International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.9, No.08 pp 246-260, 2016

Degradation regime on interaction of hydrocarbons by a co-culture of *Psuedomonas fluorescens* (MTCC:8127) and *Pseudomonas putida*(MTCC:1192)

Vishwanathan K.S and Amala Reddy*

[1]Bioprocess Engineering lab, [1]Department of Biotechnology, School of Bioengineering, SRM University, Kattankulathur, India.

Abstract : Co-cultures of *Pseudomonas putida*(**MTCC:8127**) and *Pseudomonas fluorescens* (**MTCC:1192**) cultivated as a co-culture were analysed for their abilities to degrade benzene, toluene, ethylbenzene, and xylene (together known as BTEX) under suitable growth medium. The co-culture effectively degraded of BTEX as individual hydrocarbons, present as two hydrocarbons, three hydrocarbons and as sole carbon source (BTEX).However, when glucose is present as a carbon course, the growth regime was a diauxic growth, after the deprival of the primary source, hydrocarbons were utilized effectively. There was a maximum utilization of Benzene about 93.87% when present as a sole carbon source. It is also found that benzene in any combination with toluene showed the maximum degradation but slowed down under the influence of xylene.
Key words : BTEX, Co-culture,diauxic, *Pseudomonas putida, Pseudomonas fluorescens.*

1. Introduction:

Benzene, Toluene, Ethylbenzene, and para- Xylene collectively known as BTEX are widely used as industrial solvents for organic synthesis and equipment cleansing[2,43]. They are or accidentally released into the environment in large amounts and accumulate in soil and surface water, leading to serious damage to our living conditions [3,22,33]. BTEX are the major aromatic components in many petroleum products and are frequently found in groundwater and industrial waste streams[6, 9]. BTEX enters the environment in the form of industrial discharges from petroleum refining, plastic, resins and pharmaceutical industrial effluents or oil spills[9,12,44]. BTEX also finds wide application as a base material for the preparation of styrene that is used as a solvent for coatings and in making rubber and plastic wrap[6,8]. BTEX are important industrial raw materials for paints, pesticides, resins, fiber glass unit, varnish, phenolic resin manufacture, textile unit, making of organic dyes and are also used as solvents for rubber and plastic . BTEX are highly toxic and carcinogenic compounds commonly found as contamination linked to human activities[6,8]. Among these benzene is more carcinogenic, owing to its high volatility, higher water solubility with respect to other aromatics, and high mobility. BTEX is also the majority of compounds present in crude oil[47]. The conventional physical or chemical treatment methods that are used to decontaminate the BTEX create secondary effluent problems and are not cost effective. Thus, biological treatment processes are probably the alternate effective and eco-friendly technologies for treating aqueous waste streams containing organic compounds.

Microorganisms are able to degrade BTEX under aerobic, micro-aerobic or hypoxic, as well as anaerobic conditions[30,31]. Hydrocarbons in the environment are biodegraded primarily by bacteria, yeast, and fungi[7,10,46]. Bacterial cultures give a promising solution for degradating BTEX[17,26]. Bacteria's like *Pseudomonas*

*Arthobacter, Alcaligenes, corynebacterium, Flavobacterium, Achromabacter, Micrococcus, Nocardia, Mycobacterium, Burkholderia, Sphingomonas, and Rhodococcus*were found to be involved for alkyl aromatic degradation[11,18].*Pseudomonas* genus is one of the most extensively studied bacterial genera for bioremediation[25,37.39]. Even amongst the genera, *P.putida*, since it's discovery in 1971 is one of the most widely used for bioremediation[18,34,36]. It has been shown to degrade Naphthalene, and other Polycyclic Aromatic Hydracarbons (PAHs) and other inorganic toxic substances like Cyanide and Cadmium amongst others[19,41,42]. XIt is also used in plant growth as it is able to reside in the plant seed and rhizosphere, the plant is in turn protected from plant pathogens and able to obtain vital nutrients from the bacteria[18]. It be used as a biocontrol agent as it can be used for its antagonistic damping of diseases such as Pythiumand Fusarium.

It is also used as a biopesticide against dreissenidmussels and zebra and quagga mussels[10]. It is also known for it ability to bioremediate crude oil. Studies prove that Pseudomonas species are best in degradating of organic components[29,32]. In this work, BTEX biodegradation by the co-culture of *P. putida and P. fluorescens* was studied using a synthetic medium containing BTEX as sole carbon sources[18,20]. Catabolic degradation of BTEX via two typical pathways (the *tod* and the *tol* pathways) has been extensively studied[35].

