

Optimal Conditions for Naphthalene Biodegradation by Local Bacterial Isolates

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Abstract: Polycyclic aromatic hydrocarbons (PAHs) are non-polar organic compounds consist of two or more fused benzene rings, arranged in linear, angular or clustered structures. The present study aims to find the optimal conditions for studied bacterial isolates (PS6, DR10 and Ja.2) to degrade naphthalene in liquid BH medium using the bacterial growth and biodegradation percentage of naphthalene as indicators. The three isolate were identified as *Pseudomonas putida*, *Burkholderia cepacia* and *Acinetobacter baumannii* respectively according to the results obtained from biochemical tests and vitek2 system. The optimum incubation period, temperature, pH and naphthalene concentration for *Pseudomonas putida*, *Burkholderia cepacia* and *Acinetobacter baumannii* were determined. Results showed that the maximum growth and biodegradation for naphthalene by three isolates happened after 10 days of incubation at 35°C, in BH medium adjusted to pH 7 and supplemented with 100 mg/l of naphthalene. The *Pseudomonas putida* gave highest biodegradation of naphthalene compared with other two isolates.

Keywords: Biodegradation; Naphthalene; Bacteria

1. Introduction

In the last years, extensive urbanization and rapid industrialization has resulted in the accumulation of a large number of xenobiotic chemicals into the natural environment. The use of numerous aromatic compounds in explosives, dyestuffs, pharmaceuticals and pesticides and the growth of industrialization have contributed very much in the environmental pollution and have attracted considerable attention continuously. Petroleum exploitation, exploration, transportation, consumption, attendant spills and disposal often lead to release of hydrocarbon pollutants into the environment with serious ecological problems (Okoh, 2006).

Polycyclic aromatic hydrocarbons (PAHs) are lipophilic compounds that show high affinity for organic matter, they formed by two or more fused benzene rings in linear, angular or luster arrangements (Cheung and Kinkle, 2001). The simplest structure consists of 2 rings (naphthalene) and very complex structures can be formed by more than 6 rings and alkyl groups. These compounds and their derivatives are produced from incomplete composting of organic materials or from compost of natural process, such as forest fires and volcanic eruptions. Above all, they may produce as a result of human activities. In the most recent decade, some important resources of PAH pollutants have included of industrial products, transportation, waste burning, evaporation and burning of plastic residues (Mrozik *et al.*, 2003). Petroleum refineries can generate significant quantities of PAH- containing oily sludge during the processing of crude oil and landfarming is often used as an economical method of waste treatment and disposal (Ward *et al.*, 2003).

PAHs are an alarming group of substances for humans and other organisms. Many PAHs and their oxidative metabolites are highly toxic, mutagenic and carcinogenic to microorganisms as well as to higher organisms including humans (Samanta *et al.*, 2002). Naphthalene binds covalently to molecules in liver, lung tissues and kidney, thereby enhancing its toxicity; addition it an inhibitor of

mitochondrial respiration (Falahatpisheh *et al.*, 2001). Exposure of humans to naphthalene may damage or destroy some of red blood cells. This could cause to have too few red blood cells until human body replaces the destroyed cells. This problem is called hemolytic anemia (Lohmann *et al.*, 2007).

Polycyclic aromatic hydrocarbons are highly resistant molecules that do not easily break down and can persist in the environment and adsorb onto non aqueous soil particles (Haritash and Kaushik, 2009) due to their hydrophobicity, neutrality and low water solubility which decrease their availability (Mohamed *et al.*, 2012; Straube *et al.*, 2003). Because of the great number of petroleum contaminated sited requiring cleanup, many mechanical and chemical methods to reduce hydrocarbon pollution are often expensive, time consuming and not environment friendly (Atlas, 1995). Therefore, recent studies have focused on bioremediation is a cost-effective approach for decontamination of PAH-contaminated sites and has been extensively used (Xia *et al.*, 2006). Bacteria convert the pollutant organic compounds into less harmful compounds by aerobic and anaerobic respiratory reactions, fermentation, co-metabolism, and de-halogenation; using them as the only source of carbon and energy (Kafilzadeh *et al.*, 2011).

