Simplified cocktail mix and PCR conditions for amplification of extracted DNA from common bacteria, fungi and algae isolates for Microbiological studies

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Abstract: **Real-time quantitative polymerase chain reaction (qPCR) and digital PCR (dPCR) methods have revamped environmental microbiology, providing data about targeted nucleic acids of specific microorganisms found within the environment. These data are useful for the characterization of the interacting processes of targeted microbial communities. They also assess contaminant microbes within the environment (water, air, fomites). Amplification of DNA for identification of bacteria fungi or algae commonly isolated in microbiological studies is a common and constant problem in molecular analysis this may be due to various unforeseen problems like the concentration of the DNA and the Primer, the annealing temperature used or the presence of contaminate in the PCR mixture etc This study was done to address such issues.**

Here, we suggest suitable cocktail mixture protocols that can be successfully used to make high-quality qPCR and dPCR measurements of microorganisms in the environment yielding amplicons with good integrity, quality and concentrations that can be used for further analysis. DNA of varying concentrations from different samples were diluted using proposed ratios with water and used for PCR runs. The resulting amplicons were checked for their qualities and used for Sanger sequencing. The amplicons produced were of were of good quality and quantity which were successfully used for Sanger sequencing, giving sequences that were successfully blasted and found to be similar to sequences deposited in the NCBI repository with 90% similarity and above. The suggested protocols provide defined and direct mixing aliquots to be used in PCR mixtures for good amplification outcomes when working with DNA of varying concentrations.

***Keywords*: PCR, DNA concentration, Cocktail mixture, Amplicon**

I. INTRODUCTION

Polymerase Chain Reaction (PCR) was developed in 1983 by Kary Mullis (Kellenberger, 2004). It has remained a valuable and most frequently used method in molecular techniques in most Microbiological laboratories it works by making several copies of a particular DNA or targeted gene with the assistance of DNA polymerase (Clark *et al*., 2013, Najafov and Hoxhaj, 2017). Using this technique, a predetermined section of DNA sequence or fragment is copied or modified enhanced by DNA polymerase enzyme, which amplifies the specific fragments of the target DNA molecule added to the reaction (Clark *et al*., 2013). These nucleotides which are called primers contain the sequences that are complements of the target sequences in the target DNA molecule (Alberts *et al*., 2002). PCR techniques may also be used for introducing restriction enzyme sites at the ends of DNA molecules, or in changing specific bases of DNA (referred to as site-directed mutagenesis) (Hammerling, 2012). The reactions sequence in PCR is very fast so that the amplification of a DNA molecule can produce about 1 billion molecules within short periods (less than 2 hours) as the reaction is done under perfect conditions (Alberts *et al*., 2002; Clark *et al*., 2013).

Each PCR cycle has 3 basic steps: denaturation, annealing (or hybridization), and polymerization, (Hammerling, 2012; Clark *et al*., 2013). During denaturation, the double strands of the helix of the targeted genetic (DNA) materials are uncoiled, separating them by heating at high temperatures of 90° to 95°C. In the annealing phase, the oligonucleotide primers bind to their complementary bases on the single-stranded DNA. This step requires a much cooler temperature, 55°C (but this may vary depending on the primer). In the final phase, during polymerization (at 75°C), the template strand is usually read by polymerase and then rapidly paired with the appropriate nucleotides, giving rise to 2 new helices that consist of part of the original strand and the complementary strand that is assembled (Clark *et al*., 2013, Najafov and Hoxhaj, 2017). The targeted genetic material is doubled with every cycle which is repeated 30 to 40 times. When the procedure is completed, multiple identical copies (in millions) of the original specific DNA sequence are made. The amplicon copies are expected to migrate concurrently when subjected to electrophoresis to form a single band due to their similarities in electrical charge and molecular weight (Jennifer *et al*., 2012, Clark *et al*., 2013).

The extraction of quality DNA is critical for the majority of downstream applications in molecular biology but these may

have varying yields and concentrations of DNA (Dairawan and Shetty, 2020). Selecting an appropriate DNA protocol for extracting and purifying DNA is of utmost importance for most downstream applications in molecular biology. Presently, there are several chemical, mechanical and enzymatic methods that have been developed for extracting and purifying DNA from a variety of samples but the yield outcomes vary from one method to another and also among sample types. The quality of nucleic acids directly influences problems and artifacts produced by molecular biology procedures downstream. Thus, for efficient DNA amplification, for example, using the PCR method or isothermal DNA amplification, complete separation of nucleic acid strands at all lengths is required. This study aimed at providing a protocol for dilution of DNA with varied concentrations for use in cocktail mixtures that can successfully give amplicons with good integrity, quality and concentrations that can be used for further downstream analysis.

