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BIOREMEDIATION OF CHRYSENE BY ISOLATED BACTERIA FROM PETROLEUM POLLUTED SOILS IN IRAN

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ABSTRACT

The polluted soil contains mixture of petroleum hydrocarbons. Chrysene is combination of four benzene with genotoxic, mutagenic and carcinogenic features. In this research chrysene degrading bacteria is isolated from petroleum polluted soil in Iran, and some index samples are evaluated for reviewing test of degrading in laboratory conditions and chrysene concentration changes on growth of bacteria. After filtrating, the samples are cultured in environment containing mineral and chrysene hydrocarbonic material and incubated within 0-192 hours in 32 centigrade degree. After isolation bacteria, 4 samples are evaluated for testing growth curve in different concentrations of chrysene and chromatography analysis. Also in order to better screening, the isolated bacteria are cultured in the mentioned environment and therefore any bacteria which have grown in the least time are separated. Among 4 isolated bacteria, *Pseudomonas putida*, *Mycobacterium* sp, *Micrococcus varians and Bcillus coagulans* has grown in the least time respectively. Also *Pseudomonas putida* has shown the most degradation after 192 hours.

Keywords: Chrysene, Polycyclic Aromatic Hydrocarbons (PAHs), Bioremediation, *Pseudomonas putida*

INTRODUCTION

Many polluted soils and also industrial waste are polluted to hydrophobic organic compounds. Among these pollutants, PAHs is much more important which is part of petroleum pollutants and benzene rings is used in its construction **[1]**.

PAHs are carcinogenic and are stored in fat tissues of the body and in animals such as rat will cause mammary **[2]**. These hydrocarbons have mutagenic and teratogen features in lower concentrations. LOW-molecular-weight PAHs (LMW PAHs) are highly poisonous and high-molecular-weight PAHs (HMW PAHs) are genotoxic. HMW PAHs, will cause more problems in soil and sedimentary environments. Some PAHs are recognized poisonous and mutagenic by the U.S. Environmental Protection Agency **[3, 4]**.

Chrysene is a solid composition with four rings with high molecular Wight of (228.28). Its chemical formula is (C18H12) with boiling point of 448^{o}c and melting point of 245^{o}c and is insoluble in water. Solubility in organic solvents: slightly soluble in acetone, carbon disulfide, ethanol, Glacial acetic acid, diethyl ether, toluene, and hot xylene; soluble in benzene. It is used in manufacturing iron, aluminum and steel. International Agency has introduced it as a pollutant poisonous material. According the experiment which was done on chrysene in peritoneum of hamster it is observed that entering this substance to body will cause chromosome abnormality, positive reaction in evaluating gene mutation and finally cancer **[5, 6]**.

One of the ways of reducing these pollutants is bioremediation. Bioremediation is a good method for remediation of polluted environment, because in comparison with the other methods has less expenses and less pollution danger **[7]**. By isolating degrading bacteria, pollutant materials and petroleum compositions, providing microbial bank and their usage in critical situation we can take important action in reducing environment pollutants and creating a safe ecosystem **[8]**. The goal of this research is isolation and identification chrysene degrading bacteria from petroleum polluted soils in Iran and their growth kinetics assay.

MATERIALS AND METHODS

Medium and chemical materials: applied solid hydrocarbons in this research include chrysene which is purchased from Sigma

company (German). The applied medium includes blood agar, nutrient agar and medium containing basic mineral **[6]**.

Soil Samples

Samples were taken from three stations of polluted soils to petroleum in Iran, placed into sterile bottles and transported immediately in cold storage containers to the laboratory for further work **[8]**.

