Characterization of *Aeromonashydrophila* type strains isolates from industrial wastewater Hilla - Iraq

Lecturer. SuadGhaliKadhim ALahmed
MSc .Microbiology, College of Sciences
Babylon University
Babylon, Iraq
Suad_chaly2000@yahoo.com

Assist.Prof. Anwar Kadhim AL-saffar
MSc .Microbiology, College of Sciences
Babylon University
Babylon, Iraq
Anwaralsafar78@gmail.com

Lecturer. Thekraabdulaali abed al-chaabawi
MSc .Microbiology, College of Sciences
Babylon University
Babylon, Iraq
Chacky2015@gmail.com

Abstract:
Twenty one strains of motile *Aeromonas* species were isolated fromIndustrial wastewaterAl- Furat Company 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 may 2013 to October 2013 . The results showed that the natural properties of waste water were variable due to the nature of the materials dumping in the derange stream . It was found the pH ranged from (1.5 - 5.2) , conductivity reached to 3100 *M*s / cm while the higher concentration of COD, TDS , Cl , SO4 , Ca and Mg were 140 , 1953 , 335 , 738, 200 and 104 mg/l respectively.
***A. hydrophila* suspected isolale was screening by traditionally tests and then confirmed by Vitek 2 system and PCR technique (16S r RNA gene). Inthis study *A. hydrophil*strains are highly resistance to several antibiotics that was considered as pathogen for human being causing several disease.**

Keywords: *Aeromonashydrophila*, Wastewater, Al-Furat Company.

I.Introduction

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 was discharged into rivers, lakes and coastal areas . this resulted in serious pollution problems in the water environment and caused negative effects to eco-system and humans life.[1,2]

The chemical industry is critical for the economic development of any country chemicals are a signify cant contributor to national economies across the life cycle - from extractionto disposal - is essential al to avoid signify cant risks to human health and the environment . [3,4]

Industrial Wastewater leads to septic conditions in the environment and consequently leads to the deterioration of surface and groundwater quality and pollutes the soil. wastewater is rich with nitrogen and phosphorus (N, P) and leads to the phenomena of Eutrophication. Industrial wastewater is rich withorganic matter which consumes oxygen in aquatic environment . wastewater may contains toxic gases and volatile organic matter.[5,6].

the chemicals industry has made good progress reducing its overall environmental footprint, chemicals can also create a negative impact on human health and the environment when their production and use are not managed responsibly. Although the impacts are complex and often unknown or sometimes open to debate, some negative effects are well documented, such as chemicals found in the environment that are persistent, bio accumulative and/or toxic (*e.g.* PCBs, dioxins). Most recently, concern has been expressed about chemicals which interfere with the normal function of hormonal systems of humans and animals (*i.e.* endocrine disrupters), and substances which impact on children's health . [7,8]

The chemicals industry is very diverse, comprising basic or commodity chemicals; speciality chemicals derived from basic chemicals (adhesives and sealants, catalysts, coatings, electronic chemicals, plastic additives, *etc.*); products derived from life sciences (pharmaceuticals, pesticides and products of modern biotechnology); and consumer care products (soap, detergents, bleaches, hair and skin care products, fragrances, *etc.*). The global chemicals industry today produces tens of thousands of substances (some in volumes of millions of metric tonnes, but most of them in quantities of less than 1000 tonnes per year. [9, 10] .

The term pollutant is a broad term that refers to a wide range of compounds, from a superabundance of nutrients giving rise to enrichment of ecosystems to toxic compounds that may be carcinogenic, mutagenic, or teratogenic. Chemicals - with units on all the major organic and inorganic building blocks such as ethane,sulfuric ,hydrolytic acid . Effects on ecosystem resources can include contaminate on of air, water, and soil, as well as adverse effects on wildlife. Human health effects can include both acute and chronic diseases and disorders. [11].

Recently, research for new and innovative technologies has centered on the biological treatment methods [12]. Bioremediation is the use of microorganisms to break down toxic and hazardous compounds in the environment [13]. It generally utilizes microbes

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(bacteria, fungi, yeast, and algae), although higher plants are used in some applications.

Microorganisms have the ability to accommodate a variety of pollutants, both organic and inorganic ..