II. METHODOLOGY

Bacterial profiling is determined by using broad-range amplification and analyzing sequences of 16S rRNA genes (Ztemanick *et al*., 2017; Kayla *et al*., 2021). Each DNA sample was amplified in triplicate along with an index-specific negative PCR control (single mastermix; termed "standard protocol" throughout). Each reaction contained MasterMix (One Taq Quick-Load 2X Master Mix by New England Biolabs), and the cocktail mix reaction volume is shown in table 2.1. Cycling conditions were also presented in Table 2.3. After thermal cycling, the amplicons were assessed by agarose gel electrophoresis (pooled triplicates and negative control independently) for appropriately sized bands from the DNA template and no evidence of amplification from the negative controls. The DNA was subjected to the following cocktail mix and condition for the PCR utilizing the extracted DNA.

Table 2.1 cocktail mix for PCR analysis using DNA with different concentrations

	Below 50 (ng/µl)	**Below 100 (ng/µl)**	**100-400 (ng/µl)**	**500-1000 (ng/µl)**	**1000-4000 (ng/µl)**
Master mix	12 µL	12 µL	12 µL	12 µL	12 µL
10mMol forward primer	2 µL	2 µL	2 µL	2 µL	2 µL
10m Mol reverse primer	2 µL	2 µL	2 µL	2 µL	2 µL
undiluted DNA	5 µL	3 µL	1.5 µL	1.0 µL	.5 µL
H_2O	10 µL	15 µL	17.5µL	18 µL	18.5 µL
Total	35 µL	35 µL	35 µL	35 µL	35 µL

Table 2.2 Primers sequences

Primer	**type**	**sequence**	**Reference**
16S	**27F**	**5'-AGAGTTTGATCMTGGCTCAG-3'**	(Tao *et al*., 2016)
	1492R	**5'-TACGGTACCTTGTTACGACTT**	
ITS	**ITS1F**	**5'-TCCGTAGGTGAACCTGCGG-3'**	**(**White *et al*., 1990**)**
	ITS2R	**5'-GCTGCGTTCTTCATCGATGC-3'**	
18S	**18S-F**	**5'-AACCTGGTTGATCCTGCCAG-3'**	**(**Nina *et al*., 2017**)**
	18S-R	**5'-CACCACAGACTTGCCCTCCA-3'**	

Table 2.3 PCR CONDITIONS FOR ITS, 16S and 18S

	16S		**ITS**		**18S**	
	Temp.	**Time**	**Temp.**	**Time**	**Temp.**	**Time**
Initial den.	**95˚c**	**5 mins**	**94˚c**	**4 mins**	**94˚c**	**5 mins**
Den.	**94**	**30 sec**	**94˚c**	**30 sec**	**94˚c**	**30 sec**
Ann. tempt	**58**	**30 sec**	**56**	**45 sec**	**53**	**30 sec**
Extension	**72˚c**	**45 sec**	**72˚c**	**1 min**	**72˚c**	**45 sec**
No. of circles	**25**		**35**		**35**	
Final Extension	**72˚c**	**7mins**	**72˚c**	**10min**	**72˚c**	**7mins**
Hold tempt	**10˚c**	∞	**4˚c**	∞	**4˚c**	∞

III. RESULTS

The concentration of DNA:

DNA of varying concentrations was selected f algal, fungal and bacterial cultures and loaded on 1% gel as shown in fugures1, 2 and 3. In figure 1, A had the lowest concentrations of below 100 (ng/µl), while B had a concentration of below 1000 (ng/µl) and C had a concentration of above 1000 (ng/µl). In figure 2, A had the lowest concentrations of below 50 (ng/µl) for bacteria cultures, while B (bacteria), D (bacteria) and F (fungal) had a concentration of below 100 (ng/µl) C, E and G had concentrations of between 500- 1000 (ng/µl), while H and I had concentrations above 1000 (ng/µl)

PCR products:

The resulting amplicons of the 17 samples sequenced from the suggested protocols as shown in figures 3, 4 and 5, showed that amplicons were successfully obtained after running the PCR using the stated number of cycles. The fungal amplicons (650bp size) obtained were almost of similar concentrations (figure 3) and passed quality-control tests and were successfully used for Sanger sequencing. Similarly, the bacterial amplicons (1500bp size) obtained were almost of similar concentrations (figure 5) and passed quality-control tests and were successfully used for Sanger sequencing. Although differing concentrations were obtained for the Amplicons obtained from the algal DNA (figure 4) they all passed the Q.C test and were used for Sanger sequencing and good DNA sequences were obtained.

