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Adaptation and optimization of various physico-chemical conditions for pyrene degradation by bacteria isolated from the Rhizospheric soil of *Morus alba*

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Key words: Adaptation, Physico-chemical parameter, Optimization, Biodegradation

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Abstract

Isolation from contaminated sites using classical enrichment technique is of common practice and give promising results but with a narrow range of microbial community. In this study, wild bacteria were isolated and adopted through changing growth media and exposure to UV-light for 15minutes. Various physico-chemical conditions were optimized for pyrene degradation by isolate M_3 with 1000ppm concentration, optimum temperature of 35°C, p^H 7 and shaking speed of 120 rpm. Isolate M_3 degraded 76.31% pyrene with 0.970 OD value and 2.7×10^{-31} $CFUmL^{-1}$ after 120 hours. Metabolites formed during degradation process on comparison with authentic standards were identified as phenanthrene, catechol, salicylaldehyde and salicylic acid. Plasmid isolation and curing showed a fractional reduction in pyrene degradation by this isolate. Experiment was performed in triplicate and data was analysed by 2-way ANOVA and level of significance 0.002.

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Introduction

Pyrene is a benzene rings compound was first isolated from coal tar is also a byproduct of gasification and incomplete combustion processes. It is a ring structure of a number of high molecular weight (HMW) polyaromatic hydrocarbons (PAHs) like benzo (a) pyrene. These compounds have tumer inducing effects in laboratory animals when get entered in their body through food, air and skin contact. Animal studies showed that mice that were fed pyrene and benzo (a) pyrene developed nephropathy and an increase in the weight of the liver and experienced reproductive problems, birth defects and decrease in their body weight was also seen. In addition, there were some slight changes in the blood, damage to skin, body fluids and the immune system [ATSDR 1990].

Microbial degradation is supposed to be one of the principal mechanisms of PAH removal from soils and is affected largely by contaminant bioavailability and catabolic ability of indigenous microbial populations [Macleod and Semple, 2006; Doick *et al.,* 2005; Li *et al.,* 2008; Vinas *et al.,* 2005]. Gram-positive species has been most widely studied for degrading pyrene by using it as a sole carbon and energy source. Other pyrene degrading strains include *Rhodococcus* sp., *Bacillus cereus*, *Burkholderia cepacia* [Juhasz *et al.*, 1997].

Adaptation processes, which occur as a result of an increase in the hydrocarbon-oxidising potential of the microbial community, allow the development of microbial populations with the ability to degrade PAHs. As previously reported, adaptation processes enhance the degradative capability of microbes. The mechanism of adaptation depends not only, on the interaction of microbes with soil but with the amount of bioavailable contaminants as well [Kastner *et al.,* 1998]. Previous exposure of indigenous microbes to PAHs can influence degradation capabilities, as suggested by a number of studies [Sartoros *et al.* 2005; Puglisi *et al.,* 2007; Ye *et al.* 2011]. The presence of one contaminant effect the accessibility of the other in a specified area as contaminants always exists in groups [Khan *et al.,* 2006; Kumar *et al.* 2010].

The aim of present work is to study pyrene degradation by bacterial isolates from rhizospheric soil of contaminated area.

Materials and Methods

Chemicals and Media

Analytical grade pyrene (99%), salicylic acid, salicylaldehyde, catechol, gentisic acid, 1-hydroxy-2, naphthoic acid and 2-hydroxy-1, naphthoic acid, 6, 7-benzo-caumarin, 1-naphthol and were purchased from Sigma-Aldrich and Fluka (St. Louis, MO, USA). Other chemical and media components were of high purity and analytical grade from Oxide, Merck and BDH. Nutrient agar and Mineral salt medium was used for initial screening. The composition of PNR and PNRG (PNR + 5m M glucose) per liter of distilled water [Pan *et al.*, 2008; Ebrahimi *et al.*, 2012; Khan *et al.*, 2006], is as; PN (20x) 50ml used as 50mL/L : KH_2PO_4 13.6 % (w/v), $(NH_4)_2SO_4$ 2.4 % (w/v), NaOH 2.5 % (w/v) and R salts used as 7ml/L $MgSO_4.7H_2O$ 8 % (w/v), $FeSO_4.7H_2O$ 0.2 % (w/v), HCl 0.4 % (w/v), Agar (2 %) was used as solidifying agent.