Bioremediation has already proven itself to be a cost-effective and beneficial addition to chemical and physical methods of managing wastes and environmental pollutants. New bioremediation approaches are emerging based on advances in molecular biology and process engineering. Recently developed rapid-screening assays can identify organisms capable of degrading specific wastes and new gene-probe methods can ascertain their abundance at specific sites. [14]. Bacterial survival in stressful environments is an intriguing biological problem with applications toward understanding pathogenic and environmentally important micro-organisms. [15 , 16] .

Aeromonashydrophila, a gastrointestinal pathogen of humans, was shown to exhibit a significant adaptive acid tolerance response (ATR) capable of protecting cells from severe acid at a pH of 3.5.

Adaptation required protein Synthesis since treatment with chloramphenicol during adaptation to pH *5-0* prevented the development of acid tolerance. The adaptation to acid environment was found to be a non-transient phenomenon. Also, iron was not required for acid adaptation in *A. hydrophila*to adapt and survive in acid environments by producing 'protective proteins. The adaptation and survival of this pathogen in low pH may provide valuable information about its ability to withstand acid environments in nature and in the human gastrointestinal tract [17] .

the genus *Aeromonas* are good examples of such bacteria. Regardless of the ubiquity of aeromonads in aquatic environments and the possibility to develop antimicrobial resistance, the patterns of resistance of *Aeromonas* genus present in wastewater are not fully documented in scientific literature.In this study, the ability of chitosan-immobilized *Acinetobacter*haemolyticus as biosorbent for chromium (VI) biosorption in batch system was investigate. Optimized parameter namely pH, contact time, biosorbent dosage and initial metal concentration obtained from the experiment was then applied for electroplating wastewater treatment.[18]

Chemicals are an integral part of daily life in today's world. There is hardly any industry where chemicals are not used and there is no single economic sector where chemicals do not play an important role.The natural and the zymogene microorganisms(which come in waters by pollution) constitute a diverse microbiota adapted to the different physical - chemical wastewater conditions, being very important for biodegradation [19].

II.Materials and Methods

- study of physical - chemical wastewater:

The present study carried out from May 2013 to October 2013 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 [20] Total hardness, T.D.S , calcium, magnesium, and alkalinity were determined according[21].

- Bacterial isolates:

Aeromonashydrophila, *A. caviae* and *A. sobria* were isolate from industrial wastewater of the Al-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 [22,23].

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. hydrophila* was carried out against different antibiotics (Ciprofloxacin, Gentamycin, ,Rifampin, Colstin, Rifampin, Tetracycline, Cefoyoxime 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 [24].

- Bacterial DNA isolates.

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

-Gel electrophoresis

The extracted DNA was analyzed by DNA gel electrophoresis as described by [25] .

Molecular Identification:

A polymerase chain reaction (PCR) technique was used to identify *A.hydrophila*by amplify genes of *16Sr RNA* 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[26]. The primers selection according to [22],[27] recommendations and used for diagnosis *A.hydrophila*. These primers synthesized by AccuOligo- Bioner Company, Korea, as shown in table (1)

Table 1: The Sequence of Forward and Reverse Primers

Primer Type	Primer Sequence	Product Size
Forward *16Sr RNA* –F	5-CCAGCAGCCGCGGTAATACG-3	300 bp
Reverse *16Sr RNA* – R	5-TACCAGGGTATCTAATCC-3	

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% agarose gel and each well has been loaded with 10ml of PCR product.

Electrophoresis runs at 80 volt/cm for 1hr. loaded in first well on 1% agarose gel and each well has been loaded with 10ml of PCR product [28].

Table 2:Amplication Conditions

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

III.RESULT & DISCUSSION

Result optioned from this work showed that increased in pollutants due tothe presence ofacidsandbases andinorganicsaltsraised .Fig (1:A,B,C,D,E,F,G,H,I,J,K,L and M) summaries the physical and chemical characteristics of studied area .pH value was noticed , the sampling monthes varying from (1.5 - 5.2) ,these result also recorded electrical conductivity ranged between (1005 -3100) while wastewater salinity ranged between (643 – 2058) . The present study shows closely values of chemical dissolved oxygen sites .The lowest COD(45 mg/l) was recorded while the highest value was (140 mg/l) .

Total hardness ranged between (320 -663 mg/l) while calicium and magnesium hardness (65 – 200 mg/l) , (57 - 104 mg/l) respectively and T.D.S (629 – 1953 mg/l) .

Total alkalinity ranged between (57 - 128 mg/l) while nitrate ranged between (3.1- 8.2 mg/l)

The result recorded sulphate range between (235 – 738 mg/l) while phosphate ranged between (0.11 -0.53 mg/l). Finally recorded range between (126 - 335 mg/l).

