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Isolation and identification of 2,5-dichlorobenzoic acid degrading bacteria from soils and sediments of landfill

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ABSTRACT

One of the biggest problems at present century that we face with, are wastes and how to use them correctly. Chlorobenzoates are the big xenobiotics that are mediator in making of insecticides, dyes and drugs. They are entered into environment through residue destruction and analysis of PCBs by human. The purpose of this research is isolation and identification of 2,5-dichlorobenzoic acid (DCBA) degrading bacteria and examining the growth of them in presence of this compound and also evaluating and justifying biodegradation of mentioned compound in landfill. Sampling was done from different places of landfill in spring and summer of 2010. Amount of 4.0g of sample was added to inorganic medium containing 2,5 -DCBA, then aeration was done every day for one minute. For identifying bacteria, the common biochemical tests were used. The amount of released colure was measured with UV. The measurement of the organic material 2,5-DCBA in samples of culture of 50 days was then examined by HPLC. The number of bacteria in medium containing 2,5-DCBA and the medium without this compound was accounted. In medium without 2,5-DCBA, the number of bacteria was more than the mineral medium containing this material. In spring, most of degrading bacteria was gram negative while in summer was gram positive. Resistant bacteria to 2,5-DCBA was identified and isolated as: Bacillus sp., Corynebacterium, Enterobacter, Klebsiella, Citrobacter, Neisseria. The most quantity of degradation of 2,5-DCBA was belonged to Entrobacter 62% and Corynebacterium 48% and least quantity determined by Bacillus sp.*14% bacterium. The results of the research also showed the advantage of primary enriching method for better isolation of bacteria in compare with direct culture of them.*

Key words: Landfill, Biodegradation, 2,5-DCB, *Corynebacterium*, HPLC.

INTRODUCTION

One of the big problems that we are face with at present century is cities residues. We have to use them in such a way that they would not be troublesome for environment. The landfill was made in 1920 and 1930. It is the easiest way to bury the organic and inorganic wastes. They were then covered with soil layers to keep them out of reach of insects and rodents. Decomposition is slow in landfill. Aerobic and facultative anaerobic microorganism attacks to residues that cause decomposition during which CO2 gas is produced. The buried substances are harmful and cancer-causing for humans and can come into food chain. The produced gas can also cause destroy of plants and ecosystem and firing and can enter into surface and ground waters and cause pollution [1,2]. We also can use this gas as consuming gas and for producing electricity. DCBs are macro xenobiotices which are distributed on the soils and sediments. 2, 5-dichlorobenzoeic acid (2,5-DCBA) is used for producing insecticides, dyes, poison, drugs and

antiseptics. They are degraded by microorganisms such as *Micrococcus*, *Flavobacterium* , *Alcaligenes*, and *Pseudomonas.* Biodegradation in such a way is simple and economical and cause removing of pollution from the environment. In microbial metabolism process, the break of the aromatic ring occurs through ortho and Para approach. This cause release of CO2 and the chlorine atoms will be separated on presence of oxygen. The benzene ring breaks by dioxygenase enzyme and be converted to catechol that finally converted to acetyl co-A and pyruvic acid , then enter to krebs and bacteria's metabolic cycles [3,4]. Hence, biodegradation has been considered as a safe method for environment. However, up to now, use of the microorganisms or their products in cleaning up of pollution and biodegrading on landfill haven't been investigated in Iran. However, bacteria are distributed in the soil and water ecosystem, but scientists have reported that compatible and native organisms of pollution area with chlorobenzoic acid compounds are more effective than other organisms in biodegradation. Rate of degradation in polluted area with 2,5-DCBA is depend on factors such as microbial population in landfill, type and quantity of pollution and chemical and geological conditions of landfill. There are several methods for increasing in rate of biodegradation in landfill. Most important of them are included of:

1-Inducing of microorganism of environment with adding oxygen and nutrients into polluted area (biostimulation).
2- Inoculation of soils and polluted area with microorganism that are able of degradation (bioaugmentation) [5].

In 1990, Hichey and Focht isolated bacteria of *Pseudomonas aeruginosa* JB2 from polluted soil with 2,5-DCBA that could degrade a wide range of benzoic acid halogens. According to this research catechol was the main mediator catabolism of DCBA [6]. In degradation of 2,5-DCBA dioxygenase enzyme self-starting remove chlorine atom from ortho carbon position. In 1995, Fava et al measured the degradation of 2,5-DCBA with the HPLC system and substrate stoichiometry of medium [7]. In 1999, pavlu et al investigated the activities of 1,2- dioxygenase enzyme on all strains of *Pseudomonas* [8]. These enzymes are transferred to specific strains by plasmids. Certain substances including manganese, copper and cobalt are essential for the dioxygenase enzyme. These factors increase the survival of the degrading organism such as xenobiotics in the bioremediation. Such complement increase the degrading of chloro benzoate (CBs) [7]. In swiss a study on sewage soil revealed that *Alcaligenes* sp. was capable of degrading CBs as the main source of carbon and energy. This bacteria attack to aromatic compounds by several mechanisms. In some substance the chlorine atom is replaced with a group of hydroxyls before breaking ring [3].

