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RESEARCH ARTICLE

ACINETOBACTERBAUMANNII: ISOLATION, IDENTIFICATION AND ANTIMICROBIAL RESISTANCE

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ABSTRACT

Acinetobacter is a common type of bacteria found in many places in the environment, including water, soil, and sewage. Ten strains of *Acinetobacterbaumannii* were isolated from Industrial wastewater Al-Furat Company in Hilla- Iraq. The Study aimed to evaluate the possible ecological effect of the industrial waste water released from Company. Samples were monthly taken started from June 2014 to December 2014 .

The results showed that properties of industrial wastewater variable due to Sampling different months In addition to sampling of neutral and soda tank . It was found the pH ranged from (6.5 – 11.9) (6.5 – 9.9) , conductivity reached to (4660- 4280)Ms / cm while the higher concentration of COD, TDS , Cl , SO4 , Ca and Mg were (150- 160), (2936- 8552) , (4831- 4931) , (593 - 810), (724-757) and (367 - 399) mg/l respectively .

Acinetobacterbaumannii suspected isolate was screening by traditionally tests and then confirmed by Vitek 2 system and PCR technique (*rpoB*gene). In this study *A.baumannii*strains are highly resistance to several antibiotics that was considered as pathogen for human being causing several disease.

INTRODUCTION

Wastewateris simply that part of the water supply to the community or to the industry which has been used for different purposes and has been mixed with solids either suspended or dissolved. Wastewater is 99.9% water and 0.1% solids. (Kadrivelu *et al*., 2001).

Industrial wastewater is one of the important pollution sources in the pollution of the water environment. During the last century a huge amount of industrial wastewater wasdischarged into rivers, lakes and coastal areas. This resulted in serious pollution problemsin the water environment and caused negative effects to the eco-system and human's life (Elizabeth *et al*., 2004).

Industrial pollution and waste pose potential threats to human and ecological health if not properly managed. The concerns range from toxic effects on fetuses and children to the health implications of low-level exposures to multiple pollutants and the degradation of habitats and ecosystems(Henk *et al*., 1992). Basic inorganic are relatively low cost chemicals used throughout manufacturing. They are produced in very large amounts, some in millions of tonnes a year, and include chlorine, sodium hydroxide, sulfuric and nitric acids and chemicals for fertilizers (Geoffrey, 1988).

Wastewater contains pathogenic microorganisms lead to dangerous diseases to humans and animals Hazardous matter such as heavy metals that are toxic Produces odorous gases and bad smell (Valentina, 2006).

Physical characteristics of industrial wastewater vary depending on the type of industry. The measurement of the concentration of waste organic materials in a wastewater is important in the design of the treatment plant and in the control of its operation (Ademiluyi *et al*., 2009).

The principal area of concern is wastewater with a high content of enteric pathogens, including bacteria, viruses, and helminthes, which are easily transmitted through water (simon, 1999).

Among microbial communities involved in different ecosystems such as soil, freshwater, wastewater and solid wastes, several strains belonging to the genus of *Acinetobacter* have been attracting growing interest from medical, environmental and a biotechnological point of view. Bacteria of this genus are known to be involved in biodegradation, leaching and removal of several organic and inorganic man-made hazardous wastes. It is also well known that some of *Acinetobacter* strains produce important bio products(Ying *et al*., 2007).Wastewater of petrochemical industries contains high amounts of emulsified aliphatic or aromatic hydrocarbons

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that lead to the contamination of almost all environmental resources(Jose *et al*., 2009).

Industrial wastewater characteristics vary with each type of discharge. *Acinetobacterbaumannii*, which causes a wide range of infections, including pneumonia and blood-stream infections (Lenie *et al*., 2007).

Acinetobacter species have been found in clinical specimens but not all are considered to be clinically significant. One important question is where does *A. baumannii*come from Furthermore, are there environment (Abdel El-Haleem, 2003).

The genotypic identification of *Acinetobacter* species can be achieved by whole-genome fingerprinting, restriction analysis of a particular DNA sequence and DNA-sequence determination For identification of a strain, the genotype that is obtained by one of these methods is compared with alibrary of reference strains (Antunes *et al*, 2011).

There have also been several reports of community-acquired infections involving various *Acinetobacterspp*., including *A. baumannii*, mainly in patients with some type of co-morbidity, e.g., diabetes mellitus, alcohol abuse or chronic obstructive pulmonary disease(Simor *et al*, 2002).Most of these reports originate from tropical or sub-tropical areas. It has also been suggested that the humid environment of these areas predisposes individuals, especially those with the above mentioned co-morbidities, to *Acinetobacter* infections (Abdel El-Haleem, 2003). The ongoing modifications of the global climate that have occurred over the last decades as a result of various human interventions (i.e., global warming) may cause changes in the epidemiology of community-acquired *Acinetobacter* infections, making them more frequent occurrences in other areas of the world.

