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Physico-Chemical and Microbial Analysis of Drinking Water Samples of Perumalpuram, Tirunelveli, India

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Abstract: ***Water was an important resource to all living organisms, most ecological systems, human health, food production and economic development. The safety of drinking water was affected by various chemical and microbiological contaminants leadings to serious health problems. In the present study, it was found that physical and chemical parameter of all the water samples were within the desirable limit, as recommended by WHO and BIS. The heavy metals Fe, Pb, Cr, Cu and Zn used in the study were within the prescribed limit. In the bacterial analysis, Bacillus sp., Bacillus subtilis, Staphylococcus aureus were isolated, whereas in the fungal analysis, Candida albicans, Aspergillus niger and Aspergillus flavus were isolated. One bacterial and one fungal colony were subjected to sequencing analysis. In16S rRNA sequencing, based on the molecular taxonomy and phylogeny, the bacterium was identified as Bacillus subtilis and designated as Bacillus subtilis strain STC2 (MN496131). In 18S rRNA sequencing, based on the molecular taxonomy and phylogeny, the fungus was identified as Aspergillus flavus and designated as Aspergillus flavus STC3 (MN496132).***

Keywords: drinking water, Physico-chemical, AAS, 16S rRNA sequencing, 18S rRNA sequencing

I. INTRODUCTION

Water is one of the essential and precious natural resources. It is necessary in the life of all living organisms from the simplest plant and microorganisms to the most complex living system. Safe drinking water is essential to life and safe supply of water must be available to people [1]. In most of the developing countries, the growth of population preceded the development of an infrastructure capable of handling water and waste water, which lead to widespread contamination of the groundwater by domestic and industrial effluents [2, 3]. In spite of the fact that India is one of the wettest countries, it was in the middle of a serious water problem[3]. The major reasons are high population density, time and variability of rainfall and increased depletion and contamination of its surface water and groundwater. Climate change was also affecting the hydrological cycle [4]. The World Health Organisation revealed that 75% of all diseases in developing countries happen from polluted drinking water. Therefore water quality concerns were often the most important component for measuring access to improved water sources [5]. Acceptable quality showed the safety of drinking water in terms of its physical, chemical and bacteriological parameters. International and local agencies had established parameters to determine biological and physicochemical quality of drinking water [6].

The inorganic chemicals hold a greater portion as contaminants in drinking water in comparison to organic chemicals [7]. A part of inorganics were in mineral form of heavy metals. Heavy metals tended to accumulate in human organs and nervous system and interfered with their normal functions. In recent years, heavy metals such as lead (Pb), arsenic (As), magnesium (Mg), nickel (Ni), copper (Cu), and zinc (Zn) had received significant attention due to health problems [8]. Moreover, the cardiovascular diseases, kidney-related problems, neurocognitive diseases, and cancer were related to the traces of metals such as cadmium (Cd) and chromium (Cr) as reported in epidemiological studies [9]. The Pb was known to delay the physical and mental growth in infants [8]. So the objective of this study was to collect water samples from Perumalpuram region, Tirunelveli. The physical and chemical parameters were checked. Microbial analysis was done to know the presence of coliform bacteria and fungi.

II. METHODOLOGY

The raw and purified drinking water samples were collected in sterilized containers from five sampling points in Perumalpuram (8º41'54.9"N 77º44'25.1E) during July and August, 2019. The water temperature, pH, odour, turbidity and taste were analysed immediately on the spot after the collection, whereas the analysis of remaining parameters were done in the laboratory. The pH of water sample was measured with a portable pH meter. The odour and taste of the water were detected by smelling and drinking the water, respectively. The turbidity was checked visually. The total hardness of water samples was carried out by using titration method with EDTA solution [10]. For total suspended solid (TSS), 100 ml of the water sample was filtered through a pre weighed filtered paper. The filtered papers were dried at 103 to 105°C in Hot air oven and TSS was determined by the following formula [5].

