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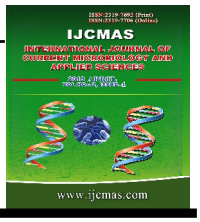

Original Research Article

Comparison of bacterial population in industrial and agricultural soils

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Keywords

Soil bacteria; industrial soils; agricultural soils; PCR; 16S rRNA gene sequencing.

A B S T R A C T

This study focused on the isolation, characterization and identification of some common soil bacterial strains with the aim of investigation of population diversity and comparison of species variation in industrial and agricultural soils. A total of 15 bacteria were isolated from four soil samples collected from Dhaka Export Processing Zone (DEPZ) at Savar (S_E), Tannery area at Hajaribagh (S_T), Dhaka, and two agricultural fields of Dhamrai (S_D) and Kushtia (S_K) in Bangladesh. After characterization of the isolates morphologically, physiologically and biochemically, they were identified provisionally as different species of Bacillus, Micrococcus, and Pseudomonas. For further confirmation by using PCR technique and 16S rRNA gene sequencing 4 isolates were identified as Micrococcus luteus strain P4_3 (from S_E) and Bacillus megaterium strain H2 (from S_T) from industrial soils and Bacillus amyloliquefaciens strain SCSAAB0007 (from S_D) and Bacillus subtilis strain 1320 (from S_K) from agricultural soils. The result of this experiment showed no sharp difference in population diversity of the soils collected from industrial and agricultural soils. Under the above technique the partial 16S rRNA gene was amplified by polymerase chain reaction (PCR) using gene specific primers (universal primer) and the PCR products were purified and sequenced. Sequence analysis was done using computer based searching procedure through BLAST program.

Introduction

Soil is a complex and dynamic biogeochemical system comprising tens of thousands to millions of bacterial species. Environmental stress may reduce this bacterial diversity. Pollution of the biosphere by heavy metals due to industrial, agricultural and domestic activities has created a serious problem for the safe and rational utilization of soils because metals cannot be naturally degraded like organic pollutants and persist in the ecosystem having accumulated in different parts of the food chain (Igwe *et al*., 2005). Heavy metals at elevated concentrations are known to effect the composition of soil microbial population and their associated activities

qualitatively and quantitatively, which may directly influence the soil fertility. On one hand, the number of microorganisms in soil depends on total content and concentrations of particular forms of heavy metals. On the other hand, it is conditioned by several other factors, such as the granulometric composition of soil, quantity and quality of organic matter, pH, total exchange capacity, nutrient availability, moisture, temperature, and oxygen availability (Das *et al*., 1997). Metals influence microorganisms by harmfully affecting their growth, morphology, and biochemical activities, resulting in decreased biomass and diversity (Malik and Ahmed, 2002). Several researchers using isolation-based techniques have demonstrated that heavy metal contamination can cause shifts in microbial populations (Roane and Kellogg, 1996).

Microbiological parameters such as the number, weight and activity of microorganisms can be good indicators of soil contamination with heavy metals (Brookes, 1995). Habitats that have high levels of metal contamination show lower numbers of microbes than uncontaminated habitats (Florea and Busselberg, 2006). The after-effect of the observed heavy metal (Cr, Zn and Cd) pollution influenced the metabolism of soil microbes in all cases (Gasper *et al*., 2005).

As it is mostly known that the industrial soils are contaminated by many of heavy metals whereas the agricultural soils are not affected at large extent, current experiment has done to give a sight on common presence of bacterial population found in industrial and agricultural soils and to make a comparison between them.

Materials and Methods

Collection of soil samples

A total of four soil samples were collected two from industrial soils of Dhaka Export Processing Zone (DEPZ) at Savar (S_E) and tannery area at Hazaribagh (S_T), Dhaka, and two from agricultural soils of Dhamrai (S_D) and Kushtia (S_K) in Bangladesh from the surface (0-15 cm depth) in plastic bags aseptically.

Isolation of bacteria

For isolation of bacteria serial dilution plate technique was carried out (Greenberg *et al*., 1980) and subsequent dilutions were made up to 10^5 times. Each diluted samples were spread over the surface of nutrient agar plates in duplicate and incubated at 37^oC for 72 hours. Colonies differing in morphological characteristics were selected and used for further studies. Three consecutive streaking of each culture were done to ensure purity of the strains.