Acinetobacterbaumannii is a significant worldwide nosocomial pathogen with a particular ability to develop antimicrobial resistance and cause nosocomial outbreaks of infection (Simor *et al*, 2002). This organism frequently causes infections associated with medical devices, e.g., vascular catheters, cerebrospinal fluid shunts or Foley catheters (Cisneros and Rodriguez, 2002; Joly-Guillou, 2008).Biofilm formation is a well-known pathogenic mechanism in such infections (Rajamohan *et al*, 2009).

The degree of polymorphism of housekeeping protein-encoding genes, such asthe *recA*, *gyrB*, and *rpoB* genes, has been found higher than that of the non-proteinencoding*16S*rDNA gene . Accordingly, sequence analysis of these genes provides a method with a better level of resolution for the identification and taxonomic classification of various bacterial species. Sequence analysis of four zones of the RNA polymerase β-subunit (*rpoB*) gene and its flanking spacers has been proposed as a useful molecular method for identification of *Acinetobacter* species (La Scola *et al*, 2006).

A. baumannii is a bacterium that appears to have a propensity for developing antimicrobial resistance extremely rapidly. Moreover, this resistance is multiple, causing serious therapeutic problems. Practices in ICUs contribute to the development of antimicrobial resistance in *A. baumannii* because the use of antimicrobials per patient and per surface area are significantly higher in this part of the hospital (Dijkshoorn *et al*, 2007).

Susceptibility of *A. baumannii* to antimicrobials is considerably different among countries, among centers and even among the wards of a given hospital. These differences may reflect different patterns of antimicrobial usage and different epidemiological situations, including antimicrobial control measures and policies. The differences in resistance patterns among isolates emphasize the importance of local surveillance in determining the most adequate therapy for *A. baumannii* infections (Ling *et al*, 2001).

The known resistance mechanisms of *A. baumannii* to antimicrobials are: the production of broad-spectrum *β*-lactamases, aminoglycoside-modifying enzymes, changes in outer membrane porins and alterations in penicillin-binding proteins (PBP). Antimicrobial resistance has been tracked to plasmids, transposons and chromosomes (Delcour, 2009).

MATERIALS AND METHODS

The present study carried out from June 2014 to December 2014 pH , electric conductivity (by EC meter type HANNA) , salinity (calculated from EC value) chemical oxygen demand were measured at the field according to standard method (APHA,1985) Total hardness, T.D.S , calcium, magnesium, and alkalinity were determined according(Parson *et al*, 1984).

Bacterial isolates

10 *A. baumannii* were isolate from industrial wastewater of theAl-Furat Company from Hilla. Bacterial isolates were identified to level of species and subspecies by using the morphological and traditional biochemical tests according to standard methods described by (Holt *et al*, 1994; Macfaddin, 2000).

Vitek System Identification

The Vitek 2 system assay has been used to confirm identification of all bacterial isolates. This system performed according to the manufacturer's instructions (Biomerieux Company, France).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of *A.baumannii* was carried out against different antibiotics (Gentamycin, Ciprofloxacin, Rifampin, Amikacin, Tetracycline, Cefotaxime and Ampicillin) using disc diffusion method on Muller-Hinton agar medium. The cultured was incubated at 37 C o for 18 hr under aerobic condition and bacterial growth inhibition zones around the discs were measured. Results were interpreted as recommended by (CLSI, 2010).

Bacterial DNA isolates

This method was performed according to the genomic DNA purification Kit supplemented by the manufacturing company.

The extracted DNA was analyzed by DNA gel electrophoresis as described by (O,Connell, 1984)

Molecular Identification

A polymerase chain reaction (PCR) technique was used to identify *A.baumannii* by amplify genes of *rpoB* gene from genomic DNA. DNA extraction from Gram negative bacteria was performed according to the genomic DNA purification kit supplemented by the manufacturing company (Geneaid/Taiwan). Gel electrophoresis has been used for detection of DNA by UV transilluminator (Sambrook and Rusell, 2001). The primers selection accordingto recommendations and used for diagnosis *A.baumannii*. These primers synthesized by AccuOligo- Bioner Company, Korea, as shown in (Table 1).

Table 1 The Sequence of Forward and Reverse Primers

Primer name	Primer Sequence	Product size(bp)
rpoB	F: TAYCGYAAAGAYTTGAAAGAAG R: CMACACCYTTGTTMCCRTGA Y:C or T M:any nucleotide	350

PCR Mixture solution was according to information of manufacturing company (Master mix, Geneaid/Taiwan) and PCR Program conditions was listed in (Table 2). Ten ml standard molecular weight of DNA ladder was loaded in first well on 1.5% agarose gel and each well has been loaded with 10ml of PCR product.

