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OPTIMIZATION OF POLYMERASE CHAIN REACTION CONDITIONS FOR META GENOMIC ANALYSIS OF SEWAGE WATER FROM A SHRIMP PROCESSING INDUSTRY OF VISAKHAPATNAM, EAST COAST OF INDIA

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ABSTRACT: Collection and storage of water samples and methods of extraction of DNA are crucial steps for metagenomic studies of sewage/water samples. This study was aimed at microbial metagenomic analysis of sewage water samples from a shrimp processing industry in Visakhapatnam, east coast of India. In this study the Cetyltrimethylammonium Bromide (CTAB) method was adopted to extract DNA from the water samples. Unfortunately, all efforts became futile at the time of gel check, as a result a study was undertaken to optimize the Polymerase Chain Reaction (PCR) conditions with the extracted DNA. Exposure of DNA sample to 53.8ºC temperature for 30 seconds and 54ºC for 30 seconds and 30 thermo cycles at each temperature were proved best to conduct Horizontal Gel Electrophoresis, leading towards metagenomic DNA extraction and analysis.

Keywords: Polymerase Chain Reaction, metagenomics, marker DNA, taq polymerase, gel electrophoresis, CTAB method.

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1. INTRODUCTION

Meta genomics is the study of DNA of microbial populations in the environmental samples, which yields large genetic pool that can be used for the recovery of novel genes, to find out new metabolic pathways and their products and also to study the biodiversity index of microbial population of an

ecosystem without carrying out laboratory culture of microorganisms [1, 2, 3]. Sampling is one of the crucial steps in metagenomic analysis. The collection and storage of the collected water samples or soil samples may affect the quality and quantity of the results [4,5]. This study was initiated with an objective to analyze the biodiversity of microbial populations in the drained water of a shrimp processing industry namely the Southern Sea Foods, Pendurti, Visakhapatnam, through metagenomic analysis. Owing to the difficulties faced at the stage of gel check and rarity of this kind of work published, the work was undertaken in two phases, the first phase is to optimize the PCR conditions at which the maximum base pairs of DNA fragments can be obtained, which leads to the metagenomic analysis as a second phase. This study will save the time and also useful to the researchers working on the metagenomics of coastal water samples, coastal sediment samples, water samples of aqua farms and water samples of hatcheries in the analysis of microbial biodiversity of genetic material in the above samples.

2. MATERIALS AND METHODS

2.1. Sample collection and processing

Water sample was collected from the Southern Sea Foods Limited (17º 49' 59.88'' N; 83º 12' 0.00'' E), Pendurti, Visakhapatnam, Andhra Pradesh. While the plant is at function, 10L of sewage water from shrimp processing section was collected in sterile plastic screw capped containers in December 2019, January 2020 and February 2020 transported to laboratory within 3 hours of its collection as the processing plant is 20km away from the laboratory. Immediately after bringing the sample to laboratory, 5L of sample was filtered through 5.0 µm of cellulose filter paper (Sartorius Q5) to remove large particles then through 0.22µm GF/F filter paper (Sartorius Q2) in succession until 10g of filtrate (with filter paper) obtained. All kinds of microbial cells on the filter paper were processed to obtain the DNA. The remaining 5L sample was stored at -80ºC for further use.

2.2. The extraction protocol for DNA from sewage water samples

The filter paper with microbial samples (weighing 10g) were removed from the filtration unit with sterilized forceps and dropped into 200ml of 0.12 M sodium phosphate buffer (NaP), pH-8.0 and 2.5 g of sodium dodecyl sulphate and incubated at 70ºC for 1 hour. The lysis of the filter paper should be greater than 90% in the mixture of buffer solution. The mixture was then centrifuged at 6000g in a centrifuge Eppendorf model 5810R for 30min and the supernatant was removed in to a beaker. Four extractions were made with the above buffer mixture. All supernatants were pooled and again centrifuged at 16000 g for 2min to remove all the cell debris, the debris of the filter paper and other particulate matter. Then the sample extracts were concentrated by adding 0.3M sodium acetate to the sample. Then the extract was precipitated by adding two volumes of ethanol to that of extract and store at -20ºC overnight. The extract was again centrifuged in 0.12 M NaP at pH- 6.8 for 7 times, until 5ml pellet formed in the centrifugation tube. The pellets suspended on the top of the centrifuge tube were collected with a 60ml syringe without needle.