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$$\text{TSS (mg/L)} = \frac{\text{filter post weight - filter pre weight x } 1000}{\text{V sample (ml)}}$$

TDS was measured using combined TDS and conductivity meter model 4200 whereas TS was measured from the two parameters of TDS and TSS and it was determined by the following formula:

$$\text{TS (mg/L)} = \text{TDS (mg/L)} + \text{TSS (mg/L)}$$

Electrical Conductivity was measured using combined TDS/conductivity meter model 4200. The analysis of five heavy metals in drinking water samples such as Copper, Chromium, Lead, Iron and Zinc were carried out based on ASTM standards [8], which were approved by APHA using Flame Atomic Absorption Spectrometer (AA-6300). The standard solution for each tested element was prepared according to its concentration and used to calibrate the system before analysing each water sample. The results were recorded on a computer connected to the AAS system.

Most Probable Number (MPN) test was done to identify the presence of coliform bacteria. Isolation of other bacteria was carried out by conventional serial dilution of the water, followed by spread plate technique. Nutrient agar and Mac Conkey agar (Himedia) were used for isolation and enumeration of bacteria [11].

The plates were incubated at 37°C for 24 hours. The isolated bacterial strain was identified by morphological and biochemical tests as per Bergey's manual and one *Bacillus* sp. was confirmed by 16S rRNA gene sequencing. 16S rRNA analysis was performed using ABI PRISM® big dye terminator version 3.1 cycles sequencing kit and ABI 3130 Genetic Analyser. The unknown sequence of the isolate was compared by alignment against 16S rRNA sequences available in the GenBank Database. The ~1.5 kb rRNA fragment was amplified using high fidelity PCR polymerase and the PCR product was sequenced bi-directionally using the primers (27F-5'AGAGTTTGATCTGGCTCAG3' and 1492R-5'TACGGTACCTTGTTACGACTT3'). The nucleotide sequences were compared with other 16S rRNA sequences, maintained by GenBank database, using the NCBI-BLAST program. The sequences were aligned by CLUSTAL W and the phylogenetic tree was constructed using MEGA X with the Maximum Likelihood method. All these analysis were performed on a bootstrapped dataset containing 1000 replicates.

Isolation of fungi was carried out by conventional serial dilution of the water, followed by spread plate technique. Potato dextrose agar (Himedia) was used for the isolation and enumeration of fungi [12]. The plates were incubated at 37°C for 48 hours. Lactophenol cotton blue staining was done to identify the fungal species and a fungus was confirmed by 18S rRNA gene sequencing. 18S rRNA analysis was performed using ABI PRISM® big dye terminator version 3.1 cycles sequencing kit and ABI 3730XL Genetic Analyser.

The unknown sequence of the isolate was compared by alignment against 18S rRNA sequences available in the GenBank Database. The ~1.5 kb rRNA fragment was amplified using high fidelity PCR polymerase and the PCR product was sequenced bi-directionally using primers (ITS1- 5'TCCGTAGGTGAACCTGCGG3' and ITS4-5' TCCTCCGCTTATTGATATGC3').

Nucleotide sequences were compared with other 18S rRNA sequences, maintained by GenBank database, using the NCBI-BLAST program. The sequences were aligned by CLUSTAL W and the phylogenetic tree was constructed using MEGA X with the Maximum Likelihood method. All these analysis were performed on a bootstrapped dataset containing 1000 replicates.

III. RESULTS AND DISCUSSION

Since the time of collection of water samples to the time of experimental analysis, many physical and chemical reactions would change the water quality.

In order to reduce the change in water sample, it was preserved soon after the collection. The values of the physico-chemical parameters of the drinking water samples were shown in Table 1. Physical parameters like odour, turbidity, taste and colour was agreeable in all the drinking water samples.

The pH was a measure of the hydrogen ion concentration in water. Drinking water with a pH ranging 6.5 to 8.5 was generally acceptable.

Acid water (pH was below 6) tended to be corrosive to plumbing and faucets. Alkaline waters (pH above 8.5) were less corrosive and it tended to have a bitter or soda-like taste. In this study, the concentration of hydrogen ion (pH) ranged between 6.93 to 7.31 and all the water samples analysed have concentration within the safe limit of 6.5 to 8.5 standard set by the WHO and BIS. Therefore, it indicated that the measured pH values of the drinking water samples were within permissible value; which will not cause any harmful effect to the consumers.

