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Exploring the Fundamentals of Molecular Biology: From DNA Extraction to Gene Cloning and Biochemical Analysis

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Abstract: *Molecular biology is essential for understanding biological systems, with DNA extraction, polymerase chain reaction (PCR) analysis, gel electrophoresis, and microbial biochemical testing serving as key methodologies. This study explores the extraction of DNA from bacterial, fungal, and plant samples, highlighting challenges and optimized protocols for high-quality nucleic acid retrieval. PCR analysis is examined in genetic analysis, disease detection, and molecular cloning, emphasizing primer specificity and real-time PCR advancements. Gel electrophoresis is discussed as a crucial tool for verifying PCR products and genetic modifications. Microbiological techniques such as Gram staining and biochemical assays aid in bacterial identification, particularly in sugarcane tissues and fish gut microbiota, providing insights into microbial interactions. Gene cloning methodologies, including restriction enzyme digestion, ligation, and PCR-based cloning, are explored for their applications in biotechnology and medical research. Despite advancements, challenges persist in nucleic acid purification and amplification efficiency. This study summarizes fundamental molecular biology techniques, their applications, and ongoing improvements, offering insights into future research directions.*

Key Words: *Molecular biology, DNA extraction, PCR analysis, gel electrophoresis, gene cloning, bacterial identification.*

1. INTRODUCTION:

Molecular biology has significantly transformed our understanding of biological processes. The study of nucleic acids, particularly DNA and RNA, has revolutionized forensic science, genetic engineering, clinical diagnostics, and biotechnology. Nucleic acid analysis is an essential tool in diverse applications, including the detection of infectious diseases, genotyping, forensic identification, and agricultural breeding programs [1]. The ability to extract and analyze DNA from various biological sources enables advancements in molecular epidemiology and gene manipulation, thereby expanding our knowledge of genetic and epigenetic regulatory mechanisms [2].

The first step in molecular analysis is DNA extraction, which varies depending on the biological source and intended downstream applications. For instance, isolating DNA from plant tissues requires overcoming unique challenges posed by secondary metabolites such as polyphenols, which interfere with nucleic acid integrity [3]. Similarly, fungal DNA extraction is complicated by rigid cell walls composed of chitin and polysaccharides, necessitating mechanical disruption or enzymatic digestion for effective lysis. Various protocols have been developed for different species, such as the rapid and hazardous-reagent-free methods for *Arabidopsis thaliana* [4] and improved techniques for the encapsulated yeast *Cryptococcus neoformans*, where capsule relaxation and bead beating are required for successful extraction [2].

DNA extraction from bacteria follows a different approach due to their distinct cell wall composition. Gram-positive bacteria possess a thick peptidoglycan layer, requiring enzymatic digestion or mechanical disruption before lysis, whereas Gram-negative bacteria can be lysed more easily using detergent-based methods [5]. Extraction of bacterial DNA plays a crucial role in the identification and analysis of microbial populations in various environments, including soil, water, and host-associated microbiomes. Efficient isolation methods are necessary for downstream applications such as sequencing, phylogenetic analysis, and microbial ecology studies.

Once extracted, DNA is subjected to polymerase chain reaction (PCR) amplification. PCR-based methods play a crucial role in molecular cloning, where recombinant DNA technology is used to insert, replicate, and manipulate

genetic material within host cell. This technique is fundamental in genetic engineering, enabling applications such as gene expression studies, functional genomics, and transgenic organism development. Advances in PCR technology, such as high-fidelity DNA polymerases and optimized reaction conditions, have enhanced the accuracy and efficiency of gene cloning [6]. The use of real-time PCR (qPCR) has revolutionized gene expression analysis, providing quantitative insights into biological processes at the molecular level.

PCR analysis is followed by gel electrophoresis, which enables the separation and visualization of amplified DNA fragments. DNA samples are loaded into an agarose gel matrix and subjected to an electric field, causing negatively charged DNA molecules to migrate toward the positive electrode. The migration rate depends on fragment size, allowing the estimation of DNA length based on molecular weight markers [6]. Ethidium bromide is commonly used to stain the DNA, providing a fluorescence-based method for fragment visualization. Gel electrophoresis is critical for verifying the success of PCR amplification and for confirming the presence of specific genetic markers in diagnostic applications.

