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Morphological and biochemical characterization of effective bio-agents against *Xanthomonas axonopodis pv. punicae*

Ramesh Ippikoppa, Kiran Kumar KC, RK Mesta, Kulapathi Hipparagi, HM Pallavi and S Raghavendra

Abstract
The study focuses on the morphological and biochemical characterization of thirteen representative isolates with effective antagonistic properties against *Xanthomonas axonopodis pv. punicae*, a pathogen causing disease in pomegranate plants. The isolates were obtained from rhizospheric samples of soil were taken in the Maharashtra and Karnataka regions that produce pomegranates. The morphological traits, colony characteristics, gram staining, starch hydrolysis, gelatin liquefaction, spore determination, levan formation, citrate utilization, growth in 7% NaCl, siderophore production, hydrocyanic acid production, and indole acetic acid production were used to identify and characterize the isolates. Additionally, the effect of temperature and pH on the growth of the isolates was examined. The results revealed distinct morphological and cultural characteristics among the isolates, with some exhibiting traits similar to Pseudomonas fluorescens and others to the Bacillus genus. Optimal growth conditions were identified at a temperature of 30 °C and a pH of 7.0 for specific isolates. This comprehensive characterization provides insights into the potential applications of these isolates for biocontrol and agricultural practices.

Keywords: Pomegranate, Morphological characterization, Biochemical characterization, *Xanthomonas axonopodis pv. punicae*, *Pseudomonas fluorescens*, *Bacillus*, Biocontrol

Introduction
Pomegranate (*Punica granatum* L.) holds significant importance as a fruit crop within the Punicaceae family, with its cultivation tracing back to ancient times across the Mediterranean regions of Asia (Holland *et al*., 2009) [5]. Flourishing in arid and semi-arid regions worldwide, its roots are deeply anchored in Iran, while its influence extends to Spain, Morocco, Egypt, Afghanistan, and other Mediterranean nations (Singh *et al*., 2006) [18]. Notably, India has embraced pomegranate cultivation, covering a total cultivation area of 1.43 lakh ha, with Maharashtra leading at 99,140 ha, closely followed by Karnataka at 19,040 ha. The impressive production figures stand at 1773.66 mt, primarily dominated by Maharashtra (13,13,370 MT) and Karnataka (2,04,640 MT). Interestingly, Tamil Nadu claims the lead in productivity (34.43 Mt/ha), followed by Telangana, Maharashtra, Andhra Pradesh, and Karnataka (Anonymous, 2019) [1].

Despite ranking second in both area and production, Karnataka finds itself in the fifth spot in terms of productivity. This lower productivity is attributed to the persistent onslaught of pests and diseases, including bacterial blight, wilt, anthracnose, leaf and fruit spots, and nematodes. Among these afflictions, bacterial blight disease, orchestrated by *Xanthomonas axonopodis pv. punicae*, emerges as a primary impediment, exacting a significant toll on production and productivity.

The emergence of bacterial blight disease in 2001, initially documented by Manjula and Khan (2002) [6] in specific parts of Karnataka and Maharashtra, marked the beginning of its rapid spread across pomegranate-growing regions in India. However, despite its profound impact, studies on bacterial blight remain relatively sparse within the Indian context. A noteworthy contribution by Benagi *et al*. (2009) [2] assessed disease severity during mrig bahar, revealing a range between 0.67% and 94.80% across diverse pomegranate-growing zones. The symptomatic expression of bacterial blight was meticulously examined by Ramesh Ippikoppa *et al*. (2017) [14], illuminating the intricate manifestations of the disease. Leaves bear the initial brunt, displaying minute water-soaked lesions that evolve into brown to dark brown spots, often encircled by a diffused water-soaked margin or a yellow halo.

Over time, these spots converge on a single leaf, giving rise to irregular, expanding lesions. Afflicted leaves eventually distort, turn yellow, and ultimately defoliate. On main stems, twigs, and branches, elongated bluish-black lesions emerge, which later transform into rough, cankerous, dark brown patches. The bark's gradual deterioration manifests through cracking, drying, and eventual decay. Fruits, too, succumb to the disease, with initial appearances of small, diffuse oily lesions that progressively turn brown and become encircled by diffused water-soaked zones. The lesions, once circular, expand into irregular, dark brown patches. The effect on the fruit rind is conspicuous, resulting in cracks that adopt L, Y, or star shapes. As the disease advances, these lesions incite longitudinal splits in affected fruits, thus exposing arils.

Addressing this disease within field conditions necessitates the exploration of various bactericides available in the market. Despite their effectiveness, concerns persist over chemical residues on fruits. The limitations of chemical interventions extend beyond their insufficiency in comprehensive disease management, encompassing pollution and ecological hazards. Furthermore, the export of pomegranates to discerning markets in Europe and the Americas requires rigorous adherence to grading standards, particularly regarding pesticide residue toxicity. This, in turn, casts doubt on the use of antibiotics and bactericides. Hence, the urgency to develop alternative, eco-friendly disease control measures has risen to the forefront.

