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Isolation and Characterization of a Bacterial Strain for Aniline Degradation

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ABSTRACT

Introduction and aim: aniline is one of the amino-aromatic environmental pollutants which is used in various industries and raises various diseases in human. One of the effective ways in removal of aniline from waste can be their biological uptake from contaminated areas. Bacteria living in contaminated areas enable to remove aniline from different ways. Municipal waste and wastes in Peimanieh Hospital -Jahrom contain compounds containing aniline. This study aims to isolate and characterize a bacterial strain for aniline degradation from municipal waste and wastes in Peimanieh Hospital -Jahrom and determine resistance range of these bacteria.Materials and methods: in this study, sampling was fulfilled from six municipal waste stations and wastes in Peimanieh Hospital -Jahrom during two consecutive seasons of the year. To isolate and characterize resistant bacteria, medium PNR containing 100 ppm aniline was used. Common biochemical tests and PRC were fulfilled to identify isolated bacteria, and resistance range of these bacteria through MIC, MBC and growth Kinetics was evaluated in various concentrations of aniline and antibiogram tests. Results: bacteria Pseudomonas aeruginosa ،Klebseilla pneumonia، Klebsiella rhinoscleromatis ،Klebsiella oxytoca، Escherichia coli and Citrobacter were isolated and characterized by means of morphology and molecular and biochemical tests. All the isolated resistant bacteria were gram-negative bacteria, and some bacteria enabled to impose aniline in high concentrations which the highest resistance has been related to Klebseilla pneumonia with concentration MIC 6400 ppm and MBC 6500 ppm; further, these bacteria had the highest rate of growth by presence of 5000 ppm. Conclusion: isolated bacteria especially Klebsiella pneumoniae were a good candidate for removal of existing aniline in pollutant wastes.

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INTRODUCTION

Aniline and its derivatives are of the most important amino-aromatic environmental pollutants and are used in drugs, insecticides, herbicides and so forth. Overpopulation has caused increasing economic and industrial activities, resulting in expansion of a wide range of pollutants in environment and environmental cycles of organisms, including humans. Aniline raises various diseases in human including diseases of the lung, skin, eye, kidney, liver and so forth. To resolve such problems, currently much time and cost are spent which cannot meet a large amount of pollution. In recent decades, use of microorganisms in biological removal of these contaminants has been drawn into attention. Bacteria use aniline as a source of carbon, energy and nitrogen, considering the fact that the bacteria using aniline degrade this substance and transform it to Catechol, and then Catechol is metabolized through a series of enzymes. Further, aniline is degraded through several physicochemical processes such as evaporation, spontaneous oxidation, light oxidation and chemical binding.

Municipal and hospital wastes contain aniline and its derivatives, which biofilm reactors with moving bed or Moving Bed Biofilm Reactor (MBBR)are used for municipal and industrial waste treatment, but this method is not efficient to refine aniline-containing waste. This is in a way that use of chemical and biological methods has been more efficient for treatment of aniline; biological removal of aniline due to high cost of chemical methods is the best method.

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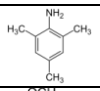
Aromatic amines:

Aromatic amines are aromatic rings containing an amino group in which NH_2, NH or nitrogen groups have been bonded to aromatic hydrocarbon. These compounds generally consist of one or more benzene rings. Aniline is the simplest aromatic amines and other members of this group are known as aniline derivatives, which a variety of these compounds can be observed in table 1[6].

Table 1: Aniline derivatives and their molecular structure.

aniline derivatives	
structure	Aromatic amines
	Aniline
	α-toluidine
	2,4,6- trimethylaniline
	Anisidine
	3-trifluoromethylaniline
	N-methylaniline

Aniline:

Aniline, phenyl amine or amino benzene is an organic compound of primary aromatic amines with formula C_6H_7N and molecular weight of 13/93 g mol. This compound was produced from alkaline distillation of indigo dye for the first time by Unverdoben in 1826, and later Fritsche called it aniline in 1840. Structure of aniline was specified in 1843 by Hofmann The melting temperature of aniline and its boiling temperature is 6 ° C and 184 ° C, respectively; also the vapor pressure of this substance is 0/65hPa in 25° C [8]. This substance consists of a phenyl group connected to the amino group and is used as a substance for most of industrial chemical substances. Further, the main use of aniline is in production of polyurethane. Aniline like many of volatile amines has an unpleasant smell like rotten fish, and it is easily flammable, so that the main feature of this substance is large flames and smoke and spicy and sharp smell. Aniline is a clear, colorless and oily liquid at room temperature. This substance exposed to air and light changes to brown color and is the solution in alcohol and perfume. Further, this substance is slightly soluble in water so that its solubility is 35 grams per liter at 20 ° C, which this increases solubility of contaminants in water. With regard to short lifetime of aniline in water, soil and air, remaining this substance in environment is far from imagination (Sanders,1979; Lyons,1984; Howard,1989). With regard to relatively low vapor pressure and high solubility in water, it is predicted that existing aniline in environment is generally a solution in water. Microbial degradation is an important process for survival of aniline in water, yet Photooxidation (in the water) and biological degradation (in water)are also drawn into attention [2,3]. Existing aniline in soil results in biodegradation, oxidation and binding to components of the soil The half-life for the photodegradation of aniline in air is estimated to be 3.3 hours.

