the selected bacterial isolates and their consortium in promoting seed vigour and germination.

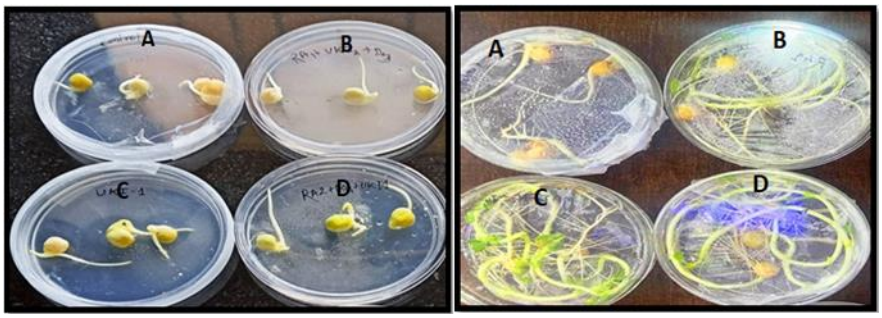


Figure12. Results of seed vigour assay (i) after 3 days of incubation (ii) after 10 days of incubation. Where (A) control (B)- D01+RA2+UKI2 (C) RA2 and (D)UKI2

***In vivo* Pot Trials Evaluation**

In the in vivo pot trials, pea plants were subjected to treatments with different strains of *Bacillus*, including DO1, RA2, UKI-2, UKI-1, and RA1, individually as well as in consortium form. All tested isolates and the consortium exhibited significant enhancements in leaf length, root length, shoot length, and fresh weight compared to untreated control plants (Figure 12) (Table 2).

The consortium comprising *Bacillus* isolates RA2, DO1, and UKI-2 demonstrated the most pronounced plant growth-promoting effects in vivo. This consortium resulted in a remarkable increase of 46% in leaf length, 39% in shoot length, 49% in root length, 28% in fresh weight, and a total plant length increase of 40% compared to the control. Subsequently, the individual application of RA2 also displayed notable enhancements, with an increase in leaf length by 38%, shoot length by 33%, root length by 47%, fresh weight by 24%, and total plant length by 36% relative to the control.

Table 2. Plant Growth and Survival Statistics.

Treatments	**No. of plants**	**No. of plants dead**	**No. of plants survived**	**Percent survival**	**% Increase in Plant Length**
Control	05	02	03	60	00
DO1	05	01	04	80	32 (Root), 25 (Shoot), 29 (Leaves)
RA1	05	03	02	40	08 (Root), 11 (Shoot), 3 (Leaves)
UKI2	05	02	03	60	17 (Root), 21 (Shoot), 12 (Leaves)
UKI1	05	02	03	60	07 (Root), 14 (Shoot), 13 (Leaves)
RA2	05	00	05	100	47 (Root), 33 (Shoot), 38 (Leaves)
RA2+DO1+UKI2	05	00	05	100	49 (Root), 39 (Shoot), 46 (Leaves)
DO1+RA1+UKI1	05	02	03	60	19 (Root), 16 (Shoot), 08 (Leaves)

Polyphasic Identification: Biochemical Characterization

Biochemical characterization of the highly potential isolate RA2 was conducted using the pre-sterile biochemical kit KB009 HiCarbohydrate by HIMEDIA, comprising 35 carbohydrate utilization tests and 1 control. The results revealed that isolate RA2 exhibited positive reactions for lactose, maltose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, mannitol, adonitol, alpha-Methyl-D-glucoside, esculin, citrate, catalase, and malonate (Table 3).

According to the Bergey's Manual of Systematic Bacteriology/Determinative Bacteriology (Holt et al., 1994), the biochemical characteristics of isolate RA2 align with the

characteristics of the *Bacillus* genus, specifically Gram-positive, endospore-forming rods that are catalase-positive.

Table 2 Biochemical charecterization of selected isolate RA2

S. No.	Test name	Original color of the medium	Reaction	Positive Reaction	Negative Reaction
	Control	Red	-ve	-	Red
1	**Lactose**	Red	+ve	Yellow	-
2	**Xylose**	Red	-ve	-	Red
3	**Maltose**	Red	+ve	Yellow	-
4	**Fructose**	Red	-ve	-	Red
5	**Dextrose**	Red	+ve	Yellow	-
6	**Galactose**	Red	+ve	Yellow	-
7	**Raffinose**	Red	+ve	Yellow	-
8	**Trehalose**	Red	+ve	Yellow	-
9	**Melibiose**	Red	+ve	Yellow	-
10	**Sucrose**	Red	+ve	Yellow	-
11	**L-Arabinose**	Red	-ve	-	Red
12	**Mannose**	Red	-ve	-	Red
13	**Inulin**	Red	-ve	-	Red
14	**Sodium**	Red	-ve	-	Red
15	**Glycerol**	Red	-ve	-	Red
16	**Salicin**	Red	-ve	-	Red
17	**Dulcitol**	Red	-ve	-	Red
18	**Inositol**	Red	-ve	-	Red
19	**Sorbitol**	Red	-ve	-	Red
20	**Mannitol**	Red	+ve	Yellow	-
21	**Adonitol**	Red	+ve	Yellow	-
22	**Arabitol**	Red	-ve	-	red
23	**Erythritol**	Red	-ve	-	red
24	**alpha-Methyl-D-glucoside**	Red	+ve	Yellow	-
25	**Rhamnose**	Red	-ve	-	Red
26	**Cellobiose**	Red	-ve	-	Red
27	**Melezitose**	Red	-ve	-	Red
28	**Alpha-Methyl-**	Red	-ve	-	Red
29	**Xylitole**	Red	-ve	-	Red
30	**ONPG**	Colourless	-ve	-	Colourless
31	**Esculin**	Cream	+ve	Black	-
32	**D-Arabinose**	Red	-ve	-	Red
33	**Citrate**	Green	+ve	Blue	-
34	**Malonate**	Light green	+ve	Blue	-
35	**Sorbose**	Red	-ve	Red	Red