Purpose of this study is isolation and identification of 2,5-DCBA degrading bacteria, survey of bacteria grow curve at presence of this compound and to assay biodegrading of this substance on shiraz landfill (Iran).

MATERIALS AND METHODS

Sampling: with enough and true knowledge about the activities and the available services in the landfill site of Shiraz, we selected some certain parts of the landfill for sample collection to isolate 2,5-DCBA bacteria (Fig.1):

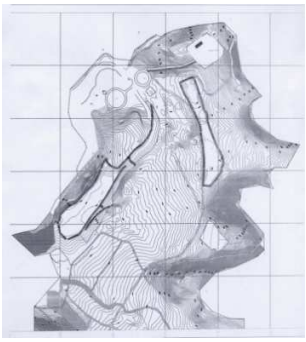


Fig1-Site map of landfill

1- *-The dry sediments at the floor of the ponds*
2- *The lagoon*
3- *Sediments*
4- *The landfill drainage*
5- *The landfill surface soil*

In spring and summer of 2010 sample collection was done from station number 5. On the whole, 20 cases were studied during each season. The samples were collected in fully sterilized glass containers. They were then

transported to the laboratory in the vicinity of ice after maximum of 4 hours delay to be tested with bacterial culture and measuring the 2,5-DCBA degradation .

Bacteria and the culture medium:
Sampling preparation:
The culture medium used for this study was a type of mineral salt medium containing the following compounds: $[NH_4]_2$ SO_4 0.1gr/l , KH_2PO_4 2gr/l , $FeSO_4$. 7 H2O 0.005 gr/l , $MgSO_4$ 7 H_2O 0.01 gr/l, Na_2 HPO_4 3gr/l, Na_2CO_3 0.1 gr/l, $Ca(NO_3)_2$ 0.0 gr/l , $MnSO_4$ 0.002gr/l, yeast extract 0.008 gr/l [7].

The above minerals were added separately to 1000 ml of twice distilled water. After dissolving each minerals the next mineral was added respective (pH=7). Then an amount of 100ml of mineral salt medium was sterilized in erlene. Then, 0.14 gram of 2,5-DCBA dissolved in acetone was added to each erlen. The amount of 4 grams of the sample was added to each erlen. Then incubation was done for 15 days. During this period, one minute aeration was conducted each day. After observing turbidity, injection to the solid basis medium containing organic material was done.

Isolation and Identification:
Single colonies were purified on blood agar medium and following tests were conducted on them: catalase test, gram stain, oxidase test, biochemical test single sugar test and enzyme investigation test. Pure bacteria were culture and incubated in LB medium. The scale of MIC was calculated on molar Hilton agar medium (made in Germany).

Bacteria counting and determination of growth curve:
To determine the growth curve of isolated bacteria in the presence and absence of 2,5-DCBA, the colony counting procedure was utilized . Different dilutions of the bacteria were prepared, which then were cultured on a solid nutrient agar medium using a glass sprier. The number of bacteria was given by CFU/ml [3].

