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Isolation and identification of bacteria from industrial effluent

Zeeshan Ali[1], Rekha Sirohi[2#], Umesh Kumar[3] and Mohammad Rehan[4]

[1,2,4] Department of Biochemistry, Sir Syad Faculty of Science, Mohammad Ali Jauhar University, Rampur

[3]Department of Paramedical Sciences, Subharti Medical College, Swami Vivekanand Subharti University, Meerut

[#]Corresponding author: email id rsirohi27@gmail.com

Contact no: +91- 9719355766

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Abstract

The technology based on the use of micro-organisms, in this case bacteria isolated from sites contaminated by industries, represents an alternative option for the treatment of wastewater before discharge into the environment. The present study deals with isolation, identification and characterization of the unknown bacteria present in the industrial effluent. A total of nine bacteria were isolated from industrial effluent using nutrient agar medium. Based on the morphological, biochemical the isolated bacteria were identified as the species of *Pseudomonas* and *E. coli*. In this context we also tested the isolated bacteria for minimum inhibitory concentration and we found: Zenflox, Azithromycin and Erythromycin were showing maximum MIC value for *Pseudomonas* compared with *E. coli.* While in another set of experiment we found Ofloxacin has maximum MIC value for *Pseudomonas*, this result reveals that normal micro flora is also developing drug resistance activity.

Key Words: *Pseudomonas*, *E. coli*, Antibiotics, Biochemical Characterization, Minimum Inhibitory Concentration

Introduction

Due to population, the world is constantly in need of water. Earth's population has tripled in the last century while water consumption has enlarged **(1)**. Most of the major challenges of today's civilization are pollution **(2)**. Industrial effluents are an important factor in aquatic pollution **(3)**. It has been detected that one-third of the total water in India is water Pollution comes in the form

of industrial effluents, solid wastes, and other toxic wastes **(4)**. Industrial waste sampling involves the collection of various physical forms of waste like solid, liquid, semi-solid, etc. Groundwater sources are contaminated due to untreated industrial effluents **(5)**. Many waterborne microorganisms are infectious to humans as well as animals and cause many diseases **(6)**.

The majority of the bacterial species isolated from skins as well as from hides skins during various stages of the leather making process are non-pathogens, however, a number of species that are considered as pathogens or potential pathogens such as bacillus cereus, Escherichia coli, and *pseudomonas aeruginosa*, were isolated from hides and skins **(7).** Pathogens present on hides and skins, and in the tannery effluent may infect tannery personnel and contaminate the environment through discharge of the effluent or disposal of the solid waste. Moreover during conventional leather making processing, due to the extreme environmental conditions, the probability of bacteria surviving on hides in reduced.

The pollution of the environment with toxic heavy metals is spreading throughout the world along with industrial progress. In the past few decades, uncontrolled urbanization has caused a serious pollution problem due to the disposal of sewage and industrial effluents to water bodies. Unlike many other pollutants, heavy metals are difficult to remove from the environment. Heavy metals are recognized to be powerful inhibitors of biodegradation activities. These metals cannot be degraded, and are ultimately imperishable **(8).**

Microbes play an important role in the bio-geochemical recycling of heavy metals and in cleaning up or remediation of metal-contaminated environments **(9**). Microorganisms have adopted a variety of mechanisms for adapting to toxic heavy metals. The evaluation of resistance to metals is a complex process. Aquatic microbes become resistant to metals as a result of contamination with effluents. The significant increase of Multiple Metal Resistant [MMR] bacteria is observed in various aquatic systems (**10).** Human diseases caused by such bacteria could be difficult to treat with drugs. The resistance development may be due to nonspecific mechanism involving gene regulation of plasmids and chromosomes, which may be transferable to other microbes due to the presence of a resistance [R]-factor **(11)**. To survive in metal-stressed conditions, bacteria have used various types of mechanisms to tolerate heavy metal ions. These mechanisms include the efflux of metal ions outside the cell and reduction of the heavy metal ions in a less toxic state. The objective of this study is to isolate and characterize microorganisms from tannery waste water which have heavy metal resistant potential, so we can use those microorganisms in treatment of heavy metals by metabolizing the heavy metals into simpler forms which are not harmful for living beings **(12).**

Heavy metal pollution of water is a major environmental problem facing the modern world. The global heavy metal concentration in various environments is increasing in the number of industries **(13).** Most of the industrial waste water contains heavy metals like cadmium, lead, zinc, cobalt, and chromium. Among heavy metals chromium is the major pollutant of the leather tanning industry and is toxic to plants and animals around the environment. The damage to the

environment by the hazardous tannery effluent is becoming an acute problem in several countries **(14).**

The industrial effluent released directly or indirectly in to natural water resources, mostly without proper treatment, pose a major threat to the environment. Among the different forms of chromium is the most toxic and carcinogenic due to its high solubility in water, rapid permeability through biological membrane with intracellular proteins and nucleic acid. In this paper an effort has been made to remove heavy metals from tannery effluent using microorganism **(15).** The evolution of metal resistance a complex process which may involve a variety of mechanisms. Aquatic microbes' become resistant to antibiotics and metals as a result of contamination with effluents .Antibiotic resistance in bacteria is more frequently associated and strongly correlated with meal résistance. Bacterial species had been isolated from drinking water that was tolerant to toxic heavy metal. Contamination of the surface water and groundwater with hexavalent chromium (Cr^{6+}) is an issue of potential concern due to its toxicity **(16).** It is well known for its toxic, mutagenic, carcinogenic on human beings and other living organisms and is classified under priority pollutants in many countries (**17*).*** Hexavalent chromium is a transition metal which is highly toxic and carcinogenic compound and is one of the major sources of pollutant. Chromate is generated as a byproduct of a large number and is discharged into the environment through the disposal of the wastes from the industries those engaged in welding, paper, pigment & wood production, leather tanning, chrome plating, metallurgical metal finishing, textiles & ceramics and thermonuclear weapons manufacturing etc **(18).** The wide spread use of chromium indiscriminate the disposal of byproducts and wastes from industrial processes have created serious problems of the environmental pollution in the urban areas and the other ecosystems associated with industrial discharge.

