Appraisal of diversity and functional attributes of thermotolerant wheat associated bacteria from the peninsular zone of India

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Abstract The biodiversity of wheat associated bacteria was deciphered from the peninsular zone of India. A total of 264 isolated bacteria were analyzed through amplified ribosomal DNA restriction analysis (ARDRA, using three restriction enzymes Alul, Msp I and Hae III, which led to the clustering of these isolates into 12–16 groups for the different sites at >75% similarity index, adding up to 70 groups). 16S rRNA gene based phylogenetic analysis, revealed that all the bacteria belonged to three phyla Proteobacteria, Firmicutes, and Actinobacteria of 32 distinct species of 15 genera namely: Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Delftia, Enterobacter, Exiguobacterium, Klebsiella, Methylobacterium, Micrococcus, Paenibacillus, Pseudomonas, Rhodobacter, Salmonella and Staphylococcus. Representative strains from each cluster were screened in vitro for plant growth promoting traits. Among plant growth promoting activities, siderophore producers were highest (15%), when compared to indole acetic acid producers (13%), Zn-solubilizers (11%), P-solubilizers (11%), ammonia (10%), hydrogen cyanide producers (9%), biocontrol (8%), N2-fixers (7%), 1-aminocyclopropane-1-carboxylate deaminase (6%), GA producers (6%) and K-solubilizers (5%). Among 32 representative strains, Alcaligenes faecalis, Arthrobacter sp., Bacillus subtilis, Delftia acidovorans, Methylobacterium mesophilicum, Methylobacterium sp., Pseudomonas poae, Pseudomonas putida, and Pseudomonas stutzeri exhibited more than six

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1. Introduction

Biodiversity is an important element of environmental conservation and is central to agriculture production. Globally wheat (*Triticum aestivum* L.) is the most important cereal crop. It is grown across a wide range of environments around the world and has the highest adaptation among all the crop species (Naresh et al., 2014). Wheat is grown in India in an area of about 30 million hectares (mha), with a production of 93 million tons. The normal national productivity is about 2.98 tones ha⁻¹. Currently, India is the second largest producer of wheat in the world after China with about 12% share in total world wheat production. Now, India is surplus and in a position to export wheat in the International Market and can earn foreign exchange (http://www.fao.org/docrep/006/y4011e/y4011e04.htm). Global warming and its associated effects are expected to impose abiotic stresses, such as extremes of temperatures, drought and flooding, that are bound to have adverse effects on food production. Climate change affects agriculture and the food production system in many ways (Godfray et al., 2011). Crop production is affected by climatic variables such as rising temperatures, changing precipitation regimes and increased atmospheric CO₂ levels. It is also affected by biological variables such as the lengths of the crop growth periods and the crop cycle.

Wheat is growing in six agro-ecological zones in India, in which peninsular zone exhibits unique characteristics in the distribution and variation in the meteorological fields compared to other parts of the country. Epiphytic, endophytic and rhizospheric bacteria have been shown to promote plant growth directly, e.g. by the fixation of atmospheric nitrogen, solubilization of minerals such as phosphorus, potassium and zinc, production of siderophores and plant growth hormones such as cytokinin, auxin and gibberellins. Several bacteria support plant growth indirectly, *via* production of antagonistic substances by inducing resistance against plant pathogens (Glick, 2015; Verma et al., 2015a; Egamberdieva et al., 2015). It has gained importance over the use of chemical fertilizers on account of deleterious effect of the latter on soil and plant health (Glick, 2015). Divergent bacterial genera are vital components of soils and plant growth. They are involved in various biotic activities of the soil ecosystem to make it dynamic for nutrient turn over and sustainable for crop production (Hayat et al., 2010). Indeed, the bacteria lodging around/in the plant roots, shoots and leaves are more versatile in transforming, mobilizing, and solubilizing the nutrients (Hayat et al., 2010). Therefore, the rhizobacteria are the dominant deriving forces in recycling the soil nutrients and consequently, they are crucial for soil fertility (Glick, 2015).

Wheat (*Triticum aestivum* L.) is a major staple food crop for more than one third of the world population and is the main staple food of Asia. The wheat associated bacterial diversity inhabiting low temperature (Mishra et al., 2011; Verma et al., 2015a), drought (Verma et al., 2014), acidic soil (Verma et al., 2013) and salinity (Egamberdieva et al., 2008; Tiwari et al., 2011) has been extensively investigated in the past few years with a focus on culture dependent techniques. However there are no reports available on thermotolerant bacterial diversity associated with wheat. The present study investigated on understanding the diversity and distribution of culturable bacteria associated with wheat growing in the peninsular zone of India. Bacteria were isolated, identified using 16S rRNA gene sequencing and characterized for plant growth promoting attributes at high temperature. The use of thermotolerant bacteria as biofertilizers and biocontrol agents would be of great use in Indian agriculture under high temperature conditions.

2. Material and methods

2.1. Sample collection and physico-chemical properties

The wheat plant with rhizospheric soil was collected from different sites in the peninsular zone of India. Forty-one samples were collected from five different sites in the peninsular zone namely: Dharwad, Nashik, Warangal, Coimbatore and Krishna Nagar, whose detail is provided in Table 1. Collected samples in sterile polythene bags were labeled and transported on ice stored at 4 °C, until samples process quickly to the laboratory (Table 1). The pH and electrical conductivity of the samples were recorded at sampling sites. Methods of soil organic carbon, total nitrogen, exchangeable cations and

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Table 1: Geographic details and physico-chemical characteristics of samples collection sites.

| Sampling sites | GPS coordinate | No. of samples | Temperature (°C) | pH | Conductivity (mS cm⁻¹) |
|---------------|----------------|---------------|------------------|----|-----------------------|
| Nashik        | 19° 59' 00" N: 73° 48' 05" E | 16 | 35.5–35.7 | 6.8–7.9 | 261–805 |
| Warangal      | 18° 01' 05" N: 79° 35' 02" E | 18 | 33.2–42.5 | 7.1–8.5 | 376–392 |
| Krishna Nagar | 23° 24' 01" N: 88° 33' 05" E | 12 | 35.2–45.6 | 7.3–8.3 | 345–365 |
| Dharwad       | 15° 28' 02" N: 75° 01' 01" E | 17 | 35.8–47.5 | 6.9–8.1 | 237–255 |
| Coimbatore    | 10° 59' 33" N: 76° 57' 41" E | 14 | 34.9–47.8 | 6.8–8.4 | 266–287 |
available phosphorus was analyzed as followed in our earlier studies (Yadav et al., 2015e).