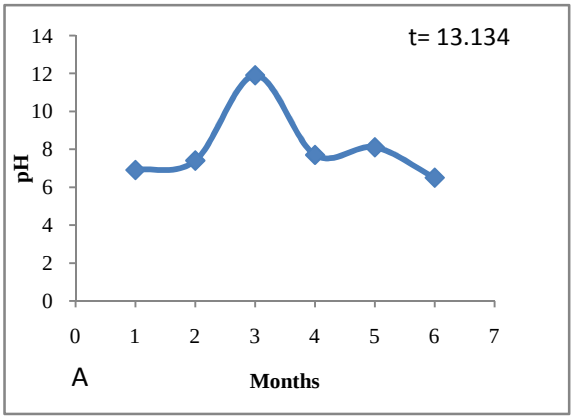
Electrophoresis runs at 70 volt/cm for 2hr. loaded in first well on 1.5% agarose gel and each well has been loaded with 10ml of PCR product (Pospiech and Neumann, 1995).

Table 2 Amplication Conditions

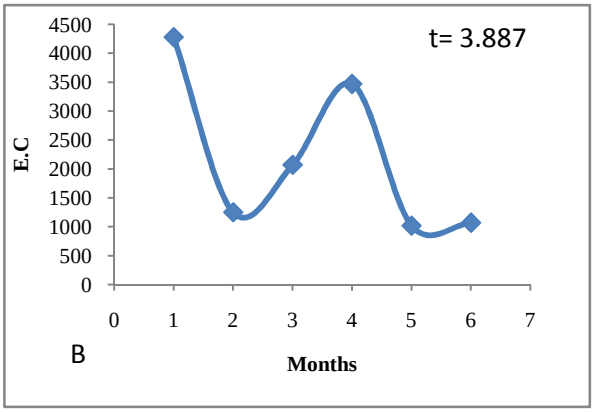
Steps	Temperature	Time	No. of cycle
Initial denaturation	94 °C	3 min	30 cycle
Denaturation	94 °C	30 sec	
Annealing	52 °C	30 sec	
Extension	72 °C	30 sec	
Final	72 °C	10 min	

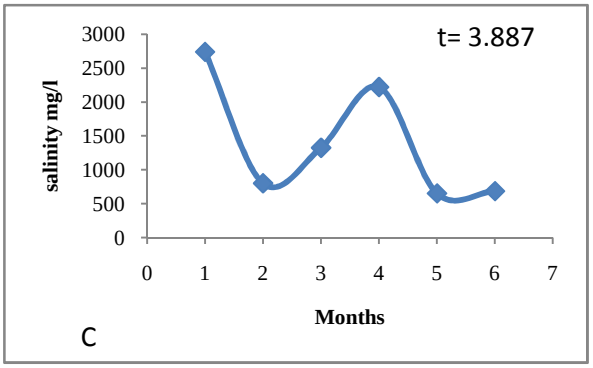
RESULT

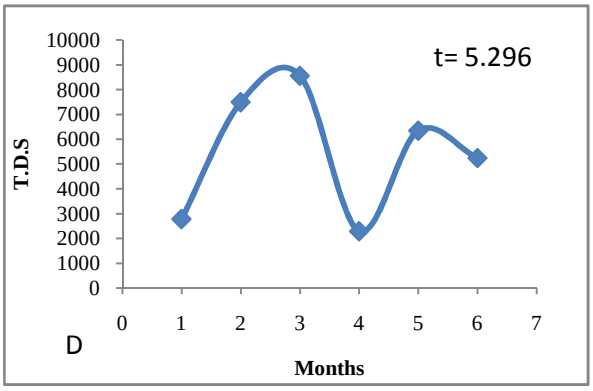
Result obtained from this work showed that increased in pollutants Chemical industrial wastes usually contain organic and inorganic matter in varying concentration. It contains acids, bases, toxic materials, and matter high in chemical oxygen demand. Fig (1,2) summaries the physical and chemical characteristics of study area.

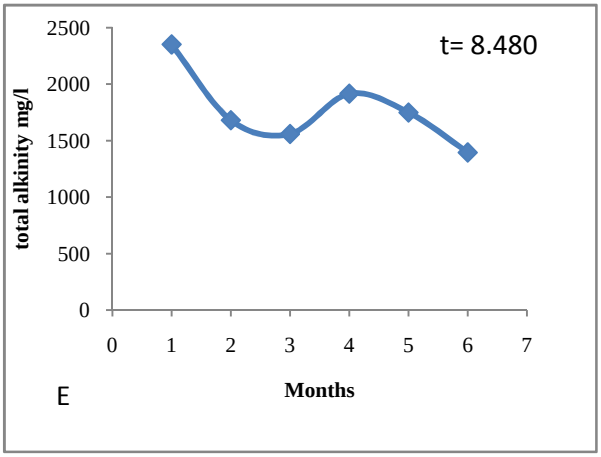


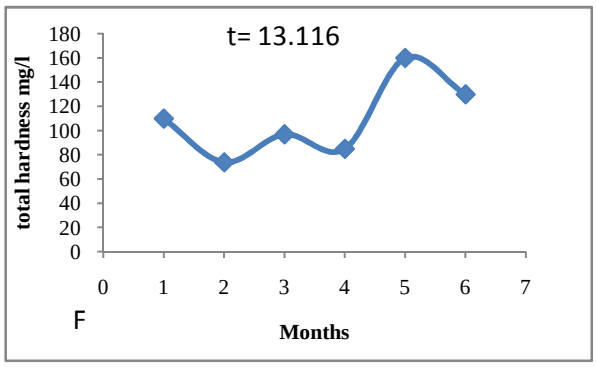
pH value was noticed , the sampling monthes varying from (6.5 – 9.9) (6.5-11.9) these parameters for neutral and soda tank respectively, these result also recorded electrical conductivity ranged between (2286 - 4660) (1017 - 4280) while wastewater salinity ranged between (1463- 2982) (651 - 2739) . The present study shows closely values of chemical dissolved oxygen sites .The lowest COD (30 , 32 mg/l) was recorded while the highest value was (150 , 160 mg/l).