Production of aniline:

Aniline is generally produced from Benzene at two stages in industry.

First stage: Benzene using concentrated mixture of nitric acid and sulfuric acid is nitrated at temperature of 50 to 60 ° C and converted to nitrobenzene.

Second stage: nitrobenzene which is a solid product is generally hydrogenised at temperature of 200 to 300 ° C by presence of a different metal catalyst (figure 1).

Fig. 1: Production of aniline.

Other methods for production of aniline include: production of aniline of chlorobenzenes using ammonolysis, production of benzene of Bromobenzene by means of substitution reaction, production of aniline

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of Acetanilide through hydrolysis in acidic environment, production of aniline of Nitrobenzene by hydrogenation of vapor phase catalytic.

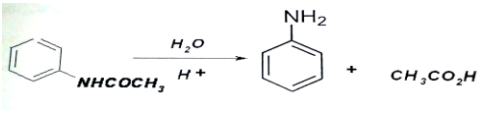

Fig. 2: Production of aniline from Acetanilide.

Fig. 3: Production of aniline from Nitrobenzene.

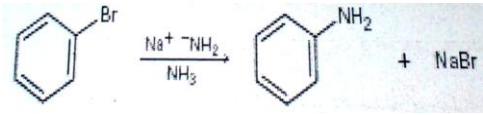

Fig. 4: Production of aniline from Bromobenzene.

Aniline reactions:

Aniline compounds chemically have been entered into a large discussion, because this compound has been available for researchers from a long distance. In following, some of various classes of aniline reactions have been represented.

Oxidation:

Oxidation of aniline is one of the reactions which has been largely entered into discussion. In aniline, Oxidation occurs by presence of Nitrogen which can raise new N-C bonds. Production of Azobenzene can be resulted from oxidation in alkaline solutions, yet production of Violaniline will be resulted in acidic medium by presence of arsenic acid. Further, Chromic acid has converted this compound to a kinnon, yet Chlorates by presence of certain metal salts especially vanadium transform aniline to black aniline. Further, oxidation of aniline by persulfate leads to production of Polyanilines compounds(figure 5); these polymers represent aniline-rich reactions.

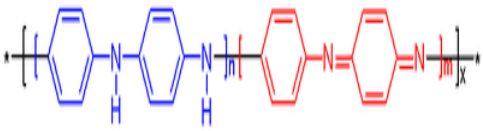

Fig. 5: Production of Polyanilines by oxidation of aniline.

Electrophilic reactions of carbon:

Aniline derivatives such as phenols are suspected to creation of electrophilic substitution reactions, which finally result in increasing the ability of electron donating of aromatic rings in this substance. For instance, the reaction of aniline with sulfuric acid at a temperature of 180 ° C results in production of Sulfanilic acid ($H_2NC_6H_4SO_3H$) and finally converts to Sulfonamides. Sulfonamide is one of Sulfa drugs which has been widely used as an antibacterial agent in the early 20^{th} century.

Reaction at nitrogen:

These reactions include carbon disulfide derivatives, acylation, N-alkylation and diazotization.

Sources of aniline:

No natural source has been recognized for aniline. However, no sufficient information has been available, some have stated that this substance can enter into environment through human resources during the stages of production, storage, transport and removal of aniline from the compounds containing aniline derivatives, as well as through climate change in other countries and due to degradation of some of chemical pesticides.

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Impacts of aniline on human:

Aniline through inhalation of steam, ingestion or absorption through the skin can have feature of toxicity. If human is exposed to high concentration of aniline, he will be subjected to cyanosis , headache , nausea , vomiting , tachycardia, dizziness , ataxia , weakness , dizziness and drowsiness , kidney failure , atrophy of the liver and cirrhosis , epilepsy , cancer , allergic skin reaction and death. Further, these compounds can raise menstrual disorders, ovarian dysfunction and spontaneous abortion. Long contact with little amount of aniline might raise the same symptoms.

aniline break-down products in body such as Phenolic acid in urine represent toxic impact of aniline in body, which its value can be measured. Aniline causes toxicity of spleen through which Splenomegaly, increasing red blood cells, hyperplasia, fibrosis, and primary tumors of the spleen. Inhalation alanine (7-22 ppm) for several hours results in negligent damages, but results in unpredictable disorders in case of being subjected to alanine(100-160 ppm) for one hour(Khezri, 2012). International Agency for Research on Cancer (IARC) has not known aniline as a carcinogen for human, but Environmental Protection Agency (EPA) has known aniline as carcinogen.