Strain Characterization

Isolate RA2, identified as the most prominent among all plant growth-promoting rhizobacteria (PGPR) strains, was selected for further characterization through 16S rRNA gene sequencing. Initially, biochemical tests were conducted, followed by 16S rRNA gene sequencing analysis.

The blast search of the National Centre for Biotechnology Information (NCBI) database revealed that strain RA2 exhibited maximum similarity (94.27%) with *Bacillus megaterium* partial 16S rRNA gene, specifically with isolate N-P-27 under accession number LS999513.1 (Figure 13). Consequently, the potential PGPR isolate RA2 was conclusively identified as *Bacillus megaterium*.

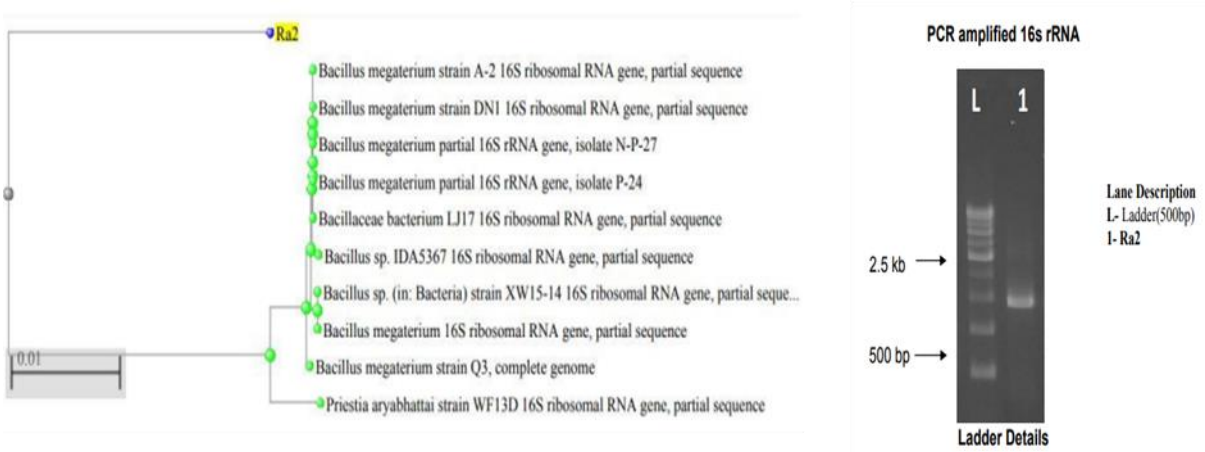
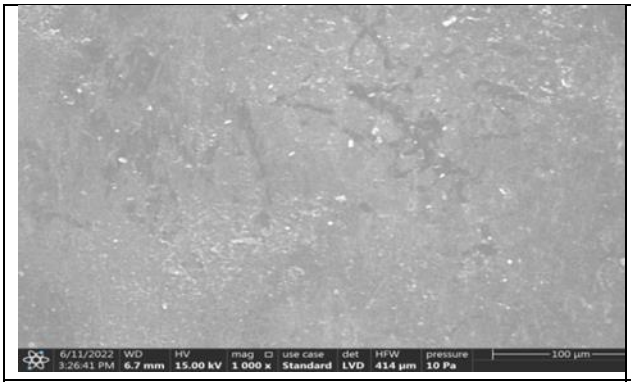


Figure 13. Phylogenetic analysis of *Bacillus* isolate RA2 using Mega X by neighbour-joining method.