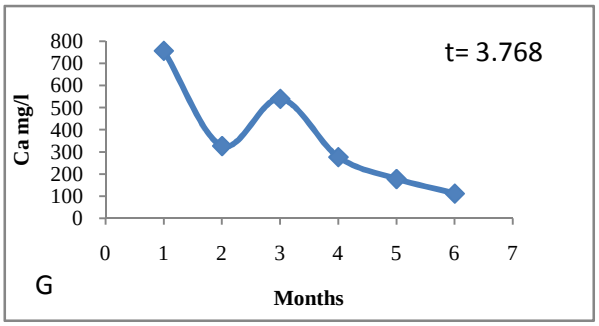


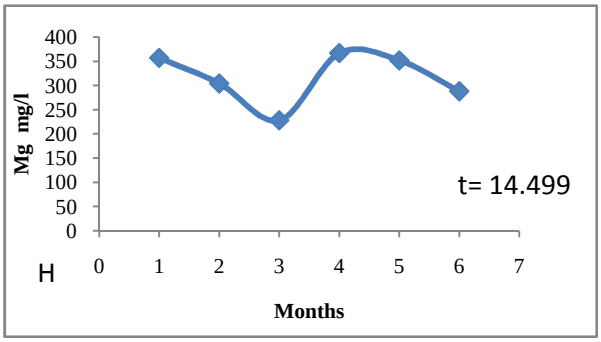


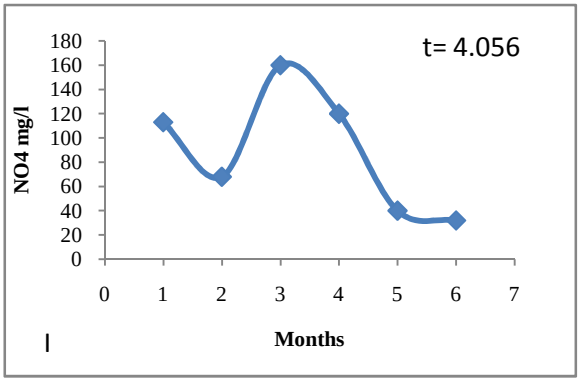


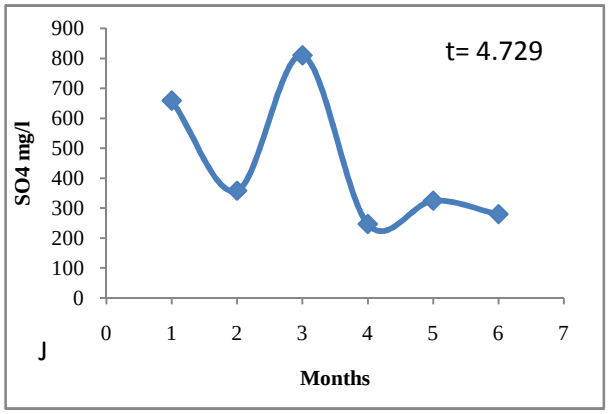


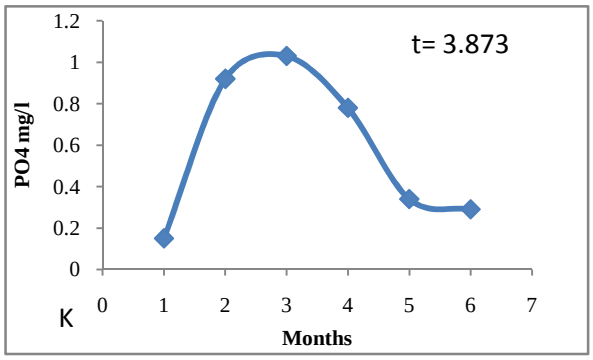


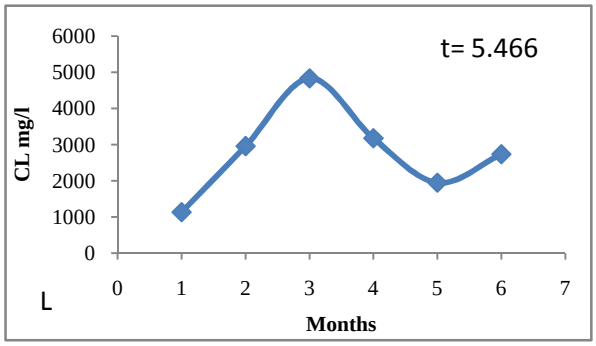


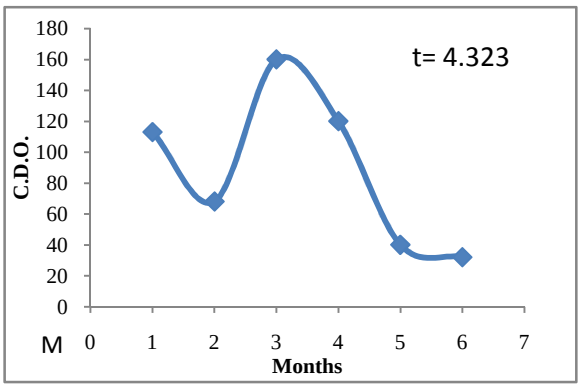






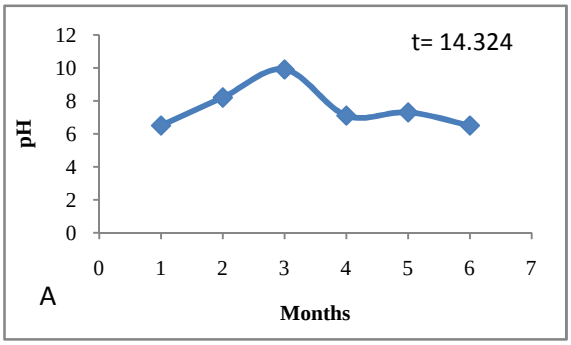


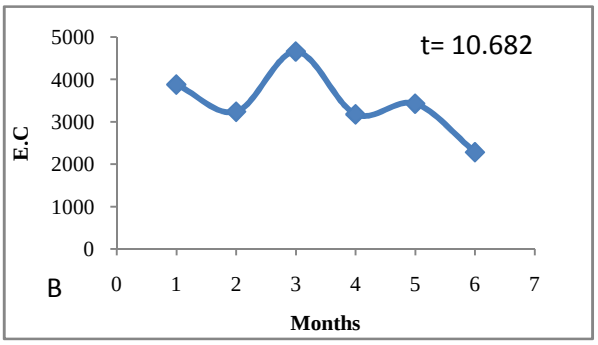




Fig(1:A,B,C,D,E,F,G,H,I,J,K,Land M) Monthly variation of pollutions industrial wastewater of soda tank.

Total hardness ranged between (1390 -2507 mg/l) (1397 -2351 mg/l) while calicium and magnesium hardness (206– 724 mg/l) (112– 757 mg/l) , (221- 399 mg/l) (228- 367 mg/l) respectively and T.D.S (1491– 2936 mg/l) (2287– 8552 mg/l). Total alkalinity ranged between (67- 136 mg/l) (74- 160 mg/l) while nitrate ranged between (1.7- 6.5mg/l) (1.5- 9.3 mg/l) The result recorded sulphate range between (102– 593 mg/l) (247- 810mg/l) while phosphate ranged between (1.07- 0.14 mg/l) (0.15- 1.03mg/l) .finally chrolred recorded range between (1005- 4931mg/l) (1134- 4831mg/l).

