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Isolation, screening and phenotypic characterization of biofilm-forming bacteria from farm pond water of semi-arid North Gujarat, India

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ABSTRACT

Bacteria that grow as surface-attached, matrix-enclosed communities are increasingly viewed as superior candidates for agricultural inoculants, because the biofilm mode of life confers desiccation tolerance, protection from predation and a competitive advantage during root colonisation. Standing farm water bodies represent an under-explored reservoir of such organisms in semi-arid regions. In this study, water was sampled from two farm ponds on the campus of Sardarkrushinagar Dantiwada Agricultural University, Gujarat, India, and fifteen morphologically distinct bacteria (R1–R15) were recovered on nutrient agar. All fifteen isolates were screened for biofilm formation by the tube staining assay, the quantitative microtitre plate assay and the Congo red agar assay. The three methods were fully concordant and identified five strongly adherent isolates, recoded RBF1–RBF5, with OD_{570} values of 0.659–0.789 against a cut-off optical density of 0.038 (i.e. greater than $4 \times OD_c$). The five isolates were rod-shaped, uni-cellular and catalase positive; RBF1 and RBF3 were Gram positive and RBF2, RBF4 and RBF5 Gram negative, an assignment independently confirmed by the 3 % KOH string test. Colonies were small, off-white to light yellow, mostly irregular in form with dry, leathery surfaces. All five isolates utilised citrate and none utilised adonitol, arabinose, lactose or sorbitol; nitrate was reduced by RBF1, RBF2 and RBF5. Extracellular amylase was produced by RBF1 and RBF2 only, and lipase by none of the isolates. Physiologically, biofilm formation peaked at 35 °C and at 10 % sucrose in all five isolates, and was greatest at pH 7 for RBF3, RBF4 and RBF5, whereas RBF1 and RBF2 formed most biofilm under mildly acidic conditions (pH 5). The five isolates therefore combine robust, reproducible adherence with a broad physiological operating window, and constitute a phenotypically defined collection suitable for further evaluation as biofilm-based bioinoculants.

Keywords: Biofilm, Congo red agar, Crystal violet, Extracellular polymeric substances, Microtitre plate assay, Pond water, Phenotypic characterization

1. Introduction

A biofilm is an assemblage of microbial cells irreversibly associated with a surface and enclosed within a self-produced matrix of extracellular polymeric substances (EPS) composed predominantly of polysaccharides, proteins, extracellular DNA and lipids (Flemming *et al.*, 2010) [1]. Biofilms develop on a wide range of living and inert surfaces, from plant roots and animal tissues to water distribution pipes and natural aquatic systems, and their architecture is governed by the nature of the substratum, the hydrodynamics of the system, nutrient availability and grazing pressure. Formation proceeds through a well-described sequence in which planktonic cells attach to a conditioned surface, organise into micro-colonies, and finally encase themselves in a differentiated extracellular matrix from which cells subsequently disperse to colonise new niches (Hall-Stoodley *et al.*, 2004; Davey *et al.*, 2000) [3, 2].

The biofilm mode of growth is not merely an architectural curiosity. Cells within a biofilm are markedly more resistant to antimicrobial agents, desiccation and protozoan predation than their planktonic counterparts, and the matrix facilitates horizontal gene transfer and the concentration of secreted metabolites in a defined microenvironment (Costerton *et al.*, 1999; Jefferson, 2004) [4, 5]. In soil, these properties underpin the ecological role of biofilms in maintaining nutrient turnover and in sustaining plant growth (Burmølle *et al.*, 2012) [6].

These same properties have prompted a reappraisal of plant growth-promoting rhizobacteria (PGPR). Interactions among PGPR have been explored largely in the planktonic state, yet the behaviour of these organisms as root-associated biofilms is only beginning to be understood (Angus *et al.*, 2013) [7]. Biofilm-forming PGPR express plant growth-promoting traits, including diazotrophic nitrogen fixation, indole-3-acetic acid synthesis, phosphate and iron solubilisation and ammonia production, more strongly than non-

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biofilm-forming strains, and they produce substantially higher amounts of antimicrobial compounds (Pandian *et al.*, 2017; Backer *et al.*, 2018; Ajijah *et al.*, 2023) [9, 10, 8]. Critically, native biofilm-producing PGPR retain their performance under field conditions, where many laboratory-selected planktonic inoculants fail (Timmusk *et al.*, 2005) [11]. Strains isolated for their biofilm-forming ability have since been shown to promote growth in tomato under water deficit (Haque *et al.*, 2020) [12], in sunflower under salinity (Yasmeen *et al.*, 2020) [13] and in wheat under field conditions (Rafique *et al.*, 2024) [14].