2.2. Isolation and enumeration of bacteria

Wheat associated bacteria were isolated from leaves, shoots, roots and rhizospheric soils using different growth media (Supplementary Table S1). The culturable bacteria were isolated from rhizospheric soil through the enrichment method, using the standard serial dilution plating technique. Epiphytic and endophytic bacteria were isolated as methods described in our earlier studies (Verma et al., 2015a). Plates were incubated at 30–50 °C for the growth of bacteria. Colonies that appeared were purified by repeated streaking on their respective medium plates. The pure cultures were maintained at 4 °C as slant and glycerol stock (25%) at −80 °C for further use.

2.3. Molecular characterization and amplified rDNA restriction analysis

Genomic DNA was extracted by the method as described earlier by Verma et al. (2016b). 16S rRNA gene sequence was amplified using universal primers pA (5′-AGAGTTT GATCCTGGTCGAG-3′) and pH (5′-AAGGAGGTGATC CAGCCGCA-3′). The amplification program employed in a 100 μL composed: each primer at a concentration of 1 μM, 5 U of Taq DNA polymerase, 1.5 mM MgCl2, 90 ng template DNA and 10 μL 10X Taq buffer and amplification conditions used were as described earlier (Yadav, 2015). The PCR product was purified by PCR purification kit (Qiagen USA). Aliquots of purified 16S rDNA PCR products were digested separately with three restriction endonucleases Alu I, Hae III and Msp I in 25 μL reaction volumes, using the manufacturer’s recommended buffer and temperature. Restricted DNA was analyzed by horizontal electrophoresis in 2.5% agarose gels. The gels were visualized and gel images were digitalized. Strong and clear bands were scored for similarity and clustering analysis was undertaken using NT SYS-2.02e package (Numerical taxonomy analysis program package, Exeter software, USA). Similarity among the isolates was calculated by Jaccard’s coefficient and the dendrogram was constructed using UPGMA method as described earlier (Suman et al., 2015b).

2.4. Sequencing of 16S rRNA gene and phylogenetic analysis

PCR products of partial 16S rRNA gene were sequenced with fluorescent terminators (Big Dye, Applied Biosystems) and run in 3130xl Applied Biosystems ABI prism automated DNA sequencer at Xcelris Labs Ltd. Ahmedabad (India). The DNA sequence was double checked by sequencing both strands using primers pA and pH for forward and reverse reactions, respectively. The partial 16S rRNA gene sequences of the isolated strains were BLAST searched on the NCBI GenBank website to identify the closest matches. Sequence alignment and comparison was performed, using the program CLUSTAL W software. One sequence from each group was selected as a representative operational taxonomic unit (OTU). The phylogenetic tree was constructed on the aligned datasets using the neighbor-joining method implemented in the program MEGA 4.0.2 software (Tamura et al., 2007).

2.5. Nucleotide sequence accession numbers

The partial 16S rRNA gene sequences of 32 bacteria were submitted to NCBI GenBank under the assigned accession numbers KF054914-KF054942, KF054945 and KF573002-KF573003.

2.6. Statistical analysis for bacterial diversity

In order to compare the bacterial diversity among five different sites in the peninsular zone, the 16S rRNA gene sequences of the isolates showing ≥97% sequence similarity were grouped into the same OTU (phylogotype). The Shannon index (H), Evenness (J) Simpson’s index (D) and Chao-1 were calculated as described earlier (Yadav et al., 2015c). Using 16S rRNA gene sequences, a rarefaction curve was generated to compare the relative diversity and coverage of each sample. Principal coordinate analysis (PCA) was performed for different physico-chemical properties and distribution ratio, using the XLSTAT program (http://www.xlstat.com).

2.7. Phenotypic characterization

Representative isolates from each cluster were screened for tolerance to temperatures (30–70 °C), pH (3–11), and salinity (3–20% NaCl concentration) as describes earlier (Yadav et al., 2015e). Wheat associated bacterial isolates were initially screened for direct PGP attributes which included the production of phytohormones indole-3-acetic acid, gibberellic acid and 1-aminoclopropane-1-carboxylate (ACC) deaminase as described earlier (Verma et al., 2016b). Solubilization of phosphorus, potassium and zinc was carried according to methods described by Pikovskaya (Pikovskaya, 1948), Hu et al. (2006) and Fasim et al. (2002) using Pikovskaya agar, Aleksandrov medium and nutrient agar medium supplemented with 0.5% tricalcium phosphate, 0.2% potassium aluminosilicate minerals and 0.1% insoluble zinc compounds (ZnO, ZnS, Zn3(PO4)2 and ZnCO3) respectively. The bacterial strains were screened for their ability to utilize the 1-Aminoclopropane-1-carboxylate (ACC) as the sole nitrogen source, a trait that is a consequence of the activity of the enzyme ACC deaminase (Jacobson et al., 1994). The bacterial strains were spotted on MDF (modified Dworkin and Foster medium) agar plate, MDF agar plate supplemented with 0.3 g L−1 of ACC and MDF agar plate with ammonium sulfate 0.3 g L−1. After 72 h of incubation, plates were observed for growth. All the representative strains were also screened for indirect PGP attributes which included the production of siderophores (Schwyn and Neilands, 1987), ammonia (Cappucino and Sherman, 1992), and HCN (Bukker and Schippers, 1987). All assays were done in triplicate.
2.8.2. Quantitative estimation

Representative strains were further screened quantitatively for the solubilization of phosphorus, potassium and zinc; biological nitrogen fixation and indole acetic acid production as methods described in our earlier studies (Verma et al., 2016b). Gibberellic acid production was estimated by colorimetric method of Holbrook et al. (1961). One milliliter bacterial culture was inoculated in 50 mL nutrient broth in Erlenmeyer flasks (100 mL), and incubated at 30 °C in a rotary shaker at 180 rpm. The bacterial cultures were harvested and centrifuged at 10,000g for 30 min. The culture supernatant was acidified (pH 2.5 using 2.0 N HCl) and extracted by adding an equal volume of ethyl acetate in three stages to obtain 45 mL extract. The extract was evaporated to 5.0 mL and gibberellic acid was estimated by colorimetric method using 100 μg/ml gibberellic acid (Sigma, USA) as standard.