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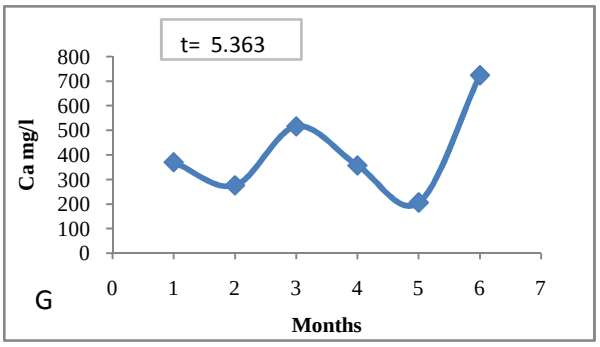
E.C
5000
4000
3000
2000
1000
0
0 1 2 3 4 5 6 7
Months
B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Ca mg/l
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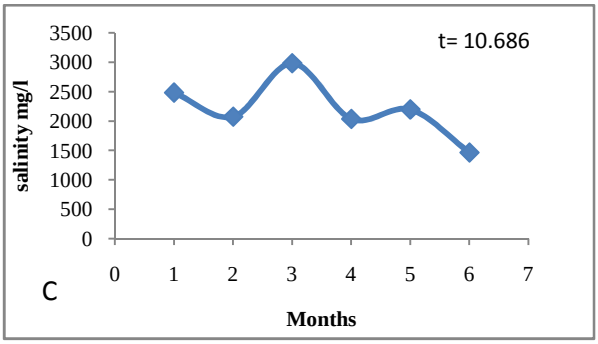
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7
Months
G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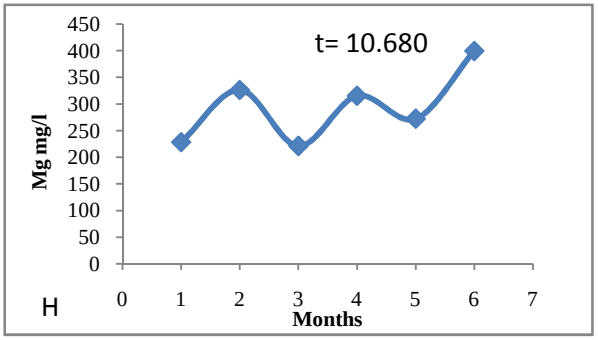
3000
2500
2000
1500
1000
500
0
0 1 2 3 4 5 6 7
Months
C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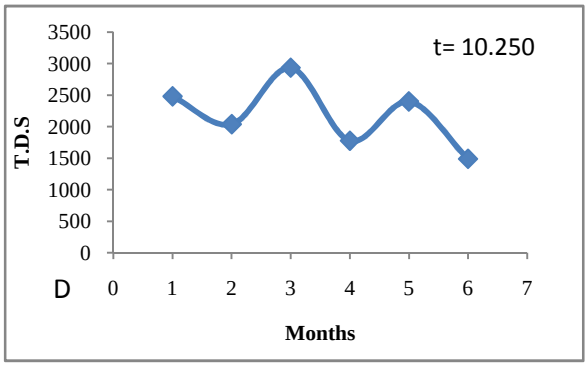
250
200
150
100
50
0
0 1 2 3 4 5 6 7
Months
H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T.D.S
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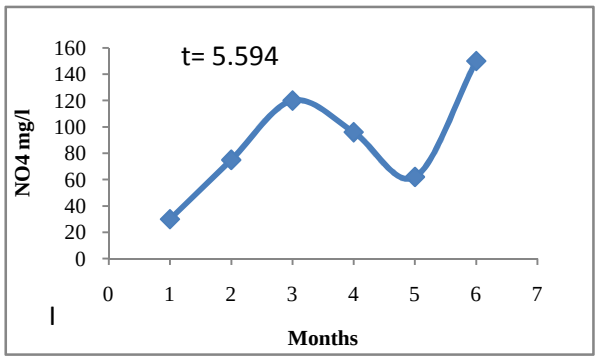
500
0
