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Characterization and 16S-RFLP-based genetic diversity of *Bacillus* sp. isolated from chromite mine soil of Sukinda, Odisha

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Abstract

In order to obtain the diversity, 10 predominant bacterial isolates (CSB 1-10) capable of growing in chromite mine soil of Sukinda, Odisha were characterized by means of morphological, biochemical analyses and all the bacterial isolates were identified as *Bacillus* species. Profiling of genomic DNA and 16S rRNA amplified gene product exhibited a difference in their band quality. Further, genetic variability among the *Bacillus* species were studied by restriction fragment length polymorphism (RFLP) analysis using two restriction enzymes (*EcoRI* and *HindIII*). Restriction digestion of PCR amplified 16S rRNA gene with *EcoRI* was fragmented into ~ 700bp and 900 bp, whereas, *HindIII* gave common bands of molecular size ~ 1500 bp.

Key words: Bacillus species, 16S rRNA, RFLP, chromite mine soil, genetic divergence.

Introduction

A large number of different microorganisms such as bacteria, fungi, actinomycetes, protozoa and algae are commonly found in heavy metal contaminant soils. Of these, bacteria are by far the most common type of soil microorganisms possibly because they can grow rapidly and have the ability to utilize a wide range of substances as either carbon or nitrogen sources. Among the different groups of bacteria, *Bacillus* sp. are the mostly dominating and highly efficient towards the resistance and reduction of hexavalent chromium (Cr VI). Phenotypically, the genus *Bacillus* is a large and heterogeneous collection of aerobic, rod-shaped, Gram-positive (to Gram-variable), endospore-forming bacteria [1] The diversity that exists in this genus is demonstrated by the enormous range of genomic guanine-plus-cytosine contents (32 to 69 mol %), as well as the variety of interesting phenotypes, that are found among the various *Bacillus* species [2]. Due to their ability to form spores and withstand a range of variable environmental conditions, *Bacillus* spp. adapt easily to diverse habitats [3]. The diverse physiology of *Bacillus* spp. requires elaborate biochemical tests for their identification [4].

Further, 16S rRNA gene sequencing is a common technique for diversity study, largely due to the mosaic composition of phy-logenetically conserved and variable regions within the gene [5; 6]. 16S rRNA sequence has hypervariable regions, where sequences have diverged over evolutionary time. Now a day's comparative 16S rRNA gene using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analyses have been gradually increasing because of their relative simplicity and rapidity [7]. The objectives of the present work was to characterise and assess 16S-RFLP-based genetic divergence of 10 *Bacillus* sp . Isolated from chromite mine environment of Sukinda, Odisha.

Materials and methods

Cr (VI) tolerant bacteria were isolated from soil samples of chromite mine of Sukinda, Odisha, India using dilution plate technique method. A total of 23 morphologically distinct bacterial colonies were isolated, purified by repeated sub-cultured and screened for Cr (VI) tolerance with increasing concentrations of Cr (VI) on nutrient agar plates. Among these, 10 highly Cr (VI) tolerant isolates CSB-1-10, and subjected to morphological and biochemical characterization (indole production, methyl red test, starch hydrolysis, glucose utilization and acid gas production etc.) were performed according to the Bergey's Manual [8] to identify the bacterial isolates.

Bacterial DNA extraction and amplification

In order to study the genomic divergence of the strains molecular analysis were carried out. For this purpose bacterial isolates CSB (1-10) were grown on nutrient broth medium (g L^{-1}: peptone 5.0; NaCl. 3.0; beef extract 3.0) for 24 h at 35 °C. The total genomic DNA was extracted from each of the bacterial isolates [9]. DNA concentrations were estimated by 0.8 % (w/v) agarose gel electrophoresis technique in 1X TAE buffer at 100 V for 1-2 h run, strained with ethidium bromide. The 16S rRNA genes of the isolated DNA were amplified by PCR using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3').

PCR-RFLP based on 16S rRNA gene

An aliquot of PCR product was digested separately with restriction endonucleases *ECoRI* and *HindIII* according to the manufacture's guide. The restricted bands were separated by horizontal gel electrophoresis in 2% (w/v) agarose gels in 1X TAE buffer at 100 V for 1-2 h run, strained with ethidium bromide and the patterns were visualized.

