The Optimum Conditions for Naphthalene Biodegradation by Filamentous Fungi

Duha B.Mohammed*[1], Saad Husein Khudeir[1], Muna H.Al-Jubouri[2]
[1]Ministry of Science and Technology. Baghdad, Iraq.
[2] College of Science, University of Baghdad, Baghdad, Iraq.

Abstract

Recent studies have proved the important role of fungi in the biodegradation of oil pollutants. The present study aims to find the optimal conditions for the fungi to get the best rate of the biodegradation of the polycyclic aromatic hydrocarbon (PAHs) (Naphthalene) compounds. Soil samples were taken from 18 different sites polluted with oil wastes and cultured then obtained 312 isolated fungi from 64 replicates Primarily screening were done on fungal isolates on solid media containing naphthalene the results revealed that 25 fungal isolates gave good growth, 47 fungal isolates gave Moderate growth, 66 gave weak growth and 147 fungal isolates gave no growth on Naphthalene solid media.

Then secondary screening were done on 25 fungal isolates the results showed that most active isolates were T1,MST2 and RS which were then identified as *Aspergillus niger*, *Trichoderma viridi*, *Fusarium verticelloides*, The results showed that the best time for Naphthalene biodegradation was 8 days for all fungal isolates (*Aspergillus niger*, *Trichoderma viridi*, *Fusarium verticelloides*) the best Naphthalene concentrations decline from 100 ppm to 12.22ppm was for *Fusarium verticelloides* , the best pH value for naphthalene degradation was 7 for all fungal isolate. The best Naphthalene concentrations decline from 100 ppm to 12.4 ppm was for *Fusarium verticelloides*, the best temperature for Naphthalene biodegradation was 30 ^{0}C for all fungal isolates, the concentrations of Naphthalene decline from 100 ppm to 12.6 ppm for *Fusarium verticelloides*, The results showed that the best concentration for Naphthalene biodegradation was 100ppm for all fungal isolates, the concentrations of Naphthalene decline from 100 ppm to 12.6 ppm for *Fusarium verticelloides*.

Keywords: Filamentous fungi; biodegradation; PAHs; Naphthalene; Optimum conditions.

الظروف المثالية للتفكك الحيوي لمركب النفثالين بواسطة الفطريات الخيطية

ضحى بهاء محمد*[1]، سعد حسين خضير[1]، منى حمودي الجبوري[2]
[1]وزارة العلوم والتكنولوجيا، بغداد، العراق، [2] كلية العلوم، جامعة بغداد، بغداد، العراق.

الخلاصة

اثبتت الدراسات الحديثة اهمية الفطريات في المعالجة البايولوجية للملوثات النفطية .تهدف هذه الدراسة الى ايجاد الظروف المثلى للفطريات للحصول على افضل معالجة بايولوجية للمركبات الاروماتية متعددة الحلقات ومنها النفثالين.اخذت مواقع تربة ملوثة بالمخلفات النفطية (18) وبواقع (64) نموذج تم الحصول على

*Email:Duhabahaa@Yahoo.com

(312) عزلة فطرية قادرة على النمو في البيئة الملوثة بالمخلفات النفطية .اجريت غربلة اولية للعزلات الفطرية بتنميتها على وسط الاملاح المعدنية الصلب الحاوي نفثالين. اعطت نتائج الغربلة 174 عزلة لم تنمو على الوسط و66 كان نموها ضعيف ،47 اعطت نمو متوسط في حين 25 عزلة نمت بشكل جيد والتي اجري عليها غربلة ثانوية باستخدام وسط الاملاح المعدنية السائل الحاوي نفثالين بتركيز 100 ppm والمحضن بالحاضنة الهزازة بدرجة 30 ^{0} م لمدة 7 ايام وتم الحصول على العزلات T1,Mi2,Ru والتي تعتبر افضل العزلات في تفكيك النفثالين بعدها شخصت مظهريا ومجهريا باستخدام المجهر المركب وكانت على التوالي .. *Aspergillus niger*, *Trichoderma viridi, Fusarium verticelloides*, درست الظروف المثلى لهذه الفطريات لتفكيك النفثالين باستخدام وسط الاملاح المعدنية السائل والحاوي نفثالين بتركيز 100 ppm .اعطت نتائج الظروف المثلى ان افضل وقت لتفكيك النفثالين كان 8 يوم ، افضل pH كان 7 ،افضل درجة حرارة كانت 30 ^{0}م وافضل تركيز كان 100 ppm للعزلات الثلاثة

Introduction

The use of fossil fuels for energy and raw material in the past century has led to a wide spread environmental pollution. Among these pollutants are polycyclic aromatic hydrocarbons (PAHs), which are considered a potential health risk because of their possible carcinogenic and mutagenic activities [1].

