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Isolation and identification of aniline degrading bacteria from sediments of Kharg island in Persian Gulf

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ABSTRACT

Aniline and its derivatives are one of an important group of bio-environmental aminoaromatic contaminants. Their presence in the environment is a serious threat and danger for human health and other beings. These combinations are very resistant against degrading. The purpose of this study was isolation and identification of aniline degrading bacteria from sediments of Kharg Island and investigation of their growth in presence of this substrate. Sampling was done from different regions of Kharg Island sediments in spring and summer. Sample was added in mineral medium containing 4oo mg/lit of aniline concentration and aeration was done every day. In order to acute identification, usual biochemical tests and PCR method were used. Bacterial number was counted in the medium containing aniline and growth curve of bacteria was drawn on the basis of obtained number. MIC test was done too. The abundance percentage of positive-gram bacteria was more than the negative- gram bacteria. *Aniline resistant bacteria to such as Corynebacterium, Vibrio, Staphylococcus, Pseudoalteromonas, Marinobacter, Pseudomonas, Photobacterium, Bacillus, Salmonella were isolated and identified. The average of bacteria's number was* 8.9×10^{8} *and* 2.06×10^{10} *CFU/ml in spring and summer respectively. Photobacterium damselae showed the lowest rate of growth. Results showed that aniline biological degradation by isolates was considerable and the Pseudoalteromonas arctica was selected in 2000 mg/lit MIC range as the superior bacterium.*

Key words: Kharg Island, Sediments, Biodegradation, Aniline, PCR.

INTRODUCTION

Aniline with *C6H5NH2* chemical formula is one of the most important substances in chemistry [1]. The annual production of aniline in America is about 80000 tons and it is 475000 in China. In countries where aniline is not produced, this substance is imported from other countries for providing the necessity of industry. In Iran, regarding the closig of aniline production plants, average imports of this substance is 38 tons [2,3]. Aniline and its derivatives are used as mediators in most of different operational fields, such as producing of colors, plastic, herbicides and insecticides and etc. [1,4]. Activities of extracting of crude oil hydrocarbons often causes environmental pollution which can lead to dangerous results for animate and inanimate combinations of ecosystems [5,6].

If aniline is distributed in soil, it can rapidly enter to the underground water or evaporate to an average extent. Half life time of aniline is between 20-30 days in air and lower than 100 days in other cases that is due to hydroxyl radicals production by chemical reactions when it is distributed to the air in considerable rate. So it can be toxic for beings living on earth and aquatic ecosystems [7].

From this information it is necessary to cleaning polluted area from these compounds immediately. Bioremediation is an economical and efficacious option which is based on microorganisms ability to severance the contaminations

into harmless metabolites or into innocuous substances like carbon dioxide, water or biomass. Different microorganisms have potential to remove hydrocarbon contaminants, such as some bacteria, fungi, yeasts and microalgae. Among this lot, Bacteria have the best operation to degrade these compounds due to high compatibility and producing proper enzymes to degrade contaminations. If there is no suitable enzyme, bacteria can't attack to hydrocarbon or degrade it completely [8]. A large number of aniline degrading bacteria have been identified such as *Rhdococcus, Acinetobacter , Pseudomonas* and etc. [9,10].

Aerobic degradation of aniline is begun by dioxygenase enzyme that converts it to catechol. In the subsequent step, catechol is changed to metabolits such as 2-hydromoconic semialdehyde. Some bacterial genes can degrade aniline under aerobic conditions with adequate oxygen, so that aniline changes to compounds which enter to the Krebs cycle and they are used for producing energy [9,11,12].
Until now, extensive studies have been done on aniline biological degrading by environmental isolates. Hongsawat et al. (2011) isolated *Acinetobacter baylyi* Gfj2 from the soil which had been polluted with herbicides having aniline combinations [13]. Nwinyi et al. (2008) isolated two strains of *Rhodococcus*, which can degrade aniline in Nigerian soil [14]. In the other research Wang et al. (2011) isolated *Candida tropicalis* strain AN_1 as aniline degrading bacteria from sewages [15].

The purpose of this study was isolation and identification of aniline degrading bacteria from sediments of Kharg Island in Persian Gulf and their growth kinetics assay in presence of this substrate.

MATERIALS AND METHODS

Isolation of aniline degrading bacteria:
Kharg Island is in terms of geographical location in 29°15` Latitude and 50° 20`Longitude. Table1 show names of investigated stations.

