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ISOLATION AND IDENTIFICATION OF 3-CHLOROBENZOIC ACID DEGRADING BACTERIA FROM KHARG ISLAND SEDIMENTS AND THEIR GROWTH KINETICS ASSAY

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ABSTRACT

Chlorobenzoates are one of the important groups of chloroaromatic contaminators which are mostly used as the herbicides. Distribution of these substances in the environment due to their toxic effects is an enormous anxiety. For this reason, elimination of these combinations has been considered through biodegradation. The purpose of this study was isolation and identification of 3-chlorobenzoic acid degrading bacteria from sediments of Persian Gulf (Kharg Island) and to investigation their growth kinetics. Sampling was done from 4 stations in two seasons. Isolation of degrading bacteria was done on mineral medium base containing 0.37 gr/lit 3-chlorobenzoic acid. For identification of bacteria, usual biochemical tests and PCR method were used on the basis of gene 16S rRNA. In order to determine the growth curve of bacteria in the presence of this substrate, the colony counting method was used and also the minimum inhibitory concentration (MIC) was measured. Eight negative-gram genera and 3 positive-gram genera were isolated as 3-chlorobenzoic acid degrading bacteria. Maximum abundance percentage of isolated bacteria was related to *Corynebacterium*. The most resistant isolates to 3-chlorobenzoic acid were *Acinetobacter, Vibrio parahaemolyticus* and *Vibrio diabolicus* respectively with MIC rate, 5, 2.5 and 2.5 gr/lit. The results obtained from this study show that different species of bacteria can degrade 3-chlorobenzoic acid that the most important one are *Acinetobacter* and *Vibrio*.

Keywords: 3-Chlorobenzoic acid, Kharg Island, Biodegradation, *Acinetobacter*, PCR

INTRODUCTION

The chlorinated aromatic chemicals used as the herbicides, insecticides, solvents, fluent-making and etc. Also, chlorinated aromatic are produced in various industries such as paper and pulp factories. Many of these chemicals has been introduced as primary contaminants by Environmental Protection Agency (EPA). These substances enter the environment in large amount while they are very toxic and resistant against degradation. Therefore, their distribution in the environment is an enormous anxiety for humans and wildlife **[1, 2]**. One of the important groups of these contaminators are chlorobenzoates used as herbicides. In addition to anthropogenic chemicals, so many kinds of acidic halogen aromatic combinations are produced naturally in soil and aquos environments **[3, 4, 5]**.

Polychlorobiphenyls (PCBs) are very toxic existed to large amounts in soils and sediments. They are strong organic combinations and were used in industrial operations extensively from the middle of 1970. Nowadays, they are known as harmful contaminants for the environment **[6, 7, 8]**. Chlorobenzoates especially 3-chlorobenzoic acid, are the mediators produced during biodegradation of Polychlorobiphenyls with some chlores that are considerable in the destruction process. This matter that whether the chlorinated aromatic combinations are destructive for the environment or not, is directly depends on chemical structure of the combination **[9, 10, 11]**.

Using biodegradation method is a useful replacing and economical way along using common technology and expensive expenses. Chemical and physical conditions and existence of microorganisms with special catabolic ability is effective in contaminants degradation. Among these factors, molecular oxygen has an important role in determining these combinations fate **[4, 12]**. Until now, different benzoic acid degrading microorganisms have been isolated under aerobic and anaerobic conditions. For example, fungous species such as *Rhodotorula glutinis* and other fungi similar to fermant, mildew *Penicillinum frequentans* and bacteria like *Rhodopseadomonsa palusris*, *Alcaligenes denitrificans*, several kinds of *Pseudomonas denitrificant* **[13-16].**

The available evidences show chlorobenzoic acid degradation under aerobic and anaerobic conditions. **Morimoto *et al.*, 2000**, **[10]**, became successful in isolation of 5 strains of bacterium *Burkhordria* with using 3-chlorobenzoic acid degradation ability from forest soils of New Zealand **[10]**. Moreover,

