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Isolation, Characterization and Screening of Bacterial Isolates from Similipal Biosphere Reserve Forest Soil for their Metal Tolerance Capacity and Extracellular Enzymatic Activities

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ABSTRACT

In total 50 bacteria were isolated from 15 soil samples collected from Similipal Biosphere Reserve through spread and pour plate technique. The CFU of bacteria per gram of soil ranged between 10^6 and 10^9. The isolates were identified and assigned to the genera *Bacillus* (38, 76%), *Pseudomonas* (8, 16%), *Micrococcus* (2, 4%) and *Geomicrococcus* (2, 4%). Isolates were characterized for their growth in different media and pH. The isolates grew well at acidic pH, although growth was observed over a wide pH range. Surprisingly a high degree of multiple antibiotic resistance (0.142-0.428) was reported while studying the antibiogram pattern of the isolates. Isolates were screened for their metal tolerance capacity against heavy metals such as Hg, Cd, Cr, Ni, Cu, Zn, Co and Pb. When the isolates were screened for the production of industrially important enzymes by the plate assay method it was reported that 25 (51.02%), 24 (48.97%), 18 (36.73%), 18 (36.73%) and 28 (57.14%) of the isolates were positive for amylase, protease, lipase, phosphatase and DNase, respectively. Soil bacteria growing at acidic pH, having metal tolerance capacity and producing industrially important enzymes could be novel elements for different industrial processes.

Keywords: *Bacillus*, *Geomicrococcus*, heavy metal, *Micrococcus*, *Pseudomonas*, soil bacteria

INTRODUCTION

Soil bacteria of different ecological habitats adapt by changing their physiology and by producing specific components within the cell. The preeminent role of these microorganisms in soil is to take part in different biogeochemical cycles for their mineralization capacities. In addition to this, many soil bacteria possess different unique characters and offer a great potential for their biotechnological exploitation. In this respect, Similipal Biosphere Reserve (SBR) is a densely forested hill-range in the heart of the District of Mayurbhanj in Orissa (India) lying close to the easternmost end of Eastern Ghats. Located in the Mahanandian biographical region and within the biotic province, Chhotanagpur plateau, it spreads over an area of 2750 km^2 and is located at latitude 20°29′ to 22°09′ N and longitude 86°37′ E. A large variation in climate, soil temperature, minerals and geology in SBR over the millennia might have produced a wealth of biological diversity in microbes as microorganisms are remarkably adaptive, and can be found in all sorts of inhospitable environments (Rath 1996; Stephen *et al.* 2000). SBR soil is rich in organic content (Bahuti 2006; Bahuti *et al.* 2006; Panda 2006) and its varied climatic conditions offers great potential for a large degree of microbial diversity. This prompted us to study the bacterial flora of this virgin forest soil and possible characterization for their biotechnological and/or commercial applications, if any, in terms of secondary metabolites and enzymes. This investigation was carried out with the following aims: i) physicochemical analysis of the soil; ii) isolation and enumeration of bacteria from collected soil samples; iii) identification and possible characterization of the isolates; iv) study of antibiogram pattern of selected isolates; v) study and evaluation of special features of these isolates in terms of their metal tolerance capacities and enzymatic activities for commercial exploitation. To the best of our knowledge this scientific investigation is the first of its kind, with reference to the study of microbial (soil bacteria) wealth from SBR, Orissa, India.

MATERIALS AND METHODS

Media used

Nutrient agar (NA), MacConkey agar (MA), soil extract agar (SEA), eosine methylene blue agar (EMB), water agar (WA), triple sugar iron agar (TSIA), mannitol motility test medium, peptone water (PW), Mueller Hinton agar (MHA), urea agar (UA) and Simmons citrate agar (SCA), were used for isolation, enumeration, identification and study of the growth characters. Starch agar (SA), skim milk agar (SMA), Pikovaskya's agar (PA), tributyrin agar (TA) and DNase test agar were used to study different extracellular enzymatic activities of the isolates. All media, except for SEA, were procured from Hi-Media, Mumbai, Pvt. Ltd., India, and prepared as per manufacturer's instructions. SEA medium was prepared in the laboratory as per Barua and Barthakur (1998).

Antibiotics used

Antibiotic discs of amikacin (Ak 15), bacitracin (B 10U), chloramphenicol (C 30), gentamycin (G 30), polymyxin (Pb 300U), tetracyclin (T 30), vancomycin (V 10), were procured from Hi-Media, Mumbai, Pvt. Ltd., India, and used to study the antibiogram pattern of the isolates.

