



RESEARCH ARTICLE

Uncovering the microbial diversity of Chak-hao rice: Isolation and characterisation of indigenous beneficial microbes in Manipur

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Abstract

Chak-hao (*Oryza sativa* L.), an aromatic black glutinous rice native to Manipur, is valued for its unique fragrance and high nutraceutical value, including iron, vitamin E and antioxidants. The present study aimed to isolate and characterise beneficial rhizospheric and endophytic microbes that could enhance Chak-hao rice cultivation. A total of 170 bacterial and 55 fungal isolates were obtained from different Chak-hao varieties collected from the farmers' fields across four districts of Manipur. These isolates were screened for plant growth-promoting traits such as phosphate solubilisation, indole-3-acetic acid (IAA) production, nitrogen fixation and antagonistic activity against harmful fungal pathogens. The study showed 51.94 % phosphate solubilisation, 41.26 % produced IAA and 9.2 % exhibited nitrogen-fixing ability. Based on multiple plant growth promoting traits, 11 promising isolates were selected for molecular characterisation. The 16S ribosomal DNA (16S rDNA) sequencing identified these isolates as belonging to *Bacillus*, *Enterobacter* and *Pseudomonas* genera, with *Bacillus* species being predominant. Microbial inoculation significantly improved grain yield, straw yield and aroma-associated compounds compared to the untreated control ($p \leq 0.05$, Duncan's multiple range test). The study reveals the potential of indigenous beneficial microbes as eco-friendly biofertilisers and biocontrol agents for sustainable Chak-hao rice cultivation, quality grain improvement and conservation of unique Chak-hao rice germplasm Manipur.

Keywords: Chak-hao; endophytic bacteria; plant growth-promoting microorganisms; rhizospheric microorganisms

Introduction

Chak-hao, a traditional aromatic rice of Northeast India, represents a unique but less-explored genetic resource within one of the richest biodiversity hotspots of the country. However, such ecologically sensitive regions are highly vulnerable to biodiversity loss and ecosystem imbalance if the valuable flora is not sustainably explored and utilised. This black scented rice of Manipur is celebrated for its mild nutty flavour, distinctive aroma and high anthocyanin content (1). Traditional foods prepared from black rice have been found to have high nutritional value with significant antioxidant activity ranging from about 70–85 % 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, total phenolic content of 200–450 mg gallic acid equivalents (GAE) /100 g, protein content of 4.7–13.49 %, fat content of 1.76–5 %, iron content of 3–5 mg/100 g and zinc content of 2–3 mg/100 g, showing its high nutritional quality over many conventional rice varieties (2). A recent study reported that pigmentation in black rice results from the activity of the *Kala 4* gene, which is necessary for anthocyanin synthesis (3). No systematic work on the improvement of Chak-hao of Manipur has been undertaken so far. However, germplasm collection, characterisation and evaluation of these rice cultivars have been made. There is a high demand for Chak-hao in the domestic market, having possibilities for export (4). As a result, there is a need for the improvement of these cultivars for higher production.

Chak-hao is susceptible to many rice pathogens of North-East India. There is a need to explore the microbial potential in rhizosphere and root endophytes of Chak-hao. The rhizosphere is central to microbial and nutrient dynamics and describes the zone of soil surrounding roots of plant species which release organic substances. Plant growth-promoting rhizobacteria (PGPR) play an important role in improving plant growth and fitness through direct mechanisms like increasing mineral nutrients and modulating phytohormones and indirectly suppressing pathogens, by modifying the rhizosphere microbial community (5). Interactions between plants and soil microorganisms profoundly affect plant growth by secreting growth regulators, solubilising minerals and providing nitrogen, leading to enhanced agronomic efficiency (6). Root colonisation of free-living PGPR were considered as one of the early signalling events to coordinate the production and release of compounds related to plant growth promotion, nutrition and induced systemic resistance (ISR) (7). Rhizospheric microorganisms can improve the crop health by controlling diseases and promoting plant growth which is sustainable for food security and safety (8).

Endophytes are naturally occurring beneficial microorganisms that colonise healthy plant tissues without causing any harm and most endophytic fungi serve as potent biocontrol agents against harmful phytopathogens (9). They exhibit multiple antifungal mechanisms such as producing lipopeptides, antibiotics

and lytic enzymes, competing for nutrients and space, parasitising pathogen cells and producing siderophores to restrict pathogen growth. Additionally, they induce systemic resistance in host plants, enhancing broad-spectrum disease protection (10, 11) and control pathogens by increasing disease resistance, reducing pathogen virulence, secreting antimicrobial substances in plants, potentially minimising the need for agrochemicals (12). Indeed, intensive work has shown that endophytic microorganisms have the potential to control rice pathogens, offering an eco-friendly alternative to synthetic chemicals capacity to control pathogens (13). *Metarhizium anisopliae*, an entomopathogenic fungus, is found to contribute to plant nutrient uptake and considered as a plant endophyte. Visual evidence of its endophytic relationship with maize roots was demonstrated using molecular tools and fluorescent labelling (14).

Long-term over-application of chemical fertilisers influences the beneficial microorganisms in the rice rhizosphere. This causes a change in the population dynamics of soil rhizobacteria leading to the soil becoming infertile, slowing down of plant growth, reduction in yield and ultimately depletion of the quality and aroma of rice grains (15). These studies highlight the significant role of the rhizospheric microbiome in enhancing rice aroma, plant growth and yield. Root exudates from plants attract various bacterial species, leading to a higher abundance of microbial communities in the rhizosphere compared to bulk soil. Consequently, the rhizosphere becomes enriched with diverse functional groups of microorganisms, including phosphate solubilisers, iron reducers, nitrogen fixers, methane cyclers and fermenters (16, 17) This results in lower pH, phosphatase and urease activities in the rhizosphere compared to bulk soil, leading to higher available phosphate, total nitrogen content and organic carbon in the rhizosphere, making it crucial for plant growth and yield (18).

Strains of *Acinetobacter* sp. and *Enterobacter ludwigi* were found to elevate the aroma level in the Basmati-370 rice variety indicating a link with the microbiome and the rice aroma along with its yield (19). Bacterial isolates viz. *Pseudomonas mosselii*, *Microvirga* sp., *Paenibacillus rigui* and *Paenibacillus graminis*, improved root and shoot growth in a rice genotype-dependent manner (20). Although Chak-hao rice is nutritionally and culturally important, very little is known about its associated rhizospheric and endophytic microbial communities and their potential contribution

to plant growth, aroma, yield and grain quality. More specifically, there has been no systematic study on the diversity and plant growth promoting potential of indigenous microorganisms associated with Chak-hao grown under the unique agro-ecological conditions of Manipur. Filling this gap is important to develop sustainable and eco-friendly strategies for the improvement, productivity and conservation of this valuable traditional rice germplasm. Thus, the present study was conducted to (i) study the microbial diversity and molecular characterisation of endophytic and rhizospheric plant growth promoting micro-organisms from roots of Chak-hao, (ii) to determine large scale field demonstration using the most efficient endophytes and rhizospheric microbes, (iii) to study effects of endophytes on anthocyanin content of Chak-hao.