0 1 2 3 4 5 6 7
Months
D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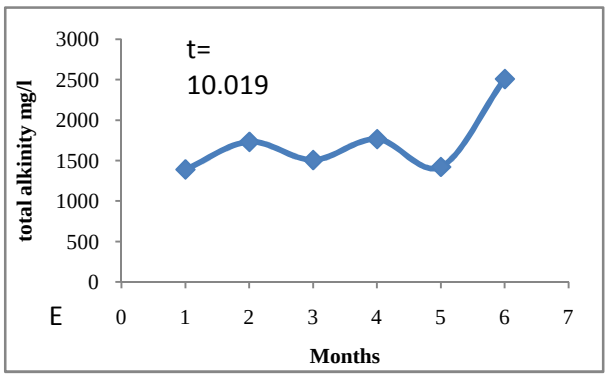
20
0
0 1 2 3 4 5 6 7
Months
I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0
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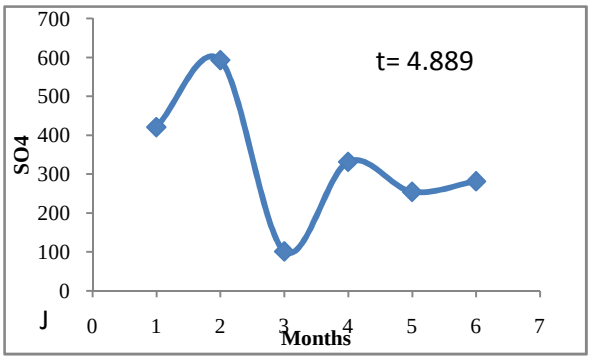
Months
E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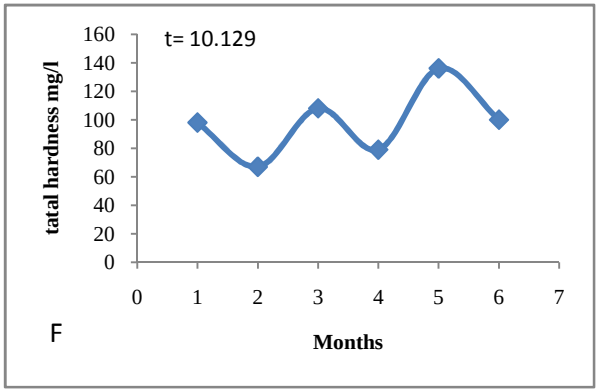
Months
J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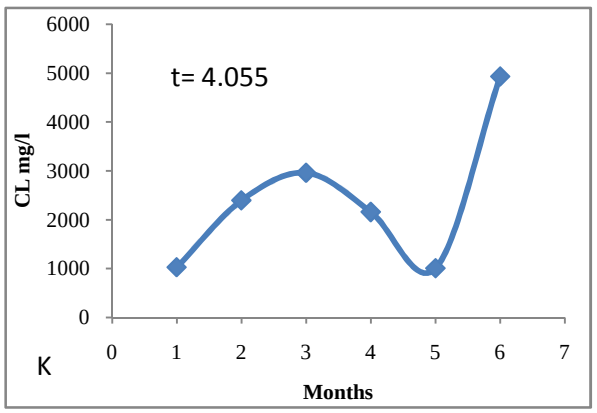
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Months
F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CL mg/l
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5000
4000
3000
2000
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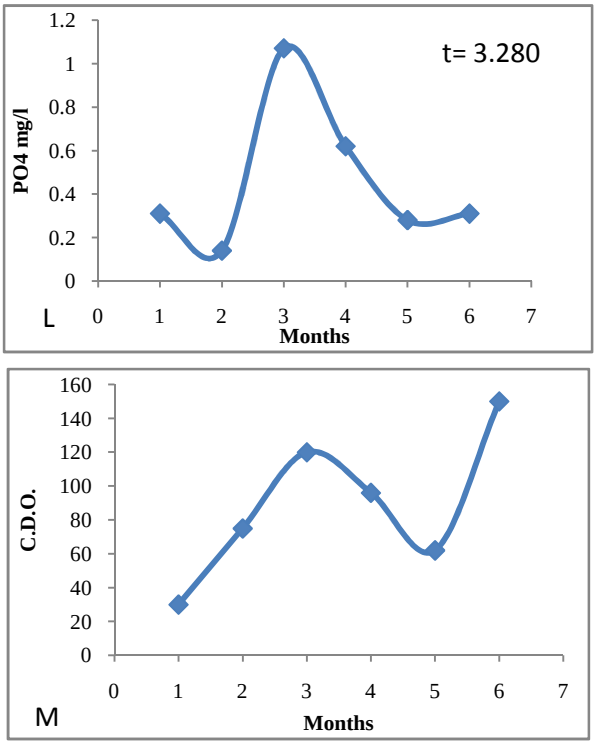
0
0 1 2 3 4 5 6 7
Months
K