Collection of soil samples

Composite soil samples were collected randomly from each plot (Barua and Barthakur 1998). Ten samples from different places in each field/plot (20 m × 20 m) were collected, mixed thoroughly,

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from where 200-250 g of soil was pooled as a composite sample, packed in pre-sterilized polythene bags and transported to the laboratory. In each plot, samples from different places were collected from different soil layers: surface layer (5 cm deep), second layer (6-12 cm deep) and third layer (12-20 cm deep). In this manner all together 15 soil samples were collected from different geographical locations extending from Gudgudia to Chahala through Bareipani and Joranda inside SBR forest.

Physico-chemical characteristic studies of soil samples

Different physico-chemical parameters such as moisture content, pH, electrical conductivity, percentage of organic carbon, nitrogen, phosphorous and potassium content of the samples were studied following the method described by Anonymous (1980) and Baruah and Barthakur (1998).

Isolation and enumeration of bacteria

Bacteria were isolated and enumerated by a serial dilution method followed by a spread, plate and pour plate technique (Cruickshank *et al.* 1972; Rath 1996). Valid plate counts were considered for enumeration. Selected colonies were picked, transferred onto NA slants, and stored at 4°C for future use.

Identification of isolates

Bacteria were identified by microscopic examination and an array of biochemical tests according to Sneath *et al.* (1986) and Butler (1986) and the schemes of Collins and Lyne (1970), and Bhat *et al.* (1971).

Growth of isolates

Isolates were grown on different media such as NA, MA, SEA, WA (contains 1.5% V/V agar powder in distilled water which served as sole source of nutrition) and cultural pattern of the isolates were compared.

***Growth of the isolates at different pHs*:** Isolates were spot inoculated on NA plates of different pH range (pH 3-11) and growth patterns were observed at 37°C.

***Antibiotic sensitivity test*:** Selected bacterial isolates were screened for their antibiogram pattern, following the method of Bauer *et al.* (1966) by disc diffusion method on MHA plates. The multiple antibiotic resistance (MAR) index of the isolates were determined (Mohapatra *et al*. 2006) in order to study their drug resistance capacities.

***Metal tolerance capacity of the isolates*:** An experiment was designed to study the metal tolerance capacity of the isolates following the method of Rath and Subramanyam (1999) with slight modifications. Resistogram was recorded by observing growth of the bacterial isolates on nutrient agar plates in presence of various concentrations of metals like cadmium ($CdCl_2$), chromium ($K_2Cr_2O_7$), cobalt ($CoCl_2$), lead ($PbCH_3COO^-$), nickel ($NiCl_2$), zinc ($ZnSO_4$), copper ($CuSO_4$), mercury ($HgCl_2$). The minimal inhibitory concentrations (MIC) were determined by observing the growth at 37°C. The MIC of a metal against an isolate was considered to be the next concentration beyond the last concentration at which the growth of the isolate was observed.

***Extracellular enzymatic activity studies*:** Extracellular enzymatic (amylase, protease, lipase, phosphatase and DNase) activities of the isolates were studied by plate assay method. Amylase, protease and phosphatase activity of the isolates were tested following the method of Rath (1996). Lipase activity was studied on tributyrin agar (tributyrin agar base with tributyrin, 1% glycerol tributyrate) plates following the methods of Kouker and Jaeger (1987). DNase activity was studied by growing the isolates on DNA (DNA at 0.3% level) agar plates following the method of Rath (1996).

RESULTS

The physico-chemical parameters of the studied samples are presented in **Table 1**. Moisture content of the samples varied from 4.3-31.8%. The soil was observed to be slightly acidic. However, negligible variability was observed with electrical conductivity among the samples. The nitrogen and potassium contents measured between 193-291 and 154-244 Kg/ha, respectively. The phosphorous ranged between 11.7-19.2 Kg/ha in these soils. The percentage of organic carbon among the samples varied between 0.598-0.985. Percentage of organic carbon, nitrogen and potassium content observed to be very high in the soil samples studied indicative of high mineralization of the organic matter in these soils. Total Plate Count (TPC) of all the samples were carried out by spread and pour plate techniques on Nutrient agar (NA) and MacCkonkey agar (MA) plates. The colony forming units per gram of soil (CFU/gm) of bacteria ranged between 10^6–10^9 and 10^6–10^8, on nutrient agar plates through spread and pour plate methods respectively (**Table 2**). However, in comparison to nutrient agar plates, fewer bacteria were observed on MA plates by both spread as well as pour plate methods. Not observing valid plate counts on MacConkey agar, in many cases, further, indicates the presence of low Gram-negative bacterial load in these samples. Bacterial growth was found to be positively associated with moisture content.

