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Molecular Analysis of Microbial Diversity of Doaba Region of Punjab From Soil

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Abstract

Assessment of the microbial diversity present in nature isthe need-of-the-hour. Not more than 10% of the bacteria can be cultured using standard culture techniques. The current study was conducted to assess the microbial diversity in soils of the Doaba region. For unravelling the microbial diversity present in this region of Punjab, the microbial population was obtained via culture in enriched media. This culturable approach providedus with 14 isolates, which could be identified using biochemical characterisation. Biochemical testing of these isolates reveals the presence of *Citrobacter, Escherichia coli, Proteus mirabilis, Providencia stuartii, and Salmonella species*.

Keywords: Doaba Region,Microbial Diversity, 16S rRNA, *Escherichia coli, Salmonella species.*

Introduction

Mother Nature is embodied with extensivebiological diversity. Even though it is not evident to our naked eye, the most diverse habitat on the earth is soil, which harboursdifferent types of microorganisms [1]. At present, the relationship between biodiversity isdetermined by the analysing the species of microbes present in the ecosystem, and functions they perform in the soil [2].One of the primary concerns is to conserve this biodiversity so that functioning in the biosphere can be sustained [3]. Thus, for measuring the genetic diversity of a bacterial species in an ecosystem, we need to isolate the pure cultures of microorganisms [4]**.**In all ecological niches and symbiotic relationships are essential to sustain the complex natural environment, but its understanding requires knowledge of thefull spectrum of microbial diversity [5]. The culturablesubset of microbesfails to fulfil this prerequisite asthe majority of the microbial biosphere belongs to uncultured organisms [6]. Thus, the new approach named "Metagenomics" has enabled us to resolve this issue of thefull spectrum of microbial diversity. As in this method, the environmental DNA is directly isolated and analysed for identifying the diverse and novel bacterial species present in the particular environmental sample [7].

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Doaba Region of Punjablies between 30°57` to 32 °7` latitude and 75° 4` to 76° 30`longitudeandcomprisesof Jalandhar District and distinct part of the Hoshiarpur and Kapurthala [8]. The alluvial deposits of the Indus – Ganga cover the land of these districts, which gets the annual-average rainfall of 600mm as these areas have the Sub-Tropical monsoon type climate [9]. The primaryoccupation of people here is Dairy farming, followed by bee-keeping, fishery, and poultry as there are 550 dairy farms, 109 bee-keeping farms, 34 poultry farms and fishery farm, which encompass the 527 Hectares of land in this district [10].Even the instant analysis of thediversity of the complex microbial communities has remained subtle, but it holds the microbial ecosystem,which is of such high diversity. Through the analysis of this diverse, complex population of microbeswill enable us to measure the composition, community structure and phylotypic richness in real-time.

However, information related to themicrobial diversity of microbes present in the Doaba region is minimal. The main objective of the study was to investigate thediversity of microbes that are present in the Doaba region. Thus, to investigate the diverse microbes traditional culturable approach and metagenomics approach was used.

Material and Methods

Material

All the chemicals were of analytical grade [Ammonium acetate (CH_3COONH_4), Chloroform ($CHCl_3$), CTAB (Cetyl trimethylammonium bromide), EDTA (Ethylenediaminetetraacetic acid), Ethanol, Hydrogen Peroxide (H_2O_2), Isoamyl alcohol, NaCl, Phenol, SDS (Sodium dodecyl sulfate), Tris-HCl and Zinc Dust], and were purchased from Central Drug House Pvt. Ltd, Delhi. Whereas the media and reagents like Agar powder for bacteriology, Agarose, EMB Agar, MacConkey Agar, MR-VP broth, Nitrate Broth, Simmons Citrate Media, Stuart's Urea Broth, Tryptone Broth, Tryptone, TSI Agar, Yeast Extract Powder, α - naphthylamine reagent, Kovac's reagent, Methyl red solution, Sulphanilic acid reagent and Voges-Proskauer reagent were purchased from the HiMedia Laboratories, Mumbai. The PCR amplifying kit was purchased from Genei India, Bengaluru.

Sampling

Two hundredgrams of soil sample was collected from land present near Wahid Sander sugar mills (31.2235° N - Latitude and 75.7658° E - Longitude) located in Phagwara (Punjab). The sample was collected in a sterile zip-lock polybag and then stored in a box containing ice

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packs. Next, the box was transported to the laboratory at Lovely Professional University for further Investigation.