Materials & Methods

This study was carried out in the Department of Biochemistry, Mohammad Ali Jauhar University, Rampur, UP India.

Sample collection: Sample was collected applying the following protocol.

- Total two water sample were collected from different site of industrial area of Rampur.
- Industrial waste water was collected in sterile container from the six inches beneath the surface of water from industrial area of Rampur at different sites.
- Sand and other fine particles present in water samples wait to be settled down before the serial dilution process.
- The water was then placed at -20^{o}c.

Isolation bacterial isolates: Isolation of bacteria were done by serial dilution agar plate method by the following method.

- 1ml of water samples were dissolved in 9 ml of sterile water and mixed thoroughly.

- 1 ml of suspension was transfer to 9 ml of sterile distill water to get serially diluted 10^{-1} to $10^{-4.}$
- Then 0.1 ml of suspension was spread on the surface of nutrient agar with help of spreader.
- Nutrient agar plates were incubated at 37^{o}c for 28-32 hours.
- Individually each isolates treated with antibiotics for detection of MDR property as method describe by **Pandey *et al*., (19).**

Biochemical characterization

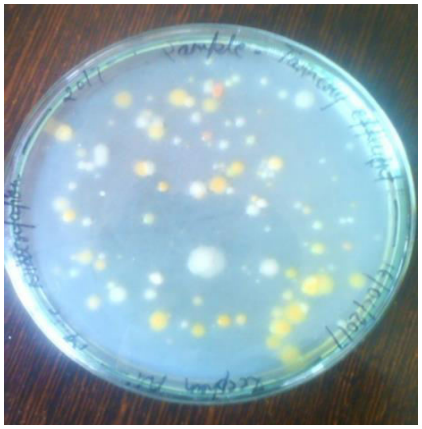
- Biochemical characterization of pesticides tolerant isolates was carried out by Hi Assorted ™ biochemical test kit (KB002-200 KT).
- Biochemical test kit can be used for screening pathogenic organism from urine, enteric specimens and other relevant clinical/environmental samples. It can also be used for validating known laboratory strain.
- The kits provide the complete list of organism that can be identified with this system is given in the identification index provided with the kit. Each Hi Assorted AM biochemical test kit is a standardized calorimetric identification system utilizing seven conventional biochemical test and five carbohydrate utilizing test.
- The tests are based on the principle of pH change and substrate utilization.
- On incubation organism undergo metabolic changes which are indicated by a color change in the media that can be either interpreted visually or after addition of the reagent.
- Biochemical characterization of recovered isolates was performed according to barge Manual of determinative bacteriology.

Minimum inhibitory concentration (MIC) of antibiotics

Minimum inhibitory concentration of antibiotics against different multidrug resistance bacterial isolates was done by spot inoculation methods by the following methods.

- We prepared different concentration of antibiotics plates at varying concentration from 0.125-8mg/ml.
- MIC were performed with help of sterilized disc congaing different concentration of antibiotics describe by the **Shafiani and Maik (20).**
- Commercial grade of antibiotics (Tetracycline, Amoxicillin, Cifixime, Zenflox, Azithromycin, Ofloxacin, Ampicillin, Erythromycin, Cefadroxil) were used. Concentration of the antibiotics was calculated on the basis of dilution method.
- Stock solution of the antibiotics were prepared in distilled water and then supplemented in nutrient agar in such manner where concentration of antibiotics varying it 0.125-8 mg/ml in a plates.
- After disc diffusion each plate were incubated at 32^{o}C for 28-32 hours. The lowest concentration of antibiotics which inhibit the growth of bacteria is known as minimum inhibitory concentration of antibiotics.

Antibiotic susceptibility



The antimicrobial sensitivity of the test strains of nine antibiotics was done using the Kirby-Bauer disc diffusion method (**21**). The commercial available antibiotic disc used for the study were tetracycline (4mg/ml), Amoxicillin (4mg/ml), Ampicillin (4mg/ml), Ofloxacin (4mg/ml), Erythromycin (4mg/ml), Cifixime (4mg/ml), Zenflox (4mg/ml), Azithromycin (4mg/ml), Cefadroxil (4mg/ml). and sterilized with Millipore filter membrane before preparation of different concentration of antibiotics and bacterial isolates were tested for sensitivity to antimicrobial agents by means of disc diffusion methods. A lawn of test pathogen was prepared by evenly spreading 100 µl inoculums with the help of a sterilized spreader on to the entire surface of the agar plate. The plates were allowed to dry before applying antibiotics disc. Then commercially available different concentration of antibiotic disc were gently placed on the agar plates, which were then left at room temperature for 1 hour to allow diffusion of the antibiotics in to the agar medium. The plates were then incubated at 37^0c for 24 hours. If an antimicrobial activity was present on the plates, it was indicated by an inhibition zone.