Inoculum preparation

All the strains were grown in Nutrient Broth according to well established protocol [Guo *et al.*, 2010].

Growth in liquid media for selection of best degraders

Growth of isolates was studied in liquid media for selection of best degraders (Khan *et al.*, 2006; Kumar *et al.*, 2010).

Optimization of various physic-chemical conditions for PAHs degradation

Different parameters such as pyrene conc, temperature, p^H, substrate (alternate carbon and energy source) and agitation speed and effect of inoculum were optimized for degradation studies. A wider range of conc i.e., 100, 500 and 1000mgL^{-1} was studied for their effect on bacterial growth and degradation. Selected p^H range was 4, 5, 6, 7, 8 and 9 and temperature 28, 30, 35, 40 and 45°C. To study the agitation effect, the isolate was subjected to different shaking speed (120, 150, 180, 200 and 220rpm) and without shaking as well.

Optimization of pyrene concentration, growth temperature, media p^H and agitation speed

The enrichment media was prepared and dispensed in 100mL volumes into 250mL Erlenmeyer flasks before autoclaving.

Prior addition of media, different concentration of pyrene dissolved in acetone 100mgL^{-1}, 500mgL^{-1} and 1000mgL^{-1} was added to flasks. Acetone was allowed to evaporate. Thereafter, flasks were inoculated with isolate M3 (pyrene) were then incubated for five days. One milliliter sample was aseptically collected from each flask and assayed for the level of microbial growth by measuring the OD (600nm). All experiments were performed in triplicate. The growth of the isolated strain on selected PAHs was observed at different temperature 28, 30, 35, 40 and 45°C for 120 hour. One mL sample was taken after 24 hours and observed for bacterial growth at 600nm. PAH degradation was optimized at different p^{H} range was 4, 5, 6, 7, 8 and 9 and maximum activity was observed at neutral p^{H} 7. To study the effect of shaking different speeds 120, 150 and 180, 200, 220rpm as well as without shaking was also observed for maximum activity. The maximum activity was observed at 120 rpm (Ahmed *et al.*, 2010; Naveenkumar *et al.*, 2010).

Adaptation through changing pyrene concentration, growth media and physical mutation

The isolate was exposed to UV-light for 15 minutes in order to check its effect on degradation of pyrene according to well established protocol (Abdelhay *et al.*, 2008).

Effect of carbon and nitrogen sources

Different carbon sources like fructose, glucose and sucrose were also added to pyrene containing media to study their effect on utilization of pyrene by bacteria. Nitrogen salts potassium, ammonium, sodium and calcium nitrate were also studied for their effect on PAHs degradability.

Inoculum concentration

Inoculum concentration is one of the important factors to be considered for the maximum degradation of PAHs. The highest inoculum concentration did not stimulate the biodegradation when compared to the minor inoculum concentrations used. 10% vv^{-1} inoculum proved to be optimum for pyrene degradation (Leahy *et al.*, 1990; Gou *et al.*, 2010).

Biodegradation Experiment

The biodegradation experiment was performed using 250ml reactor flasks containing 100ml PNR media, 10% of bacterial inoculum and 1000mgL^{-1} pyrene dissolved in acetone. Acetone was allowed to evaporate prior adding media. All samples were tested at pH 7.0 and temperature controlled at 30°C 180rpm [Othman *et al.*, 2010].

