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# Diversity and Metabolic Potential of the Dominant Culturable $N_2$-Fixing and P-Solubilising Bacteria from Tea (*Camellia sinensis* L.) Rhizosphere

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**Abstract:** The purpose of this study was to investigate the diversity of cultivable $N_2$-fixing and P-solubilizing bacteria originated from 167 rhizospheric acidic soils samples of tea. Based on the fatty acid methyl ester profiles, 34 bacterial genera were identified with a similarity index of >0.3, but 69.2% of the identified isolates belonged to five genera: *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Stenotrophomonas* and *Arthrobacter*. Among the 263 bacterial strains, 213 strains exhibited $N_2$-fixing activity and 159 were efficient in phosphate solubilisation; 134 strains were efficient in $N_2$-fixation and P-solubilisation. Most of the $N_2$-fixing and P-solubilizing bacteria isolated were Gram-positive (59.3 and 52.8%), and Gram-negative constituted only 40.7 and 47.2%. A total of 102 dominant strains were characterized by carbon sources using BIOLOG$^M$ GN2 and GP2 plates. *B. pumilus*, *B. subtilis*, *B. licheniformis*, *B. laevolacticus*, *P. fluorescens*, *P. putida*, *S. maltophilia* and *B. megaterium* were the most frequent P-solubilizing and $N_2$-fixing species in the tea rhizosphere soils. Utilization of high variety of C-sources by the $N_2$-fixing and P-solubilizing acid tolerant strains may play an important role in adapting to a variety of crop plants, and thus potentially beneficial to the growth of tea plants in that specific acidic ecosystem.

**Keywords:** Tea, Biodiversity, Plant Growth-Promoting Rhizobacteria, Biology, Carbon Source Utilization, Acid Tolerant Strains

## 1. Introduction

Soil and rhizosphere microbial communities in agro ecosystems may be affected by soil type and structure, soil pH, agro climatic conditions, plant species, plant-microorganism interaction, land use, and management [1-3]. Each plant species has a significant effect on the rhizosphere bacterial community structure due to the differences in root exudation [4], and it is thought that it may select own specific microbial populations in its rhizosphere [5]. Bacteria are the most abundant organisms that reside in rhizosphere, and are called as plant growth-promoting rhizobacteria (PGPR). They play an important role in plant growth by exerting various mechanisms such as biological nitrogen fixation, nutrient solubilisation, growth hormone and siderophore production, synergism with other bacteria–plant interactions, as well as increasing the availability of nutrients. Several studies have also reported stringent host plant specificity of rhizospheric bacteria [6]. Selection of an efficient PGPR requires an understanding of the composition and diversity of the root-associated bacteria, and characterization of its plant growth promotion (PGP)-related properties. For this reason, there has been considerable interest in examining the effect of soil type, plant species and root zone location on bacterial community structure in the rhizosphere.

Tea is a perennial leaf crop which requires more nitrogen than most other crops and N application increases both the yield and quality of tea. Over the years, productivity of the plant has been decreasing and one of the reasons for this has been attributed to the continuous use of large quantities of

chemicals in tea plantations. Many studies showed that excess amounts of N fertilizer application can cause tea orchard soil acidification [7], water pollution [8], and affect nitrification rates [9], contribute to low N use efficiency and also cause serious environmental pollution [7]. Phosphorus is the second of the main limiting factors to the productivity of tea plants and P utilization efficiency is very low in soil due to its low solubility and mobility. Hence, there is a pressing need in tea industry for utilizing either biological product completely or reducing the use of chemicals by supplementing with biological products. PGPR might increase nutrient uptake, thus reduce the need for fertilizers and prevent the accumulation of nitrates and phosphates in agricultural soils. An important requirement for the success of such applications requires selection of suitable bacteria in candidate plants appropriate for various biotechnological applications [2]. The advantage of using natural soil isolates is the easier adaptation and success when inoculated into the plant rhizosphere [10].

The study was carried out in the Gilan province on the slopes of Alborz Mountain and shores of the Caspian Sea because it presents a unique ecosystem with very high rainfall, humid climate, wide temperature fluctuations and acidic soil. Also, the effect of the tea plants on the rhizospheric bacteria has not been studied so far in this area. There is very little knowledge on the rhizosphere micro-biology of the tea plants [9, 11]. Currently, there is no information on the *diversity* and functional importance of $N_2$-fixing (NFB) and P-solubilizing bacteria (PSB) in the acidic tea soils of southern coast of the Caspian Sea. Therefore, the objective of this study is to isolate and identify the dominant cultivable PGPR from the rhizosphere of the tea grown in this region, and characterize it from the point of $N_2$-fixation, P-solubilisation and C-source utilization profiles.

# 2. Materials and Methods

### *2.1. Soil Samples, Isolation and Identification of Bacteria*

Rhizosphere soil samples were collected from 4 separate environments of tea plants production zones in the southern coast of the Caspian Sea region during the period of May 2012-September, 2013. The tea plantations was roughly divided into four region based on variations in valley, *topography, microclimates***,** acidic soil types and climate. Then have been surveyed and 167 acidic soil samples were collected. Soils samples were generally sandy loam, clay loam and sandy clay loam texture, with acid reactions (3.6-6.5), and high organic matter content (2.1-8.6%). Rhizosphere soil samples were collected carefully by uprooting the root system. Rhizospheric bacteria were isolated and identified according to the procedures described already [11]. Isolates were identified by fatty acid methyl ester (FAME) analysis using the Microbial Identification System (MIDI Inc., Newark, DE, USA). The system consisted of a Hewlett Packard Agilent 6890 GC fitted with a microprocessor containing the Sherlock software. Cells were harvested from the third and fourth quadrant streak of growth and FAME was prepared according to the standard MIS protocol. The FAME profiles were compared with the TSBA40 aerobe library.