Methods for removal of contaminants:

There are a variety of methods to remove heavy metals and aromatic compounds from various wastes. Physical, chemical and biological Treatments are of these methods.

Selection of each of these methods relies on various factors including economic feasibility, amount of waste, pollution and heavy metals and aromatic compounds.

Biological methods for degradation of aniline:

To date, several bacteria have been recognized that degrade aniline in aerobic conditions through intermediate Catechol. Aniline is transformed to Catechol through these Microorganisms through hydroxylation and deamination. Catechol through carbon cycle or meta cleavage cycle is transformed to organic compounds. Metabolization of aniline through microorganisms via meta cycle is seen in a variety of substances. Further, Aniline dioxygenase genes (AD) transform this substance to catechol. This enzyme has been developed from 5 polypeptides which are encoded through genes tdnA1, tdnA2,،tdnB ، tdnQ , tdnT. TdnB, tdnA1 and tdnA2 encode in turn large and small subunits of the terminal dioxygenase and electron transfer in proteins. tdnQ and TdnT associate to transfer of amine group from aniline and ammonia emissions. This enzyme has been seen in Pseudomonas putida and Acinetobacter which causes proliferation in Metabolization of aniline through Meta cleavage [11]. Metabolization of aniline in microorganisms through Orto cleavage path has been seen in Acinetobacter, Rhodococcus and Frateuria. Denitrifying monocrotophos-degrading Paracoccus fulfills incomplete degradation of aniline under aerobic conditions. The first bacterium known to degrade aniline completely under anaerobic conditions is sulfate-reducing Desulfobacterium aniline

Microorganisms in anaerobic conditions and by presence of CO2 fulfill metabolization of aniline aiming at providing energy and source of carbon for growth. Under such conditions, aniline through carboxylation reaction is transformed to Aminobenzoic acid-4, and finally CO_2 and Acetycoenzyme A are produced.

Traditional methods of aniline degradation:

Wide consumption of aniline in various industries has caused substantial amount of this substance is entered in wastes of these industries. Aniline and aniline derivatives are not biologically degraded, that are degraded so hard. In traditional methods, aniline is degraded through electrolysis, oxidation though ozone and adsorption through resin. In these methods, secondary metabolites like para-Methyl phenol and carboxylic acid might be produced, resulting destructive impacts in environment.

Definition of swage:

Swage is called to a collection of consumption waters for different purposes. In other words, waste water collection is conveyed as swage that can be used after collection and treatment. 99.9% of swage is developed from water and the rest from impurities and pollutants including biodegradable organic materials, suspended solids, nutrients, pathogens, heavy metals, organic compounds resistant to biodegradation and dissolved solids that each of these pollutants and their concentration rely on nature of swage.

Classification of various wastes:

The most common classification for waste relies on origin of waste production that a variety of wastes can be as follows: Household waste, commercial waste, industrial waste, wastes at general and administrative centers, hospital waste.

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Hospital waste: features and dangers:
Pathogenic microbial agents:

The major concern about hospital waste which contain enteric bacterial pathogens, bacteria, viruses and parasitic agents are easily transferred through water. Contaminated waste generated from the sections treating intestinal disease by epidemic outbreaks of diarrhea is the most important problems for environmental health. Another issue is that some pathogenic agents in hospital waste have high drug resistance, for this they have been regarded as a serious threat to health of society. Further, some of the aforementioned microorganisms might transfer their drug resistance to other pathogenic agents, whereby prevalence of infectious agents in the treatment will be much more difficult.

Hazardous chemicals:

Negligent amounts of hazardous chemicals due to disinfection and cleaning are transferred to waste collection networks, but large amounts of chemicals might enter into waste collection networks in case of not applying a suitable management.

Drug waste:

Negligent amounts of drug wastes are discharged into waste collection networks both by different hospital sections and drugstore. If a suitable management is not applied, huge amounts of drug wastes including compounds containing aromatic amines (aniline) , antibiotics and genotoxic agents will be discharged to waste collection networks.

Radioactive isotopes:

Negligent amounts of radioactive isotopes will be discharged to waste collection networks by oncology sections that will not damage to health of environment provided that a suitable management is applied. .