Extraction of PAHs

The samples were then PAHs were extracted by protocol of Shokrollahzadeh *et al* with some modifications [Shokrollahzadeh *et al.*, 2010]. The growth medium was extracted twice by dichloromethane (DCM) and acetone10mL each by shaking at 30°C for 24 hours. The extraction phase was stripped of water droplets by pipetting the upper layer after centrifugation at 12,000xg. The remaining moisture was removed by addition of 4gm anhydrous sodium sulphate (Na2SO4) and evaporated to dryness in a rotary evaporator and concentrated in a rotary evaporator to around 2ml. The samples were then filtered, using 0.45µm syringe filters. For GC-MS analysis all the flasks were incubated for 10 days at 30°C on rotary shaker incubator at 150 rpm. During incubation the residual concentration of pyrene was monitored spectrophotometrically after 10 days by liquid-liquid extraction method [Survery *et al.*, 2009]

High performance liquid chromatography (HPLC) analysis

Followed by the extraction with ethyl acetate, the samples were filtered through 0.2mm syringe filter and analyzed in a high performance liquid chromatography. HPLC (PerkinElmer) with the C18 reverse phase column was used to analyze pyrene under isocratic condition using acetonitrile: water (80:20) (vv^{-1}) as mobile phase and detection wavelength - 254nm. The flow rate of the mobile phase (acetonitrile) was maintained at 1.2mL min^{-1}. The samples volume was 2mL in each vial of HPLC tray. Metabolites were studied on the basis of retention time comparison with available authentic standards and literature values (Shokrollahzadeh *et al.*, 2012).

Gas chromatography-mass spectrometry (GC-MS) analysis

For GC-MS 2010+, Shimadzu fused silica capillary column was used for the analysis. The column temperature program was set at 100°C for 1min, 15°Cmin^{-1} to 160°C and 5°Cmin^{-1} to 300°C for 7min. The GC injector was held isothermally at 28 °C with a splitless period of 3min. Helium was used as the carrier gas, at a flow rate of 1mLmin^{-1} by using electronic pressure control.

The GC–MS interface temperature was maintained at 280°C. Standards from Sigma Aldrich were used for the PAH (pyrene) and their metabolites. GC-MS library search was used to confirm the metabolites without standards (Neelofur *et al.*, 2014).

Plasmid isolation and Agarose Gel Electrophoresis

The plasmid was isolated according to manufacturer instructions of Invitrogen mini prep kit and was electrophorezed on 0.8% agarose gel in presence of ethidium bromide (1µgml^{-1}). DNA bands were visualized under UV light under UV transilluminator and photographed [Survery *et al.*, 2009].

Results

Growth in liquid media for selection of best degraders

Isolates were studied in liquid media under static lab conditions for 72 hours and isolate M_3 with highest OD value was selected for further studies Fig. I.

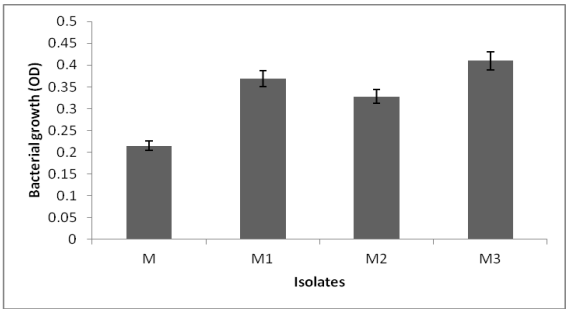


Fig. I. Screening of isolates in liquid media for selection of best degraders.

Adaptation and optimization of various physico-chemical conditions for pyrene biodegradation

Adaptation through changing PAHs concentration

Adaptation was carried out through changing pyrene concentration in the growth media. The bacterial isolates subjected to lower concentration showed medium or less growth first and then rich growth with increasing concentration as shown in Table 1.

Table 1. Adaptation of isolate through changing concentration of pyrene in the growth media.

S. No.	Isolate	Pyrene concentration in ppm on nutrient agar media											
		10	50	100	200	300	400	500	600	700	800	900	1000
1.	M	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
2.	M_1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3.	M_2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
4.	M_3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Adaptation using multiple media

When the screening media was changed from simplest nutrient agar to PNRG and then PNR with pyrene as the only carbon source showed that the growth of the isolate M_3 was not affected by media as shown in Table 2, 3 & 4.