2.Materials And Methods

2.1 Co-Culturing of P. Fluorescene and P.Putida

The both the microorganisms *pseudomonas putida* and *pseudomonas fluorescence* were co-cultured first in LB broth and then for experimental purposes in minimal salt medium whose composition is $MgSO_4.7H_2O$-0.5gms,$NH_4(SO_4)$-0.5gms,K_2HPO_4-0.5gms,KH_2PO_4-0.5gms,$MnSO_4.H_20$-10mg,$CaCl_2.2H_2O$-9.8mg,$Fe(NH_4)_2(SO_4)_2.6H_20$-8mg,$ZnSO_4$-2mg, Nitrilotriacetic acid- 20mg in 100ml of de-ionized water[23,24,28,].

2.2 Growth Standard Lipase Assay

Standard Lipase assay was performed using the titrimetric method[4,15]. For these two beakers one marked test and the other marked blank were taken. In the blank beaker 2.5ml of de-ionized water, 1ml of Tris-HClbuffer(pH-7.5) ,3ml of olive oil (as substrate) were added . In the beaker marked test , in addition to what were added in blank, 1ml of enzyme(lipase) solution was added. Both beakers were incubated at 37˚C for 30mins. After incubation 3ml of 95% ethanol was added to arrest the reaction following which they were titrated against 50mM NaOH. This was followed for various different concentrations of enzyme so as to plot the standard lipase activity curve. The activity of the enzyme was found out using the equation

$$Activity = \frac{\{(vol\ of\ NaOH)(\mathbf{Molarity}\ of\ NaOH)(1000)(2)(dilution\ factor)\}}{(volume\ of\ enzyme\ solution)}$$

2.3 Experimental Deisgn

To these the various combinations of BTEX were added. The experimental degradation were divided into group 1 which included one component only either benzene(B), toluene(T), ethyl benzene(E) or xylene(para)(X), Group 2 included 2 components in combination-BT,BE,BX,EX,TE or TX, group 3 included 3 components BTE,BTX,TEX or BEX and group 4 included all the 4 components BTEX.

2.4 Growth and Lipase Activity Measurement

Growth of the co-culture was found out by measuring optical density at 600nm in a UV-Visible spectro-photometre every 2 hours. The lipase activity was checked every 2 hours using the titrimetric method mentioned earlier[16,21].

2.5 Degradation

Degradation was carried out at room temperature(37˚c), pH of 7.5 and150rpm. Degradation of the aromatic compounds were analyzed using High performance liquid chromatography, the mobile phase for which is methanol: water in the ratio of 60:40 run at a flow rate of 2 ml/min and detected at wavelength of 210nm.The injection volume was 10µl. Percentage degradation was calculated and graph was plotted[1,38].

3. Results

3.1 Standards

The standard curve for lipase was plotted against the calculated enzyme activity in U/ml versus the prepared concentration of enzyme in mg/ml and the R^2 value was calculated to be 0.9896 which meant that the data fit well with the statistical model and were very good. The HPLC standards were run in two different mobile phases. They were 40:60 of Methanol : Water and 40:60 of 1%Acetic acid :Water. The retention times were noted as follows, Benzene-12.573 minutes and 8.231 minutes, Toluene-21.321 minutes and 11.173 minutes, Ethylbenzene-36.387 minutes and 15.386 minutes, Xylene-35.291minutes and 14.722 minutes.