Treatment of wastes containing aniline:

Municipal and hospital wastes contain aniline and its derivatives, which biofilm reactors with moving bed or Moving Bed Biofilm Reactor(MBBR)are used for municipal and industrial waste treatment, but this method is not efficient to refine aniline-containing waste. This is in a way that use of chemical and biological methods has been more efficient for treatment of aniline; biological removal of aniline due to high cost of chemical methods is the best method. In these biological methods, Acinetobacter, Rhodococcus and Frateuria are used, and removal yield goes beyond 93% in this method. Furthermore, it can use other methods such as use of aqueous emulsion membranes, Electro-Fenton process, and biological regenerative adsorption in treatment of wastes containing aniline.

Aims of study:

Isolation and characterization of a bacterial strain for aniline degradation from municipal wastes and wastes in Peimanieh Hospital -Jahrom, and examine their antibiotic resistance.

Applied aims:

Identify and find aniline-degrading bacteria from municipal wastes and wastes in Peimanieh Hospital - Jahrom in order to treat municipal and hospital wastes containing aniline.

Literature review:

Neiil *et al*., worked on growth of bacteria in aniline and factors affecting biological treatment of industrial waste. They concluded that increasing aniline to 0.25-1 g/l under conditions 0.2% NaCL with PH ranging from 5 to 7 results in removal of aniline which affects speed and rate of bacteria growth.

Kahng *et al*., examined strain HY99, aniline degrading organism, in aerobic and anaerobic ways. This organism metabolizes aniline under aerobic conditions by means of Catechol and 2- hydroxymuconic semialdehyde intermediate and under anaerobic environment by means of p-aminobenzoate. Biochemical and physiological tests indicated that this strain has a lot in common with strain Delftiaacidovorans, but strain HY99 enabled to metabolize aniline by nitrate reduction under anaerobic conditions. Using analysis 16srDNA, it was clarified that strain HY99 has had similar to bacterium Delftia acidovorans for about 96%.

In Iran, several studies on aniline-degrading bacteria have been conducted. Avatefinejad isolated aniline-degrading bacteria from sediments of Kharg Island and examined their growth kinetics. Further, Khezri [8], after isolation and identification of aniline-degrading bacteria from soil around Shiraz refinery, examined growth kinetics and resistance of aniline-degrading bacteria through MIC, MBC tests and Antibiogram tests.

Kafilzade *et al*., enabled to isolate and identify aniline-degrading bacteria from sediments of Kharg Island and examined the rate of growth and resistance of them.

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Status of sampling place:

Jahrom County is bounded to Fasa county from north, Firoozabad county from Bakhtar and south, Darab county from Khavar, Shiraz county from Northwest, Larestan from Southeast. Jahrom county has been located in 53 degrees and 33 minutes longitude and 28 degrees 30 minutes latitude, and height of 1050 meters above sea surface. Rivers Ghareaghaj Simkan and Shour are the most important rivers as this area. Weather of Jahrom County generally is hot, yet it is moderate in mountainous regions. The main routes at this region include: Larestan-Jahrom route in length of 155 km, Shiraz-Jahrom route in length of 170 km, Jahrom-Ghir route in length of 75 km.

Features of sampling stations:

Six stations were considered in these projects which are as follow:
-Station (No 1), entrance of waste of Peimanieh Hospital -Jahrom.
-Station (No 2), aerated wastewater of Peimanieh Hospital -Jahrom.
- Station (No 3), exit of wastewater of Peimanieh Hospital -Jahrom.
- Station (No 4), wastewater treatment of wastes in Peimanieh Hospital -Jahrom, aeration(No 1)
- Station (No 5), wastewater treatment of wastes in Peimanieh Hospital -Jahrom, aeration(No 5)
- Station (No 6), wastewater treatment of wastes in Peimanieh Hospital -Jahrom, aeration(No 7)

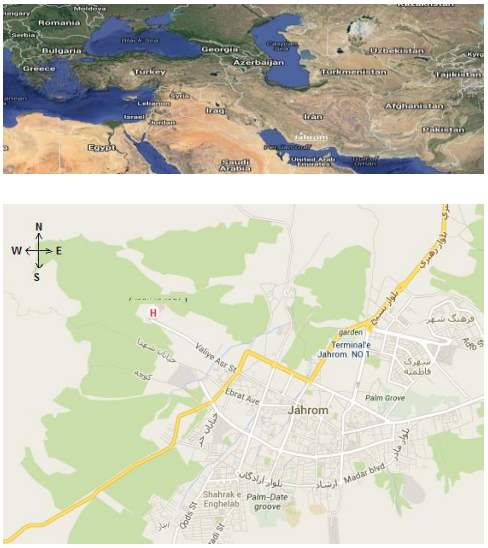

Fig. 6: Status of sampling place.