### *2.2. Biolog Carbon Substrates Utilization Patterns*

From a total of 263 rhizobacterial isolates with a similarity index of >0.3 for FAME profile match, the 102 most abundant $N_2$-fixing and/or P-solubilising isolates were tested for C-source utilization pattern and identified using Biolog system. Biolog characterization was conducted using GN2 and GP2 MicroPlates (Biolog, Inc., Hayward, CA, USA) for Gram-negative and Gram-positive bacteria, respectively. These 96-well plates contain a negative control and 95 different sole-carbon sources as well the redox dye tetrazolium violet [12]. A single colony of each gram negative and positive strains cultured on NA, respectively inoculated onto trypticase soy broth agar (TSBA) and Biolog Universal Growth Medium (BUGM). The cell suspension was then adjusted using a Biolog turbidimeter absorbance reader to a cell density of 28% transmittance. Each well of Biolog GN2 or GP2 microplates was inoculated with 150 µl of the Gram-negative or positive bacterial suspension, respectively, adjusted to the appropriate density in saline solution and incubated at 27 °C for 24 h. Following inoculation and during incubation of the plates, a respiratory burst occurs in wells where the C-source is utilized, reducing the dye in that well and resulting in a purple colour change. The plates were then read with a MicroStation photometer reader using the Biolog software MicroLog 3, Ver 4.20. The data are calculated using software on the basis of a 'dual wavelength colour statistic' as read at 590 and 750 nm.

### *2.3. Phosphate Solubilisation and Acetylene Reduction Assay*

Inorganic phosphate-solubilisation activity of the bacterial isolates was detected on Pikovskaya and NBRIP-BPB solid medium containing tricalcium-phosphate as sole source of P, as described earlier [11, 13, 14]. Soluble P in culture was then determined colorimetrically by the standard vanadomolybdophosphoric acid method. Ability of rhizobacterial isolates to grow on Döbereiner nitrogen-free culture medium indicated their non-symbiotic $N_2$-fixation ability [15, 16]. Nitrogen fixation of the isolates was also determined in nitrogen free medium by acetylene reduction assay [17]. Ethylene production was measured using a Hewlett Packard gas chromatograph (Model 6890, USA).

### *2.4. Statistical Analysis*

Overall colour development in BIOLOG plates was expressed as average well colour development (AWCD). AWCD = $\sum$ (C−R)/n, where, C is colour production within each well, R is the absorbance value of the plate′s control well, and *n* is the number of substrates (GP2 and GP2 plates, *n*=95). For principal component analysis (PCA), data were transformed by dividing the raw difference value for each

well by the AWCD of the plate in 24 h, i.e., (*C−R)*/AWCD [12]. The normalised data have been compared the analyses of the AWCD method. The substrate utilization patterns were analysed by principal components analysis (PCA). PCA is the multivariate ordination technique that was used on the correlation matrix of the C-source utilization. To reduce the dimension of the highly multivariate data sets, the final AWCD data sets were separately analysed by PCA. In addition, substrates were divided into six categories (polymers, carbohydrates, carboxylic acids, amines and amides, amino acids, and miscellaneous) and the average absorbance of each category was calculated.

# 3. Results

Two hundred and sixty-three dominant, morphologically distinct rhizobacterial isolates from 391 colonies were purified, which belonged to 34 genera and 72 species. The MIDI system identified that (SIM > 0.3) 67.3% of the bacteria isolated from the rhizosphere of tea (Table 1). However, approximately 6.6% of the isolates were not present in the MIDI Sherlock Version 4.20 library. Also about 26% of the isolates were identified with a SIM <0.3, which indicated a tentative identification, and were not included in further analysis. Predominant bacterial divisions in this soil included Actinobacteria (10.6%), Firmicutes (46.8%), and γ, β and α-subdivisions of Proteobacteria (32.3%, 8.4% and 1.9%, respectively). The bacterial population of Gram-negative bacteria (42.6%) was found to be less than that of the Gram-positive bacteria (57.4%) in the tea rhizosphere soil samples.

***Table 1.*** *Diversity and biotechnological potential of the dominant culturable $N_2$-fixing and P-solubilising bacteria from acidic soils of tea.*

| Taxonomic identification | Order | Bacterial strain FAME identification | Number of isolates[a] |
|---|---|---|---|
| *Alphaproteobacteria* | *Rhizobiales* | *Rhizobium radiobacter* | 2 (2, 2) |
| | | *Roseomonas fauriae* | 3 (3, 2) |
| *Betaproteobacteria* | *Burkholderiales* | *Burkholderia cepacia* | 5 (4, 4) |
| | | *Burkholderia pyrrocinia* | 2 (2, 1) |
| | | *Ralstonia eutropha* | 2 (2, 1) |
| | | *Alcaligenes faecalis* | 5 (3, 2) |
| | | *Achromobacter xylosoxidans denitrificans* | 4 (3, 3) |
| | | *Acidovorax facilis* | 2 (1, 2) |
| *Gammaproteobacteria* | *Xanthomonadales* | *Lysobacter enzymogenes enzymogenes* | 4 (3, 3) |
| | | *Stenotrophomonas acidaminiphila* | 3 (2, 2) |
| | | *Stenotrophomonas maltophilia* | 8 (6, 5) |
| | *Pseudomonadales* | *Pseudomonas alcaligenes* | 4 (3, 2) |
| | | *Pseudomonas agarici* | 3 (2, 2) |
| | | *Pseudomonas chlororaphis* | 2 (2, 1) |
| | | *Pseudomonas fluorescens* | 13 (10, 9) |
| | | *Pseudomonas putida* | 10 (8, 6) |
| | | *Pseudomonas stutzeri* | 2 (2, 1) |
| | | *Pseudomonas syringae atrofaciens* | 2 (2, 1) |
| | | *Pseudomonas sp.* | 3 (2, 2) |
| | | *Acinetobacter calcoaceticus* | 4 (3, 2) |
| | *Enterobacteriales* | *Erwinia-chrysanthemi* | 3 (2, 2) |
| | | *Hafnia alvei* | 3 (3, 3) |
| | | *Photorhabdus luminescens* | 2 (2, 2) |
| | | *Proteus vulgaris* | 2 (2, 1) |
| | | *Rahnella aquatilis* | 2 (1, 2) |
| | | *Serratia fonticola* | 3 (3, 2) |
| | | *Serratia marcescens* | 3 (3, 2) |
| | | *Serratia plymuthica* | 2 (2, 1) |
| *Firmicutes* | *Bacillales* | *Bacillus atrophaeus* | 2 (2, 2) |
| | | *Bacillus cereus* | 6 (5, 3) |
| | | *Bacillus coagulans* | 6 (2, 3) |
| | | *Bacillus laevolacticus* | 14 (13, 7) |
| | | *Bacillus licheniformis* | 17 (13, 7) |
| | | *Bacillus megaterium* | 7 (7, 5) |
| | | *Bacillus pumilus* | 23 (21, 11) |
| | | *Bacillus sp.* | 2 (2, 1) |
| | | *Bacillus sphaericus* | 2 (1, 1) |
| | | *Bacillus subtilis* | 22 (19, 11) |
| | | *Paenibacillus larvae* | 2 (2, 1) |
| | | *Paenibacillus lentimorbus* | 2 (2, 1) |

