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Subaran Singh
(1) Department of Seed Science & Technology, Chaudhary Charan Singh Haryana Agricultural University Hisar, Haryana
(2) Department of Molecular Biology and Genetic Engineering, GBPUAT-Pantnagar (US Nagar)-Uttarakhand, India

Reeta Goel
Department of Microbiology, GBPUAT-Pantnagar (US Nagar)-Uttarakhand

Supriya Ambawat
ICAR- All India Coordinated Research Project on Pearl millet, Agriculture University, Jodhpur, Rajasthan, India

Corresponding Author:
Subaran Singh
(1) Department of Seed Science & Technology, Chaudhary Charan Singh Haryana Agricultural University Hisar, Haryana
(2) Department of Molecular Biology and Genetic Engineering, GBPUAT-Pantnagar (US Nagar)-Uttarakhand, India

Screening of cadmium resistant isolates and evaluation of the bacterial diversity

Subaran Singh, Reeta Goel and Supriya Ambawat

Abstract
Cadmium is a non-essential heavy metal. It is well known for its toxicity, bioaccumulation and biomagnifications. It has a wide range of sources and exists in environment as well and industry in various forms. Microbes have been utilized as a bioremediation vehicle with use of genetic engineering approaches. A diverse group of microbes play an important role in this direction. In this study, six cadmium resistant isolates (710A, 710B, KNP3, KNP9, 97AN & 62BN) were used for diversity analysis. Diversification characterization was accomplished with combination of Restriction Fragment Length Polymorphism (RFLP) and Tm (melting temperature). Phylogenetic analysis after restriction digestion indicated that the six isolates viz. 710A, 710B, 62BN, 97AN, KNP3, KNP9 were grouped into three clusters at a similarity coefficient of 48%. Three isolates namely KNP3, 62BN and 710A were grouped into one cluster; KNP9 and 710B were present into second cluster while isolate 97AN was distinct and entirely separated from all other five isolates. Isolate 710A *(Pseudomonas putida)* and 710B (*Commamonas aquatica)* both exhibited highest diversification and distinct parental lineage. This study will be helpful to extract the heavy metals from the rhizosphere which will finally be useful for improving the conditions of soil for plant growth.

Keywords: Cadmium, bioremediation, diversity, gc content, heavy metal toxicity, restriction profiling

Introduction
Increasing industrialization has been accompanied by the extraction and distribution of mineral substances from their natural deposits throughout the world. These include metals as well as heavy metals. Following concentration, many of these have undergone chemical changes through technical processes and finally pass, finely dispersed in solutions by way of effluent, sewage, dumps and dust into the water, earth and air and ultimately into the food chain (Sharma and Dhaliwal, 2019) [19]. Together with essential nutrients, plants and animals also take up small amounts of contaminant heavy metal compounds and can concentrate them. As certain heavy metals such as lead, cadmium and mercury have been recognized to be potentially toxic within specific limiting values, a considerable potential hazard exists for human nutrition. Not all the traces of heavy metals in plants and animals are the results of human activity. Some arise through the absorption processes of naturally occurring soil components, as has been shown for cadmium in particular. Purely theoretically, every 1000 kg of "normal" soil contains 200 g chromium, 80 g nickel, 16 g lead, 0.5 g mercury and 0.2 g cadmium (Collins and Stotzky, 1992) [4]. Therefore it is not always easy to assign a definite cause for increased heavy metal content. The metals having a specific gravity (density) of more than about 5g/cm3 in their standard state are defined as “heavy metals”. Some metals like copper, nickel, chromium and iron are essential in very low concentrations for the survival of all forms of life and are described as essential trace elements. But, when they are present in greater quantities they become toxic like heavy metals (lead, cadmium and mercury) which are already toxic in very low concentrations and cause metabolic anomalies. Hence, the boundary between the essential and the toxic effect is somewhat problematic. Most heavy metals are toxic to cells. The toxicity of heavy metal is due to their presence in abundance and buildup. Generally, the cations and anions formed from the metals, and not the reduced metallic material (which is inert), are toxic. The toxicity is also due to their presence in abundance while their non-biodegradable nature in responsible for their concentration build up.
Cadmium is a non-essential but highly toxic metal widespread in the biosphere and known as an important environmental hazard and a potent human carcinogen (Waisberg *et al*., 2003) [20]. Exposure of bacteria to cadmium induces expression of several genes related to metal transport, heat shock response, oxidative stress response and DNA repair (Dhaliwal *et al.*, 2020) [6]. It is of particular environmental interest to understand how soil bacteria react to

toxic metals, as they represent a major biomass component in the soil. Agricultural soils are mainly contaminated with Cd from the excessive use of phosphatic fertilizers, dispersal of sewage sludge and atmospheric deposition. Cd absorption occurs because of the chemical similarity to zinc (Blayloch *et al.*, 1997) [3]. Cadmium is readily taken up by many crops including cereals, potatoes, vegetables and fruits. Thus, contamination by Cd is increasing both in human food and overall in the agricultural field.