Fig(1:A,B,C,D,E,F,G,H,I,J,K,Land M) Monthly variation of pollutions industrial wastewater of neutral tank.

In This study involved 10 of *A.baumannii* this strain dominated in industrial wastewater and further more highly resistance to several antibiotic and *A. baumannii* considered as pathogen for human being causing several disease.

Isolates were examined for colony characterization after culturing on the blood and MacConkey agar and incubated for 24 hour at 37ºC and non-pigmented with entire margin (non lactose fermenters) on MacConky agar and white to grey on the blood agar without hemolysis. Which became more mucoidal upon further incubation, microscopically appear Gram-negative, coccobacilli and paires . In terms of, initial biochemical tests *A. baumannii* showed negative for oxidase ,indole production and vogusproskaur test while the positive test appear in catalase , red methyl , simmon citrate and motility test;whereas the result in the KIA agar appear as alkaline slant , with no change to the bottom and no H2S without gas production , while the urease test gave variable results(Holt *et al*, 1994;Macfaddin, 2000). .Vitek 2 system is an efficient biochemical test to confirm identification of *A. baumannii*.

Genomic DNA was successfully extracted from *A. baumannii* isolates by using a commercial genomic DNA purification kit (Geneaid/Taiwan).The concentration and purity of extracted DNA were determined. DNA bands were confirmed and analyzed by gel electrophoresis. DNA was extracted from all isolates in the study and used as a template for polymerase chain reaction to detect rpo B gene. PCR results showed that 350 bp partial *rpoB* gene sequences were successfully obtained in Ac696F-Ac1093R primers from all ten isolates of *A. baumannii* (Fig 3).

Identified species level the ten isolates by *rpoB* gene a genotypic method which was performed for the genomic identification of *A. baumannii*. All isolates were amplified by PCR with primers, single amplicon 350 bp appeared on agarose gel electrophoresis which indicated that all isolates identified as *A.baumannii*(La Scola *et al*, 2006; Dijkshoorn *et al*,2007).

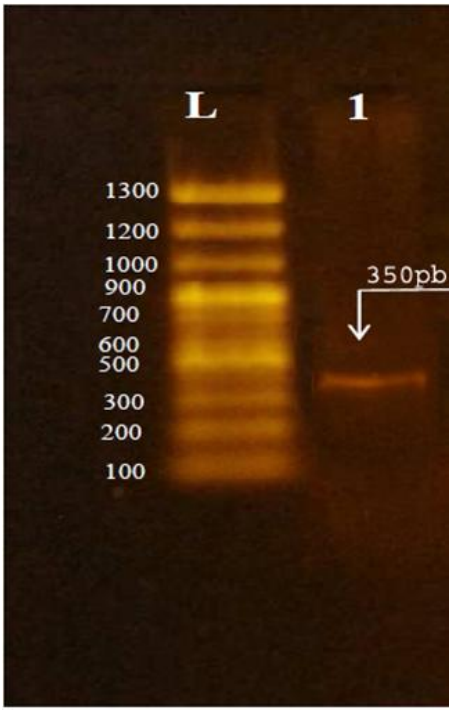


Figure 3 Electrophoresis of the amplified products of *A.baumanniirpoB*gene on 1.5% agarose gel at 70 volt for 2 hour visualized under UV afterstaining withethediumbromidLane (L): DNA Ladder 100 bp.LadderLanes: 1 isolates of *A.baumannii* show positiveresults.