PAHs consist of benzene analogs having two or more aromatic rings in various alignments. Most of PAHs are very toxic, highly teratogenic, for humans and animals [2].

There are many physical and chemical removal techniques for PAHs pollutants. But, these techniques have significant disadvantages such as their technological complexity, high cost; they produce another source of pollution and also increase the oil recovery cost .Biodegradation by microorganisms (Algae, Bacteria, Yeast and Fungi) on the contrast, is a promising option for the complete removal and destruction of PAHs contaminants [3].

Investigations into the microbial bioconversion of PAHs have shown that fungi are efficient degraders of these organic pollutants .These fungi have also been shown to deplete and detoxify PAHs in contaminated soil [1].

In oil contaminated soils, the fungal community is generally remarkably reduced to few fungal species, which are tolerant to pollutants. the predominant fungal groups belonging to Ascomycota ,Deuteromycota and Zygomycota tolerate oil contaminant soil [4] Species belonging to the genera *Trichoderma, Fusarium, Penicillium, Stachybotrys, Aspergillus, Cladosporium, Mortierella, Beauveria, Engyodontium* are some examples of fungi that have been recently described as tolerant to pollutants such as PCBs, chlorobenzoic acids (CBA), and endosulfan and that are indicated as potential bioremediation agents in soil [4-6]. In such environments microorganisms are capable of pollutants removal can constitute up to 100 % of the viable ones. In soils with relatively low levels of pollution, particular groups of fungi can become the dominant microbial population [7].

Awide variety of fungi, both lignolytic and non-lignolytic, have the capability of degrading PAHs, but in contrast to bacteria, fungi typically do not use PAHs as sole source of carbon and energy sources, but co-metabolically transform them to less toxic products [8,9].

Numerous studies show that white-rot fungi are efficient degraders of PAH, including species such as *phanerochate. chrysosporium ,Pleurotus* sp.,*Bjerkandera* sp., and *Trametes versicolor* .It is now generally accepted that ligninolytic enzymes are involved in PAHs degradation by white-rot fungi [10].

Filamentous fungi play an important role in degradation and detoxification of polycyclic hydrocarbons, including condensed aromatic ring systems as well as other xenobiotic compounds, present in polluted environments. Some of these compounds are very harmful and carcinogenic [11].

Species belonging to the genera *Trichoderma, Fusarium, Penicillium, Stachybotrys, Aspergillus,Cladosporium, Mortierella, Beauveria, Engyodontium* are some examples of this kind of fungi that have been recently described as tolerant to pollutants such as PCBs, chlorobenzoic acids (CBA), and endosulfan and that are indicated as potential bioremediation agents in soil [4].

Fungi have been shown to be relatively more successful in breaking down PAH compounds than bacteria [12].Temperature; salinity and pH are environmental variables that control the degradation

rate. Several studies found a significant correlation between temperature and PAH degradation rates [13,14]. The degradation rate increases as the temperature increases. The salinity and pH of a contaminated site may influence the species composition of PAH-degrading consortia affecting the degradation rate [14].

Temperature is another important variable that influences petroleum biodegradation. Optimum temperature dictates the rate of Naphthalene metabolism by microorganisms and also the pattern of the microbial community [15].

Temperature also has direct effect on the physical nature and chemical composition of the Naphthalene constituents [15]. When temperatures are low, Naphthalene tends to be more viscous and their water solubility is greatly reduced [16].

Low temperature will also affect microbial growth and propagation, and under normal circumstances, rates of degradation decrease accordingly [17]. This is a result primarily of decreased rates of enzymatic activity. The optimum temperature is typically in the range of 30 to 50°C. At temperature above this range, enzymatic activities are inhibited as proteins denature [18].

Further increase in temperature caused a reduction in metabolic activity of aerobic microorganisms due to the decline in oxygen solubility Bioavailability, however, was lower when the temperature rose above 50 ◦C, indicating that the adverse effect of high temperature on the cell is more important than any increase in substrate availability [19].

The rates at which Naphthalene are degraded are also determined by moisture level. The reason is simple, that water is needed for microbial growth and enzymatic/biochemical activities [20].