The activity of degrading microorganisms is dependent upon many factors, including: nutrients, oxygen, pH value, composition concentration and bioavailability of contaminants, chemical and physical characteristic of sites and the pollution history of contaminated environment (Norris 1994; Allard and Neilson 1997; Alexander 1999). A large number of microorganisms able to use PAHs of two and three rings as the sole source of carbon and energy have been reported (Bamforth and Singleton, 2005).

There is a large diversity of bacteria that are able to oxidize PAHs including: *Acinetobacter*, *Aeromonas*, *Agmenellum*, *Alcaligenes*, *Bacillus*, *Berjerinckia*, *Burkholderia*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Moraxella*, *Mycobacterium*, *Nocardioides*, *Pseudomonas*,

Rhodococcus, *Streptomyces*, *Sphingomonas*, *Stenotrophomonas*, *Vibrio*, *Paenibacillus* (Kim *et al.*, 2005; Juhasz and Naidu 2000; Daane *et al.*, 2002; Samanta *et al.*, 2002).

2. Materials and methods

Bacterial isolates

The isolates PS6, Ja2 and DR10 were isolated from soil samples contaminated with hydrocarbons which collected from different sites in Baghdad city using Bushnell-Haas medium (BHM) with the following compositions (g/l); NH_4NO_3 1.0, FeCl_3 0.05, KH_2PO_4 1.0, K_2HPO_4 1.0, MgSO_4 0.2, CaCl_2 0.02, the pH was adjusted to 7.0, then 1% of crude oil was added as carbon source, the ability of these isolates were tested for naphthalene biodegradation (Kumar *et al.*, 2006). Then the three isolates were identified according to colonies morphology, biochemical tests and the VITEK 2 system was used to confirm their identification.

Determination of naphthalene concentration

The remaining concentration of naphthalene in liquid BH medium was recorded at a wavelength of 277 nm using UV spectrophotometer, after each experiment, the isolates were separated by centrifuge 10 ml of medium at 10,000 rpm for 20 minutes and allowed to settle for 30 minutes to get a clear supernatant. Then 5 ml of the clear supernatant was extracted with 5 ml of hexane for 10 minutes in a separating funnel. The solvent layer in a separating funnel was collected and poured into the cuvettes of the spectrophotometer and the absorbance was recorded and the concentration of naphthalene was determined according to standard curve of pure naphthalene (Rahbari *et al.*, 2011).

Determination of bacterial growth

The growth of studied isolates in flasks of liquid BH medium after each experiment was measured at 600 nm using spectrophotometer (Kumar *et al.*, 2006).

Optimal conditions for naphthalene biodegradation

Effect of incubation period

To determine the best incubation period for each isolate, 25 ml of liquid BH medium, pH 7 was put into flasks and sterilized at 121°C for 15 min. After sterilization, the flasks were supplemented with 50 ppm of naphthalene, then the flasks were inoculated with bacterial isolates (one isolate for each flask) and incubated in shaker incubator 150 rpm, 30°C at different periods (2, 4, 6, 8, 10, 12 and 14 days), the flasks without inoculum were prepared as positive control for each isolate, also triplicate flasks for each experiment were used. The bacterial growth and the biodegradation rate were determined.

Effect of temperature

Twenty five milliliter of liquid BH medium, pH 7 was put into flasks and sterilized at 121°C for 15 min. After sterilization, the flasks were supplemented with 50 ppm of naphthalene, then the flasks were inoculated with bacterial isolates (one isolate for each flask) and incubated in shaker incubator 150 rpm at different temperatures (25, 30, 35, 40 and 45 °C) for 10 days, the flasks without inoculum were

prepared as positive control for each isolate, also triplicate flasks for each experiment were used. The bacterial growth and the biodegradation rate were determined.

