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Isolation and Characterization of Triclosan Resistant Bacteria from Sewage Water Sample

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ABSTRACT

The widespread use of triclosan, an antimicrobial agent, commonly found in household products, raised concern regarding its potential to select for resistant bacteria in environmental settings. Despite this, triclosan is still a useful drug for treating several infectious organisms. Examining microorganisms that have become resistant to triclosan is the goal of this investigation. Sewage water and hospital drainage water was collected, much possibility of finding resistant bacteria there. In order to comprehend adaptation processes of microorganisms, it's essential to isolate and characterize bacteria resistant to triclosan. The process of isolation entails acquiring bacterial strains from a variety of environmental sources, sewage water samples, inoculating them with triclosan at varying dilutions, and then incubating them for one week at 37^0C in a medium containing mineral salts. To get pure isolation cultures, were sub-cultured on nutrient agar medium, physical characteristics, morphological structures, growth patterns, and their chemical characteristics were used for characterization. To identify the precise genetic factors causing triclosan resistance, genomic analysis will also be carried out. The results revealed significant presence of triclosan-resistant bacteria, isolates showed varying levels of resistance to triclosan, with some exhibiting cross-resistance to other antibiotics, suggesting the potential for multi-drug resistance. It have consequences for public health regulations and the creation of substitute antimicrobial tactics in addition to advancing our scientific knowledge of triclosan resistance. We can more effectively negotiate the complicated terrain of antibiotic resistance and strive towards long term solutions by clarifying the complex interaction between bacteria and triclosan.

Keywords: *antimicrobial agent, resistant, sewage, triclosan*

1. INTRODUCTION

A widely used antimicrobial agent named Triclosan is the key ingredients of variety of personal care and household products including soaps, toothpaste, plastic items, fabrics, deodorants and other products which is claiming to be antibacterial [3-4,8,10,19,20]. Chemically, this aromatic compound contains phenols and ethers as a functional group which shows sparingly soluble nature with water [6,17]. Use of triclosan recently been linked to a number of negative effects on human health and the environment, including skin irritation, allergens or the terrestrial and aquatic environments ecotoxicity [5]. Triclosan after has the ability to react with free chlorine in water to produce the carcinogenic chloroform.

2,4-Dichlorophenols are the other substances that resulted from their interaction, once exposed to UV light, the majority of these compounds converts it to dioxins, which are believed to be a disruptor of endocrine system [14]. Triclosan is widely and persistently used in the environment, and as a result, its presence could be seen in drinking water, soil, waste water, breast milk, blood, urine. Sediments, treatment plants of waste water [4,11].

As a result of fertilizing agricultural soils with biomass derived from treatment plants of waste water, triclosan was found there [10]. Conversely, however, triclosan can also be degraded into methyl-triclosan, which is more stable and has a greater potential for bio-accumulation [14]. It combats Gram-positive strains quite well, but by altering its formulation, it can also combat yeasts and Gram-negative strains more effectively [7,15]. To enhance its permeability against the outer membrane of Gram-negative bacteria, for example, it has been demonstrated that triclosan and EDTA can be used together [13].

Research indicates that removal of upto 79% of the initial TCS flowing into a treatment plants of wastewater is achieved by biodegradation, with activated sludge absorbing 15% of it [16]. Sewage sludge is one of the main sources of Triclosan in water ways, waste water systems of municipal sewage cause TCS to accumulate in sewage sludge [1,9]. Sewage water represents a significant reservoir of microbial diversity and serves as a hotspot for the dissemination of antibiotic resistance genes.

The present work aims to isolate triclosan triclosan resistant bacteria from sewage water samples collected from different locations which is then subjected to their characterization and identification. Understanding the prevalence and characterization of triclosan -resistant bacteria in sewage water is essential for assessing environmental risks and informing mitigation strategies.