2.3. Purification of DNA

HiPur® water DNA purification kit was used for isolation and purification of microbial DNA from the collected sewage water samples (Table 1 & 2).

Table 1: Hi pur® water DNA purification kit for isolation of DNA from bacterial and fungal species in water

Product code	Reagents provided	MB547		
		20 Preps (mL)	50 Preps (mL)	250 Preps (mL)
DS0014	Gram positive lysis solution (GPLA)	6	15	75
DS0015	Lysis solution (AL)	6	15	75
DS0010	Lysis solution (C1)	6	15	75
DS0031	Prewash solution (PWB)	12	30	150
DS0012	Wash solution concentrate (WS)	4	10	50
DS0040	Elution buffer (ET) [10mM Tris-Cl pH 8.5]	4	10	50
MB086	Proteinase K	10mg	25mg	125mg
DS0003	RNase A solution (20mg/ml)	0.5	1.25	6.25
MB098	Lysozyme	0.5	1.25	6.25
DBCA03	HiElute Miniprep spin column (capped)	20nos	50nos	250nos

Pellet of 1.5ml was transferred with a 60ml disposable syringe without needle from the centrifuge tube into a capped 2.0 ml collection tube. The remaining volume of pellet stored at -80ºC for successive uses. To remove the micro debris attached to the DNA strands, the pellet was re-suspended in 180µl lysis solution (AL; product code: DS0015 I. HiPur® water DNA purification kit)

2.4. Cell lysis & extraction

20µL of proteinase K solution (0.2mg/mL^{-1}) prepared adopting the instructions given in the kit and added to the pellet, mixed well and incubated for 30 minutes at 55ºC. Then added 200µL of lysis solution (C1; product code DS0010, HiPur® water DNA purification kit) vortexed thoroughly for 15 minutes. Then loaded the obtained lysate on to Hi-Elude mini prep spin column capped, present in the DBCA16 collection tube (product code; DBCA O3; I. HiPur® water DNA purification kit). Then centrifuged at > 6500g for one minute, placed the spin column in the same 2.0 mL collection tube, when uncapped the liquid flow through which was discarded.

2.5. Washing

A volume of 500µL of prewash solution (PWB; product code D50031, HiPur® water, DNA purification kit) was added to the column and centrifuged at 6,500 rpm for one minute. The DNA was washed with 500 µL of prewash solution at least 5 times before final elution. Then the column

is washed with 500 µL of diluted wash solution and centrifuged for three minutes between 13000 and 16000 rpm. The flow through liquid was discarded and centrifuged the tube again at the same rpm for an additional 1 min to dry the column. Then the column was placed in a new 2.0 ml collection tube (uncapped) and pipetted 100µl of the elution (ET; product no: DS 0040 I. HiPur® water DNA purification kit – Hi media) directly into the column and incubated for one minute at room temperature and centrifuged at 6500g (10,000rpm) for one minute and transferred the obtained pure genomic DNA to a new 2.0 ml collection tube (capped) and stored at 2ºC for further analysis. The purity and concentrations of these fractions were monitored by spectroscopic scans from 320 to 220 nm wave length on Perkin-Elmer Model- Lamda 35 UV-Vis spectrophotometer. 10 µg of DNA was yielded per liter of sewage water sample from the shrimp processing plant under study. HiPur® water DNA purification kit was tested before DNA extraction of sample according to the predetermined specifications to ensure the product quality.

2.6. PCR

Hi Chrom PCR kit was used to carry out Polymerase Chain Reaction. The specifications of PCR master mix was given in the table-2.