aerobic degradation of chlorobenzoic acids has been studied in waters and sewages. **Gallego *et al*., 2012, [17]**, identified *Pseudomonas putida* as the indigenous 3-chlorobenzoic acid degrading bacterium from polluted river Riachuelo in Buenos Aires. Also, **Kroonemans *et al*., 1999, [4]**, isolated bacteria *Pseudomonas palustrid* strain DCP3 and *Alcaligenes* strain L6 as 3-chlorobenzoic acid degrading bacteria from polluted freshwater sediments **[4, 17]**. In studies done on the sediments, freshwater and estuarine, benzoate has been recognized as mediator of 3-chlorobenzoic acid biodegradation. Chorobenzoic acids are biodegraded under anaerobic conditions by microorganisms consortium in order to use carbon and energy for microbial growth. In the other research **Townsend *et al*., 1997, [18]**, investigated 3-chlorobenzic acid anaerobic degrading bacteria and also sulfate effect range of degrading this toxic combination by isolated microorganisms from chloroaramatic polluted sediments **[18]**. Some of the studies have explained 3-chlorobenzic acid converts to CO_2, methane, chlore and benzoate as intermediates. Therefore, chlorobenzoic acids degrade as electron acceptor under anaerobic conditions. Also 3-chlorobenzoic acid degradation has been inferred under denitrification conditions, sulfate reduction and iron reducing conditions. In multiple studies which were done on anoxic phototrophic and *Rhodopseudomonas palustris*, complete degradation of 3-chlorobenzoic acid to CO_2 and cellular biomass in the presence of light and absence of oxygen have been shown **[19]**.

The purpose of this research is to isolation and identification of 3-chlorobenzoic acid degrading indigenous bacteria from Kharg Island sediments in Persian Gulf, their growth kinetics assay in order to obtain some 3-chlorobenzoic acid degrading bacteria for polluted sediment purification.

MATERIALS AND METHODS

Sampling

Sampling was done during 2 seasons “spring and summer’’ from 4 selected stations around Kharg Island **(Figure 1, Table 1)**.

Samples were gathered from 2-3 cm depths of sediments and were transferred to the laboratory near ice within a maximum period of 24 hours.

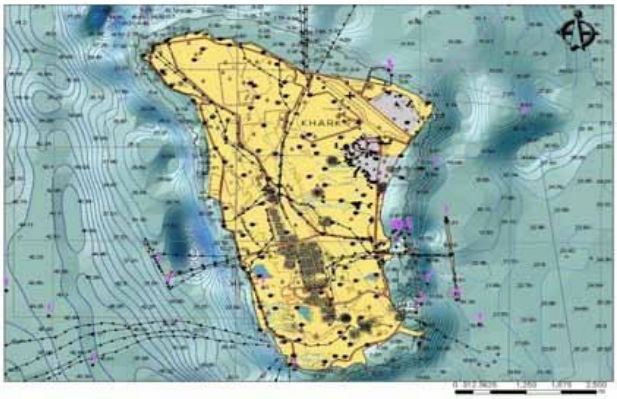

Figure 1: The place of Sampling Sediments

Table 1: Specification of Sampling

Station	Sampling location	Geographical	Geographic Situation	
			Latitude	Longitude
1	The coast of T dock	East	29°13`	50° 20`
2	The coast behind gas	South	31°15`	50° 18`
3	The coast of Azarpad	West	29°13`	52° 20`
4	The cast of coastal park	North	29°15`	50° 18`

Medium and Cultures Conditions

Mineral salt medium (MSM) were used in this study with following components: *NaCl 20gr*, *$MgCl_2$ 3gr*, *$CaCl_2$ 0.15gr*, *NH_4Cl 0.53gr*, *Na_2SO_4 0.14gr*, *KH_2PO_4 0.34gr*, *K_2HPO_4 0.43gr*.

10 g of each stations' sediments was added to 500 ml MSM medium containing 0.37 gr/lit of 3-chlorobenzoic acid as sole source of carbon and energy to performing enrichment operation. The enriched medium was incubated on the rotating shaker in dark conditions at 30°C for 10 days **[3]**.

Isolation and Identification of 3-Chlorobenzoic Acid Degrading Bacteria

Luria Bertani (LB) broth and Thiosulfate Citrate Bile Salts Sucrose (TCBS) mediums were used for isolating bacteria. Plates were incubated at 30°C for 1-2 days bacterial colonies were purified on the LB agar medium. The strains of isolation bacteria were identified via gram stain, microscopic studies and of biochemical tests such as oxidase, catalase, bacterium's motility, salty tests, sugar tests such as fermentation of lactose, sucrose, glucose and etc. Also PCR method was used for accurate identification **[3]**.

Genotyping

Molecular identification of some isolated was done on the bass of Sequencing gene 16S rRNA according to the following procedure.

DNA Exploitation

Exploitation of DNA form the bacteria was performed according to the producer company's instruction (Cinnagen, Iran).