Effect of pH values

The best pH for naphthalene biodegradation by each isolate was determined using 25 ml of liquid BH medium, the flasks of liquid BH medium were prepared in different pH values (5, 6, 7, 8 and 9) and sterilized at 121°C for 15 min, then the flasks were supplemented with 50 ppm of naphthalene inoculated with bacterial isolates (one isolate for each flask) and incubated in shaker incubator 150 rpm at 35°C for 10 days, the flasks without inoculum were prepared as positive control for each isolate, also triplicate flasks for each experiment were used. The bacterial growth and the biodegradation rate were determined.

Effect of naphthalene concentration

The effect of naphthalene concentration on biodegradation was studied for each isolate using 25 ml of liquid BH medium, pH 7 was put into flasks and sterilized at 121°C for 15 min, then the flasks were supplemented with different concentrations of naphthalene (100, 200, 300, 400, 500, 600 and 700 ppm), inoculated with bacterial isolates (one isolate for each flask) and incubated in shaker incubator 150 rpm at 35°C for 10 days, the flasks without inoculum were prepared as positive control for each isolate, also triplicate flasks for each experiment were used. The bacterial growth and the biodegradation rate were determined.

3. Results and discussions

Identification of bacterial isolates

The results obtained from tests of bacterial identification (morphology of isolates, biochemical tests and VITEK 2 system) showed that the three isolates PS6, DR10 and Ja2 belong to *Pseudomonas putida*, *Burkholderia cepacia* and *Acinetobacter baumannii* respectively.

Optimal conditions for naphthalene biodegradation

Effect of incubation period

The results depending on the growth rates and biodegradation of naphthalene in bacterial cultures of *Pseudomonas putida*, *Burkholderia cepacia* and *Acinetobacter baumannii* showed that the best time for naphthalene biodegradation was 10 days for all studied isolates, where the growth rate of isolates were 0.310, 0.266 and 0.235 respectively as in fig.(1-a), also the biodegradation of naphthalene by *pseudomonad putida* was 73.22%, while by *Burkholderia cepacia* and *Acinetobacter baumannii* were 64.81% and 53.46% respectively as in fig.(1-b). Also the results showed that the growth rate for three isolates were increased gradually from 2 to 10 days of incubation and then decreased slightly due to accumulate the intermediate compounds and metabolic substances such as fatty acids, ketones, esters, glycolipid and alcohols, these leads to decrease the pH and oxygen, then appearance the decline phase (Schmitt *et al.*, 1996; Walker *et al.*, 1976c).

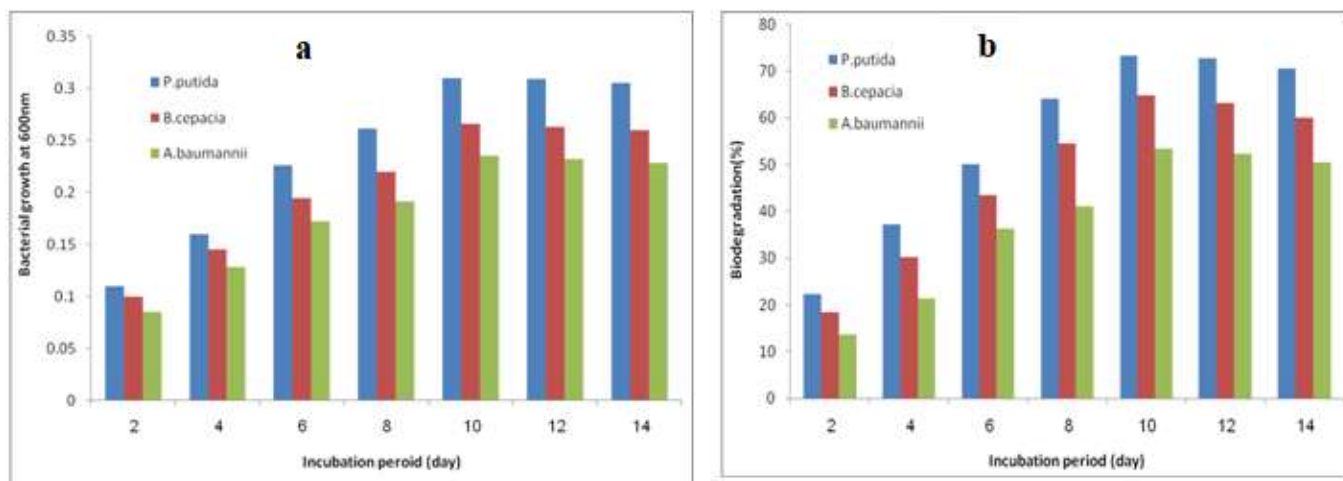


Figure 1: Effect of incubation period on bacterial growth (a) and degradation of naphthalene (b) by three isolates in liquid BH medium

