Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(4):1519-1524

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Flourescence in situ hybridization analysis of hot spring microbes using rRNA targeted oligonucleotide probes

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ABSTRACT

Analysis to detect microbes in natural environment requires isolation and subsequent identifications of isolated strain. Success isolation relied on the availability of suitable culture media and growth conditions. Identification mainly based on biochemical tests involving fermentation of carbohydrate substrates, detection of a limited amount of microbial enzymes or other analysis methods. However at most 0.1 to 10% of microscopic ally observed organisms can be grown in the laboratory. Hence many novel microbes, which may be medically and industrial important remains undiscovered. Application of molecular methods has made it possible to identify microorganisms in natural and enrichment samples without the requirements of laboratory cultivation. One such method is whole cell in with hybridization technique based on rRNA sequence analysis. In this study microbial diversity in hot spring has been analyzed by this method. The technology involves genus, species or individual strain specific oligonucleotide probes complementary to rRNA sequences. Successful hybridization leads to identification of the microorganism. Visualization of the microbial cells done by Epifluorescence microscopy.

Keywords: Oligonucleotide probe, Thermophiles, Fluorescence in with Hybridization, rRNA

INTRODUCTION

A complete structural and functional characterization of an ecosystem requires the knowledge about metabolic ,ecological and genomic diversity of microbes present in the environment. But enormous physiological and phylogenetic complexities associated with microbial communities hinder proper determination and understanding of such communities. In reality; for the microbial world there is a vast gap in knowledge, particularly apparent for bacteria and other prokaryotes.Studies revealed that a gram of soil contains millions of microorganisms and still only 1 % of them is identified. Investigations show that there exists 3, 00,000 to 1 million species of prokaryotes on the earth, yet only few thousands bacteria are described in Bergey's manual. Earlier studies were limited within normal boundaries of normal environments. But further discoveries show that the microbial life also exists in harsh environments like high salt deserts, hot springs, volcanic areas, polar ice and even in the depths of oceans. Extreme environments provide favorable habitat for the growth of complex microbial community. These communities are morphologically dominated by photosynthetic bacteria, but also include microbes engaged in most major biochemical pathways including the sulfide, nitrogen and carbon cycles. Among these bacteria can be found at greater extremes of temperatures, acidity, alkalinity, pressure and salinity than any other microbe .All these clearly show the astounding range of habitats and metabolic diversity present in the microscopic world.

The development of molecular methodologies for the assessment of microbial diversity has revolutionized the study of microbial communities. Recently, several approaches have been developed to obtain gene sequences and thereby identify microorganisms in natural samples without the requirement of laboratory cultivation.[1,2,3] These approaches are based on molecular evolutionary studies of ribosomal RNA.Introduction of molecular tools allowed

the study of microbes without prior cultivation, dramatically changed the perceptions of the phylogeny and the diversity of life.[4,5]Novel molecular methods offered new ways of in with study of microbes in various environments. By analyzing relevant genes obtained directly from a particular habitat can be identified (to some extent); characterized and counted. There are various molecular methods of phylogenetic identification, like 16S rRNA sequencing, nucleic acid hybridization, genomic DNA hybridization; polymorphs based procedures and quantitative DNA hybridization. [6]

Normal hybridization requires the isolation of DNA or RNA, separating on a gel, blotting it into nitrocellulose and probing it with a complimentary sequence. But in case of in with hybridization the probe is tagged with fluorescent chemicals or radioisotopes and used to detect specific sequence nucleotide within cells or tissue. Provided the sequence of the targeted DNA or RNA is known previously to design the probe. [7, 8]

The objective of this project work was study of community structure by utilization of molecular technique namely fluorescence in with hybridization (FISH) [9].In this method fluorescence labeled probes are targeted to specific rRNA sequences of specific type of microbes present in hot spring sediments. The study also detects microbes in enrichment culture of aerobic thermophiles by FISH.