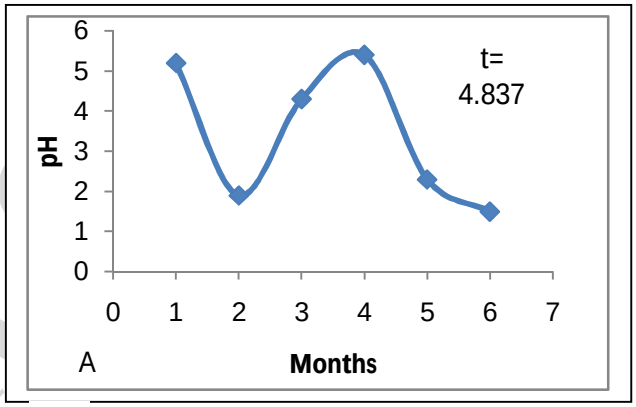
Connectivity is defined as a numerical value refers to the ability of water to carry electric current and this value depends on the concentration of ions and equal dissolved in water is acid and inorganic bases and salts dissolved in water are good conductors of electrical current. As for the salinity 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 whicha broad range by the values of pHin the terminal is due to the clear influence of the industrial wastewater company.

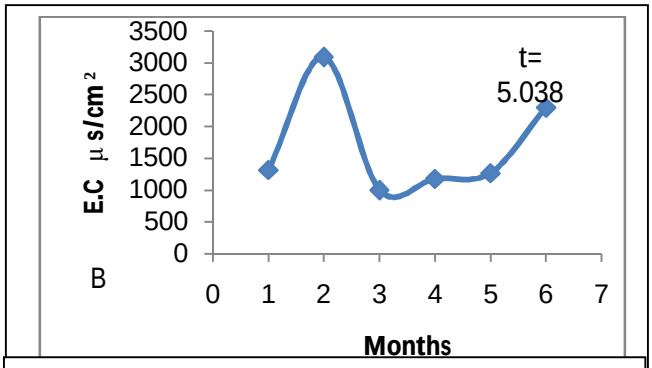
As for the solids in the wastewater of the main contaminants are found in different shapes and different amounts can be dissolved in water consisting of negative ions for vehicles combined with the positive ions of the elements may be solids suspended in the water column itself. Was measured by chemical oxygen requirement (COD) through oxidation of organic matter oxidizing chemicals has been observed through the current study COD rates at all stations exceeded the determinants of international

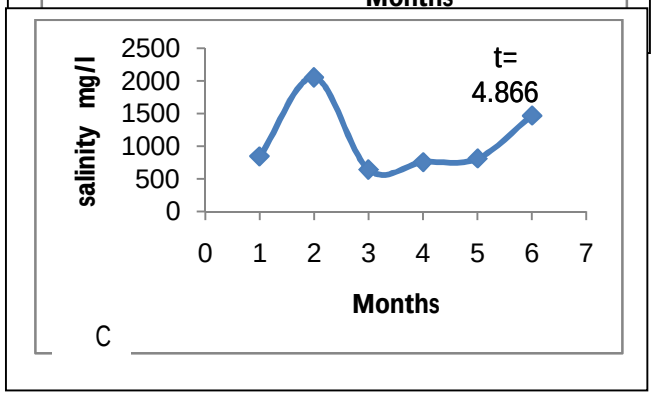
The waste water is under study in station were very hardness .Thus, for the ionic calcium and magnesium as its frequent values came with total hardness. The concentration hardness closely associated with the concentration of TD Sand salinity as the TD Sis a carbonate and bicarbonate and chloride and sulfate and nitrate and so diumand potassium, calcium, and magnesium, and an increase of the concentration of TDS increasingly saline and hardness.

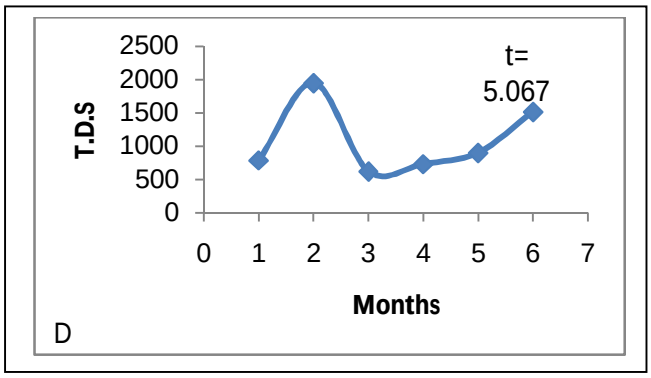
Sulphates with limited solubility in wastewater, so there are usually low concentrations in surface water with the exception of areas rich in the findings of the current study .The high sulphate in industrial waste water. The reason for this may be due to the large number of chemicals containing sulfates raised by factories and laboratories such as sulfuric acid, as well as the launch quantities offerrous sulfate andiron sulfide increased to asksulfatesodium.