Dispersion Detection of Degrading Bacteria

From each station, different dilution were provided and cultured on agar chrysene media and after incubating, related colonies to each sample is calculated and the results are announced based on cfu/g **[8].**

Isolation and Identification of Chrysene Degrading Bacteria

First of all, samples are cultured in mineral medium which includes mineral compositions including: (KH_2PO_4, Na_2HPO_4, $MnSO_4$, NH_4Cl, $MgSO_4$, $CaCl_2$ and trace elements:($MnCl_2$, $CoCl_2$, H_3BO_3, Na_2MoO_4, $NiCl_2$, $FeCl_2$, HCl) Both were sterilized separately by autoclaving at 121^0C for 15 min. 0.2 ml ethyl acetate solution (containing 0.1% chrysene) incubated in 32^oc for 14 days. In case of observing tarnishing in each of cultured pipe, appropriate amount of pipes containing sample on agar chrysene plate level will be transferred and chrysene degrading bacteria will be isolated.

Morphological and biochemical tests carried out include gram staining, motility, colonial morphology, methyl red and Voges-Proskauer tests, production of oxidase, catalase, indole, gelatin liquefaction, starch hydrolysis and sugar utilization **[6]**.

Selection of Best Degarder Strain

After isolating bacteria, cultured in basic mineral medium together with substrate chrysene and bacteria which grew in the least time were selected **[8].**

Growth Kinetics

Erlen were taken for each bacteria and were and added 90 ml of mineral medium to specified amount of bacteria were added to each erlen and therefore to each 0.2 cc were added different concentrations of solution (0.1, 0.2, 0.3 and 0.4 g/l of chrysene) and will be incubated in 32 centigrade degree and measured with spectrophotometer in wavelength of 600 nanometer within 24 hours per day in a week **[9].**

Chromatography Analysis

At the beginning each bacteria is cultured in 10 milliliter of normal ciling or nutrient broth medium and will be incubated in 32^0C within one day. Then 5ml of the solution will be added to 90 ml of medium containing basic mineral and will be incubated within one week to ten days and will be centrifuged in

order to separating sediments. The sediments will be separated and n-hexane will be added to it and will be shaken until the remained chrysene is dissolved in solvent. Then it will kept constant to separate to phase. The above phase will be separated for testing with HPLC device **[10]**.

RESULTS AND DISCUSSION

Among three sampled stations, the third station has the least amount of bacteria and the second station has the most one. The average of total calculated bacteria from three stations is $16.571*10^4$ and the average of total degrading bacteria is $22.3969 *10^3$. The mentioned results are meaningful.

In this research 10 sample of bacteria are isolated that 4 samples belong to *Pseudomonas*, 3 samples belong to *Mycobacterium*, 2 samples belong to *Micrococcus* and 1 sample *to Bacillus* **(Figure 1)**. Four index samples based on microbiological and biochemical tests include:

Bacillus coagulans, *Pseudomonas putida*, *Mycobacterium* sp, *Micrococcus varians.* The test showed that in comparison with the othe bacteria *Pseudomonas* is grown up in the least time(less than 30 hours) in medium containing basic mineral and is determined as the most powerful strain. Growth curve of *Mycobacterium* sp, *Bacillus coagulans*, *Micrococcus varians* shows that some bacteria can growth in the presence of low concentrations of chrysene and use it as the only source of carbon and energy. Different studies also confirm the above study. The results of test shows that when the concentration of chrysen is increased, the bacteria cannot grow and because of lack of appropriate carbon resource or poisonous of chrysene for microorganism, they will be destroyed. Growth of *Pseudomonas putida* in 0.1% concentration in 120^{th} hours is increased into climax and in 0**.**2% concentration in 72^{nd} hour is decreased in comparison with the other hours The results of growth kinetics of Pseudomonas putida in comparison with the other bacteria in concentrations of 0.1, 0.2, 0.3 and 0.4 g/l showed that in case of increasing the concentration of chrysene, considering its poisonous, there will be no negative effect on the growth of the bacterium. This matter shows that *Pseudomonas putida* is the most powerful bacterium in chrysene degradation **(Figure 2)**. In growth curve of *Mycobacterium* sp **(Figure 3)** in 0.1 g/l concentration in 192^{nd} hour, growth of the bacterium is in its climax. In comparison with *Pseudomonas putida*, by increasing concentration of chrysene, the growth of the bacterium is decreased but the