Table1: Names of investigated stations

Station's name	Station's no
coast of T dock	1
coast behind gas station	2
coast of Azar pad dock	3
coast of coastal park	4

For bacteria isolation from sediment samples, 10 gr of sediments each station is added to 250 ml of Mineral Salt Medium containing 400 mg/lit of aniline concentration then they were incubated for 10 days at 30°C. During this time, aeration was done several times every day. After this period 1ml of liquid medium was cultured on the TCBS and LB solid mediums. For bacterial purification obtained colonies were cultured on LD solid medium and incubated for 48-hour at 30°C [5].

Identification of aniline degrading bacteria:
Bacteria were submitted for further studies by performing gram reaction test. In order to characterization various biochemical tests were done. Results of these tests were coincidence with bergy's manual of systematic bacteriology.

PCR method:
DNA exploitation was done according to Cinnagen company kit program (DNP kit, CinnaGen, Iran). The density of substances used in this work in PCR was the followings:

dNTP: 1µl; MgCl2: 10 µl; Buffer x10PCR: 5 µl; primer: 2 µl; pattern DNA: 2 µl; DNA polymer Taq: 2/ µl.

For PCR performing, the following instruction is given to the Thermal Cycler System:

94◦c in 4 minutes and 94◦C in 45 seconds , 56.50◦C in 45 seconds; 72◦C in 60 seconds; 30 cycles ; 72◦C in 10 seconds.

Electrophoresis and tracking the PCR products:
In order to observe DNA bands, PCR products on the1.5% agar gel were located on the electrophoresis tank. Afterwards, DNA bands were transferred to the UV Transilluminator System.

Sequence of 16S rRNA:

For determining the gene as final result, an amount of 50 µl from PCR products were transferred to the Macrogen company of southern Korea.

Counting bacteria:
Counting the bacteria was done by using Total Viable Plate Count or TVPC method. In this method serial dilution of isolates suspension were prepared by physiological serum and were cultured in the LB agar medium. The incubated plates have been cultured for 48 hours at 30◦C and numbers of colonies were counted [16].
Evaluating the growth kinetics:
To determine the growth curve of indicator bacteria in the presence of aniline, the colony count method (colony count) was utilized. A suspension of each bacteria were prepared and added to 100 ml of basal mineral medium. Sampling was then done from microbial suspension daily to make different dilutions. One hundred microliter of each dilution was transferred on LB agar and then was spread completely using a sterile glass rod on the plate surface [16].

Minimum inhibitory concentration (MIC):
This test was done in order to obtain a density of lead which prevents the growth of bacteria. A bacterial suspension equal to McFarland standard was provided and 0.1 ml of it was inoculated into LB broth medium including different concentrations of aniline. After incubating for 48 h in 30°C, the last concentration of aniline which had inhibited the bacterial growth was considered as the minimum inhibitor concentration.

Statistical analysis:
ANOVA and Duncan tests were used for comparing stations in the viewpoint of average difference of degrading bacteria number at the 5% level.

RESULTS

Isolation and Identification of aniline degrading bacteria:
Average of isolated bacteria was more in summer than spring. 61.11% and 54.5% of isolated degrading bacteria were positive gram in spring and summer respectively and others were negative gram bacteria. So the abundance percentage of positive-gram bacteria was more than the negative- gram ones, and they had significant difference at 5% level. In this research, bacteria such as *Corynebacterium*, *Vibrio*, *Staphylococcus*, *Pseudoalteromonas*, *Marinobacter*, *Pseudomonas*, *Photobacterium*, *Bacillus*, *Salmonella* were respectively isolated in the viewpoint of abundance percentage. Among the isolated bacteria, the highest abundance percentage of bacteria was related to *Corynebacterium* and *Vibrio* (Fig. 1 ,2, 3).