| Taxonomic identification | Order | Bacterial strain FAME identification | Number of isolates[a] |
|---|---|---|---|
| | | *Paenibacillus macquariensis* | 3 (2, 3) |
| | | *Paenibacillus polymyxa* | 5 (4, 3) |
| | | *Paenibacillus validus* | 3 (2, 2) |
| | | *Brevibacillus choshinensis* | 2 (2, 1) |
| *Actinobacteria* | *Actinomycetales* | *Arthrobacter agilis* | 2 (2, 1) |
| | | *Arthrobacter globiformis* | 5 (4, 3) |
| | | *Arthrobacter histidinolovorans* | 2 (1, 1) |
| | | *Micrococcus luteus* | 5 (4, 4) |
| | | *Micrococcus lylae* | 3 (3, 2) |
| | | *Rhodococcus erythropolis* | 3 (3, 2) |
| Others[b] | | | 22 (15, 17) |
| No library match | | | 26 |
| Unidentified[c] | | | 102 |
| Total | | | 391 (213, 159) |

[a]Numbers in parentheses indicate the number of $N_2$- fixing and P-solubilising strains where bacterial genera were detected.
[b]Others includes the genera: *Acinetobacter, Alcaligenes, Acidovorax, Arthrobacter, Bacillus, Brevibacterium, Enterobacter, Kocuria, Kurthia, Kytococcus, Microbacterium, Pantoea, Providencia, Pseudomonas, Pseudoalteromonas, Rhodococcus and Xanthomonas*. $N_2$-fixing and P-solubilising bacteria in these genera were only detected once or twice.
[c]Isolates named with a similarity index < 0.3.

Out of a total 263 isolates, 112 belonged to Gram-negative, which included 85 of γ-proteobacteria and each of 27 α- and β-proteobacteria. Major β-proteobacterial included *Burkholderia*, *Alcaligenes* and *Achromobacter*, while *Pseudomonas*, *Stenotrophomonas*, and *Serratia* dominated the γ-proteobacterial genera. The 151 Gram-positive isolates included 123 Firmicutes and 28 Actinobacteria. Major *Bacillales* recovered from tea rhizospheres included *Bacillus*, *Paenibacillus* and *Brevibacillus*, while *Arthrobacter*, *Micrococcus* and *Rhodococcus* dominated the *Actinomycetales* genera (Table 1).

Table 1 shows that 213 and 159 out of the 263 tested isolates had potential for $N_2$-fixation and P-solubilisation, in which 34 differently known bacterial genera were represented by *Bacillus* (41.6 and 34.6%), *Pseudomonas* (14.5 and 15.7%), *Paenibacillus* (5.6 and 6.3%), *Stenotrophomonas*, *Arthrobacter*, *Serratia*, *Micrococcus*, and *Burkholderia* as the predominant genera. Isolated strains were capable of nitrogenase activity, but the amounts of $C_2H_4$ varied with bacterial species and nitrogenase activity ranging from 0.14 to 0.96 nmol $C_2H_4$ $10^7$ cfu/h. These isolates showed significant differences in their phosphate solubilizing potential, their solubilisation extend ranged between 42.7-179.8 mg $L^{-1}$ in liquid medium.

Species of *Bacillus* dominated the bacterial populations, *B. pumilus* being the most abundant. *B. subtilis*, *B. licheniformis*, *B. laevolacticus*, and *B. megaterium* were other important species (Table 1). Among the Gram-positive $N_2$-fixing strains, each of the 4 isolates of *P. polymyxa*, *A. globiformis* and *M. luteus*, and each of the 3 isolates of *M. lylae* and *R. erythropolis* were confirmed as NFB. Among these bacteria**,** 4 isolates of *M. luteus*, and each of the 3 isolates of *B. coagulans*, *P. macquariensis*, *P. polymyxa* and *A. globiformis* were confirmed as PSB. Among the Gram-negative $N_2$-fixing and P-solubilizing strains, the *most dominant species* found *were P. fluorescens*, followed by *P. putida*, *S. maltophilia,* and *B. cepacia.* Several other α, β and γ-proteobacteria, such as *R. fauriae*, *L. enzymogenes, S. acidaminiphila, P. alcaligenes, A. calcoaceticus, H. alvei*, *S. fonticola,* and *S. marcescens*, were found as regular or as dominant NFB and PSB in the tea rhizosphere.

Various strains of species *B. pumilus* (23 strains), *B. subtilis* (22 strains), *B. licheniformis* (14 strains), *B. megaterium* (7 strains), *P. fluorescens* (13 strains), *P. putida* (10 strains), *S. maltophilia* (8 strains), and *B. cepacia* (5 strains) were and examined for their ability to oxidize different carbon sources. The results were compared to those obtained with principal components analysis (PCA), a common method for reducing the dimensionality of the dataset prior to analysing Biolog data. The most important C-sources used to differentiate the bacterial strains typically gave high positive or negative correlations, which were reflected in the ordination plots. The first five PCA axes accounted for 24, 11, 7, 6 and 5%, respectively; of total variation in GP2 plate data and 34, 11, 6, 5 and 4% of total variation in GN2 plate data. PCA reduced the 95 variables to 22 and 18 principal components (PC) that explained 90% of the cumulative variance in GP2 and GN2 plate, respectively. The PC analysis showed that the first five PC axes explained 53% of the total multivariate variation in GP2 plate. In GP2 plates, the first two PC axis was most highly correlated with oxidation of arbutine, D-fructose, β-methyl-D-glucoside, salicin, uridine, L-alanyl-glycine, L-pyroglutamic acid, and maltotriose.