Enrichment and DNA Extraction

For enrichment of culture, onegram of soil sample wasadded to 100mL of 2X-YT broth and grown at 37ºC for 24 hours. Next, the culture was harvested by centrifugationfor 10 minutes at 5000rpm. The cells were resuspended in 5mL of extraction buffer along with 50µl of proteinase K and 50µl of RNase. The test tubes were incubatedwith shaking for 30 minutes at 37ºC.After incubation, 0.1mL of 10% SDS was added, and the contents were mixed gently and incubatedat 65ºC for an hour in awater bath with mixing-by-inversion.Next, the lysates were spun at 6000 rpm/10 minutes. The supernatant was taken in afresh tube and organic extraction was performed using chloroform: Isoamyl alcohol (24:1). The solution wasmixedby inversions, and spun at 10,000 rpm for 10 minutes.The aqueousfraction was collected, and $1/10^{th}$ volume of 3M sodium acetate was addedfollowed by chilled ethanol (100%)in the same amount as that of the supernatant. DNA was precipitated by centrifugationat 12,000 rpm/15-20 minutes and thepelletwere washed with 70% ethanol and re-centrifuged at 10000 rpm for 10 minutes. Again, supernatantobtained was discarded, and thepelletwas air-dried. After drying, theDNA was resuspended in 200 µl of TE buffer [11].

Qualitative Analysis of DNA

After the isolation of environmental sample by enrichment method, electrophoresis was performed on 1% agarose gel for semi-quantitative analysis of the isolated DNA.

PCR Amplification

After confirmation of DNA isolation from the enriched culture, PCR amplification of the 16S rRNA gene was done. The reaction mix contained Molecular BiologyGrade H_2O (4 µl), Template DNA (8 µl), Primers (16S Universal forward and reverse primers; 1µl each) dNTP (2.5 µl) *Taq* Polymerase buffer (2.5µl) *Taq* DNA Polymerase (1 µl). The PCR reaction was performed with the following program:

Initial Denaturation	Cycling (35X)			Final Extension	Hold
	Denaturation	Annealing	Extension		
94 ^oC 4 minutes	94^oC 30 seconds	51^oC 60 seconds	72 ^oC 90 seconds	72^oC 5 minutes	4^oC

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The primers used for amplification were 452F (GACTGGGGTGAAGTCGTAAC) and 452R (TGGCTGGGTTGCCCCATTCGG). The Amplification of the isolated DNA was confirmed by running it on the 0.8% agarose gel. After the confirmation of the amplified DNA by visualising it under the UV transilluminator. The amplified product was scaled up in the one PCR tube by 2.5 timesto prepare the reaction mixture volume of 50 µl. Thus, four PCR tubes were preparedto make the scale up thevolume of 200 µl.

Isolation of Culturable Microbes

Enrichment culture of 2X-YT (Yeast Tryptone) Medium was used for isolation of pure cultures. The enriched media inoculated with soil was serially diluted upto 10^6folds dilution using sterile test tubes.The diluted culture was then spread-plated on 2X-YT Agarand kept at 37°C for incubation of 24 hours. The bacterial colonies obtained were then separated based on their size and morphology. These pure cultures were isolated and were sorted based on gram-staining [12].

Screening of Pure cultures by Biochemical Test

For the identification of the pure isolated the conventional method of biochemical testing was employed and following biochemical tests were performed for the identification of the species: IMViC test, TSI agar, MacConkey Agar, EMB Agar, Catalase, Nitrate Reduction and Urease Test were performed according to the protocol [12].

Result and Discussion

DNA Isolation and Assessment of the Isolated DNA

After performing the DNA isolation protocol, 10 µl of the isolated environmental DNA was run on the 1% agarose gel and was visualised on the 0.8% agarose gel as shown in fig:1

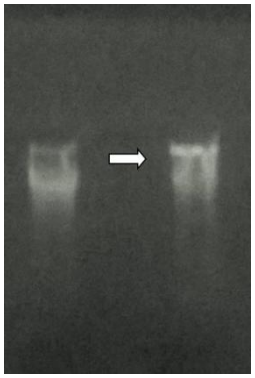

Fig 1: The genomic DNA on 0.8% agarose gel showing metagenomic DNA

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Amplification of 16S rRNA gene

The reaction mixture used for the amplification of the environmental DNA sample showed the amplification with the help of the primer 452F and 452R. The amplification was confirmed by visualising the PCR product on the 1.2% agarose gel as depicted by the band marked with anarrow, and the band was of size around 1.5kbp.

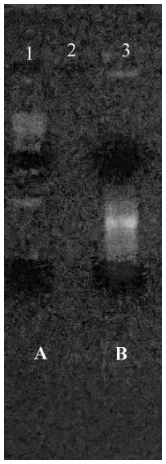

Fig 2: The PCR amplified DNA on 1.2% agarose gel, in which A showing 1Kb ladder and B showing the amplified product

On visualising the amplified DNA on the 1.2% agarose gel, it was seen that the amplified DNA was scaled up by ten times by preparing the reaction mixture of 50 µl in one PCR (thus, one PCR tubes contains the 2.5 times of the reaction mixture). The scaled-up amplified DNA was stored at 4°C.