Materials and Methods

Survey and sample collection

Rice roots and rhizosphere soil were collected from four Chak-hao varieties, namely Chak-hao Amubi, Chak-hao Angaoba, Chak-hao Porieton and Chak-hao Sempak and high yielding (HY) rice varieties viz. Tamphaphou, Sana phou and Leimaphou. The soil samples were collected from 15 different sites of each district (Table 1–4). Rhizosphere soil samples were collected from rice plants at a depth of 5–6 cm after removing the top 2 cm of surface soil. For each site, samples were obtained from four different spots. The samples were arranged diagonally and mixed thoroughly. The soil samples were air dried under shade and kept sealed in cloth bags at 4 °C in a refrigerator for subsequent use. The survey and sample collection for the present study were carried out during 2018–2019.

Determination of soil pH

Soil samples collected from different places were shade dried. A 40 g of soil samples were weighted using an electronic analytical weighing balance (Chemlab LabMart Analytical Balance; maximum capacity 220 g; readability 0.0001 g (0.1 mg); with internal calibration) and mixed with 100 mL distilled water to maintain a 1 : 2.5 soil-water ratio for the determination of pH. The suspension was stirred thoroughly with a glass rod and allowed to stand for 30 min with intermittent stirring (21). The pH meter was calibrated using a standard buffer solution of pH 7. The electrode was then immersed

Table 1. Sites of soil sample and rice varieties collected from Imphal West district

Sl. No.	Name of place	Latitude (°N)	Longitude (°E)	Altitude (ft)
1	Taothong Apheiba	24°49.300'	93°51.960'	2548
2	Tharoijamawang Leikai	24°48.806'	93°51.863'	2549
3	Salam Mamang Leikai	24°47.607'	93°51.006'	2541
4	Luker	24°48.279'	93°50.233'	2545
5	Maklang	24°48.525'	93°49.154'	2572
6	Sairemkhun	24°48.770'	93°49.190'	2559
7	Tairenpokpi	24°49.686'	93°48.381'	2609
8	Phayeng	24°50.913'	93°49.379'	2614
9	Lairenkabi	24°50.666'	93°49.953'	2587
10	Kadangbund	24°52.302'	93°48.700'	2679
11	Heibongpokpi	24°49.919'	93°51.096'	2554
12	Haorangsabal	24°49.492'	93°51.427'	2574
13	Lamsang	24°49.550'	93°51.709'	2586
14	Lambal	24°50.156'	93°51.402'	2561
15	Lamdengmakha Leikai	24°49.961'	93°52.504'	2565

ft: foot.

Table 2. Sites of soil sample and rice varieties collected from Imphal West district

Sl. No.	Name of place	Latitude (°N)	Longitude (°E)	Altitude (ft)
1	Khumidok	24°50.665'	93°58.193'	2602
2	Haotal	24°51.365'	93°58.376'	2564
3	Waiton Makha Leikai	24°52.011'	93°58.553'	2566
4	Khundrakpam	24°53.782'	93°59.012'	2555
5	Sambei	24°54.683'	93°59.211'	2560
6	Chingkhugee	24°54.196'	93°59.142'	2555
7	Tangkham	24°54.213'	93°59.126'	2568
8	Waiton	24°52.153'	93°59.542'	2525
9	Tejpur	24°52.098'	94°00.224'	2537
10	Sawombung	24°51.420'	94°00.813'	2561
11	Kangla Chingarel	24°51.452'	93°59.856'	2555
12	Kangla Sangomsang	24°51.075'	93°59.480'	2529
13	Khabeisoi	24°50.549'	93°58.939'	2540
14	Khurai Angom Leikai	24°50.116'	93°58.778'	2539
15	Kongpal Ningthoubung	24°49.369'	93°58.527'	2540

ft: foot.

Table 3. Sites of soil sample and rice varieties collected from Bishnupur district

Sl. No.	Name of place	Latitude(°N)	Longitude(°E)	Altitude (ft)
1	Utlou	24°43.820'	93°51.543'	2048
2	Kabowakching, Nambol	24°43.182'	93°48.697'	2555
3	Khoijuman	24°37.080'	93°46.475'	2546
4	Bishnupur Khablock Lamkhai	24°36.706'	93°45.715'	2584
5	Upokpi	24°35.442'	93°45.956'	2515
6	Thinunghei	24°32.547'	93°45.479'	2521
7	NaranSeinya	24°31.430'	93°45.343'	2527
8	ThinungeiManingLeikai	24°32.983'	93°45.196'	2540
9	KomKeirap	24°33.031'	93°44.858'	2550
10	Potsangbam	24°35.809'	93°45.940'	2560
11	Nachou	24°36.268'	93°45.822'	2557
12	Kwasiphai	24°36.976'	93°47.780'	2583
13	Kwairakpam	24°39.338'	93°46.163'	2516
14	TeraUrak	24°39.699'	93°46.393'	2521
15	KeinouThongthak	24°40.629'	93°47.162'	2513

ft: foot.

Table 4. Sites of soil sample and rice varieties from Thoubal District

Sl.No.	Name of place	Latitude (°N)	Longitude (°E)	Altitude (ft)
1	Tangeng	24°39.911'	93°57.981'	2532
2	Thongahanbi Kiyamsiphai	24°39.865'	93°58.168'	2561
3	Haokha	24°39.478'	93°59.204'	2558
4	Thoubal Okram	24°39.144'	93°59.531'	2539
5	Wangbal	24°36.738'	94°01.501'	2527
6	Wangbal Canal	24°36.374'	94°01.800'	2528
7	Wangjing	24°36.149'	94°02.084'	2522
8	Wangjing Mayai Leikai	24°36.159'	94°02.206'	2525
9	Wangjing Thongthak	24°36.199'	94°01.931'	2529
10	Wangbal Canal Manak	24°36.320'	94°01.828'	2538
11	Tabukhong	24°36.427'	94°01.742'	2532
12	Wangbal Lou	24°36.583'	94°01.620'	2530
13	Khangabok Part-I	24°36.986'	94°01.290'	2532
14	Khangabok Unity Park	24°37.126'	94°01.181'	2534
15	Lilong	24°41.754'	93°56.922'	2517

ft: foot.

in the soil-water suspension, and the pH was recorded from the meter display.

Isolation of endophytic and rhizospheric microbes

For the isolation of beneficial bacterial microbes, roots were washed thoroughly under running tap water to remove the attached debris. The samples were then rinsed in 70 % ethanol, surface sterilised with 0.1 % mercury (II) chloride (HgCl₂) and further washed with sterile distilled water 2–3 times to remove the surface sterilising agents (22). Serial dilutions of root homogenates and rhizospheric soil

suspensions were prepared and aliquots of appropriate dilutions were plated on nutrient agar (NA). The plates were incubated at 30°C for 48 hr. Stock cultures were preserved by glycerol stock method. While for isolation of beneficial fungal isolates, surfaced sterilised root segments were aseptically cut into 1–2 cm pieces and placed on potato dextrose agar (PDA) plates. For rhizospheric fungi, 1 g of soil sample was serially diluted in sterile distilled water and 0.1 mL aliquots was spread evenly onto PDA plates. All experiments were conducted in triplicate under aseptic laboratory conditions at 25 °C

for 5 days and emerging fungal colonies with distinct morphological characteristics were purified and maintained for further studies.