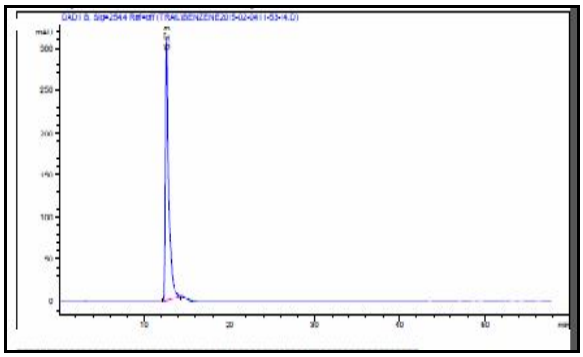


Fig 3: Benzene standard chromotogram

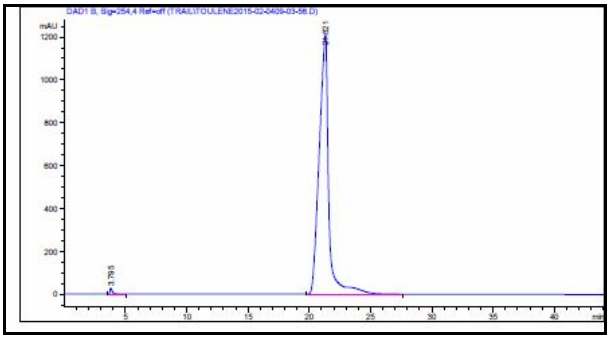


Fig 4:Toulene standard chromatogram

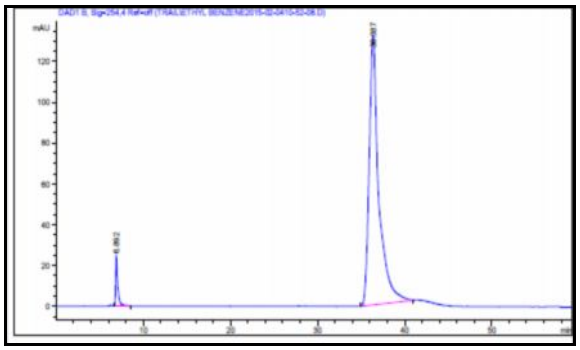


Fig 5: Ethyl benzene standard chromotogram

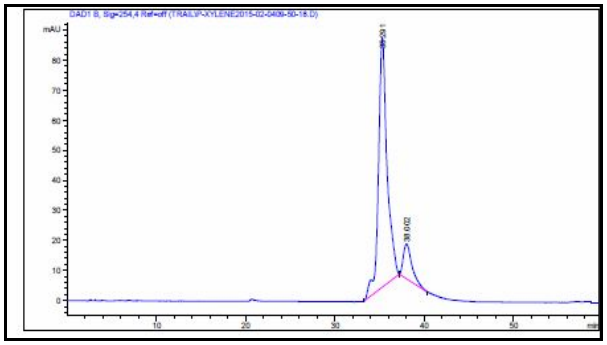


Fig 6: p-Xylene Standard Chromotogram

3.2 Growth Curve Regime:

3.2.1 Pattern of microbial co-culture growth in specific media for group I:

When one component was taken at a time, the growth curve will indicate the growth of the co-culture on that aromatic hydrocarbon alone. The log phase, which is the period of active multiplication of the bacteria, was attained slower than that of the growth when grown on a nutrient medium like LB Broth. It took an average of 8.5 hours (B – 10 hours, T,E,X – 8 hours) to get acclimatised to the media ,ie., to complete the lag phase. This was almost 4 times that of the lag phase period when grown on nutrient medium (2.3 hours). The active growth continued till 71 hours (B - 55 hours, T – 70 hours, E –90 hours, X –70 hours) and reached a maximum OD of around 0.8 for toluene. The minimum OD was seen in the flask containing xylene, which was found to be 0.6. In the nutrient medium, the maximum growth was found to be at 40 hours and was found to be an OD of 2.0. The decline phase started at 160 hours for almost all the flasks. Lipase concentration was found to maximum on the flask containing toluene and was found to be 623.8 µg/ml while the minimum was in the flask containing 509.5 µg/ml in the flask containing xylene. (Fig 1,2,3&4)