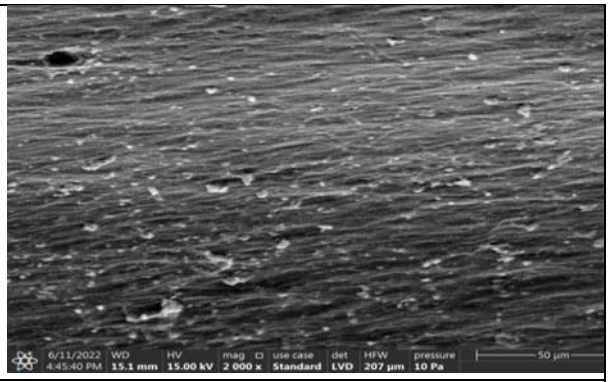
Results of Plastic Biodegradation

The predominant bacteria, RA2, were selected for detailed investigation regarding their capability to degrade low-density polyethylene (LDPE). RA2 bacterial culture exhibited significant biodegradation potential, with a degradation rate of up to 10.11% for milk polyethylene and 9.10% for vegetable polyethylene over a period of two months under laboratory conditions.

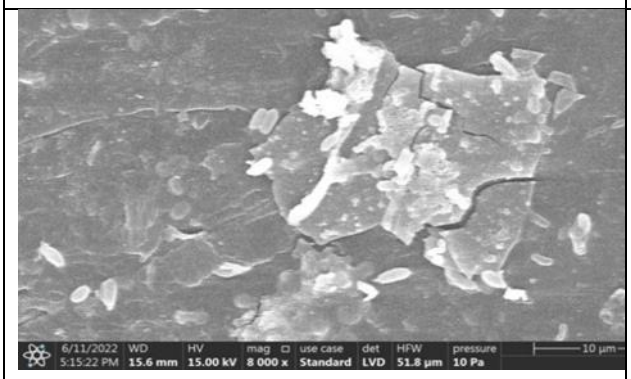
To validate these findings, scanning electron microscopy (SEM) analysis was conducted at various magnifications (Figure 14). The control polyethylene strips displayed a typical surface view, whereas those treated with the RA2 bacterial culture exhibited noticeable surface alterations, including corrosion, foldings, and cracks in comparison to the control. These alterations are likely attributed to the action of bacterial extracellular metabolites and enzymes on the surfaces of the polyethylene strips. Additionally, bacterial cells were clearly observed on the surface of the treated LDPE, further confirming the biodegradation process.



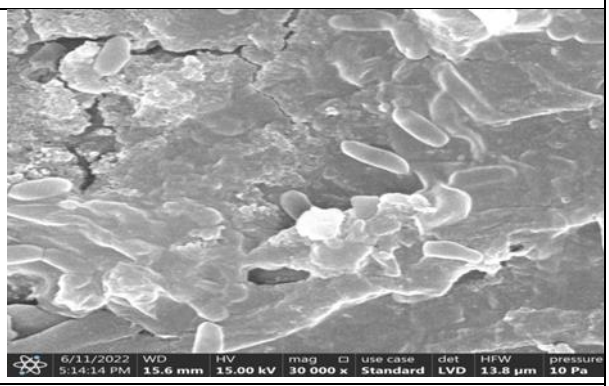
(a) Control (Milk polythene)



(b) Control (Vegetable polythene)



(c) Action of isolate RA2 on Milk polythene



(d) Action of isolate RA2 on Vegetable polythene

Figure 14. Electron micrographs showing the cells of RA2 degrading the LDPE

Bioformulation Preparation

Assessing the viability of the test organism in carrier-based preparations for prolonged storage is crucial for evaluating the suitability of the carrier material (Trivedi and Pandey, 2007). In this study, the potential isolate RA2 was effectively entrapped in two different formulations: talcum powder and sodium alginate beads (Figure 15).

Remarkably, the survival rate of isolate RA2 was found to be highest in the sodium alginate beads compared to the talcum powder-based formulation. This observation underscores the superior efficacy of the sodium alginate beads as a carrier for maintaining the viability of RA2 over an extended period.

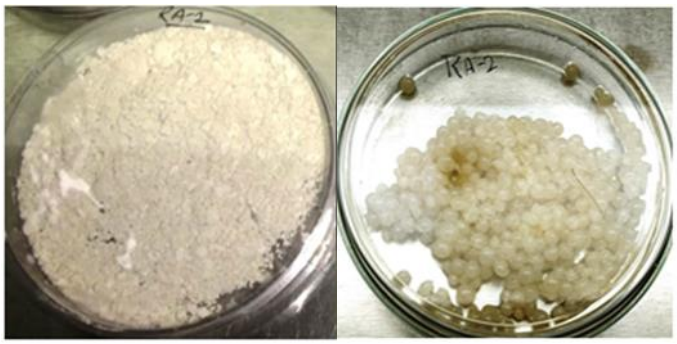


Figure 15. entrapment of isolate RA2 (i)-talcum powder based formulation (ii)- sodium alginate beads based formulation.

Conclusion

Bacillus isolate RA2 demonstrated multifaceted capabilities with significant implications across various domains. It exhibited potent antifungal activity, efficient siderophore production, and phosphorus solubilization. Additionally, RA2 displayed remarkable stress tolerance, plant growth promotion, and promising plastic biodegradation potential. Moreover, its successful encapsulation in sodium alginate beads highlights its suitability for bioformulation. These findings underscore RA2's versatility and potential applications in agriculture, environmental management, and bioremediation, warranting further research for practical implementation.

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