Biochemical and microbiological analyses is used in characterizing bacteria. Gram staining is used for bacterial classification, differentiating organisms based on cell wall composition. It is particularly useful in studying bacterial populations associated with sugarcane, where samples are collected from various plant tissues, including roots, internal tissues, leaves, and nodes. The presence and distribution of bacterial species in these regions provide insights into plant-microbe interactions, including symbiotic relationships and pathogenic infections [7]. Furthermore, biochemical tests such as catalase, oxidase, and carbohydrate fermentation assays help identify bacterial metabolic capabilities, aiding in species identification and functional characterization. These microbiological techniques have broad applications in agriculture, environmental science, and industrial biotechnology, where bacterial consortia are harnessed for beneficial purposes.

The biochemical analysis of bacteria found in the gut of fish is essential for understanding microbial ecology in aquatic environments. Gut microbiota influence host nutrition, disease resistance, and overall health, making their study relevant for aquaculture and environmental monitoring. Standard techniques include the isolation of bacterial strains, DNA extraction, and 16S rRNA sequencing for taxonomic classification. The role of gut bacteria in metabolizing organic compounds, producing essential nutrients, and modulating immune responses highlights the importance of microbiome research in aquatic biology [2]. Furthermore, understanding fish gut microbiota can aid in the development of probiotics, enhancing fish health and growth while reducing dependence on antibiotics in aquaculture systems.

Gene cloning represents involving the insertion of target DNA sequences into vector systems for replication and functional studies. Cloning methods often utilize restriction enzyme digestion, ligation into plasmid vectors, and transformation into bacterial hosts for propagation. Modern approaches, such as PCR-based cloning and Gibson assembly, offer high efficiency and precision in genetic manipulation [6]. Recombinant DNA technology is widely applied in synthetic biology, protein production, and genetic engineering, contributing to advancements in medicine, agriculture, and biotechnology. The ability to create genetically modified organisms (GMOs) has far-reaching implications in pharmaceutical production, biofuel development, and crop improvement.

Despite these advancements, challenges persist in DNA extraction and amplification, particularly in complex biological matrices. Researchers continue to refine methodologies to enhance nucleic acid purity, yield, and stability, ensuring the reliability of downstream applications. The main objectives of this study are to explore fundamental molecular biology techniques, including DNA extraction, PCR amplification, gel electrophoresis, and gene cloning, while addressing their applications, challenges, and future prospects in research and biotechnology.

2. METHODOLOGY:

- **Extraction of DNA from different samples:**

i. DNA extraction from Bacteria:

Bacterial samples were inoculated in nutrient broth from the slants for extraction and incubated for 24 hrs. Harvest the cell from the broth with the help of centrifuge, centrifuge the broth at 15000 rpm for 10 min. Discard the supernatant, to the pellet add 500 µl of lysis buffer and 120 µl of EDTA ,20 µl proteinase K. Vortex the solution for about 30 sec and incubate at 55C for 30 min and centrifuge at 15000 rpm for 5 min Transfer the supernatant to new tube, add 500 µl protein precipitation solution and vortex for about 5 min and centrifuge the solution at 15000 rpm for 5 min. Transfer the supernatant to new tube and add 600µl chilled Isopropanol to it, mix by inversion. Transfer all the contents to spin column and centrifuge at 12000 rpm for 2 min. Discard the flow through and add 600 µl chilled ethanol to it and then centrifuge at 12000 rpm for 2 min. Discard the flowthrough, perform blank centrifuge for 1 min to remove any residual ethanol. Transfer the column to new tube and add 50 µl elution buffer on the membrane. Incubate for 10-15 min and centrifuge at 12000 rpm for 2 min. Discard the column and store the DNA sample for further use [8].