EXPERIMENTAL SECTION

2. Technique of 5'-Oligolabelling of probes with fluorescence

2.1. Kinase reaction:

This step allows the attachment of a reactive phosphorothioate to the end of a deprotected oligonucleotide. The required labeling components excluding the enzyme are placed on ice to thaw. In a micro centrifuge tube on ice the reaction mixture(oligonucleotide (5'-GTTTTCCCAGTCACGAC-3' in water) 20 µl , sterile water 21.5 µl,10X T4 reaction buffer 5 µl, ATPγS 2.5 µl, T4 polynucleotide Kinase 1 µl) is prepared and mixed by gentle vortexing. The tube is incubated at 37^0C for one hour. The T4 polynucleotide Kinase was inactivated by heating for 10 minutes at 70 ^{0}C.

2.2 Coupling reaction:

This step allows the attachment of a single fluoresce in moiety to the 5'- phosphorothioated oligonucleotide. To the above reaction mixture from kinase reaction about 2 µl of 25 mM 5-Iodoacetamidofluorescein (in dimethylformanide) solution was added. The solution mixed well and incubated at 37^0C for 30 minutes. The labeled oligonucleotide so obtained was stored (at -15^0C to -30^0C) prior to purification step.

2.3 Purification of probe

The purification columns remove the unreacted fluorescent chemical and by products from fluorescein labeled oligonucleotides. The column assembly is centrifuged for 4 minutes in a horizontal centrifuge at 500-700xg to remove the void volumes in the column. It becomes dry and shows a white particulate appearance after centrifugation. About 50 µl of reaction mixture containing the labeled oligonucleotide is poured into the columns, and centrifuged at 500-700xg for 4 minutes. The oligonucleotide eluted in a solution containing: 10mM Tris, (pH-8), 1mM EDTA, 10 mM NaCl. The labeling efficiency is assayed using rapid labeling assay. This showed the fluorescence intensities of the spots of newly labeled oligonucleotide relative to the control were 60-70%.

3. Analysis of environmental sample

3.1. Sediment sample collections:

Sample collected during winter season from hot springs flowing with Tantreswar River near Tantloi (Jharkhand) and Agnikunda, hot spring at Bakreswar (West Bengal). Temperature and pH of sediment sample recorded. (Table 1)

Table: 1 Temperature and pH recorded

S.No	Area of collection	Temperature (^{0}Centigrade)	pH of sping water
1.	Tantloi,WB	68.5	9.2
2.	Agnikunda (Bakreswar)	67	9.2

3.2 FISH slide preparation:

Glass slides are dipped in concentrated HCl for one day washed and dipped in double distilled water for another day. The air dried slides washed again in 10% alcoholic Potassiun hydroxide (KOH) solution, followed by double distilled water. Dried and gelatin coated with solution containing 0.015g chromium potassium sulphate and 0.15g gelatin at 70^0C. Slides are air dried and stored at 4^0C before use.

3.3 20X SSC stock preparation:
Chemicals used are Trisodiumcitrate-2-hydrate: 88.2 g; sodium chloride: 175.3 g; Water about 1000ml.

4. Hybridization mixture preparation : Chemicals used for preparation of 50ml mixture are 0.9M sodium chloride,2.63g; 20mMTris-HCl(pH-7.6) 1ml (from 1M Tris stock); 5mM EDTA,0.093g; 0.025 SDS ,100 µl (from 10% SDS stock); 10% (v/v) formamide ,5ml; and 5-10% of Dextransulphate.

5. Preparation of wash buffer: Chemicals used for 5ml solution are 2X SSC,5ml (from 20XSSC stock); 50% formamide ,25ml; and 0.1% SDS, 500 µl (from 10% SDS stock).

6.Pretreatment of sample before FISH : About 1 ml of the sample (fixed in 4 % paraformaldehyde (8 g PFA dissolved in 100ml dist water, heated to partially dissolve then 1M NaOH added drop wise until solution is clear. Added 100ml 0.1M PBS ,mixed filtered, cooled to 4 0C before use, pH-7.4) taken in an eppendroff and sinned at 3000 rpm.The supernatant discarded and the cells washed with 1X PBS (100mM disodiumhydrogenphosphate 1.44g, 20mM 0.24g, 137mM NaCl 8g, 22mM KCl 0.2g,H_2O 1000ml, pH (7.4) for 5 minutes, and centrifuged in low rpm. After the last wash with PBS about 25ml of the sample is spread on a gelatin coated slide. The slides are air dried and dehydrated by 50%, 80%, 90% ethanol for 2-3 minutes in each solution. Air dried again prior to its use for in with hybridization