In total 50 bacteria were selected based on their colony morphology and isolated from these fifteen samples analysed and assigned with laboratory number NOU-1 through 50. From the Gram reaction it was observed that the Gram-positive forms dominated over the Gram-negative ones. The Gram-positive forms included both rods and cocci, whereas, the Gram-negative forms represented only rods.

Standard bacteriological methods were followed to study the biochemical characters of the isolates and the observations were compared with Bergy's manual of Systematic

Table 1 Physico-chemical parameters of the soil samples studied.

Sample No	Moisture content %	pH	Electrical conductivity (mmhos/cm)	Organic C (%)	Nutrients kg/ha		
					N	P	K
1	6.7	6.0	2 X 0.93	0.909	268	18.3	244
2	4.3	6.0	2 X 0.155	0.783	291	16.1	208
3	31.8	7.0	2 X 0.177	0.697	200	14.8	211
4	14.9	6.0	2 X 0.218	0.812	248	15.6	201
5	6.9	5.6	2 X 0.106	0.667	252	12.8	187
6	5.0	5.6	2 X 0.101	0.985	198	17.2	191
7	14.1	5.6	2 X 0.188	0.815	218	18.5	190
8	12.6	5.6	2 X 0.162	0.598	267	13.8	181
9	19.3	6.0	2 X 0.191	0.722	243	13.8	168
10	18.2	6.0	2 X 0.183	0.819	281	19.2	154
11	14.4	6.0	2 X 0.211	0.788	231	14.6	186
12	15.0	5.6	2 X 0.341	0.693	262	11.7	201
13	16.1	5.6	2 X 0.122	0.815	193	12.1	221
14	16.1	5.6	2 X 0.168	0.598	287	13.9	197
15	12.7	5.6	2 X 0.134	0.836	283	15.2	189

Table 2 Enumeration of bacteria of different soil samples studied from SBR*.

Sample	Bacteria CFU/g of soil			
	Nutrient agar		McConkey agar	
	SP	PP	SP	PP
1	-	2.47×10^6	8.1×10^4	3.4×10^3
2	3.6×10^8	6.9×10^6	-	-
3	6.8×10^7	2.73×10^7	5.5×10^5	1.06×10^5
4	4.2×10^8	2.35×10^6	1.2×10^6	5.8×10^3
5	1.84×10^6	4.2×10^6	-	-
6	-	2.76×10^6	-	-
7	7.3×10^6	8.1×10^6	-	-
8	1.28×10^9	5.2×10^6	-	-
9	-	4.3×10^6	-	-
10	1.83×10^7	1.47×10^7	-	-
11	-	1.22×10^7	2.35×10^5	4.2×10^3
12	1.15×10^7	9.8×10^6	-	-
13	6.0×10^7	1.08×10^8	-	-
14	5.8×10^7	1.15×10^8	-	-
15	-	1.83×10^6	1.75×10^5	1.24×10^5

* Bacteria were isolated by a 10-fold serial dilution, through spread and pour plate technique, Colony Forming Units (CFU) were calculated by multiplying plate counts with dilution factor. Data represented are mean of three experiments. - = No valid count. SP = spread plate, PP = pour plate.

Bacteriology to identify the isolates to the generic level. The same was compared with the scheme of Rath (1996) to identify the isolates up to species level. Based on the observations the isolates were identified and assigned to four different genera *viz. Bacillus*, *Micrococcus*, *Geomicroccus* and *Pseudomonas* (**Table 3**). Among the isolates, the dominated group was observed to be *Bacillus* (76%), followed by *Pseudomonas* (16%), where as, *Micrococcus* and *Geomicrococcus* observed to be present in equal composition (4% each). However, during preservation we lost the viability of one isolate NOU-50 (*Bacillus* sp), therefore, in all other experiments 49 isolates were studied.