Result and Discussion

Two different site of industrial waste water were selected for the isolation and identification of bacterial isolates. Sampling process is mentioned in the material and methods sections which are earlier described. For processing of these samples, sand and other fine particles present in water samples wait to be settled down before the serial dilution process.

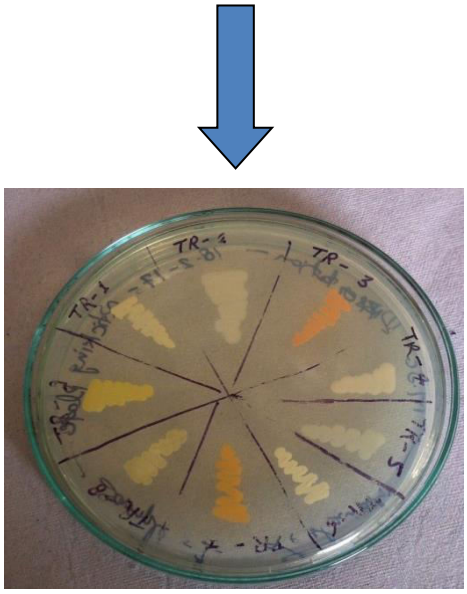


Fig 1-: Purification of bacterial isolates

A total 9 isolates in which 6 *Pseudomonas* and 3 *E. coli* bacterial isolates were isolated from industrial effluent of Rampur and were identified on the basis of morphological, cultural and biochemical characterization (Figure 1). All the isolates were gram negative rods and catalase positive. Pseudomonas gave the positive oxidase and catalase while *E.coli* was showing the metallic sheen on EMB plates. Based on comparison of these characteristics with standard description in Barge's manual of determinative bacteriology **(22)** these isolates tentatively identified as pseudomonas.

Microscopic and visual observations were used to characterize the isolated strains. Details of the morphological features of bacterial colony were recorded in the Table 4. Rod shape of all strains is identified as this comes under the species of *Pseudomonas* and E*. coli*. The biochemical characters of the bacteria were tabulated in Table 5.

To gain an insight in to the ecological status of the test system, the status of resistance of pseudomonas species against six different antibiotics. The single colonies pure culture was maintained by streak plate methods by using the following process. Inoculating loop was sterilized by putting it in flame till red hot. After cooling it down, it was dipped in to 95% ethyl alcohol and farther heated for proper sterilization. Distinguished colonies from spread plate were further streaked over three different nutrient agar plate surface by sterilized inoculating loop and then incubate for 28 to 32 h at 32° C.

A total 9 (6 *Pseudomonas* and 3 *E. coli*) bacterial isolate were showed rod shaped and gram negative in morphology. They all are aerobically positive. The texture (surface) was smooth and the appearance of *E.coli* was shiny. *E.coli* was non-pigmented (colorless). The biochemical

characterization of *E. coli* and pseudomonas isolate obtain from the industrial wasted water is given in table 4.

In our designed experiment we identified total nine isolates from industrial waste and measured the zone of antibiotics inhibition. In this context our finding reveals that Pseudomonas was showing the resistance with cifixime and azithromycin at concentration 4mg/ml (Table 1, Figure 2). Our research reveals that the antibiotics were developing tendency to resistance against bacteria which is threats for human health. In this context we also tested the isolated bacteria for minimum inhibitory concentration and we found: Zenflox, Azithromycin and Erythromycin were showing maximum MIC value for Pseudomonas compared with E. coli. While in another set of experiment we found Ofloxacin has maximum MIC value for *pseudomonas*, this result reveals that normal micro flora is also developing drug resistance activity (Table 2 and table 3). Waste water originated from domestic and industrial discharge in the river. Along with the *pseudomonas* and *E coli* is also indicator organism for water contamination. External contact and injection of bacteria from fecal contamination can cause disturbance in health. Municipal water is also responsive or E coli in the river. Certain b-lactamases are specifically induced upon growth of MDR. Strain with antibiotics and may be important conferring resistant to antibiotics as the worldwide prevalence of antibiotic resistant bacteria are on increase and may cause serious human health. The bacterium pseudomonas is one of the best free living microorganisms. It has various species pathogenic and non-pathogenic. It is widely used as indicator for fecal contamination in water bodies. The morphology of pseudomonas was based on the morphological and physiological characteristic in morphology experiment the pseudomonas was gram negative, rod shaped and the texture of pseudomonas was smooth.

Previous worker describe the antibiotic susceptibility on pseudomonas and E. coli on the basis of zone of inhibition. We also perform the same experiment and found following results. In compared to *E. coli* and all *P*seudomonas were showing the resistance with the antibiotics Zenflox, cefadroxil, cifixime. *E. coli* isolates were showing the sensitivity toward all the tested antibiotics.