Table 2: Hi- Chrom PCR master mix

Product name	Product code	Kit packing **
Hi-Chrom PCR Master Mix	MBTB089-20R	20r(0.5mL)
	MBTB089-50R	50r(1.25mL)
	MBTB089-100R	100r(0.5mL)

The DNA in the eluent was precipitated with elution buffer (ET, Product code DS0040) directly into the column incubated for 1 min at room temperature and loaded into the PCR machine Perkin Elmer model GeneAmp2400 PCR system. The initial step that was denaturation, carried out for 45 seconds at temperature 94ºC, followed by annealing which was the second step of PCR. Here, the oligonucleotide primers dissociate from their templates at the calculated melting temperature (T_m), as indicated on the PCR kit. The third step in PCR, the extension was carried out at optimal temperature given on the PCR kit (72-78 ºC) catalyzed by the thermostable polymerase. In this study, Taq Polymerase is used as thermostable polymerase. The reaction that takes place in the first cycle was, one primer extension proceeds beyond the complementary sequence of the other primer towards the binding site. This cycle was followed by the production of first molecules whose length was limited by the binding sites of the primers. Amplification of DNA starts from the third cycle onwards. The Taq polymerase polymerization rate is ~ 2000 nucleotides per minute at the optimum temperatures 72-78ºC where extension is carried out for one minute for every 1000 base pairs of product.

2.6.1. Number of cycles

In this study 30 cycles were undertaken to obtain adequate magnitude of amplification of a single copy of target sequence. Gel electrophoresis was conducted to confirm the presence of extracted DNA from the water sample. But all attempts to detect DNA through gel check were failed. So attempts were made to optimize the PCR conditions particularly at the annealing stage as the temperature at this stage, play a critical role in the PCR amplification of DNA. At high annealing temperatures, the oligonucleotide primer and the template DNA anneal poorly. Then the amplification of sample DNA will be very low. At high annealing temperatures annealing of nonspecific primers may also occur which results in the amplification of unwanted segments of DNA. Annealing is usually carried out 3ºC to 5ºC lower than the calculated melting temperature (T_m). The oligonucleotide primers dissociate 3ºC to 5ºC lower than then given T_m values, from their templates. The theoretical T_m values may differ from experimental T_m values for oligonucleotide primers of different lengths and sequences. To optimize the annealing temperature while performing PCR, a series of trail PCRs may be conducted at temperatures ranging from 2ºC to 10ºC lower of the T_m values calculated for the two oligonucleotide primers. Alternatively, the thermocycler was programmed to use progressively lower annealing temperatures in consecutive pairs of cycles [6].

In this study PCR was carried out with an initial denaturation at 94ºC for 10minutes, followed by 30 cycles of denaturation at 94ºC for 30 seconds time, followed by annealing at 53ºC for 30 seconds. Extension at 72ºC for 45 seconds, with a final extension at 72ºC for 10 minutes and final step cooling at 4ºC. In this study the initial denaturation was carried out as given above. To increase the microbial DNA concentration for gel check, the annealing process of primers and template DNA was tested at 9 different temperatures between 53ºC and 57ºC (53ºC, 53.3ºC, 53.7ºC, 53.8ºC, 54ºC, 55ºC, 56ºC, 56.5ºC and 57ºC) for 30 seconds. PCR was repeated at every test annealing temperature separately. Gel check was carried out after each experiment in order to confirm the migration of sample DNA fragments. The results were presented in the results section.

2.7. Gel check

Gel check was carried out for study of separation of microbial DNA bands by performing horizontal gel electrophoresis after PCR at every experimental annealing temperature. Standard protocol was used to conduct Horizontal Gel Electrophoresis [7]. A comb containing 8 teeth (tooth width 6.4mm x 1.5mm) was used to make the wells in the agarose gel (1.0cm thickness) plate. 50µL Marker DNA was loaded in the first well followed by loading of 50µL amplified DNA sample in the remaining 7 wells. A gel tank of 10cm length, filled with 100mL TAE buffer containing lid was used to place the gel slab. After closing the lid of the gel tank, the tray was connected to electrical leads. As the distance between the positive and negative electrodes of the gel tank used in this experiment is 10 cm, only 50V of power was applied to run the gel. The fragments of DNA migrated from the wells towards cathode was presented in the figure 1a to 1i.