Isolation of such organisms has concentrated on the rhizosphere, but standing water bodies are a rich and comparatively neglected source. Pond water supports diverse attached communities that improve water quality through nitrification, sequester heavy metals and constitute a protein-rich resource in aquaculture systems (Pandey *et al.*, 2014; Kurniawan *et al.*, 2019) [16, 15]. Bacteria recovered from such habitats have already been shown to form biofilms and to carry plant growth-promoting attributes (Merina *et al.*, 2011; Nair *et al.*, 2021) [17, 18]. Farm ponds in semi-arid regions are of particular interest because their residents experience wide diurnal and seasonal swings in temperature, pH and solute concentration, and are therefore pre-selected for the physiological robustness required of a field-deployed inoculant.

Before any such organism can be evaluated agronomically, its identity as a genuine biofilm former must be established by more than one independent method, and its phenotype must be defined. Qualitative tube staining, quantitative microtitre plate assay and Congo red agar morphotyping each detect a different facet of the adherent phenotype, and concordance between them provides considerably stronger evidence than any one assay alone (Christensen *et al.*, 1982; Stepanović *et al.*, 2000; Mathur *et al.*, 2006) [19, 20, 21]. Equally, the temperature, pH and carbon source at which adherence is maximal define the conditions under which a candidate strain will actually perform.

The present study was therefore undertaken to isolate bacteria from the water of two farm ponds in North Gujarat, to identify the strong biofilm formers among them using three independent screening assays, and to characterise the selected isolates morphologically, biochemically and physiologically.

2. Materials and Methods

2.1. Study site and collection of water samples

The investigation was carried out at the Department of Microbiology, Chimanbhai Patel College of Agriculture, Sardarkrushinagar Dantiwada Agricultural University (SDAU), Sardarkrushinagar, Banaskantha district, Gujarat, India. Water was sampled from two farm ponds on the University campus, one at the Vegetable Research Station (24° 19' 00" N, 72° 19' 00" E) and one at the Centre for Natural Resource Management, CNRM (24° 18' 43" N, 72° 16' 24" E). The pH of both water bodies ranged from 6.5 to 8.5. Samples were drawn aseptically from the centre of each pond at a depth of 45 cm; 250 mL was collected into a sterile 500 mL conical flask and transported to the laboratory, where it was held at 4 °C until processed.

2.2. Isolation of bacteria

A 10 mL aliquot of each water sample was suspended in 90 mL sterile physiological saline (0.85 % NaCl) and agitated on an orbital shaker at 100 rpm and 28 ± 2 °C for 1 h. The suspension was serially diluted to 10⁻⁵, and 0.1 mL of the 10⁻⁴ and 10⁻⁵ dilutions was spread-plated on nutrient agar and incubated at 28 ± 2 °C for 48 h. Colonies with distinct cultural morphology were picked and purified by repeated streaking on fresh nutrient agar. Pure cultures were maintained on nutrient agar slants at 4 °C.

2.3. Screening for biofilm formation

2.3.1. Tube staining assay

Qualitative biofilm formation was assessed following Christensen *et al.* (1982) [19]. Each isolate (100 µL) was inoculated into 10 mL trypticase soy broth (TSB) supplemented with 5 % sucrose and incubated for 24 h at 37 °C. Tubes were decanted, washed with phosphate-buffered saline (PBS, pH 7.3), dried and stained with 0.1 % crystal violet. Excess stain was removed with deionised water. A visible stained film adhering to the wall and base of the tube was recorded as positive (Mathur *et al.*, 2006) [21].

2.3.2. Microtitre plate assay

Biofilm biomass was quantified as described by Stepanović *et al.* (2000) [20]. Overnight cultures grown statically in TSB with 5 % sucrose at 37 °C were diluted 1:100 in the same medium, and 200 µL was dispensed into the wells of a 96-well polystyrene plate in triplicate. After 24 h of static incubation at 37 °C the contents of each well were removed by gentle tapping, the wells were washed with PBS (pH 7.3) to remove planktonic cells, and adherent biomass was stained with 0.1 % (w/v) crystal violet. Excess stain was washed off with 95 % ethanol and the plates were air-dried. Absorbance was read at 570 nm on a micro-ELISA plate reader. Uninoculated medium served as the negative control and its absorbance defined the cut-off optical density (OD_c). Isolates were classified as non-adherent ($OD \leq OD_c$), weakly adherent ($OD_c < OD \leq 2 \times OD_c$), moderately adherent ($2 \times OD_c < OD \leq 4 \times OD_c$) or strongly adherent ($OD > 4 \times OD_c$).