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Table No. 1. Physicochemical analysis of water

Test	Water samples					WHO limits	BIS limits
	A	B	C	D	E		
Colour	Colourless	Colourless	Colourless	Colourless	Colourless	Colourless	Colourless
Odour	Agreeable	Agreeable	Agreeable	Agreeable	Agreeable	Agreeable	Agreeable
Taste	Agreeable	Agreeable	Agreeable	Agreeable	Agreeable	Agreeable	Agreeable
Turbidity	Clear	Clear	Clear	Clear	Clear	Clear	Clear
pH	7.21	7.31	7.27	7.25	6.93	6.5-8.5	6.5-8.5
TDS (mg/L)	31	36	36	34	9	500	500
TSS (mg/L)	0.018	0.021	0.021	0.020	0.005	30	100-200
TS (mg/L)	31.018	36.021	36.021	34.020	9.005	500	-
EC (mS)	0.062	0.072	0.071	0.068	0.018	-	300
Total Hardness (mg/L)	4	4.5	4.5	4.4	1.2	500	300

Regarding the values of TSS, all the water samples showed less presence of contaminants, as the values ranged from 0.005 mg/L to 0.021 mg/L (Table 1). All these measured TSS values were within the permitted limits of WHO (≤ 30 mg/L). Similarly the TS values of water samples were ranging from 9.005 to 36.021 mg/L (Table 1) and the measured values were within the WHO allowed limits (500 mg/L). The results of both TSS and TS showed that the drinking water didn't cause health problem to the consumers. TDS could be taken as an indicator for the general water quality because it directly affected the aesthetic value of the water by increasing turbidity. High concentrations of TDS limited the suitability of water as a drinking source. The acceptable range of TDS was 500 mg/L. In the present study the range of TDS of analyzed water samples varied between 9 to 36 mg/L as shown in Table 1. These values were within the standard limit of WHO (500 mg/L) therefore the drinking water was safe in terms of TDS.

The ability of a solution to conduct an electrical current was governed by the migration of solutions and was dependent on the nature and numbers of the ionic species in that solution. This property was called electrical conductivity. It was a useful tool to assess the purity of water. The permissible limit for electrical conductivity (EC) was 300 µS cm-1 EC of the collected samples ranged from 0.018 to 0.072 µS cm^{-1}. This showed that the EC values of all water samples were within permissible limits and the potable water was safe in terms of EC (Table 1). In water, hardness was mainly contributed by bicarbonates, carbonates, sulphates and chlorides of calcium and magnesium. So, the hardness causing ions were calcium and magnesium. The acceptable limit of total hardness was 300 mg/L whereas the maximum limit was 600 mg/L. The hardness of analyzed water samples varied from 1.2 to 4.5 mg/L (Table 1). Durfor *et al*. 1964 had classified water as soft, moderate, hard and very hard [13]. As per this classification all the samples came under the soft category and placed within the acceptable limit values of BIS (300 mg/L) and WHO (500 mg/L).

Table No. 2. Heavy metal analysis in water by AAS

Sampling point	Iron (ppm)	Chromium (ppm)	Copper (ppm)	Lead (ppm)	Zinc (ppm)
A	0.0926	0.0022	0.0042	0.009	0.0004
B	0.1632	0.0035	0.0049	0.0078	0.0004
C	0.1556	0.0031	0.0051	0.0039	0.0006
D	0.0019	0.0016	0.0036	0.0024	0.0002
E	0.0012	0.0007	0.0016	0.0003	0.0001
WHO limits	0.3	0.05	2	0.01	-
BIS limits	0.3	0.05	0.05	0.01	5

This data (Table 2) showed that the concentration of Zn, Cu, Cr and Fe were found to be present in trace amounts in the samples but within the permissible range of metal ions as defined by WHO and BIS.