3. RESULTS AND DISCUSSION

3.1. Experimental conditions for optimization of polymerase chain reaction

Figure 1 a, b, c, d, e, f, g, h and i shows that only DNA marker bands were moved towards cathode at annealing temperature 53ºC, 53.3ºC, 53.7ºC, 53.8ºC, 54ºC, 55ºC, 56ºC, 56.5ºC and 57ºC respectively, the migration of template DNA from any of the wells was hardly expressed at annealing temperatures 53ºC, 53.3ºC, 53.7ºC, 55ºC, 56ºC, 56.5ºC and 57ºC. At annealing temperature 53.8ºC, the microbial sample DNA migrated from 6^{th} and 8^{th} wells towards cathode, marker DNA was also migrated as usual from the 1st well (Figure1 d). At annealing temperature 54ºC also, the sample DNA migrated towards cathode from 5^{th} well (Figure 1 e). This result confirmed that these annealing temperatures are favorable for amplification of the microbial DNA extracted from the sewage water samples collected from shrimp processing plants. If the annealing temperature is high, the oligonucleotide primers anneal poorly and also the template DNA. Then the amplification of sample DNA will be very low. In carrying out polymerase chain reaction experiments, sometimes amplification of unwanted segments of DNA takes place because of the annealing of nonspecific primers. Annealing is usually carried out in a range of 3ºC to 5ºC lower than the calculated melting temperature (T_m), at which the oligonucleotide primers dissociate from their templates. The theoretical T_m values may differ from experimental T_m values for oligonucleotide primers of different lengths and sequences. To obtain the best results, it is to optimize the temperature at annealing by performing a series of trial PCRs with sample DNA. Alternatively, the thermocycler has to be programmed to use progressively lower annealing temperatures in consecutive pairs of cycles [6]. In this study, from the gel checks conducted with the amplified DNA obtained through PCR at different annealing temperatures, it is clear that most DNA was migrated from the wells at annealing temperature 53.8ºC and 54 ºC which proved best PCR condition for amplification of microbial DNA of sewage water samples from shrimp processing plants. The appearance of two clear bands (6^{th} & 8^{th} wells of the gel slab) at 53.8ºC indicates the migration of DNA fragments that are pure. At 54ºC annealing temperature migration of DNA takes place but only broad single band appeared which is close to the 5^{th} well there after the migration of amplified DNA sample is diffusing and overlapping. This indicates that the DNA was migrated from the well as high molecular weight band followed by migration of contaminated genomic DNA of the microbial populations present in the sewage water samples. The sample DNA requires more purification.

In this study even after seven times washing of microbial DNA obtained in the micro spin column and the same was loaded in the thermocycler for amplification no bands of amplified DNA visualized in the gel slab. In order to avoid the interruption of amplification of DNA by the high/low GC content, the temperature at annealing process was changed. In variable temperature conditions of annealing, partial success was achieved at 53.8ºC and 54ºC (Figure 1 d and 1 e). This result is encouraging to study the microbial biodiversity of water samples through metagenomic analysis.