2.3.3. Congo red agar assay

Isolates were streaked on Mueller–Hinton agar supplemented with 0.8 g L⁻¹ Congo red dye and incubated at 37 °C for 48 h. Black colonies with a dry crystalline consistency were scored as biofilm producers, whereas red to pink colonies were scored as non-producers (Mathur *et al.*, 2006) [21].

2.4. Morphological and cultural characterization

Cell shape and arrangement were recorded after Gram staining following Cappuccino *et al.* (1992) [22], using a bright-field compound microscope under oil immersion (E200, Nikon, Japan); images were captured and cell dimensions measured with NIS-Elements Documentation software (Nikon, Japan). The Gram reaction was independently verified by the 3 % KOH string test. A loopful of 18–24 h growth was emulsified in a drop of 3 % KOH on a glass slide for up to 1 min, and the loop was lifted slowly; development of a viscous strand indicated a Gram-negative cell wall, while the absence of stringing indicated a Gram-positive one. Cultural characteristics (colony size, pigmentation, form, elevation and margin) were recorded from well-isolated colonies on nutrient agar after 48 h.

2.5. Biochemical characterization

IMViC and carbohydrate utilisation tests were performed with a ready-to-use kit (KB001-10KT, HiMedia, India) according to the manufacturer's protocol. A single colony was inoculated into 5 mL brain heart infusion broth and incubated at 35–37 °C for 4–6 h until turbidity reached ≥ 0.1 OD at 620 nm. Each well was inoculated with 50 µL of the suspension and the plates were incubated at 35–37 °C for 18–24 h, after which Kovac's reagent, methyl red reagent and Barritt's reagents A and B were added to the indole, methyl red and Voges–Proskauer wells respectively. Reactions were read as colour changes per the manufacturer's chart. Catalase activity was assayed by transferring a single colony from a 5-day Luria–Bertani agar plate to a glass slide and adding 2–3 drops of 3 % hydrogen peroxide; immediate effervescence was recorded as positive. Nitrate reduction was tested by inoculating 5 mL sterile nitrate broth and incubating at 25 °C for 8 days, after which two drops each of sulfanilic acid and α -naphthylamine reagents were added to 1 mL of culture; development of a pink to red colour indicated reduction of nitrate to nitrite. Uninoculated broth served as the control.

2.6. Extracellular enzyme assays

Amylase and lipase production were assessed by plate assay on substrate agar (Silambarasan *et al.*, 2010; Sheshadri *et al.*, 2011) [24, 23]. For amylase, starch agar (meat extract 3 g, soluble starch 2 g, peptic digest of animal tissue 5 g, agar 15 g per litre) was inoculated and incubated at 37 °C for 24–48 h, then flooded with iodine solution; a clear halo around the colony against the blue-black background indicated starch hydrolysis. For lipase, phenol red agar (peptone 5 g, yeast extract 3 g, CaCl₂ 1 g, agar 15 g per litre, adjusted to pH 7.4 with 0.1 M NaOH) was autoclaved, cooled to 60 °C and supplemented with 10 mL olive oil and phenol red (1 mg mL⁻¹). Plates were incubated at 37 °C for 48 h; a shift of the medium from pink to yellow, reflecting the pH drop caused by liberated fatty acids, indicated lipase activity.

2.7. Physiological characterization

The influence of temperature, pH and sucrose concentration on biofilm formation was examined by the microtitre plate assay described in Section 2.3.2, modified as follows. For temperature, 200 µL of diluted culture was dispensed and plates were incubated at 28, 35, 40 and 45 °C for 24 h. For pH, overnight cultures were diluted 1:100 in TSB adjusted to pH 5, 7 or 11 and incubated at 37 °C for 24 h. For carbon source, TSB was supplemented with sucrose at 5, 10, 15 or 20 % and plates were incubated at 37 °C for 24 h. In every case the wells were washed, stained with crystal violet, dried and read at 570 nm as before. Biofilm production was classified as high (OD > 0.500), moderate (OD 0.100–0.500) or poor (OD < 0.100).

2.8. Statistical analysis

All assays were performed in triplicate and the results are presented as means. Where treatments were compared, data were subjected to analysis of variance and means were separated by Duncan's New Multiple Range Test (DNMRT) at the 5 % level of significance.

3. Results

3.1. Isolation of bacteria from pond water

Fifteen bacterial colonies with distinct cultural morphology were recovered from the two pond water samples and purified to single-colony cultures. These were designated sequentially R1 to R15. Isolates R1 to R7 originated from the pond at the Vegetable Research Station and isolates R8 to R15 from the pond at the Centre for Natural Resource Management (Table 1).