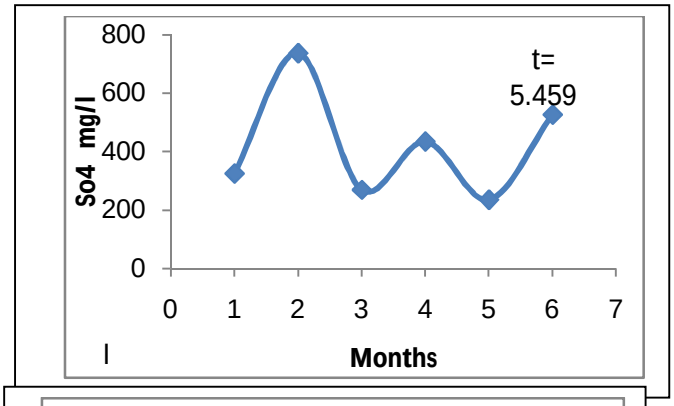
The chlorides have recorded high values. The concentrations of chlorides in Iraq are generally higher than in the countries of the world. The nitrates within the parameters for wastewater and that the change in the concentration of nitrates during the study period due to several reasons, including changes in the amount and the presence of water and the growth of plants, chapter sheat, add pollutants and denitrification. The phosphate has recorded high values himat all stations during different months of the study period and so may be attributed to the occurrence of pollution in the waste water detergent phosphorus-rich. These results agree with other studies [5, 29, 30, 31].