2. MATERIALS AND METHODS

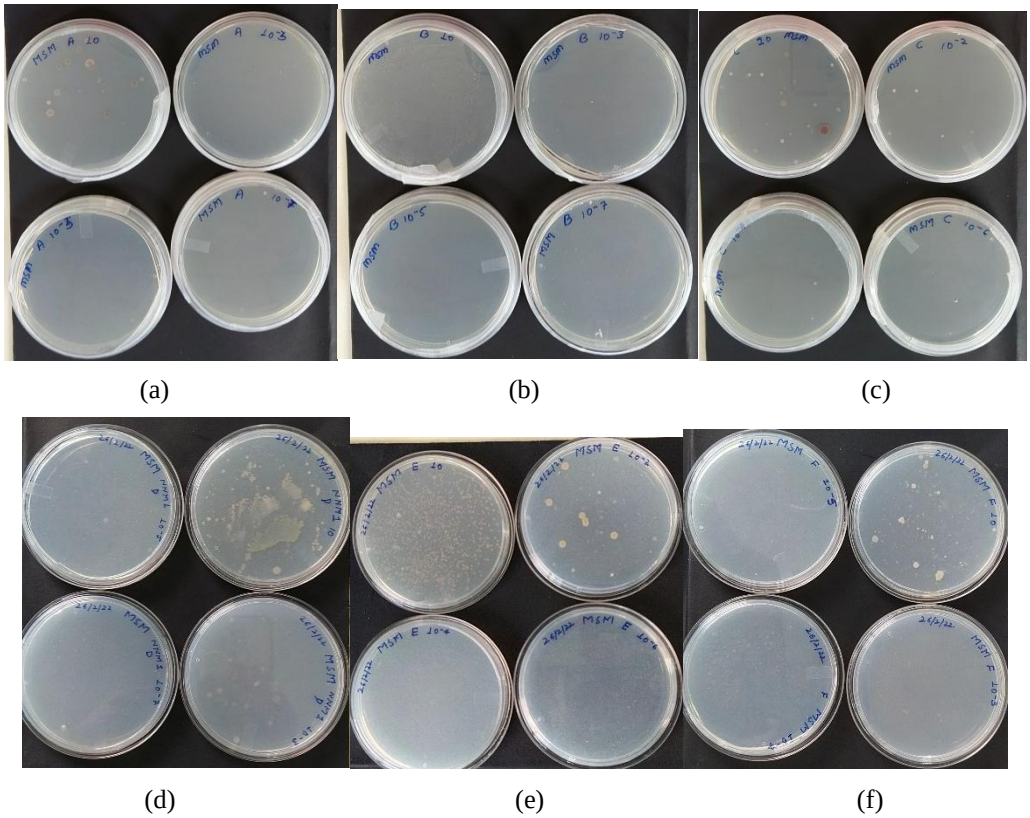
2.1 Sample collection:

Sewage water samples were collected from six different sites in sterile falcon tubes with the help of gloves . Two samples were collected from hospital drainage and four of the samples were collected from municipal sewage. One of the hospital sample was collected from Sadar Hospital Ranchi, Jharkhand and one from Sadar Hospital, Madhubani, Bihar. 2 samples were collected from 2 different locations of Ranchi and remaining two were collected from 2 different locations of Madhubani, these four samples were taken from Municipal sewage **[Fig.1]**. The collected samples were labelled as A,B,C,D,E and F respectively and transported to laboratory and stored in refrigerator to maintain the sample integrity.

Fig.1: Showing 5 different sites of sample collection i.e., hospital and municipal sewage.

2.2 Isolation of triclosan resistant Microorganisms:

The collected sewage water samples were serially diluted upto 7 dilutions i.e; Mineral salt medium containing 0.01% triclosan instead of glucose was prepared and autoclaved since TCS is insoluble in water so, TCS is weighed separately and dissolved in ethanol which is syringe filtered then added to culture media in LAF, autoclaved media was poured to sterile petri plates avoiding bubbles. Plates were incubated 1 day to check for contamination then inoculated, alternate dilutions of samples were inoculated to the media plates and spreaded evenly with the help of spreader. Inoculated plates were incubated in incubator at 37 degree celsius for upto 1 weeks and monitored on a daily basis. After proper incubation microbial growth could be observed **[Fig.2]** and purified to obtain single isolated colonies of each different strains which was preserved and maintained for further experimental works.



(a) (b) (c)

(d) (e) (f)

Fig.2: Showing isolation of triclosan resistant bacteria from 6 different sewage water sample A,B,C,D,E,F on mineral salt medium with triclosan at different dilutions i.e., 4 dilutions of each samples were taken in images a,b,c,d,e,f respectively.

2.3 Morphological Characterisation:

2.3.1. Based on physical appearance:

Triclosan resistant colonies were examined for morphological characteristics based on difference in their physical appearances bacterial isolates were categorized. Enumeration of bacterial isolates was done under Quebec colony counter and based on different parameters such as colour, shape. Size, texture, opacity etc they were also categorized morphologically.

2.3.2. Gram's staining:

Gram's staining is also done to categorize them as gram-positive or gram-negative. The basis for the Gram's stain response is the variation in the chemical make up of the cell wall of bacteria. The peptidoglycan layer of gram-positive cells is thick, while that of gram-negative cells is thin. The various reagents of this reaction which include crystal violet as a primary stain, Gram's iodine as a mordant which forms a complex called CVI Complex after that ethanol is used in place of decoloriser which is counterstained by Safranin which gives the end point of the reaction as a result; that can be examined under a microscope.