60 samples were sequenced from the modified protocol, and only 5 samples failed to amplify when this protocol was followed. In addition, 10 samples were amplified in triplicate using each protocol to assess technical variability and similar results were obtained.

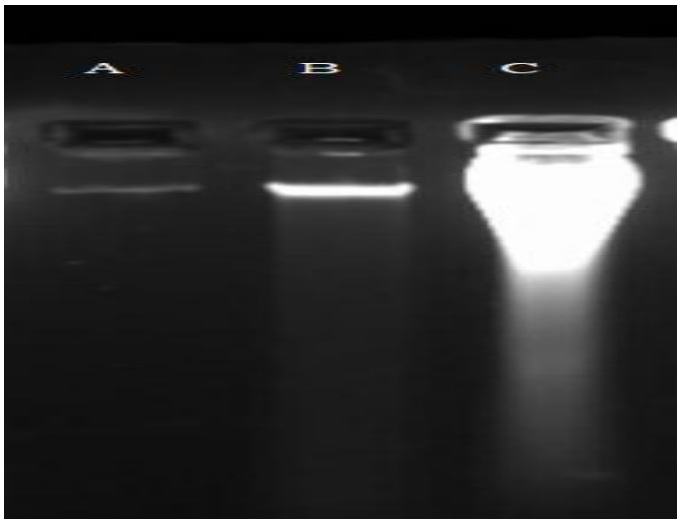

Figure1: Extracted DNA of Algal cultures with varying concentrations loaded on 1% gel

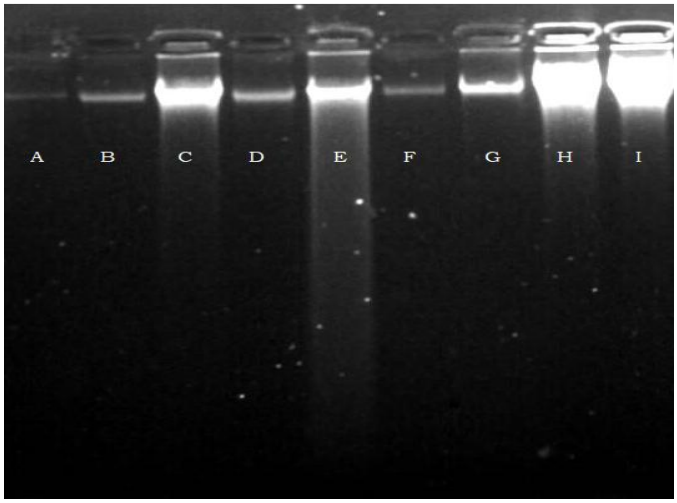

Figure 2: Extracted DNA of Bacterial and fungal cultures with varying concentrations loaded on 1% gel