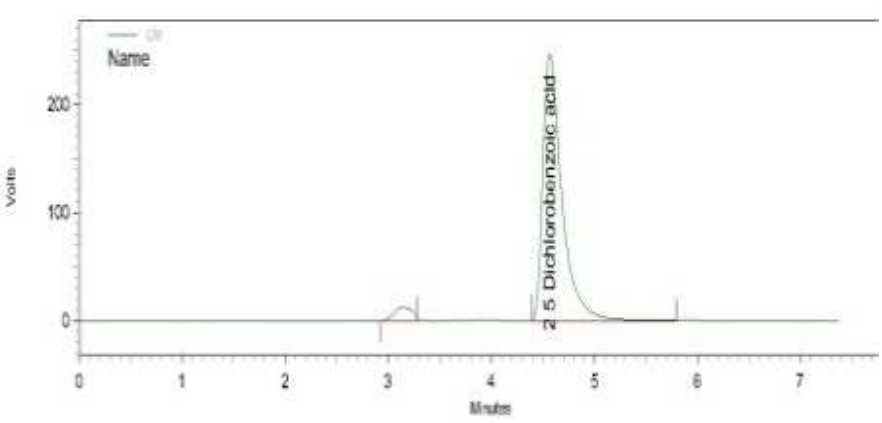
Chemical analysis:
Chloride determination: inorganic chloride was determined turbimetrically by measuring AgCl precipitation. Samples were diluted 1:2 in MSM, acidified with 10 *ml* of 10 N H_2SO_4 and centrifuged (5 min at 2250 rpm) to remove material that precipitated due to acidification alone . Each sample and standard as zeroed against itself at 525 nm on a UV spectrophotometer to minimize background variation. Precipitation of AgCl was then measured by adding 10 *ml* of 0.1 M $AgNO_3$ (in 5M H_3PO_4) and immediately reading the sample A525. Chloride as quantified by reference to a standard curve that was linear from 0.01 mM to 1M blanks, consisting of MSM alone, were free of interferences due to precipitation of medium components. 2,5-DCBA concentrations were determined by detector Knouer 2500 at 250nm , Vacuum Degasser, 20 M loop flow 1 ml/min high-performance liquid chromatography(HPLC), Knouer Smartlin Pump1000, UV Moving phase : eluting to the method of isocratic was done in reaction to 35% of A phase and 65% B phase. A: (500ml HPLC grade water was added 5ml acetic acid), B: HPLC grad methanol (fig.2) [7].

Preparing of the standard:
Preparing of standard was pour 42.4 mg standard 2,5-DCB in a 50ml volumetric flask and it becomes bulky with 80 methanol , then transfer 10ml of this solution to a 50ml volumetric flask and it becomes bulky with 80 methanol then a few milli liter of this solution has passed from a 45*M*m filters syringe head as injected HPLC set with mentioned condition a the part of A .

RESULTS

The number of bacteria in the medium without 2,5-DCBA (4.3×1012 cfu/g or cfu/ml) was more than medium containing 2,5-DCBA . Frequencies of 2,5-DCBA resistant bacteria was 55% in sediments station and %0 in surface soil station. These stations were the most contaminated and uncontaminated areas of the landfill, respectively (Table1). Different bacterial genera such as *Entrobacter, Corynebacterium, Klebsiella, Nisseria, Citrobacter, Bacillus sp.* were identified as 2,5-DCBA resistant bacteria.

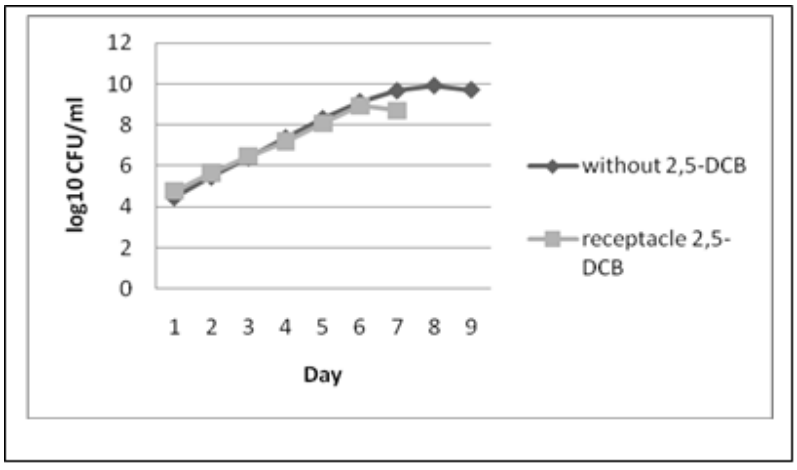
Fig.2. Chromatography 2,5-DCBA from analysis HPLC



UV Results

Pk #	Name	Retention Time	Area	Area Percent	Height
1		3.150	149236	4.550	12708
2	2 5 Dichlorobenzoic acid	4.617	3530583	95.450	246861

Fig. 3. Growth curve *Entrobacter* (with and without 2,5-DCBA)



The quantity of released chloride by bacteria was determined with UV spectrophotometer. The biggest amount of released chloride was belonged to E*ntrobacter*. Degradation of 2,5-DCBA was probe with HPLC. The most quantity of degradation of 2,5-DCBA was belonged to *Entrobacter 62%* and *Corynebacterium* 48% and least quantity determined by *Bacillus* sp.*14%* bacterium (fig7). The results of live cells counting and evaluation of bacteria growing curve at present and absence of 2,5-DCBA showed that isolated bacteria are able using 2,5-DCBA as carbon and energy source. The great quantity of counted cells for *Entrobacter* log 10, 8.95 cfu/ml, was at 50 days of experiment. The 2,5-DCBA is degrading by motive microbes crowd under aerobic conditions. The most important of degradation cases: *Klebsialla , Entrobacter, Nisseria, Corynebacterium* , B*acillus* sp (fig 3,4,5). The best bacterial degradation for deletion of 2-5-DCBA was gram negative and gram positive crowd together (fig 6).