It is clear from this study, the enhanced growth rate was observed even after changing media from that containing glucose to one having no readily available carbon source. Isolate M_3 when grown in PNR media with only pyrene as sole carbon source showed enhanced growth at 1000ppm.

Table 2. Adaptation of isolate through changing growth media.

S. No.	Isolate	Pyrene concentration in ppm on PNRG media											
		10	50	100	200	300	400	500	600	700	800	900	1000
1.	M	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++
2.	M_1	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+
3.	M_2	+++	+++	+++	+++	+++	+++	++	++	++	+	+	+
4.	M_3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Table 3. Adaptation of isolate through changing growth media.

S. No.	Isolate	Pyrene concentration in ppm on PNR media											
		10	50	100	200	300	400	500	600	700	800	900	1000
1.	M	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	++
2.	M_1	+++	+++	+++	+++	++	++	++	++	++	+	+	+
3.	M_2	+++	+++	+++	++	++	+	+	+	+	+	-	-
4.	M_3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

Table 4. Adaptation of isolate through changing growth media.

		Growth media (1000ppm pyrene)		
S. No.	Isolate	Nutrient agar	PNRG	PNR
1.	M	+++	++	++
2.	M_1	+++	++	+
3.	M_2	+++	+	-
4.	M_3	+++	+++	+++

Adaptation by physical mutation

The bacterial isolate when exposed to UV light to 15 minutes showed enhanced growth at 1000ppm of pyrene as shown in Fig. II. It was observed that the bacterial growth rate was enhanced on exposure to UV-light for 15 minutes.

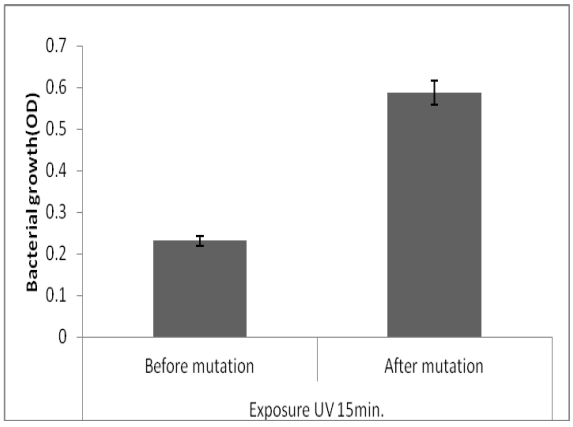


Fig. II. Effect of UV-light on growth of isolate M_3 on pyrene.

Optimization of pyrene concentration, temperature and p^H of the growth media for pyrene degradation

There was elevated growth rate of isolate M_3 with increasing levels of pyrene from 100mgL^{-1} to 1000 mgL^{-1}. Isolate M_3 showed a concentration dependent growth on pyrene with 1000mgL^{-1} as optimum concentration as shown in Fig. III. (A). The growth of isolate M_3 was monitored at different temperature range and 35°C optimum was recorded for pyrene degradation as shown in Fig. III. (B).

It was confirmed from our study, that neutral p^H favoured both extensive bacterial growth and degradation of the pyrene (Fig. III. (C).

The optimized shaking speed was 120rpm for our isolate as shown in Fig. III. (D). No substantial difference was observed in pyrene degradation in cultures with and without glucose addition as shown in Fig. III. (E).

However, addition of different nitrogen sources have no significant effect on growth of bacteria and degradation activity of pyrene (Fig. III. (F).