effect of increasing chrysene concentration in comparison with *Micrococcus* and *Bacillus* is lower **(Figure 4, 5)**. Because of weakness of *Bacillus coagulans* in degrading chrysene and /l is near to zero **(Figure 5)**. Also the results of chromatography show that *Pseudomonas putida* has degraded 95 % of chrysene with concentration of 0.1 g/l within 10 days, *Mycobacterium* sp 74%, *Micrococcus varians* 58% and *Bacillus coagulans* 42%. Today using microorganism in cleaning environment is important. By applying non-manipulated genetic microorganism especially bacteria, we can find an environment without cancerous pollutants. Meanwhile by creating optimal conditions we can increase the efficiency of bacteria into 6 times and clean the most level of pollutions by theses microscopic creatures in the least time. If genetic engineering is done on *Pseudomonas* bacterium in order to exit from disease mode it seems that it is appropriate for different degrading enzymes and this feature helps them to degradation of 4 ring chrysene **[11]**. Also analysis of chromatography confirms these results and shows that *Pseudomonas putida* has the most capacity of chrysene degradation in comparison with the other bacteria. Many studies have been done for measuring amount of aromatic

poisonous of chrysene as the sole carbon source of bacterium, growth in concentration of 0.4g

eliminating PAHs in environment **[11, 12]**. In executed tests chrysene is the sole source of carbon and because chrysene is a part of aromatic hydrocarbons with high molecular weight its microbial degradation is harder **[13].**

The result of present research shows that when the concentration of the chrysene is increased, bacterium cannot grow and will be destroyed because of lack of appropriate carbon source and poisonous of chrysene for microorganism. Among isolated bacteria, *Pseudomonas putida* shows the most degrading features in different concentrations of chrysene which is because of stability of this bacterium in different environments. On the other hand, *Pseudomonas* includes

compounds degradation and also bacterial growth kinetics.

In **2006 Nwanna *et al*** isolated chrysene degrading bacteria in petroleum soils and isolated samples were *Micrococcous varians, Alcaligenes faecalis, Acinetobacter anitratratus, Acinetobacter mallei*. But the results of present research includes

Pseudomonas, *Micrococcus*, *Bacillus* and *mycobacterium*. Growth kinetics of bacteria was also considered in different concentrations of chrysene **[6]**. In 1995 **Caldini *et al*** studied the ability of *Pseudomonas fluorescents* for chrysene degradation and other four ring hydrocarbons in polluted soils to petroleum and also reviewed growth kinetics of these bacteria. The results of present research confirms that in this research 4 bacteria are belong to *Psedomonas* **[11].**

In 2011 **Yu- bin *et al*** isolated chrysene degraing bacteria and the results showed that they were *Paracoccus* and the gene related to chrysene degradation in this bacterium was separated. Growth curve of this bacterium were estimated in the presence of chrysene and also degradation of this substance was evaluated through GC device. The results of the test were not same **[12]**.

In 2009 **Igvo *et al*** studied chrysene degradation by *Alcaligenes faecalis* from oil of diesel engines together with product of surfactant. The results of present research does not confirm their result **[14].** In 2011 **Nayak *et al*** studied chrysene degradation through *Pseudoxanthomonas* sp from place of producing gas of coal. Measuring amount of chrysen degradation was done through MS and HPLC, TLC devices. In current research *Pseudoxanthomonas* was not isolated and also HPLC was used for measurement **[15].**

