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Screening and identification of bacterial strains from milk industrial waste using 16s rRNA ribotyping

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Abstract
PCR based 16S rRNA gene profiling or ribotyping is a widely used method to accurately identify and differentiate bacterial species and strains. In the present study authors have attempted to identify bacterial species growing in waste material of milk industry. For this purpose waste material was collected form the Ballabgarh Co-operative Milk Producer Union plant situated at Ballabgarh, Haryana, India. Bacterial DNA was isolated from purified bacterial culture and subsequently utilized to PCR amplify 16S rRNA region. Sanger's method of sequencing and other computational methods were employed to screen the homology of amplified PCR products against known bacterial species.

Keywords: 16S rRNA, ribotyping, *bacillus subtilis*, *bacillus pumilus*

1. Introduction

Milk, in simple terms, can be defined as lacteal secretion from mammalian animals. These animals, such as cow, buffalo, goat etc, have mammary glands which produces milk during or after pregnancy. Milk is consumed worldwide as a highly nutritive drink. Chemically cow milk contains 87.7% water, 4.8% carbohydrates, 3.9% fats and 3.2% protein [1, 2, 3]. Raw milk harbors a variety of microbiological organisms such as *Bacillus cereus*, *Listeria monocytogenes*, *Salmonella spp*., *Escherichia coli*, *Staphylococcus aureus, Yersinia enterocolitica, Campylobacter jejuni* etc. The micro fauna of raw milk is often responsible for several health concerns [4]. Pasteurization and other milk processing techniques are used to eliminate these microbes before human consumption.

Milk and microorganisms especially bacterial species have long and interesting associations. Bacterial strains are regularly used to transform raw milk into fermented delights. Bacteria can be defined as unicellular simple microscopic organisms having primitive/prokaryotic type of cell organization. They lack histone proteins and consequently packaging of DNA into chromosome like structure is missing. Bacteria can replicate or reproduce autonomously through various methods such as vegetative (budding, fragmentation, binary fission), asexual (endospore) and sexual (conjugation, transformation, transduction) methods. Ecological and molecular adaptations of bacteria allow them to dominate over wide range of habitats such as human gut, hot sulfur springs, deep oceans, arctic snow etc [5, 6, 7, 8]. Analysis of ecological niche of bacterial life domain reveals immensely significant role in of bacteria in maintenance of soil composition through cycling of nutrients. On the other hand, bacteria are successfully utilized for commercial scale fermentation processes and production of antibiotics. In biological science, bacteria are utilized as model organisms (as in case of *E. coli*) pertaining to small and sequenced genome size, short life cycle, easy maintenance and high fecundity.

Genus Bacillus is constituted by rod shaped and Gram positive bacteria which can be free living or parasitic. Mostly these are obligate aerobes or facultative anaerobes. Members of genus Bacillus are commercially valued for their property of secretion of various enzymes. For example, Bam H1 (a restriction enzyme) and 'barnase' (an antibiotic protein) are obtained from *Bacillus amyloliquefaciens* [9, 10, 11]. *Bacillus thuringiensis* has especial significance as it is the source of CRY genes (which code for an insecticidal protein) which have been utilized to generate many insect-resistant transgenics particularly in maize and cotton [12].

The accurate identification of different bacterial species and strains is immensely important in research and industry. Until recently many characteristics are used in classifying and identifying microbes. For the sake of simplicity these characteristics have been divided into two groups. One group is based on classical characteristics which include cellular morphology (cell shape, cell size, cellular arrangements and colonial morphology on selective, non-selective and differential media), staining property (Gram staining, acid fast staining), growth pattern (rapidity of growth and effect of temperature), biochemical properties (formation of distinct end products, production of acids from various carbohydrates, presence of certain bacterial enzymes). Second group is based on PCR based 16S rRNA ribotyping. PCR- ribotyping is a method which is based on polymorphism in the 16S rRNA intergenic spacer region. It is now known that 16S rRNA markers can generate unique polymorphism which can accurately differentiate between different bacterial species and strains [13, 14]. Computational investigation of rRNA gene sequences is widely used to identify lineage of unknown bacterial species [15]. In ribotyping technique first the DNA is isolated from bacteria. This DNA is then purified and later on PCR amplified using 16S rRNA gene primers. The PCR product is cloned in a sequencing vector and the sequence of