Materials and methods

Screening of the isolates for naphthalene biodegradation

Primary screening

Fungal isolates from 64 soil samples were tested for their ability to degrade naphthalene using solid mineral salts medium as in and 0.1 ml from naphthalene solution containing 100ppm was spreaded on solid medium agar plates then methanol evaporated by left plates for 1 hr. inside sterile hood, white thin layer formed, then the plates were inoculated with fungal disk 7mm from7 days old culture of fungal isolates. The plates were incubated at $28C^{o}$ for 7 days to allow growth of test fungi. They were then examined for their growth formation around the fungal test. The growth diameter was measured in each case. [21].

Secondary screening

The fungal isolates that gave biggest growth formation in primary screening were selected as the best degrader, and were grown in 100 ml Erlenmeyer flasks containing 25 ml liquid mineral salts medium with 100ppm naphthalene, pH was adjusted to 7, then autoclaved at $121^{0}C$ for 15 min, the sterilized media were inoculated with one fungal disk (7mm) from7 days old culture of fungal isolates. Duplicate for each isolate and control, then flasks were incubated in shaker incubator 120rpm for 7days at $30^{0}C$ [22].then naphthalene residue and total petroleum hydrocarbon were determined.

Determination of Naphthalene concentration by HPLC

After incubation, the flasks were removed and the cultures centrifuged at 10000 rpm for 20 min, then filtrates through whatman no.1 10ml of hexane was added to 25 ml of mineral medium and was shaken for 30 min in separate phennel then 1ml of upper phase (hexane) was shifted to the sterile tubes and was used for HPLC analysis [23]. HPLC analyses were performed with reverse-phase column C18 (Syknm Chromatography Products, Germany). Separation was achieved by isocratic elution in acetonitrile: water (70:30) respectively, with a flow rate of 1.0ml/min and UV absorbance detector set at 279nm.

Determination of total petroleum hydrocarbon (TPH) by Horiba.

After incubation the flasks were removed and the cultures centrifuged at 10000 rpm for 20 min. then filtrates through whatman no.1, 25ml of CCl_4 was added to 25 ml of mineral medium and was shaken for 30 min in separate phennel then 5ml of upper phase CCl_4 was shifted to the sterile tubes and was used to determine TPH using Horiba analysis [24].

Identification of Fungal Isolates

On the basis of their colony and morphological characteristics, the fungal isolates were identified. Lacto phenol solution was used as mounting fluid. The slides were observed under microscope and fungi were identified by following the mycological literature [25].

Optimal conditions for naphthalene biodegradation [26]

Incubation period.

To determine the best incubation period for each isolate ,(100 ml) Erlenmeyer flasks containing(25ml) of liquid mineral salts medium as in with(100ppm) Naphthalene pH was adjusted to 7 and autoclaved at 121^0C for 15 min ,then flasks Inoculated with 7mm fungal disk from selected isolates (one isolate for each flask).the flasks were incubated in shaker incubator120 rpm $30C^o$ at different periods(2, 4, 6, 8, 10, 12, 14 days),Experiment done in duplicate and flasks without inoculation used as control for each isolate**.**

Naphthalene concentration

To determine the best concentration for each isolate ,(100 ml) Erlenmeyer flasks containing(25ml) of Liquid mineral salts medium with different concentrations(100 ,200 ,300 ,400 ,500, 600 ,700 ,800 ppm), pH was adjusted to 7 and the media were autoclaved at 121^0C for 15 min, then flasks Inoculated with 7mm fungal disk from selected isolates (one isolate for each flask). Then flasks were incubated in shaker incubator 120 rpm at $30C^o$ for 8 days **.**Experiment done in duplicate and flasks without inoculation as control for each isolate.

Optimum pH

To determine the best pH for each isolate ,(100 ml) Erlenmeyer flasks containing (25ml) of Liquid mineral salts medium with(100ppm) naphthalene, the media were prepared at different pHs value (5 ,6 ,7 ,8) and the media were autoclaved at 121^0C for 15 min, then flasks Inoculated with 7mm fungal disk from selected isolates (one isolate for each flask).the flasks were incubated in shaker incubator 120 rpm at $30C^o$ for 8 days**.** Experiment done in duplicate and flasks without inoculation used as control for each isolate**.**

Optimum temperature

To determine the best temperature for each isolate ,(100 ml) Erlenmeyer flasks containing (25ml) of Liquid mineral salts medium with(100ppm) naphthalene, pH was adjusted to 7 and the media were autoclaved at 121^0C for 15 min, then flasks Inoculated with 7mm fungal disk from selected isolates (one isolate for each flask) and were incubated in shaker incubator 120 rpm at different temperatures (25 ,30 ,35 ,40, 45 ^{0}C) for 8 days **.**Experiment done in duplicate and flasks without inoculation as control for each isolate.