2.9. Antagonistic property

In vitro antagonistic activity of bacterial isolates was evaluated against three fungal pathogens Fusarium graminearum ITCC 1856, Rhizoctonia solani ITCC 2775 and Macrophomina phaseolina ITCC 3134 according to the method described by Sijam and Dikin (2005). The fungal pathogens involved in root-rot complex in crops, were obtained from the Indian Type Culture Collection (ITCC), Indian Agricultural Research Institute, New Delhi, India. The fungal strains were inoculated on the potato dextrose agar plates and allowed to grow from 3–5 days. Bacterial strains were spot inoculated on potato dextrose agar plates already inoculated with fungal colony. Control plates with only the mycelia plug were set up and, when the pathogen had grown across these control plates, the diameter of growth in the challenge plates was measured. Triple culture assays were repeated three times per isolate and for each fungus.

3. Results

3.1. Isolation of wheat associated bacteria

The populations of bacterial isolates were enumerated in wheat plant samples collected from five diverse sites in the peninsular zones of India (Table 1). A total of 264 bacteria were isolated from Nashik (48), Warangal (43), Krishna Nagar (59), Dharwad (62) and Coimbatore (52). The significant variations were observed among the culturable bacterial populations of each sample on different media and at different sites (Supplementary Table S1). The abundance of bacteria varied from 1.4 × 10^3 to 7.2 × 10^6 CFU g^-1 soils, 1.9 × 10^6 to 6.32 × 10^6 CFU g^-1 leaves and 1.2 × 10^6 to 2.58 × 10^6 CFU g^-1 roots with the lowest and highest values recorded in Nashik and Warangal, respectively. Among the different media used, tryptic soy agar supported the highest population of bacteria 7.2 × 10^6 CFU g^-1 soil and Jensen’s agar medium the least growth for bacteria 1.4 × 10^3 CFU g^-1 soil. The highest population and number of bacteria (172) were isolated from the rhizospheric samples, whereas the least number of bacteria (26) was recovered from putative endophytic samples (Supplementary Table S1).

3.2. Molecular characterization and phylogenetic analysis

PCR amplification of 16S rRNA gene followed by ARDRA with three restriction endonucleases was carried out to look for the species variation among the morphotypes selected. The 16S rDNA amplicons were digested with three restriction enzymes, which generated profiles having 3–7 fragments ranging in size from 100 to 850 base pairs. The dendrogram was constructed for each site to determine the percent similarity among the isolates. The isolates were grouped into clusters; and the number of clusters ranged from 12–16 adding up to 70 groups (Table 2). All 70 strains were sequenced and BLAST analysis showed >97–100% similarity with the sequences available in GenBank (Table 3), that revealed the identification of 32 distinct species of fifteen genera namely: Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Delftia, Enterobacter, Exiguobacterium, Klebsiella, Methylobacterium, Micrococcus, Paenibacillus, Pseudomonas, Rhodobacter, Salmonella and Staphylococcus. The eleven isolates showing <96% similarity in partial 16S rRNA gene sequences within the GenBank database need further characterization and validation to confirm their taxonomic position. The phylogenetic tree of 32 identified species of bacteria and related genera were constructed to determine their affiliations (Fig. 1a and b).

Analysis of the 16S rRNA gene sequences revealed that 32 strains belonged to three phyla namely Proteobacteria (22 strains), Firmicutes (10 strains) and Actinobacteria (2 strains) (Fig. 1a and b). The Proteobacteria were further distributed into 3 class, as α-proteobacteria (4 strains), β-proteobacteria (7 strains) and γ-proteobacteria (11 strains). Analysis of the 16S rRNA gene sequences revealed that 65%, 29% and 6% bacteria belong to three phylum namely Proteobacteria, Firmicutes and Actinobacteria respectively, with 32 distinct species of 15 genera (Fig. 2a and b). The Proteobacteria was the most pre-dominant phylum followed by Firmicutes. Arthrobacter from Actinobacteria, Bacillus from Firmicutes and Pseudomonas from Proteobacteria were the most frequently recovered genera, respectively (Table 2; Fig. 1).

3.3. Statistical analysis

All 264 isolates from the five different sites of wheat growing in the peninsular zone were categorized into 32 clusters, based on a similarity index of >75% of the ARDRA pattern. Sequencing of representative isolates from each cluster led to the identification of 32 distinct strains. Of the 32 strains identified, four strains, Bacillus amyloliquefaciens, Bacillus subtilis, Methylobacterium sp. and Pseudomonas aeruginosa were common to all five sites (Table 2). Shannon’s diversity index ($H = 2.73$) and species richness was the highest for Krishna Nagar, whereas Warangal recorded the lowest value ($H = 2.42$). These observations are supported by bacterial diversity parameters, such as Simpson’s index, Chao-1, Evenness, and Shannon Entropy and individual rarefaction curves (Table 2). The individual rarefaction curves for all the samples of five sites indicated that the bacterial population were the least diverse in Warangal followed by Coimbatore and most diverse in Krishna Nagar followed by Dharwad (Fig. 3a). Principal coordinate analysis was used to investigate relation-
The first two dimensions of PCA (PCA1 and PCA2) explained 83.87% of the total variation, with component 1 accounting for 69.08% and component 2 for 14.79% of the variance (Fig. 2b). Correlation analysis proved the existence of significant relationship between the different parameters such as temperature, pH, conductivity, organic carbon, available nitrogen, phosphorus, potassium, zinc, exchangeable sodium, dehydrogenase and alkaline phosphatase of sampling sites. The first two factorial axes of biplot represented 26.15–46.29% variance in the data (Fig. 3c).