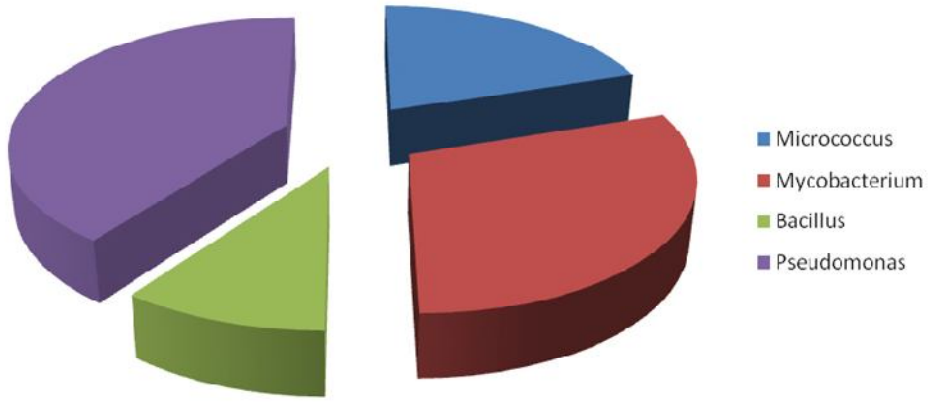

Figure 1: Chrysene Degradation Percentage

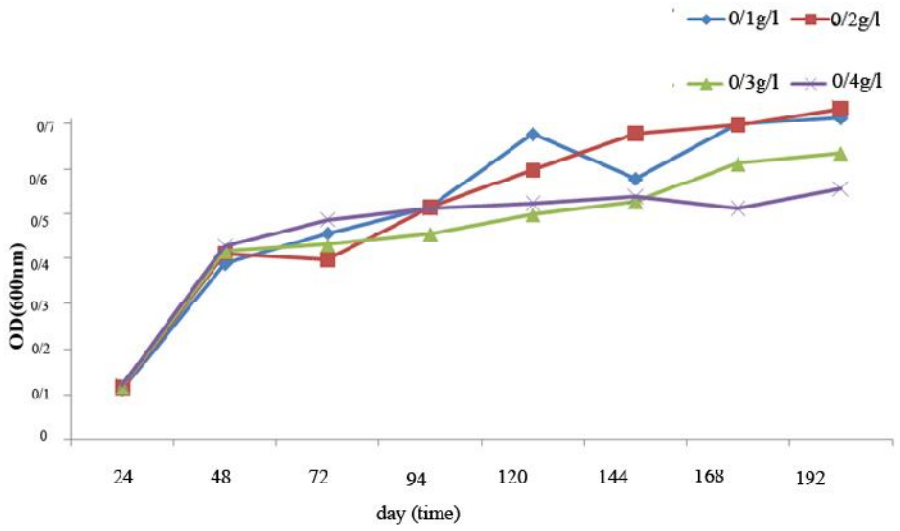

Figure 2: Growth Curve of *Pseudomonas putida*

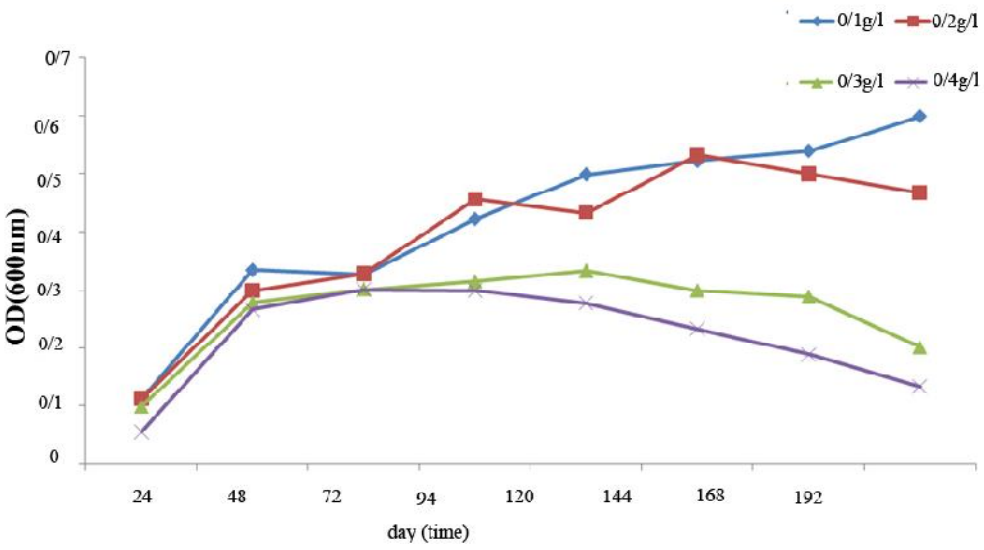

Figure 3: Growth Curve of *Mycobacterium sp.*

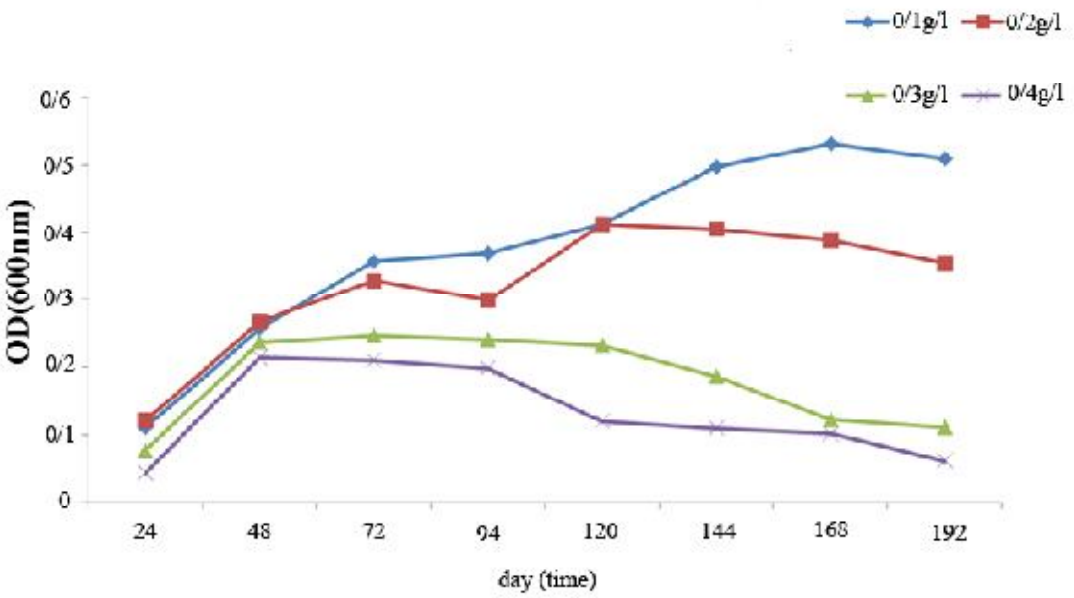

Figure 4: Growth Curve of *Micrococcus varians*

