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Full Length Research Paper

Evaluation of biodegradation of 2-chlorobenzoic acid by isolated bacteria from landfill soils in Shiraz, Iran

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Chlorobenzoic acids are one of the environmental pollutants which contaminate the soils and waters. Microorganisms have been used for removing pollution in the area of oil spill, nuclear waste, leakage from waste landfill, factory waste, and heavy metals. The aim of this study was to identify 2-chlorobenzoic acid degrading bacteria from the soils around the landfill centers (landfills) in Shiraz city (Iran) and to evaluate their degradation rate. In this study, the surface soils of landfill from various stations were collected in the winter and spring and were inoculated to mineral base medium containing 0.1% of 2-chlorobenzoic acid and then incubated for one week at temperatures of 30°C. After three to four times sub culturing, bacteria were cultured on solid mineral medium, and then, they were incubated. Identification of bacteria using biochemical tests revealed 10 genus which comprised seven Gram-negative and three Gram-positive bacteria. These bacteria were considered as 2-chlorobenzoic acid resistant or degrading bacteria. Evaluation with high performance liquid chromatography (HPLC) confirmed that *Pseudomonas* species, *Enterobacter* species, *Acinetobacter* species, and *Corynebacterium* species were able to degrade 4.53, 4.48, 35, and 7.21% of 2-chlobenzoic acid in one week, respectively. Therefore, this study confirms the potential of these bacteria for use in biodegradation of 2-chlorobenzoic acid.

Key words: 2-Chlorobenzoic acid, bioremediation, *Pseudomonas*, landfill, high performance liquid chromatography (HPLC).

INTRODUCTION

Broad spectrums of synthetic chemical which have chlorine aromatic nuclei are entered into environment as solvents, pesticides, and herbicides. Despite of concerns regarding their toxicity to humans and wildlife along with their relative stability in sediments and soils, they are still widely used (Higson and Focht, 1990). Chlorobenzoic acids (CBAs) are xenobiotics which are widely distributed in soils and sediments.

Chlorobenzoic acids are used in agriculture as herbicides (2,3,6-trichlorobenzoic acid is a kind of herbicide) or pesticides due to chlorine atom in their ortho position (Field and Sierra-Alvarez, 2008; Hickey and Sabet, 2001; Francisco et al., 2001; Hickey and Focht, 1990). They are also common metabolites of aerobic degradation of many chlorinated pollutants like polychlorinated biphenyls (PCBs) and alkylbenzene, chlorotoluene or halogenic compounds like pentachlorobenzyl alcohol. In the two past decades, treatment of contaminated soils has become a public concern. However, a serious environmental problem is soil polluted by waste leaking from the containers carrying waste in landfill sites. Concentration of these pollutions is hundreds or thousands times bigger than pollution which comes from agriculture. The most important risk of xenobiotic compounds is their stability in the environment (Monferran et al., 2005). Resistance of these compounds to biodegradation is due to their nature of being xenobiotic and replacement of halogen atom. However, it highly depends on the number and position of

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halogen atoms (Banta and Kahlon, 2007). Fortunately, the use of wide catabolic and metabolic capability of microorganisms has provided the possibility of converting these pollution to low hazardous or safe products which eventually joins the natural geochemical cycle (Fulthorpe et al., 1998). Some microorganisms, including bacteria, and fungi and even plants have been isolated which can degrade chlorobenzoic acids. Chlorobenzoic acid degrading bacteria has been isolated from the soil with PCBs (Baggi et al., 2005). 2-Chlorobenzoic acid is stable, flammable, and powerful oxidizing agent which has a chemical formula $C_6H_4ClCOOH$ and molecular weight of 156.57 g/mol. It is prepared as white powder or crystals.

Baggi and Zangrossi (2001) isolated a microbial consortium from Milan landfill soils which were capable of degrading 2-chlorobenzoic acid during the 48 h. Yun et al. (2009) reported that *Rhodococcus erythropolis* is able to degrade 2-chlorobenzoic acid in the medium containing glucose alone. It has been reported that strain of *Pseudomonas*, and two engineered strains of *Burkholderia cepacia* are able to use 2-chlorobenzoic acid and use this compound as carbon source and energy (Fava et al., 1996; Urgun-Demirtas et al., 2003). The aim of study was to isolate 2-chlorobenzoic acid degrading bacteria from landfill soils in Shiraz city (Iran) in two seasons of winter and summer and the evaluation of their bioremediation using HPLC as well.