3.4. Phenotypic characterization

All 264 bacteria were screened for tolerance to different abiotic stresses and results are represented by representative strains.

Table 2 Distribution and diversity indices of bacteria isolates from different sites in the peninsular zone of India.

| Bacterial isolates                        | Nashik | Warangal | Krishna Nagar | Dharwad | Coimbatore |
|------------------------------------------|--------|----------|--------------|---------|------------|
| *Achromobacter spanius*                  | 3*     | 2        | 5            | 5       |            |
| Alcaligenes faecalis                     |        |          |              |         |            |
| Alcaligenes sp.                          | 2      | 3        | 5            | 5       |            |
| Arthrobacter sp.                         | 3      | 3        | 5            | 5       |            |
| Bacillus altitudinis                      |        |          |              |         |            |
| Bacillus amyloliquefaciens               | 5@     | 2        | 3            | 5       | 4          |
| Bacillus flexus                          | 3      | 4        | 3            | 2       |            |
| Bacillus licheniformis                   | 5      | 3        | 5            | 4       |            |
| Bacillus mojavensis                      |        |          |              |         | 5          |
| Bacillus siamensis                       |        |          |              |         |            |
| Bacillus subtilis                        | 3      | 2        | 3            | 5       | 4          |
| *Delftia acidivorans*                    |        |          |              |         | 5          |
| Delftia lacustris                        |        |          |              |         |            |
| Delftia sp.                              | 5      | 5        |              | 3       | 4          |
| *Delftia tsuruhatensis*                  |        |          |              |         |            |
| Enterobacter asburiae                    |        |          |              |         | 5          |
| Enterobacter hormaechei                  |        |          |              |         | 6          |
| *Exiguobacterium acetylicum*             |        |          |              |         |            |
| Klebsiella oxytoca                       |        |          |              |         |            |
| Micrococcus luteus                       | 5      |          | 4            |         |            |
| Methylobacterium sp.                     | 2      | 3        | 5            | 5       | 4          |
| *M. mesophilicum*                        |        |          |              |         | 5          |
| *Paenibacillus amylolyticus*             | 2      |          |              |         |            |
| *Pseudomonas aeruginosa*                 | 3      | 4        | 5            | 5       | 4          |
| Pseudomonas japonica                     |        |          |              |         |            |
| Pseudomonas poae                         |        |          |              | 2       |            |
| Pseudomonas putida                       |        |          |              | 2       | 4          |
| *Pseudomonas stutzeri*                   |        |          |              | 5       | 2          |
| Rhodobacter capsulatus                   |        |          |              | 3       |            |
| Rhodobacter sphaeroides                  |        |          |              |         | 4          |
| Salmonella bongori                       |        |          |              |         | 5          |
| *Staphylococcus succinu*                 |        |          |              |         |            |
| Total isolates                           | 48     | 43       | 59           | 62      | 52         |
| Species richness                         | 14     | 12       | 16           | 15      | 13         |
| Chao-1                                    | 14     | 12       | 16           | 15      | 13         |
| Niche-specific                           | 02     | 03       | 02           | 04      | 05         |
| Simpson’s (D)                            | 0.91   | 0.90     | 0.93         | 0.92    | 0.91       |
| Shannon (H)                              | 2.57   | 2.42     | **2.73**     | 2.63    | 2.52       |
| Evenness (J’)                            | 0.93   | 0.94     | 0.95         | 0.93    | 0.96       |

