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Research Paper

ISOLATION AND CHARACTERIZATION OF BACTERIA (*Bacillus sp.*) FROM CONTAMINATED WATER SAMPLE

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Abstract

The present investigation was carried out to isolate and chraterization of bacteria from polluted and non polluted water samples. The colony forming units (c.f.u) of contaminated water samples was found to be more significant in contaminated water than the fresh water. Pure culture of two selected isolated bacterial isolates were carried out and observed that the isolate 1 shows positive against H_2S production, starch hydrolysis and catalase test and negative against Urease test whereas the isolate 2 shows positive against starch hydrolysis, catalase and urease test and negative against H_2S production. Carbohydrate fermentation test shows positive against glucose, sucrose, xylose and lactose for isolate 1. Isolate 2 shows positive against glucose and lactose source only and could not utilise the sucrose and xylose to carry out fermentation. The bacteriological analysis revealed that the *Bacillus* sp was present in contaminated water. Hence, it is very much necessary to recommend the proper sanitation, regular treatment, supervision of water sources and regular bacteriological assessment of all water sources for consumption as a drinking water.

Key words: Water cycle, biochemical test, antibiotic sensitivity, *Bacillus* sp., Bioremediation.

INTRODUCTION

Water pollution is a major global problem which requires ongoing evaluation and revision of water resource policy at all levels. it has been suggested that pollution is the leading worldwide cause deaths and diseases, and that it account for the deaths of more than 14,000 people daily. About 90 percent of water in the cities of is polluted, in addition to the acute problem of water pollution in developing countries.

Developed countries also continue to struggle with pollution problem, water is typically referred to as polluted when it is compared by anthropogenic contaminant and either does not support a human use such as drinking water, or undergoes a marked shift in its ability to support its constituent biotic communities, such as fish. Natural phenomena such as volcanoes, algal bloom, storms and earthquake also causes major changes in water quality and the ecological status of water.

Many of the major problems that humanity is facing in the twenty-first century are related to water quantity or water quality issue. These problems are going to be major aggravated in the future by climate change, resulting in higher water temperature, melting of glaciers, and an intensification of the water cycle.

Bacteria are microscopic, single celled organisms. They are so small that five million could be placed on the head of a pin. Under favourable conditions they can reproduce rapidly and can form colonies that are visible without magnification. Bacteria can utilize a large variety of habitats and can survive and adapt to almost all conditions present on planet earth. They have been so successful that they are the most numerous life forms on the planet. Most bacteria are beneficial and responsible for important environmental processes such as decomposition, nutrient cycling and the breakdown of environmental toxins. Some bacteria, however, are pathogenic (or disease causing) and result in human health problems.

The nature of the bacteria present in these sites was identified by microscopy, as well as a series of biochemical tests based on bacterial metabolism. By investigating fluctuations in these microbial environments, especially information about numbers, biomass, and activity, it becomes possible to more rapidly evaluate the state of health of other dependent ecosystems. The water cycle represents an obvious mode of disease transmission, which makes water supply sanitation the most essential feature for the prevention of infection. Recent studies by the World Health Organization (2014) have determined that more than approximately four million people die worldwide from water associated diseases, mostly intestinal infections, every year.

According to Olstadt et al., (2012) estimated Total Escheria coli (EC) in water samples collected from different sampling areas (Higher Income Group, Minimum Income Group, Lower Income Group, Juggi Jhopari and Industrial Area) of entire

Kanpur during seasonal variation (Summer, Rainy and Winter) which revealed the unsanitary condition of water sources. Result showed that Maximum EC were in JJ area water samples and were minimum from contamination in HIG area. However, MIG, LIG and IA areas behaved intermediary.