When the isolates were grown on different media such as Nutrient agar, MacConkey agar, Soil extract agar and water agar to study their growth characteristics it was observed that all the isolates grew luxuriantly on nutrient agar plates followed by five times diluted NA medium. Only the Gram-negative isolates grew on plates containing MA medium. Twenty seven (53.1%) and 39 (79.59%) of the isolates grew when cultured on SEA and five times diluted SEA medium respectively. None of the isolates, however, grew on water agar medium where agar was the sole source of energy. Similarly, while studying the growth characters of the isolates on NA with a wide rage of pH (pH3-pH11) it was observed that most of the isolates showed luxuriant growth within pH 6-9. One isolate NOU-10 (*Bacillus* sp) grew over a wide pH range (3-9) while, three (6.12%) isolates, NOU-14, NOU-22 (*Bacillus* sp.) and NOU-34 (*Pseudomonas aeruginosa*) showed growth over wide pH range (3-11). Gram-positive strains (*Micrococcus* and *Geomicroccocus* sp.), on the other hand, grew in a narrow pH range (5-8). Surprisingly, four (8.16%) and 26 (53.06%) of the isolates grew at pH 4 and 11, respectively.

Table 3 Identification of the isolates.

Genera	Number	Percentage
Bacillus sp.	38	76
Geomicroccus sp.	2	4
Micrococcus sp.	2	4
Pseudomonas sp.	8	16

Selective isolates were screened to study their antibiotic sensitivity pattern by disc diffusion method and the multiple antibiotic resistance (MAR) index was determined by using the formula of Mohapatra *et al*. (2006). The antibiogram pattern of the isolates is presented in **Table 4**. The sensitive degree of the isolates towards the antibiotics studied can be graded as T > Ak > G > C > V, respectively. NOU-28 a strain of *Bacillus* represented sensitivity to all the antibiotics used. A high degree of resistance was observed against bacitracin and polymyxin-B and the MAR index of the isolates ranged between 0.125–0.625. Since the strains are isolated from natural forest soil, the development of MAR among these isolates is the matter of concern.

The resistogram of the isolates towards metal tolerance is presented in **Table 5**. It is observed that 43 isolates (87.75%) tolerated 10-30 µg/ml of Hg in the medium

Table 4 Antibiotic sensitivity pattern of the isolates.

Isolates	Antibiogram		
	Sensitive to	Resistant to	MAR index
NOU-13	T(16), V(15), G(22), Ak(23), C(27)	B, Pb	0.285
NOU-14	T(22), V(25), G(26), Ak(18)	B, Pb	0.285
NOU-15	T(11),V(18),G(22), Ak(20),C(29)	B, Pb	0.285
NOU-17	T(19), V(17),G(21),Ak(20), C(29)	B, Pb	0.285
NOU-18	T(11), G(12), Ak(19)	V, C, Pb	0.428
NOU-28	T(23), V(28), B(11), G(26), Ak(24), C(30), Pb(13)	-	-
NOU-31	T(31), V(17), G(25), Ak(26), C(27), Pb(11)	B	0.142
NOU-32	T(20), V(17), G(23), Ak(23), C(29), Pb(12)	B	0.142
NOU-36	T(21), V(16), G(21), Ak(24), C(22)	B, Pb	0.285
NOU-37	T(27), V(17),G(27), Ak(26), C(29), Pb(14)	B	0.142
NOU-41	T(29), V(20), B(11), G(26), Ak(26), C(30), Pb(12)	-	-
NOU-44	T(15), V(15), G(19), Ak(22), C(28)	B, Pb	0.285
NOU-45	T(15), V(13), G(19), Ak(21)	B, C, Pb	0.428
NOU-46	T(18), V(15), G(20), Ak(19), C(13)	B, Pb	0.285
NOU-48	T(16),V(15), G(22), Ak(24), C(25)	B, Pb	0.285

Values in parenthesis represent zones of inhibition or sensitivity in mm. Ak, amikacin; B, bacitracin; C, chloramphenicol; G, gentamycin; Pb, polymyxin B; T, tetracycline; V, vancomycin; MAR, multiple antibiotic resistance.

Table 5 Resistogram of the isolates toward different heavy metals*.