Niche-specific bacteria; @common bacteria at all sites.
| Nearest phylogenetic relative | Strain number | Relative distribution (%) | Solubilization |
|-------------------------------|---------------|---------------------------|----------------|
|                               |               |                           | Phosphorus | Potassium | Zinc# |
| Achromobacter spanius         | IARI-NIAW2-15| 1.1                       | 61.9 ± 0.2 | –         | 2.4 ± 0.1 |
| Alcaligenes faecalis          | IARI-NIAW1-6 | 3.8                       | 55.9 ± 1.4 | –         | 3.3 ± 0.2 |
| Alcaligenes sp.               | IARI-NIAW1-8 | 6.8                       | 63.0 ± 1.0 | –         | 1.6 ± 0.9 |
| Arthrobacter sp.              | IARI-NIAW1-4 | 7.2                       | 66.0 ± 0.7 | –         | 2.0 ± 1.1 |
| Bacillus altitudinis          | IARI-NIAW1-38| 3.8                       | 45.7 ± 1.1 | 23 ± 0.5 | 4.3 ± 0.2 |
| Bacillus amyloliquefaciens    | IARI-NIAW1-23| 12.5                      | –          | –         | –         |
| Bacillus flexus               | IARI-NIAW2-25| 8.0                       | 47.8 ± 0.1 | –         | –         |
| Bacillus licheniformis        | IARI-NIAW2-3 | 8.7                       | 45.6 ± 1.0 | –         | –         |
| Bacillus mojavensis           | IARI-NIAW2-23| 3.8                       | 43.9 ± 0.7 | –         | –         |
| Bacillus siamensis            | IARI-NIAW1-21| 6.1                       | –          | 27 ± 0.9 | 2.6 ± 0.1 |
| Bacillus subtilis             | IARI-NIAW1-13| 11.7                      | 47.9 ± 1.4 | 17 ± 0.9 | 4.5 ± 0.2 |
| Delftia acidovorans           | IARI-NIAW1-20| 3.8                       | 47.2 ± 1.4 | 33 ± 0.5 | 5.0 ± 0.6 |
| Delftia lacticrís             | IARI-NIAW1-34| 3.8                       | 43.7 ± 0.9 | –         | 1.1 ± 0.4 |
| Delftia sp.                   | IARI-NIAW1-31| 7.2                       | –          | 12 ± 0.8 | 2.4 ± 0.8 |
| Delftia tsuruhatensis         | IARI-NIAW1-15| 3.0                       | –          | –         | 2.0 ± 1.3 |
| Enterobacter asburiae         | IARI-NIAW2-21| 3.8                       | –          | –         | 1.5 ± 0.1 |
| Enterobacter hormaechei       | IARI-NIAW2-34| 4.5                       | –          | –         | –         |
| Exiguobacterium acetylicum    | IARI-NIAW2-27| 3.8                       | –          | –         | –         |
| Klebsiella oxytoca            | IARI-NIAW2-11| 3.8                       | –          | –         | –         |
| Micrococcus luteus            | IARI-NIAW1-1 | 4.9                       | –          | –         | –         |
| Methylobacterium sp.          | IARI-NIAW2-37| 13.6                      | 41.6 ± 0.1 | 27 ± 0.9 | 3.6 ± 0.1 |
| Methylobacterium mesophilicum | IARI-NIAW1-41| 3.8                       | 43.2 ± 1.1 | 29 ± 0.5 | 3.4 ± 0.2 |
| Paenibacillus amylolyticus    | IARI-NIAW2-33| 0.8                       | –          | –         | –         |
| Pseudomonas aeruginosa        | IARI-NIAW1-2 | 11.0                      | –          | 33 ± 1.2 | 1.6 ± 0.7 |
| Pseudomonas japonica          | IARI-NIAW2-24| 2.3                       | 64.6 ± 0.9 | –         | 2.6 ± 0.5 |
| Pseudomonas poae              | IARI-NIAW2-1 | 1.5                       | 34.4 ± 1.2 | –         | 3.6 ± 1.2 |
| Pseudomonas putida            | IARI-NIAW1-16| 9.8                       | 54.6 ± 0.9 | –         | –         |
| Pseudomonas stutzeri          | IARI-NIAW1-3 | 3.0                       | 51.6 ± 1.0 | –         | 2.1 ± 1.1 |
| Rhodobacter capsulatus        | IARI-NIAW1-9 | 3.8                       | –          | –         | –         |
| Rhodobacter sphaeroides       | IARI-NIAW1-7 | 9.1                       | 45.0 ± 1.2 | –         | –         |
| Salmonella bongori            | IARI-NIAW2-19| 6.8                       | 55.7 ± 0.5 | 28 ± 1.2 | –         |
| Staphylococcus succinus       | IARI-NIAW2-28| 1.1                       | 40.4 ± 1.1 | –         | –         |

| Strain number | Production | Other activities |
|---------------|------------|------------------|
|               | IAA        | Sidero#           | GA | HCN | NH₃ | ACC | N₂F | BC  |
| IARI-NIAW2-15| 27.8 ± 1.2 | 1.0 ± 0.1         | –  | –   | +  | –   | 11.5 ± 1.5 | –   |
| IARI-NIAW1-6 | 21.4 ± 1.3 | 4.9 ± 0.1         | –  | –   | +  | +   | 17.5 ± 1.0 | +   |
| IARI-NIAW1-8 | 20.4 ± 1.1 | 2.1 ± 0.1         | 45.5 ± 1.8 | +  | –  | –   | –   | –   |
| IARI-NIAW1-4 | 11.4 ± 1.5 | 2.6 ± 0.1         | –  | +   | +  | –   | –   | –   |
| IARI-NIAW1-38| 16.6 ± 1.0 | 3.5 ± 0.2         | –  | –   | +  | –   | –   | –   |
| IARI-NIAW1-23| 2.5 ± 0.1  | 2.5 ± 0.1         | –  | +   | +  | –   | –   | –   |
| IARI-NIAW2-25| 2.6 ± 0.1  | 2.6 ± 0.1         | –  | –   | –  | –   | –   | –   |
| IARI-NIAW2-3  | –          | –                | +  | +   | +  | –   | 55.5 ± 1.5 | –   |
| IARI-NIAW2-23| 15.6 ± 0.7 |                 | 22.6 ± 1.5 | +  | –   | –   | 45.3 ± 1.2 | –   |
| IARI-NIAW1-21| 6.6 ± 1.0  | –                | 12.2 ± 0.3 | +  | –   | –   | 23.5 ± 1.3 | +   |
| IARI-NIAW1-13| 8.75 ± 1.2 | 1.8 ± 0.2         | –  | +   | –   | +   | –   | –   |
| IARI-NIAW1-20| 17.8 ± 1.2 | 2.1 ± 0.1         | 15.5 ± 1.5 | +  | –   | –   | 31.5 ± 1.6 | +   |
| IARI-NIAW1-34| –          | 1.5 ± 0.1         | –  | +   | –   | –   | 13.5 ± 1.7 | +   |
| IARI-NIAW1-31| –          | 1.2 ± 0.2         | –  | +   | –   | –   | –   | +   |
| IARI-NIAW1-15| 22.8 ± 0.8 | 3.1 ± 0.1         | –  | +   | –   | –   | –   | +   |
| IARI-NIAW2-21| 28.6 ± 1.0 | 4.1 ± 0.2         | –  | +   | +   | –   | –   | –   |
| IARI-NIAW2-34| –          | 3.2 ± 0.2         | –  | –   | –   | –   | –   | +   |
| IARI-NIAW2-27| 17.2 ± 1.1 | 2.2 ± 0.5         | 11.8 ± 0.3 | –  | +   | –   | –   | –   |
| IARI-NIAW2-11| 12.2 ± 1.0 | 3.2 ± 0.5         | 10.1 ± 0.5 | –  | +   | –   | –   | –   |
| IARI-NIAW1-31| 18.2 ± 1.0 | 4.2 ± 0.5         | 33.3 ± 0.6 | –  | +   | +   | –   | –   |
| IARI-NIAW2-37| 25.5 ± 1.2 | 3.6 ± 0.1         | 17.1 ± 0.8 | +  | +   | –   | –   | +   |
| IARI-NIAW1-41| 31.6 ± 0.5 | 4.1 ± 0.2         | 43.1 ± 1.2 | +  | –   | –   | –   | +   |
| IARI-NIAW2-33| 27.2 ± 1.0 | 2.4 ± 1.2         | 32.1 ± 1.6 | –  | +   | +   | –   | –   |
| IARI-NIAW1-2  | –          | 2.2 ± 1.0         | 18.1 ± 0.5 | –  | +   | –   | –   | –   |