ii. Isolating Genomic DNA from fish, Snail, Insect, Bee, Parasite, Tick:
In a centrifuge tube, mix 500 µl of Nuclei Lysis Solution with 120 µl of 0. 5M EDTA solution (pH 8. 0) for each sample. Cool this mixture on ice, where it will become hazy. Prepare a 1. 5 ml microcentrifuge tube with 0. 5–1 cm of fresh or thawed fish tissue. Grind the tissue into a fine powder using a chilled mortar and pestle and move it to the microcentrifuge tube. Add 600 µl of EDTA/Nuclei Lysis Solution and 20 µl of Proteinase K to the tube. Shake gently while incubating at 55°C overnight, or for three hours while shaking, vortexing once each hour. Check that the tissue is fully broken down. Add 3 µl of RNase Solution to the lysate and mix by flipping the tube. Incubate at 37°C for 15 to 30 minutes and cool the sample for five minutes. Add 200 µl of Protein Precipitation Solution, vortex for 20 seconds, then chill on ice for five minutes. Centrifuge at 15,000 rpm for 5 minutes, remove the supernatant, and transfer to a new tube with 600 µl of isopropanol. Invert gently to form DNA threads, centrifuge at 15,000 rpm for 1 minute, and decant the supernatant. Wash the DNA pellet with 600 µl of 70% ethanol, centrifuge again, and carefully aspirate the ethanol. Let the pellet air dry for 10-15 minutes. Rehydrate the DNA with 100 µl of DNA Rehydration Solution at 65°C for an hour or at room temperature or 4°C overnight. Store the DNA between 2 and 8°C [8.9].

iii. DNA Extraction from Plant:
Transfer the ground plant tissue into a polypropylene tube. For each 100 mg of homogenised tissue, add 500 µl of CTAB Buffer. Stir and fully vortex. Place the tube in a water bath at 60°C for 30 minutes. Centrifuge the homogenate for 5 minutes at 15,000 rpm. Transfer the supernatant into a fresh tube. Add 5 µl of RNase A solution and incubate at 37°C for 20 minutes. Add an equal proportion of phenol, chloroform, and isoamyl alcohol (25:24:1). To separate the phases, vortex the sample for 5 seconds before centrifuging it at 15000 rpm for 1 minute. Transfer the aqueous top phase into a fresh tube. Repeat the extraction until the top phase is clear. Transfer the top aqueous phase into a new tube. Add 0.7 litre of cold isopropanol and incubate at -20°C for 15 minutes to precipitate the DNA. Centrifuge the sample at 15000 rpm for ten minutes. Decant the supernatant without disturbing the particle, then wash with 500 µl of ice-cold 70% ethanol. Centrifuge at 15,000 rpm for 2 minutes. Decant the ethanol. Air drying is used to remove any remaining ethanol. Dry the pellet long enough to eliminate the alcohol without totally drying the DNA. Dissolve the DNA pellet in 20 µL TE buffer. The pellet may need to be warmed before dissolving. Store for future use [10, 11].

iv. DNA Extraction from Fungi:
Transfer the fungal mycelium to a polypropylene tube. For each 100 mg of mycelia, add 500 µl of CTAB Buffer. Stir and fully vortex. Place the tube in a water bath at 60°C for 30 minutes. Centrifuge the homogenate for 5 minutes at 15,000 rpm. Transfer the supernatant into a fresh tube. Add 5 µl of RNase A solution and incubate at 37°C for 20 minutes. Add an equal proportion of phenol, chloroform, and isoamyl alcohol (25:24:1). To separate the phases, vortex the sample for 5 seconds before centrifuging it at 15000 rpm for 1 minute. Transfer the aqueous top phase into a fresh tube. Repeat the extraction until the top phase is clear. Transfer the top aqueous phase into a new tube. Add 0.7 litre of cold isopropanol and incubate at -20°C for 15 minutes to precipitate the DNA. Centrifuge the sample at 15000 rpm for ten minutes. Decant the supernatant without disturbing the particle, then wash with 500 µl of ice-cold 70% ethanol. Centrifuge at 15,000 rpm for 2 minutes. Decant the ethanol. Air drying is used to remove any remaining ethanol. Dry the pellet long enough to eliminate the alcohol without totally drying the DNA. Dissolve the DNA pellet in 20 µL TE buffer. The pellet may need to be warmed before dissolving. Store for future use [12].

v. DNA Extraction from Stool Sample:
Take 250 mg of stool sample and mix it with 1ml of TE buffer. Vortex it well and centrifuge at 8,000 x g for 3 minutes. Discard the liquid on top and mix the pellet with 500 µl of Lysis Solution. Transfer 200 µl of this mix to a new tube for lysis. Add 20 µl of Proteinase K Solution to the 200 µl of the resuspended solution, mix, and heat for 30 minutes at 55°C. If RNA-free DNA is needed, add 25 µl of RNase A solution, mix, and let it sit for 5 minutes before proceeding. Next, add 200 µl of Stool Lysis Buffer, vortex, and heat at 70°C for 10 minutes. Add 250 µl of Inhibitor Removal Solution, vortex, and cool for 5 minutes. Centrifuge for 1 minute, then transfer the liquid to a new tube. Add 200 µl of Binding Solution, mix, and load onto the HiElute Miniprep Spin Column. Centrifuge, discard the liquid, and wash twice with diluted Wash Solution. Finally, transfer the column to a new tube, add 200 µl of Elution Buffer, and centrifuge again. Store the resulting liquid in a new tube [13].