Subhadradevi et al., (2003) studied river water from different parts of western Tamil Nadu, India samples were collected in the period between January – March 2012 and various physico-chemical and microbial analyses were performed based on standard methods. The comparative results showed the pH (7.5 to 10.0), DO (8 - 16 mg/ml), BOD (2.5 - 7.5 mg/L), COD (14.5 - 15 mg/L), total hardness (100-520 mg/L), calcium (80- 200 mg/L), magnesium (20- 320 mg/L), number of bacterial colonies(100- 120 CFU) and number of fungal colonies(30-45 CFU) thus concluded that low pH values obtained in the river water samples, which may consequently affect the bacterial counts. Agarwal et al., (2012) reported that a comparative study of antimicrobial properties of four varieties of Piper betel; namely Desawari, Desi, Bangladeshi and Jaleswar, cultivated in India. Cold Aqueous, Methanolic , Ethanolic, and Ethyl Acetate extracts of dried leaves of all the four varieties of Piper betel at a final concentration of 500 mg/ml were tested against pathogenic microorganisms such as *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* using agar well diffusion method.

According to Mohadikar et al., (2011) Regional Forensic Science Laboratory, Jodhpur, Rajasthan, India The evaluation of water quality for a specific water body is typically based on the major uses for that source. The acceptable amount of faecal coliform bacteria in water used for irrigating vegetable crops is therefore different from the guideline that is applied to waters used for swimming or drinking. Regions of extensive agriculture and sewage outfalls have the potential to introduce pathogens into surface waters which must be removed by water treatment before direct consumption. The guidelines for water quality stipulated by Alberta Environment indicate acceptable levels of indicator organisms that can be present to minimize the possibility of waterborne disease. Livestock (especially beef cattle) can be important sources of these microorganisms, especially during periods of high runoff. Agricultural inputs are generally considered to be non- point sources because of their wide

geographic distribution and are difficult to control. Point sources such as sewage outfalls are much easier to regulate and treat.

Pathak et al.(2012) reported that the physic-chemical status and anthropogenic activity of two water bodies at Sagar city. The maximum colonies being observed during summer and monsoon season. Rainfall is one of the factor which affects the bacterial densities. Thakur et al (2012) reported the characterization of water contamination bacteria in drinking water samples in Himachal Pradesh , India. Chandra et al . (2006) analyzed The seasonal physico-chemical and microbial quality of Gola river water after confluence of pulp paper mill waste. The study revealed that it has enhanced 20- 30 times pollution load of BOD, COD, TDS, TSS, sulphate, chloride, sodium, nitrate, potassium, lignin and phenol after mixing of pulp paper mill waste with river water in all season. Further, it induced the bacterial growth by increasing most probable number value of E. coli was 1.57 x 10(4), 1.6 x 10(4), 1.37 x 10(4) and SPC count was 1.68 x 10(4), 1.64 x 10(4),1.67 x 10(4)/100 ml during summer, monsoon, winter respectively. The monsoon season showed presence of FC and TC indicated the thermo-tolerant and disease causing group of bacterial population in effluent and its sequence was observed as monsoon>summer>winter. This indicated the growth of many pathogenic and non -pathogenic bacteria for health hazards. Ramteke et al., (2005) reported that the microbiological analysis of river water indicated that population of bacteria in the monsoon was maximum followed by winter and summer. The faecal coliform bacteria were isolated in three seasons from most sampling sites of Pavana river, while population of faecal coliforms in monsoon was more compared to the other seasons. Begum et al., (2005) reported that overball bacterial count was highest in river, followed by well, supply water and tubewell. The critical differences showed that the variation in bacterial count between any two sources was highly significant. However, the variation in bacterial load between supply water and tube well was not significant. Olatunji et al.(2011) studied physicochemical and bacteriological evaluation of pollution in the Unity Road stream segment of Asa River in Ilorin, Nigeria. The water pH was found to range from 6.32 to 6.43 with a mean temperature range of 24.3 to 25.8 °C. Other physicochemical parameters monitored including total suspended solids, total dissolved solids, biochemical oxygen demand and chemical oxygen demand values exceeded the recommended level for surface water quality. Results of bacteriological analyses including total heterotrophic count, total coliform and thermo

tolerant coliform counts revealed a high level of faecal pollution of the river. It was inferred that the downstream As a River is polluted and its aquatic biota is bacteriological contaminated and unsafe for human and animal consumption. Ostensvik et al. (2004) isolated cytotoxic *Bacillus* spp. belonging to the *B. cereus* and *B. subtilis* groups from surface waters of Norwegian.