All strains tested *Bacillus pumilus* utilized 19 carbon sources (for example, arbutine, D-cellobiose, D-fructose, D-mannitol, D-mannose, and etc.) (Table 2). From the BIOLOG carbon sources, carbohydrates (gentiobiose, α-D-glucose, D-sorbitol and 3-methyl-glucose), amino acids (D-alanine, L-alanine and L-serine) and miscellaneous carbon sources (2,3-butanediol, 2'-deoxy adenosine, inosine and uridine) as well as polymers (dextrin), and carboxylic acids (methyl pyruvate and mono-methyl-succinate) other carbon sources were the most preferred (Table 2). *B. pumilus* utilized miscellaneous carbon sources at a higher rate than did the other strain tested

(Figure 1). All $N_2$-fixing and P-solubilizing *Bacillus subtilis* tested utilized 22 carbon sources. Of these, fifteen were carbohydrates (arbutine, D-fructose, maltose, sucrose, etc.), two were amino acid (L-asparagine and L-glutamic acid), three were miscellaneous carbon sources (salicin, glycerol, uridine), and one was a carboxylic acids (pyruvic acid) and the polymers (β-cyclodextrin). Of the substrates to GP2 plates, each of the seventeen carbon sources were used by 50% or more strains of *B. subtilis*, but twenty-nine carbon sources were not utilized by any of theirs (Table 2). *B. subtilis* utilized carbohydrates carbon sources at a higher rate than did the other strain tested (Figure 1).

***Table 2.*** *Substrate utilized as carbon sources by $N_2$-fixing and P-solubilising B. pumilus, B. subtilis, B. megaterium and B. licheniformis strains as determined by Biolog GP2 Microplate assays.*

| Well | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Bacillus pumilus (23 strains)* | | | | | | | | | | | | |
| A[b] | 0[a] | 0 | 0 | 57 | 22 | 0 | 0 | 48 | 0 | 100 | 17 | 17 |
| B | 30 | 0 | 100 | 100 | 100 | 0 | 17 | 0 | 96 | 9 | 100 | 0 |
| C | 0 | 0 | 0 | 30 | 100 | 100 | 0 | 0 | 0 | 0 | 74 | 0 |
| D | 100 | 0 | 13 | 100 | 22 | 0 | 100 | 100 | 0 | 96 | 0 | 100 |
| E | 22 | 100 | 48 | 0 | 17 | 26 | 0 | 13 | 17 | 0 | 39 | 17 |
| F | 0 | 17 | 22 | 30 | 100 | 91 | 78 | 22 | 100 | 9 | 26 | 22 |
| G | 9 | 52 | 65 | 35 | 100 | 100 | 26 | 13 | 91 | 17 | 57 | 100 |
| H | 100 | 78 | 91 | 100 | 91 | 0 | 35 | 0 | 0 | 0 | 0 | 17 |
| *Bacillus subtilis (22 strains)* | | | | | | | | | | | | |
| A | 0 | 0 | 0 | 100 | 32 | 0 | 0 | 18 | 77 | 91 | 5 | 64 |
| B | 55 | 0 | 100 | 100 | 100 | 0 | 64 | 0 | 100 | 27 | 100 | 0 |
| C | 0 | 0 | 100 | 100 | 100 | 100 | 10 | 0 | 0 | 0 | 91 | 86 |
| D | 100 | 0 | 64 | 100 | 10 | 0 | 82 | 100 | 0 | 100 | 0 | 100 |
| E | 36 | 100 | 100 | 0 | 32 | 5 | 0 | 5 | 50 | 0 | 10 | 23 |
| F | 0 | 10 | 23 | 14 | 91 | 91 | 23 | 0 | 100 | 23 | 14 | 27 |
| G | 0 | 27 | 36 | 10 | 100 | 100 | 10 | 10 | 86 | 18 | 41 | 100 |
| H | 64 | 59 | 77 | 95 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 41 |
| *Bacillus megaterium (7 strains)* | | | | | | | | | | | | |
| A | 0 | 86 | 71 | 100 | 86 | 0 | 0 | 57 | 57 | 86 | 0 | 43 |
| B | 100 | 0 | 100 | 100 | 100 | 0 | 71 | 0 | 100 | 57 | 100 | 43 |
| C | 0 | 0 | 100 | 100 | 100 | 100 | 57 | 86 | 0 | 0 | 100 | 71 |
| D | 71 | 0 | 86 | 100 | 86 | 0 | 100 | 71 | 0 | 71 | 0 | 100 |
| E | 14 | 100 | 100 | 0 | 100 | 0 | 0 | 86 | 57 | 0 | 86 | 86 |
| F | 43 | 71 | 86 | 0 | 100 | 71 | 71 | 0 | 100 | 86 | 86 | 47 |
| G | 29 | 86 | 57 | 100 | 100 | 100 | 43 | 71 | 71 | 0 | 57 | 71 |
| H | 100 | 57 | 100 | 100 | 100 | 0 | 29 | 71 | 0 | 0 | 0 | 71 |
| *Bacillus licheniformis (14 strains)* | | | | | | | | | | | | |
| A | 0 | 43 | 50 | 100 | 86 | 0 | 0 | 21 | 14 | 100 | 71 | 86 |
| B | 64 | 0 | 100 | 100 | 100 | 0 | 64 | 0 | 93 | 93 | 100 | 36 |
| C | 0 | 0 | 93 | 100 | 100 | 100 | 57 | 29 | 0 | 0 | 100 | 79 |
| D | 100 | 0 | 93 | 100 | 36 | 0 | 100 | 100 | 0 | 100 | 0 | 100 |
| E | 79 | 100 | 100 | 0 | 57 | 64 | 0 | 29 | 64 | 0 | 86 | 64 |
| F | 0 | 29 | 43 | 50 | 100 | 100 | 36 | 36 | 100 | 64 | 71 | 64 |
| G | 50 | 86 | 71 | 93 | 100 | 100 | 79 | 57 | 93 | 0 | 79 | 100 |
| H | 100 | 100 | 100 | 100 | 100 | 0 | 43 | 14 | 0 | 0 | 0 | 100 |