- **Estimation of DNA concentration using fluorometer**

Warm all assay components to room temperature before use. To prepare a Blank Sample, add 200µl of QuantiFluor ONE dsDNA Dye to an empty 0. 5ml PCR tube. For the 400ng Standard Sample, add 1µl of the provided QuantiFluor

ONE Lambda DNA standard (400µg/ml) to 200µl of QuantiFluor ONE dsDNA Dye in an empty 0. 5ml PCR tube. Vortex well and protect the tube from light. Alternatively, to reduce pipetting errors, add 2µl of the 400ng/µl DNA standard to 400µl of QuantiFluor ONE dsDNA Dye. To prepare the Unknown Sample, add 2µl of the sample to 200µl of QuantiFluor ONE dsDNA Dye, vortexing well and protecting it from light. Mix all tubes thoroughly by vortexing or pipetting without introducing bubbles, then incubate for 5 minutes at room temperature, shielded from light. Select the ONE DNA protocol on the Quantus™ Fluorometer. Calibrate by reading the blank and standard samples, then save. Enter the volume of the unknown sample and measure fluorescence, which indicates the concentration of the original sample [14].

- **PCR Analysis of DNA Sample**

PCR analysis was conducted using a Bio-Rad T100 Thermal Cycler, with specific primers and optimized annealing temperatures for each DNA sample across 35 cycles of amplification. The reaction mix included template DNA, primers, dNTPs, Taq polymerase, and buffer. The amplified products were analyzed using 1.5% agarose gel electrophoresis to confirm expected fragment sizes [1]. Different primers used for the PCR analysis of different DNA are as follows

Table 1: Primers used for PCR Analysis of each DNA Sample

Sample Type	Region	Primers	Annealing Temp (°C)	Cycles	PCR Components					
					10X Buffer (µl)	Primers (µl each)	Water (µl)	$MgCl_2$ (µl)	dNTP (µl)	Taq DNA (µl)
Bacterial DNA	16s rRNA	27F, 1492R	54	35	2.5	1	16.5	0.3	0.4	0.4
Fungal DNA	ITS	ITS 1, ITS 4	54	35	2.5	1	16.5	0.3	0.4	0.4
Plant DNA	MAT K	MatK F, MatK R	53	35	2.5	1	16.5	0.3	0.4	0.4
Bees DNA	COI	LEP F1, LEP R1	51	35	2.5	1	16.5	0.3	0.4	0.4
Fish & Snail DNA	COI	LCO 1490, HCO2198	50	35	2.5	1	16.5	0.3	0.4	0.4
Insect DNA	COI	LCO 1490, HCO2198	50	35	2.5	1	16.5	0.3	0.4	0.4

- **Gel Electrophoresis**

The PCR amplicons were allowed to run on 1.3% agarose gel for 30 min. The Gel was prepared by mixing 0.65g of Agarose in 50 ml 1X TAE buffer. The gel was melted in microwave oven till it turns into clear solution. The gel was allowed to cool to 50-55° C and 2 µl ETBR was added and mixed properly and poured in casting tray and allowed to solidify. The Gel was allowed to run for 30 min at 100 V and 110 Amp. After the complete run the gel was visualized in UV transilluminator [1].

- **Gram Staining of Bacteria found in various parts of Sugarcane**

Prepare a thin stain on a transparent, dry glass slide. Allow it to air dry before fixing with mild heat. Flood with Gram's Crystal Violet (S012) for 1 minute. (If excessive staining causes incorrect decolourization of recognized gram-negative organisms, use less crystal violet). Wash with tap water. Fill the smear with Gram's Iodine (S013). Allow it to remain for one minute. Decolourize the smear with Gram's Decolourizer (S032) until the blue dye stops flowing. Wash with tap water. Counterstain using 0.5% w/v Safranin (S027) for 20 seconds, then rinse with water. Wash with tap water. Allow the slide to air dry or blot dry between sheets of clean bibulous paper and examine under oil immersion objective [15].