PCR product is identified through Sanger's method based sequencing. Obtained sequence of the PCR product is used as query to search in gene or nucleotide database. The most significant hit is selected based on several parameters such as query coverage, percent identity and e-value. In the present study, we have been able to identify two bacterial species (*Bacillus subtilis* strain MAC-109 and *Bacillus pumilus* strain CPB11) from waste material collected form milk industrial plant named as the Ballabgarh Co-operative Milk Producer Union plant situated at Ballabgarh, Haryana, India. The present work is a preliminary survey of utility of ribotyping method for identification of bacterial species. A more in-depth study is required to ascertain the presented findings.

2. Materials and methods

2.1 Waste samples

Waste samples were collected from different sites in the Ballabgarh Co-operative Milk Producer Union plant situated at Ballabgarh, Haryana, India.

2.2 Culture and isolation of bacterial species

Waste sample (5 ml) is mixed with nutrient agar media (100 ml) and incubated with constant shaking (100 rpm, 50^{0}C) in an incubator shaker. Bacterial culture was serial diluted in following manner: In 100 ml water 0.75 gm NaCl was mixed along with 50 µl Tween 80. The saline solution was autoclaved and 9 ml of this saline solution was distributed in 10 culture tubes. In first tube 1 ml bacterial culture was mixed. Subsequently, 1 ml from tube 1 was mixed in tube 2, and so on, upto tube 10. The serially diluted cultures were plated on nutrient agar media and incubated for 72 hours. Any bacterial growth was sub-cultured and then enriched through liquid LB medium.

2.3 Isolation of genomic DNA

Approximately, 5 ml bacterial culture was centrifuged at 3000 rpm. The pellet was dissolved in 1ml of Lysis Buffer I (50 mM Tris-HCl; pH 8.0, 10 mM EDTA, 100 µg/ml RNase A) along with 20 ul of RNase A (10mg/ml stock) by vortexing or pipetting several time for efficient lysis. Subsequently, 2 ml of Lysozyme (100mg/ml) and 45 ul of proteinase K (20mg/ml) were added to the sample with mild mixing. The tube was incubated at 37ºC for 30 min in a dry bath. Later on, 0.35 ml of Bacterial lysis buffer II (200 mM NaOH, 1% SDS) was mixed by inverting tube or vortexing for few minutes. Again, the tube was incubated at 50ºC for 30 minutes in a dry bath. After incubation of bacterial cell culture was centrifuged at 12,000 rpm. One ml of the supernatant was transferred in a Genai Ultrapure™ column. Subsequent steps were followed as per manufacturer's instructions to obtain purified genomic DNA.

2.4 Amplification of 16s rRNA gene

The 16S rRNA region of the genome was amplified in a PCR machine using a forward primer and a reverse primer. The PCR protocol used for amplification reaction includes denaturation at 95^{0}C for 5 minutes, 30 cycles of 95^{0}C for 1 minutes, 58^{0}C for 1 minutes, 72^{0}C for 1 minutes, and 72^{0}C for 5 minutes. The composition of reaction mixture used to set PCR is presented in Table 1 and the sequence of primers used for PCR amplification is documented in Table 2.

Table 1: Composition used to set PCR reaction.

Component	Volume (µl)
DNA template (50ng/µl)	1.0
PCR buffer(10X)	2.0
dNTPs(10mM/µl)	2.0
Forward primer (20pM/µl)	0.5
Reverse primer (20pM/µl)	0.5
Taq polymerase	0.5
Water	13.5
Total volume	20.0

Table 2: Name and sequence of primers used for amplification.

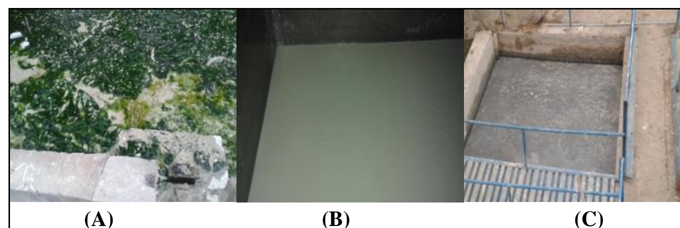
Primer name	Sequence
Forward primer	5 'CAGCAGCCGCGGTAATAC 3'
Reverse primer	5' TACGGCTACCTTACG 3'

2.5 Elution of PCR product and sequencing

Amplified PCR product was purified using Genai PURE PCR purification kit as per manufacturer's instructions. The sequencing of purified PCR products was outsourced to TCGA (The center for genomic application), Delhi, India.