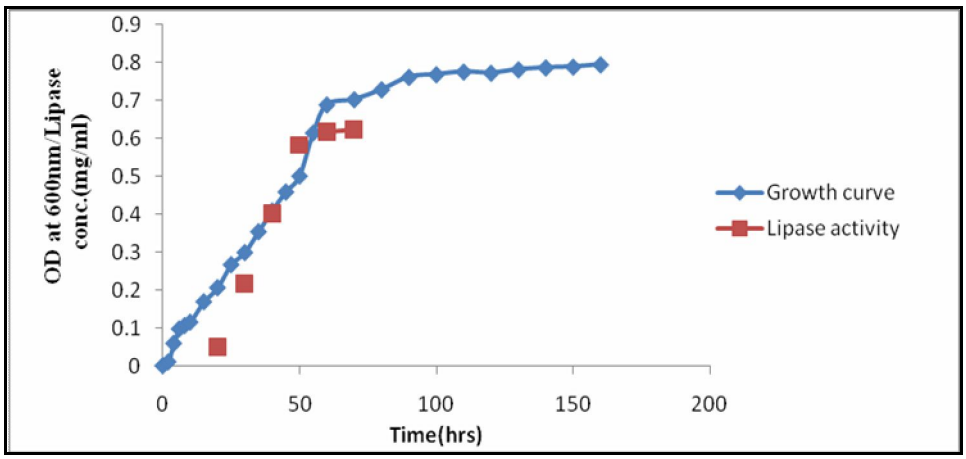


Fig 1:Growth curve of co-culture on T/Lipase activity

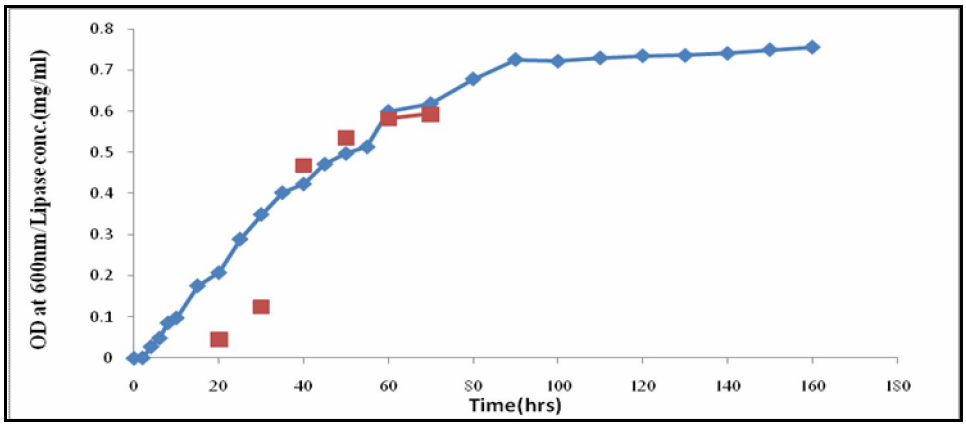


Fig 2:Growth curve of co-culture on E/Lipase activity

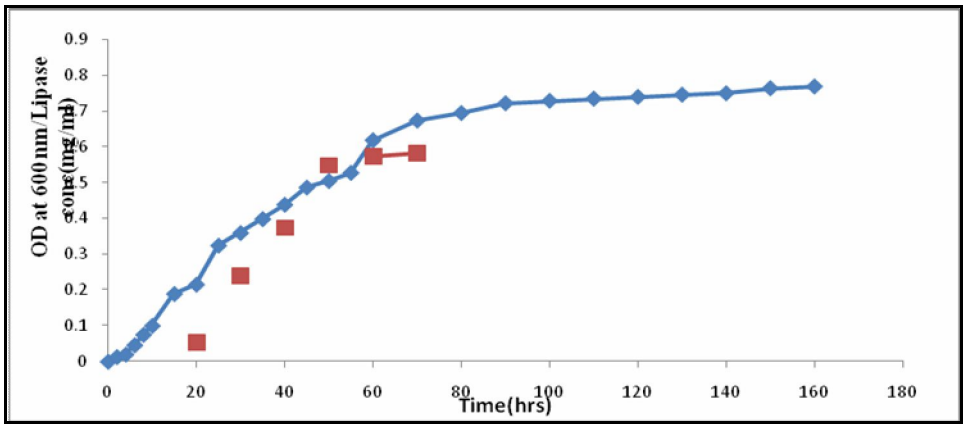


Fig 3:Growth curve of co-culture on B/Lipase activity

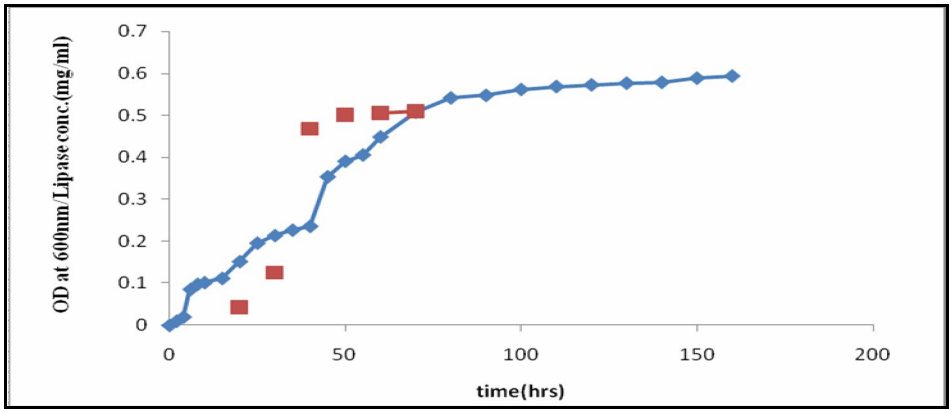


Fig 4:Growth curve of co-culture on X/Lipase activity

3.2.2 Pattern of microbial co-culture growth in specific media for group II:

When two components were taken together, the growth the maximum growth was seen in the flask containing benzene and toluene at 160 hours at an OD of 0.823 after which the OD started to decrease. The minimum growth was seen in the flask with benzene and xylene, followed by the flask with ethyl benzene and xylene, 0.583 and 0.627 respectively. The percentage degradation of the respective substrates are as follows. BE- 94.15% ,BT-93.28% ,BX-73.12% ,TE-92.84% ,TX-82.095% and EX-85.25% (Fig: 5). The maximum lipase was found in the flask containing B and T ,which was also the all-time highest, and was found to be 631.1µg/ml. The flask containing B and X had the least lipase of 461.6µg/ml. the rest of the flasks had the following concentration of the enzyme. BE- 586.7µg/ml, TX-521.4µg/ml, ET-588.3µg/ml, EX-489.4µg/ml. The flask having B and T the least time in lag phase of 8 hours and the longest to attain decline phase that is 180 hours (Fig:6).