Identification of bacterial isolates through microscopic study and biochemical tests

Morphological characteristics such as color, form, margin, elevation, and optical feature of the colonies, shape and arrangements of the vegetative cells (Pelczar and Reid, 1958) after Gram staining were observed under a phase contrast microscope from 20h old culture grown on solidified agar plates, and biochemical tests *viz.* motility, gelatin liquefaction, starch hydrolysis, indole production, oxygen requirement, oxidase test, catalase test, MR-VP test, deamination of phenylalanine, acid and gas production

from glucose, and citrate utilization using were performed as suggested by standard microbiological methods followed by Sneath *et al*. (1986); Cappuccino and Sherman (2005); Collins and Lyne (1984); Claus (1995).

Identification of the isolates by PCR and 16S rRNA gene sequencing

In order to identify the isolates based on sequence comparison, partial amplification of 16S rRNA gene was done using the primer pairs of 5'-16S rRNA: CCAGACTCCTACGGGAGGCAGC and 3'-16S rRNA: CTTGTGCGGGCCCCCGTC AATTC. Polymerase Chain Reaction (PCR) products purified through alcohol precipitation were sequenced directly using a DNA auto sequencer (Applied Bio-system 3130) using the bacterial universal primers 27f and 1492r. To prepare PCR cocktail (total 200 µl for 4 samples) sterile deionized distilled water (152 µl), taq buffer B 10X (20 µl), $MgCl_2$ (12 µl), primer forward (2 µl), primer reverse (2 µl), dNTPs 10mM (2 µl), taq DNA polymerase 5U/ µl (2 µl), and template DNA 25 ng/ µl (8 µl) were used. The thermal cycler was programmed in an oil-free thermal cycler (UNO II, Biometra) as follows: 5 min initial denaturation at 95°C, followed by 30 cycles that consisted of denaturation for 1 min at 94°C, annealing for 30 s at 55°C and extension at 72°C for 1 min and a final extension of 5 min at 72°C. After completion of cycling program the reactions were held at 4^{o}C. The PCR amplified product was analyzed by 1% agarose gel electrophoresis using TAE buffer (Pepper and Gerba, 2004). The resulting DNA patterns were examined with UV light under transilluminator, photographed and analyzed using gel documentation system (Herolabs, Germany). The sequence generated from automated sequencing of PCR amplified DNA was analyzed and compared with all accessible sequences in databases through NCBI (National Centre of Biotechnology Information) BLAST (http://blast.ncbi.nlm.nih.gov/) program to find out possible maximum similar organism through alignment of homologous sequences of known bacterial DNA.

Results and Discussion

Isolation of bacteria

A total of 15 isolates were identified based on colonial and cell morphology and biochemical characteristics and designated as mentioned in Table 1. Four bacterial colonies (E4, T8, D10 and K13) were selected randomly as one from each of four soil samples for further confirmation test by PCR and 16S rRNA gene sequencing.

Morphology of bacterial colonies

The forms of all bacterial colonies were circular; elevations were convex, effuse, and umbonate; margins were regular, erose, entire, and undulate type; surfaces of the colonies were smooth, concentric, and rough; the colors of the colonies were yellow, brown, off-white, white, orange, and light pink (Table 2).

Cell Morphology

Cell morphology such as arrangement, shape and Gram reaction were observed during simple and Gram staining of each strain. Cell shape of the strains were observed as cocci, rod, and short rod whereas cell arrangements were single, paired, tetrad, chain, and scattered; all of the isolated strains were Gram positive except two; seven isolates were non-motile and 8 were motile. Detail results for cell morphology and Gram reaction are presented in Table 2.