In this context, the concept of biological control, harnessing antagonistic microorganisms, emerges as a promising non-chemical avenue (Harman, 1991) [4]. Acknowledged for its cost-effectiveness and environmentally benign nature, this method hinges on the members of the Pseudomonas and Bacillus genera. These microorganisms have garnered attention for their potential in subduing bacterial plant diseases (Pant and Mukhopadhyay, 2001) [12]. The proposed biological approach involves collecting native bio-agent isolates from diverse rhizosphere soils, followed by evaluating their antagonistic impact against *Xanthomonas axonopodis pv. punicae*. Notably, *Pseudomonas fluorescens* stands out among these bio-agents, celebrated for its dual ability to promote plant growth and manage diseases effectively (Mazzola *et al*., 1992) [8]. The growth and proliferation of plant pathogenic bacteria are inhibited by the secondary metabolites produced by these bio-control agents, including siderophores, antibiotics, volatile chemicals, HCN, enzymes, and phytohormones (Nagarajkumar *et al*., 2004) [11]. Thus, bacterial bio-agents emerge as an ideal strategy for managing bacterial blight disease in pomegranates, effectively steering cultivation toward a sustainable and eco-friendly path.

Therefore, within this context, bacterial bio-agents are considered ideal disease management strategies for combating bacterial blight of pomegranates. Hence, the present investigation aims to collect, isolate, and morphologically and biochemically characterize effective bio-agents against *Xanthomonas axonopodis pv. punicae.*

Materials and Methods

In the previous study, rhizospheric soil samples were taken from several locations of Maharashtra state and from the key pomegranate-growing districts of Karnataka, including Bagalkot, Vijayapur, Chitradurga, and Koppal. Using the dual culture approach in *in vitro* environments, 170 isolates were collected from the rhizospheric soil and examined for their antagonistic activity against X. a. pv. punicae.57 of the evaluated isolates showed evidence of the capacity to prevent the growth of Xanthomonas axonopodis pv. punicae colonies in *in vitro* conditions. From these fifty-seven isolates, thirteen representative isolates (SM-1A, VK-6B, BK-6, KK-9A, VK-10C, KK-3A, BK-5, BK-3, CK-13A, BK-8, SM-2A, BK-7, and BK-1L) exhibited more pronounced inhibition of Xanthomonas axonopodis pv. punicae, as previously reported by Ramesh Ippikoppa *et al*. (2023) [15] (Table 1). The current work seeks to carry out morphological and biochemical characterisation of these thirteen typical isolates as a continuation of this research.

Table 1: List of the 13 bacterial bio-agent isolates that were utilized in the experiment

Sl. No.	Village	District	State	Codes
1	Sokanadagi	**Bagalkot**	Karnataka	BK-1
2	Sokanadagi	Bagalkot	Karnataka	BK-3
3	Tulasigere	Bagalkot	Karnataka	BK-5
4	Sokanadagi	Bagalkot	Karnataka	BK-6
5	Sector 70 BGK	Bagalkot	Karnataka	BK-7
6	Udyanagiri BGK	Bagalkot	Karnataka	BK-8
7	Kannur	Vijayapura	Karnataka	VK-6
8	Tidagundi	Vijayapura	Karnataka	VK-10
9	Sanehalli	Chitradurga	Karnataka	CK -13
10	Bevuru	Koppal	Karnataka	KK-3
11	Gore Hanchinal	Koppal	Karnataka	KK-9
12	Solapur (MH)	Solapur	Maharashtra	SM-1
13	Solapur (MH)	Solapur	Maharashtra	SM-2

Morphological and biochemical approaches used to characterize effective bioagent isolates

Utilizing cultural characteristics like colony shape, colony type, colony color, reaction to UV light, and biochemical characteristics like gram staining, starch hydrolysis, gelatin liquefaction, production of indole acetic acid (IAA), siderophore, and HCN, purified effective antagonistic bio-agents were identified.

Characterization of morphology

For colony formation, pure cultures of the chosen isolates were streaked separately on petri plates containing nutritional agar. The specific colonies' size, form, colony organization, and color were all evaluated.

A response to UV light

All thirteen isolates were added to the test tubes containing sterilized Kings B medium before being cultured at 30 °C for five days. Positive results were later seen when the yellowish green fluorescent pigment was exposed to UV radiation at 365 nm (Meera and Balabaskar, 2012) [9].

Gram staining: The gram staining approach was used to separate all thirteen isolates into two major categories (gram positive or negative) (Schaad, 1992) [16].

Biochemical characters

Biochemical Tests

KOH Examination (Schaad, 1992) [16].

The 3% KOH solution was combined with one drop of the bacterial suspension. Gram-positive bacteria did not display a sticky consistency when mixed with a loop, while gram-negative bacteria did.

Spore Determination (Schaad, 1992) [16]
On a slide, water droplets were used to suspend and air-dry bacterial colonies produced on agar media. The slide was stained for ten minutes with a 5.0% (w/v) aqueous solution of malachite green. Following a thorough rinse under running water and a brief dry, the slide was counter-stained with a 0.5% (w/v) aqueous solution of safranin for 15 seconds. Following another thorough rinse and blot drying, bacterial cells were observed at 40x magnification. Bacterial cells appeared red, while spores were green.

Hydrolysis of starch (Schaad, 1992) [16]
On starch agar plates, isolates were plated and cultured for five days. The Lugol's iodine solution was poured liberally onto the plates. Starch use by the bacteria was shown by the presence of distinct, colorless zones (halos) surrounding the bacterial growth and by the blue coloring of the remaining plates.