Multiplication of Gene 16S rRNA by PCR Technique

14 µl of Sterile distilled water, 2.5 µl PCR Buffer with 10 times concentrations (Cinnagen, Iran), 1 µl from primer (whith 10/mol concentration), 0.5 µl of Taq–Polymerase enzyme (Cinnagen, Iran) and 5 µl of DNA.

In order to begin Polymerasition process, Trmosaykler system was adjusted at 95^{o}C for 4 minutes. Afterwards, 35 cycles PCR was performed at 95◦C for 40 seconds, 56◦C for 30 seconds and 72◦C for40 seconds. Finally, the action of making long for the last time was done at 72◦C for 5 minutes.

Electrophoresis

For performing electrophoresis, 5 µl of 2000 size marker (Fermentance-Russia) and each samples were transferred into the contrived small wells in 1.5% agarose gel. Voltage was adjusted on 75 volt for 30 minutes. Afterwards, gel was transferred to the UV Transilluminator System (Russia-cleaver) in order to observe the created bands.

Determining Gene Sequence of 16S rRNA

50 µl from PCR product of Macrogen company *(http://www.macrogen.com)* was sent for confirming the identification of isolated strains.

Bioinformatic

All gene sequences that were obtained by using software BLAST existed in site NCBI *(http://www.ncbi.nlm.nih.gov/BLAST*) were evaluated.

Colonies Counting and Growth Kinetics Evaluation

Colony count method was used in order to determine the growth curve of index bacteria in the presence of 50 mg/lit 3-chlorobenzoic acid during 9 days. Counting colonies was done by Total Viable Plate Count (TVPC) method. Serial dilutions of samples were provided by the physiological serum and samples were cultured on the surface of LB agar medium. Samples were incubated for 48 hours then bacterial numbers (cfu/g) were counted on the LB agar medium **[20]**.

Minimum Inhibitory Concentration (MIC)

The Minimum inhibitory concentration for index bacteria in, 0.37, 0.74, 1.5, 2.5, 5, 10, and 20 gr/lit concentrations of 3-chlorobenzoic acid were investigated and the most resistant degrading bacteria was identified.

Statistical analysis:

Excel and SPSS software were used for drawing graphs and data analyzing respectively. Also statistical tests like

Analysis of Variance (ANOVA) and Duncan test were done and significant differences were determined at 5% level.

RESULTS

The number of isolated bacteria was higher in summer than in spring. Comparing two societies (summer and spring) by t-test showed there were no significant differences at %5 level. The percentage of negative and positive gram bacteria isolated in the investigated seasons showed that the highest number of isolated bacteria in two seasons related to positive -gram bacteria with 60% in spring and 66.66% in summer. Also abundance percentage of the percentage of negative -gram bacteria was 40% and 33.33% in spring and summer respectively **(Figure 2)**. Abundance percentage of isolated bacteria in of spring and summer were different from each other. The highest number of isolated bacteria in spring and summer related to the stations No. 1 and 4; also the lowest number of isolated bacteria in spring related to stations No 1 and 2 and the lowest number of isolates in summer related to station No. 4. Comparing the differences of stations in both seasons showed no significant at 5% level in viewpoint of isolated bacterial number. **Figures 3 and 4** show *Corynebacterium* has the highest abundance percentage in both seasons spring and summer.

The average number of 3-chlorobenzoic acid resistant bacteria with 50 mg/lit concentration were 2.04×10^9 and 2.09×10^9 cfu/ml in spring and summer respectively.

Growth curves of the investigated strains show similar growth pattern and also indicate *Acinetobacter* sp. and *Vibrio diabolicus have the best growth and Corynebacterium* sp. has the lowest growth rate **(Figure 5, 6, 7).**

The MIC rate was observed 5gr/lit for *Acintobacter*, 2.5 gr/lit for *Vibrio parahaemolyticus* and 2.5 gr/lit for *Vibrio diabolicus*.

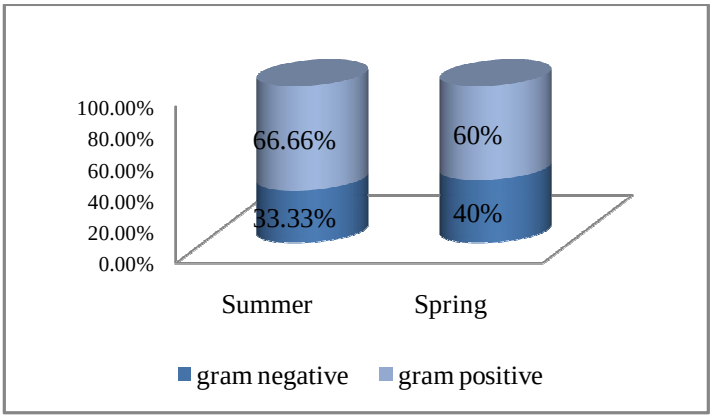

Figure 2: Abundance Percentage of Positive –Gram and Negative –Gram Bacteria in Summer and Spring