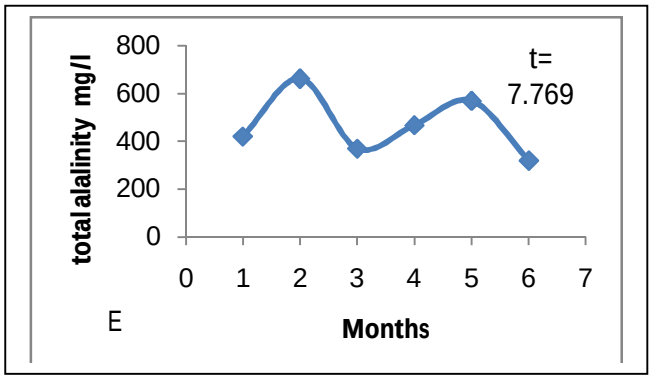


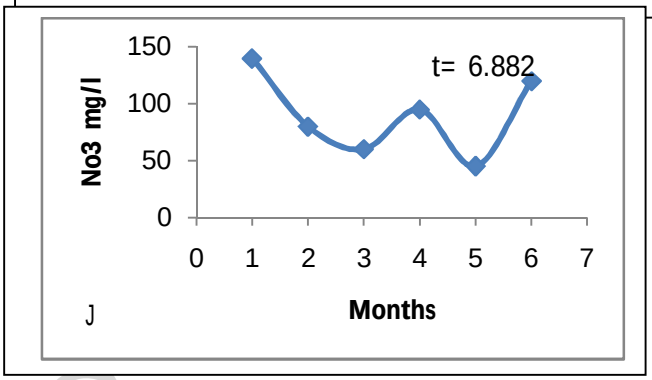


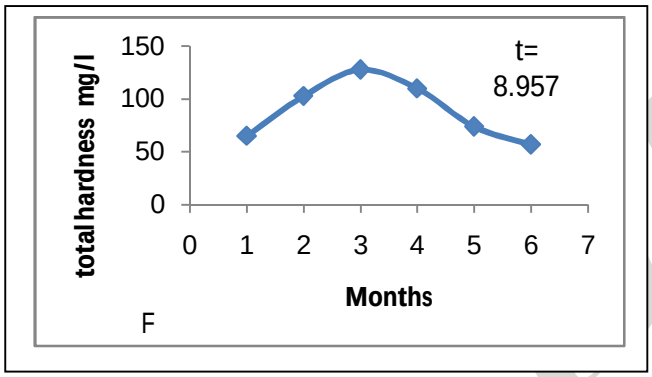


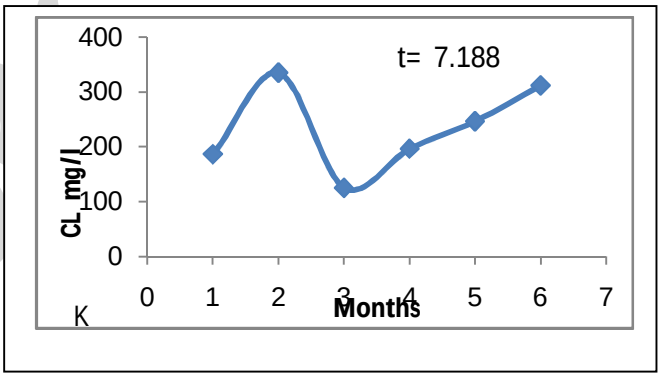

T.D.S
2500
2000
1500
1000
500
0
0 1 2 3 4 5 6 7
Months
t= 5.067
D

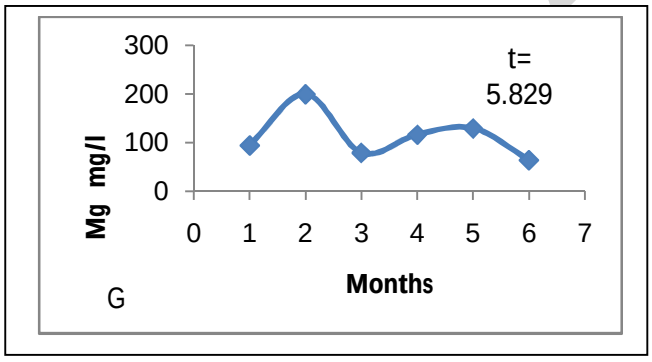

So4 mg/l
800
600
400
200
0
0 1 2 3 4 5 6 7
Months
t= 5.459
I

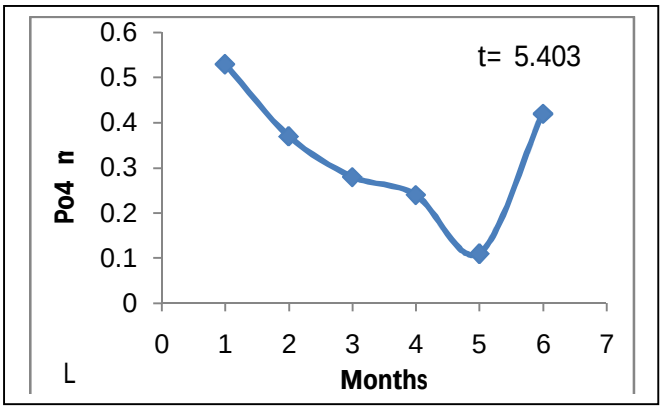

total alalinity mg/l
800
600
400
200
0
0 1 2 3 4 5 6 7
Months
t= 7.769
E

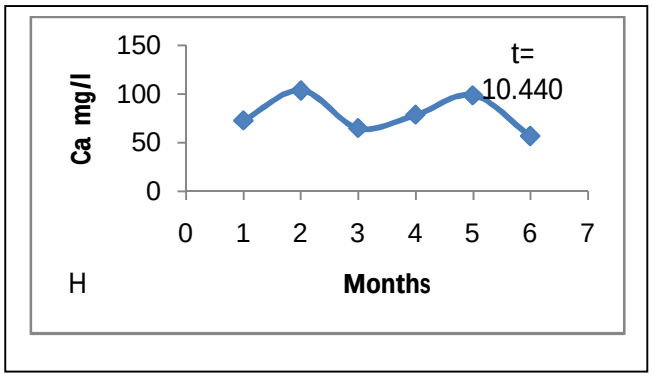

No3 mg/l
150
100
50
0
0 1 2 3 4 5 6 7
Months
t= 6.882
J

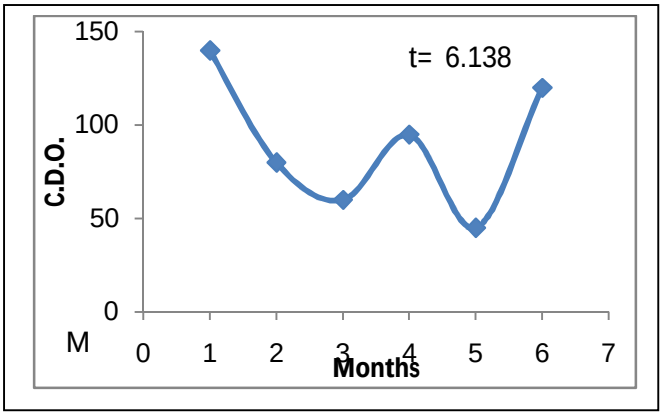

total hardness mg/l
150
100
50
0
0 1 2 3 4 5 6 7
Months
t= 8.957
F


Cl mg/l
400
300
200
100
0
0 1 2 3 4 5 6 7
Months
t= 7.188
K


Mg mg/l
300
200
100
0
0 1 2 3 4 5 6 7
Months
t= 5.829
G


Po4 n
0.6
0.5
0.4
0.3
0.2
0.1
0
0 1 2 3 4 5 6 7
Months
t= 5.403
L


Ca mg/l
150
100
50
0
0 1 2 3 4 5 6 7
Months
t= 10.440
H


C.D.O.
150
100
50
0
0 1 2 3 4 5 6 7
Months
t= 6.138
M

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

In This study involved 21 bacterial strains, including 9 *Aeromonashydrophila*, 7 *A.caviaeand* 5 *A. sobria*, this research has show that *A. hydrophila* strain dominated in industrial wastewater and further more highly resistance to several antibiotic and *A. hydrophila*considered as pathogen for human being causing several disease. .*Aeromonas*organisms are nonsporulating Gram negative rods that are ubiquitous inhabitants of fresh water sources. These hardy organisms multiply and grow under a variety of conditions and temperatures.

A.hydrophila showed a yellow shine colour on TCBS agar, pale (non -lactose fermenters) on the MacConkey agar, smooth, convex, rounded, *β*-hemolytic colonies and pale white to grey colour on blood agar[22]. In terms of, initial biochemical tests *A. hydrophila*showed a positive result to each of catalase, oxidase, Indole, methyl red, simmonecitrateand gelatin liquefaction. The current results of the biochemical tests in this study are almost finding in the other researchers reports [17] While, *A. hydrophila*was gave variable results to vogues-proskauer. Vitek 2 system is an efficient biochemical test to confirm identification of *A. hydrophila*[19].