Liquefaction of gelatin (Schaad, 1992) [16]
Gelatin medium was made by dissolving the components in steam, dispensing 4–10 ml portions into tubes, and sterilizing the tubes for 12–15 minutes at 121°C. The tubes were quickly chilled without tilting and maintained at a cold temperature until inoculation. Stabbing inoculation was used to introduce cells from a 24-hour agar slant into the gelatin medium, which was subsequently incubated at 27 °C. The tubes were kept at 4 °C for 30 minutes following 3 days before the results were recorded. If the material flowed easily with slight tilting, gelatin hydrolysis had occurred, which was a good result. The outcomes were contrasted with non-inoculated media that had undergone comparable incubation and cooling.

Formation of Levan (Schaad, 1992) [16]
On nutritional agar treated with 5% sucrose (w/v), levan formation was discovered. After three to five days of incubation, the development of convex, white mucoid colonies suggested that the experiment was successful.

Citrate Utilization (Schaad, 1992) [16]
Simmons' citrate agar was dispensed into test tubes, autoclaved at 121°C for 15 minutes, and cooled in a slanted position. Bioagents were inoculated and incubated, and after 24-48 hours, a deep Prussian blue color indicated citrate utilization, while the original green color indicated non-utilization.

Growth of bioagents in 7% NaCl (Schaad, 1992) [16]
After being infused with nutrition broth containing 0.5% glucose and 7% NaCl, bioagent cultures were cultured on an orbital shaker. Daily observations were made, with increased turbidity in the broth indicating bioagent growth compared to an uninoculated control.

Production of Siderophores
The Chrome Azurol S (CAS) plate assay was used to evaluate the quality of siderophore production by all isolates (Schwyn and Neilands, 1987) [17]. At 28 °C, isolates were cultured for 48 hours after being spotted onto cetrimide agar. After incubation, the bacterial growth was covered with a thin layer of CAS reagent in 0.7% agar. Followed by another 24-hour incubation at 28°C. The presence of a yellow-orange color zone around the colonies indicated siderophore production.

Hydrocyanic Acid Production
An altered approach based on Wei *et al*. (1991) [20] was used to estimate the generation of hydrocyanic acid (HCN). On TSA that had 4.4 g/L of glycine added as a supplement, bacterial strains were grown. The lids of each petri dish were covered with filter paper strips dipped in a picric acid solution (0.5% picric acid, 2% sodium carbonate). The filter paper was tested for color changes after four days of incubation at 28°C; a brown tint indicated the presence of HCN.

The production of indole acetic acid
The standard procedure described by Bric *et al*. (1991) [3] was used to evaluate the synthesis of indole acetic acid (IAA). Bioagents were infused with 500 g/mL of tryptophan or not infused with tryptophan in nutrient broth, and then incubated at 30 °C for 24 hours. Each tube had five milliliters of culture removed, and for 15 minutes it was centrifuged at 10,000 rpm. 2 mL of the supernatant's aliquot was transferred to new tubes. Followed by the addition of 100 µL of 10 mM orthophosphoric acid and 4 mL of Salkowski's reagent (1 mL of 0.5M FeCl3 in 50 mL of 35% perchloric acid). The mixture was incubated at room temperature for 30 minutes, and the development of a pink color was considered a positive result.

Effects of temperature and pH on the development in particular bioagent isolates
Effect of Temperature: This study aimed to determine the optimal temperature requirements for the growth of four selected isolates using Nutrient Agar (NA) as the basal medium. A loopful of a 72-hour-old bacterial culture was serially diluted with 9 ml of sterile water. One milliliter of a 10^5 dilution was plated onto the surface of nutrient agar medium in sterilized petri plates, and the suspension was evenly spread using a sterilized spreader. The inoculated plates were then incubated at different temperature levels: 0°C, 5°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C for 72 hours. Observations were made regarding colony development on the plates at specific temperature levels. Colony counts were recorded, and the data were analyzed using appropriate statistical procedures.

Effect of pH: The impact of hydrogen ion concentration on the growth of all thirteen isolates was examined by adjusting the pH of the Nutrient Agar (NA) medium to various levels: 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, and 11.0, using suitable phosphate buffers. A loopful of a 72-hour-old bacterial culture was serially diluted with 9 ml of sterile water. One milliliter of a 10^5 dilution was plated onto the surface of the medium adjusted to various pH levels. The suspension was spread uniformly over the medium using a sterilized spreader. The inoculated plates were then incubated at a temperature of 30°C for 72 hours. After the incubation period, observations were recorded regarding colony development in media with different pH levels. Colony counts were recorded and statistically analyzed.

Results
The morphological, cultural, and biochemical characterisation of thirteen successful isolates was done.

Cultural and Morphological Aspects
Table 2 displays the findings in relation to several morphological and cultural features.

Gram Staining
Nine isolates (VK-6B, KK-9A, VK-10C, KK-3A, BK-5, BK-3, CK-13A, SM-2A, and BK-1L) out of the thirteen isolates showed a positive Gram staining reaction. A negative response was shown by the remaining four isolates (SM-1A, BK-6, BK-8, and BK-7). Gram-positive bacteria retained the crystal violet dye, while a counterstain (safranin) imparted a red or pink color to all Gram-negative bacteria.

Fluorescence Under UV Light
Only three of the thirteen effective isolates (BK-6, BK-8, and BK-7) showed a fluorescent pigment that was yellowish-green under 365 nm UV light. The remaining isolates did not exhibit this fluorescence (Fig 1).