Screening for plant growth promoting traits

For isolation of phosphate solubilising bacterial and fungal strains, 1 g of each field moist soil samples was suspended in 9 mL of sterilised distilled water and mixed vigorously. Serial dilutions were prepared using dilution plate technique. Each dilution was plated on National Botanical Research Institute's phosphate (NBRIP) agar plates containing 16 g agar, 10 g glucose, 5 g $\text{Ca}_3(\text{PO}_4)_2$, 5 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.25 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g KCl, and 0.1 g $(\text{NH}_4)_2\text{SO}_4$ per litre of distilled water. The NBRIP medium containing tri calcium phosphate (TCP) act as sole phosphorus source for selectively screening the bacteria which have the ability to release soluble inorganic phosphate (23). The pH of the media was adjusted to 7 using HCl. The plates were incubated 4–5 days in an incubator at 30 °C. The colonies with clear halos were considered to be phosphate solubilising colonies and they were then further purified by re-streaking on the fresh NBRIP agar plates at 30 °C (24). Bacterial strains that exhibited clear zones on the agar plates were selected as phosphate solubilising organisms for further studies.

For the detection of indole-3-acetic acid (IAA) production, each isolate was grown in nutrient broth (NB) medium and shaken for 12 hr at room temperature. Then 1 mL overnight culture was transferred to minimal medium supplemented with 1 mL L-Tryptophan and incubated at room temperature for 48 hr with occasional shaking. The 48 hr culture was then centrifuged at 10000 rpm for 10 min at 4 °C and indolic compound production was measured as indole-3-acetic acid (IAA) equivalents. The measurement was performed as follows: 2 mL of culture supernatant was placed into test tubes containing 4 mL of Salkowski reagent (1 mL 0.5 M FeCl_3 + 50 mL HClO_4 50 % (v/v)). The tubes containing the mixture were allowed to stand for 30 min to 60 min in the dark at room temperature for colour development. The intensity of colour was spectrophotometrically measured at 530 nm using a standard curve for calibration by using authentic IAA (25).

Screening for nitrogen fixation was done only for bacterial isolates by stab inoculating the cultures in nitrogen free bromothymol blue semi-solid media and incubated for 3 to 5 days. Liquid nitrogen-free bromothymol blue (NFB) medium (500 mL) composition was as follows: 5 g L^{-1} malic acid; 0.5 g L^{-1} K_2HPO_4 ; 0.2 g L^{-1} MgSO_4 ; 0.1 g L^{-1} NaCl; 0.02 g L^{-1} $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 2 mL L^{-1} micronutrient solution (0.04 g L^{-1} $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.12 g L^{-1} $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.40 g L^{-1} H_3BO_3 , 1.0 g L^{-1} $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and 1.175 g L^{-1} $\text{MnSO}_4 \cdot \text{H}_2\text{O}$); 2 mL L^{-1} bromothymol blue solution (5 g L^{-1} bromothymol blue in 0.2 N KOH); 4 mL L^{-1} iron-ethylenediaminetetraacetic acid (EDTA) solution (16.4 g L^{-1} Fe-EDTA); 1 mL L^{-1} vitamin solution (10 mg biotin and 20 mg pyridoxal-hydrochloride (HCl) in 100 mL distilled water); and 4.5 g L^{-1} potassium hydroxide (KOH). Owing to the high pH, the components were added in the above order to avoid precipitation of iron and other salts. The pH was adjusted to 6.5 and the medium was sterilised at 121 °C for 15 min (26, 27). The antagonism activity against phytopathogens viz. *Fusarium oxysporium* and *Rhizoctonia solani* was done by using dual culture technique. Phylogenetic analysis of the selected strains was done. The present study focused on preliminary qualitative screening to identify microbial isolates capable of phosphate solubilisation, IAA production and nitrogen fixation. Future studies will include detailed quantitative biochemical analyses to better evaluate the efficiency of

these promising isolates.

Field trial of bacterial isolates

Five potential antagonistic bacterial isolates, namely (T1-*Bacillus megaterium*, T2-*Enterobacter asburiae*, T3-*Bacillus flexus*, T4-*Pseudomonas palleroniana*, T5-*Bacillus subtilis*) and their consortium (mixture of all the five isolates in equal proportion) were evaluated for the field experiment. The experiment was conducted in a randomised block design (RBD) with seven treatments including the uninoculated control, with three replications for each treatment. Each experimental plot measured 2.5 m × 2.5 m. The Chak-hao rice seeds were sown at a spacing of 15 cm × 20 cm and fertilisers were applied at the recommended dose of 75 : 40 : 40 kg/ha of nitrogen: phosphorus: potassium (N : P : K). The soil pH of the experimental field was 5.5, which was found to be suitable for the cultivation of Chak-hao rice. The bacterial isolates were inoculated in NB and incubated for 3 days to get fully grown cultures. It was then centrifuged (10000 rpm, 10 min). The pellets were collected and washed thrice with sterile distilled water (SDW) and culture suspensions were prepared using SDW. Black rice was surface sterilised with 70 % ethanol for 5 min, followed by 0.2 % sodium hypochlorite for 5 min and rinsed four times with SDW. Surface sterilised seeds were soaked in the cell suspensions and kept under shaking conditions (150 rpm, 30 °C) for 2 hr (28). Seeds of Chak-hao Poreiton rice variety were thoroughly washed several times with sterile distilled water and surfaced sterilised prior treatment. The seeds were separately inoculated with the cultures of five selected strains at 5 g/kg seed, with an inoculum concentration of 10^6 colony-forming units (CFU)/g and the treated seeds were sown in the field. For the control, seeds were treated with sterile distilled water. The crop was grown by following standard agronomic practices, with irrigation as per requirement and the plants were harvested after they reached the maturation stage of 145–150 days (29). Grain yield and straw yield were recorded from each treatment plot. Gas chromatography-mass spectrometry (GC-MS) analysis was performed to identify aroma-related, antioxidant-associated and other bioactive metabolites influenced by microbial inoculation in Chak-hao rice. A 10–20 g of harvested grain samples were finely ground and subjected to GC-MS analysis after solvent extraction. The obtained chromatographic peaks were compared with standard reference compounds for identification and estimation of volatile and bioactive metabolites. Data obtained from field experiments were subjected to analysis of variance (ANOVA) and treatment means were compared using Duncan's multiple range test (DMRT) at $p \leq 0.05$ to determine significant differences among treatments. Statistical analysis was carried out using SPSS software version 21.0.

Results and Discussion

The survey revealed that Chak-hao varieties generally showed lower productivity (1.5–3.0 t ha^{-1}) and longer crop duration (130–160 days) compared to HY cultivars, which yielded 3.5–6.0 t ha^{-1} within 120–140 days (Table 5). Chak-hao were mainly grown in Imphal west, Imphal east and Bishnupur districts. The high yielding (HY) rice varieties obtained were Tamphaphou, Sana phou and Leimaphou. The details of sampling locations and rice varieties collected are presented in Tables 1–4. Similar performance gaps between traditional aromatic rice and improved varieties have been reported

Table 5. Yield of the normal aromatic rice grown in Manipur

Sl. No	Name of rice	Area production	Duration	Location
1	Chak-hao Amubi	2.5–3.0 tonnes	130–150 days	Imphal-west, Bishnupur
2	Chak-hao Angaoba	2.5–3.0 tonnes	140–160 days	Thoubal
3	Chak-hao Poreiton	1.5–2.5 tonnes	130–150 days	Imphal-east, Imphal-west
4	Chak-hao Sempak	1.5–2.5 tonnes	130–150 days	Imphal-east, Imphal-west
5	Tamphaphou (HY)	5.0–6.0 tonnes	120–130 days	Imphal-west, Imphal-west
6	Sana phou (HY)	3.5–4.5 tonnes	130–140 days	Imphal-west, Imphal-west
7	Leimaphou (HY)	4.0–5.0 tonnes	125–130 days	Imphal-west, Imphal-west