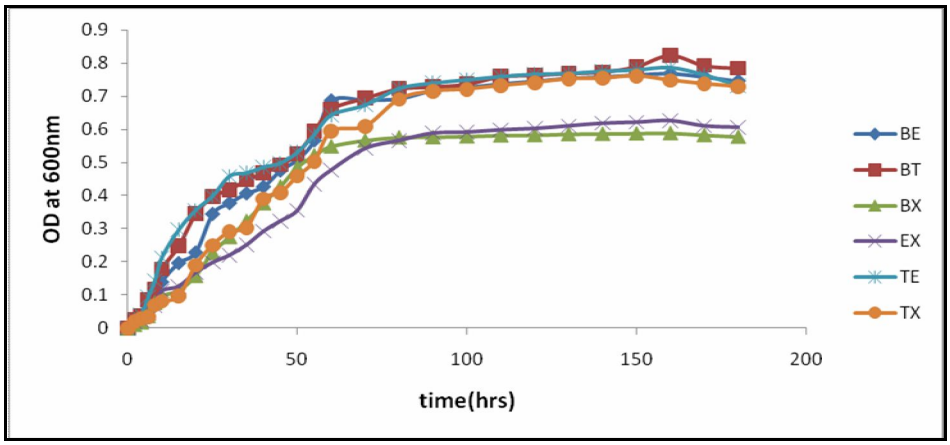


Fig 5:Growth curve of co-culture on Group 2

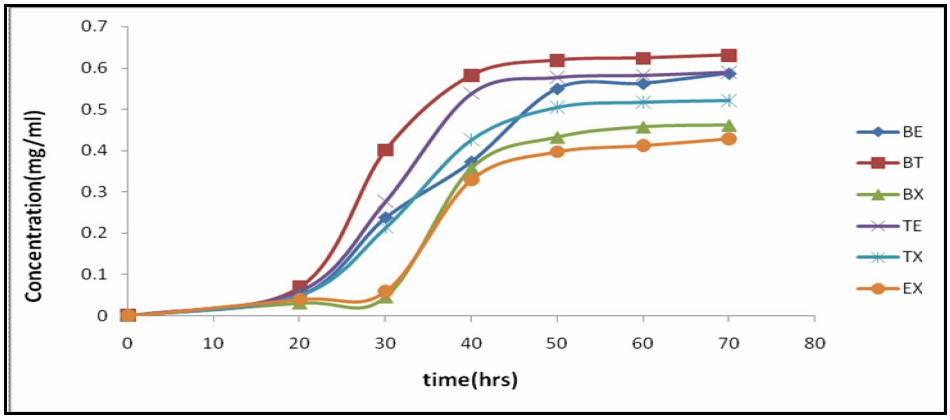


Fig 6:Lipase activity of co-culture on Group 2

3.2.3 Pattern of microbial co-culture growth in specific media for group III:

There are four ways of taking those components three at a time. They are BET, BTX, BEX and TEX. Maximum growth was seen in the flask that lacks xylene. It was found to be an OD of 0.831 at 600nm. It was followed by BTX which was 0.801. The second leastgrowth was seen in the flask containing TEX, which was 0.742 .The least growth was found the flask that did not have toluene. At 160 hours, the OD in that flask was found to be as low as 0.572 (Fig:7). The maximum lipase concentration was found in the flask which had maximum growth in this group and was found to be 582.6µg/ml. The lipases in The flask BTX and TEX were present in very close concentration of 516.8µg/ml and 526.7µg/ml respectively. The least lipase 457.2 µg/ml ,was found in the flask where there was least growth , that is BEX(Fig:8).

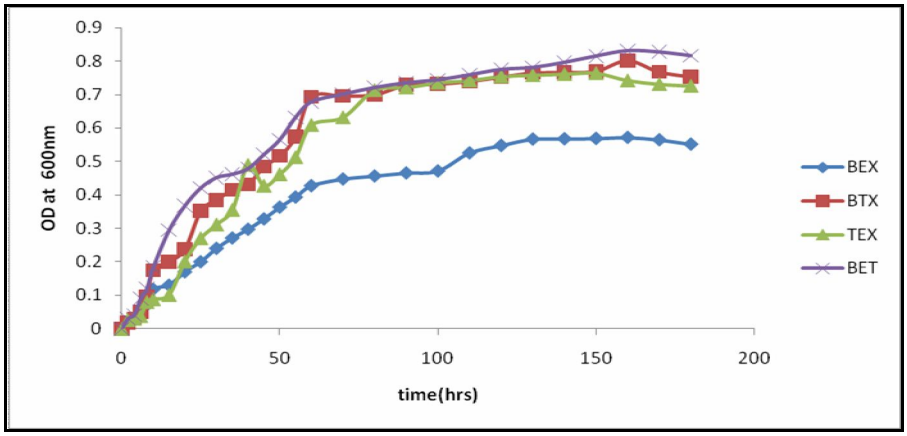


Fig 7:Growth curve of co-culture on Group 3

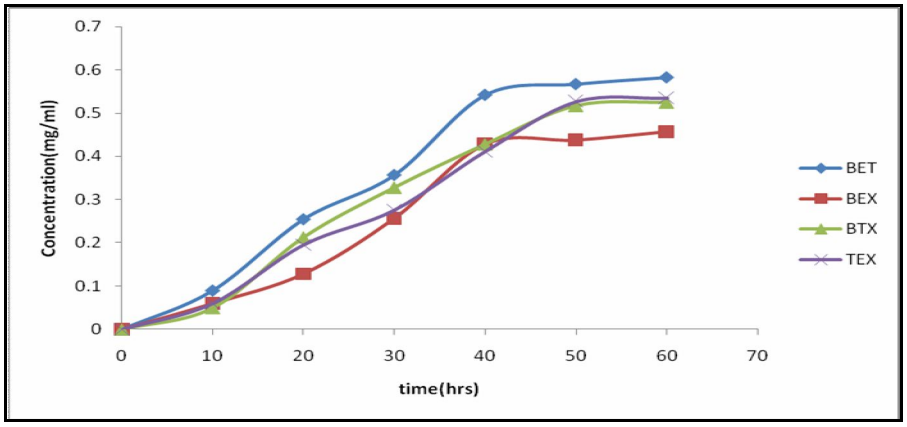


Fig 8:Lipase activity of co-culture on Group 3

3.2.4 Pattern of microbial co-culture growth in specific media for group IV:

This is the group where all the four components are taken at once. Here the log phase was attained at 10 hours and continued till 60 hours. There was rapid growth ended with a maximum OD of 0.778.The stationary phase continued till 160 hours after which it started to decline rapidly(Fig:9).The lipase in this mixture was found to be as concentrated as 597.1µg/ml (Fig: 9).