[a]Percentage of strains giving positive results

[b]BIOLOG GP MicroPlate: A1:water, A2:α-cyclodextrin, A3:β-cyclodextrin, A4:dextrin, A5:glycogen, A6:inulin, A7:mannan, A8:tween 40, A9:tween 80, A10:N-acetyl-D-glucosamine, A11:N-acetyl-β-D-mannosamine, A12:amygdalin, B1:L-arabinose, B2:D-arabitol, B3:arbutine, B4:D-cellobiose, B5:D-fructose, B6:L-fucose, B7:D-galactose, B8:D-galacturonic acid, B9:gentiobiose, B10:D-gluconic acid, B11:α-D-glucose, B12:m-inositol, C1:α-D-lactose, C2:lactulose, C3:maltose, C4:maltotriose, C5:D-mannitol, C6:D-mannose, C7:D-melezitose, C8:D-melibiose, C9:α-methyl-D-galactoside, C10:β-methyl-D-galactoside, C11:3-methyl-glucose, C12:α-methyl-D-glucoside, D1:β-methyl-D-glucoside, D2:α-methyl-D-mannoside, D3:palatinose, D4:D-psicose, D5:D-raffinose, D6:L-rhamnose, D7:D-ribose, D8:salicin, D9:sedoheptulosan, D10:D-sorbitol, D11:stachyose, D12:sucrose, E1:D-tagatose, E2:D-trehalose, E3:turanose, E4:xylitol, E5:D-xylose, E6:acetic acid, E7:α-hydroxybutyric acid, E8:β-hydroxybutyric acid, E9:γ-hydroxybutyric acid, E10:p-hydroxy-phenlyacetic acid, E11:α-ketoglutaric acid, E12:α-ketovaleric acid, F1:lactamide, F2:D-lactic acid methyl ester, F3:L-lactic acid, F4:D-malic acid, F5:L-malic acid, F6:pyruvic acid methyl ester, F7:succinic acid mono-methyl ester, F8:propionic acid, F9:pyruvic acid, F10:succinamic acid, F11:succinic acid, F12:N-acetyl L-glutamic acid, G1:L-alaninamide, G2:D-alanine, G3:L-alanine, G4:L-alanyl-glycine, G5:L-asparagine, G6:L-glutamic acid, G7:glycyl-L-glutamic acid, G8:L-pyroglutamic acid, G9:L-serine, G10:putrescine, G11:2,3-butanediol, G12:glycerol, H1:adenosine, H2:2'-deoxy adenosine, H3:inosine, H4:thymidine, H5:uridine, H6:adenosine-5'-monophosphate, H7:thymidine-5'-monophosphate, H8:uridine-5'-monophosphate, H9:D-fructose-6-phasphate, H10:α-D-glucose-1-phosphate, H11:D-glucose-6- phosphate, H12:D-L-α-glycerol phosphate.

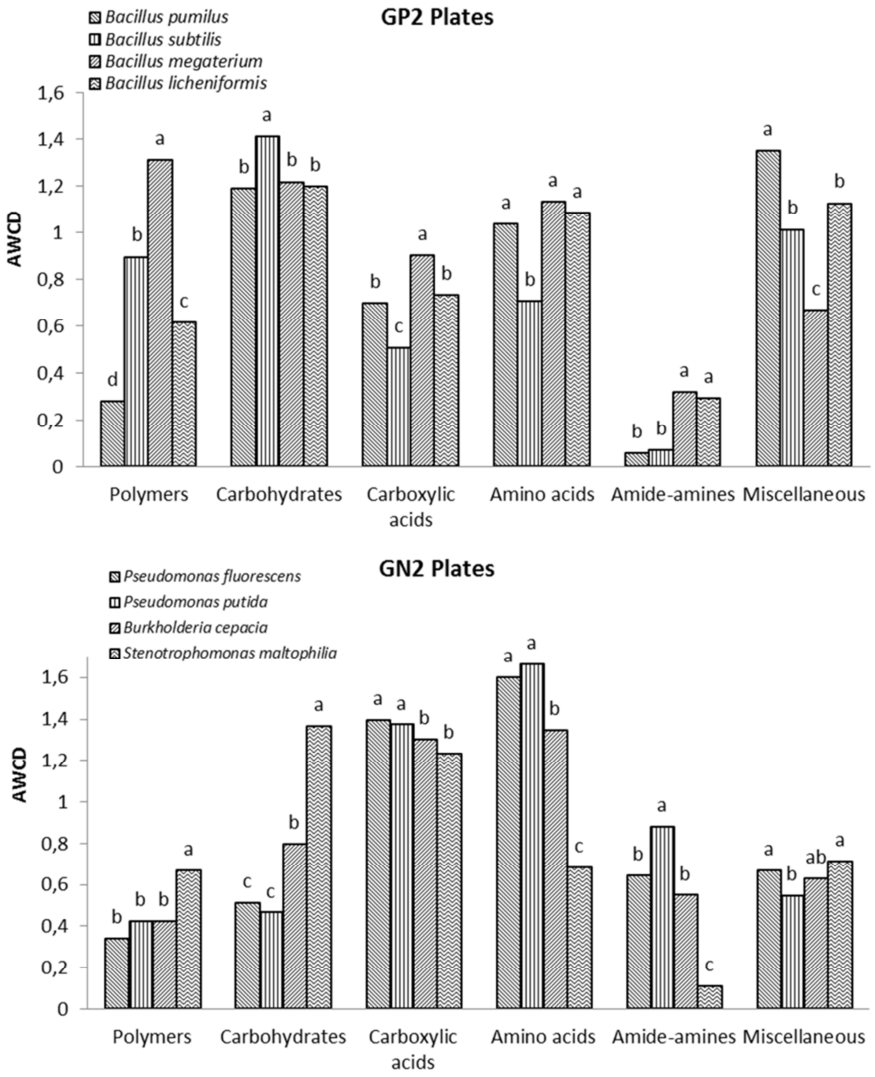


***Figure 1.*** *Average well colour development in the six groups of carbon compounds in the Biolog GP2 and GN2 plate by selected species from acidic tea rhizosphere soils.*