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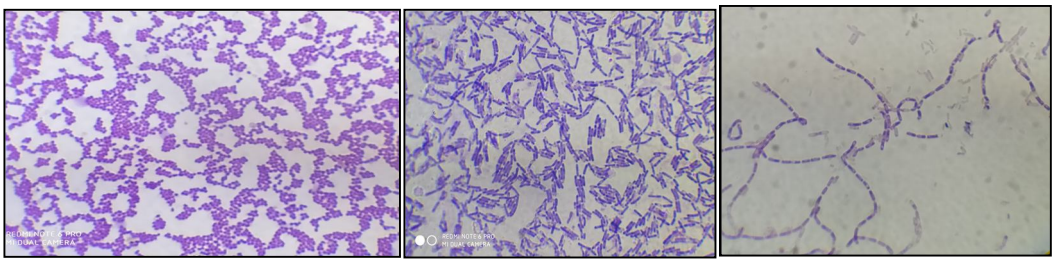
Table No. 3. Total enumeration of bacteria and fungi

Sampling point	Bacteria		Fungi		Coliforms
	CFU/100ml	Identification	CFU/100ml	Identification	
A	10±2	Staphylococcus aureus, Bacillus sp.	0	-	-
B	957±50	Bacillus sp.	13±2	Candida albicans, Aspergillus niger	-
C	35±6	Staphylococcus aureus, Bacillus sp.	0	-	-
D	162±14	Bacillus sp.	9±3	Aspergillus sp.	-
E	11±3	Streptobacillus, Bacillus sp.	0	-	-

Almost all the water samples contained *Bacillus* sp. and two samples contained *Staphylococcus aureus*. *Bacillus* sp. was white in colour with irregular margin. They were motile, gram positive rods, catalase positive, oxidase positive, indole test negative, MR negative, VP positive, nitrate reduction positive and citrate positive. *Staphylococcus aureus* was white, round, pin point colonies in Nutrient agar. It was non-motile, gram positive cooci, catalase positive, oxidase negative, indole negative, MR positive, VP positive, nitrate reduction positive and citrate positive.



A B C

Fig.1. Microscopic images of gram positive bacteria (A – *Staphylococcus aureus*, B – *Bacillus* sp., C – *Streptobacillus*)

Khaled and El-Gay in 2017 isolated Bacillus subtilis from tap water [14]. Armon et al., in 2014 reported the presence of Bacillus spores in drinking water [15]. Sumathi and Manonmani in 2016 reported the presence of Bacillus sp. and Staphylococcus aureus in river water of Nagapattinam district [16]. Abhishek et al., in 2017 isolated Staphylococcus aureus from drinking water sold by road side vendors in Delhi [17]. The portability of water was determined by analysing the bacteria present in it. The presence of microbial colonies might be due to the organic matter dissolved in the water bodies which render it to be slightly contaminated. Since Bacillus was a spore forming bacteria, the water should be boiled to 100ºC to kill it. In the tested water samples, bacterial pathogens like Salmonella, Vibrio cholerae, Klebsiella sp. was not detected. Surprisingly, no coliforms were detected in the water samples. In order to confirm *Bacillus* sp., the bacterium was characterized by 16S rRNA sequencing. The 16S rRNA gene sequence analysis was carried out to elucidate the taxonomic position and relationships among closely related species. The genomic DNA of the bacterium was isolated, amplified by polymerase chain reaction and analyzed by agarose gel electrophoresis. It showed a molecular weight of approximately 1.5 kilo base pairs when compared with standard markers. The result of the sequencing of the bacterium was obtained in the form of rough electrophotogram under automated sequencer.

Partial sequence of 16S rRNA of *Bacillus subtilis* strain STC2

CTCAGGCTCCTTGTTCGACTTACTGAGCCATGATCAAACTCTACGGTATACCTTGCTTACGACTGACTGAGCCAGGAT
CAAACTCTACCGGGACCTTATTCCCCGCGGCATGATGCGCGATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCA
GACTGCGATCCGAACTGAGAACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTGTTCTGTCCATTGT
AGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAG
TCACCTTAGAGTGCCCAATGAATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTC
ACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCCTATCTCTAGGATTGTCA
GAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCG
TCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGG
CGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGC
TTTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACC
GCTACACGTGGAAATTCCACTCTCCTCTGCTGCACTCAAGTTCCCCGGTTTCAATGAGCCTCCCCGGTGGAGCCGGGG
GCTTTCACATCAGAATTAAGAAAGCGGCTGCGAG

