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Enumeration of Bacterial Population of the Rhizopheric Soil by Culturable Method of Similipal Biosphere Reserve, Odisha, India

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Abstract: In *toto* 100 soil samples were collected from 10 different sites of Similipal Biosphere reserve, Odisha, India. Soil samples were subjected to physico-chemical parameter analysis. The soil observed to be acidic in nature. The organic carbon content of the soil fluctuated from site to site. The highest percentage of organic carbon was reported at the site of chahala-1 to be 0.676 during winter season. Soil bacterial population in the collected samples ranged between $2.9x10^8 \pm 9.2x10^7$ to $1.69x10^6 \pm 2.9x10^4$ CFU/gm of soil when studied through culturable methods. The highest bacterial population was observed at the site Natto-1 followed by Joranda, and Chahala-2. Phosphate solubilizing bacterial population ranged between $4.4x10^6 \pm 2.4x10^5$ to $6.9x10^4 \pm 1.2x10^3$ CFU/gm of soil. Total 450 isolates were isolated and identified by gram's reaction and through a battery of biochemical characters. Out of 201 phosphate solubilizing isolates, 30 bacterial isolates were selected for studying their enzymatic activity. Isolates showed higher activities for phosphate, starch and protein digestion on solid medium with specific substrates.

Key words: Bacteria population, phosphate solubilization

Introduction

Soil contains a variety of microorganisms including bacteria that can be found in any natural ecosystem. However, soil microbial communities are considered to hold the most diverse microbial communities in the world, with up to 10^4 bacterial species per gram and more than 30,000 prokaryotic species [3,13,17]. Rhizophere is the region of the soil and the root influenced by enhanced microorganisms, as the plant provides better site for growth of microorganisms and micro-organisms play major role in the nutrients cycle. In the rhizosphere, the microbial density is typically higher than in bulk soil and ranges up to 10^9 bacteria per gram. It is the site for harmful and beneficial activities where many key interactions take place between microbes and plant, where, bacteria are essential for the closing of nutrient and geochemical cycles such as the carbon, nitrogen, sulfur and phosphorous cycle. More microbial activity in soil ecosystem is the better site for plant growth [5]. Similipal Biosphere Reserve located in the midst of Mayurbhanj district, Odisha, India and in between 21°28' to 22° 08'N latitude and 86° 04' to 86° 37' E longitude and covering 5,578 km^2 of forest land. The Similipal Biosphere Reserve has been divided into three zones, i.e Core zone (845 km^2), Buffer zone (2174 km^2) and transitional zone (2559 km^2). The climate of Similipal is tropical with warm and humid summer, temperature over around 40° C during Peak of the season. Rainfall of about 125 mm in the monsoon. The winter temperature falls down to 5°C in some parts of the hill. This uneven geophysical condition influences the diversity of floral and faunal distribution. To the best of our knowledge, this virgin forest soil is not explored much for its

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microbial wealth, which prompted us to undertake this study to enumerate the bacterial population of the rhizospheric soil, collected from this unique system.

Materials and methods

Collection of samples

A total of one hundred soil samples were collected from ten different sites of Similipal Biosphere Reserve in rhizosphere region of Shorea robusta. The samples were collected during summer and winter seasons (fifty for each season) for a period of two years (April, 2011-March, 2013). Each soil sample represent composite of well mixed five samples unit collected from sampling plot of 20x20 meter.

Physico-chemical parameters study

Soil pH, temperature, humidity, organic carbon were determined by following standard soil analysis methods [1,4].

Isolation and enumeration of bacteria

Bacteria were isolated and enumerated by culturable methods (spread plate and pour plate technique) on basal medium (nutrient agar) and specialized (Pikovaskay's agar) medium [6]. Individual colonies of bacteria which varied in shape and colour were picked up and purified by streaking on nutrient agar. The bacterial isolates were preserved & maintained on nutrient agar slopes at 4°C and viability of the isolates was checked by routine subculture an interval of 3 months.

Identification of isolates

The selected bacterial isolates were identified on the basis of classification schemes published in Bergey's Manual of Systematic Bacteriology [12]. All the Gm +ve rods were further characterized by growing them on Hi-chrome Bacillus Agar medium based on their growth and pigmentation of the isolates, in this medium.