Results and Discussion

Diversity of highly chromium resistant bacterial communities from chromite mine environment of Sukinda, Odisha were examined using classical biochemical technique as well as molecular methods. All the isolates (CSB1-10) were Gram positive, spore forming and motile in nature. A slight but remarkable difference in phenotypic characteristics was observed among all the 10 bacterial isolates, suggesting the presence of different species. Among ten bacterial (CSB1-10) isolates, 9 showed positive

Table 1: Phenotypic characterization of 10 chromium resistant Bacillus species isolated from soil samples of Sukinda of Odisha

Characters		Bacterial isolates								
	CSB-1	CSB-2	CSB-3	CSB-4	CSB-5	CSB-6	CSB-7	CSB-8	CSB-9	CSB-10
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Cell diameter(μm)	1.57	2.45	2.79	2.56	2.69	2.56	2.63	2.75	2.76	2.69
Gram stain	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Spore	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Catalase test	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Indole production test	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Methyl red test	+ve	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	-ve
Urease	-ve	+ve	-ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve
Protease										
Gelatin	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Casein	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Lipase										
Tributyrin	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
Tween 80	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Chitin	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Strach hydrolysis	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Oxidase	+ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	-ve	-ve
Cholesterol	-ve	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve
Acid from										
Glucose	+ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve
Sucrose	+ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	-ve	-ve
Raffinose	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve
Citrate	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Utilization of										
Glucose	-ve	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Fructose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
Sucrose	+ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve
Starch	-ve	+ve	-ve	+ve	-ve	-ve	+ve	+ve	-ve	-ve

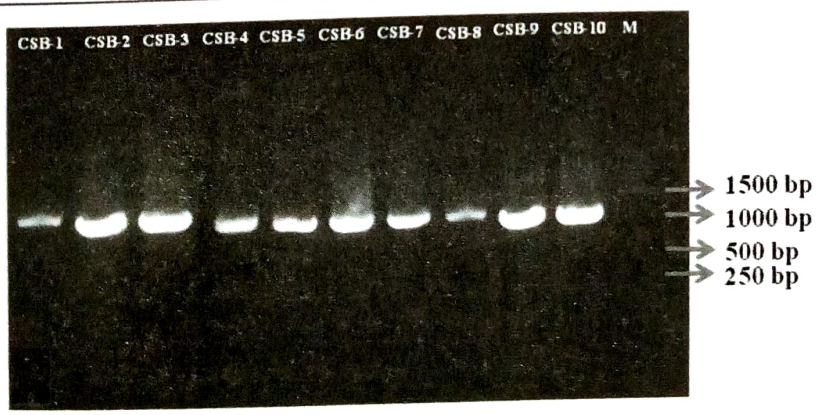

Fig. 1 PCR amplified products of 10 chromium resistant bacteria isolated from soil from chromite mine, Sukinda