Results and discussions

Samples Collection and Fungal Isolation

Sixty four (64) soil samples were collected from eighteen (18) different contaminated **sites** with oil waste, with different periods from February to August 2012.Out of these samples, a total of 312 isolates were found, also different species belongs to Ascomycota and Deuteromycota were isolated from oil contaminated soil [27] this concluded Positive for the occurrence of fungi in oil contaminated soil table-1.

Table 1- Fungal isolates from samples of soil contaminated with waste oil from different regions.

S.no	Sites of Isolates	Sites Symbol	Samples	Isolation Date	Number of
1-	Kut City	KC	4	5/2/2012	13
2-	AL- *Zaafarani*a	AL-Z	3	15/2/2012	22
3-	Jesr Diyala	JD	4	26/2/2012	10
4-	Al-Tweetha	T	5	6/3/2012	14
5-	AL-Dora/Saha	DS	4	16/3/2012	24
6-	AL-Dora Refinery	DR	3	26/3/2012	11
7-	AL-Sadr City	SC	3	6/4/2012	16
8-	Ministry of Science and Technology	MST	6	16/4/2012	25
9-	Baghdad University Fuel Station	BF	2	27/4/2012	19
10	AL-Jadriyah (Generator)	JG	2	6/5/2012	20
11-	AL-Yarmouk City	Y	4	18/5/2012	15
12-	AL-Maamun City	MC	4	29/5/2012	23
13-	Al-Shu'ala	SH	6	9/6/2012	13
14-	AL-Saydiya	SA	2	20/6/2012	12
15-	AL-Dora/Al-Mekanek	DM	3	2/7/2012	21
16-	AL-Dora/60thStret	D60	4	13/7/2012	14
17-	AL-Dora/Asia	DA	3	24/7/2012	18
18-	AL-Rustamiyah Station	RS	2	6/8/2012	22
			Total 64		**Total 312**

Screening of the isolates for naphthalene biodegradation

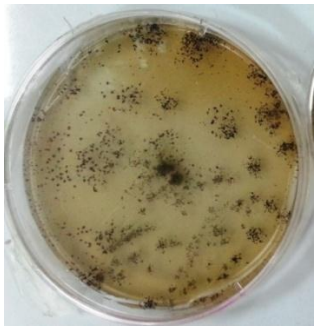
Primary screening

A total of 312 Pure fungal Isolates were tested for its ability to grow on solid MSM containing Naphthalene, The percentage of positive growth isolates was found to be 44.23% in samples collected from oil contaminated soil, while 55.77% of isolates show negative growth . the results shows 25 fungal isolates gave good growth (50-80mm diameter of fungal growth), 47 fungal isolates gave Moderate growth (20-49mm diameter of fungal growth), 66 gave weak growth (10-19mm diameter of fungal growth) and 147 fungal isolates gave no growth on Naphthalene solid media This may be attributed to the reason that fungal colonies are not growing on this medium because they do not possess the ability to degrade these compounds as a result of the lack of enzymatic system specialist, or due to low solubility of this compound which reduce availability to microorganisms [29].identical study done by [28] found failure of fungal isolates to grow on solid MSM with phenanthrene which attribute the degradation of compound containing more than one cycle is more resistant to oxidative enzymes figure-1-A,B,C,D table-2.

Table 2 -Primarily screening of fungal isolates on Solid Media with Naphthalene (100ppm).

S.no	Growth Development of Isolates	Isolates Number
1-	Good	25
2-	Moderate	47
3-	Weak	66
4-	No growth	174

Good: - Diameter of Fungal Growth (50-80mm)
Moderate: - Diameter of Fungal Growth (20-49mm)
Weak: - Diameter of Fungal Growth (10-19mm)



A-Good Fungal Growth

B-Moderate Fungal Growth

C-Weak Fungal Growth

D- No Growth

Figure 1, A, B, C, D- Fungal growth on solid media containing Naphthalene after 7 days at 28^0C

Secondary screening

Selected fungal isolates that displayed "good" growth on solid media containing Naphthalene test its ability to degrade naphthalene after a period lap for 7 days, the results showed the ability of all fungal isolates to degrade naphthalene table-3.