Table.1 Common bacteria isolated from four soil samples

Soil	Soil sample	Location	Bacterial sample
Industrial	S_E	DEPZ, Savar	E1
			E2
			E3
			E4
	S_T	Tannery Area, Hazaribagh	T5
			T6
			T7
			T8
Agricultural	S_D	Agricultural fields of Dhamrai	D9
			D10
			D11
	S_K	Agricultural fields of Kushtia	K12
			K13
			K14
			K15

Table. 2 Morphological characteristics and gram reaction of isolates

Morphological characteristics	Bacterial Samples														
	E1	**E2**	**E3**	**E4**	**T5**	**T6**	**T7**	**T8**	**D9**	**D10**	**D11**	**K12**	**K13**	**K14**	**K15**
Form	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Elevation	Convex	Effuse	Effuse	Convex	Umbo-nate	Effuse	Convex	Effuse	Convex	Convex	Effuse	Effuse	Convex	Convex	Effuse
Margin	Erose	Undu-late	Entire	Regular	Undu-late	Entire	Entire	Entire	Entire	Undu-late	Undu-late	Undu-late	Entire	Entire	Erose
Surface	Concentric	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Rough	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Color	White	White	Off White	Yellow	White	Brown	Brown	Off White	Off White	Off White	Off White	White	Light Pink	Light Pink	Orange
Shape and arrangement of cells	Rod, rounded end, occur in chain	Rod, rounded end, occur in chain	Rod, rounded end, occur in chain	Cocci, rounded end, occur in tetrads	Rod, rounded end, occur in chain	Rod, rounded end, occur in single	Short Rod, rounded end, occur in single	Rod, rounded end, occur in chain	Short Rod, rounded end, occur in single	Rod, rounded end, occur in chain	Rod, rounded end, occur in chain	Rod, rounded end, occur in chain	Rod, rounded end, occur in chain	Rod, rounded end, occur in chain	Short Rod, rounded end, occur in single
Motility	+	+	-	-	+	-	-	-	-	-	+	+	+	+	+
Gram reaction	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+

Table. 3 Biochemical characteristics and relative identified bacteria

Biochemical Characteristics	**Bacterial Samples**														
	E1	**E2**	**E3**	**E4**	**T5**	**T6**	**T7**	**T8**	**D9**	**D10**	**D11**	**K12**	**K13**	**K14**	**K15**
Oxidase test	+	-	-	+	-	+	+	-	+	+	-	-	+	+	+
Catalase Test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxygen Requirement	Facultative Anaerobes	Facultative Anaerobes	Facultative Anaerobes	Strictly Aerobes	Strictly Aerobes	Facultative Anaerobes	Strictly Aerobes	Facultative Anaerobes	Strictly Aerobes	Facultative Anaerobes	Facultative Anaerobes	Facultative Anaerobes	Facultative Anaerobes	Facultative Anaerobes	Strictly Aerobes
Gelatin Liquefaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch Hydrolysis	-	+	-	-	-	+	-	-	-	-	+	+	+	+	-
Indole Formation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VP Test	-	+	-	-	-	+	-	-	-	-	+	+	-	-	-
MR Test	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+
Deamination of Phenylalanine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Utilization of Citrate	-	+	+	+	+	-	-	+	+	-	+	+	+	+	-
Acid Production from D-Glucose	-	-	+	-	+	-	+	+	+	+	+	+	+	+	+
Gas Production from D-Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Identified Isolates	*Bacillus lentus*	*Bacillus cereus*	*Bacillus pumilus*	*Micrococcus luteus*	*Bacillus firmus*	*Bacillus pocheonensis*	*Pseudomonas pseudoalcali-genes*	*Bacillus megaterium*	*Pseudomonas pseudoalcali-genes*	*Bacillus amyloliquefaci-ens*	*Bacillus cereus*	*Bacillus cereus*	*Bacillus subtilis*	*Bacillus subtilis*	*Bacillus globisporus*

Biochemical characterization of the isolates

Results of the physiological and biochemical tests are presented in Table 3. Seven of the isolates were facultative anaerobes and 5 were strictly aerobes. All bacterial samples showed positive results for both gelatin liquefaction and catalase tests and negative results for both indole formation and deamination of phenylalanine. Six samples showed positive and 9 showed negative results for starch hydrolysis whereas 9 showed positive and 6 showed negative results for oxidase tests. Among the 15 bacteria 8 showed positive and 7 showed negative results for MR test. Eleven isolates gave negative results and 4 were positive for VP test whereas 11 were positive and 4 were negative for the test of acid production from D-Glucose. All samples showed negative results in gas production from D-Glucose.