Status of stations during sampling using Global Positioning System (GPS) was specified in order to prepare sampling at each season from a certain point.

How to prepare sampling:

In this study, sampling was fulfilled from six municipal waste stations and wastes in Peimanieh Hospital -Jahrom during two consecutive seasons(summer and autumn) of the year. Sampling from each station was prepared for three times in each season. The samples were collected for microbial culture in sterile glass jars. Amounts of temperature and PH at each station were measured and the samples immediately during less than 2 hours by maintaining 4 ° C in a flask near the ice were transferred to the laboratory. A large difference was not observed at temperature and PH in various stations, and also a substantial difference on amount of aniline was not seen in various stations. In this regard, the highest rate of pollution has been for about 84 ppb in station 1 and the least rate of pollution has been for about 24 ppb in station 4. 44 colonies were examined and six aniline-degrading bacteria by means of common biochemical tests were isolated and identified, that the name of bacteria as well as rate of their resistance to aniline through MIC and MBC has been represented in tables 5 and 6.

Firstly bacteria regarding concentration ranging from 5000 ppm to 6000 ppmMIC were examined, and all strains ranging from 5000 ppm to 6000 ppm had growth, thus MIC kept increasing in concentrations above 6000ppm. Minimum inhibitory concentration and minimum bactericidal concentration was provided for each bacterium and one liter of it was inoculated to the tubes containing one ml of various concentrations of aniline. A nonincubated as negative control and an incubated liquid environment lacking aniline as a positive control besides a series of dilutions were added. The tubes after 24 hours incubation were examined. The first tube without microbial growth has been considered with the least MIC concentration. By clarifying MIC with the

same dilution (0.1 ml) in solid medium and incubation, pilots were examined. A concentration of metal in which no colony was grown was considered as MBC

Device and materials:

All the device and materials used in this study have been represented in table 2.

Table 2: materials used in this project.

Row	Name of product	Manufacturing company
1	Blood agar	German Merck
2	LB agar	German Merck
3	LB Broth	German Merck
4	Tsi Agar	German Merck
5	Sim Agar	German Merck
6	Simmons Citrate agar	Himedia
7	Urea broth	German Merck
8	sterile swab and loop	Iran
9	Pyrex test tube	Iran
10	McFarland standard 0.5	Diaghosis Miesobidty
11	Antibiogram discs	Rosco
12	Antibiogram discs	Antibody Medicine
13	$KH2PO_4$	German Merck
14	$(NH_4)2\ SO_4$	German Merck
15	NaoH	German Merck
16	Fe_2SO_4	German Merck
17	4-Aminophenylacetic acid	German Merck
18	Glucose	German Merck
19	Agar	German Merck
20	HCL	Scharla 4-spain
21	Sampler tip	German Merck
22	$MgSO_4$	German Eulab
23	Mueller Hinton Broth	German Merck

Table 3: used devices.

Row	Name of product	Manufacturing company
1	Autoclave	Model RT-1 Raihan Medicine of Iran
2	Network Incubator	Fan Azma Gostar Iran company
3	Spectrophotometry	Japan FD-303
4	Digital scale	German KERM
5	Sampler	German Eu lab
6	Oven	German Memm ert
7	Centerfigue	Insosal Iran
8	Microscope	Japan OSK
9	Shaker (scientifica)	VEIP company
10	Thermocycler	GTG model and Biorad USA model

Table 4: Values of temperature, PH, and concentration of aniline in stations under study during two seasons (summer and autumn).

Season	Station	Temperature	PH	Amount of aniline(ppb)
Summer(2013)	1	29	7/9	84
	2	29	7/8	80
	3	28	7/8	78
	4	27	7/6	24
	5	28	7/5	29
	6	27	7/6	34
Autumn(2013)	1	27	8/3	84
	2	27	8/2	80
	3	26	8/2	78
	4	25	7/8	24
	5	25	7/6	29

Table 5: MIC test in isolated resistant bacteria.

Name of bacterium/ ppm concentration	6000	6100	6200	6300	6400	6500
Escherichia coli				Lack of growth		
Citrobacter				Lack of growth		
Pseudomonas aeruginosa			Lack of growth			
Klebseillapneumoniae					Lack of growth	
Klebsiellarhinoscleromatis				Lack of growth		
Klebsiellaoxytoca				Lack of growth		

Table 6: MBC test in isolated resistant bacteria.