Isolates	Metals							
	Hg	**Cr**	**Cd**	**Ni**	**Cu**	**Pb**	**Zn**	**Co**
NOU-1	25	300	50	500	500	2000	150	300
NOU-2	25	250	50	200	500	2000	200	250
NOU-3	15	300	50	800	300	2000	250	250
NOU-4	10	50	50	50	200	200	200	250
NOU-5	15	300	200	500	500	2000	150	250
NOU-6	25	300	50	500	500	2000	200	200
NOU-7	10	50	50	500	200	2000	300	200
NOU-8	50	250	300	500	1000	2000	1000	250
NOU-9	20	250	50	500	500	2000	250	250
NOU-10	30	300	50	500	500	2000	150	200
NOU-11	20	300	50	500	200	2000	200	300
NOU-12	25	300	100	800	800	2000	1000	300
NOU-13	20	300	500	800	500	2000	1000	300
NOU-14	20	300	300	800	500	2000	1000	300
NOU-15	<10	50	50	50	300	2000	1000	200
NOU-16	20	50	50	300	300	200	1000	200
NOU-17	20	250	50	300	500	2000	200	300
NOU-18	25	250	200	500	500	2000	300	200
NOU-19	15	300	50	500	500	2000	150	300
NOU-20	15	300	50	500	500	2000	300	200
NOU-21	20	250	50	800	500	2000	150	200
NOU-22	10	300	100	800	500	2000	300	200
NOU-23	<10	50	50	50	500	2000	500	200
NOU-24	20	250	100	500	500	2000	800	300
NOU-25	20	250	250	800	800	2000	1000	300
NOU-26	25	500	50	800	800	2000	200	300
NOU-27	<10	50	100	500	200	2000	200	200
NOU-28	25	300	50	500	500	500	200	200
NOU-29	20	250	250	300	500	2000	200	200
NOU-30	20	250	300	300	500	2000	500	200
NOU-31	30	250	50	800	800	2000	1000	300
NOU-32	<10	200	100	500	500	1000	200	300
NOU-33	15	300	300	800	500	2000	800	200
NOU-34	25	50	300	300	800	2000	300	200
NOU-35	10	300	50	500	500	2000	500	200
NOU-36	25	250	150	500	500	2000	1000	200
NOU-37	15	300	50	800	500	2000	300	300
NOU-38	30	300	800	800	500	2000	1000	300
NOU-39	20	200	50	300	500	2000	300	200
NOU-40	25	250	250	800	500	2000	800	300
NOU-41	15	300	100	300	500	2000	200	200
NOU-42	30	300	500	800	1000	2000	1000	300
NOU-43	20	250	100	500	500	2000	200	250
NOU-44	20	200	50	500	500	2000	200	250
NOU-45	25	250	500	500	1000	2000	800	200
NOU-46	15	300	50	500	500	2000	200	200
NOU-47	30	250	500	800	1000	2000	1000	300
NOU-48	15	200	50	300	500	2000	200	200
NOU-49	50	250	50	500	500	2000	200	250

* The resistogram was obtained from a spot inoculation method. Values represent MIC in µg/ml of medium.

whereas, 4 (8.16%, NOU- 15, NOU-23, NOU-27, NOU-32) were inhibited at 10 µg/ml of Hg in the medium. Highest MIC value (50 µg/ml) of Hg was observed against two isolates NOU-8 (*Pseudomonas fluorescence*) and NOU-49 (*Micrococcus* sp.). 83.67% of the isolates were inhibited at 200-300 µg/ml of Cr in the medium, whereas, seven (14.28%) isolates were inhibited at 50 µg/ml of chromium in the medium. Highest MIC value 500 µg/ml of chromium was reported against NOU-26. Although the MIC value of cadmium against 42 (85.71%) isolates was 50-300 µg/ml, five (10.20%) isolates showed a higher MIC value i.e. 500 µg/ml. A similarly higher MIC value was also observed for Ni (31, 63.26% isolates showed MIC within 200-500 µg; 15, 30.61% represented MIC of 800 µg/ml), whereas three (NOU-4, NOU-15, NOU-23) i.e. 6.12% of the isolates represented lower MIC value (50 µg/ml of Ni in the medium). Four (NOU-8, NOU-42, NOU-46, NOU-48) 8.16% of the isolates were inhibited at 1000 µg/ml of copper in the medium. But most isolates (41, 83.67%) showed the highest degree of resistance against lead i.e. an MIC value of 2000 µg/ml. Interestingly, two (NOU-4 and NOU-16, 4.08%) isolates represented a ten-times lower MIC value (200 µg/ml of lead in the medium). A variable degree of resistance was observed against Zn among the isolates even though a lower MIC value (150-300 µg/ml) was reported for 28 (57.14%) isolates, 10 (20.40%) of which were inhibited at higher concentrations of Zn in the medium (MIC 1000 µg/ml). Twenty three (46.93%), 9 (18.36%) and 17 (34.69%) of the isolates were resistant against cobalt at 200, 250 and 300 µg/ml, respectively in the medium.