In previous study, Gupta(2012) found the biodegradation efficiency of *Pseudomonas sp.S3* was 77.77% for naphthalene when used 2000 ppm after 7 days of incubation and then decline, while (Kafilzadeh and Pour,2012) found that the naphthalene was completely consumed by *pseudomonas sp.* and *Corynebacterium sp.* in medium with 400 ppm after 10 days of incubation.

Effect of temperature

Temperature has a role in biodegradation of all pollutants because it effects on enzyme activity and microbial growth (Bennett *et al.*, 2012). Results in fig. (2-a) elucidated that the maximum growth rate of three isolates in liquid BH medium with naphthalene were 0.339, 0.288 and 0.266 for *P. putida*,

B.cepacia and *A.baumannii* respectively at 35°C, also the results showed that the *P.putida* appeared the highest degradation rate for naphthalene (80.11%) at 35°C, compared with other two isolates *B. cepacia* and *A.baumannii* which were gave 70.89% and 59.72% respectively at 35°C as in fig.(2-b). Also results demonstrated progressive decrease in bacterial growth for all isolates with increasing temperature above 35°C, this because the accumulation of metabolites resulting from oxidation processes of bacterial isolates, or to absence of oxygen and nutrients (Bishnoi *et al.*, 2009).

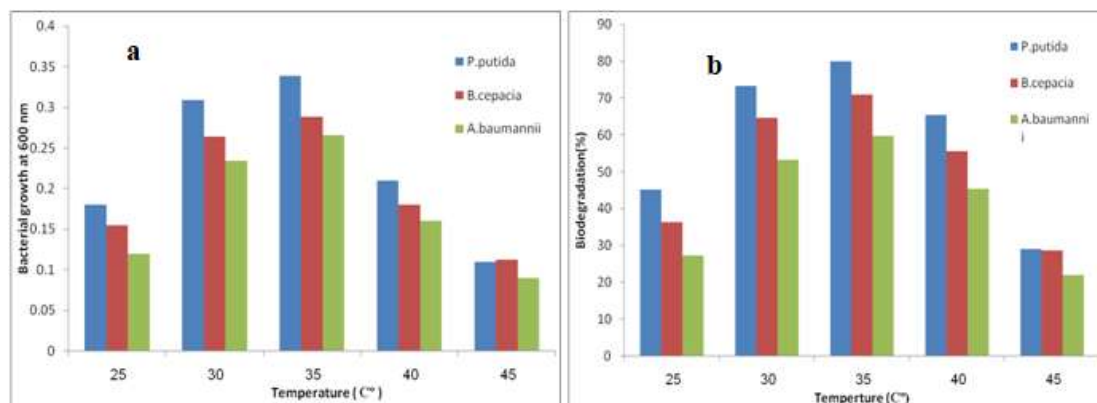


Figure 2: Effect of temperature on bacterial growth (a) and degradation of naphthalene (b) by three isolates in liquid BH medium

The high temperature decreases the solubility of oxygen and this leads to inhibit the growth of aerobic microbes and then decrease the biodegradation rate (Atlas, 1981; Margesin and Schinner, 2001). Results from Harisha and Lakshmi (2016) showed that the maximum biodegradation of spent engine oil by *P.putida* was occurred at 35°C, also Emran (2015) found that the highest rate of growth and degradation for *pseudomonas putida* occurred at 35°C, likewise the growth of *A.baumannii* as optical density with diesel oil was maximum rate at 35°C (Palanisamy *et al.*,2014), while Bishnoi *et al.*,(2009) revealed that the *pseudomonas putida* and *pseudomonas paucimobilis* gave maximum biodegradation of pyrene at 30°C. We concluded from

present study and previous studies the optimal temperature for the growth of isolates ranges between 30 – 35°C , this may depend on the nature of the isolates and the place where isolated from it.