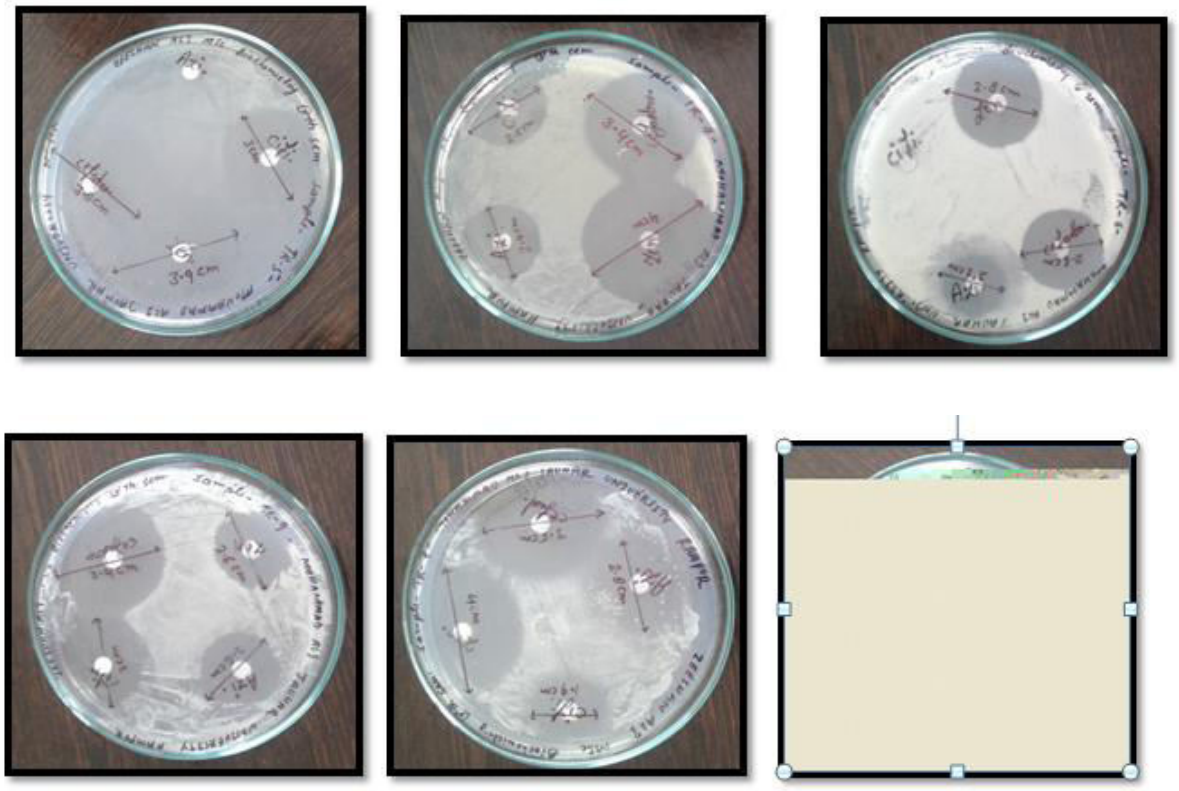


Figure: 2-: Zone of inhibition of different antibiotics at 4mg/ml concentration

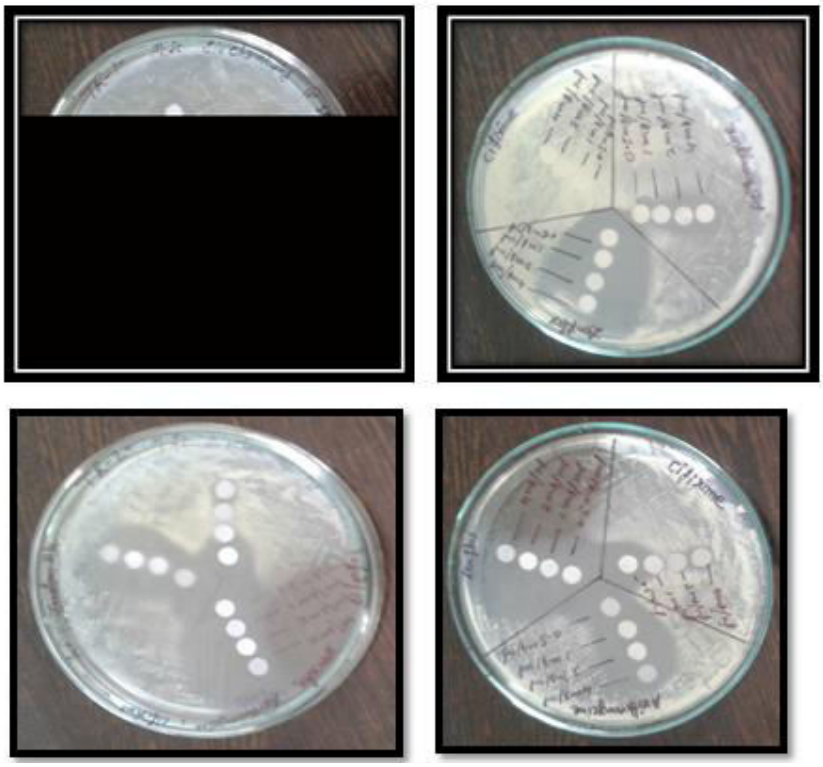


Figure 3:- MIC of different