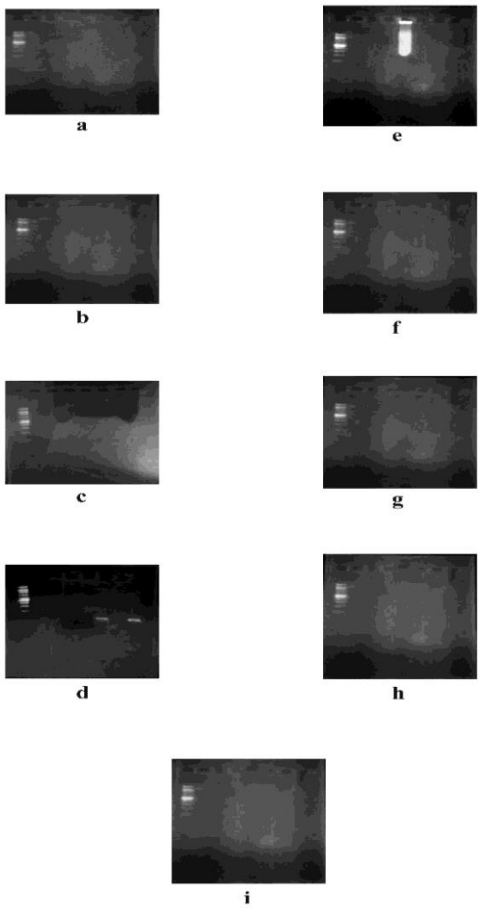

Figure 1. Agarose gel electrophoresis of DNA isolated from sewage water sample of shrimp processing plant at different annealing temperatures of PCR

Further this study opens avenues for a detailed study to optimize the PCR conditions for extraction of microbial DNA of sewage water samples.

4. CONCLUSION

This study aimed at metagenomic DNA extraction and analysis of sewage water samples from a shrimp processing industry in Visakhapatnam later turned to optimize the PCR conditions for amplification of extracted DNA from sewage water samples. This study revealed that the amplification of DNA while conducting PCR plays a very important role for further gel check studies. While conducting PCR, exposure of DNA sample to 53.8ºC annealing temperature for 30 seconds and 30 cycles are suitable for obtaining the fragments of DNA of micro and other organisms present in the sewage water.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals or humans were used for the studies that are based on this research.

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

REFERENCES

1. Handelsman J, Rondon MR, Brady SF, Clardy J, Goodman RM. Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chemistry & Biology. 1998; 5 (10): R245 - 249.
2. Zhou J, Bruns MA & Tiedje JM. DNA recovery from soils of diverse composition. Applied and Environmental Microbiology. 1996; 62 (2): 316 - 322.
3. Schmidt TM, DeLong EF & Pace NR. Analysis of a Marine Picoplankton Community by 16S rRNA Gene Cloning and Sequencing. Journal of Bacteriology. 1991; 173 (14): 4371- 4378.
4. Andrew O, Sayler GS & Barkay T. The extraction and purification of microbial DNA from sediments. Journal of Microbiological Methods. 1987; 7 (2): 57 - 66.
5. Thomas BT, Efuntoye MO, Kolawole RM, Popoola OD & Tajudeen, AO, Metagenomic tracking of microbial consortia of Cassava flakes (garri). Ife Journal of Science. 2021; 23 (2): 075 - 082.
6. Mamedov TG, Pienaar E, Whitney SE, TerMaat JR, Carvill G, Goliath R, Subramanian A & Viljoen HJ, A fundamental study of the PCR Amplification of GC-Rich DNA Templates. Computational Biology and Chemistry. 2008; 32 (6): 452 - 457.
7. Lee PY, Costumbrado J, Hsu CY, Kim YH, agarose gel electrophoresis for the separation of DNA fragments. Journal of Visualized Experiments. 2012; 20 (62): e3923 – e3928.
8. Assal N & Lin M, PCR procedure to amplify GC-rich DNA sequences of Mycobacterium bovis. Journal of Microbiological Methods. 2021; 181: 106121-127.
9. Acharya A, Blackburn A, Mohammed J, Haile AT, Hiruy AM, Werner D, Metagenomic water quality monitoring with a portable laboratory. Water Research. 2020; 184: 116112.
10. Acharya K, Khanal S, Pantha K, Amatya N, Davenport RJ, Werner D. A comparative assessment of conventional and molecular methods, including MinION nanopore sequencing, for surveying