Table (3): Antibiotic susceptibility testing of *A.hydro*

Antibiotic	Susceptibility testing
Ciprofloxacin	S
Gentamycin	R
Ampicillin	R
Rifampin	R
Tetracycline	R
Cefotoxime	R
Clostin	R

Resistance :R Sensitive : S

The results related to the antimicrobial susceptibility to the seventh tested antibiotics are given in the Table 3.The antibiotic resistance levels are expressed in percentages of the whole population and as antibiotic resistance index numbers. Due to the fact that the isolation medium already contained Gentamycin, Ampicillin, Rifampin, Colstin, Tetracycline and Cefotoxime, absolute resistance to all of these drugs was expected to be confirmed and therefore it was not practical to consider these resistance levels [32]. Where as it sensitive to Ciprofloxacin [33] .

With regard to extract DNA of this bacteriaaccording to the genomic DNA purification Kit and after the DNA extracted agarose gel electrophoresis , showed the results of this study contain only Chromosomal DNA (Fig 2).

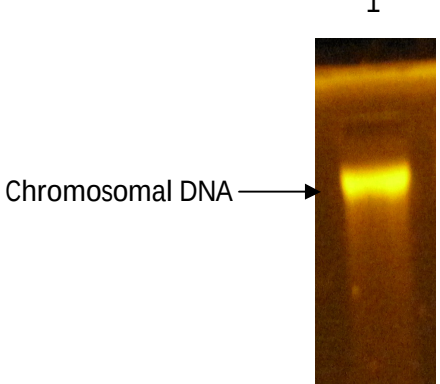


Fig (2) : Electrophoresis of whole DNA for *Aeromonashydrophila*(0.7%) agarose gel for 1.5 hr. 60 v .

In this study a polymerase chain reaction (PCR) technique was used to identify *A.hydrophila*by amplify genes of 16Sr RNA gene from genomic DNA of *A. hydrophila* isolate. This isolate has given a positive results for 16Sr RNA (300)bp (Fig 3). In addition to that, they reported the ribosomal mainly 16Sr RNA gene has confirmed to be a stable and specific molecular marker for the identification of A. hydrophila bacteria. Amplification of specific DNA by PCR provides a highly sensitive and specific tool for the detection of *A. hydrophila* from differentsources.