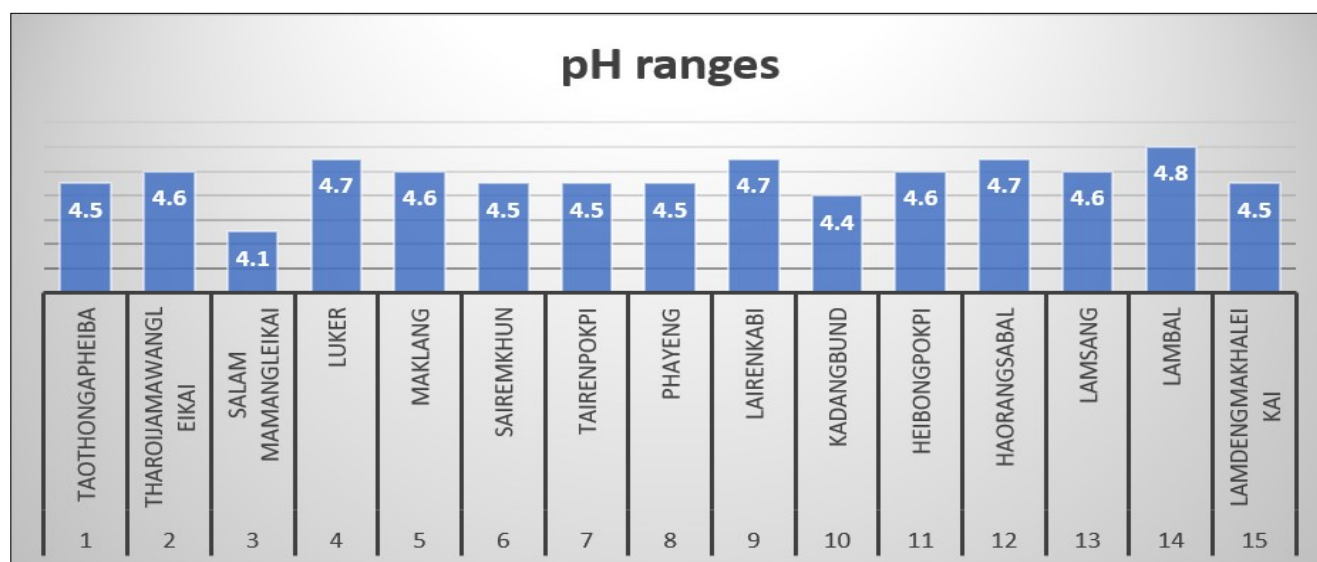
HY: indicates high-yielding varieties.

earlier, where local scented rice generally exhibits lower yield potential due to its unique genetic background and resource allocation toward aroma compounds rather than biomass production (2, 30). The diversity and abundance of microbial isolates associated with Chak-hao may be due to the complex root exudate composition and long-term adaptation of traditional rice cultivars to local agro-ecological conditions. Such microbial enrichment may contribute to improved nutrient cycling, stress tolerance and maintenance of grain quality traits characteristic of Chak-hao rice.

For determination of soil pH, the value of pH from 15 different sites of each district are shown in Fig. 1–4. The soil pH from Imphal West, Imphal East, Bishnupur and Thoubal District, ranged from 4.1 to 4.8, 4.5 to 6.6, 4.2 to 6.3 and 5.6 to 6.7 respectively. Among the four districts, Thoubal district had the highest pH and Imphal West showed the lowest. These results indicated that the sampled soils ranged from strongly to slightly acidic, depending on location. The soil pH values across the four districts varied from strongly to slightly acidic, and this variation is expected to influence soil microbial communities. The pH is an important factor that controls microbial diversity by influencing nutrient availability and microbial activity in the rhizosphere. The more acidic soils in Imphal West may favour acid-tolerant and spore-forming bacteria like *Bacillus* spp. and the relatively higher pH in Thoubal may support a wider range of rhizospheric microbes like *Pseudomonas* spp. Soil pH may also influence fungal distribution, including genera such as *Penicillium*, *Trichoderma* and *Aspergillus*. Overall, these pH differences may help explain the variation in microbial diversity observed across the study sites (31).

A total of 170 bacterial and 55 fungal isolates were obtained from Chak-hao rhizosphere, out of which 101 bacteria and 40 fungi

were from rhizosphere (rhizospheric) and 69 bacteria and 15 fungi were from rice roots (endophytic). From high yielding (HY) rice varieties, a total of 36 bacterial and 21 fungal isolates were obtained, out of which 21 bacteria and 13 fungi were from rhizosphere (rhizospheric) and 15 bacteria and 8 fungi were from rice roots (endophytic). The increase in the microbial abundance in Chak-hao roots and rhizospheric soils is similar with previous studies, which suggest that traditional rice genotypes are usually richer in microbial communities due to their diverse and complex root exudates (16, 17). Root exudates such as sugars, amino acids, organic acids, phenolic compounds and other secondary metabolites play an important role in shaping the rhizosphere microbiome by selectively attracting and supporting beneficial microorganisms around plant roots (32). Host genotype plays an important role in shaping root-associated microbiomes by influencing their root structure, exudate composition and plant-microbe interactions. Indigenous rice landraces such as Chak-hao often possess greater genetic diversity and local adaptation than modern HY cultivars, which may enhance the development of beneficial microbes involved in nutrient cycling, stress tolerance, disease suppression and methane cycling (33). Research on indigenous rice varieties has also highlighted their tendency to support a wide range of endophytic bacteria, indicating that Chak-hao offers a particularly supportive environment for beneficial microbes to thrive (34). Further evidence comes from work on wild rice species, where endophyte-inoculated plants exhibited marked improvements in growth-related traits such as overall vigour, tillering, fertility percentage, panicle production per hill and the number of filled grains when compared with uninoculated controls (35).