Effect of pH values

pH is one of the major environmental factors that effect on the availability of nutrients, contaminants and activity of biological processes by affects the metabolism of microorganisms and hence the bioremediation kinetics of contaminated soil and water (Ajoku and Oduola, 2013).The obtained results from this experiment as in Fig. (3-a) showed that the highest growth was at pH 7 for all bacterial isolates

after 10 days of incubation with 50 ppm of naphthalene and the *P.putida* isolate gave the highest growth rate 0.335, while the growth rate from *B. cepacia* and *A.baumannii* were 0.289 and 0.265 respectively. Furthermore the degradation rate of naphthalene by *p.putida*, *B. cepacia* and

A.baumannii were 80.21%, 70.73% and 59.74% respectively at pH 7 as in fig. (3-b).

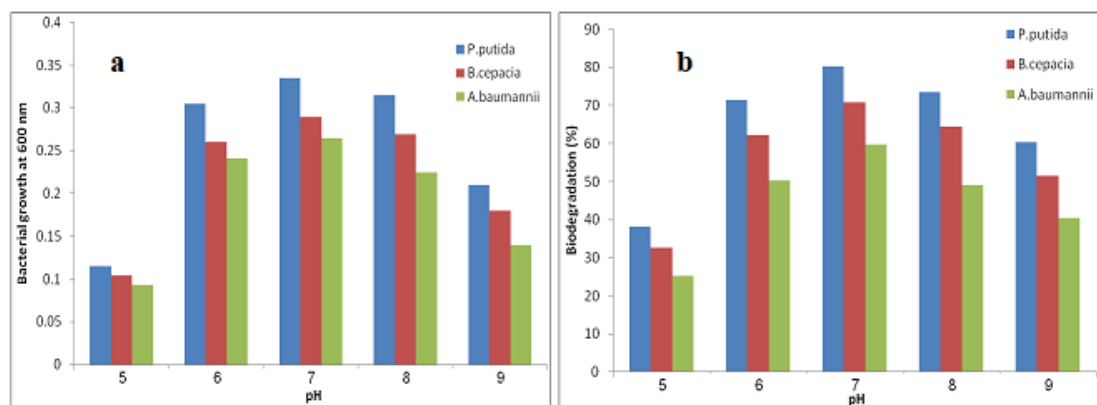


Figure 3: Effect of pHs values on bacterial growth (a) and degradation of naphthalene (b) by three isolates in liquid BH medium

The pollutants have high mobility at low pHs values, but the degradation was expected to be limited because the microbial activity was reduced, this may be also due to fact that nutrients are commonly more available at neutral pH (Chesworth, 2008), while at acidic pH the anionic nutrients interact with protons and thus less available, Furthermore at basic pH the cationic nutrients interactions with hydroxyls and thus less available, Whilst at neutral pH these interactions are reduced and so the nutrients are more available (Chesworth, 2008). Harisha and Lakshmi (2016) proved that the *pseudomonas putida* gave high growth and biodegradation rate at pH 7, also Sawadogo *et al.*, (2014) demonstrated that the optimum growth of *Acinetobacter* at pH 7.5 and 8.5 for *Pseudomonas*, While Emran (2015) found that pH 6 was suitable for the better growth of *Pseudomonas putida* grown in medium containing pesticide. Bamforth and Singleton (2005) has reported that 40 % of phenanthrene in a liquid culture at pH 5.5 was degraded by *Burkholderia cocovenenas*, but the degradation at neutral pH was 80 %. Moreover, Palanisamy *et al.*, (2014) found that the optimal biodegradation for diesel oil by *A.baumannii* was occurred at pH7 in liquid MSM.