2.4 Biochemical Characterisation:

2.4.1 With the help of biochemical test kits:

Biochemical tests were done for the isolates which were now subjected to biochemical tests for biochemical characterisation with the help of Himedia Biochemical test kits following proper protocols. Fresh culture is used to get accurate results hence, fresh culture is obtained from culture collection and subcultured to different agar plates and incubated for optimal growth until visible colony appears. The biochemical test kits KB001 and KB002 [HiMedia] were normalised to room temperature before initiating the experiments and manufacturer's instruction is followed strictly. KB001 contains some slots to check for carbon source utilization to asses the bacteria to utilize different carbon sources while KB002 is having the slots for enzymatic activities to detect the presence of specific enzymes produced by the bacteria etc. For metabolic pathway analysis both the kits are used because both the kits are having common slots for them which can be done by assessing various biochemical reactions, such as fermentation of sugars, production of acids and gas production. So, the freshly prepared cultures were inoculated to different slots of different kits and incubated at 37degree celsius then observed but in case of some kits after

incubation some reagents needs to be added and then observed according to instruction manual. Results obtained from each biochemical test was recorded based on colour change **[Fig.3]**. Compare the results with known biochemical profiles of bacteria to identify specific metabolic characteristics of the isolated triclosan resistant strains.

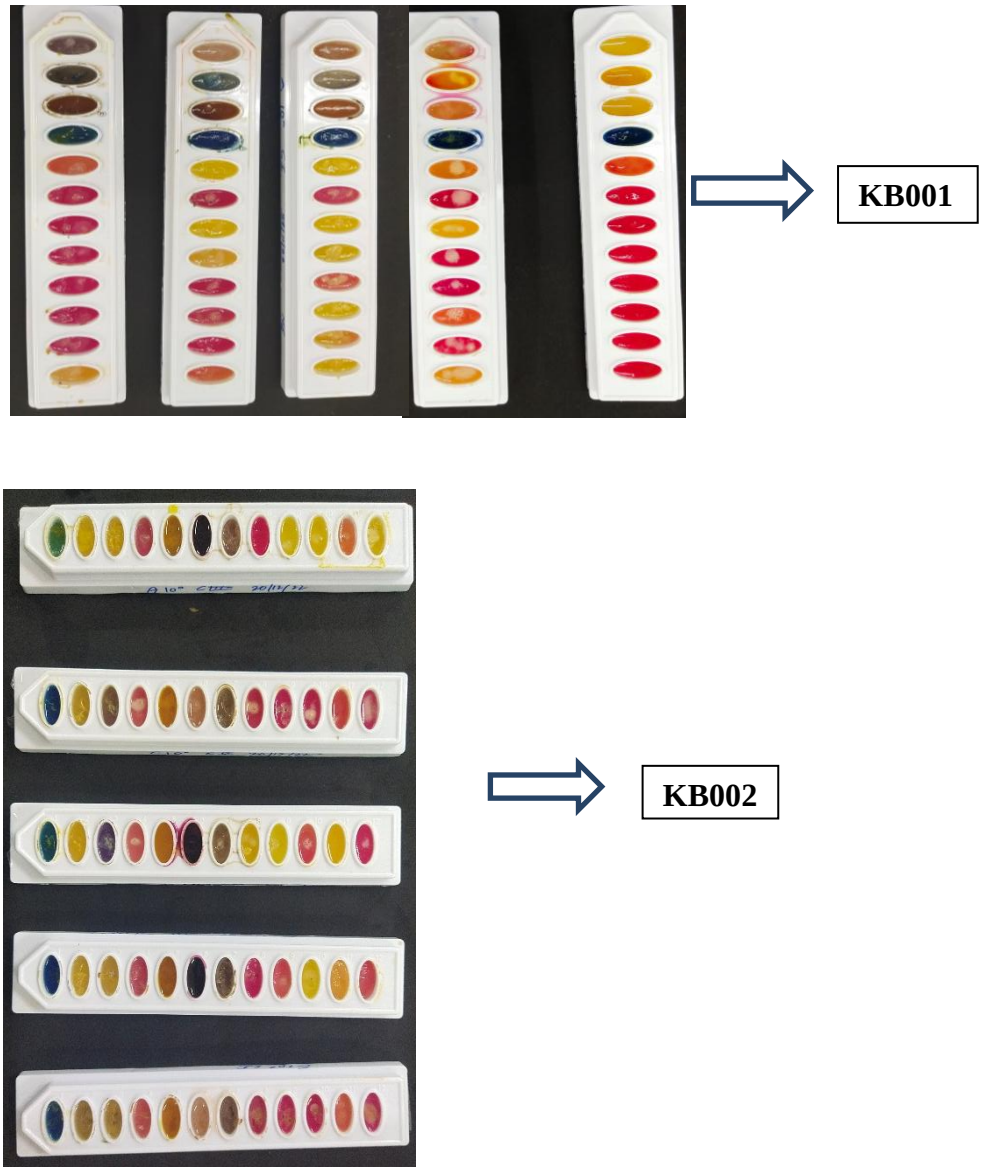


Fig.3: Showing images of biochemical test kit KB001 and KB002 after inoculation.