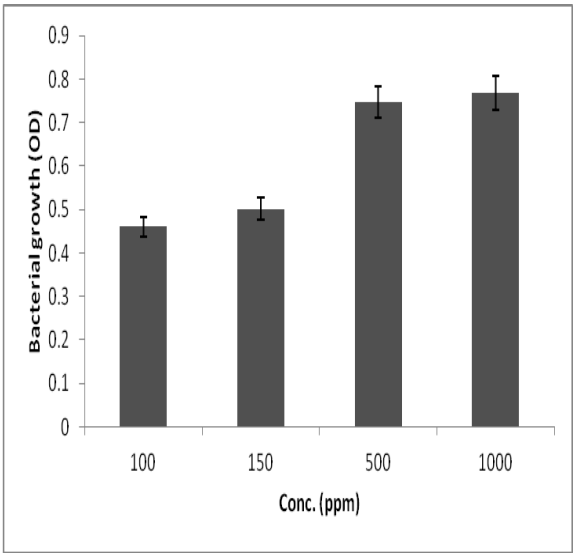


Fig. III (A). Optimization of pyrene concentration

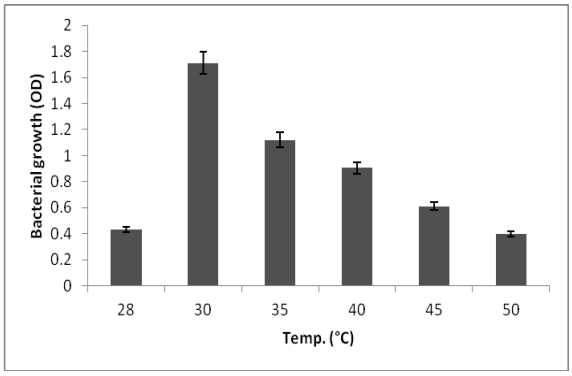


Fig. III (B). Optimization of temperature for pyrene degradation.

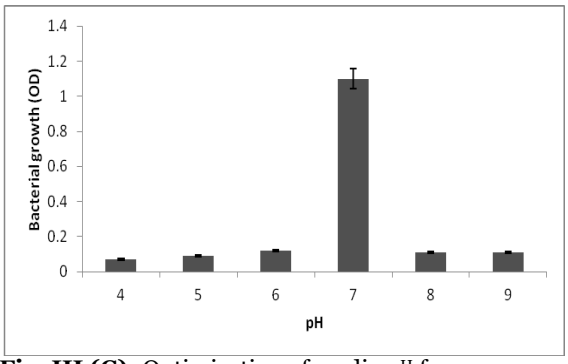


Fig. III (C). Optimization of media p^H for pyrene degradation.

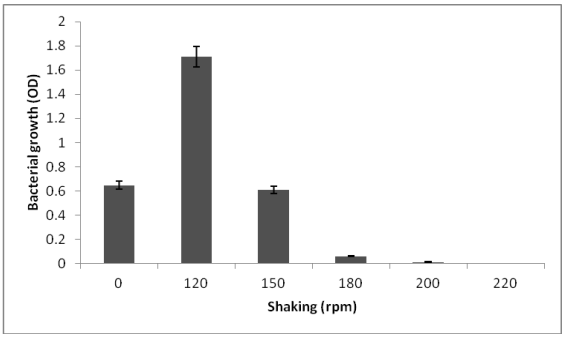


Fig. III (D). Optimization of shaking speed for pyrene degradation.

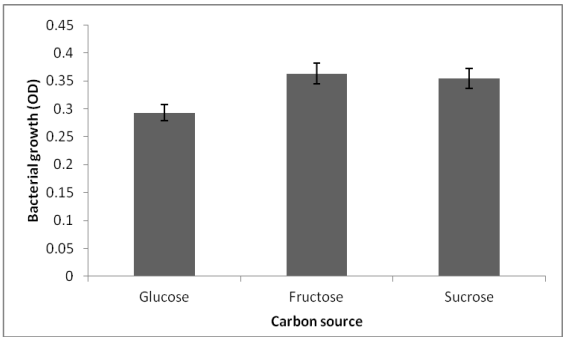


Fig. III (E). Effect of carbon source on pyrene degradation by isolate M_3.