water quality. Scitific Reports. 2019; 9 (1): 15726

11. Al-Ashhab A, Marmen S, Schweitzer-Natan O et al. Freshwater microbial metagenomes sampled across different water body characteristics, space and time in Israel. Scientific Data. 2022; 9 (1): 652.
12. Amos GC, Zhang L, Hawkey PM, Gaze WH, Wellington EM. Functional metagenomic analysis reveals rivers are a reservoir for diverse antibiotic resistance genes. Veterinary Microbiology. 2014; 171 (3-4): 441-447.
13. Bai Y, Liu R, Liang J, Qu J. Integrated metagenomic and physiochemical analysis to evaluate the potential role of microbes in the sand filter of a drinking water treatment system. PLos One. 2013; 8 (4): e61011.
14. Barrett T, Clark K, Gorekenkov V, Griboy E, Karsch-Mizrachi I, Kimelman M, Pruitt KD, Resenchuk S, Tatusova T, Yaschenko E, Ostell J. Bioproject and biosample database at NCBI: facilitating capture and organization of metadata, Nucleic Acids Research. 2012; 40 (database issue): D57-63.
15. Carrigg C, Rice O, Kavanagh S, Collins G, O'Flaherty V. DNA extraction method affects microbial community profile from soil and sediment. Applied Microbiology and Biotechnology. 2007; 77 (4): 955-964.
16. Chan OC, Liu WT, Fang HH, Study of microbial community of brewery-treating granular sludge by denaturing gradient gel electrophoresis of 16S rRNA gene. Water Science and Technology. 2001; 43(1): 77-82.
17. Chao y, Ma L, Yang Y, Ju F, Zhang XX, Wu WM, Zhang T. Metagenomic analysis reveals significant changes of microbial compositions and protective functions during drinking water treatment. Scientific Reports. 2013; 3: 3550, 1-9.
18. Daniel R, The metagenomics of soil. Nature Reviews Microbiology. 2005; 3 (6): 470-478.
19. Ferreira AJ et al. Core microbial functional activities in ocean environments revealed by global metagenomic profiling analyses. PLoS One. 2014; 9 (6): e97338.
20. Ghai R, Rodr F, McMahon KD, Toyama D, Rlnke R, Oliveria TCS, Garcia JW, Mlranda FP, Henrique-Silva F. Metagenomics of the water column in the pristine upper course of the Amazon River. PLoS One. 2011; 6 (8): e23785.
21. Gilbride KA, Lee Dy, beaudette LA. Molecular techniques in waste water: understanding microbial communities, detecting pathogens, and real time process control. Journal of Microbiological Methods. 2006; 66(1): 1-20.
22. Jayasree L, Janakiram P, Madhavi R. Isolation and characterization of bacteria associated with cultured Penaeus monodon afftected by Loose Shell Syndrome. Israeli journal of aquaculture-Bamidgeh. 2008; 6(1): 46-56.
23. Jimenez DJ, Andreote FD, Chaves D, Montan JS, Osorio-Forero C, Juncal H, Zambrano MM,

Baenal S. Structural and functional insights from the metagenome of an acidic hot spring microbial planktonic community in the Colombian Andes. PLoS One. 2012; 7 (12): e52069.

24. Lakay FM, Botha A, Prior BA. Comparative analysis of environmental DNA extraction and purification methods from different humic acid-rich soils. Journal of Applied Microbiology. 2007; 102 (1): 265-273.
25. Motro Y, Wainsztain D, Michael-Gayego A, Mathur S, Marano RB, Salah I, Rosenbluh C, Temper V, Strahilevitz J, Moran-Gilad J. Metagenomic sequencing for investigation of a national keratoconjunctivities outbreak, Israel, Eurosurveillance. 2023; 28 (31):2300010.
26. Ogram A, Sayler GS, Barkay T. The extraction and purification of microbial DNA from sediments. Journal of Microbiological Methods. 1987; 7 (2-3): 57-66.
27. Wang Y, Liao S, Gai Y, Liu G, Jin T, Liu H, Gram L, Strube ML, Fan G, Sahu SK, Liu S, Gan S, Xie Z, Kong L, Zhang P, Liu X, Wang DZ. Metagenomic analysis reveals microbial community structure and metabolic potential for nitrogen acquisition in the oligotrophic surface water of the Indian Ocean. Frontiers in Microbiology. 2021; 12: 518865.