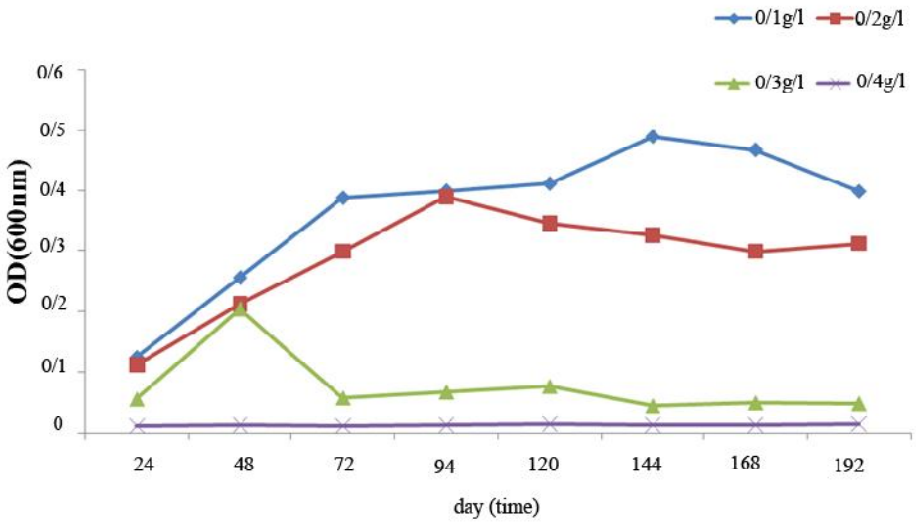

Figure 5: Growth Curve of *Bacillus coagulans*

COCNLUSION

Among isolated bacteria which were facultative anaerobe, *Psedomonas* showed the most degradation in the optimal conditions and was recognized as the most powerful bacterium in chrysen degradation.

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