Zvidzai et al., (2007) stated that traditional methods employing selective, differential and non selective media were used to isolate and identify different species of bacteria from rural water reservoirs of Mount Darwin district Zimbabwe. The colony counts from non selective nutrient agar plates give an indication of the overall level of bacterial activity from each water sample. Open deep wells shallow wells and rivers were found to be the most heavily contaminated water sources. Borehole water sources had very low total microbial loads and men absent in some of the water samples. The prevalent bacteria found were the gram negative *Escherichia coli, Shigella, Salmonella, Enterobacter aerogenes*. The presence of faecal pathogenic species in the river water and open wells poses epidemiological cases of diarrhoeal cases in the district studied. Tiwari et.,(2005) showed characteristic changes in bacterial population through potable water treatment with high TDS, TSS,BOD, and COD and reported that due to discharge of untreated sewage into the Ganga, the water quality of Ganga has been severely deteriorated and the potable nature of water is being lost. Therefore to be necessary to ensure that water treatment and distribution do not cause a shift in the composition of the bacterial population that would favour opportunistic pathogens. Shariq et al.(2016) reported Several water born disease and chronic health problem which are caused by consuming unsafe drinking water.

MATERIALS AND METHODS

A. Collection of water samples:

Two water sample were collected, one from contaminated water source and one from fresh water from college campus. The sample were brought to the laboratory and kept in refrigerator at 4^0C

B. Cultural and morphological features of the bacteria isolates

The serial dilution technique is followed for the isolation and enumeration of bacteria .The cultural and morphological features falls under the phenotypic characterisation, which were studied by adopting standard methods (Goodfellow, 1989).Morphology of colonies on plates is a characteristic feature of bacterium, which is quite useful in preliminary identification procedure. Different colony features such as configuration, elevation, margin, texture, consistency etc. were noted down by using a hand lens.

C. Gram staining

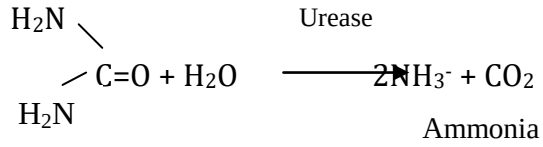
Gram staining, a differential staining technique separates bacteria into two groups, Gram positive and Gram negative. A thin smear of the bacterial culture was made on a glass slide, after heat fixing it was stained with crystal violet for one minute. After rinsing with water Grams iodine solution was added and kept for one minute. It was washed with water and then decolourised with 95% ethyl alcohol for 20 seconds. Rinsed with water and then counter stained with safranin for one minute. Rinsed with water blotted dried and then observed under the microscope. The nature of Gram's reaction was observed and morphological details were noted down. Cells were identified by the colour observed purple for Gram positive and pink or red for Gram negative cells.

D. Biochemical characterization of bacterial isolates

For biochemical characterisation, the isolate was tested for urease activity, nitrate reduction, H_2S production, Starch amylase test, Catalase activity etc. and fermentation of 5 different sugars. Identification of the bacterial isolates was carried out according to Bergey's Manual of Systematic Bacteriology (Holt *et al*., 1994).

(i) Urease test

Microorganisms with the ability to produce hydrolytic enzyme urease breaks down urea with the liberation of ammonia as shown below:



The urease test was performed by inoculating the bacterial isolates into Urease broth. Urea was sterilised by ultraviolet irradiation before adding to the sterilised medium) containing in test tubes and was observed after incubation as above. The colour change of the indicator from yellow to pink indicated a positive result due to the accumulation of ammonia which raises the pH of the medium.