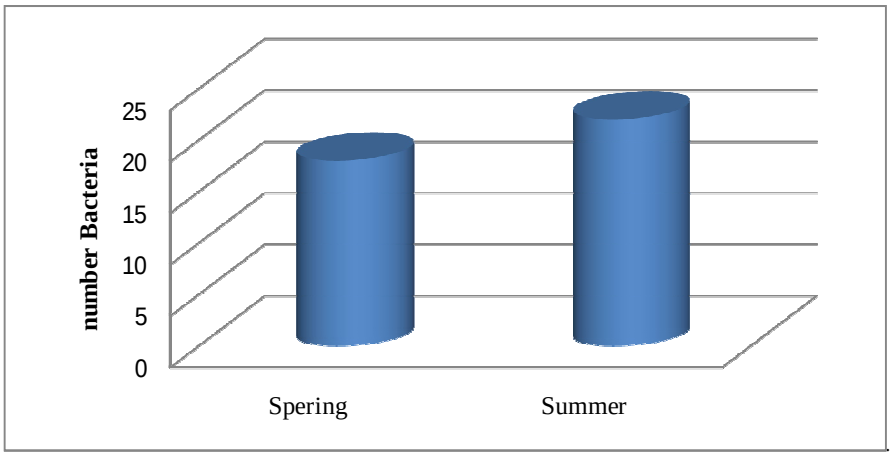

Fig.1: Number bacteria in spring and summer

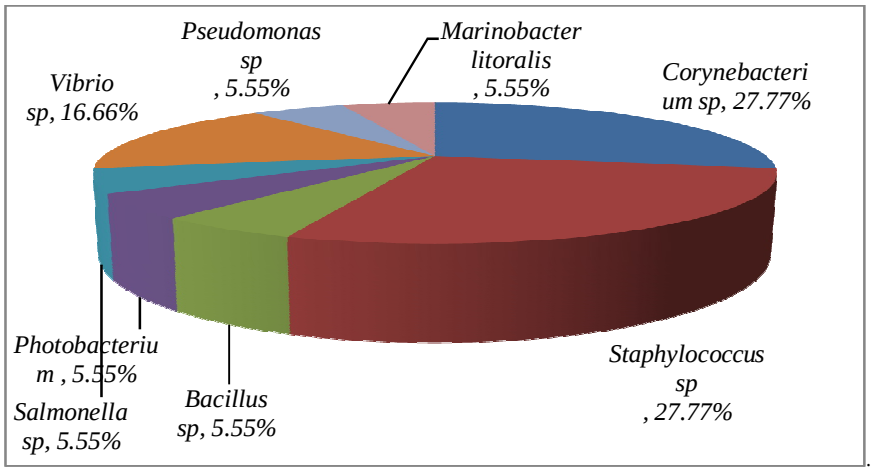

Fig.2: Percentage of isolated bacteria in spring

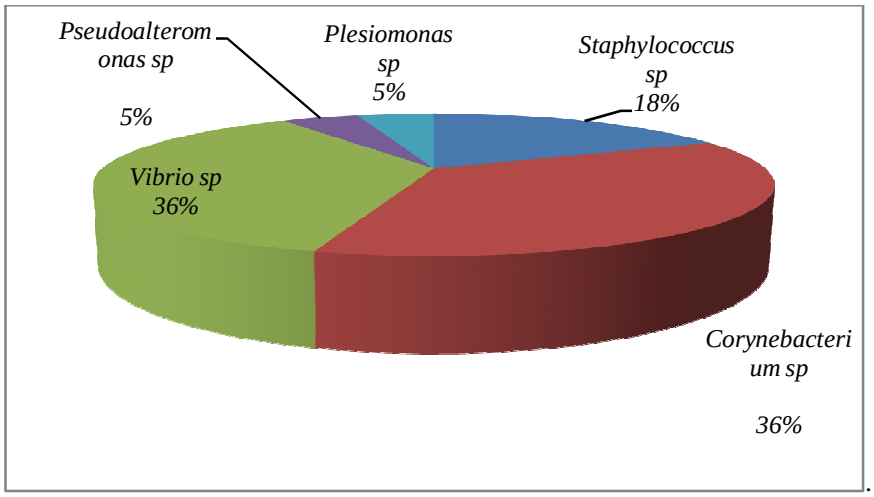

Fig.3: Percentage of isolated bacteria in summer

Isolated bacteria were different in spring and summer from investigated stations. Isolated bacteria abundance percentage in the stations were as the followings: stations no.1 and 3: 22% in spring, stations no.2 and 4: 28% in spring, also, stations no-1 and 3: 32% in summer , stations no.2 and 4: 18% in summer. The isolated bacteria from investigated stations didn't show have any differences at 5% level in seasons spring and summer.

Bacterial Counting and evaluating their growth kinetics:

The average number of aniline resistant bacteria was 8.9×10^{8} and 2.06×10^{10} CFU/ml in spring and summer respectively.

The growth curve of 4 strain through selected 8 strain bacteria, in the presence of aniline, was drawn on the basis of counted bacteria number in 9 days (Fig. 3,4,5,6).