Name of bacterium/ ppm concentration	6000	6100	6200	6300	6400	6500
Escherichia coli					Lack of growth	
Citrobacter					Lack of growth	
Pseudomonas aeruginosa				Lack of growth		
Klebseillapneumoniae						Lack of growth
Klebsiellarhinoscleromatis					Lack of growth	
Klebsiellaoxytoca					Lack of growth	

Measurement of aniline in samples:

To measure aniline in samples, HPLC device equipped with three-phase solvent systems has been used in Azma Shiraz laboratory. This device includes a column (c18) and detector (DAD), and chem.station software. The moving phase consisted of acetonitrile - water 20/80 in 1 ml/min flow, where the Detection has been fulfilled in 230 nanometer wavelength.

Isolation of aniline-degrading bacteria *from samples:*

After ending the second week of enrichment, isolation of aniline-degrading bacteria was started, that is, 0.1 ml of enriched environment was added to PNR agar environment containing 100ppm aniline, and incubated for 24-48 hours at $30^{\circ}C$ temperature.

Purification:

Purification was fulfilled on blood agar environment; thereby various colonies were isolated from PNR agar environment and transferred to blood agar environment. To use purified bacteria and keeping them on blood agar environment, around of pilot were covered with parafilm and kept in refrigerator (Kafilzadeh *et al*.,2013).

Counting bacteria:

To count colony, 10 tubes were selected and dilutions 10^{-1} to 10^{-9} were prepared from each of samples (figure 7).

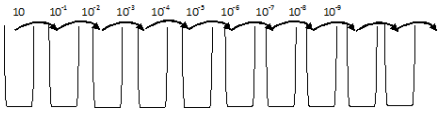

Fig. 7: Dilution of tubes for counting.

9ml amount of normal saline was poured into all tubes, and then 1cc of sample waste was added to tube(No 1) and mixed, then 1cc amount of tube(No 1) was transferred to tube(No 2) and this was continued till tube(No 10) , and this amount was poured into tube(No 10). The tube was incubated for 24 hours at temperature of $30^{\circ}C$, and then it was cultured in two environments of nutrient agar containing aniline and nutrient agar without aniline using method of Spread plate; thereby pilots were incubated for 24 hours at temperature of $30^{\circ}C$, and the pilots after 24 hours were investigated. Due to lack of aniline at control culture medium, after incubation, all the aerobic bacteria will grow at this medium. As bacteria resistant to aniline only grow at culture medium containing aniline, after emerging colonies, the pilots containing certain colonies were selected and the number of colonies was counted, and multiplied by volume(0.1 ml) and number of dilution so as to acquire the number of bacteria in control medium and medium containing aniline in terms of CFU/ml and CFU/gr. Average number of bacteria in all stations indicate that station 2 compared to other stations enjoys more bacteria at significant level($P<0.001$). on the other hand, average number of bacteria by presence of aniline compared to control lacking aniline is significantly reduced(table 7).

Identification of bacteria:

Identification of isolated bacteria using common biochemical tests regarding book "Bergey's Manual of Systematic Bacteriology" and by means of gram staining, studying morphologic features, tests for catalase, oxidase, KOH, Simmons citrate, urease tests, and other differential diagnostic tests was confirmed. 44 colonies were examined and 6 bacteria resistant to aniline were isolated and identified by means of gram staining and common biochemical tests, that the name of bacteria as well as presence or lack of isolated bacteria in various stations during two seasons of summer and autumn has been displayed in tables 8 and 9.

Frequency percent of gram-negative bacteria has been greater than gram-positive bacteria, thereby 100% of isolated resistant bacteria were gram-negative bacteria. The highest frequency percent of bacteria in various stations and seasons relate to E.coli with frequency(100%), and the least frequency percent relates to Klebseilla oxytoca with frequency(8.33%), that was isolated in stations two during summer. Bacterium E.coli was

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isolated in all seasons and sampling stations(figure 8). Further, percent of isolated bacteria in summer was greater than autumn(figure 9).

Table 7: Average number of bacteria by presence of aniline or lack of aniline in two seasons(summer & autumn(2013)).

Season	Stations	Containing aniline	Lacking aniline
Summer(2013)	**1**	191×10^3	81×10^5
	2	95×10^5	66×10^6
	3	256×10^3	94×10^5
	4	51×10^3	50×10^4
	5	32×10^3	47×10^4
	6	47×10^3	68×10^4
	1	110×10^3	80×10^5
	2	132×10^4	194×10^5
	3	141×10^3	100×10^4
	4	89×10^3	55×10^4
	5	80×10^3	49×10^4
	6	52×10^3	46×10^4

Table 8: Isolated bacteria and presence of lack of them in studied stations during summer.