7. Hybridization and post hybridization processes:
About 25 µl of hybridization mixture containing about 20ng of fluorescence labeled probe (Table 2) applied on the dried slides. Gently the mixture is spread with the tip of pipette so that all the cells come in contact with the hybridization solution. The slides are carefully placed horizontally on racks in humified box containing prewarmed solution (at 56^0C) of 50% formamide and 5X SSC. The box sealed and incubated at 56^0C for 2-3 hours. Then the slides are washed by immersing in prewarmed 25% formamide (58^0C), 2 X SSC solutions, kept on warm shaking platform. The process is continued for 30 minutes. Washed again with 2X SSC twice (30 each step) at 58^0C.The wash solution is drained and slides are aired dried .The cells are then overlaid with 10 micro liter of hybridization solution (without probe) containing only DAPI (diamidino-2-phenylindole) (1mg/ml) and kept in dark for 8 minutes. This are thoroughly washed with double distilled water for several minutes. Slides dried and visualized under epifluorescence microscope. All the steps of hybridization carried out with minimal exposure of light to minimize fluorescence bleaching.

Table: 2 Table of probes used

S.No	Probe Sequence	Specific targeted microorganism for identification
1.	-CTTGGGCCCTGCTTTGGG-	Thermus
2.	FluoroGCCTTCCCACATCGTTT-	γ-proteobacteria
3.	GCCTGCCCACTCATTGCGCACT	Cyanobacteria

8. Enrichment of Aerobic Thermophiles
A portion of the sediment samples (collected from Tantloi and Agnikunda) was inoculated in 20ml of Luria Broth and incubated at 65^0C. About 3 to 4 microliter of culture solution was later poured on 3% agar plates. Growth of colonies was observed within 6 to 7 hours

RESULTS AND DISCUSSION

The direct study of microbial communities from the sediment samples in the hot springs without prior cultivation revealed the composition of the community. From the data collected by fluorescence in situ hybridization (FISH) studies we took averages between the numbers of microbial cells present in the sample (by genus or specific probe) with the total number of cells seen through DAPI staining .Subsequent percentage calculation and comparison of these data for all microbes studied, gave the percentage of Thermus, Cyanobacteria and Gamma-proteobacteria in the geothermal spring of Tantloi and Agnikunda (Bakreshwar).

Table: 3 Percentage of microbes in hot spring

S. No.	Sample site	Total cell count in DAPI staining	Total number of cells hybridized	Percentage (%) of microbial cells in hot spring
1.	Tantloi	1463	289	19.75
2.	Tantloi	1185	391	32.99
3.	Tantloi	1544	647	41.9
4.	Agnikunda	2086	890	42.66
5.	Agnikunda	1838	1060	57.6

Fig1: Sediment samples from Tantloi (Probe specific for Cyanobacteria).

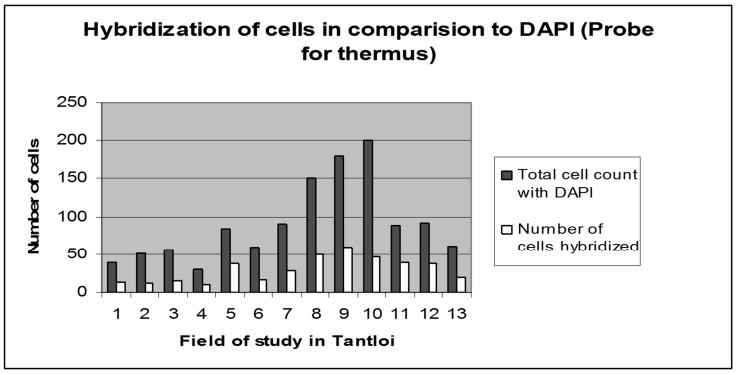


Fig 2: Sediment samples from Tantloi (Probe specific for Thermus).