MATERIALS AND METHODS

Sampling, isolation, and enrichment of bacteria

Sampling of landfill soils was conducted with completely sterile containers from five stations in two seasons. Five grams of each soil sample were added to 100 ml of base mineral medium which comprised 1 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$, 0.0 005 g $FeSO_4.7H_2O$, 2 g KH_2PO_4, 3 g Na_2HPO_4, 0.1 g Na_2CO_3, 0.01 g $CaCl_2$, 0.002 g $MnSO_4$, and 0.008 g yeast extract along with 0.1% of 2-chlorobenzoic acid as a source of carbon and energy. They were then incubated for one week in temperatures of 30°C on shaker. At the end of each week, if the turbidity was observed in the culture medium, then it was inoculated to the new medium. Subculturing was continued for 3 to 4 weeks, to insure that turbidity is just due to bacterial growth. After final passage, they were cultured on solid mineral medium and bacteria were isolated as single colonies (Baggi and Zangrossi, 2001).

Identification of isolated bacteria

To identify the isolated bacteria, biochemical tests and microscopic studies were used which were included of Gram stain, microscopic shape, oxidase, catalase, triple sugar iron agar (TSI), sulphide indole motility (SIM), citrate, lysine decarboxylase (LD)/ornithine decarboxylase (OD), O/F, urease, gelatin and starch hydrolysis, nitrate reduction, and tolerance to different concentrations of salt.

Evaluation of growth kinetics

To determine the growth curve of indicator bacteria in the presence of 2-chlorobenzoic acid, the colony count method (colony count) was utilized. A suspension of each bacteria (0.5 Mc Farland) 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 nutrient agar and then was spread completely using a sterile glass rod on the plate surface.

Analytical method

Concentration of 2-chlorobenzoic acid was determined by reverse phase HPLC, using columns C18 4.6 × 250 mm. Mobile phase included solvent of 0.1% phosphoric acid (60 ml) and acetonitrile (40 ml). Passage rate of solvent was 1 ml/min while wavelength detector was set at 254 nm. Samples were then passed through 0.2 µm filters and 20 µl of sample was injected into HPLC system.

Standard curve was prepared using six standard solutions that ranged from 0.1 to 0.6 mM in methanol (Yun et al., 2009).

Statistical analysis

The number of bacteria in different seasons and stations and their effects were statistically compared using analysis of variance (ANOVA).

RESULTS

Table 1 and Figure 1 show the number and percentage of isolated bacteria in the winter and spring. The total number of isolates in spring were higher than winter. This difference was not however significant ($P > 0.05$). Table 2 compares the percentage of Gram positive and Gram negative bacteria in spring and winter. Gram positive bacteria were higher in spring than of Gram negative, while in winter Gram negative were higher than Gram positive bacteria. There was no significant difference between the numbers of bacteria in different stations.

However, as shown in Figure 2, they are found to be decreased in stations number five, four, one, two, and three, respectively ($P > 0.05$). Figure 3 shows interaction of season and station in term of number of bacteria. Stations number five, one and two had the highest number of bacteria in spring, respectively, while stations four and three had the highest number of bacteria in winter. However, the number of bacteria in spring is generally higher than winter. Figure 4 shows the percentage of degradation of 2-chlorobenzoic acid by four isolation of four different bacteria species including *Pseudomonas* species, *Enterobacter* species, *Acinetobacter* species, and *Corynebacterium* species which were able to degrade 53.4, 48.4, 35, and 21.7% of 2-chlorobenzoic within 7 days, respectively. Figure 5 shows *Pseudomonas* chromatogram in the first and seventh day which is included of 0.54 mM and is 0.28 mM. Figure 6 shows growth kinetics rate and degrading of 2-chlorobenzoic acid by *Pseudomonas* that has reduced 0.6 to 0.28 mM during the seven days. It shows the highest rate of degradation. Bacterium *Enterobacter* is the second strong bacteria which decreased concentration of 2-chlorobenzoic acid from 0.5 to 0.39

Table 1. 2-Chlorobenzoic acid degrading or resistant bacteria in spring and winter.