Out of 95 different carbon sources, as many as 26 were oxidized by all *Bacillus megaterium* tested strains (Table 2). These include one polymer (dextrin), 16 carbohydrates (such as L-arabinose, D-fructose, D-mannitol, etc.), 2 carboxylic acids (L-malic and pyruvic acid), 3 were amino acid (L-alanyl-glycine, L-asparagine and L-glutamic acid) and four miscellaneous carbon sources. *B. megaterium* utilized polymers, carboxylic and amino acid carbon sources at a higher rate than did the other strain tested (Figure 1). All strains tested *Bacillus licheniformis* utilized 30 carbon sources. Of the substrates to GP2 plates, each of the thirty carbon sources were used by 50% or more strains of *B. licheniformis*, but twenty-one carbon sources were not utilized by any of theirs (Table 2).

The GN2 plate data PCA indicated that the first principal components explained 34% of the total multivariate variation, and the first five PC axes explained 60%. Characters with high coefficients (with loadings higher than 0.90, $p< 0.01$) in the first PC (D-melibiose, lactulose, maltose, D-cellobiose, dextrin, gentiobiose, α-D-lactose, α-ketobutyric, and quinic acid), the second PC (adonitol, L-fucose, m-inositol, and sebacic acid), and the third PC (D-galactose, L-alanyl-glycine, and D-serine) were considered the most important since these axes explain nearly half of the total variation.

All $N_2$-fixing and/or P-solubilizing *Pseudomonas putida* tested utilized 30 carbon sources (Table 3). These include one each of polymer and miscellaneous carbon source (tween 80 and bromosuccinic acid), 5 carbohydrates (L-arabinose, D-fructose, etc.), 3 amide-amine (succinamic acid, L-alaninamide and putrescine), 9 amino acids and 13 carboxylic acids. Twenty-six carbon sources were not utilized by any of the tested *P. putida* strains (Table 3). *P. putida* utilized amino acids and amide-amine carbon sources at a higher rate than did the other strain tested (Figure 1). Each of the 26 substrates on the GN2 plates were used by thirteen $N_2$-fixing and/or P-solubilizing *Pseudomonas fluorescens* (Table 3). Of these, four were carbohydrates (D-fructose, D-galactose, α-D-glucose and D-mannose), three were miscellaneous carbon sources, eight were amino acid and eleven were carboxylic acids. The substrates GN2 plates included L-arabinose, D-gluconic acid, L-alaninamide, L-

histidine, hydroxy-L-proline, L-threonine, and γ-aminobutyric acid, which were only transformed by 92% of the gram-negative *P. fluorescens.*

***Table 3.*** *Substrate utilized as carbon sources by $N_2$-fixing and P-solubilising P. putida, P. fluorescens, S. maltophilia and B. cepacia strains as determined by Biolog GN2 Microplate assays.*

| Well | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Pseudomonas fluorescens (13 strains)* | | | | | | | | | | | | |
| A[b] | 0[a] | 0 | 15 | 69 | 77 | 69 | 0 | 46 | 0 | 92 | 77 | 0 |
| B | 0 | 100 | 15 | 100 | 0 | 100 | 54 | 0 | 0 | 0 | 85 | 100 |
| C | 15 | 0 | 77 | 0 | 0 | 38 | 46 | 46 | 0 | 0 | 100 | 69 |
| D | 85 | 100 | 100 | 77 | 62 | 38 | 92 | 77 | 38 | 77 | 100 | 0 |
| E | 0 | 0 | 77 | 100 | 69 | 100 | 100 | 100 | 100 | 100 | 0 | 100 |
| F | 100 | 54 | 38 | 92 | 100 | 100 | 100 | 100 | 100 | 100 | 23 | 54 |
| G | 92 | 92 | 85 | 62 | 31 | 100 | 85 | 38 | 100 | 92 | 85 | 92 |
| H | 100 | 62 | 54 | 0 | 31 | 85 | 85 | 0 | 100 | 31 | 0 | 54 |
| *Pseudomonas putida (10 strains)* | | | | | | | | | | | | |
| A | 0 | 0 | 20 | 80 | 80 | 100 | 0 | 20 | 0 | 100 | 70 | 0 |
| B | 0 | 100 | 0 | 100 | 0 | 100 | 0 | 0 | 0 | 0 | 80 | 100 |
| C | 0 | 0 | 70 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 |
| D | 100 | 100 | 100 | 100 | 70 | 10 | 100 | 80 | 20 | 80 | 100 | 0 |
| E | 0 | 0 | 30 | 100 | 70 | 100 | 90 | 100 | 100 | 90 | 30 | 100 |
| F | 100 | 100 | 20 | 100 | 100 | 100 | 80 | 100 | 100 | 100 | 0 | 90 |
| G | 90 | 80 | 90 | 80 | 30 | 100 | 100 | 30 | 100 | 80 | 80 | 100 |
| H | 80 | 30 | 30 | 0 | 30 | 100 | 80 | 0 | 90 | 0 | 0 | 40 |
| *Burkholderia cepacia (5 strains)* | | | | | | | | | | | | |
| A | 0 | 0 | 60 | 60 | 60 | 80 | 60 | 100 | 100 | 100 | 100 | 60 |
| B | 0 | 100 | 100 | 100 | 0 | 100 | 100 | 0 | 0 | 80 | 100 | 100 |
| C | 0 | 0 | 40 | 60 | 0 | 100 | 60 | 100 | 80 | 0 | 100 | 100 |
| D | 100 | 100 | 100 | 100 | 100 | 80 | 100 | 80 | 100 | 80 | 100 | 0 |
| E | 0 | 0 | 100 | 100 | 60 | 100 | 60 | 100 | 100 | 100 | 100 | 100 |
| F | 80 | 40 | 40 | 40 | 100 | 100 | 100 | 100 | 100 | 100 | 20 | 60 |
| G | 100 | 100 | 40 | 0 | 100 | 100 | 100 | 40 | 100 | 60 | 40 | 100 |
| H | 80 | 0 | 0 | 0 | 100 | 40 | 100 | 0 | 80 | 60 | 0 | 100 |
| *Stenotrophomonas maltophilia (8 strains)* | | | | | | | | | | | | |
| A | 0 | 0 | 100 | 0 | 37 | 50 | 100 | 100 | 0 | 0 | 0 | 100 |
| B | 0 | 100 | 0 | 50 | 100 | 100 | 0 | 100 | 100 | 100 | 25 | 100 |
| C | 100 | 75 | 75 | 50 | 0 | 25 | 87 | 87 | 62 | 0 | 100 | 87 |
| D | 100 | 62 | 100 | 25 | 0 | 0 | 0 | 0 | 0 | 100 | 62 | 0 |
| E | 0 | 0 | 100 | 100 | 100 | 100 | 87 | 100 | 0 | 0 | 0 | 100 |
| F | 87 | 0 | 0 | 50 | 75 | 75 | 100 | 62 | 62 | 100 | 75 | 100 |
| G | 25 | 0 | 75 | 0 | 25 | 62 | 0 | 0 | 100 | 87 | 25 | 0 |
| H | 0 | 62 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 62 |