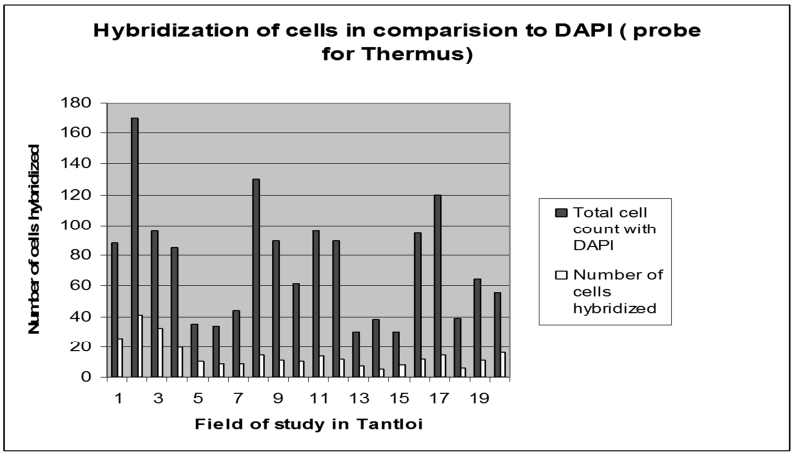


Fig 3: Sediment samples from Tantloi (Probe specific for γ-proteobacteria)

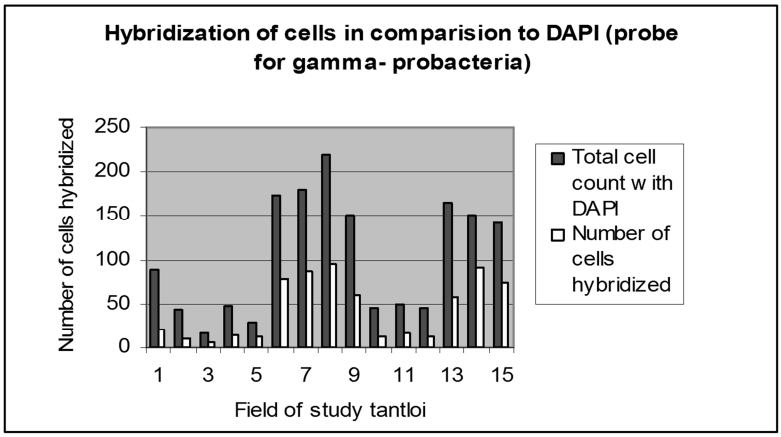


Fig 4: Sediment samples from Agnikunda (Probe specific cyanobacteria)

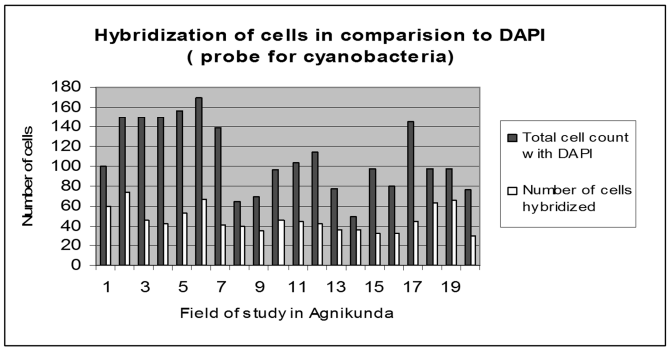
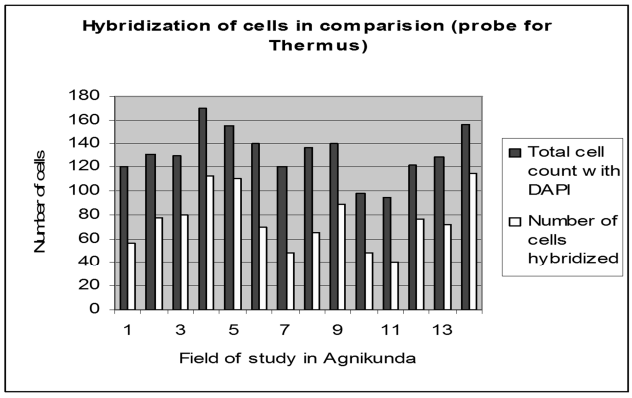


Fig 5: Sediment samples from Agnikunda (Probe for Thermus)



While analyzing the enrichment culture the presence of a specific genus of microorganism viz Thermus was found, as it was evident from FISH analysis. The microbes stained gram negative and were strictly aerobic in nature.