3. Result and discussion

The samples for this study were collected from the Ballabgarh Co-operative Milk Producer's Union Ltd. Milk Plant, situated at Ballabgarh, Haryana, India (Figure 1). The samples were stored at 4ºC for 24 hour and after that equal volume of each sample was mixed and incubated with nutrient agar media for 10-15 days in an orbital incubator shaker at 37 degrees. This resulted in exponential increase in bacteria number. Subsequently purified bacterial cultures were prepared through serial dilution method. Genomic DNA was extracted using purified bacterial cultures. Agarose gel electrophoresis (AGE) technique was used to assess the quality of extracted DNA. High quality genomic DNA samples were used to profile 16S rRNA region using PCR. The amplicons obtained through PCR were size separated on low percentage (1%) agarose gel and visualized by staining with ethidium bromide stain (Figure 2).



(A) (B) (C)

Fig 1: Picture of waste sample collection sites (A) Sediment waste site (B) Waste coming from curd preparation and (C) Outlet of milk processing unit.

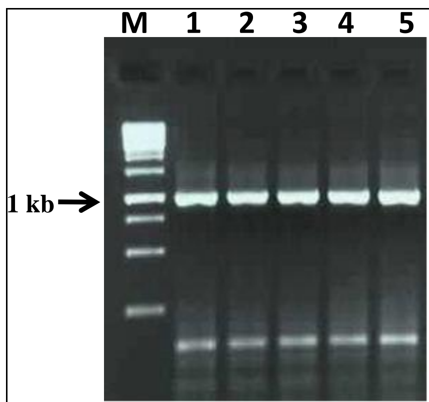


Fig 2: DNA isolated from purified bacterial culture was amplified using 16s rRNA primer. The amplified PCR product was resolved on agarose gel. (M: marker, 1- 5: bacterial DNA samples).

3.1 Sequencing of PCR product and homology analysis

The amplicons obtained by PCR were purified from agarose gel. The purified PCR products were sent to TCGA, Okhla Industrial Estate, New Delhi, India for sequencing. The sequence read obtained after sequencing of PCR products were used to stitch a consensus sequence. The consensus sequence was searched for homology in National Centre for Biotechnology (NCBI, www.ncbi.nih.nlm.gov) nucleotide database GenBank using homology search tool BLAST (Basic Local Alignment Search Tool). The best hit was selected on the basis of lowest e-value cut off, highest query coverage and identity. The complete consensus sequence and result of BLAST hit table is given below.

Sequence 1

TCCTCGCCAGGCGGTTTCTTAAGTCTGATGTGAAAGC
CCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGG
GAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACG
TGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACAC
CAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACG
CTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGAGTGC
TAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGC
TAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCG
CAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGC
ACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC
GCGAAGAACCTTACCAGGTCTTGACATCCTCTGACA
ATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGT
GACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG
AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
TGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAG
GTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGAT
GACGTCAAATCATCATGCCCCTTATGACCTGGGCTAC
ACACGTGCTACAATGGACAGAACAAAGGGCAGCGA
AACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCT
CAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAA
GCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGC
GGGGGAATAACG

Sequence 2

CTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATT
CCACGTGTAGCGGTGAAAT
CGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCG
ACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAG
CGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGT
TTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCAC
TCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGC
ATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC
CAGGTCTTGACATCCTCTGACAACCCTAGAGATAGG
GCTTTCCCTTCGGGGACAGAGTGACAGGTGGTGCAT
GGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCA
GCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGA
CAAACCGGAGGAAGGTGGGGATGACGTCAAATCATC
ATGCCCCTTATGACCTGGGCTACACACGTGCTACAAT
GGACAGAACAAAGGGCTGCAAGACCGCAAGGGTTT
AGCCAATCCCATAAATCTGTTCTCAGTTCGGATCGCA
GTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAG
TAATCGCGGATCAGC