2.4.2 Catalase test:

Some bacteria produce Hydrogen peroxide under aerobic respiration which cause lethality to the cell. To surpass this lethal condition enzyme catalase help to catalyze the reaction to give water and oxygen as bubbles which is called catalase positive test **[Fig.4]**.

Culture + 2-3 drops of H202 → Bubbles

↓

Positive

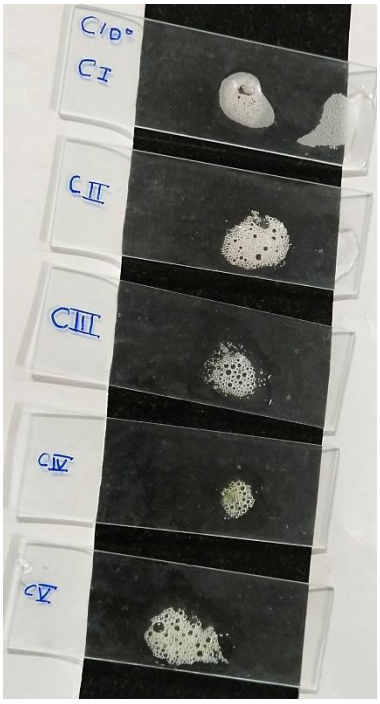


Fig.4: Showing results of catalase test with positive results of all the strains, showed bubble formation.

2.5 Growth Curve:

Experiment was started after obtaining the isolated triclosan resistant bacteria from the culture collection and subcultured overnight at 37degree celsius to get a fresh culture for initiating this experiment. Multiple flasks with media was prepared fresh depending on experimental design but in sets of two where one is containing glucose and another set supplemented with triclosan to compare growth dynamics.

An Erlen Meyer flask holding 150ml of nutrient broth inoculated from the freshly prepared overnight culture, the flask was incubated at 37degree celsius. From these flasks 1ml was taken out periodically and optical density of the cultures were calculated via spectrophotometer at 600nm to estimate the bacterial growth in presence and absence of Triclosan by comparing the respective ODs. The values of different ODs of different samples were plotted on XY axis to generate the growth curve for the triclosan resistant bacteria. The four different growth phase lag phase, log phase , stationary phase and decline phase were calculated based on graph of growth curve **[Fig.11-12.]**.

3. RESULT AND DISCUSSION

3.1 Microbial isolation:

Based on their ability to grow with triclosan and using it as a source of carbon, the microorganisms that could break down the material were isolated, halo zone exhibiters were chosen for additional investigations [Fig5.].

Total no. of HPC colonies isolated was 138 [Table 1] in which some shared similar morphology but some isolates from one of the sample was lost during further experimentation hence, remaining isolates that is 127 [Table2] was carried further. There were both gram positive and gram negative isolates among them. Few studies have been done on microorganisms that can break down triclosan. Some major genera identified were *Enterococcus*, *Paenibacillus*, *Bacillus*, *Brevibacillus* and *Pseudomonas*. Since this was not the study's goal, no further classification was investigated [6].

Triclosan has been a common addition in toothpastes, soaps, deodorants, and disinfectants for use in hospitals and homes due to its low harmful effects and overall safety for people [18]. Nevertheless, regular use of these chemicals has led to a rise in the amount of triclosan released into stabilized sludge, waste waters and receivers. Thus, the first section of the investigation confirmed the presence of TCS resistant *coliform* bacterial species in various stabilized sludge samples. No research has examined triclosan resistance in sewage sludge or at such high triclosan concentration, but other studies have shown the existence of triclosan- resistant bacteria in waste and surface water [6,12]. Compared to hydrophilic chemicals, this biocide's sorption to sludge is significantly better, since it is lipophilic. This supports our findings, which show that bacteria in sewage sludge are more resistant to extremely high TCS concentration. *Citrobacter freundii*, *Morganella morganii*, *Serratia fonticola*, and *Serratia liquefaciens* have been identified as triclosan-resistant isolates. These bacteria are commonly found in soil, waste waters, and sewage but they can also be found in food, the human or animal digestive tract,

and food itself [2]. *C. freundii* is primarily linked to respiratory and urinary tract infections, but it can also develop into an opportunistic pathogen . Prior to recent years, *M. morganii* was thought to be a minor pathogen, but it has since been determined that it is an important contributor of nosocomial infections. Human conditional pathogen *S. fonticola* and *S. liquifaciens* cause infections of the skin and soft tissues, lower respiratory tract, urinary tract and nosocomial infections [11]. The bacterial species isolated here are gram negative as well as gram positive in nature and based on various biochemical nature showed different biochemical properties.