antibiotic at the different concentration

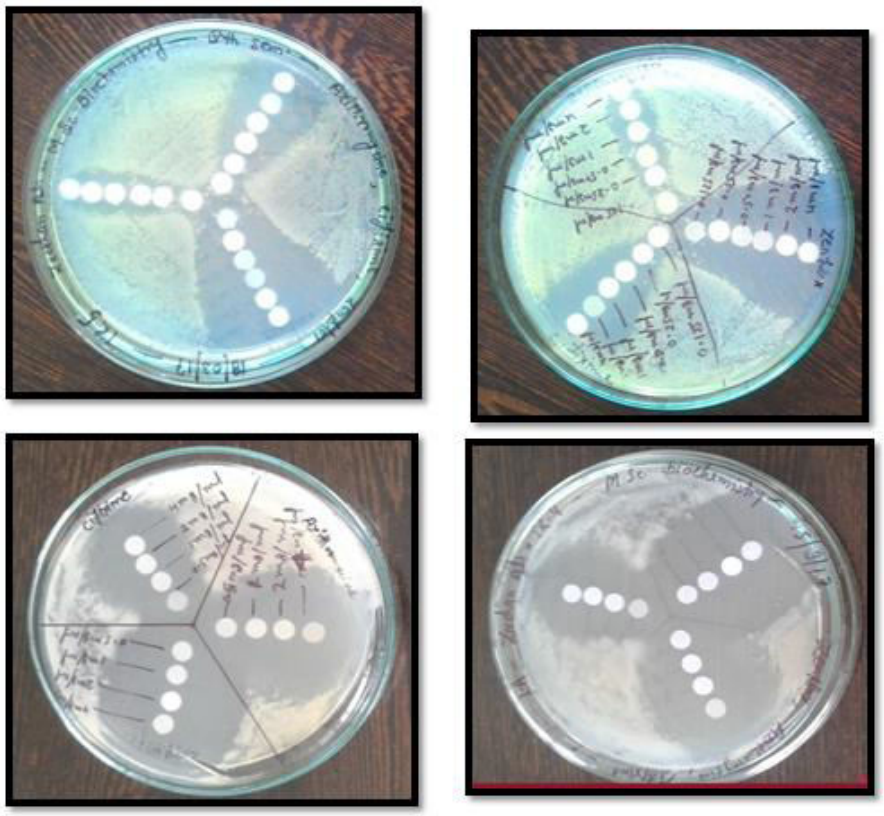
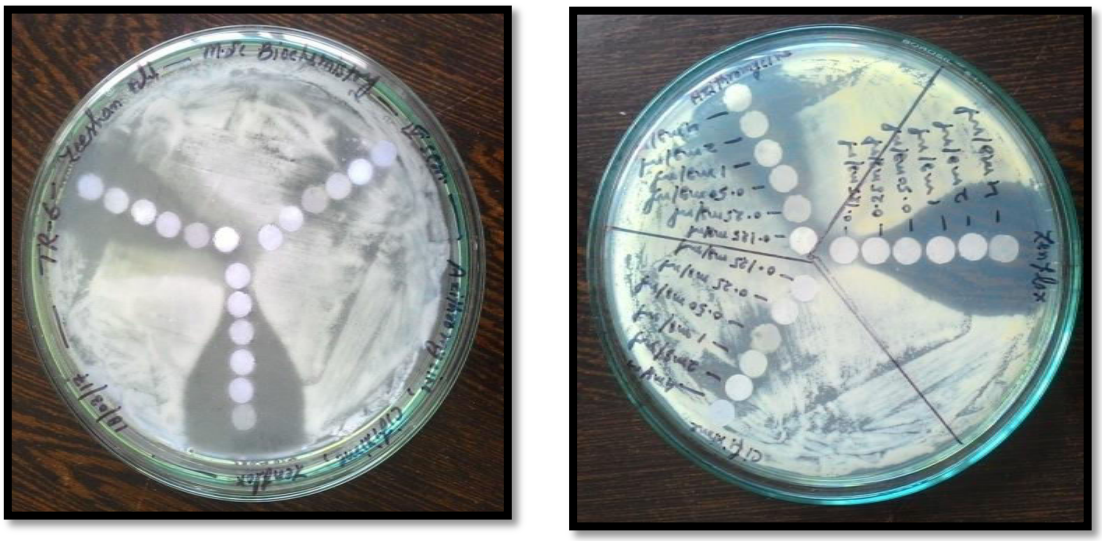


Figure 4-: MIC of different antibiotic at the different concentration.