Identification by Biochemical Tests

The Enterobacteriaceae species were screened for identifying the bacterial species based onthe result obtained from these biochemical tests. The results obtained for the IMViC test revealed that all other bacterial strains showed +/- results except the PGW2, PGW9, PGW11 and PGW14. As the PGW11 and PGW14 were able to synthesise tryptophanase whereas PGW2 and PGW9 were not able to synthesise it. PGW2, PGW9 and PGW11were unable to create the acidic environment, whereas the rest of the bacterial strains were able to create the acidic environment. All the bacterial strains showed an inability to synthesise acetyl methyl carbinol. Only PGW14 strain was unable to catabolize citrate in comparison with all other strains. These results were compared with the findings of previous literature. All the bacterial strains were found to be lactose non-fermenting except the PGW14, which was confirmed by culturing the bacterial strains all the sample on MacConkey and EMB Agar. Then all the

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bacterial strains were evaluated to check the ability of bacterial strains to ferment three different sugar and synthesise H_2S gas. On analysis, it was found that all the strains were able to catabolize all the three sugars as it changed the colour from red to yellow on both slant and butt because of the acid production. For Catalase test, all the bacterial strains showed the positive result which confirms that bacterial strains can produce catalase enzyme. Whereas, for Nitrate reduction test, PGW2, PGW4, PGW9, PGW11 and PGW12 were unable to synthesise nitrate reductase enzyme. On performing the Urease test, it was found that PGW1. PGW2, PGW3, PGW6, PGW7, PGW9 and PGW10 were urease enzyme-producing strain whereas rest were non-urease producing bacterial strain. Thus, on concluding the results of all ten-different biochemical test and comparing it with previously documented literature as well as the Bergey's Manual. The following bacterial strains, *Citrobacter amalonaticus, Citrobacter freundii, Escherichia coli, Proteus mirabilis, Providencia stuartii, and Salmonella species* were identified [13-18] and depicted in Table: 1.

Isolation of Culturable Microbes and Pure Culture Screening by Biochemical Test

Sample No.	**TSI agar**	**M.R**	**V.P**	**Catalase**	**Citrate**	**Nitrate Reduction Test**	**Urease**	**EMB**	**Mac Conkey**	**Indole**	**Species Name**	**References**
PGW1	**A/A + G**	+	-	+	+	+	+	-	-	-	*Citrobacter freundii*	13
PGW2	**A/A + G**	-	-	+	+	-	+	-	-	-	*Proteus mirabilis*	14
PGW3	**A/A + G**	+	-	+	+	+	+	-	-	-	*Citrobacter freundii*	13
PGW4	**A/A + G**	+	-	+	+	-	-	-	-	-	*Providencia stuartii*	18
PGW5	**A/A + G**	+	-	+	+	+	-	-	-	-	*Salmonella species*	15
PGW6	**A/A + G**	+	-	+	+	+	+	-	-	-	*Citrobacter freundii*	13
PGW7	**A/A + G**	+	-	+	+	+	+	-	-	-	*Citrobacter freundii*	13
PGW8	**A/A + G**	+	-	+	+	+	-	-	-	-	*Salmonella species*	15
PGW9	**A/A + G**	+	-	+	+	-	+	-	-	-	*Proteus mirabilis*	14
PGW10	**A/A + G**	+	-	+	+	+	+	-	-	-	*Citrobacter freundii*	13
PGW11	**A/A + G**	-	-	+	+	-	-	-	-	+	*Citrobacter amalonaticus*	16
PGW12	**A/A + G**	+	-	+	+	-	-	-	-	-	*Providencia stuartii*	18

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PGW13	**A/A + G**	+	-	+	+	+	-	-	-	-	*Salmonella species*	15
PGW14	**A/A + G**	+	-	+	-	+	-	+	+	+	*Escherichia coli*	17

+ve – Positive, -ve – Negative, G- gas, A/A – Glucose and Sucrose Fermenter

Table 1: Biochemical tests analysis of isolated bacterial strains from the soil

Conclusion

Our environment is the natural habitat for different bacteria and fungi that are used for the various purposes in different industries such as chemical, detergent, tannery, leather, and pharmaceutical, which require anovel enzyme to conduct different process. The microbes are not limited to the industrial purpose as they also have arole in maintaining theecological balance of the nature and ability of the soil. This study focused on revealing the microbial diversity of the Doaba region by using the culturable and non-culturable approach. The result of culturable approach reveals the presence of *Citrobacter amalonaticus, Citrobacter freundii, Escherichia coli, Proteus mirabilis, Providencia stuartii, and Salmonella species* in the soil.

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Conflict of Interest

The authors declare no conflict of interest.

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