Table 1: Showing different samples collected with their respective location and total no. of colonies isolated from the source.

S.No.	Different samples collected for isolation (source)	Total no. of colonies	No. of colonies showing halo zone
1.	Sample A- Sadar Hospital Ranchi	27	5
2.	Sample B- Municipality Sewage Ranchi	11	3
3.	Sample C- Municipality Sewage Madhubani	20	5
4.	Sample D- Municipality Sewage Madhubani	49	5
5.	Sample E- Municipality Sewage Madhubani	20	5
6.	Sample F- Sadar Hospital Madhubani	11	5

Table 2: showing total no. of colonies isolated and types of colonies isolated from the different samples.

Sample	Total no. of colonies	C1	C2	C3	C4	C5
A	27	1	12	6	5	3
B	11	5	6	Numerous	—	—
C	20	1	5	3	8	3
D	49	1	7	34	7	—
E	20	4	2	5	3	6
F	11	3	4	4	Numerous	—

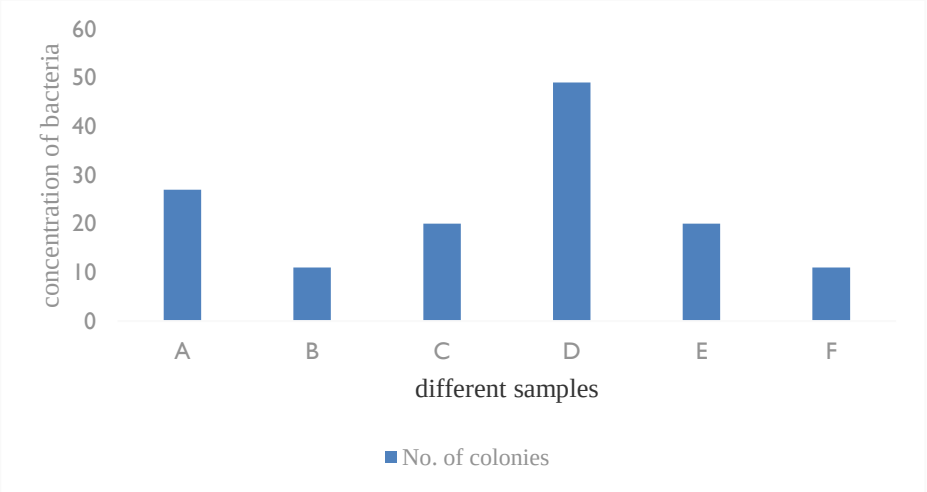


Fig.5: Graphical representation of concentration of bacteria per sample isolated.

3.2 Morphological Characterisation:

After isolation of the bacterial strains, morphological characterisation was done to distinguish the strains based on their visual properties such as their shape, size, opacity, color etc [Table3].

Table 3: Showing morphological characterisation of isolated triclosan resistant bacteria from 6 different sewage water samples on mineral salt medium with triclosan.

Sample A:

Strain	No.	Colour	Shape	Size	Opacity	Elevation
CI	1	White + Black	Round	Medium	Opaque	Elevated
CII	12	Off-white	Round	Small	Translucent	Elevated
CIII	6	White + Black	Round	Small	Translucent	Elevated
CIV	5	White + Black	Round	Small	Translucent	Elevated
CV	3	Brown	Semi-circular	Medium	Translucent	Elevated

Sample B:

Strain	No.	Colour	Shape	Size	Opacity	Elevation
CI	5	Black	Round	Small	Transparent	Flat
CII	6	Grey	Irregular	Small	Opaque	Flat
CIII	Numerous	Black	Round	Small	Transparent	Flat

Sample C:

Strain	No.	Colour	Shape	Size	Opacity	Elevation
CI	1	Brown	Irregular	Large	Translucent	Elevated
CII	5	Off-white	Round	Small	Translucent	Elevated
CIII	3	Black + White	Round	Small	Translucent	Elevated
CIV	8	White	Irregular	Medium	Opaque	Elevated
CV	3	Off-white	Irregular	Small	Opaque	Elevated

Sample D:

Strain	No.	Colour	Shape	Size	Opacity	Elevation
CI	1	Brown	Round	Medium	Translucent	Elevated

CII	7	White	Round	Small	Opaque	Elevated
CIII	34	White	Irregular	Small	Opaque	Elevated
CIV	7	Cream	Irregular	Large	Transparent	Flat