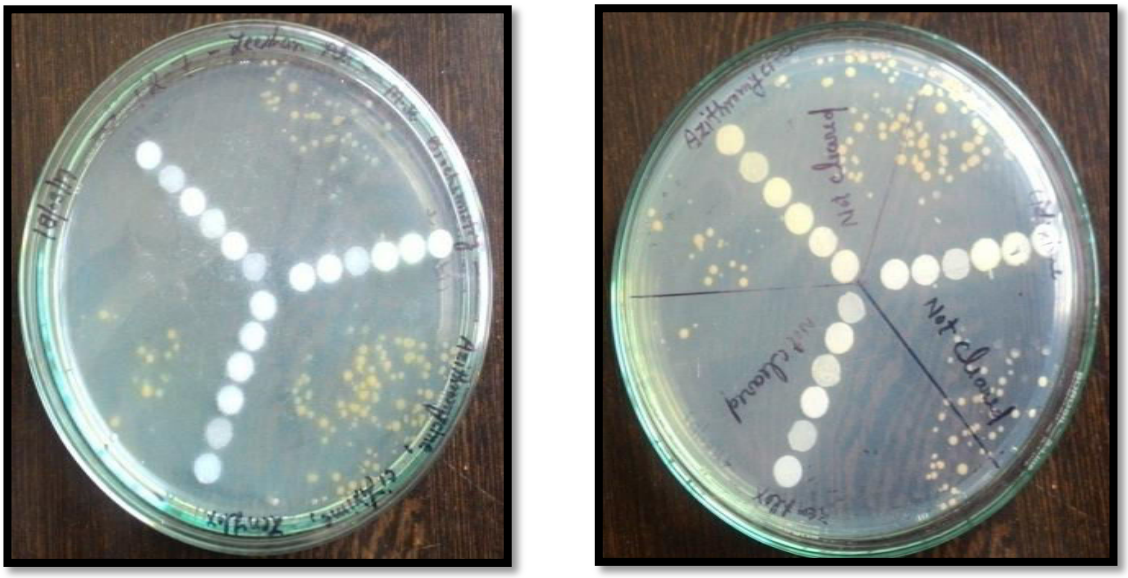


Figure 5: MIC of different antibiotic at the different concentration

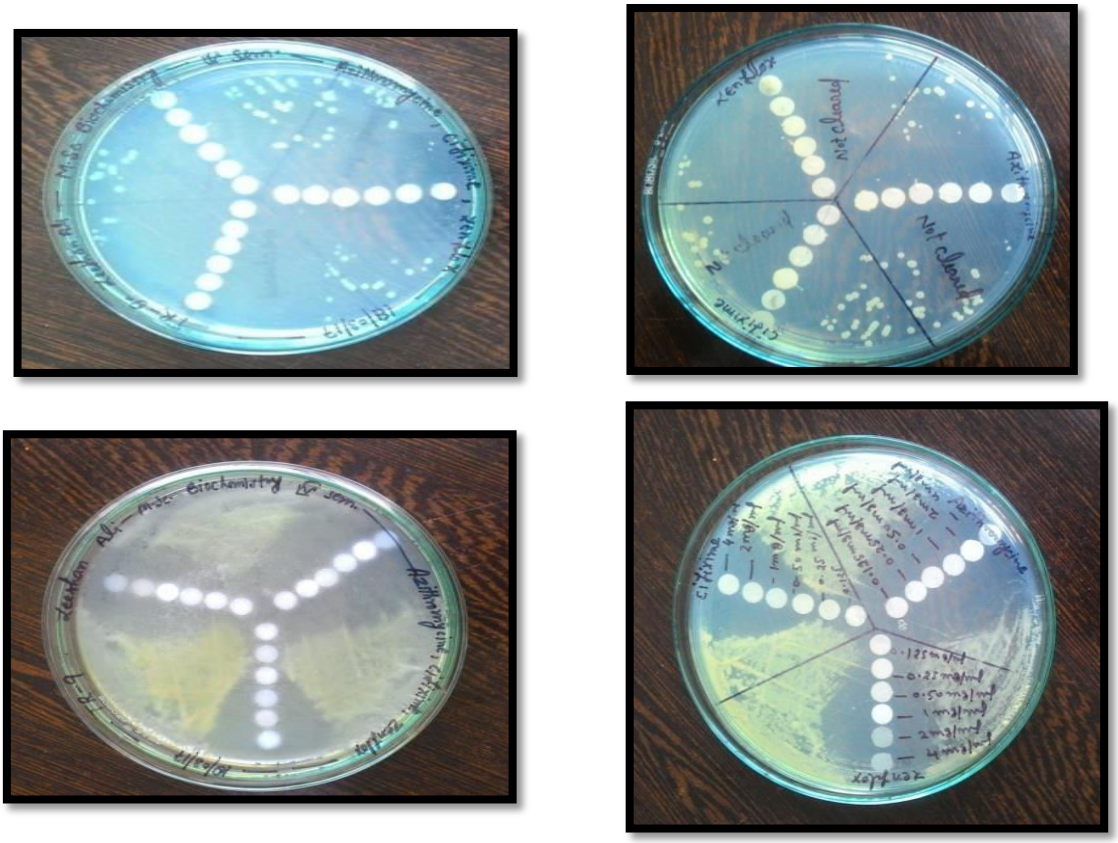


Figure 6: MIC of different antibiotic at the different concentration

Figure 7: MIC of different antibiotic at the different concentration

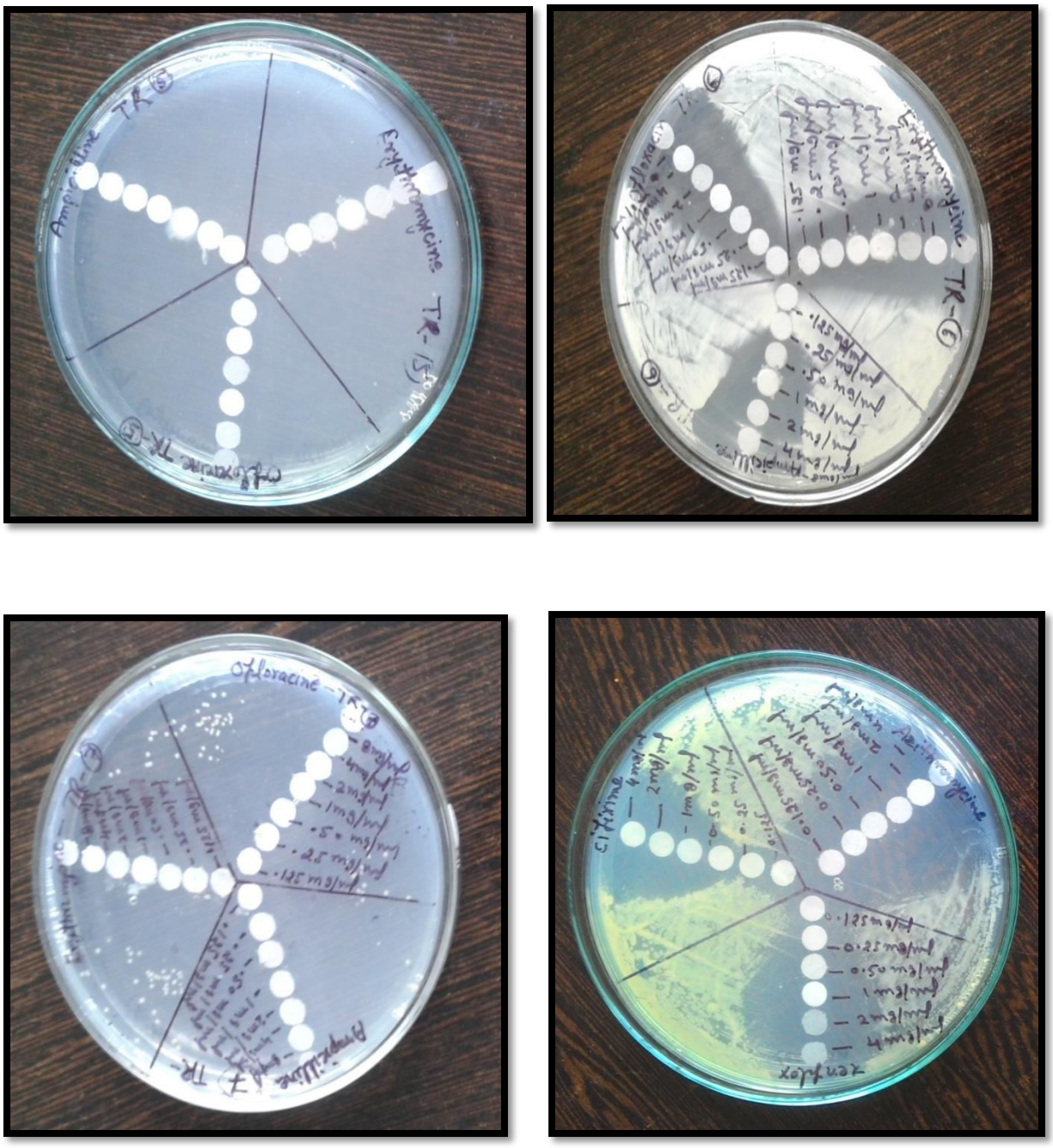


Figure 8: MIC of different antibiotic at the different concentration

Figure 9: MIC of different antibiotic at the different concentration

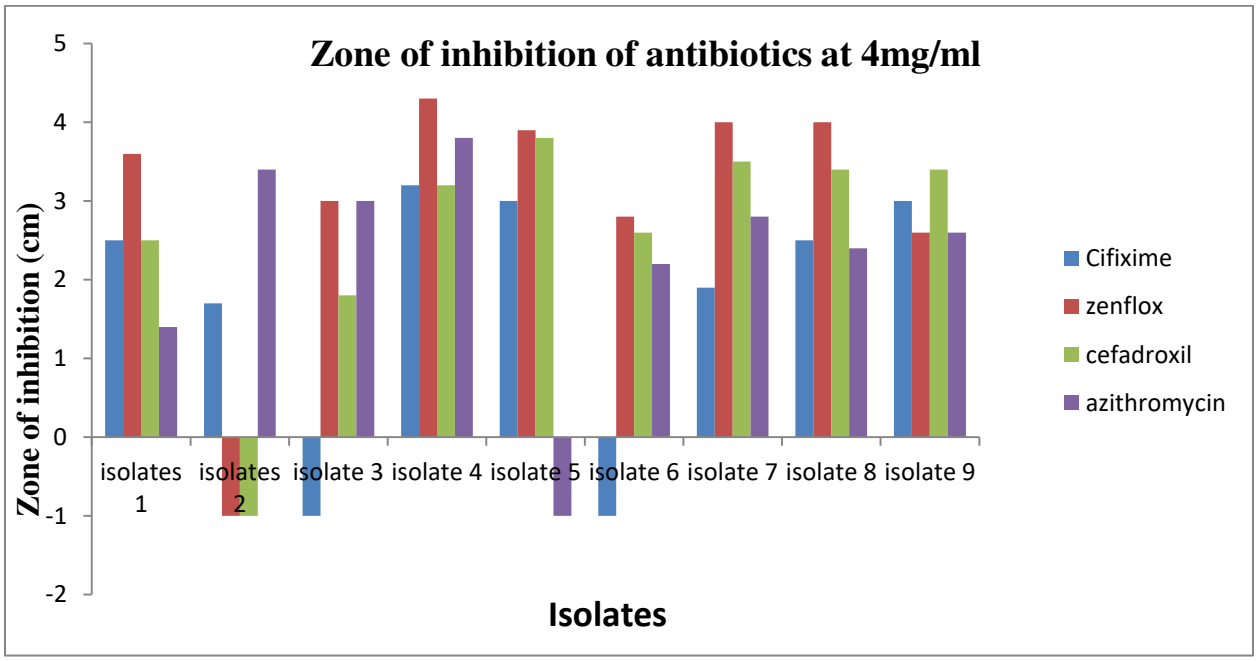


Figure 10: Graph represents antibiotics zone of inhibition

Table: 1- Antibiotic susceptibility on *Pseudomonas* and *E. coli*

SN	Antibiotic	Zone of Inhibition at 4mg/ml concentration								
		TR-1	TR-2	TR-3	TR-4	TR-5	TR-6	TR-7	TR-8	TR-9
1	Cifixime	R	1.7cm.	R	3.2cm.	3.0cm.	R	1.9cm.	2.5cm.	3.0cm.
2	Zenflox	3.6cm.	R	3.0cm.	4.3cm.	3.9cm.	2.8cm.	4.0cm.	4.0cm.	4.6cm.
3	Ofloxacin	3.3cm.	3.5cm.	R	R	R	R	R	R	R
4	Cefadroxil	2.5cm.	R	1.8cm.	3.2cm.	3.8cm.	2.6cm.	3.5cm.	3.4cm.	3.4cm.
5	Azithromycin	1.4cm.	3.4cm.	3.0cm.	3.8cm.	R	2.2cm.	2.8cm.	2.4cm.	3.6cm.