Table 1. Bacterial isolates recovered from the two farm pond water samples.

Vegetable Research Station pond	Centre for Natural Resource Management pond
R1	R8
R2	R9
R3	R10
R4	R11
R5	R12
R6	R13
R7	R14
—	R15

3.2. Screening for biofilm formation

All fifteen isolates were screened by the tube staining assay, the microtitre plate assay and the Congo red agar assay. The three methods were fully concordant (Table 2). In the tube assay, five isolates R1, R5, R8, R11 and R12 gave a strongly positive reaction after 24 h, visible as a deep purple, continuous layer of stained biomass adhering firmly to the inner wall and base of the tube, indicating both surface attachment and substantial extracellular matrix production. The remaining ten isolates failed to retain crystal violet after washing and showed neither a film nor a ring along the glass, consistent with an absence of sessile growth under the conditions tested.

The microtitre plate assay placed the same five isolates unambiguously in the strongly adherent class. The negative control gave a stable baseline absorbance of 0.0376, so the thresholds for weak, moderate and strong adherence were 0.038, 0.075 and 0.150 respectively. Isolate R12 gave the highest biofilm biomass (OD_{570} 0.789), followed by R11 (0.743), R8 (0.719), R5 (0.688) and R1 (0.659); each of these values exceeds the strong-adherence threshold by a factor of more than four (Table 3).

On Congo red agar, the same five isolates produced black colonies with a dry, crystalline consistency, the morph type diagnostic of biofilm production, whereas the remaining ten isolates produced smooth red colonies.

Because the three independent assays agreed completely, the five positive isolates were retained for detailed characterization and were re-coded RBF1 (R1), RBF2 (R5), RBF3 (R8), RBF4 (R11) and RBF5 (R12).

Table 2. Screening of the fifteen pond water isolates for biofilm formation by three independent assays.

Isolate	Tube assay	Microtitre plate assay	Congo red agar assay
R1	+	Strong	Black, dry crystalline
R2	—	Non-adherent	Red
R3	—	Non-adherent	Red
R4	—	Non-adherent	Red
R5	+	Strong	Black, dry crystalline
R6	—	Non-adherent	Red
R7	—	Non-adherent	Red
R8	+	Strong	Black, dry crystalline
R9	—	Non-adherent	Red
R10	—	Non-adherent	Red
R11	+	Strong	Black, dry crystalline
R12	+	Strong	Black, dry crystalline
R13	—	Non-adherent	Red
R14	—	Non-adherent	Red
R15	—	Non-adherent	Red

+, positive; —, negative. Classification in the microtitre plate assay follows Stepanović *et al.* (2000) [20] relative to the cut-off optical

density of the uninoculated control ($OD_c = 0.0376$).

3.3. Morphological and cultural characterization

Microscopic examination after Gram staining showed that all five isolates were rod-shaped and occurred singly rather than in chains or clusters. Isolates RBF1 and RBF3 retained the crystal violet–iodine complex and appeared purple, and were therefore Gram positive, whereas RBF2, RBF4 and RBF5 were decolourised and counter-stained pink to red, and were therefore Gram negative.

Because Gram staining of environmental isolates can be misleading when decolourisation is imperfectly controlled, the Gram reaction was verified by the 3 % KOH string test. Isolates RBF2, RBF4 and RBF5 produced a viscous strand on lifting the loop, confirming a Gram-negative cell wall, while RBF1 and RBF3 gave no stringing, confirming a Gram-positive cell wall. The two methods agreed for all five isolates (Table 4).

Table 3. Quantitative biofilm formation by the five selected isolates in the microtitre plate assay.

Original code	New code	OD570	Classification
R1	RBF1	0.659	Strong
R5	RBF2	0.688	Strong

Original code	New code	OD570	Classification
R8	RBF3	0.719	Strong
R11	RBF4	0.743	Strong
R12	RBF5	0.789	Strong
Control	—	0.0376	—

$OD_c = 0.0376$; $2 \times OD_c = 0.075$; $4 \times OD_c = 0.150$. Weak, $OD_c < OD \leq 2 \times OD_c$; moderate, $2 \times OD_c < OD \leq 4 \times OD_c$; strong, $OD > 4 \times OD_c$. Values are means of three replicates.

On nutrient agar the isolates formed small, compact colonies with dry, often leathery surfaces, giving a conical appearance. Pigmentation ranged from off-white through creamy-white to light yellow. Four of the five isolates (RBF1, RBF2, RBF3 and RBF4) produced colonies of irregular form, whereas RBF5 was circular. Elevation ranged from flat (RBF1) through slightly raised (RBF2) to raised (RBF3, RBF4, RBF5), and colony margins were irregular in RBF1, entire in RBF2 and RBF5, and undulate in RBF3 and RBF4 (Table 5).