Phosphate solubilizing studies of the isolates

Phosphate solubilizing activity of the isolates was tested on Pikovskaya's agar (PA) containing tricalcium phosphate as insoluble phosphate source. Pikovskaya's agar plates were prepared as per manufacturer's (Hi-Media Pvt. Ltd. Mumbai, India) instructions. Freshly grown cultures of bacteria were spot inoculated on PA plates by the help of a sterile loop. Inoculated plates were incubated at 37°C. Formation of a halo zone around the colonies is indicative of positive phosphatase activity. Solubilizing index (SI) of the isolates was determined by using the formula [11] as follows:

$$SI = \frac{\text{Halo zone + Colony diameter}}{\text{Colony diameter}}$$

Amylase activity (starch hydrolysis)

Amylase activity of the phosphate solubilizing bacterial isolates (30) was initially tested on Starch Nutrient Agar plates. Freshly grown cultures on NA slants of the isolates were transferred onto Starch Nutrient Agar plates by the help of a sterile loop. After 24 hrs (37°C) of incubation, the plates were exposed to iodine vapour, entire plate turned blue and a clear zone around the colony indicated positive amylase activity and/or starch hydrolysis by the isolates [10].

Protease activity (protein hydrolysis)

To test proteolytic activity (30 phosphate solubilizing bacterial isolates), the organisms were grown on Sikimmed Milk Agar Plates. Sikmmed milk (Sikmmed milk 1 %, Dextrose 2 % and Agar 1.5 %) plates were prepared and the isolates were spot inoculated by the help of a sterile loop taking from freshly grown NA of bacteria respectively. The plates were incubated at 24 hrs (37°C). The plates were observed after incubation period, a clear zone around the colony indicates positive protease activity [10].

Results and discussion

In this study, total 100 soil samples were collected from 10 different sites of the ecosystem (Table-1). Ten samples from each sites were analyzed (5 in each season of winter and summer). The physico-chemical parameters of the soil samples were studied (Table 1). The soil samples were found to be acidic in nature. Among the sites, high acidity, moisture content and organic carbon percentage were observed in soil sample collected from Chahala (Table 1). Similarly, lowest moisture content and organic

Table 1. Physico chemical parameters of soil samples collected from different sites of Similipal Biosphere Reserve

Collection site	Moisture content % (winter)	Moisture content % (summer)	Temperature (winter)	Temperature (summer)	Organic C % (winter)	Organic C % (summer)	Acidity (winter)	Acidity (summer)	Salinity (winter and summer)
Natto-1	20	16	19	24	0.644	0.654	5.36	5.4	0.5
Natto -2	19.8	15	19.2	25	0.612	0.632	5.64	5.8	0.5
Chahala-1	21.4	20.4	14	16	0.676	0.656	5.08	5.1	0.5
Chahala-2	23.6	21.6	14.8	18	0.6375	0.6415	5.14	5.1	0.5
Gurguria-1	14.6	12.6	17.5	22.5	0.564	0.555	6.1	5.9	0.7
Gurguria-2	14.8	12	16.9	19	0.516	0.51	6.12	6.0	0.5
Bareripani	12.8	10	18	20	0.428	0.422	5.92	5.8	0.5
Joronda	23.4	19.1	15.6	20.2	0.622	0.63	5.52	5.4	0.5
Pithahata-1	17.6	16.2	15.6	19.6	0.58	0.588	6.04	5.9	0.5
Pithahata-2	18.8	15.2	15.9	20.9	0.572	0.55	5.98	5.8	0.5

carbon percentage were recorded in the site Bareripani. Several workers [7,9,15] reported that soil pH is determined by availability of organic substances and microbial activities. Therefore, less pH and more organic carbon was observed to be in the site Chahala, followed by Natto and Joranda (Table 1, 2) could be attributable to highest microbial activities at this site.

The bacterial isolation and enumeration were done by spread plate and pour plate methods. Soil bacterial population of Similipal Biosphere Reserve (collected soil samples) ranged from $2.9x10^8 \pm 9.2x10^7$ to $1.69x10^6 \pm 2.9x10^4$ CFU/gm of soil. The highest population was observed at the site Natto followed by Joranda, Chahala. But, the bacterial population was less in the soil samples collected from Bareipani with pH 5.92 and less organic substances. In our study, we observed that the bacterial population was directly correlated to the organic carbon and moisture content of the soil (fig. 1) as reported by earlier researchers [16]. Forest soil rich in microbial flora, their activities in soil leads to the fertility of soil. With this aim an attempt was made to enumerate the population of phosphate solubilizing bacteria in these soil samples on Pikovaskya's agar medium and we observed the presence off a high population of phosphate solubilizing bacteria ($4.4x10^6 \pm 2.4x10^5$ to $6.9x10^4 \pm 1.2x10^3$ CFU/gm of soil). In agreement to our observation presence of high numbers of phosphate-solubilizing bacterium (3×10^6 cfu g^{-1}) was also reported in rhizophric soil of *Piper betel* in Karnatak, India [14].