Colony Color
Different colony colors could be seen in the thirteen efficient isolates. In particular, colonies from isolates SM-1A, BK-6, BK-8, and BK-7 were creamy white; those from isolates VK-6B, VK-10C, BK-5, and BK-3 were dull white; and colonies from isolates KK-9A, KK-3A, CK-13A, SM-2A, and BK-1L were bright white.

Table 2: Morphological characters of effective isolates of bacterial antagonists

Sl. No.	Bio agents	Gram staining	Colony Colour	Colony Shape	Colony Nature	Colony Margin /Edge	Shape of the cell	Colony Surface	Colony Elevation	Reaction to UV light
1	SM-1A	Negative	Creamy white	irregular	Non Spreading	Undulate	Rod	Smooth	Umbonate	Negative
2	VK-6B	Positive	Dull white	filamentous	Spreading	Filamentous	Rod	Wrinkled	Convex	Negative
3	BK-6	Negative	Creamy white	Rhizoid	Non Spreading	Lobate	Rod	Glistering	Flattened	Positive
4	KK-9A	Positive	Bright White	Filamentous	Spreading	Filamentous	Rod	Wrinkled	Raised	Negative
5	VK-10C	Positive	Dull white	Filamentous	Spreading	Filamentous	Rod	Dry	Flattened	Negative
6	KK-3A	Positive	Bright White	Rhizoid	Non Spreading	Lobate	Rod	Smooth	Umbonate	Negative
7	BK-5	Positive	dull white	Filamentous	Spreading	Filamentous	Rod	Dry	Flattened	Negative
8	BK-3	Positive	dull white	Filamentous	Spreading	Filamentous	Rod	Dry	Flattened	Negative
9	CK-13A	Positive	Bright white	Filamentous	Spreading	Filamentous	Rod	Dry	Flattened	Negative
10	BK-8	Negative	Creamy white	Rhizoid	Non Spreading	Lobate	Rod	Glistering	Flattened	Positive
11	SM-2A	Positive	Bright white	Filamentous	Spreading	Filamentous	Rod	Smooth	Raised	Negative
12	BK-7	Negative	Creamy white	Rhizoid	Non Spreading	Lobate	Rod	Glistering	Raised	Positive
13	BK-1L	Positive	Bright white	Filamentous	Spreading	Filamentous	Rod	Dry	Pulvinate	Negative

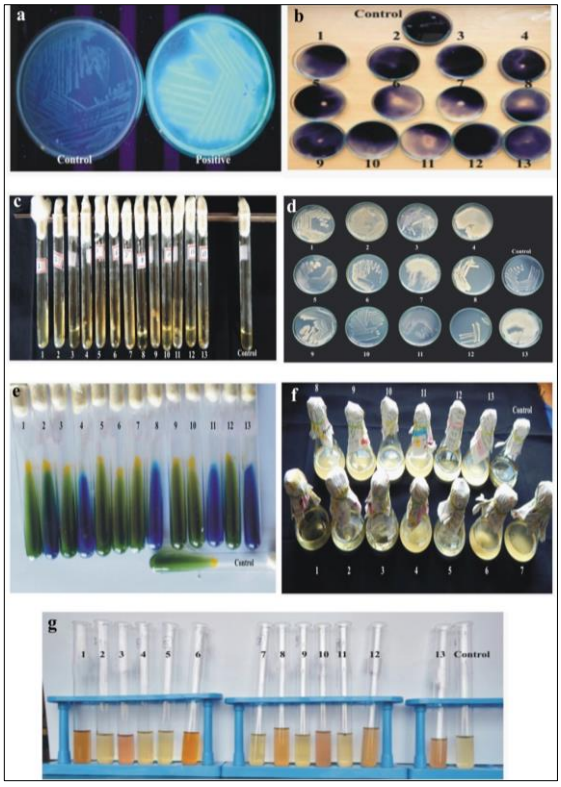

Fig 1: Morphological and Biochemical characters of effective isolates of bacterial bio-agents; The yellowish green fluorescent pigment under UV light (9a); Starch Hydrolysis (9b); Gelatine liquefaction (9c); Levan formation (9d); Citrate utilization (9e); Growth at 7% NaCl (9f); IAA production (9g)

Colony Shape
SM-1A, one of the thirteen isolates, had colonies with irregular shapes. The colonies of the isolates VK-6B, KK-9A, VK-10C, BK-5, BK-3, CK-13A, SM-2A, and BK-1L were filamentous, whereas the colonies of the isolates BK-6, KK-3A, BK-8, and BK-7 were rhizoid-shaped.

Bio-Agents' Nature
During their growth phase, the isolates SM-1A, BK-6, KK-3A, BK-8, and BK-7 displayed a non-spreading character. During their growth period, the remaining isolates (VK-6B, KK-9A, VK-10C, BK-5, BK-3, CK-13A, SM-2A, and BK-1L) showed a spreading character.

Colony Margin/Edge
Colony growth at the margin was observed as undulated for SM-1A, filamentous for VK-6B, KK-9A, VK-10C, BK-5, BK-3, CK-13A, SM-2A, and BK-1L, and lobate for BK-6, KK-3A, BK-8, and BK-7.

Cell Shape
Rod-shaped cells were observed across all thirteen effective isolates.