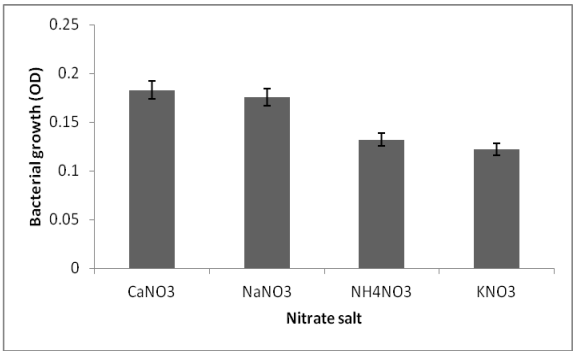


Fig. III (F). Effect of nitrogen source on pyrene degradation by isolate M_3.

Growth of isolate M_3 at the expense of pyrene is shown in Fig. IV. The growth was slower in first 24 hours which increase to 0.609 OD value in 96 hours. The growth rate of 0.97 was observed after 120 hours with cell viability of (2.5×10^{-31}) shown in Table. 5.

76.31% pyrene was degraded by this isolate in 120 hours of incubation. In first 24 hours the degradation rate was slower and about 9.08% and 11.20% was degraded in incubation period of 24 hours and 48 hours respectively. About 50% of pyrene was disappeared in 96 hours with 23% degradation in last 24 hours as shown in Fig.V.

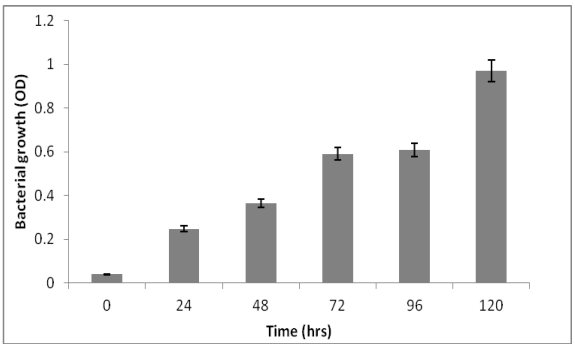


Fig. IV. Growth of Isolates M_3 on pyrene.

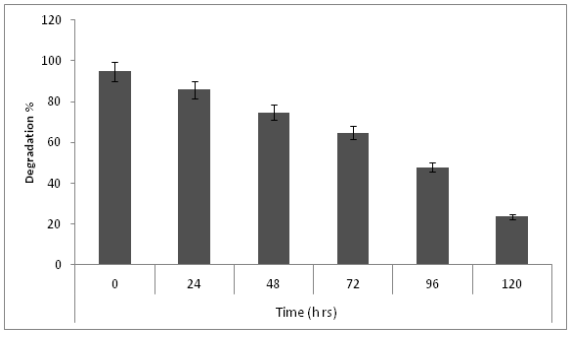
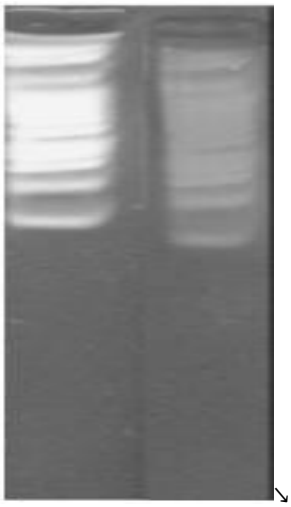


Fig. V. Degradation of pyrene by isolate M_3.

Table 5. CFUmL^{-1} of isolate M_3.

Time (Hrs).	Isolate	0	24	48	72	96	120
CFUmL^{-1}	M_3	1.3×10^{-6}	1.6×10^{-11}	1.8×10^{-18}	2.1×10^{-23}	2.4×10^{-26}	2.7×10^{-31}

If we look at the HPLC chromatogram after 24 hours there was a sharp single peak with retention time of 2.943 and after 48 hours two other peaks with 4.269 and 4.609 start to appear. After 72 hours two sharp peaks with retention time of 9.25 and 10.08 apperared, this disappeared on further degradation for 24 hours and appeared with retention time of 8.21 and 9.006. Two other peaks appeared with 8.002 and 8.55 which remained unaffected even after 48 hours of incubation. In order to study the further fate of pyrene by the strain was allowed to degrade it for 240 hours and analyzed by GC-MS as shown in chromatogram with some unknown peaks. Plasmid isolation was also carried out from isolate M3 (Fig.VI) and after curing showed a small reduction in the rate of pyrene degradation



1kb Ladder↙ ↘Plasmid M_3

Fig. VI. Isolated plasmid and 1Kb ladder.