Microorganisms and plants have been extensively used for decontamination of heavy metals. Microbial methods of environment purification and/or clean-up are known to be the most promising because of their safety, efficiency and cost-effectiveness and bioremediation strategies have drowned lot of attention and they have been proposed as attractive alternatives. Some microorganisms can absorb and concentrate heavy metals thus, providing resistance (Roane *et al.*, 2001) [17]. The ability of micro-organisms to survive and reproduce in a metals habitat may depend on genetic as well as physiological adaptations. The former leads to the widespread appearance of metal resistant organisms. Soils and sediments or solubilizing metals are useful to facilitate their extraction. The known mechanisms of heavy metal toxicity include induction of oxidative stress and interfering with protein folding and function (Nies, 1999) [13]. However, the mechanism of heavy metal resistance and its genetic basis varies with the microbe and the metal in question. A natural isolate from contaminated habitat will be an ideal candidate to work on heavy metal resistance mechanism (Olson and Thornton, 1982) [14]. It is better to use indigenous microorganisms as they have already been adapted to survival requirement in the polluted soil. Bacteria have developed a variety of resistance mechanisms to counteract heavy metals stresses. These mechanisms include the formation and sequestration of heavy metals complexes, reducing of metals to a less toxic species and direct efflux of metal out of the cell (Outten *et al.*, 2000) [15].

The ability of biochemical and molecular methods to identify and characterize natural culturable bacterial community isolated from polluted environment, and the potential exploitation of cadmium-resistant bacterial strains in bioremediation processes aimed at heavy metal removal from contaminated environments. Several remediation techniques have been used for removal of heavy metals from various environments (Lianwen *et al.*, 2018; Selvi *et al.*, 2019; Xu *et al.*, 2019) [9, 19, 22]. Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. The G+C content of a bacterial chromosome is an important index for the identification and classification of bacteria by calculating its Tm (melting temperature) (Mandel *et al.*, 1970) [10]. An *in silico* analysis of metal binding proteins and identification of putative metal binding motifs for the ions of cadmium, cobalt, zinc, arsenic, mercury, magnesium, manganese, molybdenum and nickel give the functional characterization of diversity among bacterial isolates. 2D gel analysis study provides comparative functional diversity among heavy metal resistant micro-organisms. In this research work, we studied on different bacterial isolates showing varying degree of resistance at different concentrations of cadmium. For the diversity analysis, restriction profiling and Tm (melting Temperature) curve study was done. These results showed a diverse pattern among bacterial isolates for their resistance against cadmium.

2. Materials and methods

2.1 Collection of bacterial isolates

All cadmium resistant bacterial isolates (710A, 710B, KNP3, KNP9, 62BN, 97AN) were procured from the Department of Microbiology, GBPUAT, Pantnagar (Table1).

2.2 Bacterial genomic DNA extraction and RFLP analysis

The genomic DNA was extracted from bacterial cultures during their mid log phase ($O.D_{600}$-0.60) as described by Agarwal *et al.* (2001) [1] and RFLP pattern was studied using different restriction endonucleases. The reactions were carried out in a total volume of 20 µl containing 10X RE buffer, 1µg/µl of DNA and 1U of enzyme (Table 3).

2.3 G+C content estimation using Tm evaluation

The melting temperature (Tm) of all six isolates (710A, 710B, KNP3, KNP9, 62BN, 97AN) was evaluated with UV-Visible Spectrophotometer (Perkin Elmer, Lambda 35) and further using Tm value, mol % G+C content was calculated by an empirical formula as reported by Mandel *et al.* (1970) [10]:

$$\text{mol\% G+C}x = [\text{mol\% G+C}r + 1.99(Tm_x - Tm_r)]$$

(Where *x* is the unknown organism and *r* is the reference organism)

In this formula, *E.coli* was taken as a reference organism, having Tm value 85.4±0.5 and G+C content 50-51 mol%.