**Fig. 1.** Level of pH range of soil samples collected from Imphal West district.

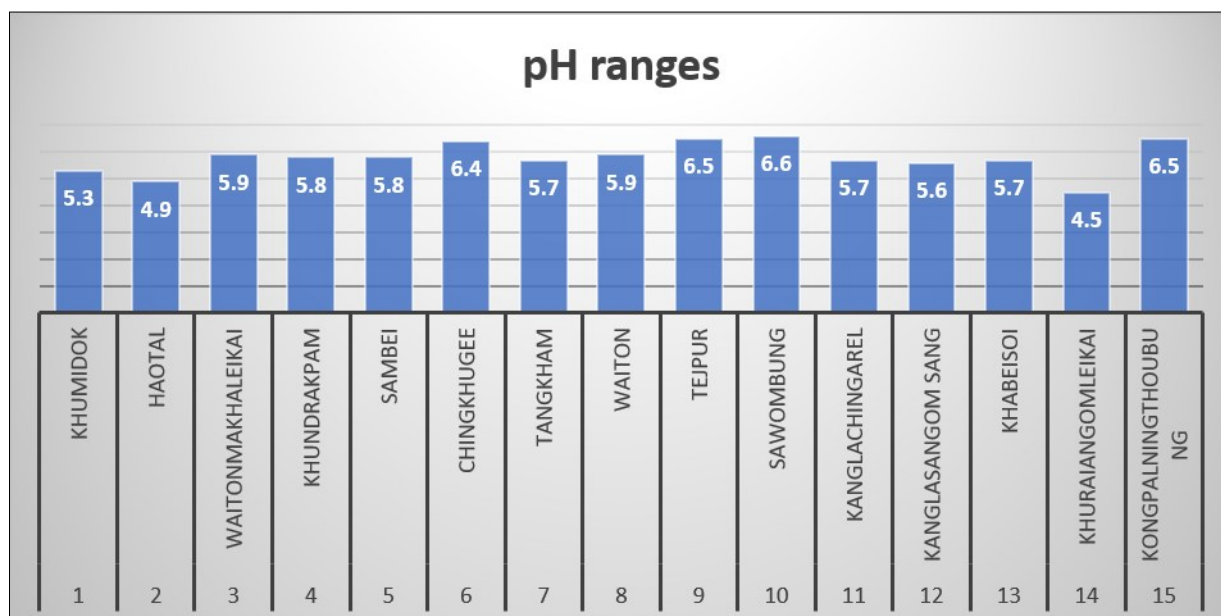


Fig. 2. Level of pH range of soil samples collected from Imphal East district.

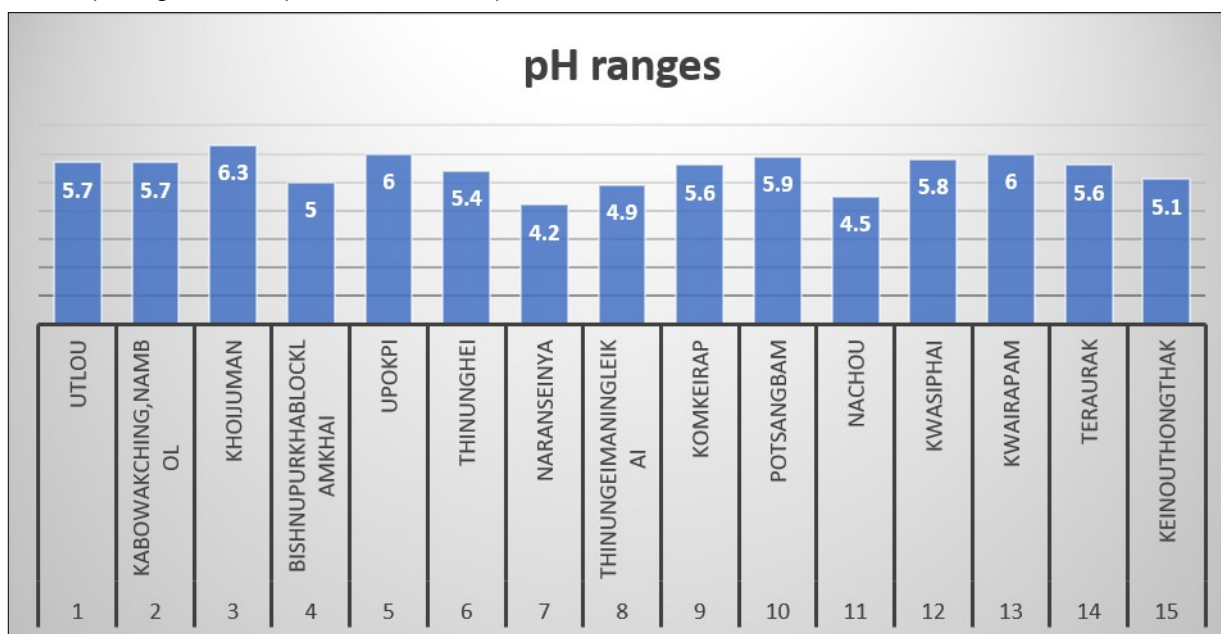


Fig. 3. Level of pH range of soil samples collected from Bishnupur district.

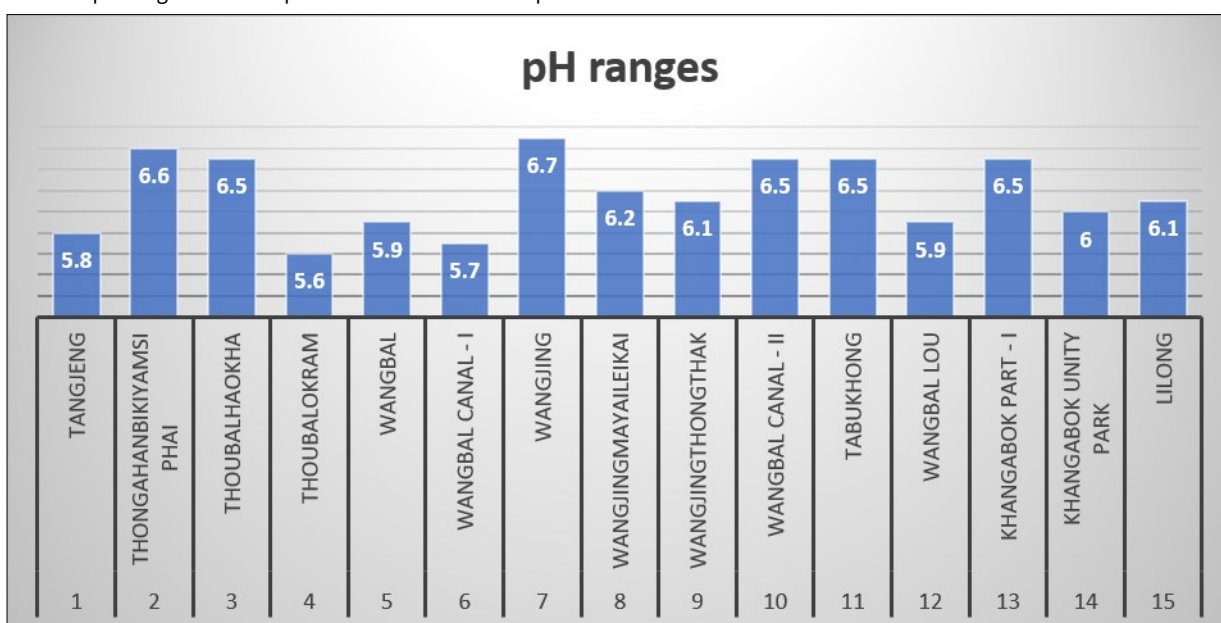


Fig. 4. Level of pH range of soil samples collected from Thoubal district.

Screening for plant growth promoting traits

Screening of plant growth promoting (PGP) traits namely phosphate solubilisation, IAA production and nitrogen fixation was conducted for 170 bacterial isolates. For IAA production assay, pink colour development in the broth cultures under treatment of Salkowski's reagent indicate positive IAA production. Nitrogen-fixing ability was evaluated using nitrogen-free bromothymol blue medium, where a colour change from yellow to green-blue indicated positive nitrogen fixation. For phosphate solubilisation assay in National Botanical Research Institute-bromophenol blue (NBRI-BPB) medium, halo-zone formation around the colony indicates phosphate solubilisation. A 51.94 % of the bacterial isolates and 30.2 % of fungal isolates were phosphate solubilisers showing halo-zone formation in NBRI-BPB agar medium and 9.2 % of the bacterial isolates showed pellicle growth and colour change in nitrogen free bromo-thymol blue semi-solid medium and 41.26 % of the bacterial isolates reacted with Salkowski reagent indicating IAA production (Fig. 5 and 6). For fungal isolates only phosphate solubilisation is accounted for primary screening (Fig. 6). Thus, based on the above primary screening, a total of 55 bacterial and 23 fungal isolates were selected.