[a]Percentage of strains giving positive results

[b]BIOLOG GN MicroPlate: A1:water, A2:α-cyclodextrin, A3:dextrin, A4:glycogen, A5:tween 40, A6:tween 80, A7:N-acetyl-D-galactosamine, A8:N-acetyl-D-glucosamine, A9:Adonitol, A10:L-arabinose, A11:D-arabitol, A12:D-cellobiose, B1:i-erytritol, B2:D-fructose, B3:L-fucose, B4:D-galactose, B5:gentiobiose, B6:α-D-glucose, B7:m-inositol, B8:α-D-lactose, B9:lactulose, B10:maltose, B11:D-mannitol, B12:D-mannose, C1:D-melibiose, C2:β-methyl-D-glucoside, C3:D-psicose, C4:D-raffinose, C5:L-rhamnose, C6:D-sorbitol, C7:sucrose, C8:D-trehalose, C9:turanose, C10:xylitol, C11:pyruvic acid methil ester, C12:succinic acid mono-methil ester, D1:acetic acid, D2:Cis-aconitic acid, D3:citric acid, D4:formic acid, D5:D-galactonic acid lactone, D6:D-galacturonic acid, D7:D-gluconic acid, D8:D-glucosaminic acid, D9:D-glucuronic acid, D10:α-hydroxybutyric acid, D11:β-hydroxybutyric acid, D12:γ-hydroxybutyric acid, E1:p-hydroxy-phenlyacetic acid, E2:itaconic acid, E3:α-ketobutyric acid, E4:α-keto glutaric acid, E5:α-keto valeric acid, E6:D, L-lactic acid, E7:malonic acid, E8:propionic acid, E9:quinic acid, E10:D-saccharic acid, E11:sebacic acid, E12:succinic acid, F1:bromosuccinic acid, F2:succinamic acid, F3:glucuronamide, F4:L-alaninamide, F5:D-alanine, F6:L-alanine, F7:L-alanyl-glycine, F8:L-asparagine, F9:L-aspartic acid, F10:L-glutamic acid, F11:glycyl-L-aspartic acid, F12:glycyl-L-glutamic acid, G1:L-histidine, G2:hydroxy-L-proline, G3:L-leucine, G4:L-omithine, G5:L-phenylalanine, G6:L-proline, G7:L-pyroglutamic acid, G8:D-serine, G9:L-serine, G10:L-threonine, G11:D, L-carnitine, G12:γ-aminobutyric acid, H1:urocanic acid, H2:inosine, H3:uridine, H4:thymidine, H5:phenylethyl-amine, H6:putrescine, H7:2-aminoethanol, H8:2,3-butanediol, H9:glycerol, H10:D, L, α-glycerol phosphate, H11:α-D-glucose-1 phosphate, H12:D-glucose-6 phosphate.

All $N_2$-fixing and phosphate-solubilizing *Burkholderia cepacia* tested utilized 47 carbon sources (Table 3). Of these, fourteen were carbohydrates, eighteen were carboxylic acids, thirteen were amino acid, two were amide-amines group carbon sources, and one was a phosphorylated compound (Table 3). All *Stenotrophomonas maltophilia* strains tested utilized 27 carbon sources. Of these, eleven were carbohydrates, ten were carboxylic acids, four were amino acids, and one was a miscellaneous carbon source and polymer. However, use of the amide-amine and amino acids was limited to eight gram-negative *S. maltophilia* isolates, which was a utilized carbohydrate and polymer at a higher rate than did the other strain tested (Table 3, Figure 1).

Of the substrates common to GN2 and GP2 plates, each of the eleven carbon sources (arbutine, D-cellobiose, α-D-glucose, D-mannitol, D-mannose, D-psicose, sucrose, D-

trehalose, pyruvic acid, L-asparagine, and L-glutamic acid) were used by all of Gram-positive isolates. Ten of these substrates (D-fructose, α-D-glucose, D-mannose, citric acid, α-ketoglutaric acid, L-lactic acid, propionic acid, succinic acid, L-glutamic acid, and L-serine) were used by all of the Gram-negative strains. Only three (α-D-glucose, D-mannose, and L-glutamic acid) substrates were utilized by all Gram-positive and negative tested species.