Identification of bacteria

All 450 isolates were characterized by Grams staining and grouped as Gm+ve rod (282), Gm-ve rod (98), Gm+ve cocci (51) and Gm-ve cocci (19). Further, all grams positive rods (282) were characterized on Hichrome Bacillus agar media and based on their growth patterns and pigmentation isolates were identified as *B. subtilis* (20), *B. megaterium* (10), *B. coagulans* (10), *B. thuringiensis* (5), *B. cerius* (10) in Fig. 2. All the Gm-ve rods were tentatively identified and assigned to genera *Pseudomonas*. It was observed that *Bacillus* to be the dominant phosphate

Table 2. Soil bacterial population of Similipal Biosphere Reserve by culturable method

Collection site	Nutrient agar				Pikovaskay's agar			
	Spread Plate winter	Spread plate summer	Pour plate winter	Pour plater summer	Spread Plate winter	Spread plate summer	Pour plate winter	Pour plate summer
Natto-1	$2.9x10^{8}\pm$ $9.2x10^{7}$	$1.6x10^{8}\pm$ $8.8x10^{7}$	$2.7x10^{8}\pm$ $1.1x10^{7}$	$7.94x10^{7}\pm$ $2.0x10^{5}$	$3.94x10^{5}\pm$ $2.6x10^{4}$	$4.1x0^{5}\pm$ $2.8x10^{4}$	$4.58x10^{5}\pm$ $2.0x10^{4}$	$2.96x10^{5}\pm$ $9.8x10^{4}$
Natto -2	$6.53x10^{6}\pm$ $2.2x10^{5}$	$6.9x10^{7}\pm$ $2.8x10^{5}$	$1.69x10^{7}\pm$ $2.3x10^{5}$	$7.06x10^{5}\pm$ $1.8x10^{4}$	$3.64x10^{5}\pm$ $1.3x10^{4}$	$6.88x10^{4}\pm$ $2.6x10^{3}$	$2.94x10^{5}\pm$ $1.1x10^{4}$	$1.96x10^{5}\pm$ $7.8x10^{3}$
Chahala-1	$7.3x10^{7}\pm$ $8.7x10^{4}$	$8.8x10^{5}\pm$ $2.7x10^{4}$	$7.27x10^{6}\pm$ $1.6x10^{5}$	$5.65x10^{6}\pm$ $4.2x10^{4}$	$3.56x10^{5}\pm$ $5.7x10^{4}$	$3.0x10^{5}\pm$ $2.9x10^{4}$	$3.2x10^{5}\pm$ $1.9x10^{4}$	$2.59x10^{5}\pm$ $2.7x10^{4}$
Chahala-2	$8.7x10^{7}\pm$ $1.0x10^{6}$	$3.25x10^{6}\pm$ $3.8x10^{5}$	$7.22x10^{7}\pm$ $1.1x10^{6}$	$6.65x10^{7}\pm$ $3.4x10^{6}$	$2.7x10^{6}\pm$ $1.3x10^{5}$	$6.08x10^{5}\pm$ $1.5x10^{5}$	$4.4x10^{6}\pm$ $2.4x10^{5}$	$6.56x10^{5}\pm$ $2.4x10^{5}$
Gurguria-1	$1.010^{7}\pm$ $1.1x10^{5}$	$6.68x10^{7}\pm$ $9.3x10^{6}$	$3.9x10^{7}\pm$ $4.2x10^{6}$	$7.4x10^{6}\pm$ $8.9x10^{4}$	$3.5x10^{6}\pm$ $2.9x10^{6}$	$6.58x10^{5}\pm$ $2.1x10^{4}$	$2.2x10^{6}\pm$ $1.7x10^{4}$	$5.0x10^{5}\pm$ $1.1x10^{4}$
Gurguria-2	$1.8x10^{7}\pm$ $8.8x10^{5}$	$4.58x10^{6}\pm$ $3.0x10^{4}$	$2.2x10^{7}\pm$ $9.3x10^{5}$	$3.69x10^{6}\pm$ $2.7x10^{4}$	$3.5x10^{5}\pm$ $1.4x10^{4}$	$5.54x10^{5}\pm$ $4.9x10^{4}$	$3.2x10^{5}\pm$ $6.9x10^{4}$	$7.34x10^{5}\pm$ $1.2x10^{4}$
Bareripani	$1.69x10^{6}\pm$ $2.9x10^{4}$	$1.86x10^{6}\pm$ $5.3x10^{4}$	$3.62x10^{7}\pm$ $1.6x10^{5}$	$6.8x10^{5}\pm$ $1.7x10^{4}$	$2.25x10^{6}\pm$ $1.8x10^{5}$	$6.9x10^{4}\pm$ $1.2x10^{3}$	$1.8x10^{5}\pm$ $1.7x10^{4}$	$5.6x10^{4}\pm$ $2.6x10^{3}$
Joronda	$1.0x10^{8}\pm$ $2.5x10^{5}$	$7.42x10^{5}\pm$ $1.8x10^{4}$	$5.0x10^{7}\pm$ $3.9x10^{5}$	$6.6x10^{6}\pm$ $3.2x10^{4}$	$6.94x10^{5}\pm$ $8.4x10^{4}$	$1.26x10^{5}\pm$ $6.4x10^{4}$	$2.58x10^{5}\pm$ $4.7x10^{4}$	$5.96x10^{5}\pm$ $2.6x10^{4}$
Pithahata-1	$5.95x10^{7}\pm$ $1.4x10^{5}$	$7.3x10^{6}\pm$ $1.2x10^{4}$	$7.14x10^{7}\pm$ $2.1x10^{5}$	$4.34x10^{5}\pm$ $9.0x10^{3}$	$1.92x10^{6}\pm$ $5.7x10^{4}$	$3.2x10^{4}\pm$ $9.7x10^{3}$	$2.0x10^{6}\pm$ $4.1x10^{4}$	$2.610^{4}\pm$ $1.0x10^{3}$
Pithahata-2	$4.0x10^{7}\pm$ $1.4x10^{5}$	$6.8x10^{5}\pm$ $1.7x10^{3}$	$3.27x10^{7}\pm$ $1.7x10^{5}$	$7.4x10^{6}\pm$ $8.9x10^{4}$	$4.0x10^{5}\pm$ $2.4x10^{4}$	$4.46x10^{4}\pm$ $2.6x10^{3}$	$4.2x10^{5}\pm$ $1.0x10^{4}$	$5.68x10^{4}\pm$ $1.8x10^{3}$