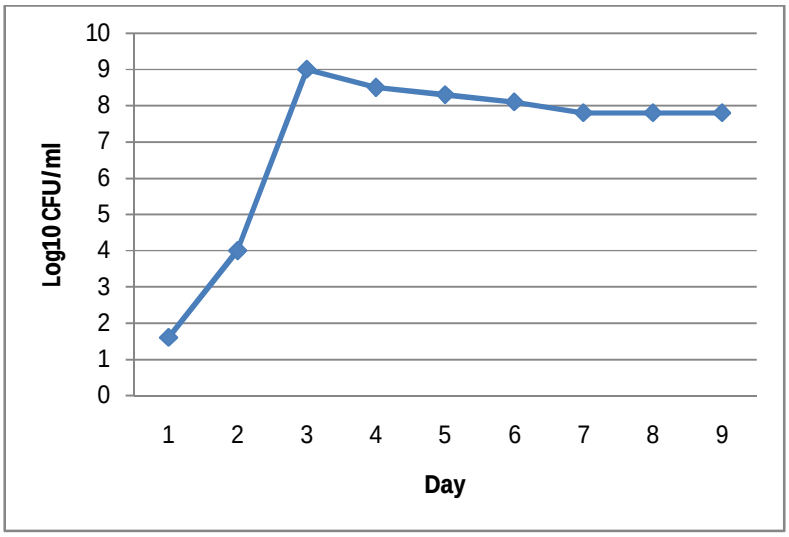

Fig.3: Growth curve of *Photobacterium damselae* during 9 days

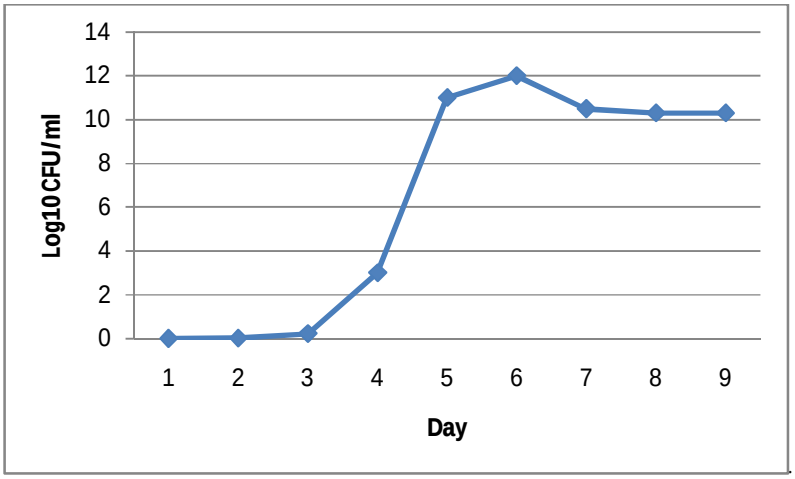

Fig. 4: Growth curve of *Pseudoalteromonas arctica* during 9 days

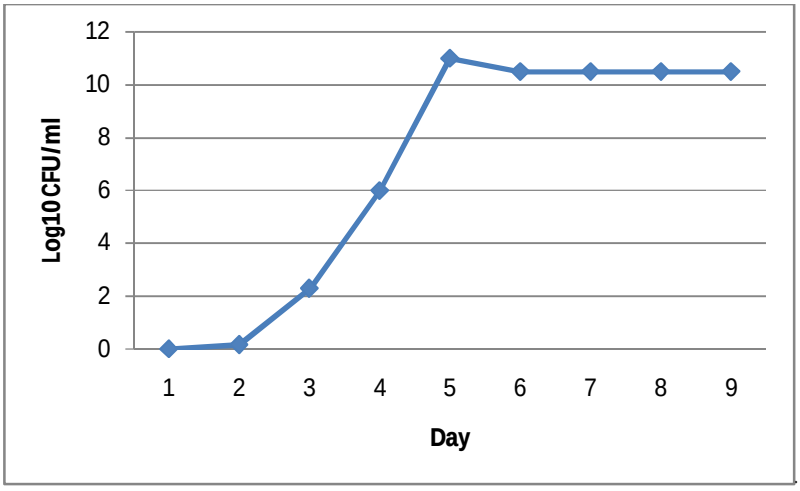

Fig.5: Growth curve of *Vibrio parahaemolyticus* during 9 days

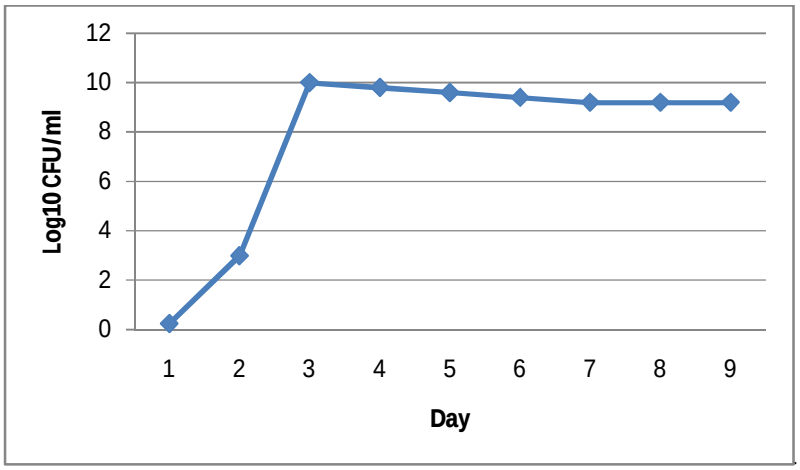

Fig.6: Growth curve of *Corynebacterium* during 9 days

DISCUSSION

Bioremediation with suitable bacterial strains is an effective and economical option that is based on microorganisms ability to degrade contaminations. Bacterial degradation is a major pathway for the removal of contaminant from the nature but we can prompt this process with isolating efficient bacteria from contaminated sites and use them for purification such place [13,14,17]. In the current research different bacteria have been isolated as the aniline degraders such as *Corynebacterium*, *Vibrio*, *Staphylococcus*, *Pseudoalteromonas*, *Marinobacter*, *Pseudomonas*, *Photobacterium*, *Bacillus*, *Salmonella*.
Sylvie et al. (2008) studied biological destruction of organic contaminants such as aniline by halophilic microorganisms. Urata et al. (2004), isolated aniline degrading bacteria from natural environment sediments of Japan in their researches [17,18]. In the current study, sediment samples from the Persian Gulf to aniline degrading bacteria were isolated and identified during the spring and summer.

Kahng et al. (2000), isolated aniline degrading microorganisms from investigated soil in Sothern Korea. Bacteria were identified by using PCR method gene 16S rRNA [19]. In the current study, aniline degrading bacteria were identified via usual biochemical tests and PCR method with 16S rRNA gene.
Urata et al. (2004) categorized the aniline degrading bacteria in 8 groups from Japan environmental sediments that seven groups of them were related to negative- gram bacteria [17].

Hinteregger et al.(1992), Urata et al.(2004), Tanaka et al.(2009), Vangnai et al. (2007), Quanfeng et al.(2005), Meyers et al.(1992), defined *Pseudomonas* as aniline degrading bacterium [9,17,20,21,22,23]. In the present research, *Pseudomonas* also was determined as the aniline degrading bacterium.