# 4. Discussion

In total, 263 bacterial strains were identified representing 34 different genera, and 72 species. The dominant bacteria associated with acidic tea rhizosphere belonged to the genera *Bacillus* and *Pseudomonas*. Similarly, [11, 18] found *Bacillus* followed by *Pseudomonas* to be the dominant species in the acidic tea soil. This is important because *Pseudomonas* and *Bacillus* are the two most common PGPR that can enhance the biomass, nitrogen and phosphorous uptake, and crop yield [19]. *Bacillus* species were found to be well adapted to the rhizosphere of established tea bushes [20] and can be characterized with the ability to tolerate the unfavourable conditions [21]. Nevertheless, there have been few investigations concerning *Bacillus* in acidic soils. Survival of these bacterial species under adverse environmental conditions was probably due to their spore forming property. Gram-positive bacteria were the most abundant, in agreement with previous studies [11, 16]. In contrast, other studies show a higher level of Gram-negative species in the rhizosphere relative to Gram-positive species [22, 23].

In this study was evaluated to represent the culturable diversity of diazotrophs and phosphobacteria, and thus potentially beneficial to the growth and survival of tea plants in that specific acidic ecosystem. The composition of the rhizobacterial community associated with plant roots is influenced by a variety of sites, soil pH and type, and environmental factors. The soils of the sites sampled in the present study had pH values that ranged from 3.6 to 6.5. Soil pH was the characteristic most closely related to members of the genus *Bacillus*, *Pseudomonas* and *Paenibacillus*. Hartman *et al.* [1] and Beneduzi *et al.* [3] reported [3, 12] reported that pH was the major soil factors affecting diversity of soil diazotrophic and bacterial communities. This study indicated that soil pH and habitat had a strong influence on the diversity of NFB and PSB species. PGPR strains isolated from the rhizosphere of different crops have been developed in different acidic soils [24], and soil pH is the important characteristics associated with tea rhizosphere [20]. The pH tolerance investigations have highlighted the fact that the tested strains possess wide ecological tolerance values.

It has been investigated the diversity and composition of NFB and PSB, naturally colonizing a mild climate with high precipitation and acidic soil, in the tea growing region. A number of bacterial species belonging to genera *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Stenotrophomonas, Arthrobacter, Serratia*, and *Burkholderia* were the most common NFB and PSB in the tea rhizosphere. All genera encountered, according to this study, have been already described as PSB and/or BNF bacteria, such as *Pseudomonas* [4, 25, 26], *Bacillus* and *Paenibacillus* [27, 28], *Burkholderia* [4, 24, 29], and *Arthrobacter* [10]). *B. pumilus* was the most dominant NFB and PSB in the acidic tea rhizosphere, followed by *B. subtilis*, *B. licheniformis*, *B. laevolacticus*, *P. fluorescens*, *P. putida*, *S. maltophilia* and *B. megaterium.* Among the rhizobacteria, a total of 81.0 and 60.5% isolates showing $N_2$-fixation and P-solubilisation were considerably lower than those of previous study [2, 25], while higher than others [4, 6, 16]. These NFB and PSB could serve as efficient biofertilizer candidates for improving the N and P-nutrition of crop plants.

A total of 102 $N_2$-fixing and P-solubilizing isolates were *tested* for *their* ability to oxidize carbon substrates. These strains, belonged to *B. subtilis*, *B. licheniformis*, *B. megaterium*, *P. fluorescens*, *P. putida*, *S. maltophilia* and *B. cepacia* have been reported as good phosphate solubilisers, nitrogen fixers and plant growth promoters [10, 11, 18, 22, 23, 25, 30]. Biolog GP2 and GN2 MicroPlates were also used to in order to determine if there are differences in the carbon utilizations of selected strains of these species. It may be possible to afford a competitive advantage for different purposes by providing them with a substrate that they can readily use as a C-source. The pattern of substrate utilisation could be used to determine differences in the physiological functions of PGPR in soil because the utilization of available carbon is the key factor governing microbial growth in soil.

Gram-negative species have utilized the carboxylic acids, amino acid and carbohydrate as the carbon source at a higher rate than did the other substrates (Figure 1). Carbohydrates, amino acid and miscellaneous carbon sources were also utilized predominantly by Gram-positive *Bacillus* species. There were large differences in the C utilizations of *B. pumilus*, *B. subtilis*, *B. licheniformis* and *B. megaterium*, which were arranged according to the order of best C-sources utilized by all *B. licheniformis* strains. Also, D-cellobiose, gentiobiose, maltose, turanose, thymidine and 2, 3-butanediol were utilized well by most of the *Bacillus* species and which was not used by any gram-negative *P. fluorescens* and *P. putida* isolates.

In GP2 plates, the most discriminating substrates were the arbutine, D-fructose, β-methyl-D-glucoside, uridine and salicin associated with PC1, carbohydrates maltotriose and turanose associated with PC2 and the acid carboxylic methyl pyruvate associated with PC3. In GN2 plates, the most discriminating substrates were the lactulose and D-melibiose associated with PC1 and carbohydrates adonitol and L-fucose associated with PC2, for which there were consistent differences in the degree of substrate oxidation among the selected gram negative $N_2$-fixing and/or P-solubilizing strains. Gram-positive NFB and PSB appeared to favour carbohydrates and amino acids, and Gram-negative bacteria appeared to favour carboxylic and amino acids as carbon sources.

## 5. Conclusion

Present study describes the dominant culturable diversity and metabolic potential of root-associated $N_2$-fixing, P-solubilizing, and different C source-utilizing bacteria of tea plant in the southern coast of the Caspian Sea region for the first time. It is likely that strains that are better adapted to a specific rhizosphere environment are more competitive than the strains that were isolated from a different environment. Moreover, the ability to utilize a broad range of C-sources and the specific organic compounds NFB and PSB in the rhizosphere was shown to provide a selective advantage to the bacterial strain, and could have a high potential for use as a biofertilizer in agriculture. Also, the present results suggest that the Biolog microplate assay could be readily used, not only for the study on C-source utilization but also for the identification and classification of PGPR species.

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