These results are consistent with earlier reports that rice-associated microbes frequently express multiple plant growth-promoting (PGP) traits (6, 9). Strong PGP activity including phosphate solubilisation, IAA and siderophore production among endophytes were isolated from wild and cultivated rice (1), supporting the present findings. The high proportion of phosphate-solubilising and IAA-producing isolates shows that the Chak-hao rhizosphere have functionally active microbial communities capable of enhancing the nutrient availability and root development.

Screening based on antagonism against fungal phytopathogen

All 55 bacterial and 23 fungal isolates were subjected to antagonism assays against fungal phytopathogen viz. *Fusarium oxysporum* and *Rhizoctonia solani*. Out of 55 bacterial isolates, 16.36 % and 58.10 % showed antagonism against *Fusarium oxysporum* and *Rhizoctonia solani* respectively (Fig. 7). These results agree with earlier findings showing strong antifungal properties in *Bacillus*, *Paenibacillus* and *Microbacterium* isolated from rice roots (36). These also match reports that Chak-hao rhizosphere harbours *Bacillus* spp. with significant antagonistic activity against major rice pathogens (28), supporting the efficacy observed in the current study.

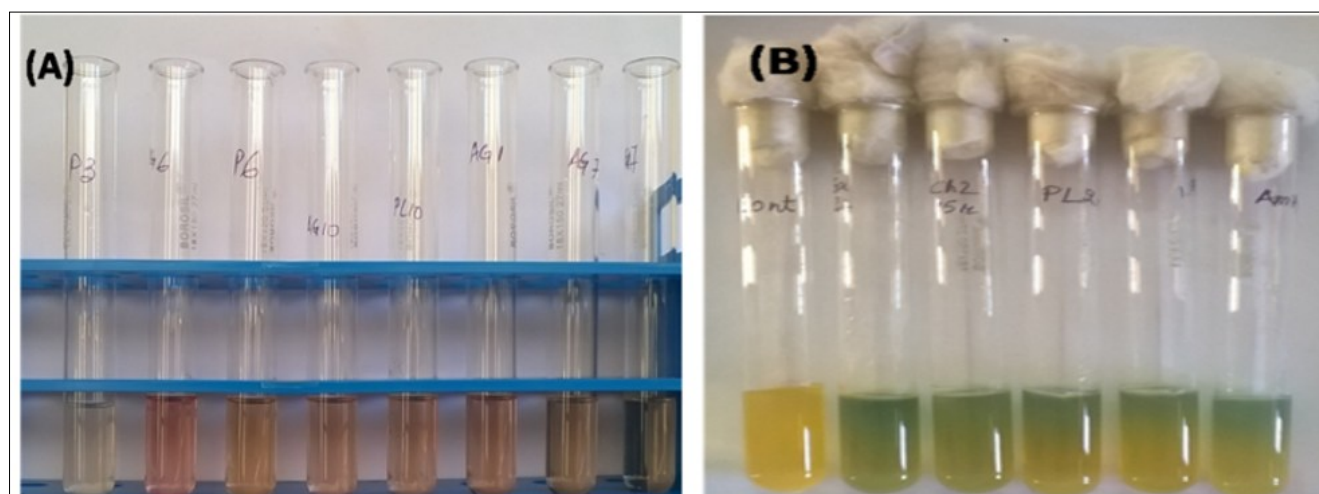


Fig. 5. Screening of plant growth-promoting traits (A) indole-3-acetic acid production assay (B) nitrogen fixation assay.

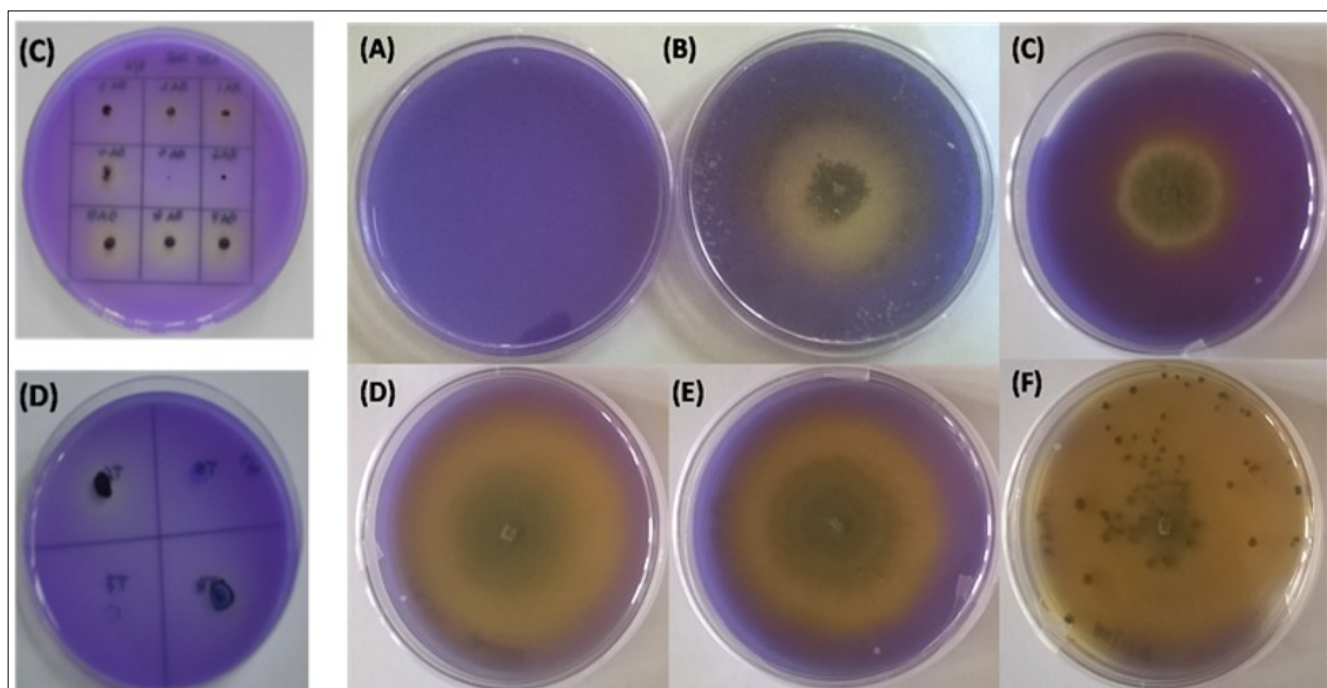


Fig. 6. Screening of bacterial (C, D) and fungal isolates for phosphate solubilisation in National Botanical Research Institute-Phosphate Buffered medium (A): control plate; (B–F): halo-zone formation around the colony that indicate phosphate solubilisation.