Identification of Bacterial isolates through Morphological and Biochemical tests

Considering all observed characters of the isolates, identification of Gram positive bacteria was done following Bergey's Manual of Systematic Bacteriology (Vol. 2) (Sneath *et al.,* 1986) and the results are mentioned in Table 3. The isolated organisms showed minor differences in biochemical characters from those cited in that text.

Identification of Bacterial isolates through Morphological and Biochemical tests

Considering all observed characters of the isolated organisms, identification of Gram positive bacteria was done following Bergey's Manual of Systematic Bacteriology (Vol. 2) (Sneath *et al.,* 1986) and the results are mentioned in Table 3. The isolated organisms showed some minor differences in biochemical characters from those cited in that text.

Identification of selective isolates by PCR and 16S rRNA gene sequencing

For the confirmation test by PCR and 16S rRNA gene sequencing four isolates were selected as one from each of four soil samples. 16S rRNA PCR products of 4 isolates were sent for sequencing and studied using BLAST in NCBI database. After using the BLAST programs against similar sequences in the NCBI databank the isolate E4 was affiliated to *Micrococcus luteus* strain P4_3 (99% similarity), isolate T8 to *Bacillus megaterium* strain H2 (99% similarity), isolate D10 to *Bacillus amyloliquefaciens* strain SCSAAB0007 (99% similarity), and isolate K13 to *Bacillus subtilis* strain 1320 (99% similarity). Partial sequences of 16S rRNA gene of four isolates were as follows:

***Micrococcus luteus* strain P4_3**

GGAGACAGACGGTCTGGCATGCTTCCAGGCGGGTCACGTGTAATGCGTCTAGCTGCGGCGCGGAAACCGTGGAATGGTCCCCACA
CCTAGTGCCCAACGTTTACGGCATGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCATGCTTTCGCTCCTCAGCGTCAGTTACA
GCCCAGAGACCTGCCTTCGCCATCGGTGTTCCTCCTGATATCTGCGCATTCCACCGCTACACCAGGAATTCCAGTCTCCCCTACTG
CACTCTAGTCTGCCCGTACCCACCGCAGATCCGGGGTTAAGCCCCGGACTTTCACGACAGACGCGACAAACCGCCTACGAGCTCT
TTACGCCCAATAATTCCGGATAACGCTCGCACCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTCTTCTGCAGG
TACCGTCACTTTCGCTTCTTCCCTACTGAAAGAGGTTTACAACCCGAAGGCCGTCATCCCTCACGCGGCGTCGCTGCATCAGGCTT
TCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGAGGGAAGTCTGGATGCCTCCCGGAAGAAATCTGGGGATTCCCCCCCGAA
AGGAATTTGGTATGCCCCCCCCAAAGAATTTGGGCATCCTTTAGCA

***Bacillus megaterium* strain H2**

CCTTAGGTCTGCATACTCTCCAGGCGGAGTGCTGACTGCGTTAGCTGCAGCACTAAAGGGCGGAAACCCTCTAACACTTAGCACT
CATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTACAGACCAAAAA
GCCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCGCTTTTCTCTTCTGCACTCAAGTT
CCCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGCGCGCTTTACGCCCAAT
AATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGG
TACAAGCAGTTACTCTTGTACTTGTTCTTCCCTAACAACAGAGTTTTACGACCCGAAAGCCTTCATCACTCACGCGGCGTTGCTCC
GTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGAGGGAAGTCTGGATGAAAACGT

***Bacillus amyloliquefaciens* strain SCSAAB0007**

TCCCCCAGGAATCTGAAACTACTCTCCAGGCGGAGTGCTGACTGCGTTAGCTGCAGCACTGAGGGGCGGAAACCCCCTAACACTT
AGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGAC
CAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACT
CAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTTACG
CCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCG
TCAAGGTGCCGCCCTATTTGAACGGCACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGCG
TTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGA

***Bacillus subtilis* strain 1320**

GGCGAATGACGAGTCTACTCTCCAGGCGGAGTGCTTATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCAC
TCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAG
AGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGT
TCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTTACGCCCAA
TAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAG
GTACCGCCCTATTCGAACGGTACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCT
CCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGAGGGAATCTCTGAAGCCTCCCGTAGGGATCCGGGGCGC
CCCCCCAAGGGATTCCGGGGCTCCCCCGGAAGATTCGGGGATTACCGAAT

Sampling sites were selected with the aim to isolate common soil bacteria and to make comparison among the population diversity from both polluted and non-polluted sites for which the best option was to locate metal contaminated industrial soils and uncontaminated agricultural soils. There was no significant difference in the presence of bacterial species in four soil samples as because in industrial sites, bacteria are continuously exposed to different heavy metals and other pollutants, thus giving rise to survival of stress tolerant strains. Even some of the strains which were not stress tolerant and are available in agricultural soils may become tolerant, possibly, due to mutations. *Micrococcus, Bacillus* and *Pseudomonas* was found available in industrial soils whereas *Bacillus* and *Pseudomonas* were also common in two agricultural soils. This study showed that two industrial soils include the bacterial species of *B. lentus, B. cereus* (Qing *et al.*, 2007), *B. pumilus* (Jaysankar *et al.*, 2008), *M. luteus, B. firmus, B. pocheonensis, P. pseudoalcaligens, B. megaterium* and two agricultural soils include the bacterial species of *P. pseudoalcaligens, amyloliquefaciens, B. cereus, B. subtilis,* and *B. globisporus.* From this study it was observed that same bacterial species *i.e. Bacillus cereus* and *Pseudomonas pseudoalcaligenes* were found in both industrial and agricultural soils. Same results

were showed by Bahig *et al.,* (2008). It indicates that same bacterial species can assist in both polluted and unpolluted environment leading to increased incidence of stress tolerant species. The abundance of *Bacillus* in all types of soil samples was possibly due to the spore structure in the *Bacilli* group which increases its power of uptake and resistance against the stresses. However, other results have also reported that the diversity of *Bacilli* was greatest in contaminated soil (Sagardoy and Salerno, 1983). From the previous results the diversity of *Bacilli* was greatest in both sites of soils irrigated with wastewater which indicated the polluted soils and with canal water which indicated the non-polluted soils (Malik *et al.,* 2002).

A number of chromium-resistant microorganisms have been reported, including *Pseudomonas* spp. (Mondaca, 1998), *Bacillus* spp. (Campos *et al.,* 1995). However, most of them have been isolated from tannery sludge, and industrial sewage. Almost similar observations were found also by Sevgi (2010) from an industrial area in Turkey. *Pseudomonas aeruginosa* was isolated on the basis of morphological, biochemical and 16S rRNA gene sequencing from sewage water (Raja, 2009). In the present study *Micrococcus luteus* is also found in industrial soil of DEPZ area. Congeevaram *et al.,* (2007) also showed same result as *micrococcus* to present in industrial soil. Species of *Pseudomonas* (Desai *et al*., 2008) and *Bacillus* spp. (Zahoor and Rehma, 2009) are also found in stress condition of hypersaline environment. High levels of heavy metals in hypersaline condition can affect both the qualitative and the quantitative structure of microbial communities. *Pseudomonas* and *Bacillus* spp. are very common in agricultural soils as observed in current study. These results are in agreement with the data obtained by Laila *et al.,* (2011) and Sagervanshi *et al.,* (2012). *Pseudomonas mendocina* strain PC19 was isolated from agricultural soil treated with organophosphates by Najia *et al*., (2012).

Soil is one of the most important environments for microbes and is easily exposed to many pollutants, and evaluating the effects of pollutants on the microbial population is much valuable. Recently, the discharge of industrial wastewater from different resources containing heavy metals and other pollutants has resulted in a population increase of the resistant bacteria. This is why there is no significant variation in microbial composition of industrial and agricultural soils. Identification of bacterial species present in contaminated sites is firstly essential to go for further analysis to study on bacterial resistance in stress condition. It is also proposed that the resistance ability of the isolates could be exploited in considering the isolates as possible candidates for the decontamination of polluted sites.

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