Name of bacterium	Station 1	Station 2	Station 3	Station 4	Station 5	Station 6
Escherichia coli	+	+	+	+	+	+
Citrobacter	+	+	+	-	-	+
Pseudomonas aeruginosa	+	+	-	+	-	-
Klebseilla pneumonia	+	+	+	-	-	-
Klebsiella rhinoscleromatis	+	+	-	-	-	-
Klebsiella oxytoca	-	+	-	-	-	-

Table 9: isolated bacteria and presence of lack of them in studied stations during autumn.

Name of bacterium	Station 1	Station 2	Station 3	Station 4	Station 5	Station 6
Escherichia coli	+	+	+	+	+	+
Citrobacter	+	+	+	-	-	-
Pseudomonas aeruginosa	-	+	-	+	-	-
Klebseilla pneumonia	+	+	+	-	-	-
Klebsiella rhinoscleromatis	+	+	-	-	-	-
Klebsiella oxytoca	-	-	-	-	-	-

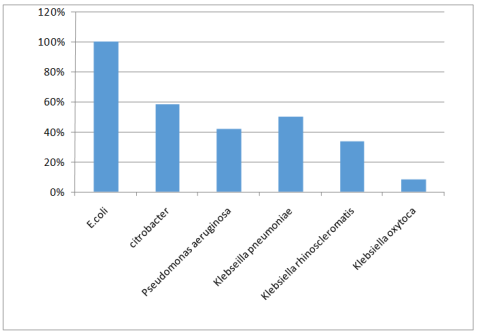

Fig. 8: Frequency percent of isolated bacteria in various seasons and stations.

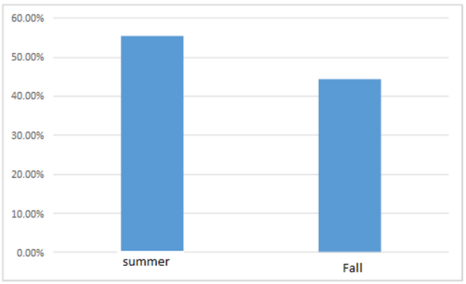

Fig. 9: Comparing seasons in terms of percent of isolated bacteria.

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PCR method:

To identify strain, polymerase chain reaction (PCR)was used in Gerash University of Medical Science.

Extraction of DNA:

To fulfill Pcr, 16srRNA gene was used, such that firstly 500µl distilled water was poured into tubes to extract DNA, and then the tubes were kept closed after incubation of bacteria inside them, that finally they were centrifuged at speed of 4500 rpm for 10 minutes. Then, tubes were taken and liquid above was discharged and 20µl lisozima was added to each tube, and then the mixture was vortexed, in a way that the drops surrounding tubes were conducted with Microspin. Drops were incubated for 30 minutes at temperature of 37°C. Then, 10µl Ributinase was added to each tube and once more the mixture was vortexed and conducted with microspin, and placed at temperature 55°C for 2-3 mintures. After this stage, the protocol was continued as follow:

-adding 400µl of buffer Lysis, and vortex for 20 seconds.
-adding 300µ of solution Precipitation, and vortex for 5 seconds.
-transferring the solution to filter tube of Spincolumn using sampler
-centrifuging tubes with speed of 1300 rpm for 1 minute and removal of substrate solution
-adding 400µl of buffer Wash II to filter tube and centrifuging with round 13000 rpm for 1 minute and removal of substrate solution
- centrifuging filter tubes with round 13000 rpm for 1 minute to drain them

One tube(1.5cc) was taken and its tip was removed, and the filter part was transferred accurately and 30 µl buffer of Elution°C65 was poured into filter. The door of tube was closed, and incubated for 3-5 minutes at temperature of 65°C. then, the tube was centrifuged for 1 minute so as to isolate DNA from filter and transfer it to tube 1.5 cc.

PCR conditions:

Materials and amounts consumed in PCR are as follows:

Buffer (PCR-buffer) 10X	3µl
$MgCl_2$	1 µl
dNTP	1 µl
primer F	1 µl
primer R	1 µl
Taq polymerase enzyme	5 µl
Cdna	5 µl
Water	7.5 µl
Final volume	20 µl

Table 10: Temperature for device PCR to identify bacteria.

Initial denaturation	Reproduction			Final Elongation
	denaturation	Primer binding	Elongation	
95°C5minutes	95°C1minute	58-59°C1 minute	72°C1 minute	72°C1 minute
1 cycle	35 cycles			1 cycle

Studying Kinetics of growth of resistant bacteria in two consecutive seasons:

After all stages and identification of aniline-degrading bacteria, Kinetics of growth of bacteria at four various concentrations of aniline was examined in order to evaluate ability of growth of various bacteria, where the selected concentrations are as follow(table 11):

100, 150, 200 and 250 λ aniline are about 2000, 3000, 4000 and 5000 concentration.

Table 10: Amount and concentration of aniline in Kinetics of growth medium.