In the KOH string test, + denotes formation of a viscous strand (Gram negative) and – denotes no stringing (Gram positive).

Table 4. Microscopic characteristics and Gram reaction of the five biofilm-forming isolates.

Isolate	Cell shape	Cell arrangement	Gram reaction	3 % KOH
RBF1	Rod	Single cells	Positive	–
RBF2	Rod	Single cells	Negative	+
RBF3	Rod	Single cells	Positive	–
RBF4	Rod	Single cells	Negative	+
RBF5	Rod	Single cells	Negative	+

Table 5. Cultural characteristics of the five biofilm-forming isolates on nutrient agar after 48 h at 28 ± 2 °C.

Isolate	Pigmentation	Size	Form	Elevation	Margin
RBF1	Off-white	Small	Irregular	Flat	Irregular
RBF2	Off-white	Small	Irregular	Slightly raised	Entire
RBF3	Creamy-white	Small	Irregular	Raised	Undulate
RBF4	Light yellow	Small	Irregular	Raised	Undulate
RBF5	Off-white	Small	Circular	Raised	Entire

3.4. Biochemical characterization

The five isolates were differentiated biochemically by the IMViC series, twelve carbohydrate utilisation tests, catalase activity and nitrate reduction (Table 6). Every isolate produced a distinct reaction profile,

confirming that the five represent five different organisms rather than replicates of the same strain.

Table 6. Biochemical profile of the five biofilm-forming isolates.

No.	Test	RBF1	RBF2	RBF3	RBF4	RBF5
1	Indole	–	–	+	+	+
2	Methyl red	–	–	–	+	+
3	Voges–Proskauer	–	+	–	+	–
4	Citrate utilisation	+	+	+	+	+
5	Glucose	–	–	–	+	+
6	Adonitol	–	–	–	–	–
7	Arabinose	–	–	–	–	–
8	Lactose	–	–	–	–	–
9	Sorbitol	–	–	–	–	–
10	Mannitol	–	–	–	+	–
11	Rhamnose	–	–	–	–	+
12	Sucrose	+	–	–	+	+
13	Catalase	+	+	+	+	+
14	Nitrate reduction	+	+	–	–	+

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No.	Test	RBF1	RBF2	RBF3	RBF4	RBF5
15	Amylase	+	+	–	–	–
16	Lipase	–	–	–	–	–

+, positive reaction; –, negative reaction. Tests 1–12 were performed with the HiMedia IMViC and carbohydrate utilisation kit (KB001-10KT).

Citrate was utilised by all five isolates, and none utilised adonitol, arabinose, lactose or sorbitol. Indole was produced by RBF3, RBF4 and RBF5; the methyl red reaction was positive in RBF4 and RBF5 only; and the Voges–Proskauer reaction was positive in RBF2 and RBF4. Among the remaining carbohydrates, glucose was fermented by RBF4 and RBF5, mannitol by RBF4 alone, rhamnose by RBF5 alone, and sucrose by RBF1, RBF4 and RBF5.

All five isolates evolved oxygen bubbles immediately on addition of hydrogen peroxide and were therefore catalase positive. Nitrate reduction, indicated by development of a cherry-red colour after addition of the diazotisation reagents, was positive in RBF1, RBF2 and RBF5 and negative in RBF3 and RBF4.

3.5. Extracellular enzyme production

After incubation on starch agar and flooding with iodine, isolates RBF1 and RBF2 produced prominent clear zones of hydrolysis surrounding the growth, confirming extracellular amylase activity. In RBF3, RBF4 and RBF5 the dark blue starch–iodine complex extended to the colony margin, indicating no detectable starch degradation.

On phenol red–olive oil agar, none of the five isolates produced a yellow zone of clearance; the medium surrounding every colony remained deep pink, indicating that no fatty acids were liberated and that lipase activity was absent in all five isolates under the conditions tested.

3.6. Physiological characterization

3.6.1. Effect of temperature

Biofilm formation increased markedly at 35 °C, which was the optimum for every isolate tested. At this temperature RBF3 gave the highest biofilm biomass (OD_{570} 0.977), closely followed by RBF2

(0.965), RBF5 (0.880), RBF4 (0.849) and RBF1 (0.840). At 28 °C all five isolates retained a moderate capacity to form biofilm; RBF2 (0.612) and RBF5 (0.596) were the most adaptable at this lower temperature, while RBF1 gave the lowest value (0.485). Raising the temperature to 40 °C reduced biofilm production substantially across the whole set, with values clustering narrowly between 0.455 (RBF4) and 0.520 (RBF2). The constraint became more pronounced at 45 °C, where most isolates reached their lowest recorded yields and RBF4 fell to a minimum of 0.401; RBF3 alone retained a slight tolerance at this temperature (Table 7).