	Winter (first season)		Spring (second season)	
Gram positive	**Degrading or resistant bacterium/total**	**Percentage**	**Degrading or resistant bacterium/total**	**Percentage**
Corynebacterium sp.	7/20	35	10/25	40
Staphylococcus sp.	1/20	5	2/25	8
Bacillus sp.	1/20	5	4/25	16
Gram negative				
Pseudomonas sp.	2/20	10	1/25	4
Vibrio cholerae	1/20	5	1/25	4
Vibrio mimicus	2/20	10	2/25	8
Vibrio parahaemolyticus	2/20	10		
Alcaligenes fecalis	1/20	5		
Acinetobacter sp.	1/20	5	2/25	8
Enterobacter sp.	-		1/25	4
Neisseria sp.	1/20	5	2/25	8
Salmonella sp.	1/20	5	-	

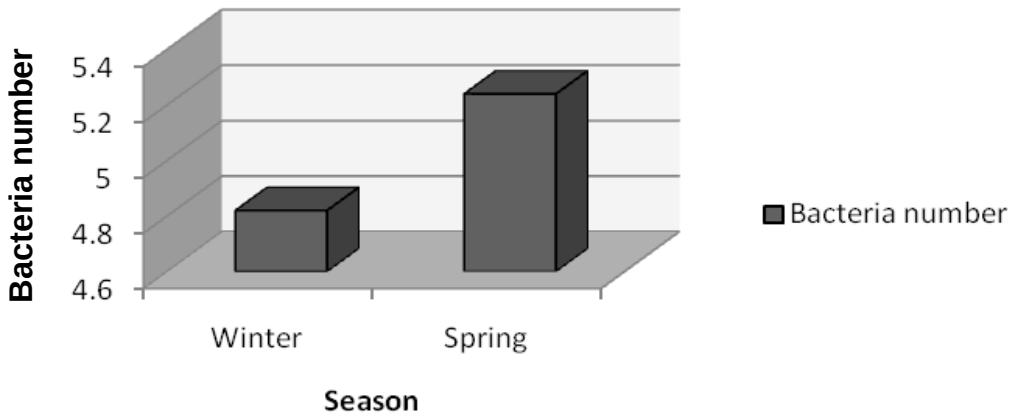

Figure 1. Comparison of seasons in term of bacteria number.

Table 2. Comparison of percentage of Gram positive and Gram negative bacteria in spring and winter.

Bateria	Winter (%)	Spring (%)
Gram positive	45	64
Gram negative	55	36

mM in third and fifth days (Figure 7). *Acinetobacter* was the third bacterium that decreased concentration of 2-chlorobenzoic to 0.31 mM after 2 weeks (Figure 8). The only Gram positive bacterium that has been investigated was *Corynebacterium*, that is, the weakest degrading bacteria. It has reduced the concentration of 2-chlorobenzoic acid to 0.47 mM after seven days (Figure 9).

DISCUSSION

In current study, microorganisms which could use 2-chlorobenzoic acid as only source of carbon and energy were isolated. Morphology and physiology of isolated bacteria along with biochemical tests identified them as *Pseudomonas, Enterobacter, Acinetobacter, Alcaligenes, Vibrio, Salmonella, Neisseria, Bacillus, Corynebacteria*,

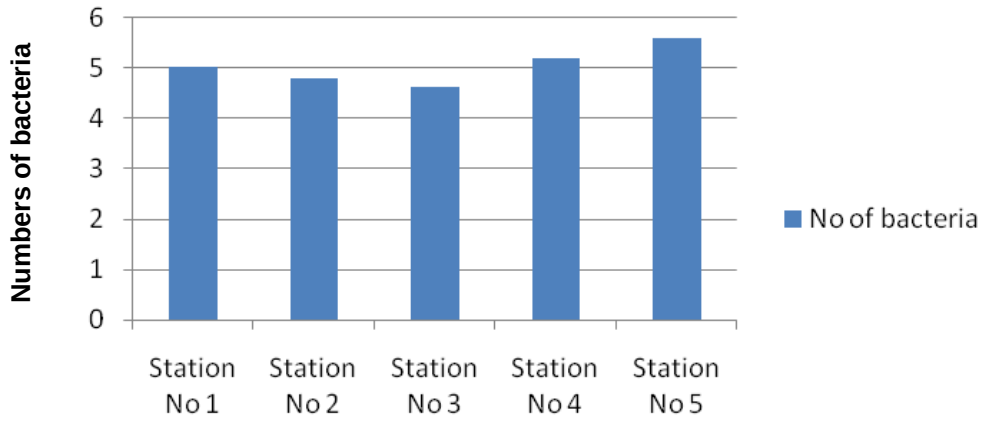

Figure 2. Comparison of stations in term of bacteria number.