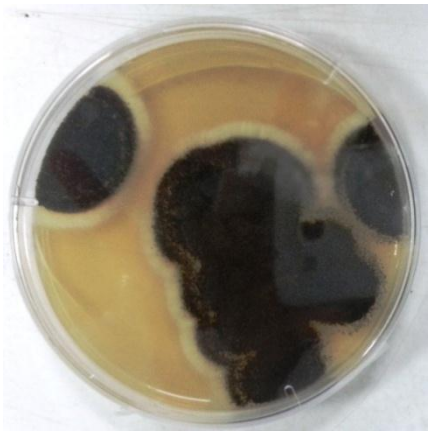
According to secondary screening results we selected the most active sites isolates which were (T1, MI2 and RU)**.**

Table 3- Secondary screening of Active fungal isolates in liquid mineral salt medium with 100ppm naphthalene, pH 7, 120 rpm.for7 days

S.no	Isolates Names(Strains)	Naphthalene Concentrations (ppm) by HPLC	TPH Amount (ppm) by Horiba
1.	JD1	24.5	25
2.	JD 2	41.6	42.3
3.	T1	12.4	12.9
4.	T2	22.3	22.9
5.	DS1	31	31.9
6.	DS2	56.85	57.1
7.	DS3	23.1	24,61
8.	DS4	34.54	35.4
9.	DR1	51.68	52
10.	DR2	36.0	37.9
11.	SC	22.9	23.2
12.	MST1	19.9	20.1
13.	MST2	11.9	12.2
14.	MST3	25.9	26.2
15.	JG	18.4	18.9
16.	Y1	22.1	22.8
17.	Y2	27.8	29.4
18.	MC	31.5	35.9
19.	DM	19.5	20.1
20.	D601	22.4	23.7
21.	D602	34.22	37.2
22.	DA1	32.6	33.7
23.	DA2	52.68	53.3
24.	RS	12.3	12.8
25.	SH	30.05	31.5

Identification of Fungal Isolates

the initial examination of the fungal colonies developing were done by using a dissecting microscope, and attended the slides for these colonies for the purpose of study their characteristics under an optical microscope compound. The results showed that most active isolates were *Aspergillus niger* figure-2-a,b *Trichoderma viridi* (3a and b), *Fusarium verticelloides* figure-4-a,b respectively [25]. These isolates were used for completion of experiments.

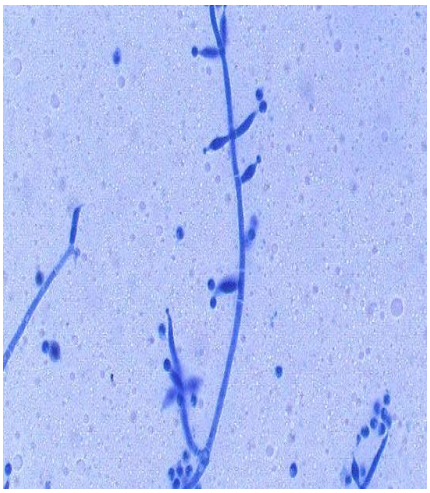


a- Macroscopic feature of *Aspergillus niger*

b- Microscopic feature of *Aspergillus niger*

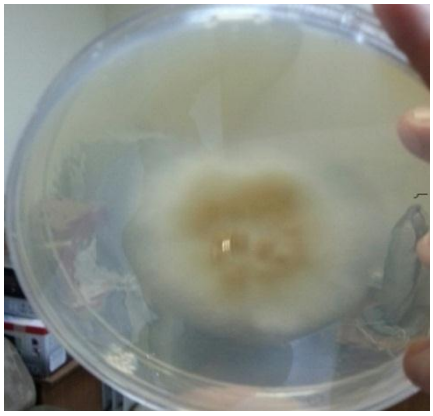
Figure 2, a, b:-Macroscopic and microscopic features of *Aspergillus niger*