Fig 4. Growth curve *Corynebacterium* (with and without 2,5-DCBA)

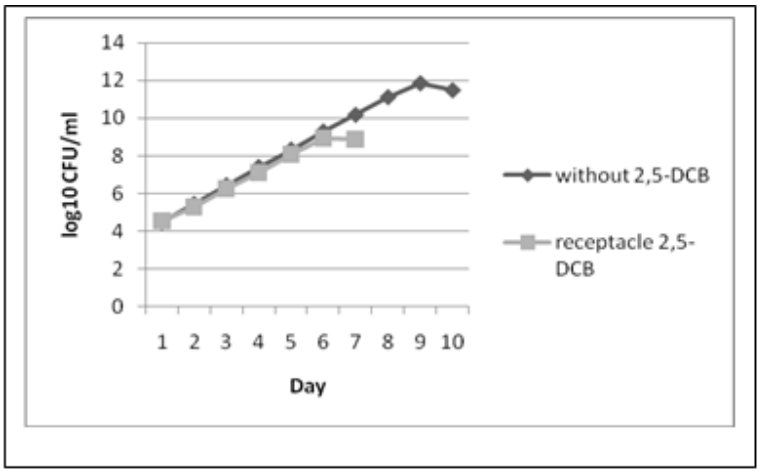


Fig .5. Growth curve *Klebsiella* (with and without 2,5-DCBA)

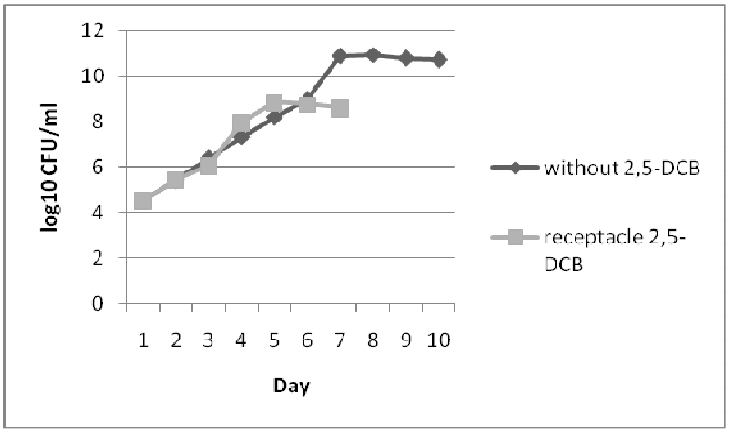


Fig. 6. Chloride release from 2,5-DCBA by different bacteria

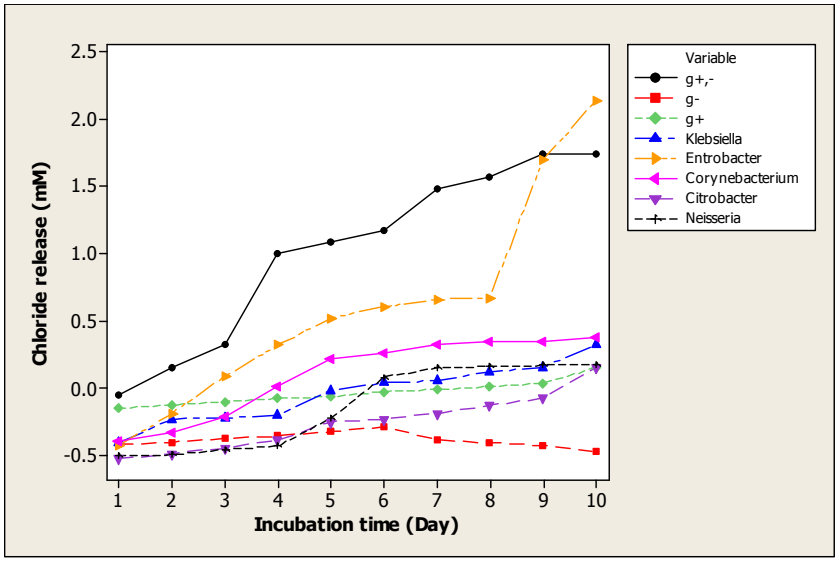
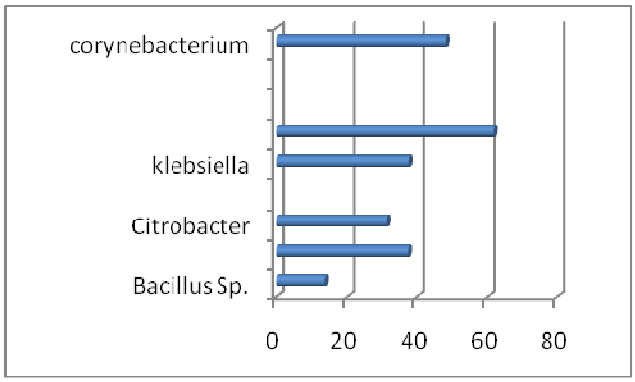