Susceptibility of all *A. baumannii* isolates to 7 antibiotics data presented in (Table 3) shows a high level resistance of *A. baumannii* isolates to most of the antibiotics under test.

Table 3 Antibiotic susceptibility testing of *A.baumannii*.

Antibiotic	Susceptibility testing
Ciprofloxacin	R
Rifampin	R
Amikacin	R
Cefotaxime	R
Tetracycline	R
Ampicillin	R
Gentamycin	S

Resistance :R Sensitive : S

The present study revealed that all *A. baumannii* isolates had resistance Ciprofloxacin, Rifampin, Amikacin, Cefotaxime, Tetracycline, Ampicillin. Where as it sensitive to Gentamycin.

DISCUSSION

Most recently, concern has been expressed about chemicals which are characterised by persistence in the environment, resistance to degradation, and acute and chronic toxicity. Further, some of these can be transported over long distances through the atmosphere or aquatic systems and pollute areas where they have never even been used . Connectivity is defined as a numerical valuerefers to the ability of water to carry electric current and this value depends on the

concentration of ions and equal dissolved in water is acid and inorganic bases and salts dissolved in water are good conductors of electrical current. As for the salinity of the wastewater study area is very high water salinity irrigation water according to the classification based on the U.S. Salinity Laboratory classification. The extent to which abroad range by the values of phin the terminals is due to the clear influence of the industrial wastewater company.

As for the solids in the wastewater of the main contaminants are found in different shapes and different amounts can be dissolved in water consisting of negative ions for vehicles combined with the positive ions of the elements may be solids suspended in the water column itself.
Was measured by chemical oxygen requirement(COD)through oxidation forganicmatter oxidizing chemicals has been observed through the current study COD rates at all stations exceeded the determinant so finer national The waste water is under study in station were very hardness .Thus, for the ionic calcium and magnesium as its frequent values came with total hardness. The concentration hardness closely associated with the concentration of TDS and salinity as the TDSisacarbonate and bicarbonate and chloride and sulfate and nitrate and sodium and potassium, calcium, and magnesium and an increase of the concentration of TDS increasingly saline and hardness.

Sulphates with limited so lubility in wastewater, so the reareusuallylow concentrationsin surface water, with the exception of areas rich in the findings of the current study.The high sulphate in industrial waste water. There as on for this may be due to the large number of chemicals containing sulfatesraised by factories and laboratories such as ulfuricacid, as well as the launch quantities of ferrous sulphate and ironsulfide increased to asksulfatesodium The chlorides have recorded high values.(APHA, 1985; Fahid, 2003 ; Isoken *et al*, 2012 and Alahmed and AL-saffar, 2014).

Acinetobacter is a common type of bacteria. There are at least 25 different species of *Acinetobacter*. All isolates primarily identified as *A.baumannii* ,by culturing the samples on MacConkey agar which yielded rapid and lactose fermenters while on blood agar they appeared white to grey and non-hemolytic, bacterial cell appeared as Gram-negative coccobacilli, catalase positive, oxidase negative. In addition using Vitek 2 system was carried out as confirmatory test for the identification of this organism. Accordingly, identified species level the eleven isolates by *rpoB* gene a genotypic method which was performed for the genomic identification of *A. baumannii* . The application of *rpoB* gene identification of *Acinetobacter* species has several advantages over phenotypic identification. *rpoB* gene considered rapid and reliable and universally applicable method for identification of most of the *Acinetobacter* genomic species, thus contribute to better understanding of the clinical importance and epidemiology of *A. baumannii*. *A. baumannii* of isolates arecommonly resistant to multiple antimicrobial drug classes and have the ability tosurvive in the environment for prolonged periods of time. The antimicrobial susceptibility of *A. baumannii* was determined by the disk diffusion method in accordance with the clinical and laboratory standards institute guidelines (2010) depending on a diameter of inhibition zone (mm) as shown in (Table 3). Resistance mechanism that is found in many bacteria that can conferreduced susceptibilities to a number of antibiotic classes is the loss of OMPs that facilitate the transport of the antibiotic molecules across the cell membrane (Delcour, 2009). Porins are pore forming proteins on the outer membrane of bacteria.

CONCLUSION

This study included bacteriologic and genetic parts to investigate *Acinetobacterbaumannii* which represents one of the important causing agents of industrial pollution, PCR technique was found to be a useful method for detection of *A.baumannii* by *rpoB*gene; so as vitek 2 correctly identified this bacteria isolates to the species level. All isolates of *A.baumannii* showed multiple drugresistance.

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