3.6.2. Effect of pH

Biofilm formation was assessed at pH 5, 7 and 11. Neutral conditions were optimal for the majority of the isolates: RBF3, RBF4 and RBF5 all reached their maximum at pH 7, with RBF5 recording the highest overall absorbance of the experiment (OD_{570} 0.925). Under alkaline conditions biofilm formation was reduced relative to pH 7 but remained appreciable, with RBF3 (0.509) and RBF4 (0.357) retaining a substantial adherent biomass at pH 11. Two isolates departed from this pattern: RBF1 and RBF2 formed most biofilm under mildly acidic conditions, RBF1 peaking at pH 5 with an absorbance of 0.552 (Table 8).

3.6.3. Effect of sucrose concentration

All five isolates formed biofilm at every sucrose concentration tested, and in each case production was maximal at 10 % sucrose. RBF2 maintained a strong adherent phenotype across the whole range, peaking at 10 % with an absorbance of 0.970 and retaining high biofilm biomass at 15 % (0.840) and 20 % (0.759). The remaining four isolates (RBF1, RBF3, RBF4 and RBF5) showed weak to moderate formation, characterised by a steady rise from 5 % to 10 % sucrose followed by a sharp decline at 15 % and 20 % (Table 9).

Table 7. Effect of incubation temperature on biofilm formation (OD_{570}) by the five isolates.

Isolate	28 °C	35 °C	40 °C	45 °C
RBF1	0.485 ^b	0.840 ^a	0.472 ^c	0.418 ^d
RBF2	0.612 ^b	0.965 ^a	0.520 ^c	0.463 ^d
RBF3	0.558 ^b	0.977 ^a	0.498 ^c	0.520 ^d
RBF4	0.524 ^b	0.849 ^a	0.455 ^c	0.401 ^d
RBF5	0.596 ^b	0.880 ^a	0.512 ^c	0.439 ^d
S.Em. (±)		0.013		
C. D. (0.05)		0.041		

(Note: Means with different superscript letters are significantly different from each other at the 5% level of significance)

Table 8. Effect of medium pH on biofilm formation (OD_{570}) by the five isolates.

Isolate	pH 5	pH 7	pH 11
RBF1	0.552 ^b	0.478 ^d	0.261 ^c
RBF2	0.241 ^e	0.283 ^e	0.115 ^d
RBF3	0.332 ^d	0.682 ^b	0.509 ^a

Isolate	pH 5	pH 7	pH 11
RBF4	0.412 ^c	0.645 ^c	0.357 ^b
RBF5	0.738 ^a	0.925 ^a	0.266 ^c
S.Em. (±)	0.013	0.010	0.008
C. D. (0.05)	0.041	0.031	0.024

(Note: Means with different superscript letters are significantly different from each other at the 5% level of significance).

Table 9. Effect of sucrose concentration on biofilm formation (OD_{570}) by the five isolates.

Isolate	5 % sucrose	10 % sucrose	15 % sucrose	20 % sucrose
RBF1	0.481 ^b	0.652 ^d	0.281 ^b	0.167 ^b
RBF2	0.619 ^a	0.980 ^a	0.412 ^a	0.290 ^a

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Isolate	5 % sucrose	10 % sucrose	15 % sucrose	20 % sucrose
RBF3	0.460 ^c	0.727 ^c	0.260 ^b	0.183 ^b
RBF4	0.408 ^d	0.690 ^{cd}	0.234 ^b	0.190 ^b
RBF5	0.482 ^b	0.871 ^b	0.334 ^{ab}	0.207 ^{ab}
S.Em. (±)	0.003	0.023	0.001	0.004
C. D. (0.05)	0.001	0.067	0.022	0.085

(Note: Means with different superscript letters are significantly different from each other at the 5% level of significance).

4. Discussion

Five of the fifteen bacteria recovered from two semi-arid farm ponds, a recovery rate of one in three, were strong biofilm formers. This proportion is comparable with that reported for other aquatic habitats: Nair *et al.* (2021) [18] found that seven of twelve isolates from a water treatment tank formed biofilm in the tube assay within 24 h, and Merina *et al.* (2011) [17] recovered biofilm-forming *bacilli* from a marine seagrass habitat. Standing farm water therefore appears to be a productive source of adherent bacteria, and one that is logistically far simpler to sample than the rhizosphere.