(continued on next page)
Table 3 (continued)

| Strain number | Production | Other activities |
|---------------|------------|-----------------|
|               | IAA        | Sidero$^6$  | GA  | HCN | NH$_3$ | ACC | N$_2$F | BC |
| IARI-NIAW2-24 | –          | 3.1 ± 0.1 | –   | –   | –     | –   | –     | –  |
| IARI-NIAW2-1  | 15.2 ± 1.1 | 1.6 ± 0.2 | 4.1 ± 0.1 | 9.8 ± 0.5 | +   | +   | –     | +  |
| IARI-NIAW1-16 | 70.8 ± 1.5 | 1.8 ± 0.1 | –   | –   | –     | –   | –     | –  |
| IARI-NIAW2-29 | 20.8 ± 1.5 | 1.8 ± 0.1 | –   | –   | –     | –   | –     | –  |
| IARI-NIAW1-3  | 25.4 ± 1.2 | –          | –   | –   | –     | –   | –     | –  |
| IARI-NIAW1-9  | 69.1 ± 0.5 | 4.6 ± 0.1 | –   | –   | –     | –   | –     | –  |
| IARI-NIAW1-7  | 18.2 ± 1.1 | 4.4 ± 0.1 | –   | –   | –     | –   | –     | –  |
| IARI-NIAW2-19 | 66.7 ± 0.5 | 2.5 ± 0.2 | –   | –   | +     | –   | –     | –  |
| IARI-NIAW2-28 | 66.1 ± 0.7 | 2.4 ± 0.1 | –   | –   | +     | –   | –     | –  |

IAA, Indole 3-acetic acid (µg g$^{-1}$ protein day$^{-1}$); phosphorus (mg L$^{-1}$); potassium (mg mL$^{-1}$); GA, gibberellic acid (µg mL$^{-1}$); HCN, Hydrogen cyanide, ACC, 1-aminocyclopropane-1-carboxylate; N$_2$F, N$_2$-fixation; $^6$Numerical values are mean ± SD of three independent observations; # Radius of halo zone in mm; (−), negative for the attributes; (+), positive for the attributes.

from each cluster at different sites. Among the representative 32 strains, tolerant to a temperature of 50 °C or more 15 strains could grow at up to 65 °C. Among 32 strains, majority of strains could tolerate 5–8 pH. Three strains B. amyloliquefaciens IARI-NIAW1-23, Enterobacter asburiae IARI-NIAW2-21, and P. aeruginosa IARI-NIAW1-2 could be grouped as acido-thermotolerant bacteria. Five strains Bacillus siamensis IARI-NIAW1-21, P. aeruginosa IARI-NIAW1-2, Pseudomonas poae IARI-NIAW2-1, Staphylococcus succinus IARI-NIAW2-28 and Delftia acidovorans IARI-NIAW1-20 could tolerate 15% NaCl said to be halo-thermotolerant bacteria (Supplementary Table S2).

3.5. Plant growth promoting attributes

All 264 isolates were screened for direct and indirect PGP traits and results were represented by 32 representative isolates. Of 32 representatives, 20, 9 and 18 strains exhibited solubilization of phosphorus, potassium and zinc, respectively (Table 3). Out of 32 representatives, 25 strains produced indole-3-acetic acid, while only 12 strains produced gibberelllic acid (Table 2). Nitrogen fixation and ACC deaminase activity were exhibited by 13 and 11 strains, respectively. Of 32 representatives 29, 19, 16 strains exhibited activity for siderophore, ammonia, HCN and biocontrol respectively (Table 3).

The strain Arthrobacter sp. IARI-NIAW1-4 solubilized the highest amount of phosphorus (66.0 ± 0.7 mg L$^{-1}$) followed by Pseudomonas japonica IARI-NIAW2-24 (64.6 ± 0.9 mg L$^{-1}$). Methyllobacterium mesophilicum IARI-NIAW1-41 exhibited the highest solubilization of potassium (Table 3). Pseudomonas putida IARI-NIAW1-16 showed the highest IAA production (70.8 ± 1.5 µg g$^{-1}$ protein day$^{-1}$) followed by Rhodobacter capsulatus IARI-NIAW1-9 (69.1 ± 0.5 µg g$^{-1}$ protein day$^{-1}$). Alcaligenes faecalis IARI-NIAW1-6 showed the highest production of siderophore (4.9 ± 0.1 mm). Sixteen strains showed antagonistic activity against Fusarium graminarum, Rhizoctonia solani and Macrophomina phaseolina (Table 3). Among thirty-two strains, ten strains were identified as A. faecalis, Arthrobacter sp., B. siamensis, B. subtilis, D. acidovorans, M. mesophilicum, Methyllobacterium sp., P. poae, P. putida, and Pseudomonas stutzeri exhibiting more than six different plant growth promoting activities at high temperatures (Fig. 3).

4 Discussion

Over the past decades, climate change has directly affected the plant growth with different abiotic stresses and change ecosystems. For instance, thermoderterant microbes used as plant growth promoting to protect the diverse stresses have become more production and yield in many crops. The extreme environment of high temperature harbors microbial diversity, which may be fundamental for the maintenance and conservation of global genetic resources. Our present study deciphers the diversity of microbial communities coupled with the epiphytic, endophytic and rhizosphere of wheat crops and their PGP attributes. A total of 264, wheat allied bacterial isolates were obtained from different sites in the peninsular zone of India. To the best of our knowledge, this is the first report, which elucidates the bacterial diversity associated with rhizosphere, endophytic and epiphytic of wheat crops and their PGP attributes at high temperature condition.