b. Macroscopic feature of *Trichoderma viridi*

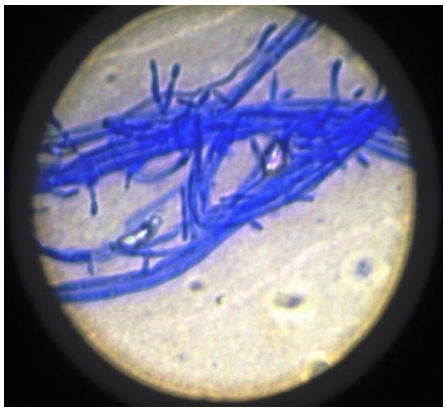


a. Microscopic feature of *Trichoderma viridi*

Figure 3,a,b:-Macroscopic and microscopic features of *Trichoderma viridi*



a Macroscopic feature of *Fusarium verticelloides*



b Microscopic feature of *Fusarium verticelloides*

Figure 4, a, b:-Macroscopic and microscopic features of *Fusarium verticelloides*

Optimal conditions for naphthalene biodegradationTime

The results showed that the best time for Naphthalene biodegradation was 8 days for all fungal isolates (*Aspergillus niger*, *Trichoderma viridi*, *Fusarium verticelloides*) the concentrations of Naphthalene decline from 100 ppm to 11.55 for *Trichoderma viridi*, 12.1 ppm for *Aspergillus niger* and to 12.22ppm for *Fusarium verticelloides* figure-5.

After 8 days, degradation occur but less than in 7 and 8 days because survival complex compounds such as nutrients, or low pH due to the accumulation of metabolites resulting from oxidation processes which are products of self toxicity (Autotoxic) cells, or to a lack of oxygen and lack of ventilation [30].

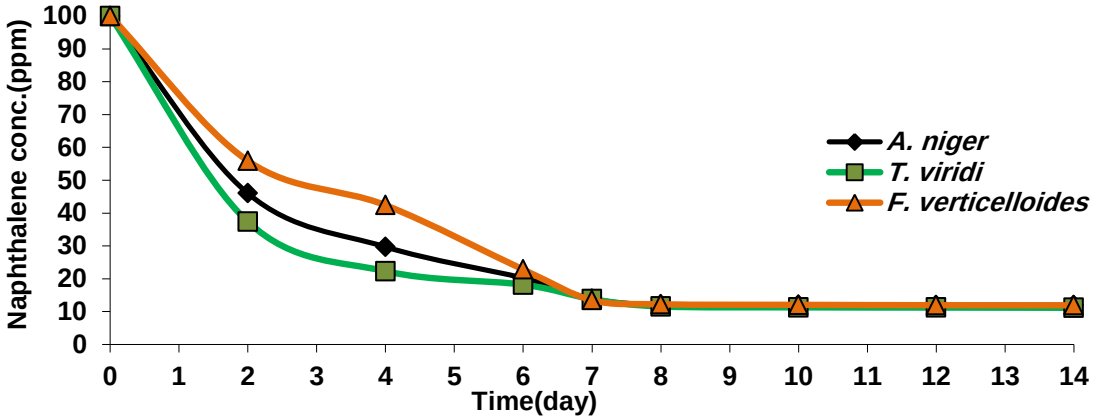


Figure 5- Optimal time for naphthalene biodegradation

Other researchers studied the effect of incubation period on PAH degradation. [31]. Display that *Aspergillus sclerotiorum* presented the best results of pyrene and benzo[a] pyrene degradation after 4 and 8 days, respectively**.**

In a previous study reported in the literature, the ascomycete *Cyclotirium* sp. was the most efficient fungus in degrading pyrene, with a rate of pyrene transformation of 48.5% after 6 days [12]**.**The zygomycete *Mucor racemosus* was able to degrade more than 40% of anthracene after 8 days [32].

pH

pH Culture medium can affect microbial diversity and activity and therefore investigations into the effect of enzyme activity .the results showed the best pH value for naphthalene degradation was 7 for all fungal isolate. The concentrations of Naphthalene decline from 100 ppm to 11.3 for *Trichoderma viridi*, 12.2 ppm for *Aspergillus niger* and to 12.4 ppm for *Fusarium verticelloides* figure-6.