Sample E:

Strain	No.	Colour	Shape	Size	Opacity	Elevation
CI	4	Brown	Round	Medium	Opaque	Elevated
CII	2	Brown	Irregular	Medium	Opaque	Elevated
CIII	5	Pale-white	Irregular	Medium	Translucent	Flat
CIV	3	White	Round	Small	Translucent	Elevated
CV	6	White	Round	Medium	Translucent	Flat

Sample F:

Strain	No.	Colour	Shape	Size	Opacity	Elevation
CI	1	Brown	Round	Medium	Translucent	Elevated
CII	7	White	Round	Small	Opaque	Elevated
CIII	34	White	Irregular	Small	Opaque	Elevated
CIV	7	Cream	Irregular	Large	Transparent	Flat

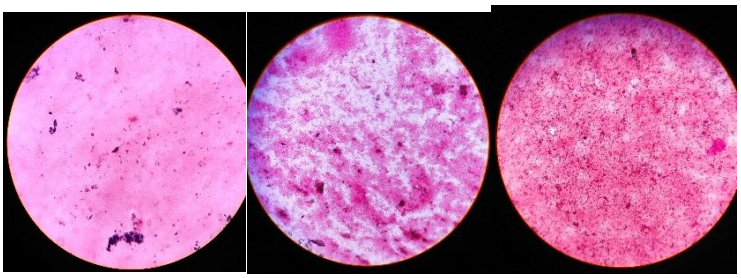
3.3 Gram's staining:

After morphological characterisation the bacterial strains were distinguished on the basis of cell wall composition which is done via Gram's staining and the remaining strain were gram's stained whose results are given below [Table4-8] **[Fig6-10.]**.

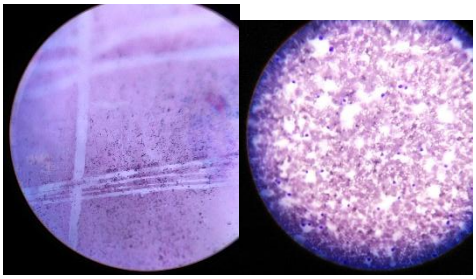
Table 4: Showing results of Gram's staining of sample A.

S.no.	Colony	Colour	Shape	Result
1.	CI	Purple	Round	Positive
2.	CII	Pink	Round	Negative
3.	CIII	Pink	Rod	Negative
4.	CIV	Purple	Round	Positive

5.	CV	Purple	Round	Positive



CI CII CIII

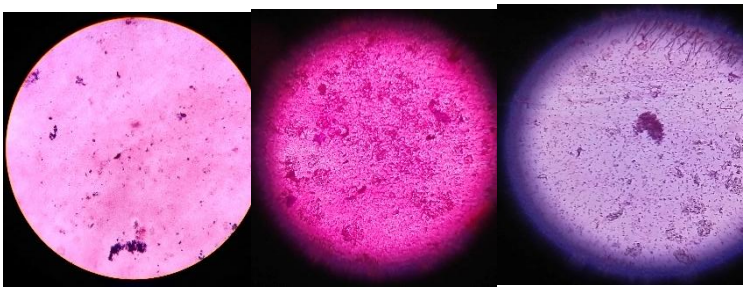


CIV CV

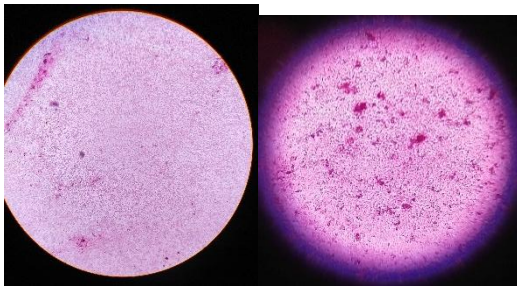
Fig.6: Showing results of Gram's staining of sample A at 40X.

Table 5: Showing results of Gram's staining of sample C.

S.no.	Colony	Colour	Shape	Result
1.	CI	Purple	Round	Positive
2.	CII	Pink	Round	Negative
3.	CIII	Purple	Rod	Positive
4.	CIV	Pink	Round	Negaitive
5.	CV	Purple	Round	Positive