Colony Surface
Isolate surfaces appeared smooth for SM-1A, KK-3A, and SM-2A, wrinkled for VK-6B and KK-9A, dry for VK-10C, BK-5, BK-3, CK-13A, and BK-1L, and glistening for BK-6, BK-8, and BK-7.

Colony Elevation
Colony elevation varied, with isolates SM-1A and KK-3A being umbonate, VK-6B, BK-6, VK-10C, BK-5, BK-3, KK-13A, and BK-8 being convex, and BK-7 and BK-1L being raised and pulvinate respectively.

Biochemical Examinations
Table 3 and Fig. 1b–1g provide the results of the biochemical testing.

KOH Test
Four isolates (SM-1A, BK-6, BK-8, and BK-7) had a gummy look after being exposed to 3% KOH, according to observations regarding the gummy appearance. This appearance was absent in the other nine isolates (VK-6B, KK-9A, VK-10C, KK-3A, BK-5, BK-3, CK-13A, SM-2A, and BK-1L).

Determination of Spores
Five isolates (VK-6B, KK-9A, BK-3, SM-2A, and BK-1L) were shown to be able to produce green spores, according to microscopic examination. The other isolates (SM-1A, BK-6, VK-10C, KK-3A, BK-5, CK-13A, BK-8, and BK-7) only had red-colored cells and failed to form green spores.

Starch Hydrolysis
The clear, colorless zones surrounding their growth and the blue coloration in the rest of the plates demonstrated that nine isolates (VK-6B, KK-9A, VK-10C, KK-3A, BK-5, BK-3, CK-13A, SM-2A, and BK-1L) were capable of hydrolyzing starch. Four isolates (SM-1A, BK-6, VK-10C, and BK-7) did not exhibit colorless zones around their colonies because they were unable to hydrolyze starch.

Liquefication of gelatin
Nine isolates (SM-1A, VK-6B, KK-9A, VK-10C, KK-3A, BK-5, CK-13A, SM-2A, and BK-1L) showed a positive response to the gelatin liquefaction test, producing hydrolyzed gelatin that remained liquid even at low temperatures (4°C for 30 minutes). The remaining four isolates (BK-6, BK-3, BK-8, and BK-7) displayed negative responses and remained solid, as did the uninoculated control medium.

Table 3: Biochemical characters of effective isolates of bacterial bio-agents

Sl. No	Isolates Bio-chemical characters	SM-1A	VK-6B	BK-6	KK-9A	VK-10C	KK-3A	BK-5	BK-3	CK-13A	BK-8	SM-2A	BK-7	BK-1L
1	Gummy appearance on 3% KOH	Positive	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Negative	Positive	Negative
2	Starch hydrolysis	Negative	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Positive	Negative	Positive
3	Gelatine liquefaction	Positive	Positive	Negative	Positive	Positive	Positive	Positive	Negative	Positive	Negative	Positive	Negative	Positive
4	growth @7% NaCl	Negative	Positive	Negative	Positive	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Positive
5	HCN production	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
6	Levan formation	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
7	Spore formation	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Positive	Negative	Negative	Positive	Negative	Positive
8	Citrate utilization	Negative	Positive	Negative	Positive	Negative	Negative	Negative	Positive	Negative	Negative	Positive	Negative	Positive
9	IAA production	Positive	Negative	Positive	Negative	Negative	Positive	Negative	Positive	Negative	Negative	Positive	Negative	Positive
10	Siderophore production	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative

Levan Formation
After five days of incubation, convex, white mucoid colonies appeared on all thirteen isolates, indicating levan formation.

Citrate Utilization: Only five isolates (VK-6B, KK-9A, BK-3, SM-2A, and BK-1L) were able to use citrate after 48 hours of incubation, resulting in a profound Prussian blue color change. Eight other isolates (SM-1A, BK-6, VK-10C, KK-3A, BK-5, CK-13A, BK-8, and BK-7) did not use citrate and maintained their natural green color.

Growth of bioagents in 7% NaCl
In nutrient broth that had been added with 0.5% glucose and 7% NaCl, the growth response of the thirteen isolates was assessed. For incubation, the inoculated flasks were set up on an orbital shaker. Nine flasks containing different isolates (VK-6B, KK-9A, KK-3A, BK-5, BK-3, CK-13A, BK-8, SM-2A, and BK-1L) showed turbidity after three days of incubation. The absence of turbidity in the other four isolate-containing flasks (SM-1A, BK-6, VK-10C, and BK-7), however, showed that no growth had occurred in those flasks (Fig. 1f).

Siderophore Production
Qualitative assessment of siderophore production for all isolates was conducted using the Chrome Azurol S (CAS) plate assay, as detailed in the materials and methods (Section 3.9.2.8). Notably, none of the bacterial bio-agent isolates demonstrated the ability to produce siderophores, as evidenced by the lack of formation of a yellow-orange color zone around the colonies in the plate assay.

HCN Production
The capacity for Hydrocyanic acid (HCN) production was assessed in all thirteen isolates. Regrettably, none of the isolates exhibited the capability to produce Hydrocyanic acid, resulting in a negative reaction across the board.