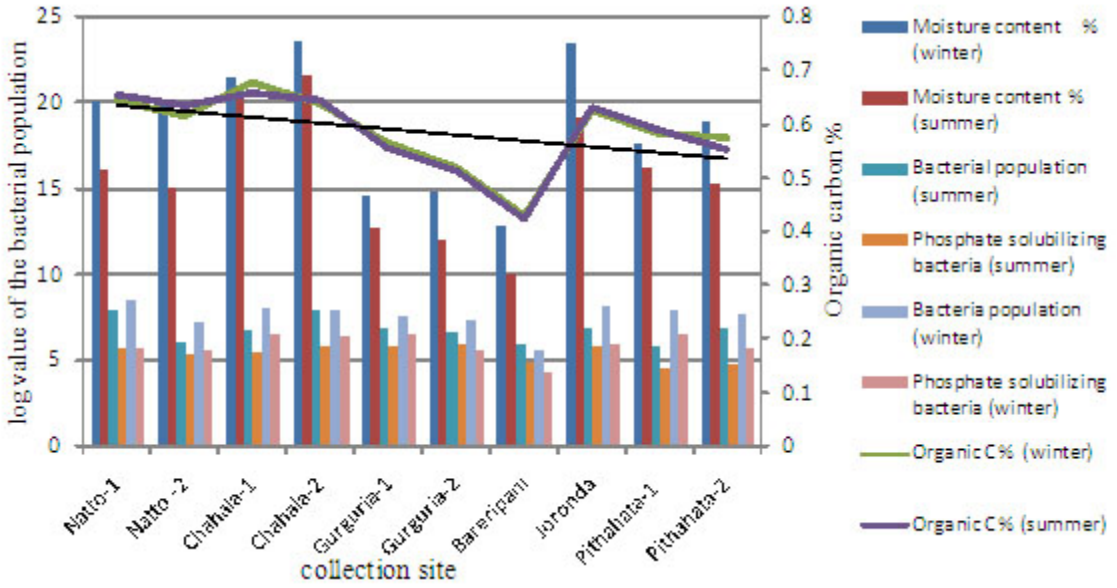

Fig. 1. Relation between bacterial population with moisture content and organic carbon