Discussion

Adaptation processes, which occur as a result of an increase in the hydrocarbon-oxidising potential of the microbial community, allow the development of microbial populations with the ability to degrade PAHs [Ye *et al.*, 2011]. These adaptation processes, although not fully understood, are considered to be controlled by a number of contaminant and soil factors including the concentration of contaminant, interactions with the indigenous microbial populations and length of contaminated soil contact time.

Microbe can adapt to a changing environment by rapid multiplication or induction of resistant genes that can cope with the degradation of pollutant present in their vicinity. Pre-exposure of indigenous microbes to PAHs can influence degradation capabilities, as suggested by a number of studies [Kastner *et al.*, 1998; Sartoros *et al.*, 2005; Puglisi *et al.*, 2007].

The selective nature of the isolation media originally used to obtain the wild-type degradative isolate from soil was investigated and mutation using UV light was performed to enhance the degradation ability. Hence, it could be concluded that exposure to UV light for 15 minutes could improve the degradation ability significantly as reported earlier. They can also make their cell resistant to contaminants by changing their genome sequencing. They possess specialized genes that can move free within genome and can transfer them within its population [Leahy & Colwell, 1990; Top & Springael, 2003].

Optimization of physico-chemical parameter like, concentration of compound, media p^H and temperature prior to any degradation experiment is helpful in obtaining desired results. The very first and important factor is concentration of contaminant in the media. As exposure of microbes to high dose of xenobiotics can adversely affect the enzymatic machinery and can be proved lethal to bacterial system. These microbes must be supplied with gradual increase in the concentration level of contaminant so they can have enough time to cope with the pollutant in their vicinity [Naveenkumar *et al.*, 2010; Abdelhay *et al.*, 2008; Rosmarie 1993]. With increasing concentration enhanced growth rate was observed on the media plates is an indication of adaptation of the isolates. As previously reported, these adaptation processes, even though not fully understood, are considered to be controlled by a number of contaminants and soil factors including the concentration of contaminant, interactions with the indigenous microbial populations and length of contaminated soil contact time [Kastner *et al.* 1998; Johnsen *et al.* 2005].

Biodegradation of PAHs can occur over a wide temperature. However, most studies tend to focus on mesophilic temperatures rather than the efficiency of transformations at very low or high temperatures. Temperature has a detrimental effect on the capability of the microorganisms to degrade a certain compound like pyrene in this study. The solubility of pyrene increases with an increase in temperature to a certain limit which makes it bioavailable. However, beyond a specific limit increase in temperature results in decrease solubility of oxygen and thus reduce the metabolic activity of aerobic microorganisms. According to our study, temperature influenced the degradation of pyrene and its degradation level was significant in the thermophilic range with the highest degradation occurring at 35°C. Isolate M_3 showed growth at 40°C is an indication, that this isolate has enzyme adaptation at highest temperature [Deeb *et al.*, 1999]. The enzymes of this microorganism might be adapted to bit high temperature [Ahmed *et al.*, 2010; Nour *et al.*, 2010; Arulazhagan *et al.*, 2011; Mukesh *et al.*, 2012]. Microbial system has no mechanism for adjusting their internal p^H is considerably affected by the p^H of their immediate environment [Nnamchi *et al.,* 2006].

The charge imbalance inside and on the surface of the cells was prevented at p^H 7 in this study. Pyrene removal by isolate M_3 was however, more sensitive to the p^H of the growth media, with the degradation of pyrene significantly inhibited at pH 4 and 5, relative to p^H 7 [Lakshmi *et al.*, 2011]. Better aeration results in ample supply of oxygen, an even distribution of nutrients and low oxygen tension were attained by our isolate M_3 at 120rpm. Consequently, adequate availability of oxygen could enzymatically incorporate atmospheric oxygen more efficiently in the aromatic ring of the of hydrophobic compounds like, pyrene and bring about better initial ring oxidation, which is typically the rate regulating step in the biodegradation of PAHs [Alias *et al.*, 2011, Park *et al.*, 1990]. The growth of our studied isolate and pyrene degradation was not affected by supplementing glucose in the media. Glucose which normally supplies an additional nutrient for bacterial growth has no significant increase in the number of bacteria in this study.