(ii) H_2S production

Hydrogen sulphide production was studied by stab inoculating the bacterial isolates in SIM (Hydrogen sulphide, indole and motility test agar) agar deep tubes and incubated as above. Blackening of the culture medium indicated the positive test for H_2S production. This black colour was due to the production of H_2S from sodium thiosulphate, which can combines with ferrous ammonium sulphate of the medium, resulting in the formation of the black insoluble compound, ferrous sulphide.

(iii) Starch hydrolysis

The test is used to differentiate based on their ability to hydrolyze starch with the enzyme α-amylase or oligo-1,6-glucosidase by breaking the glycosidic linkages between the sugar subunits. The bacterial cultures were streaked on the starch agar plates and incubated at 30^0C for 24-48 hrs. After incubation, iodine solution was flooded on the petriplates.Iodine reacts with starch and produces a blue or dark brown colour, therefore, any microbial growth hydrolysis will be revealed as a clear zone surrounding the growth.The formation of clear zone around the colony was takena as a positive test.

(iv) Catalase test

The enzyme catalase present in some microorganisms breaks down hydrogen peroxide to water and oxygen which helps them in their survival. The bacterial isolates were inoculated into Nutrient agar (NA) and then incubated for 48 hours at 30±2.0 ^{0}C and After proper growth, catalase production was determined by introducing 5-6 drops of H_2O_2 (20%) into each slides.Release of free oxygen gas (O_2) bubbles indicated a positive catalase test.

(v) Fermentation of carbohydrates

Fermentative degradation of various carbohydrates such as glucose, sucrose, lactose, maltose, fructose, galactose, arabinose, and mannitol was carried out in a fermentation tube that contained Durham's tube a small tube placed in an inverted position inside the

culture tube) for the detection of gas production. The medium used contained ingredients of nutrient broth, a carbohydrate source (Glucose, sucrose, xylose, maltose,) an indicator (phenol red) and pH-7.3. The broth was prepared separately for each set of carbon source; cultures were inoculated and incubated at 30^0C. Change of colour of the broth from red to yellow indicated the production of organic acid as a result of fermentation of the particular carbohydrate. Phenol red which was red at neutral pH (7.0) turned yellow at or below a pH of 6.8 due to accumulation of organic acid. No change in the colour when compared to the uninoculated control indicated negative for acid production.

(vi) Determination of antibiotic resistance

To determine the antibiotic sensitivity of the bacterial isolates, antibiotic discs (Hi-media) were placed on freshly prepared lawns of each isolates on nutrient agar plates. The isolates were tested for antibiotic sensitivity according to Kirby-Bauer disc diffusion method (Bauer *et al*., 1966) to 12 antibiotics. The selected antibiotics were placed on the plate and incubated at 37ºC for 24 hours. The diameter of the inhibition zones was measured to the nearest mm and the isolates were classified as resistant (R), intermediate (I) and susceptible (S) following the standard antibiotic disk sensitivity testing method.Discs containing the following antibiotics were used: Penicillin G (10 units), Polymyxin B (300 units), Streptomycin (10 mcg), Tetracycline (30mcg).

RESULTS:

Study Site Showing Contaminated Water

Table 1: C.F.U of contaminated and fresh water samples

Dilution factor	Isolated colonies of Water samples		C.F.U	
	Contaminated water	Fresh water	Contaminated water	Fresh water
10^{-1}	142±0.5	34±0.5	$142X10^{-1}$	$34X10^{-1}$
10^{-2}	78±0.3	19±0.4	$78X10^{-2}$	$19X10^{-2}$
10^{-3}	41±0.2	9±0.5	$41X10^{-3}$	$9X10^{-3}$
10^{-4}	25±0.5	5±0.3	$25X10^{-4}$	$5X10^{-4}$

Table 2: Biochemical test and identification of bacterial isolates

Biochemical test	Isolate (*Bacillus* sp1)	Isolate 2(*Bacillus* sp 2)
Gram staining	(+)ve; (rod shaped)	(+) ve; (rod shaped)
Urease Test	-	+
H_2S production	+	-
Starch hydrolysis	+	+
Catalase Test	+	+
Fermentation test		
Glucose	+	+
sucrose	+	-
Xylose	+	-
lactose	+	+
Antibiotic sensitivity test		
Penicilliin	No inhibition	No inhibition
Polymyxin B	12(I)	14(S)
Streptomycin	31 (S)	28(S)
Tetracycline	24(S)	22(S)

NI=No Inhibition, Diameter of disc =6mm

Letter in parenthesis indicate sensitivity; R = Resistant; I = Intermediate; S = Susceptible.