Indole Acetic Acid Production
The thirteen bacterial bio-agent isolates were tested for their ability to produce indole acetic acid. Six of them (SM-1A, BK-6, KK-3A, BK-3, SM-2A, and BK-1L) showed the emergence of a pink tint, demonstrating a successful generation of indole acetic acid. The lack of pink colouring in the remaining seven isolates (VK-6B, KK-9A, VK-10C, BK-5, CK-13A, BK-8, and BK-7) indicates that they were unable to generate indole acetic acid (Fig. 1g).

Temperature and pH's impact on bioagent growth
The impact of temperature
Under *in vitro* conditions, several temperature levels were investigated for their effects on the growth and proliferation

of the four efficient isolates: 0 °C, 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, 45 °C, and 50 °C. The results are presented in Table 4. Notably, the impact of temperature on Isolate-SM-1A exhibited statistical significance. Optimal growth occurred at 30 °C, yielding the highest colony count (147.3 x 10^5 cfu/ml), followed by 25 °C (137.7 x 10^5 cfu/ml) and 35 °C (89.0 x 10^5 cfu/ml). Similar trends were observed for Isolate-VK-6B, Isolate-BK-6, and Isolate-KK-9A, where the temperature of 30 °C proved most favorable for colony growth. Significantly elevated colony counts (156.3 x 10^5 cfu/ml, 150 x 10^5 cfu/ml, and 154.0 x 10^5 cfu/ml respectively) were achieved at this temperature, followed by 25 °C (142.3 x 10^5 cfu/ml, 136.7 x 10^5 cfu/ml, and 132.7 x 10^5 cfu/ml respectively) and 35 °C (89 x 10^5 cfu/ml, 87.7 x 10^5 cfu/ml, and 85.3 x 10^5 cfu/ml respectively). Notably, growth was observed within the temperature range of 15 °C to 45 °C, with failure to thrive observed at both the lowest (10 °C) and highest (50 °C) extremes.

Table 4: Effect of temperature on growth of selected bacterial bio-agents under *in vitro* condition

Sl. No.	Temperature levels (˚C)	Number of colonies of the bacterial bio-agents				Average
		SM-1A	VK-6B	BK-6	KK-9A	
1	0	0.0 (0.70)	0.0 (0.70)	0.0 (0.70)	0.00 (0.70)	0
2	5	0.0 (0.70)	0.0 (0.70)	0.0 (0.70)	0.0 (0.70)	0
3	10	0.0 (0.70)	0.0 (0.70)	0.0 (0.70)	0.0 (0.70)	0
4	15	20.0 (5.44)	29.0 (5.44)	26.3 (5.18)	21.0 (4.64)	24.07
5	20	38.3 (6.45)	41.0 (6.45)	39.0 (6.29)	42.7 (6.57)	40.25
6	25	137.7 (11.96)	142.3 (11.96)	136.7 (11.72)	132.7 (11.53)	137.35
7	30	147.3 (12.53)	156.3 (12.53)	150.0 (12.27)	154.0 (12.42)	151.90
8	35	89.0 (9.46)	89.0 (9.46)	87.7 (9.38)	85.3 (9.27)	87.75
9	40	38.3 (6.20)	38.0 (6.20)	37.7 (6.18)	38.3 (6.24)	38.07
10	45	22.0 (5.21)	26.7 (5.21)	26.0 (5.14)	25.0 (5.04)	24.92
11	50	0.0 (0.70)	0.0 (0.70)	0.0 (0.70)	0.0 (0.70)	0
S.Em±		0.07	0.04	0.05	0.06	
CD (0.01)		0.26	0.22	0.22	0.24	

Note: Figures in parentheses indicate Square root transformed values

Result of pH: The goal of this experiment was to determine how pH affected the development of four different bacterial bio-agent isolates. According to "Materials and Methods (3.10)," the study included a pH range from 3.0 to 11.0, and the results are listed in Table 5. The data highlighted a correlation between pH and bacterial colony count for Isolate-SM-1A, Isolate-VK-6B, Isolate-BK-6, and Isolate-KK-9A. An increase in pH within the range of 4.0 to 7.0 led to an augmented number of bacterial colonies, followed by a decline with further pH elevation. Optimal pH levels for maximal growth across all four isolates were determined to be 7.0 and 7.5. Specifically, the greatest colony counts (147.3 x 10^5 cfu/ml, 148 x 10^5 cfu/ml, 150 x 10^5 cfu/ml, and 136.7 x 10^5 cfu/ml respectively) were recorded at pH 7.0, followed closely by pH 7.5 (136.0 x 10^5 cfu/ml, 132.0 x 10^5 cfu/ml, 136.3 x 10^5 cfu/ml, and 126.3 x 10^5 cfu/ml respectively). At pH 4.0, Isolate-VK-6B and Isolate-KK-9A managed to yield colony counts of 4.3 x 10^5 cfu/ml and 5.0 x 10^5 cfu/ml respectively, while no colonies were detected for Isolate-SM-1A and Isolate-BK-6. Conversely, at pH 10.5, colony counts of 6.0 x 10^5 cfu/ml, 4.7 x 10^5 cfu/ml, 6.3 x 10^5 cfu/ml, and 5.7 x 10^5 cfu/ml were achieved for Isolate-SM-1A, Isolate-VK-6B, Isolate-BK-6, and Isolate-KK-9A respectively. Beyond pH 10.5, no colonies were viable. Notably, pH levels below 4.0 and above 10.5 were incapable of supporting colony growth across all four isolates.