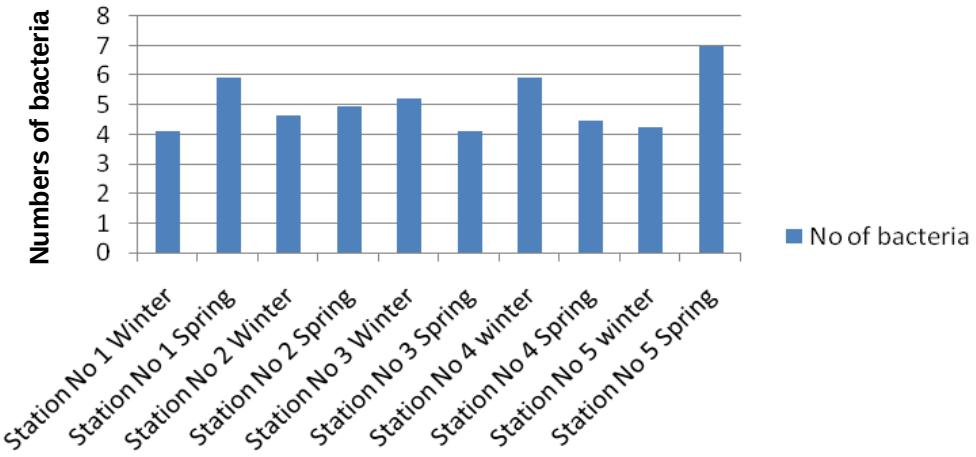

Figure 3. Comparison of interaction of season: station in term of numbers of bacteria.

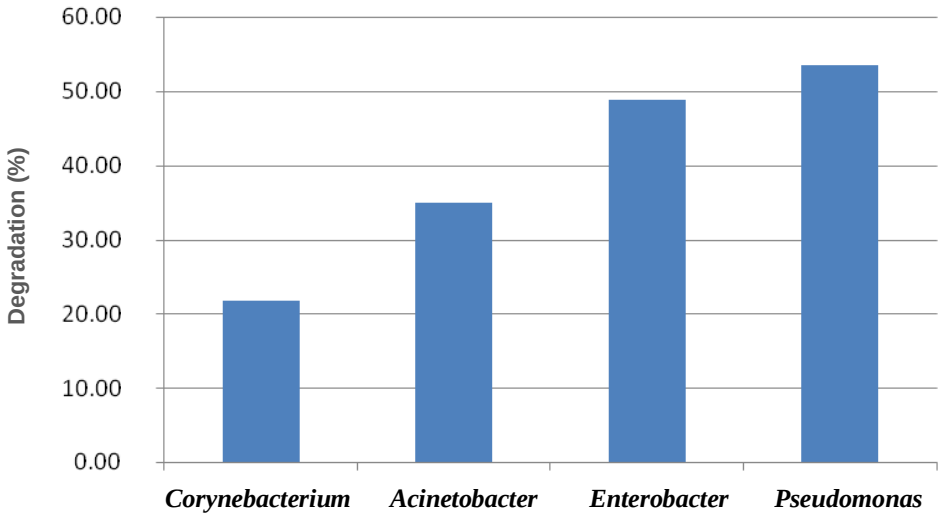

Figure 4. Percentage of degradation of 2-chlorobenzoic acid by bacteria.