Amount of aniline	Concentration
100 λ	2000 $\rightarrow$ ppm
150 λ	3000 $\rightarrow$ ppm
200 λ	4000 $\rightarrow$ ppm
250 λ	5000 $\rightarrow$ ppm

Preparation of culture and inoculation medium:

Five Erlenmeyers with capacity of 100ml per bacterium were autoclaved from culture medium Luria Bertani broth, and then bacteria suspension regarding McFarland standard 0.5 was prepared. A medium "Luria Bertani broth" without bacterium per five erlenmeyers was considered to zero out the spectrophotometer. Immediately, after adding bacterium, absorbance at 600 nm was registered for each of media [8]. Gels were stained with ethidium bromide for 10-15 minutes, that bacterium using UV was seen after washing with water(figure 10).

Bacterium 1, 2 and 3: Klebseilla pneumoni with band length 400 bp, the primer used include:
(5′-GCT CGT GTG GTG AAA TGT TGG GT-3′)
(5′-GCG ATT TCTGAA TGG GGT AAC CC-3′)
Bacterium 4: Klebsiella rhinoscleromatis with band length 550 bp, the primer used include:
(5´TAT TCA TCA GAA GCA GCA CGC AGC TGGGAG AAG CC)
(5´-GCG CTC TGG CTG GTC CAT TTA CCG GTC CCT TTG)
Bacterium 5: Klebsiella oxytoca with band length 250 bp, the primer used include:
(5-GCT GGT AAC AGT TAA GAC AGC GGC GGT AGC G)
(5-TAT TCA TCA GAA GCA GCA CGC AGC TGG)
Bacterium 6 and 7: Escherichia coli with band length 650 bp, the primer used include:
(5' GTCGGAATCGCTAGTAATCG 3')
(3' GGGTTCCCCCATTCGGA 5')

Fig. 10: results of PCR.

Registering OD6000 to examine Kinetics of bacterial growth:

After early registry of absorbance rate, tip of Erlenmeyer was soaked with cotton and placed in shaker incubator at temperature of 30 ° C, and was registered during one week for each 12 hours(Khezri, 2013).

Chart of growth for bacteria resistant to aniline at five statuses indicates that medium enjoys four various concentrations of aniline and concentration without aniline(figure 11-13).

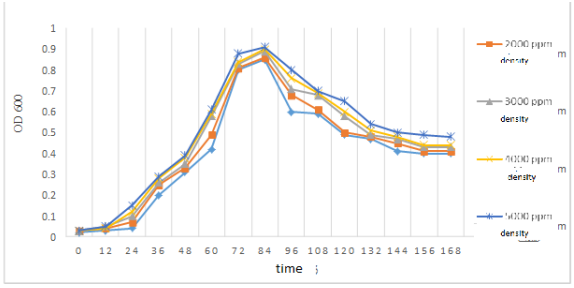

Fig. 11: Results of growth kinetics of Escherichia coli

Conclusion:

The bacteria which have more growth by presence of aniline enjoy higher resistance to various antibiotics; further, aniline has no effect on reducing growth of bacteria with high resistance likewise bacterium Klebseilla pneumonia which enjoys higher concentration of MIC and MBC, and this bacterium is a good candidate for degradation and removal of aniline from contaminated areas.

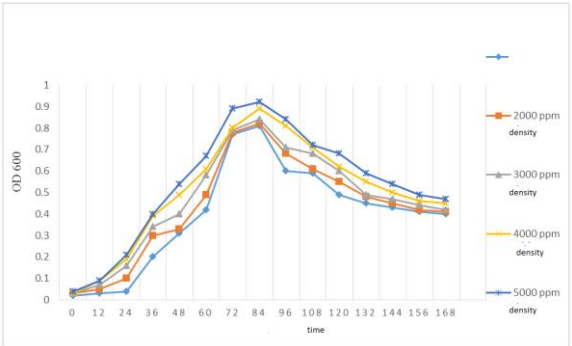

Fig. 12: Results of growth kinetics of Citrobacter.

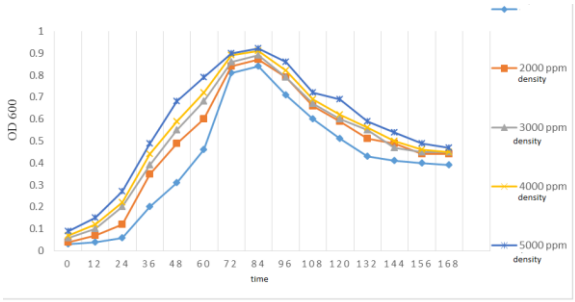

Fig. 13: Results of growth kinetics of Pseudomonas aeruginosa.

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