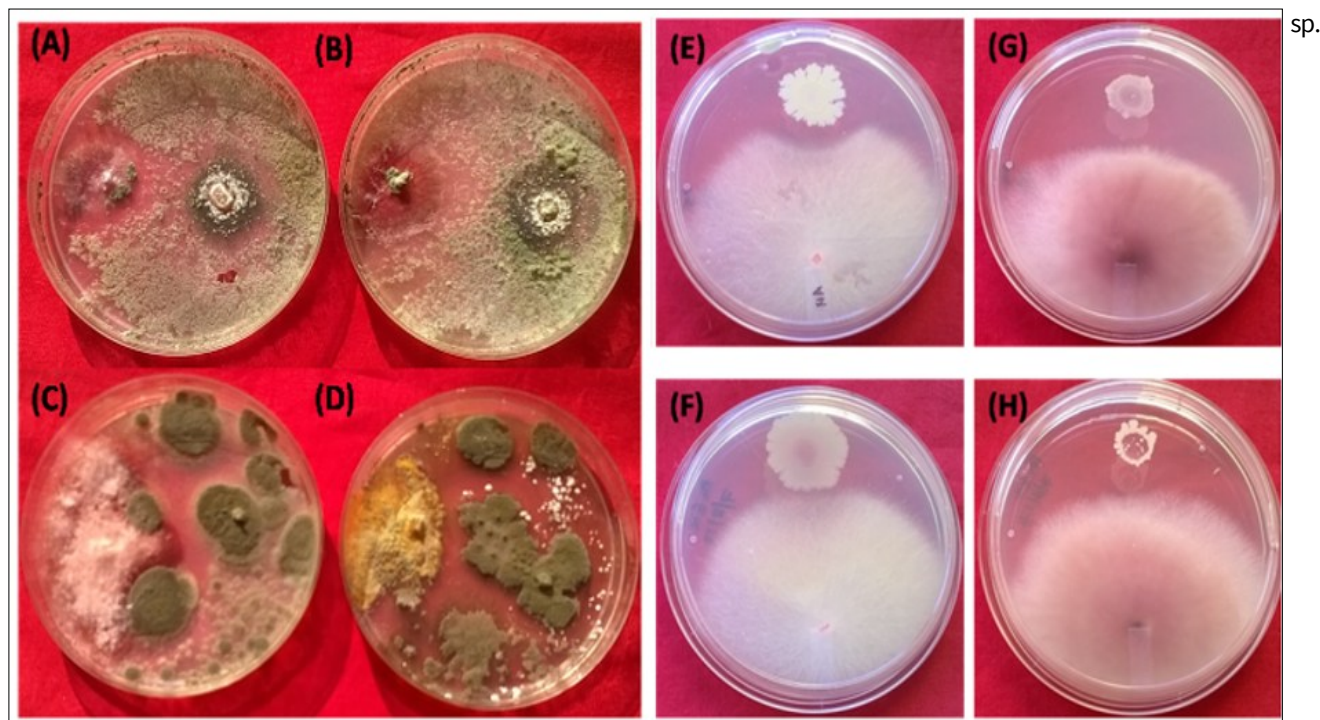


Fig. 7. Screening of fungal isolates for antagonism against phytopathogens.

(A, C): dual culture assay against *Fusarium oxysporum* showing antagonistic interaction of fungal isolates; (B, D): dual culture assay against *Rhizoctonia solani* showing antagonistic interaction; (E, F): antagonistic activity of isolates against *Rhizoctonia solani*; (G, H): antagonistic activity against *Fusarium oxysporum*. Antagonistic fungal isolates are positioned on the left side of the plates, exhibiting inhibition of pathogen growth.

In case of fungal antagonism, fungal growth was observed based on Bell's scale (37). Degree of antagonism was scored on a scale of classes 1–5. Based on this scale, a fungal test strain is said to be antagonistic to the pathogen, if the mean score for a given comparison (comparison of the test strain with different pathogen) was ≤ 2.5 but not highly antagonistic if the number was ≥ 3 . Thus, based on this scale 10 fungal isolates (3 HY and 7 Chak-hao isolates) showed the scale of ≤ 2.5 . This outcome is consistent with earlier observations that certain fungal endophytes can substantially reduce disease severity in rice while simultaneously supporting plant growth (34).

Thus, based on the above multiparametric screening, 18 bacterial and 10 fungal isolates were selected out of which 12 bacteria and 7 fungi from Chak-hao and 6 bacteria and 3 fungi were from high yielding rice variety.

Molecular identification of the selected strains and phylogenetic analysis

Genomic DNA was separately isolated from bacterial and fungal strains using HiMedia DNA isolation kits. For bacterial isolates, the 16S rRNA gene was amplified using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTACCTTGTACGACTT-3') (38). For fungal isolates, the internal transcribed spacer (ITS) region was amplified using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (39). The amplified polymerase chain reaction (PCR) products were sequenced at Excelris Pvt. Ltd. Phylogenetic analysis of sequences of bacteria and fungi were done. Comparison with GenBank databases revealed that bacterial 16S rRNA sequences (Fig. 8) are at least 98–99 % similar to those of known species of *Enterobacter*, *Bacillus* and *Pseudomonas* and fungal ITS sequences (Fig. 9) to those of *Penicillium*, *Trichoderma* and *Aspergillus*. The GenBank analysis revealed the dominance of strains belonging to *Bacillus*

and *Penicillium* sp. Earlier studies showed similarities where these genera frequently occur as rice endophytes with proven biocontrol and PGP capabilities (40, 11). The dominance of *Bacillus* spp. agrees with multiple reports (41, 42) highlighting their strong enzymatic activity, induced systemic resistance (ISR) induction and secondary metabolite production.

Field trial of selected isolates in Chak-hao rice

Field evaluation demonstrated significant improvement in both grain and straw yield of Chak-hao Porieton, particularly with *Bacillus megaterium* (T1) and *Bacillus flexus* (T3) (Table 6) which may be associated with their combined plant growth-promoting and antagonistic activities, including improved phosphate mobilisation, phytohormone production and suppression of soil-borne pathogens, thereby improving nutrient uptake and plant vigour under field conditions. *Bacillus* species are also known to tolerate harsh environmental conditions because they can form endospores, which helps them survive and colonise plant roots more effectively under field conditions. These findings align with the previous observations showing that *Bacillus* spp. such as *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus pumilus* and *Pseudomonas putida*, effectively enhance rice growth while reducing disease incidence (43–45). The *B. subtilis* were also shown to enhance the vigour index by 1574.784 and seed germination rates by 96 % (46). The consortium treatment (T6) showed less performance than the control, which may be because of the different microbial strains in the mixture, not interacting well with each other. Competition between the strains for nutrients and space could have reduced their efficacy in the rhizosphere. Also, their differences in survival and colonisation ability under the field conditions may have hindered the expected synergistic effect of the consortium. Other studies have observed that microbial consortia do not always outperform individual strains under field conditions (47).

Results of the field trial data were subjected to analysis of

Fig. 8. Phylogenetic analysis based on 16S ribosomal DNA sequencing.

Fig. 9. Neighbour joining phylogenetic tree based on internal transcribed spacer region of fungus.

Table 6. Effect of five rhizobacterial strains on the grain and straw yield of Chak-hao Porieton

Treatments	Grain yield	Straw yield
T1	2.45c	4.87e
T2	1.33a	2.8cd
T3	2.19c	5.31f
T4	1.78b	3.03d
T5	1.83b	2.63bc
T6	1.25a	2.51b
T7 (Control)	1.06a	2.08a

Values followed by different letters indicate a significant difference ($p = 0.05$) between treatments as compared with the control, by Duncan's multiple range test.

variance (ANOVA) and additionally Duncan's multiple range test ($p \leq 0.05$) for the bioassay results using SPSS Statistical Software package, version 2.1.