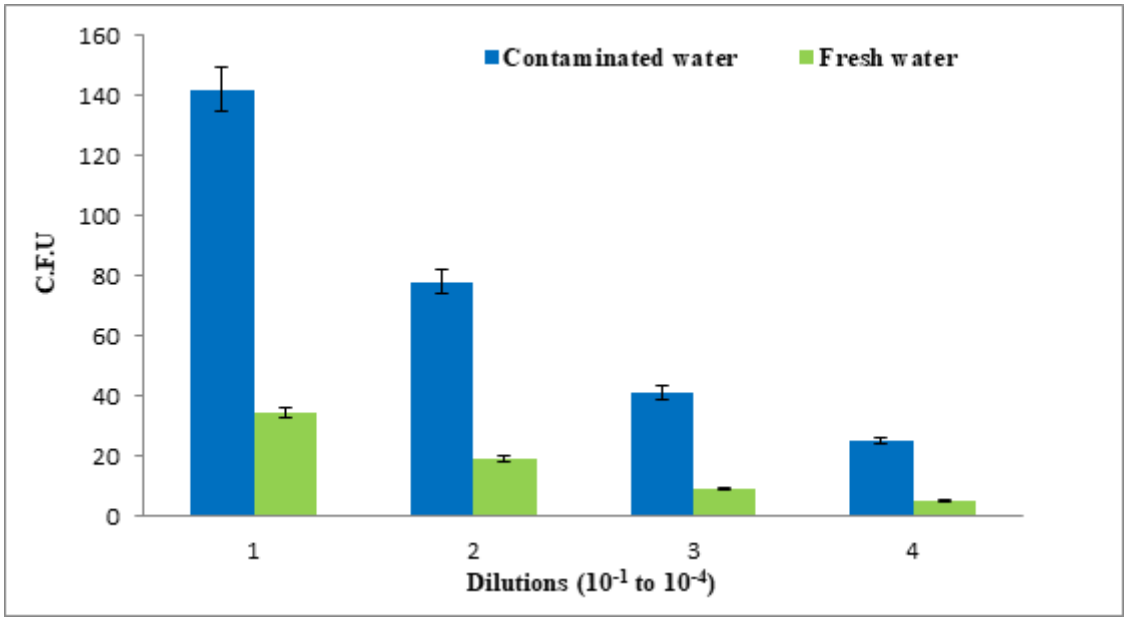


Figure 1: C.F.U of contaminated and fresh water samples

The colony forming units (c.f.u) of contaminated and fresh water have been estimated and it is observed that in contaminated water, c.f.u is significant more than the fresh water. In 10^{-1} dilution, c.f.u. of contaminated water is 142 and in fresh water is 34.In all other dilutions from 10^{-2} to 10^{-3} the bacterial colonies are recorded more in contaminated water than the fresh water sample (**Table 1; Figure 1**).

Pure culture of two selected bacterial isolates were carried out to and biochemical tests.,viz., Urease , H_2S production, starch hydrolysis and catalase test were done. The isolate 1 shows positive against H_2S production, starch hydrolysis and catalase test and negative gainst Urease test whereas the isolate 2 shows positive against starch hydrolysis, catalase and urease test and negative against H_2S production (**Plate 1**; **Table 2**).

Carbohydrate fermentation test shows positive against glucose, sucrose, xylose and lactose for isolate 1. Isolate 2 shows positive against glucose and lactose source only and could not utilise the sucrose and xylose to carry out fermentation.