- **Biochemical analysis of Bacteria found in Gut of Fish and various parts of Sugarcane** [15,16]**:**

i. Indole Test:

To perform the indole test, inoculate a tube of tryptone broth with a small amount of a pure culture and incubate it at 37°C for 24 to 48 hours. After incubation, add 5 drops of Kovac's reagent to the tube. A positive result shows a pink to

red colour at the top of the medium, called a "cherry-red ring," within seconds. If indole is not produced, the reagent layer stays yellow or becomes slightly cloudy [16].

ii. Citrate Test:
For the citrate test, use a fresh pure culture (16- to 18-hour old). Lightly streak the surface of the slant with a single isolated colony using a needle, which helps limit the amount of bacteria transferred. Do not use liquid cultures. Since citrate utilization needs oxygen, keep screw caps loose. Incubate at 35°C (± 2°C) for 18 to 48 hours, although some cultures may take up to 7 days to grow. A positive result shows growth and an intense Prussian blue colour, while a negative result shows little to no growth with no colour change, keeping the medium deep forest green [].

iii. Urease Test:
Prepare a urea agar slant and label it with your name, the organism, and the test media. Streak the entire slant with a heavy amount of bacteria using a sterile inoculating loop. Incubate at 35 to 37°C. A positive urease test is indicated by a pink colour.

iv. MR-VP Test:
For the VP test, pick isolated colonies from an 18 to 24-hour culture and inoculate broth. Incubate for 18 to 24 hours at 35±2°C. After this, transfer 2 mL of broth to a clean test tube, add 6 drops of Reagent A and 2 drops of Reagent B, and mix. A red-pink colour after 30 minutes indicates a positive result. If no colour appears, re-incubate for 24 hours and test again.
For the MR test, inoculate MRVP broth with a pure culture and incubate for at least 48 hours. Add 5 or 6 drops of methyl red reagent. A bright red colour indicates a positive result, while yellow indicates a negative result.

- **Gene Cloning**

i. Day 1: Culture Preparation:
Open the vial containing bacterial culture and resuspend the cells in 0.25 ml of LB broth.Using a sterile loop, streak the resuspended culture onto an LB agar plate. Incubate the plate overnight at 37°C to allow bacterial colony formation.

ii. Day 2: Plasmid Ligation Setup:
Pick a single colony from the overnight culture and inoculate it into 1 ml of LB broth. Incubate at 37°C overnight with shaking to promote bacterial growth. Thaw the necessary reagents on ice, including 10X Ligase Buffer, T4 DNA Ligase, Vector DNA, and Insert DNA. Prepare the ligation reaction mix as Molecular biology-grade water (2 µl), Vector DNA (3 µl) Insert DNA (10X) (3 µl), Ligase buffer (1 µl), T4 DNA Ligase (1 µl) & thus total volume is10 µl. Gently mix by tapping and incubate overnight at 16°C in a water bath for efficient ligation.

iii. Day 3: Transformation and Competent Cell Preparation

a. **Preparation of Competent Cells:**
Transfer 50 ml of LB broth into a sterile flask. Add 1 ml of overnight grown culture into the LB broth. Incubate at 37°C on a shaker (250 rpm) for 2-3 hours until OD600 reaches ~0.6. Transfer the culture into a pre-chilled 50 ml polypropylene tube and store on ice for 10 minutes. Centrifuge at 5000 rpm for 10 minutes at 4°C and discard the supernatant completely. Resuspend the bacterial pellet in 30 ml pre-chilled sterile 0.1M Calcium chloride solution. Incubate on ice for 30 minutes. Repeat centrifugation at 5000 rpm for 10 minutes at 4°C, discard the supernatant completely. Resuspend the pellet in 2 ml pre-chilled sterile 0.1M Calcium chloride solution. The competent cell suspension is now ready for transformation.

b. Transformation of Cells
Take 200 µl of competent cell suspension into four 2.0 ml collection tubes labeled as: Control, Positive Control Plasmid, Negative Control Plasmid Ligation Mix. Add 2 µl of positive control plasmid DNA to the positive control tube and 2 µl of negative control plasmid DNA to the negative control tube, mix well. Add 10 µl of ligation mix to the respective tube and mix well. Incubate all tubes on ice for 30 minutes. Perform heat shock at 42°C for 2 minutes to facilitate DNA uptake. Rapidly transfer the tubes on ice for 5 minutes. Add 800 µl of LB broth to all tubes and incubate at 37°C for 1 hour to allow expression of antibiotic resistance genes [17,18].