Fig 5: Percentage of specific thermophilic microbes in Tantloi

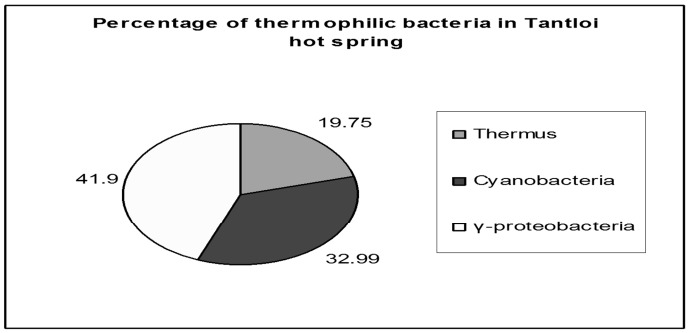
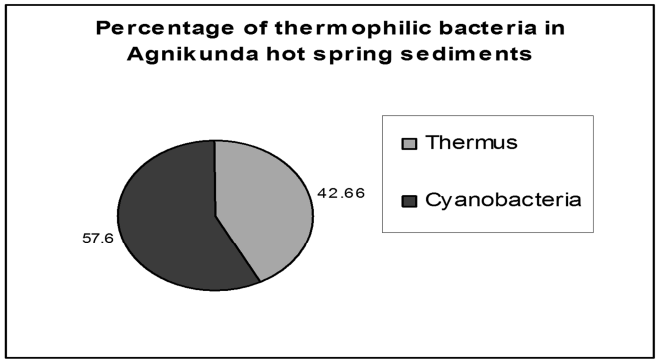


Fig 6: Percentage of specific thermophilc microbes in Agnikunda



CONCLUSION

In this study, we sought to cultivate fully aerobic thermophilic micro-organisms from hot spring sediment samples from Tantloi and Agnikunda (Bakreshwar) West Bengal. We succeeded in isolating novel strictly aerobic thermopiles. Thermophilic bacterium was isolated from temperatures about 65^0C-69^0C.The new isolated strains were found to be aerobic, rod shaped. Some coccus in shape occurring in single or paired form. Growth was optimum at 65^0C, at pH 7.2.The isolates were capable of growing on medium containing only sodium chloride (NaCl) yeast extract and tryptone. Phylogenetic analysis with fluorescence in situ hybridization revealed the presence of micro-organisms mostly belonging to the genus Thermus.

Acknowledgement
Authors wish to thank Prof Subrta.Pal , Jadavpur University, and Kolkata for his valuable advice and guidance during the research work. Also acknowledge JIS group And DSCSDEC authority for their encouragement in research work.

REFERENCES

[1] Amann,R.I,Lidwig,W.Schleifer,K.H. *Microbiol.Rev*,**1995**,59(1),143-169
[2] Amann R, Glöckner F O, Neef A. *FEMS Microbiol Rev*. **1997**;20:191–200.
[3] Burggraf S, Mayer T, Amann R, Schadhauser S, Woese CR, Stetter KO. *Appl Environ Microbiol.* **1994** Sep;60(9):3112–3119.
[4] Lee, N., P. H. Nielsen, K. H. Andreasen, S. Juretschko, J. L. Nielsen, K.-H. Schleifer and M. Wagner**.** *Appl. Environ. Microbial.* **1999,**65**:**1289-1297.
[5] Manz, W. *Methods Enzymol.* 1999. 310**:**79-91
[6] Manz,W.,Eisenbrecher,M.,Neu,T.R and Szewzyk,U., *FEMS Microbiol.Ecol.* **1998**, 25.
[7] Manz,W.,Amann,r.,Ludwig.W.,Vancanneyt,M and Schleifer,K.H. *Microbiology.***1996**,142:1097-1106
[8] Schleifer, K.H., Wagner, M. *Bioscience Microflora* **1998**, 17:15-p; 22.
[9] Stoffels,*M.*,Ludwig,W.,Schleifer,K.H. *Environ .Microbiol.* **1999**, 1:259-p;271.