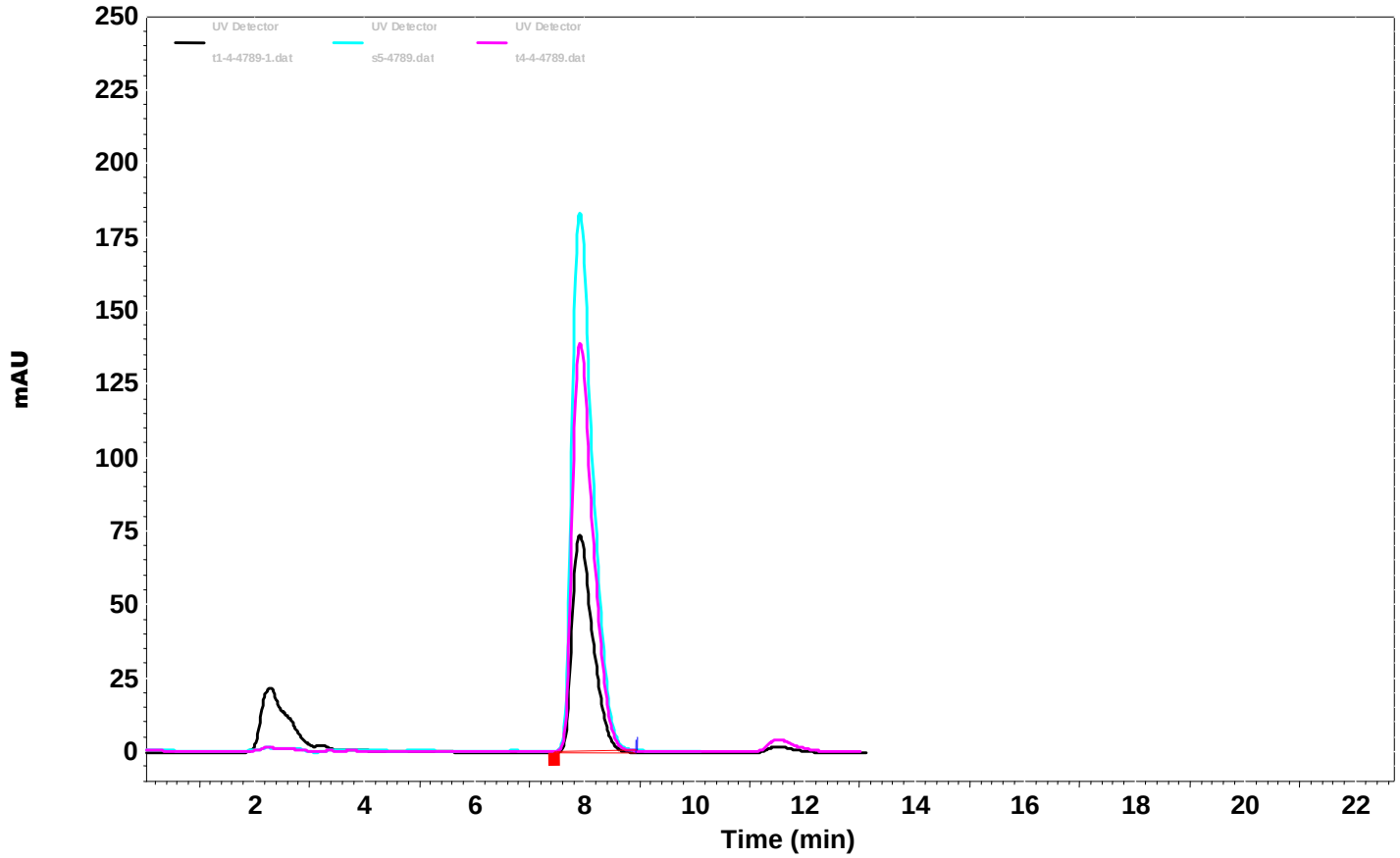

Figure 5. Chromatogram of *Pseudomonas* in the first and seventh days.