Effect of naphthalene concentration

The concentration of naphthalene affects the bacterial grow and degradation ability of microorganisms in a contaminated environment, therefore the effect of initial concentration was examined to assess the ability of bacterial isolates to tolerate different concentrations of PAHs (Cerniglia, 1992; Bennett *et al.*, 2012).

Eight concentrations from naphthalene were used in this experiment; the results showed that 100mg/l of naphthalene gave the best growth rate in all isolates after 10 days of incubation than other concentrations, where the growth rate of *P.putida*, *B. cepacia* and *A.baumannii* were 0.366, 0.320 and 0.283 respectively as in fig. (4-a). Also the *P.putida* was appeared the best ability for naphthalene degradation 86.78%, while *B. cepacia* and *A.baumannii* were 75.89% and 64.95% respectively as in fig.(4-b). So the results found that the growth rate and degradation rate of all isolates decreased when naphthalene concentrations reached to 600 and 700mg/l.

The concentration of PAHs has important effect on the potential success of microbial degradation, if the concentrations of PAHs were low, the gene necessary for the production of enzyme may not induced, and the enzymes necessary for degradation may not produce (Van Hamme, 2004), in the other side, if the concentrations of PAHs were high, this may lead to toxic effects upon the cells and toxic metabolites may accumulate in the growth medium (Morasch *et al.*, 2001). In previous study the *Pseudomonas* sp. degraded 80% of naphthalene in medium with 400mg/l of naphthalene, but the degradation rate was decreased above this concentration (Kafilzadeh *et al.*, 2011), while Kucerova(2006) proved that the *pseudomonas putida* removal was 81% of some PAH compounds after 30 days of incubation, also Zhou *et al.*, (2012) found that the two isolate of *Pseudomonas* sp CZ2 and CZ5 removed 61.8 and 64.3% of naphthalene with concentration of 1000 ppm after 48h.

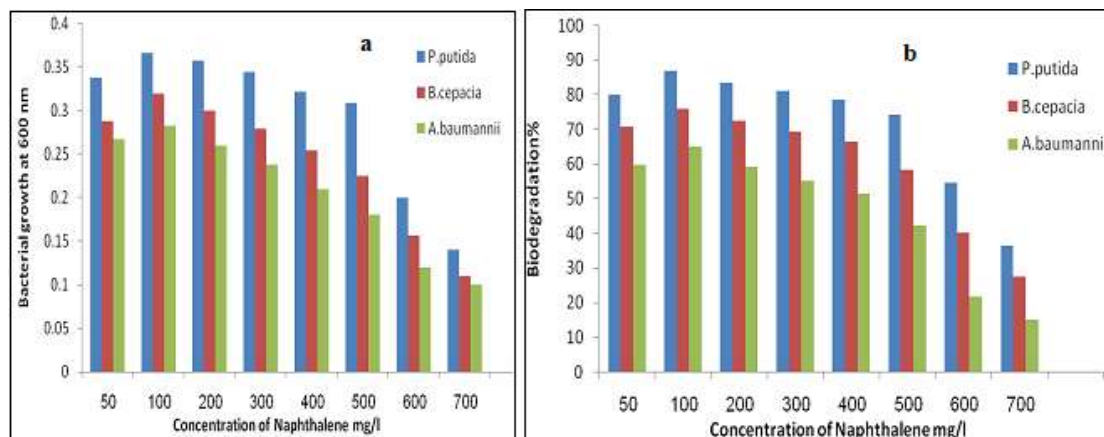


Figure 4: Effect of naphthalene concentrations on bacterial growth (a) and degradation of naphthalene (b) by three isolates in liquid BH medium

4. Conclusions

Contaminated soil with hydrocarbons considered as a good source for the isolation of naphthalene degrading bacteria. The bacterial isolates *pseudomonas putida*, *Burkholderia cepacia* and *Acinetobacter baumannii* have ability to degrade naphthalene under optimum conditions pH 7, concentration of naphthalene 100ppm, temperature 35°C and the best time was 10 days. *pseudomonas putida* showed the highest ability to degrade the naphthalene than other two isolates.

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