3. Results and discussion

The study of diversity among micro-organisms is based on their classification, taxonomical variation and evolutionary distance from the parental lineage. The classification of micro-organisms on the basis of traditional microbiological methods (morphological, physiological and biochemical) creates a blurred image about their taxonomical status and thus needs further clarification. Microbial taxonomy or systematics deals with the classification, identification and nomenclature of micro-organisms. The term taxonomy or systematics have been often used interchangeably but the two differ in their meaning while taxonomy is the theory and practice of classifying organisms, systematics refers to the study of diversity of organisms and all relationship among them including their evolutionary relatedness (phylogeny) and all possible biological interactions (Mayr and Ashlock, 1991) [11]. The first attempt of microbial classification based on ssDNA was made by Schildkraut *et al.* (1961) [18].This was a major breakthrough in the world of microbial classification and diversity paving the way towards development of the polyphasic system of classification in its present form. A bacterial species is defined as a group of strains sharing 70% or more DNA-DNA relatedness with 5^0C or less ΔTm value (Tm is the melting temperature of the hybrid) among members of the group, provided that all the phenotypic and chemo-taxonomical features.

Table 1: Description of the cadmium resistant isolates

S. No.	Strain No.	Isolation site/ location	Name of Isolate	NCBI, Accession No.	Cd Resistance Level
1.	710A	Semara Mines, Ranchi, Jharkhand	*Pseudomonas putida*	EF207715	1.0mM
2.	710B	Semara Mines, Ranchi, Jharkhand	*Commamonas aquatica*	EF207716	0.5mM

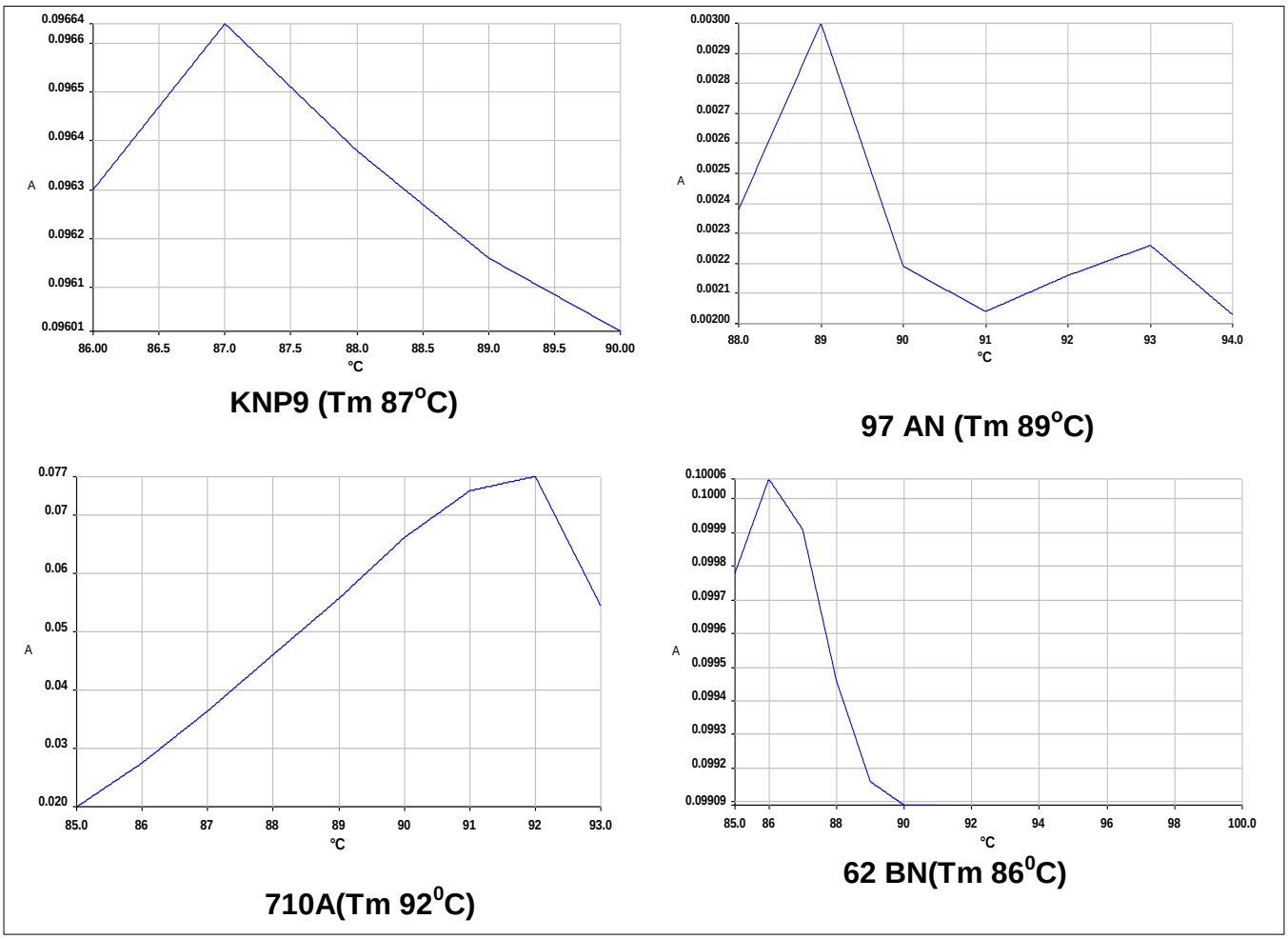
3.	62BN	Semara Mines, Ranchi, Jharkhand	*Pseudomonas putida*	EU512944	1.0mM
4.	97AN	Semara Mines, Ranchi, Jharkhand	*Pseudomonas montelli*	EU512943	1.0mM
5.	KNP3	Panki Power Plant, Kanpur, U.P.	*Proteus vulgaris*	DQ205432	1.3mM
6.	KNP9	Panki Power Plant, Kanpur, U.P.	*Pseudomonas putida*	DQ205427	1.0mM