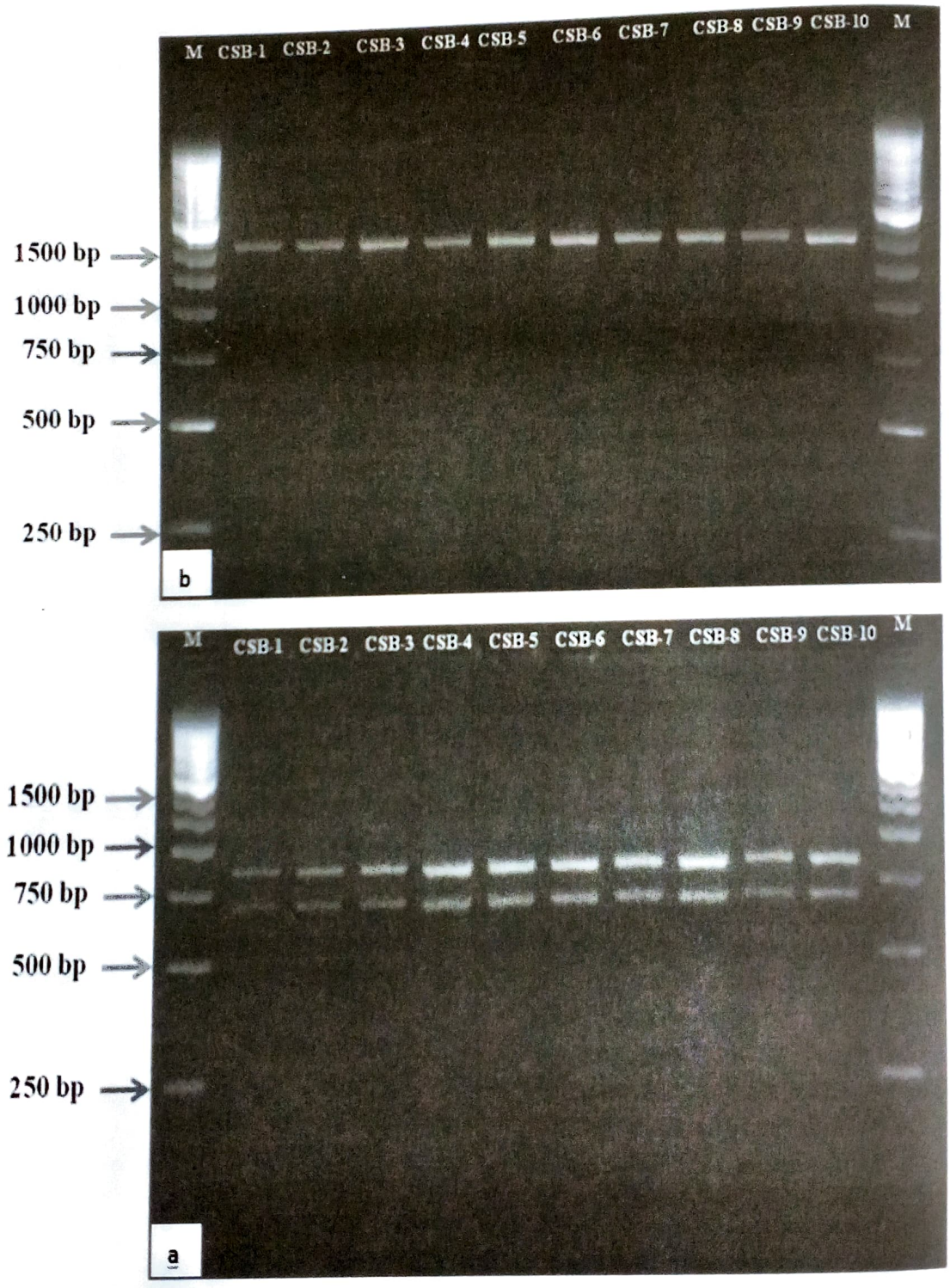

Fig.2. The restriction patterns of amplified 16S rRNA genes of ten bacteria isolated from soil samples of chromite mine using restriction enzymes; (a). *EcoRI*; (b). *Hind III* (M) 1 kb ladder.

reaction to catalase except CSB-2. All the isolates were casein positive whereas indole production test, methyl red test, urease hydrolysis and starch hydrolysis test were variable. Lipase activities (tributyrin) of the isolates were positive except CSB-1, CSB-3 and CSB-5. Tween 80 hydrolysis test was variable for the isolates. The details of the biochemical analysis are given in Table 1. Identification of the bacteria isolated from Sukinda chromite mine soil samples suggested the prevalence of the representatives of the *Bacillus* species in contaminated samples. Though all the isolates were belonging to the genera *Bacillus* but some variations were observed among them by biochemical analyses. A number of chromium tolerance bacteria reported by different authors were belonging to the genera *Bacillus* [10]. The genus *Bacillus* is ubiquitous in terrestrial and fresh water environment and is extensively distributed in the environment [12], thus reflecting its presence at each of the site studied. Also, this phenomenon is in accordance with results of several studies which established the dominance of *Bacillus* in heavy metal polluted soils [13]. Abdelatey et al. (2011) [14] has reported that Gram positive isolates showed higher metal tolerance ability than Gram-negative isolates. Carboxyl groups in Gram-positive bacteria are the main agents for the uptake of heavy metals. The sources of these carboxyl groups are the teichoic acids, associated to the peptidoglycan layers of the cell wall, whose phosphate groups are key components for the uptake of metal [15].

Restriction enzyme analysis of amplified DNA

The genomic DNA of all the ten bacterial isolates was extracted and amplified.The genomic DNA profile and PCR amplified 16SrRNA products showed difference in the band quality (Fig. 1). 16S-RFLP analysis of 10 bacterial isolates were then compared among themselves being digested with *EcoRI* and *HindIII* resriction enzymes showed stable species-specific restriction patterns. *EcoRI* restriction enzymes which generated only two, identical restriction patterns and all the strains fragmented into 4,700 bp and 800 bp (Fig. 2), whereas, *HindIII* restriction enzymes showed one common band of molecular size 4 1500 bp. Patterns of each enzyme were combined together and each strain was assigned a composite genotype [16]. This finding along with biochemical results suggests that though all of the isolates were belong to the same *Bacillus* species yet, they exhibit genetic variations among the different species. Elangovan et al. (2010) [10] observed the frequent occurrence of inter-operon variability of the 16S-rRNA gene in *Bacillus* and *Paenibacillus*. 16S-RFLP detects inter-species and inter-strain as well as inter-operon variability and enables a relatively fast multiple strain analysis per taxon. The 16S-RFLP fingerprinting also allows the construction of a database for identification purposes. Shangkuan et al. (Year missing) [16] reported that PCR-RFLP is simple to perform and has potential as a rapid method for typing and discriminating *B. anthraces* strains from other *B. cereus* group bacteria.

Conclusion

Chromite mine soil is predominantly in habited by *Bacillus* species. Characterization of bacterial species by morphological and biochemical analyses or determination of the 16S rRNA gene sequence, can be used as identification tools. The results of biochemical analysis were validated by 16S rRNA sequencing. Restriction enzymes *EcoRI* was found to be more informative enzymes as compared to *HindIII* for restriction analysis of 16S rRNA amplified PCR products obtained from chromium tolerant *Bacillus* species isolated from mine soil samples. This technique therefore, offers an opportunity to detect genetic variability among the *Bacillus* species. 16S-RFLP has shown to be a relatively simple, rapid and reliable technique to determine inter-specific variability and can be used for divergence study among the *Bacillus* species.

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