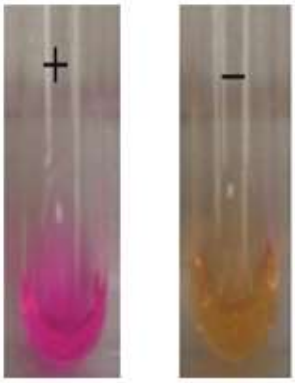
The two bacterial strains were tested for antibiotic sensitivity against Penicillin, Polymyxin B, Streptomycin and Tetracycline .The isolates were found to be multi-antibiotic susceptible to Polymyxin B, Streptomycin and Tetracycline and showed no inhibition against Penicillin antibiotic (**Plate 2**).

Pure Culture of isolate

Pure Culture in Nutrient Broth

(+) H_2S production (isolate 1)

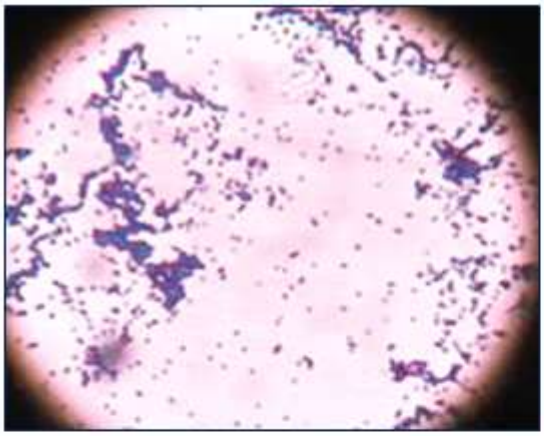
(+) Starch hydrolysis (isolates 1 & 2)



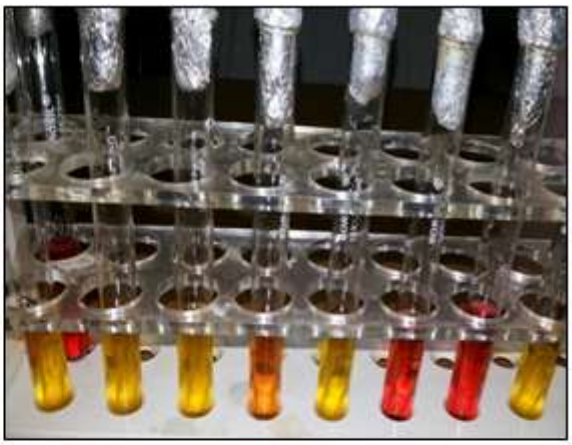
(+) Urease Test (isolate 2)

(+) ve Catalase test (isolates 1 & 2)

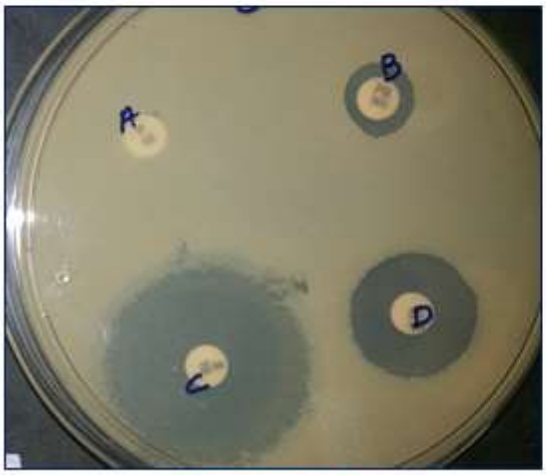
Plate 1: Positive (+) Biochemical tests of bacterial isolates