3.1 Melting curve analysis of isolated genomic DNA samples

The Tm value and GC content both have a positive correlation. Higher Tm value indicates high GC content in the specific organism, comprising genetic complexity and diversification. So, DNA sample provides valuable information about the heterogeneity among given cadmium resistant isolates. Different melting temperature and GC content of six genomic DNA samples of cadmium resistant isolates is documented in Table 2. A great amount of diversity was observed among the DNA samples from different isolates (Figure 1). 710B isolate had lowest Tm value (85^{0}C) and calculated G+C (50.5 mol %) content, showing less genomic complexity than its neighboring isolates 710A, 62BN and 97AN. But from diversity point of view, it exhibited larger variability from the other samples. Isolate 62BN, showed moderate Tm value (86^{0}C) and calculated G+C (52.5 mol %) composition and slightly higher genomic complexity than 710B but less than others. In relation to diversity, it was found close to 710B but distinct from the other isolates. KNP9 sample had moderate Tm value (87^{0}C) and calculated G+C (54.5 mol%) content, depicting less genomic complexity from its neighboring sample KNP3 but from diversity point of view, it showed moderate variability from the other isolates. Isolate 97AN has higher Tm value (89^{0}C) and calculated G+C (58.5mol %) content than 710B, 62BN and KNP9, showing higher genomic complexity but was found to be closer to KNP3 during diversity analysis. KNP3 isolate had higher Tm value (90^{0}C) and calculated G+C (60.4mol %) content than 710A, showing higher genomic complexity and comparative diversity in relation to others. 710A isolate has highest Tm value (92^{0}C) and calculated G+C (64.4 mol%) content, showing more genomic complexity than its neighboring isolates 710B, 62BN and 97AN and from diversity point of view, it exhibited larger variability from the other isolates. Thus, it is evident that 710A *(Pseudomonas putida)* and 710B (*Commamonas aquatica)* both exhibited highest diversification and distinct parental lineage from each other while they have been collected from the same location (Semara mine Ranchi, Jharkhand). Similarly, Mesbah et al. (1989) [12] used G+C content for depicting the diversity of microorganisms from each other.

Table 2: Tm (melting temperature) and G+C content of selected cadmium resistant isolates

S. No.	DNA Sample	Tm(in^{0}C)	G+C (in mol %)
1.	710B	85	50.5
2.	62BN	86	52.5
3.	KNP9	87	54.5
4.	97AN	89	58.5
5.	KNP3	90	60.4
6.	710A	92	64.4