5. RESULTS:

Fig. 1. PCR Thermal Cycler

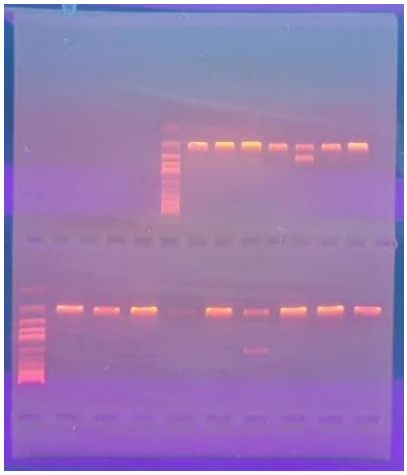
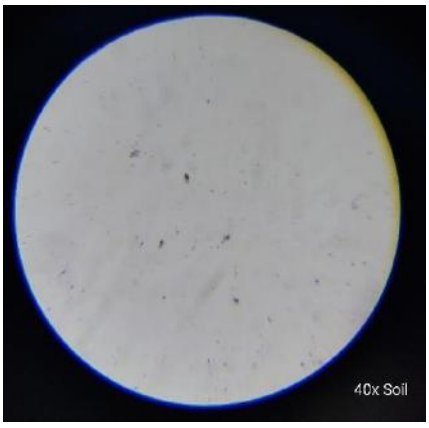
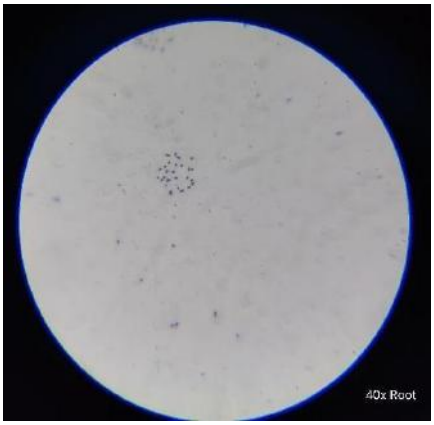


Fig. 2. Gel Electrophoresis

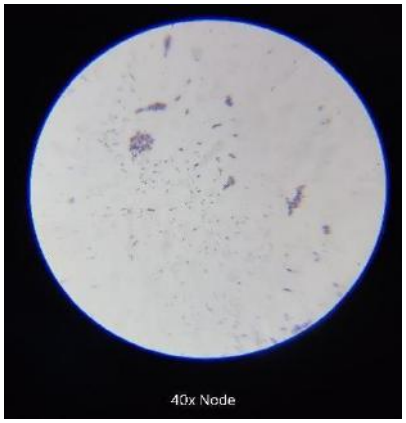
Fig.3. Quantas Fluorometer



4.(a)



4.(b)



4.(c)

Fig.4. Gram Staining results of Bacteria from (a) Soil (b) Root (c) Node of Sugarcane

Quantas Readings:

Table 2. Quantas Reading of Bacterial Samples

Bacteria	Readings (µg/ml)
1	143.19
2	157.55
3	149.23
4	79.24
5	151.70

Table 3. Quantas Reading of Parasite (Tick) Samples

Parasite (Tick)	Readings (µg/ml)
1	83.97
2	50.76
3	54.17
4	10.89
5	23.87

Table 4. Quantas Reading of Fungal Samples

Fungus	Readings (µg/ml)
1	119.14
2	152.70
3	153.58

Table 5. Quantas Reading of Insect Samples

Insect	Readings (µg/ml)
1	158.66
2	75.87
3	79.82

Table 6. Quantas Reading of Bacterial Samples

Fish	Readings (µg/ml)
1	129.28
2	184.34
3	161.45

Table 7. Quantas Reading of Plant Samples

Plant	Readings (µg/ml)
1	111.87
2	140.25
3	145.66

Table 8. Quantas Reading of Stool Samples

Stool	Readings (µg/ml)
1	1.87
2	2.74
3	7.83

Table 9. Gram Staining Results

Site	Observation
Root	Gram - ve
Node	Gram - ve
Internal Tissue	Gram - ve

Table 10. Biochemical Tests of Bacteria found in various parts of Sugarcane

Test	Result
Indole	+ve
Citrate	-ve
Urease	+ve
MR	-ve
VP	-ve

Table 11. Biochemical Tests of Bacteria found in Fish Gut

Test	Result
Indole	-ve
Citrate	+ve
Urease	+ve
MR	-ve
VP	+ve

The results from this study confirm the successful DNA extraction from various biological sources, including bacterial, fungal, plant, insect, and animal tissues. The DNA yield and purity varied among samples, with bacterial DNA showing the highest concentrations, particularly in sample 2 (157.55 µg/ml) and sample 5 (151.70 µg/ml), while stool samples exhibited the lowest DNA concentrations, with readings as low as 1.87 µg/ml. The DNA extraction protocol effectively isolated genetic material with minimal degradation, ensuring suitability for subsequent molecular applications.

PCR analysis yielded successful amplification across all DNA samples, confirming the effectiveness of primer selection and optimization. The expected amplicon sizes were observed for 16S rRNA in bacterial samples, ITS regions in fungal samples, MAT K in plant DNA, and COI in fish, snails, and insect DNA. The results were consistent across both standard extraction methods and commercial kits.

Gel electrophoresis validated the integrity of the PCR products. Clear, distinct bands corresponding to the expected fragment sizes were observed in the agarose gel, confirming the efficiency of amplification. No significant smearing was detected, indicating minimal degradation of DNA. The use of ethidium bromide staining facilitated visualization under UV light, and molecular weight markers confirmed the accuracy of band sizes.

Microbiological assays further complemented the molecular findings. Gram staining results indicated that bacterial isolates from sugarcane root, internal tissues, and nodes were predominantly Gram-negative. Biochemical tests of bacteria from sugarcane samples revealed positive results for indole and urease production but negative results for citrate utilization, methyl red (MR), and Voges-Proskauer (VP) tests. Conversely, bacterial isolates from fish gut exhibited a different biochemical profile, with negative indole results but positive VP and urease tests.

Gene cloning experiments confirmed the successful insertion of target DNA fragments into plasmids. Blue-white screening on LB agar plates supplemented with ampicillin, X-Gal, and IPTG enabled the identification of recombinant colonies. The presence of white colonies confirmed the successful uptake of recombinant plasmids, while blue colonies indicated non-recombinant plasmids. Subsequent plasmid isolation and restriction digestion analysis validated the presence of the inserted gene sequences

6. DISCUSSION

The DNA extraction form different types of samples was performed successfully. The DNA extraction from different samples is the most important steps in their phylogenetic analysis. The isolated DNA concentration is determined with the help of Quantas-fluorometer; the quantity is compared with the standard of 200 ng/µl or 200 µg/ml.

This quantity is helpful in determining the amount of template which is added in the PCR reaction. If the reading of fluorometer was greater than the desired concentration the DNA samples were diluted with Nuclease free water to reduce the concentration of DNA, if the reading was too low then the amount if DNA added to PCR reaction was increased. The DNA template was added to the PCR reaction and was set as per number of copies required. For the PCR product to be of good quality it is very essential to store all the PCR in freezer when not in use this prevents from the denaturation of chemicals such as taq DNA polymerase, dNTPs etc. The PCR analysis of all samples was performed according to the region which is to be amplified or studied such as 16s rRNA or Cytochrome oxidase 1 or ITS region. After the amplification all the reaction are run on to an agarose gel of 1.5% and the bands are compared with the molecular ladder of 1kb length. To check whether desired region is amplified the PCR reaction was run on agarose gel and viewed under UV light.

7. CONCLUSION

This study showed how molecular biology techniques work well with different biological samples. The improved DNA extraction methods provided high yield and purity for further use. PCR tests confirmed successful gene amplification, and gel electrophoresis confirmed the quality of the amplified pieces. Assays helped to understand the diversity of bacteria in sugarcane and fish gut. Gene cloning experiments showed effective DNA insertion and growth. Future studies should refine methods for complex samples and improve PCR accuracy. These results support advances in molecular diagnostics, biotechnology, and microbial ecology.

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