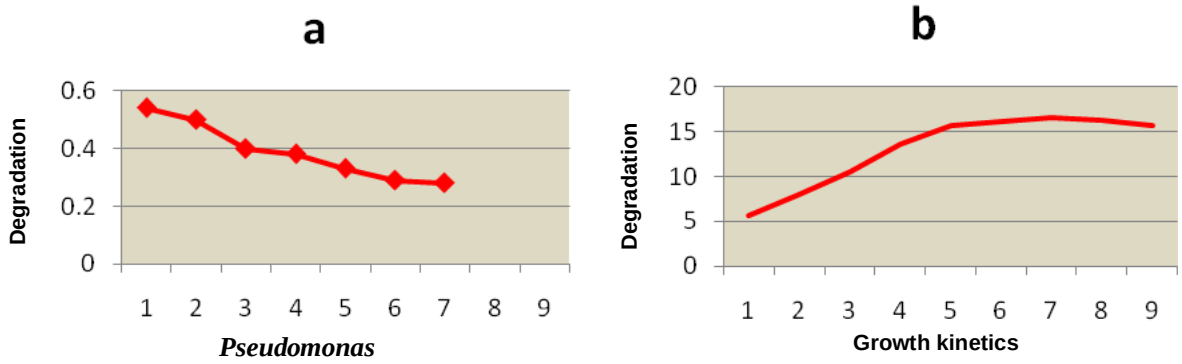

Figure 6. (a) Degradation of 2-chlorobenzoic acid by *Pseudomonas* and (b) Growth kinetics.

and *Staphylococcus*. These bacteria had the ability to endure and grow in the presence of 2-chlorobenzoic acid. *Pseudomonas* however, showed the highest rate of degradation. Bioremediation is of a promising technology for the treatment of contaminated environment with chlorobenzoic acid. This technology has been used for the successful removal of many pollutants, such as oil and related contaminants (Adebusoye et al., 2007). According to studies, bacteria which degrade2-chlorobenzoic acid are scattered in water and soil and could degrade chemicals which added to the soils. *Pseudomonas* is one of the most valuable and efficient bacteria which could degrade 2-chlorobenzoic acid. Having broad degrading genes form chromosomes and plasmid; which make this bacteria efficient for degrading of many compounds. Wang et al. (2004) used *Pseudomonas putida* GN2 containing plasmid gene coded for the oxidation of 2-chlorobenzoic acid. It led to

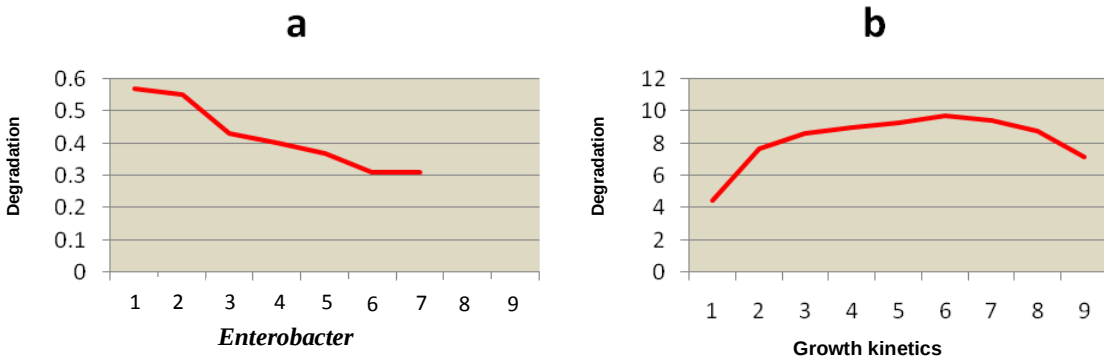

Figure 7. (a) Degradation of 2-chlorobenzoic acid by *Enterobacter* and (b) Growth kinetics.

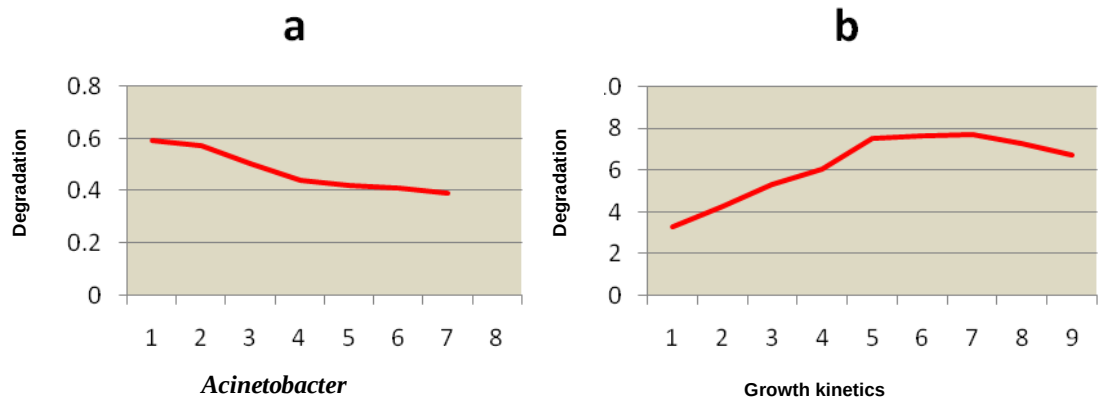

Figure 8. (a) Degradation of 2-chlorobenzoic acid by *Acinetobacter* and (b) Growth kinetics.