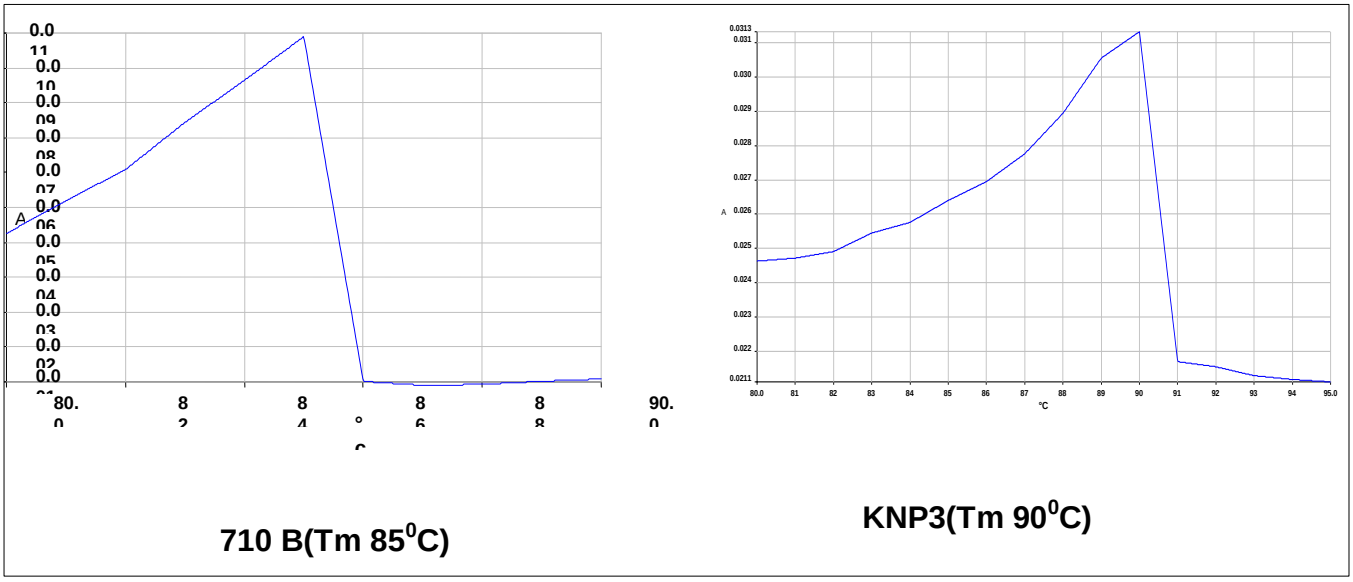


Fig 1: Melting curves of different DNA samples of the isoaltes

3.2 Restriction profiling based on RFLP

The study of identification and characterization of the microorganisms can be done with the use of restriction enzymes. These enzymes cleave the DNA at a specific end and produce various restriction patterns of variable sizes. On the basis of these restriction patterns, we can study homologies and evolutionary relationship among the organisms (Barkay *et al.*, 1985) [2]. Therefore, genomic DNA from the different cadmium resistant isolates was cleaved with six restriction enzymes as shown in Table 3. Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species (and even isolates) from each another. Similar studies for cadmium resistant were carried out by Rani *et al.* (2009) [16], Jain *et al.* (2010) [8] and Huan *et al.* (2017) [7].

Table 3: Restriction enzymes used during RFLP

Name of Isolate	Restriction Enzyme					
	***Sma* I**	***Xba* I**	***Sac* II**	***Hind* III**	***Bgl* II**	***Pas* I**
62BN	-	-	+	+	-	-
KNP3	-	-	+	+	+	-
KNP9	-	-	+	+	-	-
710A	-	-	+	+	-	-
710B	-	+	+	+	-	-
97AN	-	+	+	+	+	-

3.3 Diversity analysis

Phylogenetic inference has become routine for most studies of genetic variation among different taxa. Phylogenetic relation in the different DNA samples of cadmium resistant isolates, collected from different sites is shown in Figure 2. The *in silico* analysis of restriction profiling of cadmium resistant DNA samples collected from various sites across the country reflected a significant amount of diversity among them. The value of similarity coefficient from phylogenetic tree indicated that 48% similarity grouped all the six isolates viz. 710A, 710B, 62BN, 97AN, KNP3, KNP9 into three clusters. Out of these, three isolates namely KNP3, 62BN and 710A were grouped into one cluster; KNP9 and 710B were present together into second cluster while 97AN was entirely distinct and separated from the other two clusters and exhibited in third cluster. Isolates 710A and 62BN had a very close similarity of 60% in between them and shared less phylogenic distance while KNP3 had close relatedness (54%) with 710A and 62BN isolates of first cluster. KNP9 and 710B both were found close to each other and shared about 50% similarity.