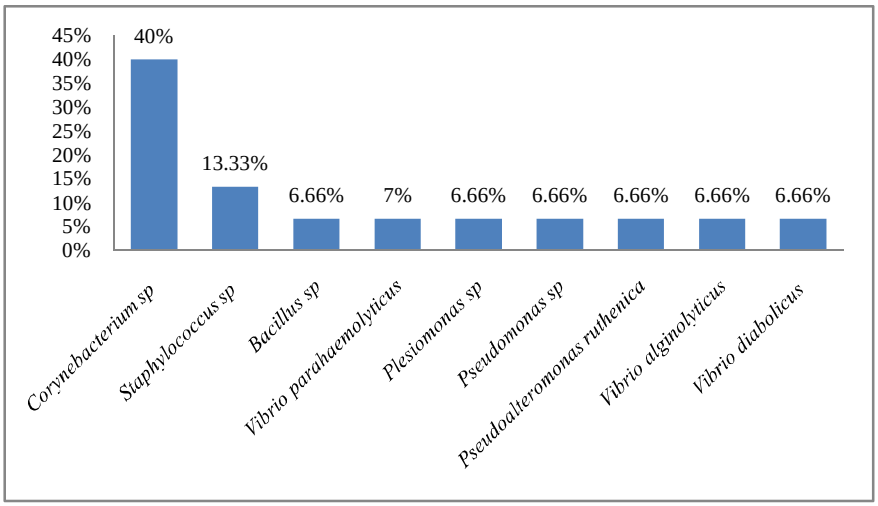

Figure 3: Abundance Percentage of Isolated Strains in Spring

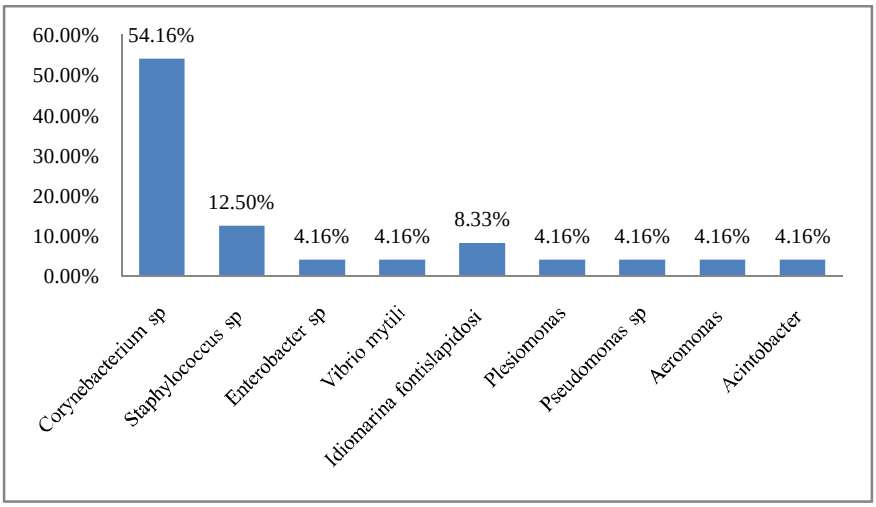

Figure 4: Abundance Percentage of Isolated Strains in Summer

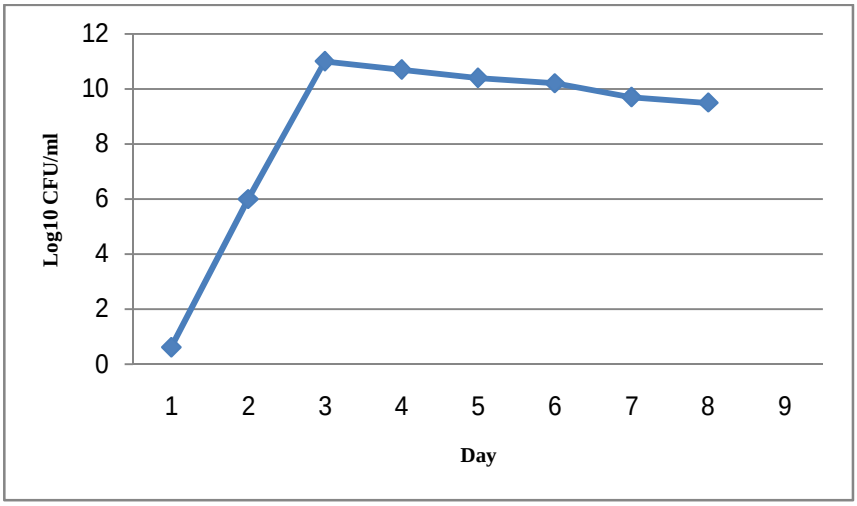

Figure 5: Growth Curve of *Corynebacterium* During 9 Days

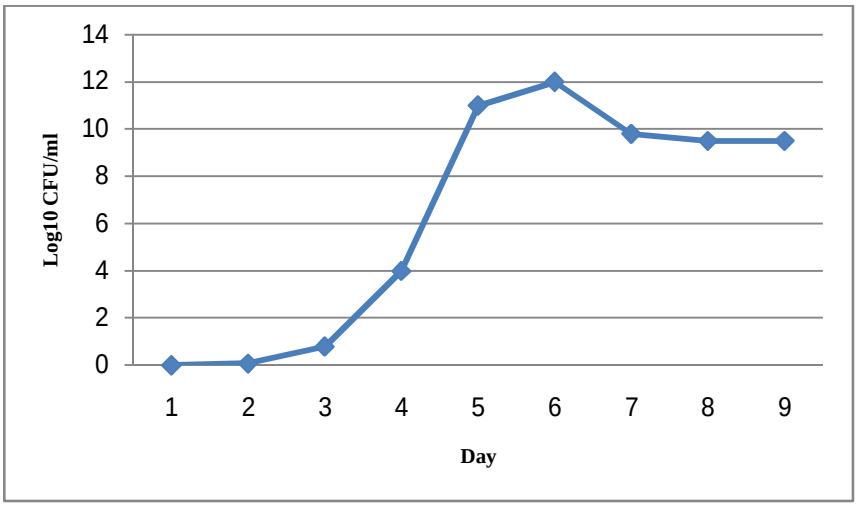

Figure 6: Growth Curve of *Vibrio diabolicus* During 9 Days

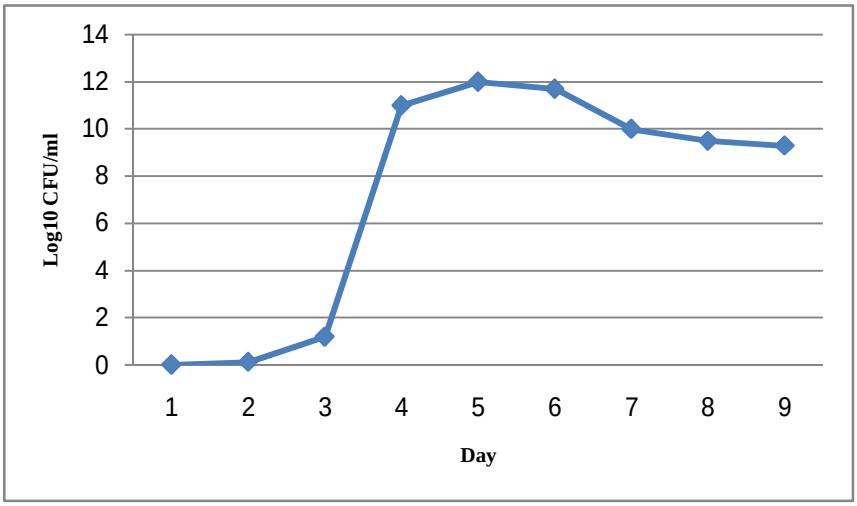

Figure 7: Growth Carve of *Acinetobacter* During 9 Days

DISCUSSION

Isolation of pollutants degrading bacteria is an important step in bioremediation. Enrichment method is a simple and effective method for isolation degrading microorganisms from different ecosystems **[21]**.

Flthrope *et al*., 1998, [22], isolated 3-chlorobenzoic acid degrading bacteria from virgin soil samples in Canada. Also **Hernandez *et al*., 1991, [23],** in order isolation of 3-chlorobenzoic acid degrading bacteria used primary enriching method **[22, 23]**. **Adebusoye, 2008, [3],** used polluted water and sediments in order to isolation of 3-chlorobenzoic acid degrading bacteria use of Sea Water base (SW) mineral salts (MS)

medium for treating sediment and water samples. In the current research, kharg Island sediments were used for isolation of 3-chlorobenzoic acid degrading bacteria by enriching on Mineral Salt Medium and strick culture methods.