CI CII CIII

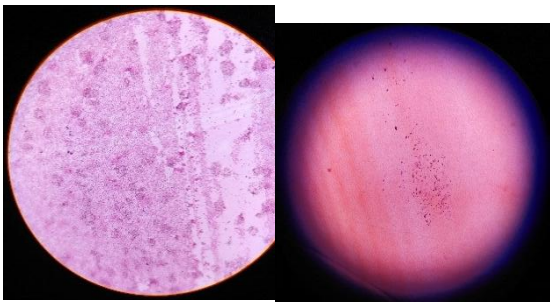


CIV **CV**

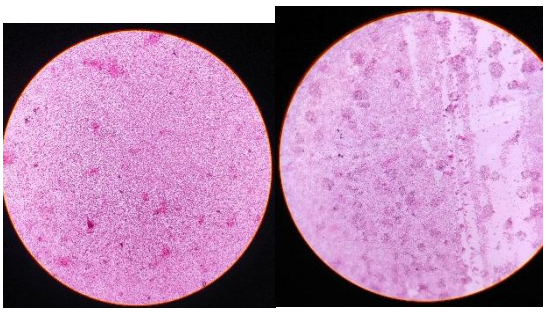
Fig7.: Showing results of Gram's staining of sample C at 40X.

Table 6: Showing results of Gram's staining of sample D.

S.no.	Colony	Colour	Shape	Result
1.	CI	Pink	Round	Negative
2.	CII	Purple	Round	Positive
3.	CIII	Pink	Rod	Negative
4.	CIV	Pink	Round	Negative



CI **CII**

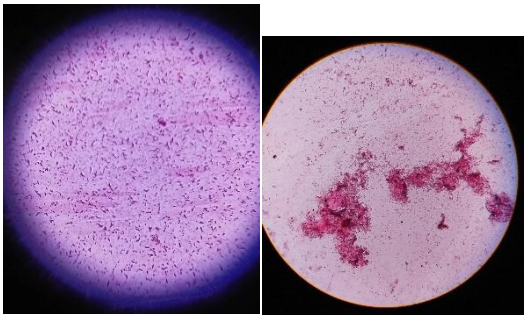


CIII **CIV**

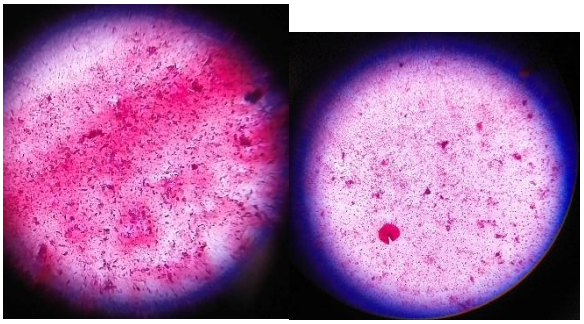
Fig.8: Showing results of Gram's staining of sample D at 40X.

Table 7: Showing results of Gram's staining of sample E.

S.no.	Colony	Colour	Shape	Result
1.	CI	Purple	Rod	Positive
2.	CII	Pink	Rod	Negative
3.	CIII	Pink	Round	Negative
4.	CV	Purple	Round	Positive



CI **CII**

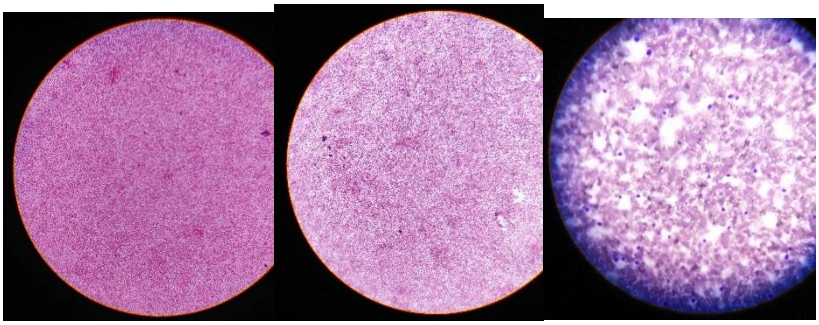


CIII **CV**

Fig.9: Showing results of Gram's staining of sample E at 40X.

Table 8: Showing results of Gram's staining of sample F.

S.no.	Colony	Colour	Shape	Result
1	CI	Purple	Rod	Negative
2.	CII	Pink	Rod	Negative
3.	CIV	Purple	Round	Positive



CI **CII** **CIV**

Fig.10: Showing results of Gram's staining of sample F at 40X.

3.4 Biochemical Characterisation:

The isolated strains after morphological characterisation and Gram's staining they must undergo biochemical characterisation based on different biochemical tests and all isolated strains were not able to survive completely so only the remaining revived strains were biochemically characterized [Table9] and further experimentation.

Table 9: Showing results of Biochemical test of all the samples.