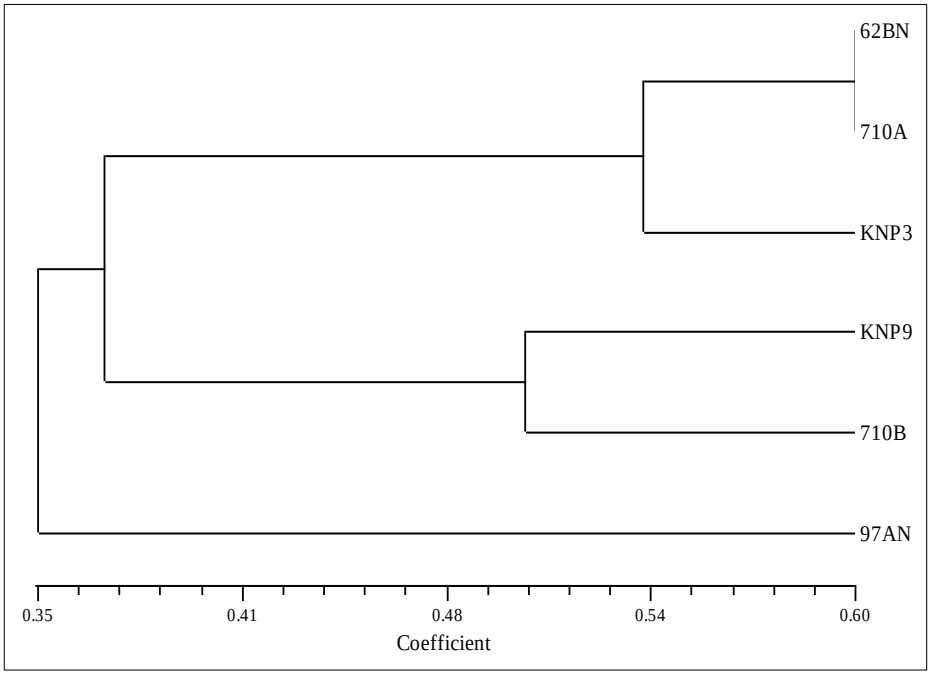


Fig 2: Phylogenetic tree revealing relatedness among the cadmium resistant bacterial isolates using NTSYSpc 2.2.

3.4 Tm versus phylogeny

A comparative study between Tm and RFLP analysis was also done. Isolate 710B had 50% similarity with KNP9 by phylogeny method but in Tm evaluation method both these had 97.65% similarity due to less ΔTm value. Similarly, in another case isolates 62BN &710A exhibited a similarity of 60% (phylogeny method) and 93.03% (Tm evaluation). KNP3 had 54% similarity with both 62BN and 710A (phylogeny method), while 97.78% similarity with 710A and 95.56% (Tm evaluation) with 62BN isolate. 97AN had only 35% similarity with all other isolates (phylogeny method), while it was found to close to KNP3 and 710A while away from the remaining isolates during Tm evaluation. In the reference of this view, this study suggested that phylogeny method is more feasible than Tm evaluation for diversity analysis. It is difficult to make accuracy with use of Tm evaluation due to less difference in their ΔTm values while there is greater variability in the data of phylogeny method (either similarity coefficient or distance matrices). It is also advisable to use more than one method for making any decision. Derakshani *et al*. (2001) [5] used DNA re-association technique to reveal the total genetic diversity of a given bacterial community.

4. Conclusion

Microbial diversity in the ecosystems exceeds, by far, that of eukaryotic organisms. Microbial diversity may also be considered to be the amount and of information, which is directly applicable to the total genetic diversity or complexity in a community. The genetic complexity or genome size of microbial community genomes can be assessed by re-association of community DNA. The total genomic complexity denotes the confines of diversity in terms of genetic information present and provides information about the overall (potential) taxonomic and functional variability at the community level. Important aspects of diversity at the ecosystem level are the range of processes, complexity of interactions, and number of trophic levels. Thus, measures of microbial diversity should include multiple methods integrating holistic measures at the total community level and partial approaches targeting structural or functional subsets. This has been possible owing to rapid development in molecular biological techniques, automation of DNA sequencing, proteomic analysis coupled with advances in bioinformatics tools. Several DNA-based typing methods provide information for delineating bacteria into different genera and species and have the potential to resolve differences among the strains of a species.

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Conflict of Interest The authors declare that they have no competing interest.

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