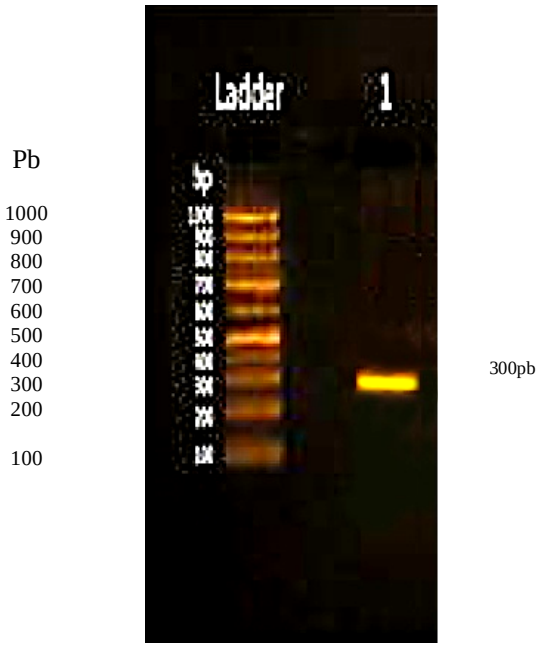


Figure 3: Agarose Gel Electrophoresis of PCR Amplified of Gene product 300 bp of *Aeromonashydrophila* Isolates for 1 hr at 80 Volt.. Lane 1 Amplify of gene product.

Industrial wastewater aeromonads were confirmed as relevant agents for antimicrobial resistance spreading in the environment. The Patterns of *Aeromonas* species and antimicrobial resistance varied over different parts of the industrial wastewater.

References

[1]-Han, C.(1994) . industrial wastewater-types, amounts and effects.department of environmental science and engineering tsinghua university ,Beijing ,china .vol 1.

[2]-Majid,S .(2010) . Experimental studies on effect of Heavy Metals presence in Industrial Wastewater on Biological Treatment . INTERNATIONAL JOURNAL OF ENVIRONMENTAL SCIENCES Volume 1, No 4.

[3]- Parsons , T. R.; Maita , Y. and Lalli . C. M. (1984). "Amanual of chemical and biological methods for sea water analysis " . Per gamon press . Ox ford .

[4]- Schlag, S. and Funada, C. (2009). Chemical Economics Handbook Marketing Research Report: Lime/Limestone. SRI. Consulting.

[5]- Fahid, R. (2003).Unit 1. Physical, chemical and biological Characteristics of Wastewater lecture notes ,The Islamic University of Gaza-Civil Engineering Department Advanced Sanitary Engineering-ECIV 5325 .pp:1-28.

[6]- Daniel, J. ; Hans, W.; Robert, W. and Donald, F. (2009). Controlling Eutrophication: Nitrogen and Phosphorus. SCIENCE VOL 324.

[7]- Berlau, J.; Aucken, H. M.; Houang, E. and Pitt, T.L.(1999) Isolation of Acinetobacter spp. including A. baumannii from vegetables: implications for hospital-acquired infections. J Hosp Infect 1999;42:201–4.

[8]- Yongli, Z. ; Carl, F. ; Marrs , C. and Chuanwu, X .(2009) . Wastewater treatment contributes to selective increase of antibiotic resistance among Acinetobacter spp. Science of the Total Environment 407 . pp: 3702–3706.

[9]- OECD .(2001). Environmental Outlook for the Chemicals Industry. Organisation for Economic co-operation and development. pp: 1-165.

[10]-Wittcoff, H.A. and Reuben , B. G. (1996), *Industrial Organic Chemicals*, Wiley-Interscience, John Wiley & Sons, Inc. New York.

[11]- Figueria, V.; Vaz-Moreira, I.; Silva, M. and Manaia, C.M. (2011). Diversity and antibiotic resistance of *Aeromonasspp.* In drinking and waste water treatment plants. CBQF. Pp: 4204-4210.

[12]- Morales-Barrera, L., Guillén-Jiménez, F.M., Ortiz-Moreno, A., Villegas-Garrido, T.L., Sandoval-Cabrera, A., Hernández-Rodríguez, C.H., and Cristiani-Urbina, E. (2008). Isolation, Identification and Characterization of a *Hypocreatawa*Strain with High Cr(VI) Reduction Potential. *Biochemical Engineering Journal*, 40, 284-292.