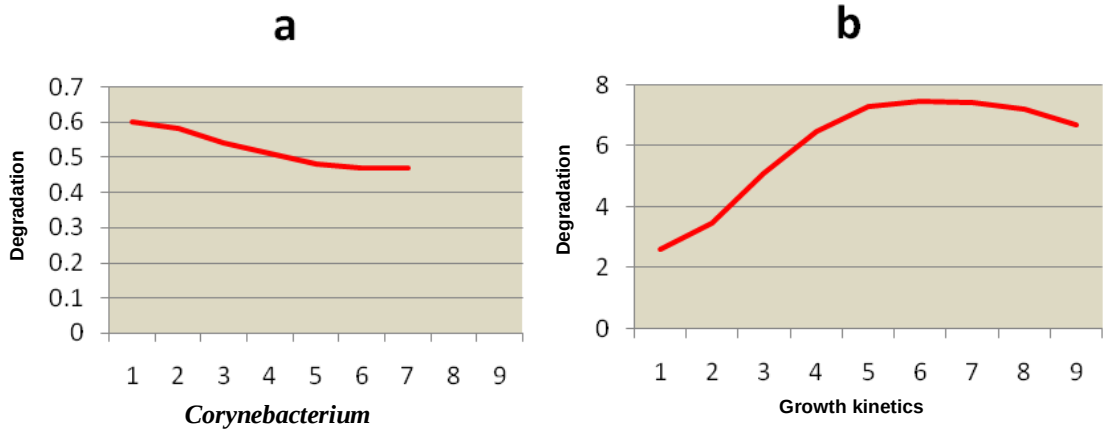

Figure 9. (a) Degradation of 2-chlorobenzoic acid by *Corynebacterium* and (b) Growth kinetics.

the removal of 500 mg/kg 2-chlorobenzoic acid within five days. Pavlu et al. (1999) examined two infected places with PCB for the presence of chlorobenzoic acid degrading bacteria. He reported that degrading bacteria were strains of *Pseudomonas*. Corbella et al. (2001) showed that *Pseudomonas aeruginosa* strain 142 is able of metabolize 2-chlorobenzoic acid in mineral medium without glucose. Baggi et al. (2005) used a microbial enrichment consortium which was included in the *Pseudomonas* strains. They reported that

2-choloromuconate is produced as intermediates in the degradation of 2-chlorobenzoic acid. They also mentioned that early attack to 2-chlorobenzoic acid is induced by dioxygenase. *Enterobacter* is a mobile Gram negative bacterium that belongs to the family Enterobacteriaceae. This bacterium was the second strong bacteria in degradation of 2-chlorobenzoic acid. *Acinetobacter*, the aerobic Gram negative bacterium is widely distributed in the soils and water as cocci or cocobacilli, and able to degrade 2-chlorobenzoic acid. *Corynebacterium* is no spore's Gram positive bacterium and has mycolic acid which is able to degrade 2-chlorobenzoic acid. Saini et al. (1998) isolated *Corynebacterium liquefaciens* with the maximum rate (70.3%) of degradation of 2-chlorobenzoic acid 3.2 mM. The results of the current study indicate that isolated bacteria had capacity of growth on 2-chlorobenzoic acid. Two-chlorobenzoic acid degrading bacteria are widely distributed in nature, particularly in landfill soil. However, further studies are needed to examine other bacteria for their ability to degrade 2-chlorobenzoic acid. Bioremediation of 2-chlorobenzoic acid by the bacteria is an efficient and fast method. Wide range of chlorobenzoic acids are produced as the final metabolites (dead-end) during the microbial degradation of aromatic compounds, such as PCBs, herbicides, and pesticides. Therefore, the study of microbial decomposition of aromatic compounds and chlorobenzoic acid, the decomposition efficiency of degrading bacteria and enrichment of their population are important for effective development of bioremediation (Raji et al., 2007). These finding have ecological importance and could be useful for improving efficiency and sustainability of bioremediation processes.

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