The principal methodological strength of this screening is that three assays with different readouts agreed completely. The tube assay detects gross adherence and matrix retention but is qualitative and prone to observer bias; the microtitre plate assay quantifies attached biomass but can register non-specific staining; and the Congo red agar assay detects the dry, rugose morphotype associated with exopolysaccharide and amyloid fibre production, a phenotype known to be regulated independently of attachment itself (Römling, 2005; Milanov *et al.*, 2015) [26, 25]. Individual assays are known to disagree, and Mathur *et al.* (2006) [21] concluded from a direct comparison of the three that agreement should not be assumed. The complete concordance observed here, across fifteen isolates rather than five, gives considerably greater confidence that RBF1 to RBF5 are genuine biofilm formers than any single assay could. The margin was also large: all five gave OD_{570} values more than four times the cut-off, with the lowest (0.659) more than seventeen times the control absorbance, so the classification does not depend on where the boundary between moderate and strong adherence is drawn.

Phenotypically the five isolates were uniform in some respects and diverse in others. All were single, rod-shaped cells and all were catalase positive. The rod morphology is consistent with the dominance of *Bacillaceae*, *Paenibacillaceae* and Gram-negative *Proteobacteria* among reported biofilm-forming PGPR (Bais *et al.*, 2004; Timmusk *et al.*, 2005; Ansari *et al.*, 2019) [28, 11, 27]. Universal catalase activity is not incidental. Catalase neutralises hydrogen peroxide and so protects DNA, lipids and proteins from oxidative damage; its presence in all five isolates is consistent with the observation that biofilm-forming bacteria generally carry stronger antioxidant defences than planktonic strains, a trait that supports persistence on root surfaces and under the salinity, drought and oxidative stress encountered in the field (Kloepper *et al.*, 1989; Flemming *et al.*, 2010) [29, 1]. Yasmeen *et al.* (2020) [13] likewise recorded catalase activity in all their biofilm-forming rhizobacteria and linked it directly to enhanced salt tolerance in sunflower.

The decision to verify the Gram reaction by an independent chemical method deserves emphasis. Gram staining depends on controlled decolourisation, and certain spore-forming genera, notably *Bacillus* and *Clostridium*, are known to stain Gram-negative despite possessing a Gram-positive cell wall. The 3 % KOH string test, which depends on the differential lysis of the Gram-negative envelope and the consequent release of viscous chromosomal DNA, supplied an orthogonal check. That the two methods agreed for all five isolates means that the assignment of RBF1 and RBF3 as Gram positive and RBF2, RBF4 and RBF5 as Gram negative is secure, and that any subsequent genomic DNA extraction protocol can be selected on a sound basis.

Colony morphology on nutrient agar small, compact colonies with dry, leathery, often conical surfaces is itself informative, since a dry rugose surface reflects abundant extracellular matrix and correlates

with the black, crystalline morph type on congo red agar. The range of forms, margins and pigments recorded here is consistent with that described by Hussain *et al.* (2013) [30] for bacteria isolated from water sources, who reported round to irregular forms, smooth to dry surfaces, and whitish, cream and yellow pigmentation in a comparable collection.

The biochemical profiles distinguished all five isolates from one another. Universal citrate utilisation combined with an inability to use adonitol, arabinose, lactose or sorbitol indicates organisms adapted to simple rather than complex carbon sources, which is consistent with a nutrient-limited pond water origin. The isolates differed in indole, methyl red and Voges–Proskauer reactions and in their use of glucose, mannitol, rhamnose and sucrose, and this combination of characters is the conventional basis for provisional generic placement (Sujana *et al.*, 2013; Sonkusale *et al.*, 2015) [32, 31]. Nitrate reduction in RBF1, RBF2 and RBF5 is of particular agronomic interest, since nitrate-reducing biofilms contribute to nitrogen cycling in both aquatic and rhizosphere environments. Yasmeen *et al.* (2020) [13] similarly used nitrate reductase activity and citrate utilisation to separate their biofilm-forming isolates.

Extracellular enzyme production was more restricted than in some published collections. Amylase was confined to RBF1 and RBF2, and lipase was absent throughout. Nair *et al.* (2021) [18] found that all seven of their biofilm-forming strains produced both amylase and lipase, and suggested that exoenzymes assist adherence to solid surfaces. The present results indicate that neither enzyme is required for strong adherence, since RBF3, RBF4 and RBF5 produced the highest microtitre plate absorbances of the set while producing neither enzyme. Attachment in these isolates is therefore likely to depend on matrix polysaccharide and surface adhesins rather than on substrate hydrolysis. The practical implication is positive: a strain that does not secrete lipase is less likely to degrade seed coat lipids during seed treatment.