[13]-Acquaah, G. (2004). *Understanding Biotechnology: An Integrated and Cyber-Based Approach.* (1st ed.). New Jersey: Pearson Education, Inc. 294-295.

[14]-Bonaventura, C. and Johnson, F. M. (1997). Healthy Environments for Healthy People: Bioremediation Today and Tomorrow. *Environmental Health Perspectives,* 105.

[15]- Foster, J. W. (1992).Beyond pH homeostasis: the acid tolerance response of *Salmonellae.,4m* Soc*Microbiol58,* 266-267.

[16]-White, S..; Tuttle, F. E.; Blankenhorn, D. D., Donald, C. and Slonczewski, J. L. (1992). pH dependence and gene structure of*inaA*in *Escbericbiacoli. J Bacteriol 174,* 1537-1 543.

[17]-Kevin, L. Karem, W. and Asim, K. B .(1994) .Adaptive acid tolerance response (ATR) in*Aeromonashydrophila* . vol 140, pp:1731-1736.

[18]-Ahmed, w. and akaria, N . (2012) .Biosorption of chromium (VI) by chitosan-immobilized *Acinetobacterhaemolyticus* . Humanities , science and engine : 24-27.

[19]-Organisa on for Economic Development (OECD) (2012). *OECD Environmental Outlook to 2050: The Consequences of inaction paris* .

[20]- James, H. and Robert, P. (2011). The Economic Benefits of a Green Chemical Industry in the United States . University of Massachusetts, Amherst. 1:343-345.

[21]- EPA . (2002) . Onsite Wastewater Treatment Systems Manual . Office of Water. Office of Research andevelopment . U.S. Environmental Protection Agency .

[22]- Mafaddin, J. F. (2000). Biochamical tests for identification of medical bacteria. 3ed. AwolterKlvwer Company.

[23]-Holt, J. G.; Kreig, N. R.; Sneath, P. H.; Staley, J. T. and Williams, S.T. (1994). Bergeys Manual of determinative bacteriology, 9th ed. Williams and Wilkins Baltimore. U.S.A.

[24]-Clinical and Laboratory Standards Institute. (2010). Performance Standards for Antimicrobial Susceptibility Testing 20th Informational supplement. Approved standard M07-A8. Clinical and Laboratory Standards Institute, Wayne, Pa.

[25]-O'Connell, M. (1984). Genetic transfer in Prokaryotes: Transformation, Transduction and Conjugation. In." A. Auhler and K. Timmis (eds)", pp. 2-13. Advanced molecular genetics. "Sipringer, Verlage, Berline".

[26]-Sambrook, J. &Rusell, D.W. (2001). Molecular cloning.A laboratory manual. 3rd ed. Cold Spring Harbor Laboratory Press, NewYork, USA.

[27]- Yogananth, N., Bhakyaraj, R., Chantchuru, A., Anbalagan,T. &MullaiNila, K.(2009). Detection of virulence gene in *Aeromonashydrophila* isolated from fish samples using PCR technique. Global JournalBiotechnology Biochemical 4: 51–53.

[28]- Dubai, N. Q. and AL-Thahab. (2013). Isolation of outer membrane protein of *Aeromonashydrophila* recovered from children with Diarrhea. Best, J. V.1,I. 2: 15-22.

[29]- Isoken, H.*et al* (2012) . Antibiotic Susceptibility Profile of *Aeromonas* Species Isolated from Wastewater Treatment Plant . The Scientific World Journal .

[30] - APHA (American Public Health Association), (1985). " Standard methods for examination of water and wastewater" . 16th ed. New York.

[31]- Dawson, D. (2005). Food borne protozoan parasites, *International Journal of Food Microbiology* 103 : 207– 227.

[32]-Vandan N,Ravindranath, S. andBandekar, J. R. (2011). Prevalence,Characterization, and Antimicrobial Resistance of *Aeromonas* Strainsfrom Various Retail Foodfrom Various Retail Food Products in Mumbai, India. Ind. J. Food Sci. 76:486–492

[33]-Vila. J.; Ruiz. J.; Gallardo, F.;Varzas, M.;Soler, L.; Figueras, M. andGascon, J. (2003). *Aeromonas* species and travellersdiarrhoea: clinical features and antimicrobial resistance. Emerging Infect. Dis. 9:552-555.

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