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The BLAST search of the 16S rRNA sequences (896 base pairs) of the bacterium showed maximum of 98.52% similarity with Bacillus subtilis strain VP4 (MN062737.1), Bacillus subtilis strain RN-2 (MN013973.1), Bacillus subtilis strain BDK7 (MH645813.1), Bacillus subtilis strain RH 21 (MH040981.1) and Bacillus subtilis strain PAH17 (MF806594.1). Based on the molecular taxonomy and phylogeny, it was identified as Bacillus subtilis and designated as Bacillus subtilis strain STC2. The nucleotide sequence of 16S rRNA gene partial sequence was deposited in NCBI under the accession number (MN496131). A Maximum likelihood method based on 16S rRNA sequence of Bacillus subtilis strain STC2 showed that, the isolate occupied a distinct phylogenetic position within the representatives of the Bacillus family (Figure 2).

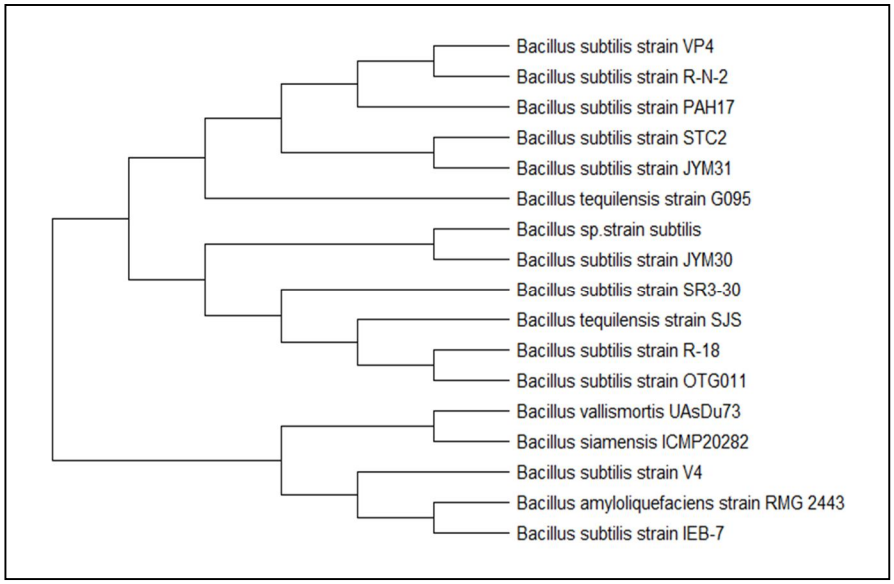

Fig. 2 Relationships between Bacillus subtilis strain STC2 and members of the genus Bacillus sp. on rooted neighbour joining tree based on 16S rRNA sequences

The Maximum Likelihood method and Tamura-Nei model was used to study the evolutionary history [18]. The tree with the highest log likelihood (-27868.12) was shown. Initial phylogenetic tree for the exploratory search were found by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances calculated by Maximum Composite Likelihood (MCL) method, and then choosing the topology with greater log likelihood value. The phylogenetic tree was drawn to scale, with branch lengths measured in number of substitutions per position [19]. This analysis involved 17 nucleotide sequences. There were a total of 1600 positions in the final dataset.

The water samples were serially diluted and plated on PDA. After incubation, fungi were grown only in two samples (B and D). In Sample B, white colour filamentous colonies with black colour in the centre and white colour pointed colonies were observed in the PDA plates. The colonies were found to be Aspergillus niger and Candida albicans. Aspergillus niger was confirmed by visual examination whereas Candida albicans was found out by gram staining. Shaista et al., in 2011 isolated Aspergillus niger and Aspergillus flavus from river water [12]. Candida albicans was isolated from drinking water by Nicholas in 2015 [20]. In Sample D, two types of fungal colonies were observed in the PDA plates. They were white colour colonies and small white-green colour fungi. The colonies were filamentous in nature. The white colony was found to be Aspergillus sp. and the other one was confirmed by 18S rRNA sequencing.