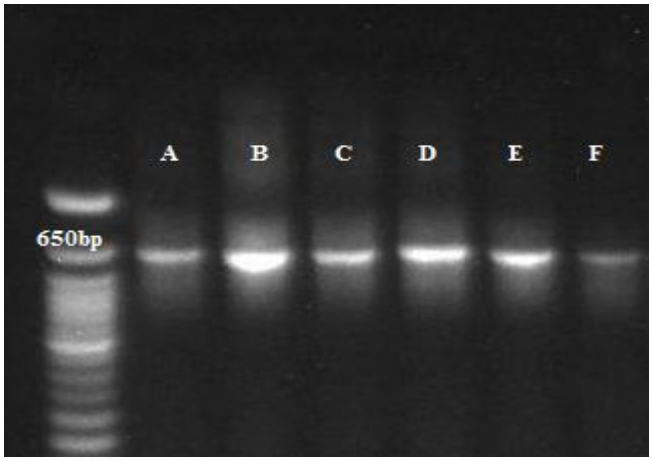

Figure 3: PCR amplicons of Fungi DNA using ITS1 and ITS4 primers

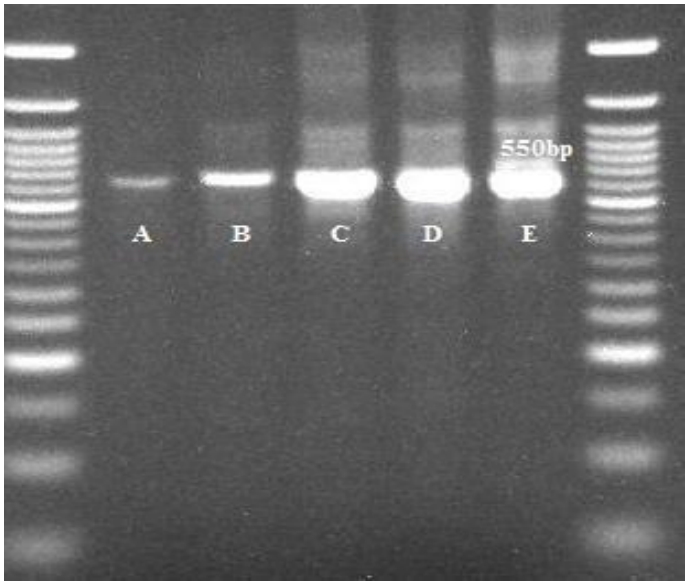

Figure 4: PCR amplicon for Algal DNA using 18S primers

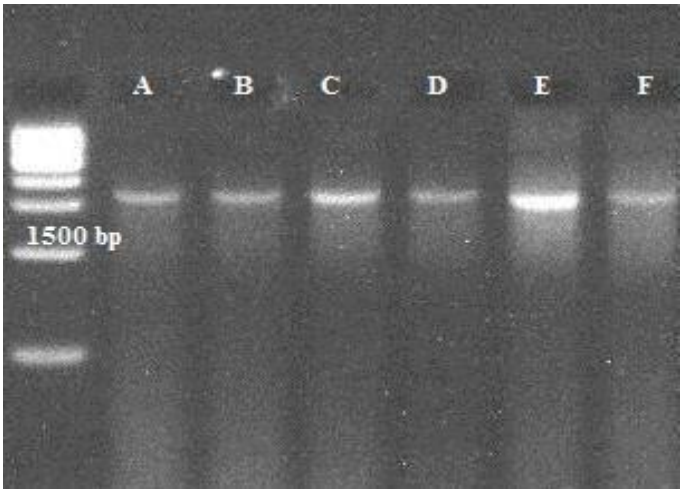

Figure 5: PCR amplicon for Bacterial DNA using 16S primers

IV. DISCUSSION

The modified protocol provides an easy method of using DNA with varying concentrations after extraction to run PCR that gives good quality and quantity of amplicon for further sequencing analysis this was similar to the report of Bu¨rgmann *et al*. 2001 and Dairawan and Shetty, 2020 (where it was stated that no single extraction procedure applies to all contexts of DNA extraction concerning producing yields with optimal purity for convenience of use).

The amplicons were able to migrate concurrently when subjected to electrophoresis forming single bands due to their similarities in electrical charge and molecular weight as is explained in the findings of Jennifer *et al*. 2012 and Clark *et al*. 2013).

The fungal amplicons (650bp size) obtained were similar to those obtained (300- 500 bp) in the findings of Fujita *et al.* 2001 and (600 and 900 bp) as seen in the work of Toju et al. 2012; Mbareche et al. 2020. Similarly, the bacterial amplicons (1500bp size) were in the same size range (~1400bp) obtained in the work of Frank *et al*. 2008 and Dos Santos *et al*. 2019. Algal amplicons (550bp size) obtained was similar to that obtained in the work of Christa et al. 2012 which was about 500bp and differed from the size of amplicon obtained in the work of The resulting amplicons from the PCR run using the above protocols were of good quality and quantity which were successfully used for Sanger sequencing this was similar to the findings of Ztemanick *et al*. 2017and Kayla *et al*. 2021. For samples that were successfully sequenced following the above protocol, the sequences were successfully blasted and were found to be similar to sequences deposited in the NCBI repository with 90% similarity and above for the fungal, bacteria and Fungi isolates. The rationale was to provide a protocol for dilution of DNA with varied concentrations for use in cocktail mixtures that are sufficiently able to obtain adequate amplification for reliable sequencing and also aimed at successfully producing amplicons with good integrity, quality and concentrations.

V. CONCLUSION

We show that the suggested cocktail protocols used were successfully used in amplifying DNA of different concentrations and can be used as a standard protocol especially when samples with varying bacterial loads are extracted giving rise to different DNA concentrations. This is likely due to the improved efficiency of PCR using non-fusion primers. Therefore, our suggested cocktail mixture protocol provides a reliable positive amplification when running PCR resulting in amplicons with good integrity, quality and concentrations that can be used for further molecular analysis.

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