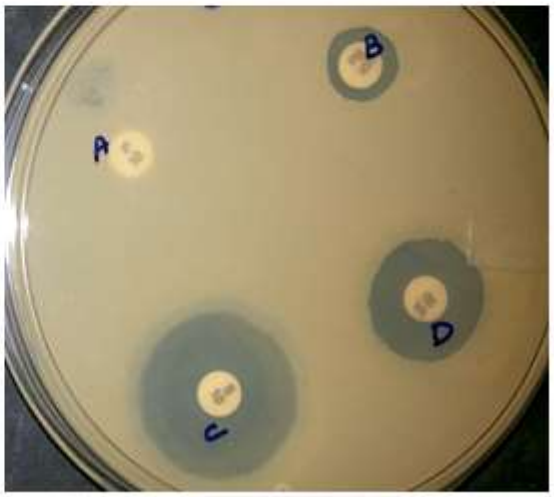
1. Gram staining of showing (+) isolate



2. Carbohydrate fermentation Test (Glucose, Sucrose, Xylose, Lactose)



Isolate 1



Isolate 2

3. Antibiotic sensitivity Test

A: Penicillin (10mcg) B. Polymyxin (300) C. Streptomycin (10mcg), D: Tetracycline (30mcg)

Plate 2: 1). Gram's staining, 2) Carbohydrate fermentation test, 3) Antibiotic sensitivity Test of isolate 1 & 2

DISCUSSIONS:

The isolated were observed to be gram (+)ve as the glycoproteins present on the outer site of Gram positive bacterial cells have more potential binding sites than the Gram negative bacteria having an outer layer of lipopolysaccharide (LPS),phospholipids and

proteins (Rathnayake *et al*., 2009; Karelova *et al*., 2011; Gupta *et al*., 2012; Issazadeh *et al*., 2013). The isolates were showed positive activity Urease, H_2S production, starch hydrolysis and catalase test. Similar observations were reported by different workers for these Gram positive strains of same bacterial strain. The premilinary results of all chemotaxonomic characters revealed that the isolated strains could be Bacillus sp. Similar biochemical activities were observed earlier by various workers (Graumann ,2012;Wazeck, 2013; Kacar and Kocyigit, 2013; Elsilk *et al*., 2014).

The presence of toxigenic *Bacillus* sp. in water sample indicates that water may play a role as a risk factor for introducing food poisoning to the various animals as the spores which are present in the source water may pass through drinking water treatment and can create health problems in human beings (Baldursson and karanis, 2011; Elangovan et al., 2006; Sundar et al., 2010., Szabo et al., 2017). Recent studies by the World Health Organization (2014) have determined that more than approximately four million people die worldwide from water associated diseases, mostly intestinal infections, every year. The major health risk is associated with the consumption of water contaminated with human and animal faecal matter. This is true for both freshwater and coastal water. Diseases associated with consumption of contaminated water include cholera, gastroenteritis, typhoid fever, shigellosis, and severe diarrhea. Bacteria such as *Clostridium perfringens*, *Shigella sonnei*, *Escherichia coli*, *Bacillus* spp. *Salmonella typhi*, and *Vibrio cholerae* are microorganisms associated with water supply known to cause diseases (Cabral, 2010, United States Environmental Protection Agency, 2012). The isolated strains were found resistant to Penicillin, Polymyxin B, Streptomycin and Tetracycline. Our results are in conformity with the results of Gao et al., (2012) who also observed the tetracycline-resistant bacteria and resistance genes in aquaculture environment. Consequently, *Bacillus* sp. present in drinking water can be transferred to food products and can be regarded as a hazard for contamination to create water pollution (Tallent et al., 2012, Bauman,2014, Tantry et al., 2015., Fletcher et al., 2017). To achieve more knowledge of water as a risk factor for contamination of water with cytotoxic *Bacillus* sp. further investigations is needed to characterize and prevent the contamination for the safe use of human consumption.

CONCLUSION:

In developing countries, the most common cause of gastroenteritis which affects humanity is due to lack of safe and clean drinking water supply. The bacteriological analysis revealed that the *Bacillus* sp is present in contaminated water. Hence, we would like to recommend the proper sanitation, regular treatment, supervision of water sources and regular bacteriological assessment of all water sources for drinking should be planned and conducted for bioremediation process.

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