BLAST hit table result

Table 3: Sequence

Accession	Description	Max score	Total score	Query coverage	Identiy
JX049345.1	*Bacillus subtilis* strain MAC-109 16S ribosomal RNA gene Partial sequence	1491	1491	100%	100%

Table 4: Sequence

Accession	Description	Max score	Total score	Query coverage	Identity
GU322345.1	*Bacillus pumilus* strain CPB11 16S ribosomal RNA gene, partial sequence	1325	1325	100%	99%

4. Conclusion

Bacterial domain of life plays significant role in ecosystem and their properties are harnessed for human benefits. First and foremost requirement of any industry or biological research program, dealing with bacteria, is accurate identification of bacterial species or strains. In this paper we demonstrate the efficiency and accuracy of 16S rRNA ribotyping method to identify bacterial species from milk waste generated at milk plant (the Ballabgarh Co-operative Milk Producer's Union Ltd. milk plant Ballabgarh, Haryana, India). Two ecologically significant bacterial species namely *Bacillus subtilis* and *Bacillus pumilus* were identified. This method can also be used to assay novel bacterial flora.

5. References

1. Arora R, Bhojak N. Physiochemical and environmental factors responsible for change in milk composition of milking animal. The international journal of engineering and science. 2013; 2(01):275-277.
2. Pal UK, Mandal PK, Rao VK, Das CD. Quality and utility of goal milk with special reference to india: An overview. Asian journal of animal sciences. 2011; 5(1):56-63.
3. National Nutrient Database for Standard Reference. Ars.usda.gov. Retrieved, 2011.
4. Ryser ET. Public health concerns. In Marth, EH, Steele JL, (Eds.), Appl. Dairy Microbiol. Marcel decker, inc. New York, 1998, 263-403.
5. Saxena R, Sharma VK. A metagenomic insight into the human microbiome: Its implications in health and disease. In D. Kumar and S. Antonarakis. Medical and health genomics, Elsevier Science. doi:10.1016/B978-0-12-420196-5.00009-5. 1998-2016, 117.
6. Rawat S. Bacterial diversity of a sulphur spring in Uttarakhand, India, Advances in applied science research. 2015; 6(4):236-244.
7. Hoehler TM, Bebout BM, Des Marais DJ. The role of microbial mats in the production of reduced gases on the early Earth. Nature. 2001; 412(6844):324-7.
8. Harding T, Jungblut AD, Lovejoy C, Vincent WF. Microbes in high arctic snow and implications for the cold biosphere. Applied and environmental microbiology. 2011; 77(10):3234-3243.
9. Paddon CJ, Hartley RW. Expression of *Bacillus amyloliquefaciens* extracellular ribonuclease (barnase) in *Escherichia coli* following an inactivating mutation. Gene. 1987; 53(1):11-9.
10. Wells JA, Ferrari E, Henner DJ, Estell DA, Chen EY. Cloning, sequencing, and secretion of *Bacillus amyloliquefaciens* subtilisin in *Bacillus subtilis*. Nucleic Acids Research. 1983; 11(22):7911-25.
11. Vanek PG, Connaughton JF, Kaloss WD. Chirikjian JG. The complete sequence of the *Bacillus amyloliquefaciens* strain H, cellular BamHI methylase gene. Nucleic Acids Research. 1990; 18(20):6145.
12. Roh JY, Choi JY, Li MS, Jin BR, Je YH. *Bacillus thuringiensis* as a specific, safe, and effective tool for insect pest control. Journal of microbiology and biotechnology. 2007; 17(4):547-59.
13. Isenbarger TA, Carr CE, Johnson SS, Finney M, Chur JM. The most conserved genome segments for life: Detection on earth and other planets. Origins of life and evolution of biospheres. DOI 10.1007/s11084-008-9148-z, 2008.
14. Kumar S, Garg B, Bhardwaj AR, Khaling M. Identification of bacterial strain form waste decay material using 16s rRNA ribotyping. Research & Reviews: A Journal of Life Sciences, 2016, 6(3).
15. Ameen A, Munir N, Ahmad J, Raza S. Isolation, identification and molecular characterization of the most prevalent bacillus bacteria in decomposition of municipal solid waste. European journal of biomedical and pharmaceutical sciences. 2016; 3(5):92-97.