Song *et al*., 2000, **[24],** isolated 3-chlorobenzoic acid degrading negative-gram bacteria from samples of American sediments and also they used analysis method of 16S rRNA gene in order to identification of isolated bacteria. In the present study in addition to differential- diagnostic tests and microscopic studies, gene analysis 16S rRNA was used by PCR in order to accurate identification of isolated bacteria.

Adebusoye and Miletto, 2010, [25], isolated positive –gram and negative –gram bacteria for degradation of 3-chlorobenzoic acid, including *Alteromonas, Marinomonas, Lysinibacillus* and *Bacillus* from sediments and polluted waters of Nijerieh. **Nishino *et al*., 1999**, **[26]**, isolated positive –gram for degradation of 3-chlorobenzoic acid from groundwater, including *Brevibacterium*,*Corynebacterium*, *Nocardia* and *Bacillus* sp. while all gram-negative bacteria isolated were *Pseudomonads*. These bacteria could mineralize approximately 54% of the CB within 7 days, with no accumulation of 3-chlorocatechol. **Demnerova *et al*., 2002**, **[6]**, isolated positive –gram and negative –gram bacteria for degradation of 3-chlorobenzoic acid from soil that had been contaminated with PCBs and CBS for 15–30 years. In the present research, positive- gram and negative-gram bacteria were also isolated as the resistant bacteria to 3CBA For example, the positive –gram bacteria: *Corynebacterium* sp., *Bacillus sp.*, *Staphylococcus* sp. and negative-gram bacteria: *Plesiomonas* sp., *Pseudomonas* sp., *Pseudoalteromonas* sp.

Kroonemans *et al*., 1999, [4], isolated *Psudomonas palustrid* strain DCP3 and *Alcaligenes* strain L6 as 3-chlorobenzoic acid degrading bacteria. **Hoskeri *et al*., 2010**, **Obayori *et al*., 2011, and Kamal *et al*., 1990**, **[2, 27, 28]** defined *Pseudomonas* sp. as 3-chlorobenzoic acid resistant bacterium. In the current study, also *Psudomonas* sp. was isolated from Kharg Island sediments.

Adebusoye and Miletto, 2010, [25], isolated 17 genera of important members of bacterial concersium as 3-chlorobenzoic acid degrading bacteria. Isolated bacteria from soil were mostly *Burkholderia* genera; while the bacteria isolated from water recognized mostly from *Alteromonas, Marinomonas, Lysinibacillus* and *Bacillus*. The sediments bacteria were very various. This research shows that various microorganisms can grow

on chlorobenzoates. *Bacillus* was isolated in the present research similar to mentioned study.

Cvancarova *et al.*, 2009, [11], isolated *Vibrio fisherii* as 3-chlorobenzoic acid the resistant bacterium as like as current research.

Peel and Wyndham, 1999, **[29]** investigated the chlorobenzoates degrading bacteria from groundwaters and surface waters of Niagara waterfall during 3 seasons of spring, summer and fall. Different bacteria were isolated such as *Protobacteria*, *Alcaligenes* sp., *Comamonas* and *Burkholderia*. In the present study, none of the mentioned bacteria were isolated as the 3-chlorobenzoic acid resistant bacteria.

Ahamad *et al.*, 2011, [30] studied the growth kinetics of the isolated bacteria called *Pseudomonas* CP4 and 3mT in 0.3 and 1.5 and 3 gr/lit concentrations of 3-chlorobenzoic acid. Also, **Gallego *et al.*, 2012, [17]**, investigated the growth kinetics of *Pseudomonas putida* , *Vibrio fisherii* and *Lactuca sativa* form a polluted river in 0.1,0.2 and 0.4 gr/lit concentrations of 3CBA, consequently, *Pseudomonas putida* was defined as the most resistant degrading bacterium. In the current research, the growth kinetics of isolated bacteria of Kharg Island polluted sediments in 50 mg/lit concentration of 3-chlorobenzoic acid was investigated and *Acinetobacter* and *Vibrio* were determined as the most resistant bacteria.

CONCLUSION

The results of this research show that the3-chlorobenzoic acid degrading bacteria have an extensive distribution in nature and sediments around Kharg Island and a large number of mentioned bacteria. According to results, *Corynebacterim* has the maximum abundance percentage and *Acinetobacter* has the maximum resistance against 3-chlorobenzoic acid.

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