Similarly, addition of nitrate salts used in this experiment act as competitive substrate in pyrene degradation as reported previously [Hadibrata & Kristanti 2012; Wick *et al.*, 2003].

The rate of bioremediation a pollutant depends on the number and type of degrading organisms, environmental conditions, chemical and nature of the compound to be degraded. Some microorganisms can consume PAHs as a source of carbon and energy [Johnsen *et al.*, 2005]. In this study the pyrene removal of 76.03% by isolate M_3 in just five days are far better than reported work on two bacterial isolates, *Enterobacter hormaechei* and *Pseudomonas pseudoalcaligenes* from petroleum contaminated sites. Pyrene was degraded by these isolates with efficiency 77.7 and 83.7% within 15 days of incubation, respectively. Results of our isolate in this study are in contrast to previously reported work for *Klebsiella pneumoniae* that degraded only 63.4 % of 20mgL^{-1} in 10 days [Hesham *et al.* 2014; Michael Stieber 1994]. *Pseudomonas stutzeri* CET 930 degradation of wastes containing pyrene degraded 71.99 % pyrene in one week, are in line with the present studies [Moscoso *et al.,* 2014].

It can be concluded from HPLC and GC-MS analysis through comparison with authentic standards and GC-MS library that the peaks with retention time in range of 4.2-4.6 may be phenanthrene formed as metabolite of pyrene. Which, on further degradation for 24 hours converted to compounds with retention time of 9.006 and 9.25. Peaks in this range of retention time (9.0-10.0) have close resemblance with that formed by catechol. While, peaks formed in the range of 8.0-8.5 retention time are mostly formed by salicyalaldehyde and salicylic acid. The results demonstrate that the degradation capability of pyrene from soil bacteria of contaminated sites has comparatively more potential to degrade HMW PAHs (pyrene) and can be applied in an efficient bioremediation process [Ping *et al,*. 2014]. Microbes that can grow on salycilate develop enhanced degradation of those PAHs which degrade these compounds through salycilate pathway [Gaskin & Bentham 2005].

Study on the plasmids in pyrene degraders demonstrated that some *Mycobacterium* sp, have no plasmid. Likewise, the presences of plasmid have no effect on pyrene degradation as clear from our studies. The strains were able to degrade pyrene after curing plasmid from these isolates. It would thus appear that, the choice of method is very important in the detection of plasmids, as most large plasmids are likely to escape detection using routine techniques used for small plasmids. It is not unlikely that plasmids are more involved in the degradation of HMW PAH than previously thought [Miller *et al.*, 2007].

After curing the isolate M3 for plasmid the degradation rate remains unaffected with a fractional decrease. It is clear that plasmid have not substantial role in pyrene degradation rather it was chromosomal mediated. The degradation rate was slower in cured isolate can be the possible explanation that plasmid may have role somewhere in degradation process.

The size of plasmid from isolate M_3 was seemed to be larger than 1Kb ladder as seen on gel electrophoresis as clear Fig. V. One of the most important factor that aid to degradative efficiency of microorganism is the presence of plasmid which play a pivotal role in obtaining novel catabolic pathways in microbial communities. Like previously reported studies, some interesting findings form *Mycobacterium* sp. Isolate from a petroleum contaminated area was capable to grow on HMW PAHs possesses a large plasmid.

But, this plasmid was later confirmed to have no role in degradation. The first reported pyrene degrader and from the studies of a large number of strains it was found that genes for pyrene degradation are localized on chromosomes [Krivobok *et al.*, 2003; Kim *et al.*, 2007].

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