Table : 2- MIC of different antibiotic at the different concentration of Antibiotics

Antibiotic	Concentration (mg/ml)	Number of isolates								
		TR-1	TR-2	TR-3	TR-4	TR-5	TR-6	TR-7	TR-8	TR-9
Ofloxacin	8 mg/ml	+	+	+	+	+	+	+	+	+
	4 mg/ml	+	+	+	+	+	+	+	+	+
	2mg/ml	+	+	+	+	+	+	+	+	+
	1mg/ml	+	+	+	+	+	+	+	+	+
	0.5mg/ml	+	+	+	+	+	+	+	+	+
	0.25mg/ml	+	+	+	+	+	+	+	+	+
	0.125mg/ml	+	+	+	+	+	+	+	+	-
Ampicillin										
	8mg/ml	+	+	+	+	+	+	+	+	+
	4mg/ml	+	+	+	+	+	+	+	+	-
	2mg/ml	+	+	+	+	+	+	+	+	-
	1mg/ml	+	+	+	+	+	+	+	+	-
	0.5mg/ml	+	-	+	+	+	+	+	+	-
	0.25mg/ml	+	-	+	+	+	-	+	+	-
	0.125mg/ml	+	-	+	+	+	-	+	+	-
Erythromycin										
	8mg/ml	+	+	+	+	+	+	+	+	+
	4mg/ml	+	+	+	+	+	+	+	+	+
	2mg/ml	+	+	+	+	+	+	+	+	-
	1mg/ml	-	+	+	+	+	+	+	+	-
	0.5mg/ml	-	+	+	+	+	+	+	+	-
	0.25mg/ml	-	+	+	+	+	+	+	+	-
	0.125mg/ml	-	+	+	+	+	+	+	+	-

Table : 3- MIC of different antibiotic at the different concentration of Antibiotics

Antibiotic	Concentration (mg/ml)	Number of isolates								
		TR-1	TR-2	TR-3	TR-4	TR-5	TR-6	TR-7	TR-8	TR-9
Zenflox	8 mg/ml	+	+	+	+	+	+	+	+	+
	4 mg/ml	+	+	+	+	+	+	+	+	+
	2mg/ml	+	+	+	+	+	+	+	+	+
	1mg/ml	+	+	+	+	+	+	+	+	+
	0.5mg/ml	+	+	+	+	+	+	+	+	+
	0.25mg/ml	-	-	-	-	+	+	+	+	+
	0.125mg/ml	-	-	-	-	+	-	+	+	-
Azithromycin										
	8mg/ml	+	+	+	+	+	+	+	+	+
	4mg/ml	+	+	+	+	+	+	+	+	+
	2mg/ml	+	+	+	+	+	+	+	+	+
	1mg/ml	+	+	+	+	+	+	+	+	+
	0.5mg/ml	+	-	+	+	+	+	+	+	-
	0.25mg/ml	-	-	-	-	+	+	+	+	-
	0.125mg/ml	-	-	-	-	+	+	+	+	-
Cifixime										
	8mg/ml	-	+	+	+	+	+	+	+	+
	4mg/ml	-	+	+	+	+	+	+	+	+
	2mg/ml	-	+	+	+	+	+	+	+	-
	1mg/ml	-	+	+	+	+	+	+	+	-
	0.5mg/ml	-	+	+	+	+	+	+	+	-
	0.25mg/ml	-	-	-	-	+	+	+	+	-
	0.125mg/ml	-	-	-	-	+	+	+	+	-

Table : 4- Morphological and physiological characteristics of different isolates

	An outline of the morphological and physiological characteristics of isolate			
Isolate no.	**Morphology**	**Gram staining**	**Growth an aerobically**	**Pigmentation**
1	Rod	-ve	+ve	Colorless
2	Rod	-ve	+ve	Colorless
3	Rod	-ve	+ve	Colorless
4	Rod	-ve	+ve	Colorless
5	Rod	-ve	+ve	Colorless
6	Rod	-ve	+ve	Colorless
7	Rod	-ve	+ve	Colorless
8	Rod	-ve	+ve	Colorless
9	Rod	-ve	+ve	Colorless

Table : 5- Biochemical characterization of *Pseudomonas* and *E. coli*

Isolate no	**Indole test**	**Methyl red test**	**Vogues proskeur test**	**Simmons test**	
				Ammonium citrate test	**Ammonium acetate test**
1	-Ve	+ve	-Ve	+ve	-ve
2	-Ve	+ve	-Ve	+ve	-ve
3	-Ve	+ve	-Ve	+ve	-ve
4	-Ve	+ve	-Ve	+ve	-ve
5	-Ve	+ve	-Ve	+ve	-ve
6	-Ve	+ve	-Ve	+ve	-ve
7	+ve	+ve	+ve	+ve	-ve
8	+ve	+ve	+ve	+ve	-ve
9	+ve	+ve	+ve	+ve	-ve

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