The physiological data define the operating window within which these organisms will perform. A temperature optimum of 35 °C in all five isolates, with substantial biofilm still formed at 28 °C and measurable biofilm retained at 40 and 45 °C, matches the thermal regime of North Gujarat soils during the kharif and summer seasons and indicates that adherence will not collapse under field heat. Sonkusale *et al.* (2015) [31] reported an analogous pattern, with maximum biofilm at 37 °C, retention at 50 °C in some isolates, and minimal formation at 25 °C.

The pH response was more revealing. That RBF3, RBF4 and RBF5 peaked at pH 7 accords with the general finding that neutral conditions favour cell attachment and EPS matrix production (Sonkusale *et al.*, 2015; Cai *et al.*, 2013) [31, 33]. More interesting is the retention of appreciable biofilm at pH 11 by RBF3 and RBF4, and the departure of RBF1 and RBF2, which formed most biofilm at pH 5. Alkaline calcareous soils predominate across much of North Gujarat, so alkali-tolerant adherence is a directly useful trait, while acid-tolerant isolates broaden the range of soils in which the collection as a whole could be deployed. Taken together the five isolates cover a pH span from 5 to 11, which is unusually wide for a set of this size.

Sucrose supported biofilm formation in all isolates with a consistent optimum at 10 %, above which formation declined sharply in four of the five. The decline is most plausibly osmotic: at 15 and 20 % sucrose the water activity of the medium falls far enough to impose a solute stress that outweighs the benefit of additional carbon for matrix synthesis. RBF2 was the exception, maintaining absorbances of 0.840 and 0.759 at 15 and 20 % respectively, which points to superior os

motolerance. Since osmotic and salt stress tolerance is one of the central arguments for using biofilm-forming inoculants in the first place (Qurashi *et al.*, 2012; Ansari *et al.*, 2019; Ajjah *et al.*, 2023) [34, 27, 8], RBF2 merits particular attention in any subsequent evaluation under saline or drought conditions.

The wider relevance of this collection lies in the accumulating evidence that biofilm-forming rhizobacteria outperform planktonic inoculants where it matters. Biofilm-forming strains express plant growth-promoting traits more strongly, produce more antimicrobial metabolites and survive better in competitive soil (Pandini *et al.*, 2017; Backer *et al.*, 2018) [9, 10], and they maintain performance under field conditions where laboratory-selected planktonic strains commonly fail (Timmusk *et al.*, 2005; Haque *et al.*, 2020; Yasmeen *et al.* 2020 and Rafique *et al.* 2024) [11, 12, 13] have each demonstrated growth promotion by biofilm-forming isolates in tomato, sunflower and wheat respectively. The five isolates reported here have been defined phenotypically to a standard sufficient to support that next step.

Two limitations should be stated. First, the isolates are characterised phenotypically and have not been assigned to species here; molecular identification based on 16S rRNA gene sequencing is required before any taxonomic claim can be made, and is reported separately. Second, biofilm screening was conducted at 37 °C on a sucrose-rich laboratory medium, conditions that optimise the assay but do not reproduce the rhizosphere; adherence on root surfaces *in situ* remains to be demonstrated.

5. Conclusion

Fifteen bacteria were isolated from the water of two farm ponds in semi-arid North Gujarat, and five of them recoded RBF1 to RBF5 were identified as strong biofilm formers by three independent and fully concordant assays. The five isolates are rod-shaped, single-celled and uniformly catalase positive; two are Gram positive and three Gram negative, as confirmed by both Gram staining and the 3 % KOH string test. Each has a distinct biochemical profile, all utilise citrate, three reduce nitrate, two produce extracellular amylase and none produces lipase. Biofilm formation is maximal at 35 °C and 10 % sucrose in every isolate, and the set collectively spans pH 5 to pH 11, with RBF2 additionally tolerating sucrose concentrations up to 20 %. Farm pond water is thus confirmed as a convenient and productive source of robust biofilm-forming bacteria, and RBF1 to RBF5 constitute a phenotypically defined collection suitable for molecular identification and for evaluation as biofilm-based bioinoculants.

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Author contributions

[Author One] conceived and performed the experiments and drafted the manuscript. [Author Two] supervised the work and revised the manuscript. [Author Three] contributed to data analysis and interpretation. All authors read and approved the final manuscript.

Declaration of competing interest

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

Data availability

All data generated or analysed during this study are included in this published article.

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