Partial sequence of 18S rRNA of *Aspergillus flavus* STC3

CTGTACCTTAGTTGCTTCGGCGGGCCCGCCATTCATGGCCGCCGGGGGCTCTCAGCCCCGGGCCCGCGCCCGCCGGAG
ACACCACGAACTCTGTCTGATCTAGTGAAGTTGATTGTATCGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGT
TCCGGCATGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCAGAATTCCGTGAATCATCGAGTCTTTGA
ACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGCGAGCGTCATTGCTGCCCATCAAGCACGGCTTGTGTGT
TGGGTCGTCGTCCCCTCGGGGGGGACGGGCCCCAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGATGGGGCT
TTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGAACGCAAATCAATCTTTTTCCAGGTTGACCTCGGATCAGGT
AGGGATACCCGCTGAACTTAAGCATATCAATAAGGCGGAGGA

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The BLAST search of the 18S rRNA sequences (521 base pairs) of the bacterium showed maximum of 100% similarity with Aspergillus flavus strain A26R (MN095167.1), Aspergillus flavus 119 (MN345959.1), Aspergillus flavus strain 113 (MH345953.1), Aspergillus flavus strain 106 (MH345946.1) and Aspergillus flavus strain 73 (MH345913.1). Based on the molecular taxonomy and phylogeny, it was identified as Aspergillus flavus and designated as Aspergillus flavus STC3. The nucleotide sequence of 18S rRNA gene partial sequence was deposited in NCBI under the accession number (MN496132). A Maximum likelihood method based on 18S rRNA sequence of Aspergillus flavus STC3 showed that, the isolate occupied a distinct phylogenetic position within the representatives of the Aspergillus family (Figure 3). This phylogenetic analysis involved 12 nucleotide sequences. There were a total of 991 positions in the final data set.

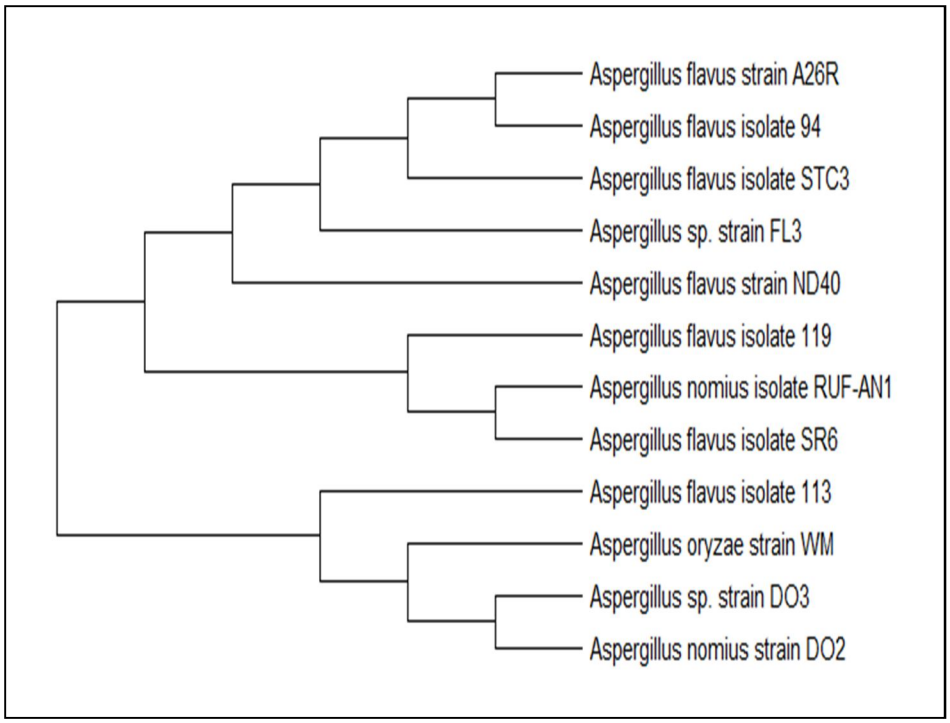

Fig. 3 Relationships between Aspergillus flavus STC3 and members of the genus Aspergillus sp. on rooted neighbour joining tree based on 18S rRNA sequences

IV. CONCLUSION

The values of all the physiochemical parameters and heavy metal analyses were found within the permissible limits of the WHO and BIS guideline for drinking water and no pathogenic microorganisms present in the drinking water samples therefore, the water qualities of the study sites were suitable for drinking and other domestic purposes.