Table 5: Effect of pH on growth of selected bacterial bio-agents under *in vitro* condition

Sl. No.	pH levels	Number of colonies of the bacterial bio-agents				Average
		SM-1A	VK-6B	BK-6	KK-9A	
1	3.0	0.0 (0.70)	0.0 (0.70)	0.0 (0.70)	0.0 (0.70)	0.00
2	3.5	0.0 (0.70)	0.0 (0.70)	0.0 (0.70)	0.0 (0.70)	0.00
3	4.0	0.0 (0.70)	4.3 (2.19)	0.0 (0.70)	5.0 (2.34)	2.33
4	4.5	0.0 (0.70)	7.7 (2.85)	0.0 (0.70)	9.0 (3.07)	4.17
5	5.0	10.0 (3.23)	13.0 (3.68)	9.0 (3.07)	14.3 (3.85)	11.58
6	5.5	15.0 (3.94)	27.7 (5.31)	19.3 (4.45)	28.0 (5.34)	22.50
7	6.0	37.7 (6.17)	45.7 (6.80)	32.0 (5.70)	46.0 (6.82)	40.33
8	6.5	79.0 (8.91)	87.3 (9.38)	71.0 (8.46)	82.0 (9.09)	79.83
9	7.0	147.3 (12.15)	148.0 (12.19)	150.3 (12.28)	136.7 (11.72)	145.58
10	7.5	136.0 (11.68)	132.0 (11.52)	136.3 (11.70)	126.3 (11.27)	132.67
11	8.0	104.0 (10.22)	109.7 (10.50)	90.0 (9.51)	107.3 (10.38)	102.75
12	8.5	75.0 (8.69)	79.0 (8.91)	74.0 (8.63)	87.3 (9.38)	78.83
13	9.0	47.3 (6.91)	48.0 (6.97)	46.0 (6.82)	41.3 (6.47)	45.67
14	9.5	27.7 (5.31)	27.7 (5.31)	32.0 (5.70)	31.3 (5.64)	29.67
15	10.0	16.0 (4.05)	14.7 (3.89)	22.3 (4.77)	14.3 (3.85)	16.83
16	10.5	6.0 (2.54)	4.7 (2.28)	6.3 (2.60)	5.7 (2.47)	5.67
17	11.0	0.0 (0.70)	0.0 (0.70)	0.0 (0.70)	0.0 (0.70)	0.00
S.Em±		0.09	0.09	0.08	0.10	
CD (0.01)		0.34	0.35	0.24	0.40	

Note: Figures in parentheses indicate Square root transformed values

Discussion

In this study, we observed distinct morphological and cultural characteristics among the thirteen isolates. Colonies displayed varying colors, with four isolates exhibiting creamy white colonies, four displaying dull white colonies, and five showing bright white colonies. Colony shape analysis revealed that eight isolates had a filamentous shape, four had a rhizoid shape, and one demonstrated an irregular shape. Spreading habits on growing media indicated that seven isolates exhibited a spreading nature, while the remaining isolates displayed a non-spreading nature. Regarding colony margin, four isolates had a lobate margin, eight isolates had a filamentous margin, and one had an undulate margin. All isolates were characterized by rod-shaped cells. Surface characteristics included wrinkled surfaces for two isolates, glistering surfaces for three, smooth surfaces for three, and dry surfaces for the rest. Elevational differences among colonies were noted, with three isolates having a raised elevation, two an umbonate elevation, one a convex elevation, one a pulvinate elevation, two with a raised elevation, and the remainder with a flattened colony surface. Regarding fluorescence under UV light, only three isolates exhibited positive fluorescence, while the rest did not fluoresce.

Preeti *et al*.'s (2011) [13] study, which involved the isolation of 28 bacterial species from rhizosphere soil, is very similar to our own work. The isolated organisms were mucoid colony surfaces on motile rods. Four of these isolates (Pseudomonas) were classified as gram-negative, and the remaining isolates (Bacillus) as gram-positive. The isolates exhibited distinct cultural characteristics on solid media, echoing our findings. Additionally, the investigation by Manjunatha and Naik (2013) [7] aligned with our study's focus. Two fluorescent Pseudomonas bacterial isolates (RPF-13 and RPF-81) from rhizosphere soil were described. According to our observations, these isolates were determined to be Gram-negative, rod-shaped bacteria that produced fluorescent pigment on King's B medium and displayed fluorescence under UV light.

To characterize thirteen chosen efficient bacterial bio-agent isolates, a wide range of biochemical tests, including gram staining, KOH test, spore determination, starch hydrolysis, gelatin liquefaction, levan formation, citrate utilization, growth in 7% NaCl, siderophore production, hydrocyanic acid production, and indole acetic acid production, were used. Gram staining results indicated that four out of thirteen isolates (SM-1A, BK-6, BK-8, and BK-7) exhibited a gram-negative nature, displaying a gummy appearance upon exposure to 3% KOH and an inability to hydrolyze starch. The remaining nine isolates (VK-6B, KK-9A, VK-10C, KK-3A, BK-5, BK-3, CK-13A, SM-2A, and BK-1L) were gram-positive and did not display gummy appearance on KOH test, yet were capable of starch hydrolysis. Endospore formation was observed in isolates VK-6B, KK-9A, BK-3, SM-2A, and BK-1L, which also exhibited citrate utilization. Conversely, the remaining eight isolates failed to show endospore formation and were unable to utilize citrate. Siderophore production and hydrocyanic acid production were not detected in any of the isolates. However, levan formation was a common trait among all isolates. Notably, isolates BK-6, BK-3, BK-8, and BK-7 showed a negative response to gelatin liquefaction, while SM-1A, BK-6, VK-10C, and BK-7 were unable to grow in 7% NaCl.