All the isolates were screened for commercially important extracellular enzymatic activities by a plate assay method and the findings are presented in **Table 6**. It is reported that 25 (51.02%), 24 (48.97%), 18 (36.73%), 18 (36.73%) and 28 (57.14%) of isolates showed different enzymatic activities such as amylase, protease, lipase, phosphatase and DNase, respectively. Strain NOU-23 (*Bacillus* sp.) showed highest amylase activity (zone size of 20 mm), followed by

Table 6 Enzymatic activities of the strains*.

Isolate	Activity (Zone sizes in mm)				
	Amylase	**Protease**	**Lipase**	**Phosphatase**	**DNase**
NOU-1	-	8	10	-	8
NOU-2	-	-	11	3	-
NOU-3	12	16	28	6	8
NOU-4	-	-	-	-	-
NOU-5	11	17	34	9	7
NOU-6	12	15	32	8	7
NOU-7	-	15	-	-	-
NOU-8	-	-	-	6	-
NOU-9	-	-	-	-	9
NOU-10	13	15	34	9	11
NOU-11	11	16	31	6	7
NOU-12	11	17	-	-	13
NOU-13	14	14	-	-	13
NOU-14	15	13	17	-	14
NOU-15	-	-	-	-	-
NOU-16	-	-	-	-	-
NOU-17	-	-	-	-	13
NOU-18	7	-	-	-	9
NOU-19	-	-	9	-	6
NOU-20	12	19	37	10	6
NOU-21	8	6	-	-	5
NOU-22	12	16	31	6	5
NOU-23	20	-	24	7	-
NOU-24	-	-	-	-	-
NOU-25	14	19	-	-	13
NOU-26	16	12	-	-	14
NOU-27	-	-	-	-	-
NOU-28	10	9	-	-	7
NOU-29	-	-	-	-	6
NOU-30	16	-	-	-	15
NOU-31	16	11	-	-	8
NOU-32	-	-	-	-	5
NOU-33	14	17	26	8	-
NOU-34	-	-	-	-	-
NOU-35	13	18	29	8	-
NOU-36	-	-	-	-	10
NOU-37	12	16	27	5	9
NOU-38	12	14	-	7	-
NOU-39	-	-	-	-	-
NOU-40	15	10	-	-	9
NOU-41	-	-	-	-	-
NOU-42	8	16	16	8	-
NOU-43	-	-	-	-	11
NOU-44	-	-	-	-	10
NOU-45	-	-	-	7	-
NOU-46	17	-	36	6	-
NOU-47	-	14	-	5	-
NOU-48	-	-	4	-	-
NOU-49	-	-	-	-	-

* Enzymatic activities were studied by plate assay method with respective substrates. Zone sizes represent mean of three experiments.

NOU-46 (*P. fluorescence*, a zone size of 17 mm) and least amylase activity was reported by strain NOU-18 with a zone size of only 7 mm, when studied through starch-iodine plate method. NOU-20, a *Bacillus* strain, showed highest activity in terms of zone sizes for both lipase and phosphatase. But NOU-26 showed maximum DNase activity. Eight (16.32%) isolates [NOU-3 *(P. aeruginosa*) and NOU-5, 6, 10, 11, 20, 22, 37 (*Bacillus* sp.)] were positive for all five enzymes studied. None of the *Micrococcus*, *Geomicrococcus* and *P. fluorescence* isolates were positive for DNase activity.

DISCUSSION

Since the soil samples studied reported to be acidic, a large number of microbial communities are expected to be present, as acidic soil and litter decomposition favours the growth and multiplication of soil microorganisms (Kenedy and Papendick 1995). The Colony Forming Units of the bacterial isolates ranged between 10^6-10^9, which corroborates with findings of several groups (Gledhill and Casida 1969; Boer *et al*. 2003; Latour *et al.* 2003). Further, plant diversity affects the bacterial community, more specifically soil bacterial genera such as *Bacillus* and *Pseudomonas* (Stephen *et al.* 2000). In this respect the Similipal Biosphere Reserve, with its rich diversity of plant species reveals *Bacillus* as the dominant genera followed by *Pseudomonas*, *Micrococcus* and *Geomicrococcus* as observed in this investigation. Growth, viability and metabolism of microorganisms are directly related to the environment from which they have been isolated. In contrast to this, we reported that few isolates grew at alkaline pH, even though the strains were isolated from acidic soil. However, the majority of the isolates were able to grow at acidic pH. Since the litter decomposition was reported to be very high in these soils (Bahuti 2006; Bahuti *et al.* 2006; Panda 2006), the isolates required high and specialised nutrients for growth as observed in this study i.e. the isolates grew luxuriantly