V. ACKNOWLEDGMENT

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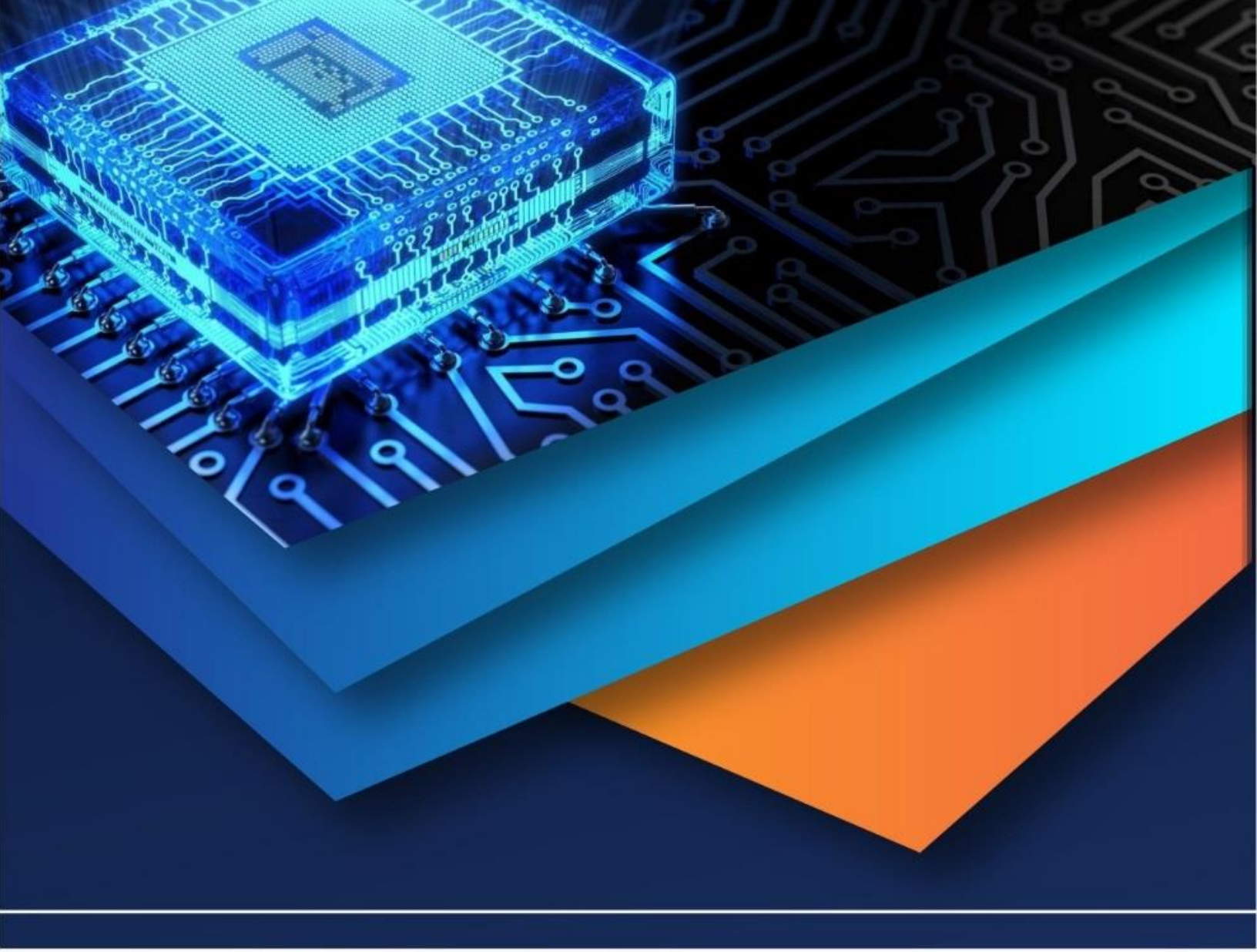

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