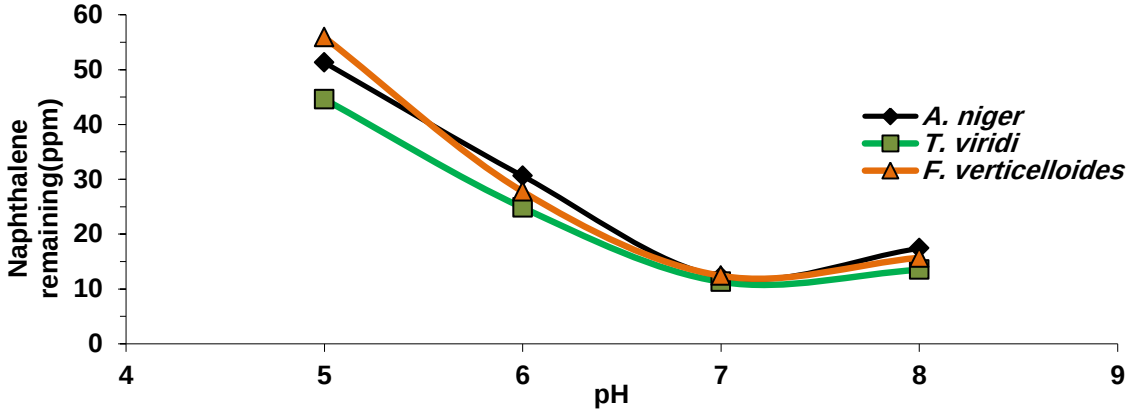


Figure 6- optimal pH for naphthalene biodegradation

In general, most heterotrophic bacteria and fungi favor a neutral pH level, and extremes in pH can adversely affect PAH degradation [33]. Hydrocarbon degradation was found to be optimal when the pH level was within the range of 6.5 and 8.0 [34]. These results are consistent with most studies, where microorganisms favored growth at pH levels ranging from 6.0 to 8.0 [35].

At pH 7, the mineralization of oily sludge in soil is also improved, thus, enhancing the overall biodegradation process [36].More than 90% of Naphthalene was degraded when the pH of the culture medium ranged from 6.0 to7.5. [37].

These results are consistent with most studies,where microorganisms favored growth at pH levels rangingfrom6.0to8.0. Increased acidity is most likely associated with the production of acidic metabolites, such as benzoic acid and phthalate during the biodegradation of Naphthalene [38].

Temperature

Temperature can play an important role in the biodegradation of organic pollutants by microorganisms [39].

The results showed that the best temperature for Naphthalene biodegradation was 30 ^{0}C for all fungal isolates, the concentrations of Naphthalene decline from 100 ppm to 11.3 for *Trichoderma viridi*, 12.5 ppm for *Aspergillus niger* and to 12.6 ppm for *Fusarium verticelloides* figure-7.

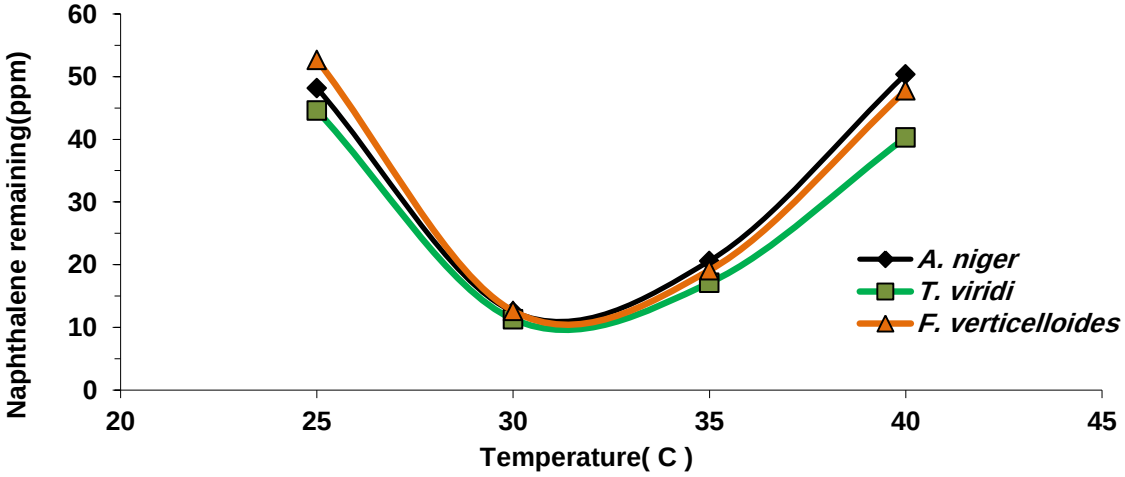


Figure 7- Optimal temperature for naphthalene biodegradation

Biodegradation of hydrocarbons has been reported from temperatures as low as 0˚C to as high as 70˚C [39]. At the optimum temperature 30^{0}C the solubility of naphthalene increases, this makes naphthalene bioavailability molecules for microorganisms [40]. Bioavailability, however, was lower when the temperature rose above 40 ◦C, indicating that the adverse effect of high temperature on the cell is more important than any increase in substrate availability [41]. As the temperature decreases, generally the rate of biodegradation also decreases due to reduced enzymatic activity [42].