Sequencing of 16S rRNA gene of the representative strain from each cluster of all five different sites was identified and selected 32 distinct species were taken up for phylogenetic analysis. Partial sequencing of the smaller subunit of 16S rRNA gene assigned and all the representative bacteria could be grouped into 3 phyla, Actinobacteria (6%), Firmicutes (29%) and Proteobacteria (64%) (Fig. 4a). Among the five sites analyzed, at four sites all three phyla were present whereas in Coimbatore only members of phyla Proteobacteria and Firmicutes are present (Fig. 4b). Many species of Bacillus, Pseudomonas and Methyllobacterium were found to be common to all five different sites. Apart from dominant and common species of bacteria at different sites, niche specific bacterial species were also identified at all the sites. These niche specific bacteria were represented by Bacillus altitudinis, Bacillus mojavensis, D. acidovorans, Delftia tsuruhatensis, and M. mesophilicum in Coimbatore; E. asburiae, Enterobacter hormaechei, R. capsulatus and Rhodobacter sphaeroides in Dharwad; Exiguobacterium acetylicum, P. japonica and P. poae in Krishna Nagar; A. faecalis, Delftia lacustris and Micrococcus luteus in Warangal; Acromobacter sparnius and Paenibacillus amylolitycicus in Nashik (Table 2; Fig. 4e). Other reports are also available on niche-specific bacterial diversity from different habitats (Pandey et al., 2013; Kumar et al., 2014a,b; Verma et al., 2016b; Yadav et al., 2015a–c, 2016).
Figure 1  Phylogenetic tree showing the relationship among 32 bacteria isolates, 16S rRNA gene sequences with reference sequences obtained through BLAST analysis. The sequence alignment was performed using the CLUSTAL W program and trees were constructed using neighbor joining with algorithm using MEGA4 software (Tamura et al., 2007).
Distribution ratio of each phylum showed a clear difference between each sites. The wheat growing in Nashik and Krishna Nagar showed genera and species that are more diverse than other three sites in the peninsular zone of India (Table 4). Among 32 distinct species genera Bacillus have been reported as the most dominant in Nashik (33.3% distribution ratio) followed by Krishna Nagar (32.2% distribution ratio) (Table 4). Among three phyla, Proteobacteria were most dominant followed by Firmicutes. Of 32 representative strains, 5 distinct species with two genera Rhodobacter and Methylobacterium belonged to α-proteobacteria while three strains Alcaligenes, Achromobacter and Delftia belonged to β-proteobacteria. The most predominant class of Proteobacteria is γ-proteobacteria consisting of 10 distinct species distributed into...
three orders: Pseudomonadales with five strains P. aeruginosa, P. stutzeri, P. putida, P. poae and P. japonica; Enterobacteriales with three strains E. asburiae, E. hormaechei and Klebsiella oxytoca (Table 4; Fig. 1). Second dominant phylum is Firmicutes, included four families; Bacillaceae with three strains B. altitudinis, B. amyloliquefaciens and Bacillus flexus; Bacillales Incertae Sedis, Paenibacillaceae and Staphylococcaceae with one strain Exiguobacterium antarcticum; P. amylolyticus and S. succinus. Two strains belonged to the phylum Actinobacteria, represented by Arthrobacter sp. and M. luteus (Table 4; Fig. 1).

Different groups of bacterial isolates were investigated from wheat such as Arthrobacter, Bacillus and Alcaligenes. The epiphytic bacterial strains identified as B. amyloliquefaciens, A. faecalis and P. poae were most dominant in phyllosphere. B. amyloliquefaciens and P. poae were identified as epiphytic PGPB for the first time herein, from wheat growing at arid land and high temperature environments (Joo et al. 2005). A. faecalis first time reported from ammonium removal by heterotrophic nitrification-aerobic denitrification. It is a niche specific bacterium isolated from wheat growing in Warangal, Telangana. It is a peach colored thermotolerant bacterium that could tolerate 5% NaCl and produced IAA, siderophore and ammonia; solubilized phosphorous and zinc; fixed nitrogen; exhibited ACC deaminase activity and showed antagonistic against Fusarium graminearum, Rhizoctonia solani and Macrophomina phaseolina at high temperatures. P. poae was earlier reported from phyllosphere of grasses (Behrendt et al., 2003) while first time reported here from wheat phyllosphere. It is a light yellow colored thermotolerant and alkalitolerant bacterium that could tolerate 10% NaCl and produced IAA and siderophore; and solubilized phosphorus and zinc at high temperatures.

In the present investigated endophytes, A. spanius, B. flexus, D. acidovorans, Delftia sp., D. lacustris, M. luteus and P. aeruginosa were isolated and identified from wheat. A.

Figure 2 (a) Rarefaction curves of observed OTUs in the five samples from the peninsular zone of India; (b) principal coordinate analysis of the diversity indices (H) of the 16S rDNA PCR-ARDRA profiles of the five sites in relation to 16S rRNA gene sequences, Component 1 and Component 2 accounted for 14.79% and for 69.08% of the total variation, respectively. (c) Biplot showing relationship between different sampling sites and temperature, pH, conductivity, organic carbon, available NPK and zinc, exchangeable sodium, dehydrogenase and alkaline phosphatase.

Figure 3 Characterization of ten bacteria endowed with different plant growth promoting attributes.
D. acidovorans and D. lacustris were isolated first time from internal tissue of wheat. A. spanius have been earlier isolated from clinical, fresh water and phenol (Coenye et al., 2003; Juárez-Jiménez et al., 2010). Firstly reported from wheat internal tissues, it is a light yellow colored thermotolerant that could tolerate 7% NaCl and produced IAA, siderophore and ammonia; solubilized phosphorus and zinc and also exhibited nitrogen fixation. The potential of endophytic bacteria exert several beneficial effects on host plants, such as stimulation of plant growth, nitrogen fixation and induction of resistance.