Fig.7. Percentage of 2,5-DCBA bacterial degradation



Table(1): Percent and mean number of 2,5-DCBA resistant bacteria in different stations

Station	Percent of 2,5-DCB resistant bacteria	Mean number of 2,5-DCB resistant bacteria (cfu/ml)	Mean of total bacteria (cfu/ml)
Dry sediments of pond	22.04	1.02×10^{5}	5.49×10^{10}
Lagoon	1.62	2.37×10^{7}	5.34×10^{9}
Sediments	55.21	2.30×10^{5}	5.04×10^{10}
Landfill drainge	21.11	2.64×10^{5}	$1.151 0^{11}$
Landfill surface soil	0	0	0

DISCUSSION

As mentioned in the previous sections, contamination with aromatic compounds and 2,5-DCBA is hazardous to the environment and surface and ground water resources. Released choloride radical could cause cancer in human. Hence, the biodegradation of 2,5-DCBA from residues in very crucial. There are various types of approaches for organic waste treatment including physic-chemical methods. However, these methods are not so effective since they are high costly, and they produce great amounts of intermediate products. Nowadays, biodegradation is considered to have great advantages for eliminating the aromatic compounds. Some bacteria, especially *Pseudomonas* and *Alcaligenes* are important in biodegradation. In current study, six genera of degrading bacteria were isolated. Among these bacteria, four of them were selected to be effective on degrading of mentioned compound. Many investigators have reported the isolation of chlorobenzens utilizing bacteria, such as *Alcaligenes* [9], *Pseudomonas, Burkholderia, Xanthobacter, Rhodococcus, Acidovorax, Bordetella sp.* [10] and *Psedomonas aeruginosa JB2* [8,11]. Isolated bacteria in present study were not same as results from other studies. This is maybe due to difference places which were used for sampling. Hickey and Focht in 1990 described the isolation and characterization of *Pseudomonas aeruginosa* , which used different CBAs as growth substrates, specifically 2,5-DCBA [6]. Isolated bacteria in current research are also capable of using 2,5 DCBA as only sources of carbon and energy.

Fava et al in 1995 reported *Pseudomonas* sp. strain CPE2, which was capable of dechlorinating 2,5-DCBA. They were selected from a polychlorinated-biphenyl-degrading aerobic mixed bacteria culture. Bacteria could release radical from chlorine in anaerobic condition and break benzene ring and convert to catechol and use from residue carbon [7]. Johnson et al in 2002 investigated the bacteria growth on the cyclic aromatic compounds using the micro plate method. They utilized dye wst-1 in their research [12]. However, so far there is no research which has been done at the presence of 2,5-DCBA benefiting from the micro plate method. A fixed microbial consortium has a vast flexible analysis of DCBs. When 2,5-DCB are added to culture simultaneously, following the first stage of decomposition during 48 hours, 2,5-DCB are degraded. If 3-CB, 2,3-DCB, 3,4-DCB and 3,5-DCB are added to the culture medium simultaneously, the growth is entirely ceased [3]. Despite other studies, bacteria in current study could degrade some of 2,5-DCBA during 50 days. Pavlu et al in 1999 reported that CBs are macro- xenobiotics which are distributed all over soil and sediments. Chlorobenzoates transmit chlorine atoms to ortho position; the activity which is fatal for planets. In fact, 2,3,6-TCB is deadly to planets by itself [8]. In one research, all gram positive and gram negative bacteria were cultured together and then compared each other. In some bacteria such as:

Citrobacter ,*Nisseria* , *Corynebactrium* , after 50 days of incubation with 2,5-DCBA , a red pigment was obtained while some other researchers believe that this red pigment is probably result of accumulation of chlorocatechols which are suffering from auto-oxidation [9,13,14]. In this study, temperature, pH and source of oxygen was not involved in formation of pigments. This activity of bacteria may be due to formation of quinone radicals during polymerization of dichlrocatechol.

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