The isolates BK-6, BK-8, and BK-7 may be related to P. fluorescens based on morphological traits such creamy white colonies, rod-shaped cells, positive responses to UV light, the KOH test, levan formation, and IAA generation, along with negative gram staining and endospore formation. The diverging UV light sensitivity of isolate SM-1A, however, raises the possibility that it may belong to a different species of Pseudomonas. In contrast, the nine isolates that were found to be positive for gram staining, starch hydrolysis, gelatin liquefaction, growth in 7% NaCl, and endospore formation as well as morphological characteristics like white to dull white colonies, filamentous shape, spreading nature, and rod-shaped cells may belong to the genus Bacillus.

Comparisons with previous studies yielded intriguing insights. Preeti *et al*. (2011) [13] identified similar patterns, grouping gram-negative isolates exhibiting characteristics such as cream-white colonies, mucoid surface, undulate margin, regular and irregular forms, abundant growth, negative gram staining, negative endospore staining, and rod-shaped bacterial cells as *Pseudomonas*. Additionally, Meera and Balabaskar's work in 2012 with effective native isolates of *P. fluorescens* corroborated our findings in terms of gram staining, starch hydrolysis, gelatin liquefaction, catalase test, oxidase test, and fluorescent pigmentation. IAA production was universally positive among the isolates (Meera and Balabaskar, 2012) [9]. Mulla *et al*. (2013) [10] categorized isolates from different zones of Karnataka as P. fluorescens based on gram reaction, gelatin liquefaction, and indole production.

In the evaluation of varied temperature levels for the growth of the four selected bacterial bio-agent isolates, it was determined that an optimal temperature of 30°C facilitated the maximum growth for all isolates, exhibiting the highest average colony count of 151.9 cfu/ml. Nevertheless, these isolates displayed growth across a broad range of temperature levels, spanning from 15 to 45 °C, with no growth observed at the extreme temperature limits of 50 °C and 5 °C.

Analysis of the impact of pH on the growth of the chosen bacterial bio-agent isolates revealed an interesting trend. The growth, indicated by the colony count, exhibited an increase as the pH of the medium escalated from 4.0 to 7.0. Beyond a pH of 7.0, however, the growth declined with a rise in pH. The most prolific growth was observed at pH 7.0, with an average colony count of 145.58 cfu/ml, which was significantly higher compared to other pH levels. Notably, colony counts were found to be significantly lower at the lowest (pH 4.0, 2.33 x 10^5 cfu/ml) and highest (pH 10.5, 5.6 x 10^5 cfu/ml) pH levels. These results align with those of Wang *et al*. (2002) [19], who investigated the pH and temperature preferences of Bacillus subtilis. Similar results showed that the pH value of 7 and the temperature range of 25–30 °C were optimal for the bacterium's growth.

Conclusion

The present study embarked on a comprehensive exploration of thirteen bacterial antagonists (SM-1A, VK-6B, BK-6, KK-9A, VK-10C, KK-3A, BK-5, BK-3, CK-13A, BK-8, SM-2A, BK-7, and BK-1L), meticulously chosen based on their outstanding performance. These isolates underwent meticulous morphological and biochemical characterization, shedding light on their diverse traits and potential applications. Among these antagonists, BK-6, BK-7, and BK-8 exhibited morphological traits closely aligned with Pseudomonas fluorescens. Their distinct features, such as

creamy white colonies, rod-shaped cells, positive reactions under UV light, KOH test, levan formation, and indole acetic acid production, coupled with negative gram staining and endospore formation, strongly support their classification within the Pseudomonas genus. In contrast, the remaining nine antagonists exhibited characteristics indicative of the Bacillus genus. These isolates demonstrated positive outcomes in gram staining, starch hydrolysis, gelatine liquefaction, growth in 7% NaCl, and displayed morphological traits such as white to dull white colonies with filamentous shapes, a spreading nature, rod-shaped cells, and, in certain instances, endospore production. Our thorough investigations into temperature and pH preferences yielded crucial insights. Optimal growth conditions were identified at a temperature of 30 °C and a pH of 7.0 for a specific subset of bacterial antagonists, namely SM-1A, VK-6B, BK-6, and KK-9A, where they exhibited the highest average colony counts (151.9 and 145.33 x 10^5 cfu/ml, respectively). These findings underscore the importance of meticulous environmental control when harnessing these bacterial antagonists for biocontrol and agricultural applications. Hence, the systematic characterization of morphological and biochemical attributes, combined with insights into optimal growth conditions, provides a comprehensive understanding of the potential of these bacterial antagonists across diverse applications. The clear differentiation of isolates into Pseudomonas fluorescens and Bacillus genus forms a solid foundation for targeted usage and further research in the realms of biocontrol and agriculture. These findings enrich our understanding of these bacterial antagonists, paving the way for their effective implementation and management strategies across varied agricultural contexts. As we continue to harness the potential of these bacterial antagonists, their role in promoting sustainable agricultural practices becomes increasingly promising.

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