Figure 4 Abundance of different bacteria; (a) distribution of phylum and group in the samples surveyed; (b) distribution of total bacteria in five sampling sites; (c) distribution of different genera in five sampling sites in the peninsular zone of India.
to plant pathogens (Suman et al., 2015a). There were obvious differences among the rhizosphere, endophytic and epiphytic bacterial communities in terms of CFU count and phylotype. Lower bacterial diversity was observed in the phyllospheric compared to rhizospheric and endophytic. Pink-pigmented facultative methylotrophs (PPFMs) M. mesophilicum, and Methylobacterium sp. were identified as epiphytic. In our present study, A. faecalis, B. amyloliquefaciens and P. poae were isolated as epiphytic whereas A. spanius, B. flexus, D. acidovorans, Delftia sp., D. lacustris, M. luteus and P. aeruginosa as endophytic only. Alcaligenes genera was recovered from both epiphytic and rhizospheric.

Wheat associated PGPB have a high potential for agriculture because they can improve plant growth, under limiting or stress conditions of temperatures. PGPB can directly facilitate the proliferation of their plant host through the production of the stimulatory phytohormones. IAA is a phytohormone, a type of best characterized auxin, which is essential for the growth and development of plants. Along with phytohormone production, plant growth promotion is known to be mediated by a variety of mechanisms including the solubilization of phosphorus, potassium and zinc; production of ammonia, siderophores and HCN (Verma et al., 2015a; Tilak et al., 2005; Verma et al., 2016b). There are considerable populations of P- or K-solubilizing bacteria in soil and in plants rhizosphere. P-solubilizing bacteria (PSB) have the ability to solubilize inorganic phosphate compounds, such as tricalcium phosphate (Vyas et al., 2009; Yadav et al., 2015d) in the present study P-solubilization activity was exhibited by many genera such as Alcaligenes, Arthrobacter, Actinobacter, Bacillus, Delftia, Methylobacterium, Pseudomonas, Rhodobacter, Staphylococcus and Salmonella. K-solubilizing bacteria (KSB)

| Sampling sites | Phylum | Genera | Distribution ratio (%) |
|---------------|--------|--------|------------------------|
| Dharwad (8 Genus) | Actinobacteria (22%) | Arthrobacter | 1.6 |
| | Firmicutes (11%) | Bacillus | 25.8 |
| | α-proteobacteria (22%) | Rhodobacter | 14.5 |
| | β-proteobacteria (11%) | Methylobacterium | 8.06 |
| | γ-proteobacteria (34%) | Alcaligenes | 8.1 |
| | | Pseudomonas | 16.1 |
| | | Salmonella | 8.1 |
| | | Enterobacter | 17.7 |
| Krishna Nagar (10 Genus) | Actinobacteria (22%) | Arthrobacter | 3.4 |
| | Firmicutes (34%) | Bacillus | 32.2 |
| | | Exiguobacterium | 8.5 |
| | | Staphylococcus | 5.1 |
| | | Methylobacterium | 8.5 |
| | | Alcaligenes | 5.1 |
| | | Delftia | 6.8 |
| | | Pseudomonas | 23.7 |
| Coimbatore (10 Genus) | Firmicutes (28%) | Bacillus | 32.1 |
| | | Staphylococcus | 8.9 |
| | | Methylobacterium | 16.1 |
| | | Delftia | 21.4 |
| | | Pseudomonas | 7.1 |
| | | Salmonella | 7.1 |
| Nashik (10 Genus) | Actinobacteria (20%) | Arthrobacter | 6.3 |
| | Firmicutes (30%) | Bacillus | 33.3 |
| | | Paenibacillus | 4.2 |
| | | Staphylococcus | 4.2 |
| | | Methylobacterium | 4.2 |
| | | Alcaligenes | 4.2 |
| | | Delftia | 10.4 |
| | | Pseudomonas | 6.25 |
| Warangal (9 Genus) | Actinobacteria (12%) | Arthrobacter | 11.6 |
| | Firmicutes (12%) | Bacillus | 25.6 |
| | | Methylobacterium | 7.0 |
| | | Alcaligenes | 11.6 |
| | | Delftia | 11.6 |
| | | Pseudomonas | 14.0 |
| | | Salmonella | 7.0 |
| | | Klebsiella | 11.6 |
were found to resolve potassium, silicon and aluminum from insoluble minerals. To the our best of knowledge, *Delftia* and *Salmonella* are first time reported as potassium at high temperatures. The K-solubilizing bacteria may have use in the amelioration of K-deficient soil in agriculture at high temperatures. In the present investigation diverse groups of bacteria were characterized for nitrogen fixation, such as *Achromobacter*, *Alcaligenes*, *Bacillus*, *Delftia*, *Providencia*, *Pseudomonas*, *Rhodobacter* and *Salmonella*. *A. faecalis*, *B. subtilis*, *D. acidovorans* and *M. mesophilicum* that showed more than six plant growth promoting activities. Selected nitrogen fixing, P and K-solubilizing bacteria could be effectively used as biofertilizers in the place of chemical fertilizers. NPK could increase soil productivity to improve sustainability of agriculture production.

In conclusion, utility of such thermotolerant bacterial strains in the context of temperature, considering the unique crops growing in the climatic conditions of the high-temperature agricultural eco-systems. Such systems require situation-specific microbial inoculants that withstand extremities of temperature tolerance and retain their functional traits for PGP. The PGP potential of the bacterial strain dealt in this study requires further evaluation and validation before its use as bio-inoculants. The selection of native functional plant growth promoting microorganisms is a mandatory step for reducing the use of energy intensive chemical fertilizers. The strain reported in this study seems to be an ideal candidate for promotion as a bio-inoculant, due to its high temperature tolerance and multiple traits for plant growth promotion. PGP thermotolerant bacteria are another important isolates that could be developed as suitable inoculants for summer season crops grown in the arid, sub-arid, high plateau and high temperature regions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.jsbhs.2016.01.042.

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