S.No.	Name of Test	Result (A)	Result (C)	Result (D)	Result (E)	Result (F)
1.	Indole	Negative	Negative	Negative	Positive	Negative
2.	Methyl Red	Negative	Negative	Negative	Negative	Negative
3.	Voges Proskauer	Positive	Positive	Negative	Negative	Negative
4.	Citrate Utilisation	Positive	Positive	Positive	Positive	Positive
5.	Glucose Utilisation	Positive	Positive	Positive	Negative	Negative
6.	Adonitol	Negative	Negative	Negative	Negative	Negative
7.	Arabinose	Positive	Positive	Negative	Negative	Negative
8.	Lactose	Negative	Negative	Negative	Negative	Negative
9.	Sorbitol	Negative	Negative	Negative	Positive	Negative
10.	Mannitol	Negative	Negative	Negative	Negative	Negative
11.	Rhamnose	Negative	Negative	Negative	Negative	Negative
12.	Sucrose	Positive	Negative	Negative	Negative	Positive
13.	Lysine	Negative	Negative	Negative	Negative	Negative
14.	Ornithine	Negative	Positive	Positive	Negative	Negative
15.	Urease	Positive	Positive	Positive	Positive	Positive

16.	Phenyl alanine	Negative	Negative	Negative	Negative	Negative
17.	Nitrate	Positive	Negative	Positive	Positive	Negative
18.	H2S production	Positive	Positive	Positive	Positive	Positive

3.5 Growth curve:

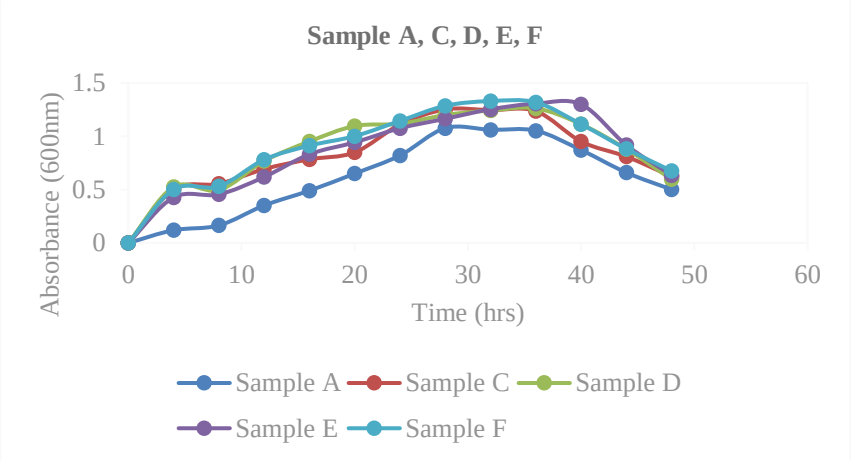


Fig.11:Showing results of growth curve in absence of Triclosan of all the samples A, C, D, E and F in different growth phases.

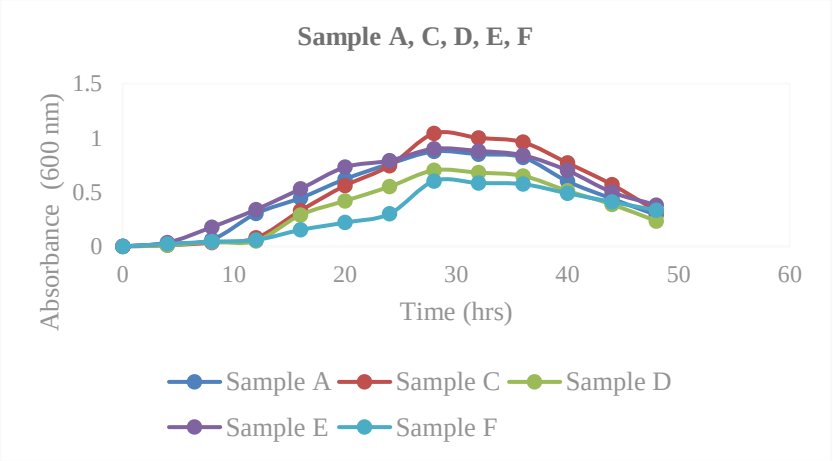


Fig.12: Showing results of growth curve in presence of Triclosan of all the samples A, C, D, E and F in different growth phases.

4. CONCLUSION

This study focuses on isolated and biochemically characterized triclosan-resistant bacteria from sewage water samples highlighting the growing concern of antimicrobial resistance in environmental settings. The presence of resistant strains suggests the potential for horizontal gene transfer, posing risks to public health and ecosystems. The finding emphasize the need for stricter waste water management and reduced triclosan usage to mitigate resistance spread. Further molecular studies are recommended to understand the genetic mechanisms underlying resistance. Overall this research underscores the urgency of monitoring environmental antibiotic resistance to prevent the emergence of more resilient and potentially pathogenic bacterial strains.

Conflict of Interest: The author declares that we have no conflict of interest.

5. ACKNOWLEDGEMENT

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