on NA and SEA medium. Rath (1996) reported the growth of thermotolerant bacteria, including both *Bacillus* and *Pseudomonas* species, in water agar medium, where agar was the sole source of nutrients and energy. However, here we failed to report the growth of any isolate on WA, which indicates that in addition to environmental factors nutritional composition of the habitat from where the microorganisms are isolated affects their growth and metabolism, too. We observed the development of multiple antibiotic resistance (MAR index 0.142-0.428) among these isolates that corroborates with the findings of other scientists (Panda 2006; Patra 2007). However, it is difficult to speculate any mechanism/factor responsible for development of drug resistance among these isolates, as these ecosystems are virgin and unexplored.

We observed a high degree of metal resistance of these isolates towards various heavy metals including Cd, Cr, Ni, Zn, Hg, etc. The reports of Rath and Subramanyam (1999) regarding metal resistogram of *Bacillus* and *Pseudomonas* towards heavy metals corroborates with our findings. Resistance of soil bacteria to different heavy metals is well documented in the literature (Al-Aoukaty *et al.* 1991; Appanna and Finn 1995; Appanna and Peerr 1996; Rath and Subramanyam 1999). Biotransformation, metal efflux and intracellular sequestration are some of the defense mechanisms invoked by microbes confronted by metal challenges (Kasan and Stegmann 1987; Silver *et al.*1989). Industrial pollution and acid rain have led to a sharp increase in the bioavailability of metal ions in different ecosystems. The use of bacteria to remove metal ions from different contaminated sites offers a great potential. However, some of these processes are hindered (Appanna and Peerr 1996; Rath and Subramanyam 1999; Noorwez and Satyanarayana 2002; Gupta and Mukerji 2002) due to the inhospitable conditions of the contaminated sites such as high temperatures, acid or alkaline pH, etc., which inhibit the growth and metabolism of the microorganisms being used to remediate them. As a matter of fact, the isolates studied in this study offer a great potential for use in bioremediation, as the isolates were able to grow at a wide range of pHs and showed a high degree of resistance towards heavy metals.

Enzymes from soil bacteria offer great challenges for their use in different industrial processes (Panda 2006; Patra 2007). In this investigation we documented the extracellular enzymatic activity of *Bacillus*, *Pseudomonas*, *Micrococcus* and *Geomicroccus* spp. with respect to amylase, protease, lipase, phosphatase and DNase. Rezanka (1991) reported that lipase production is common among Gram-positive bacteria in nature in comparison to their Gram-negative counterparts. In contrast, we observed lipase activity of *P. aeruginosa* (Gram-negative isolates NOU-1, NOU-3). It is well established that the logarithm of lipase activity is linearly related to the zone diameter thereby, fulfilling the requirement of a valid agar diffusion assay (Lawrence *et al.* 1967). In our studies 18 (36.73%) had extracellular phosphatase activity. Phosphate-dissolving soil microorganisms play an important role in correcting the phosphate balance in crops, increase the concentration of soluble phosphates in soil and made them available in ecosystem through plants. The presence of a high phosphorous content in these soils as recorded in our previous experiments during the study tempted us to speculate that it could be attributable to the mineralizing capacity of these isolates to release bound phosphate from rocks and bones in the soil. Amylase, and protease of bacterial origin, finds great potential application in different industrial processes (Rath 2000; Gomes and Steiner 2004). Heavy metals usually bind to essential biomolecules, more specifically to the enzymes inside the cell and inhibit normal biological activities (Poole and Gadd 1989). Since the isolates studied in this investigation represented extracellular enzymatic activities as well as metal tolerance towards heavy metals, they could be novel sources for different industrial processes.

In conclusion, we isolated and characterized bacteria from the Simlipal Biosphere Reserve, India, forest soil with metal-tolerant capacities and with extracellular enzymatic activities, speculating their use in different industrial processes and bioapplications. Studies such as this are a prerequisite for tapping the biotechnological applications of these microbes from these unique environments.

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