Gas chromatography-mass spectrometry profiling of aroma and antioxidant-associated compounds in Chak-hao

Gas chromatography-mass spectrometry profiling of Chak-hao rice showed the presence of several volatile and bioactive metabolites, such as fatty acids, fatty acid esters, alcohols, terpenoids and antioxidant-associated compounds. Major compounds identified were N-hexadecanoic acid, pentadecanoic acid and L-(+)-ascorbic acid 2,6 dihexadecanoate with higher peaks of retention time in these compounds (Table 7) (Fig. 10). Higher levels of important aromatic and antioxidant-related compounds were observed in treatments inoculated with beneficial microbes (T1, T3, T5 and T6) than in the uninoculated control (T7), indicating that microbial

inoculation had a positive effect on the biochemical profile of Chak-hao rice (Table 8). Significant differences were observed between the treatment as compared to the control while there was no significant difference in grain yield between the consortia treatment (T6) and the control (T7). This enhancement may be attributed to improved nutrient availability, phytohormone production and modulation of plant metabolic pathways by plant growth-promoting microorganisms, which can stimulate the synthesis of secondary metabolites associated with aroma and antioxidant activity. Similar volatile metabolites, including fatty acids, esters, aldehydes and alcohols, have also been reported in aromatic and pigmented rice varieties in previous studies (19, 48). This is a preliminary study and final conclusions can only be drawn after further investigation of the specific aroma-related compounds and their responses to microbial application.

Table 7. Gas chromatography-mass spectrometry analysis of the Chak-hao Porieton showing the one peak retention time and probable compound

Treatment Name	Retention time	Compound name	SI	Function
T1R1	34.194	N-hexadecanoic acid	≥ 0.8	Experimentally measured binding affinity data (Kd) for protein-ligand complexes derived from PDB
		L-(+)-ascorbic acid 2,6-dihexadecanoate		1. Antioxidant activity against superoxide dismutase like activity after 15 min by NBT spectrometric assay 2. Antioxidant activity assessed as DPPH free radical scavenging activity after 30 min
		Pentadecanoic acid		qHTS assay to identify small molecule agonists of the peroxisome proliferator-activated receptor delta (PPAR δ) signalling pathway: Summary

SI: similarity index; PDB: protein data bank; NBT: nitroblue tetrazolium; DPPH: 2,2-diphenyl-1-picrylhydrazyl; qHTS: quantitative high-throughput screening; Kd: dissociation constant.

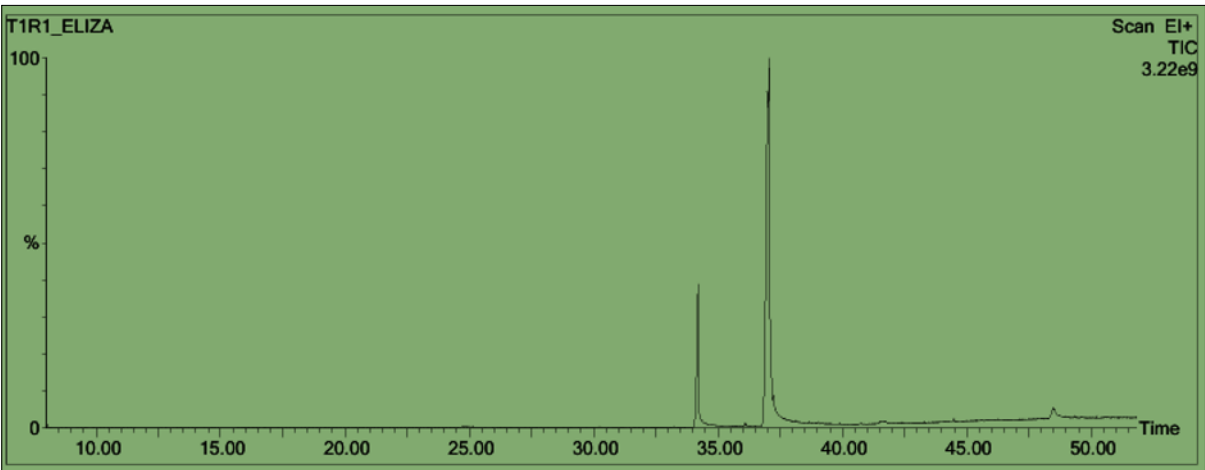


Fig. 10. Gas chromatography-mass spectrometry analysis of the Chak-hao Porieton.

Table 8. Gas chromatography-mass spectrometry analysis of the Chak-hao Porieton

Sl. No	RT	Treatments					
		T1	T3	T5	T6	T7	
1	34.194	119,228,240.00	211,721,840.00	244,942,528.00	219,313,472.00	82,758,408.00	Peak value
2	37.05	526,871,424.00	785,685,440.00	896,957,568.00	883,766,528.00	371,638,624.00	
3	37.22	83,562,384.00	180,796,576.00	193,815,280.00	180,488,112.00	98,216,560.00	

Conclusion

The present study showed that Chak-hao rice harbours a diverse community of endophytic and rhizospheric microorganisms with significant plant growth-promoting and biocontrol potential. The indigenous microbial isolates primarily belonging to *Bacillus*, *Enterobacter*, *Pseudomonas*, *Penicillium*, *Trichoderma* and *Aspergillus* exhibited significant functional traits including phosphate solubilisation, indole-3-acetic acid (IAA) production, nitrogen fixation and antagonism against major rice pathogens. Field trials demonstrated that selected isolates, especially *Bacillus megaterium* and *Bacillus flexus*, significantly improved the grain yield, straw yield and aroma-associated biochemical compounds of Chak-hao rice. Gas chromatography-mass spectrometry analysis also showed higher antioxidant and aromatic metabolites in microbe- treated plants, which indicates positive microbial effect on nutritional and quality traits. These findings highlight the potential of indigenous plant growth-promoting rhizobacteria and endophytes as eco-friendly bionoculants for sustainable cultivation, biocontrol potential against harmful pathogens and conservation of unique traditional rice germplasm, by improving the productivity and quality in Chak-hao rice. The study also provides a basis for the development of effective microbial bioformulations that can support sustainable and low-input rice cultivation in Northeast India. Future studies should focus on development of efficient microbial consortia and bioformulations for wider adoption. Multi-location field trials in different agro-ecological zones of Northeast India will help validate their effectiveness. Advanced metagenomic studies could further uncover functional pathways involved in aroma enhancement and stress tolerance. Integration of these microbial communities with organic and geographic indication (GI)-based cultivation practices have great potential for conserving Chak-hao biodiversity as well as improving the farmer income and soil health.

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Authors' contributions

BS supervised the study, developed the overall concept and reviewed the manuscript draft. ET conducted experiments and prepared the initial manuscript. CN performed data analysis and contributed to editing and moderation. NTA revised the language and grammar. HMD carried out the calculations and assisted in manuscript revision. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used Elicit AI and Grammarly to improve language quality, perform grammar correction, assist with paraphrasing and identify relevant literature. All scientific content, data analysis, interpretation of results and conclusions are solely the responsibility of the authors. After using these tools, the authors reviewed and edited the content as needed and took full responsibility of the content for publication.

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