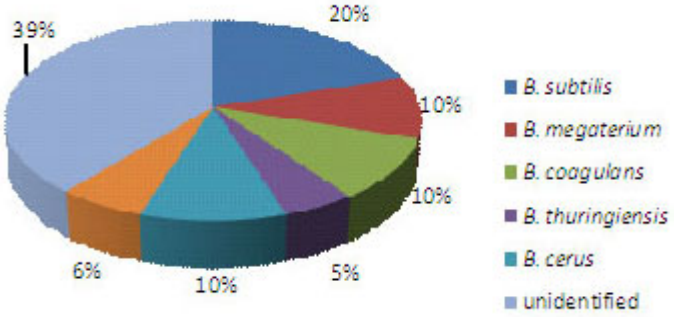

Fig. 2. Identification of *Bacillus* species

solubilizing microorganisms in the soil. Soil bacteria like *Bacillus* and *Pseudomonas* with phosphate solubilizing activities are also isolated by several workers [8,16] that corroborates with our findings.

Screening of isolates for enzymatic activities

While studying the phosphatase activity of the isolates on Pikovaskay's agar medium it was observed that out of 450 isolates 201 (44.7 %) bacteria showed distinct halo zone on PA medium, representing phosphate solubilizing activities. Microorganisms have common feature to break down complex organic material into simplest and increase soil fertility. In this regard, the amylase and protease activities of the microorganisms are another significance characters [2]. Out of 201 phosphate solubilizing bacteria, 30 isolates were selected for studying their enzymatic activities (amylase and protease). When these isolates were screened for amylase and protease activities it was observed that bacterial isolates (S1-5, S7-1,S1-1) were positive for protease and S3-1, S5-1, S6-2 and S16-1for amylase activities. But isolates S16-2, S8-1 and S4-2 were positive for both amylase and protease activities. Enzyme activity such as this are required for soil bacteria to degrade complex organic matters to simpler forms in order to increase soil fertility.

Through this investigation we place in record that the Similipal forest soil harbors a high population of soil bacteria with phosphate, starch and protein digesting activities. Though, it is a preliminary investigation studies such as this, is a prerequisite to tap the unique potential of these bacteria for their application in agriculture.

Table 3. Enzymatic activities of selected isolates

Isolates	Enzyme activity (SI)		
	Phosphatase	Protease activity	Amylase activity
S_{1-1}	1.172±0.07	-	-
S_{1-2}	1.291±0.09	-	-
S_{1-5}	1.133±0.01	2.0±0.2	-
S_{1-7}	1.223±0.02	-	-
S_{1-8}	1.162±0.09	-	-
S_{1-9}	1.173±0.03	-	-
S_{1-11}	1.2±0.04	1.8±0.2	-
S_{2-3}	1.21±0.03	-	-
S_{3-1}	1.21±0.06	-	1.1±0.1
S_{4-2}	1.114±0.01	1.6±0.3	1.3±0.3
S_{4-6}	1.124±0.04	-	-
S_{5-1}	1.258±0.05	-	1.3±0.2
S_{5-2}	1.209±0.07	-	-
S_{6-1}	1.15±0.09	-	-
S_{6-2}	1.101±0.03	-	1.4±0.4
S_{6-4}	1.129±0.03	-	-
S_{7-1}	1.927±0.06	7.3±0.4	-
S_{7-2}	1.143±0.03	-	-
S_{7-4}	1.156±0.01	-	-
S_{8-1}	1.261±0.08	-	-
S_{8-2}	1.216±0.1	1.4±0.2	1.1±0.1
S_{9-1}	1.108±0.01	-	-
S_{9-2}	1.19±0.04	-	-
S_{9-6}	1.144±0.02	-	-
S_{10-1}	1.597±0.02	-	-
S_{10-2}	1.167±0.072	-	-
S_{10-3}	1.185±0.049	-	-
S_{11-2}	1.126±0.042	-	-
S_{16-1}	1.326±0.083	-	1.3±0.2
S_{16-2}	1.219±0.021	6.3±0.4	2.0±0.2

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