Sorensen et al. (2002), isolated one halophile strain that were called *Marinobacter* (DPV2) from aniline polluted desert in the northern part of Naghaveh desert in Israel [24]. Wang et al. (2007) isolated aniline degrading bacteria from waters with high pollution level in China. In this research, the bacteria such as *Alcaligenes*, *Bacillus*, *Pseudomonas* were isolated [25]. Ahmed et al. (2009) after their researches on the investigated soils, informed that bacterium *Staphylococcus aureus*; strain ST1, is one of aniline degrading bacteria [5].

Growth curve of isolates in presence of aniline is shown in fig 3 to 6. In the main, isolated bacteria had a rapid growth in presence of aniline. This curve confirm to bacterial schematic growth curve. Isolated bacteria had no lag phase in presence of this substrate except *Pseudoalteromonas*. Bacteria reached to the maximum optical density at 3th to 6th days of incubation. So the growth curve showed ascendant situation. Eventually bacterial growth rate was decreased with time as a result of aniline depletion as a sole carbon source and also increasing the toxicity of secondary metabolic. So there was a decline in cell number after these days.

Wang et al. (2011), investigated the growth kinetics of *Candida tropicalis* AN1 isolated from aerobic granular sludgein in 100 and 500 mg/lit of aniline concentration during 7 days via OD method and also effect of initial aniline concentration on the biodegradation of aniline by AN1 was studied using five different aniline concentrations: 100, 200, 400, 600 and 800 mg/lit. When the initial aniline concentration was below 800 mg/lit, the strain AN1 could successfully degrade aniline added and the degradation rates depended on the initial concentrations [15]. Zhang et al. (2007) investigated the growth kinetics of degrading bacteria of aniline namely *Delftia* sp. AN3, *E.*

coli JM109 and *E. coli* JM109-AN1 in 300 mg/lit of aniline concentration during 5 days; so that they defined *E.coli* JM-ANI as the most resistant bacteria [26]. In the present study, the growth kinetics of isolated bacteria was investigated in 600 mg/lit of aniline concentration and *Pseudoalteromonas arctica* was selected as the most resistant bacterium.

CONCLUSION

As a whole, this research shows that the degrading types of isolated aniline from Kharg Island sediments have potential ability for aniline degradation. Bacteria such as *Pseudoalteromonas* and *Vibrio* have the highest degrading power. So we can suggest them as appropriate organisms for using in critical water pollution by this pollutant especially in marine environments.

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