At high temperature a reduction in metabolic activity of aerobic microorganisms due to the decline in oxygen solubility [3]. In addition, the properties of hydrocarbons may be altered at higher temperatures resulting in increased rates of diffusion, solubility and volatilization [40,42].The fungi, *Fusarium folciparum*, *Trichoderma* spp, *Trametes versicolor*,*Pleurotus ostreatus* degredade 5 PAHs anthracene, fluoranthene, pyrene, chrysene and phenanthrene under lab condition [43].

Concentration

Effect of initial naphthalene concentrations on biodegradation was also examined to assess fungal isolates capacity to tolerate different concentrations of naphthalene.

The results showed that the best concentration for Naphthalene biodegradation was 100ppm for all fungal isolates, the concentrations of Naphthalene decline from 100 ppm to 11.3 for *Trichoderma viridi*, 12.5 ppm for *Aspergillus niger* and to 12.6 ppm for *Fusarium verticelloides* figure-8.

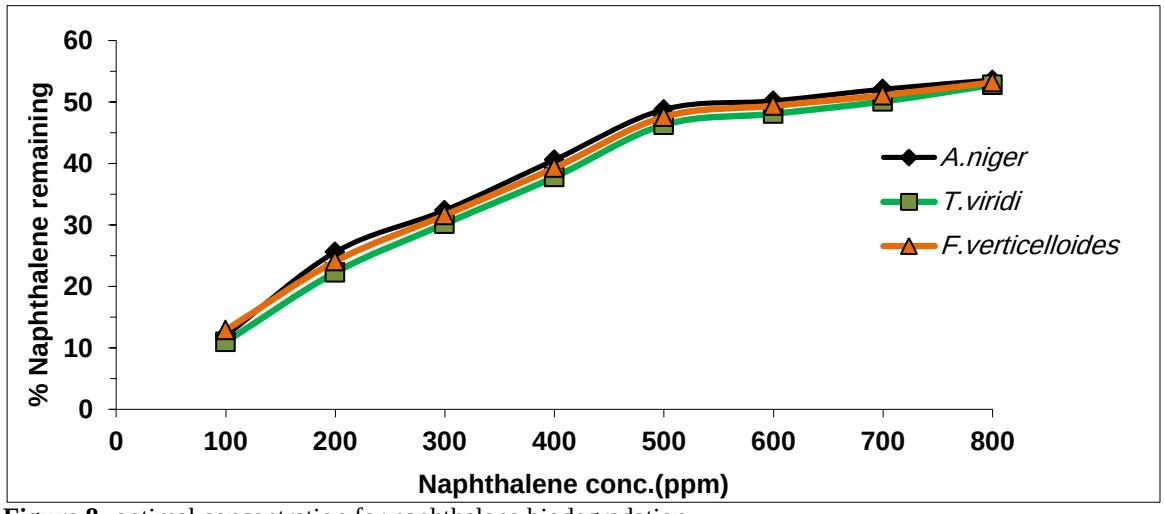


Figure 8- optimal concentration for naphthalene biodegradation

The concentration of a PAH also has a crucial impact on the potential success of microbial degradation. If PAH concentrations are too low, genes necessary for enzyme production may not be induced, and the enzymes necessary for degradation may not be produced [36]. However if PAH concentrations are too high, toxic effects can be exerted upon the cell and toxic metabolites may accumulate in the growth medium [44].

Fusarium spp. degraded 55% of 100ppm anthracene when the fungal isolate incubated in 100rpm under lab condition [45]. *Trametes versicolor* degraded 61% of 300ppm Phenanethrene when the fungal isolate incubated under lab condition [45].

PAHs adapted fungal strain *Aspergillus niger*, *Trichoderma viridi*, *Fusarium verticelloides*, isolated from the soil of petroleum refinery, have ability to degrade naphthalene, under optimum conditions pH 7, concentration of naphthalene 100ppm, temperature 30^0C best time was 8 days. Degradation rate was found high by these fungal isolates.

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