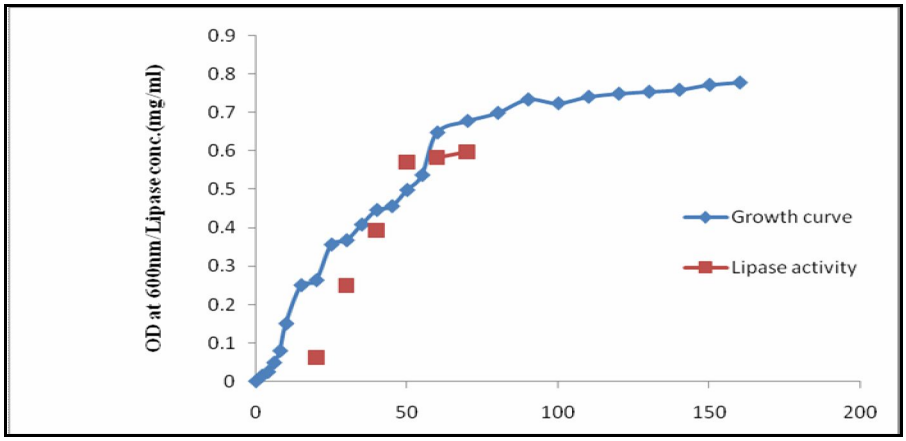
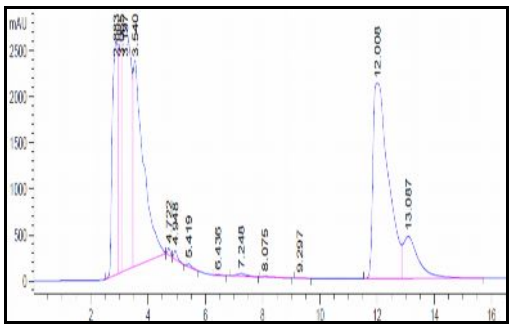


Fig 9:Growth curve and lipase activity of co-culture in group 4

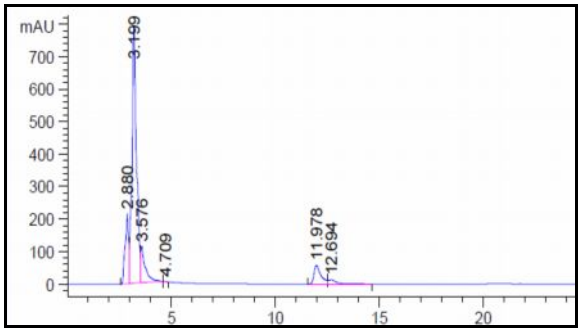
4. Degradation Studies:

4.1 Group I: Reduction pattern of B,T,E&X with respect to time

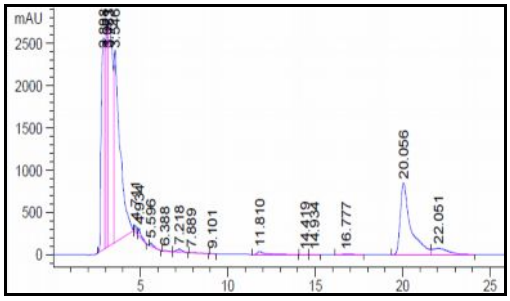
Benzene,Toluene, Ethyl benzene and xylene was observed for degradation pattern in High performance liquid chromatography with the mobile phase of methanol:water in the ratio of 60:40 ,run at a flow rate of 2 ml/min and detected at wavelength of 210nm.The above said hydrocarbons were observed for degradation by comparing the initial concentration in Day1 and the final concentration of substrates in Day7 respectively.The degradation was found to be 93.87%, 77.78%, 85.79 % and 77.93 % for B,T,E and X respectively (Fig10).



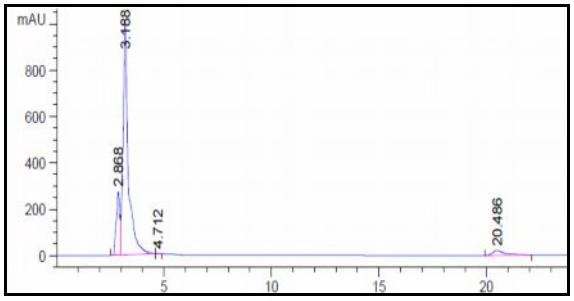
Degradation of Benzene in DAY1



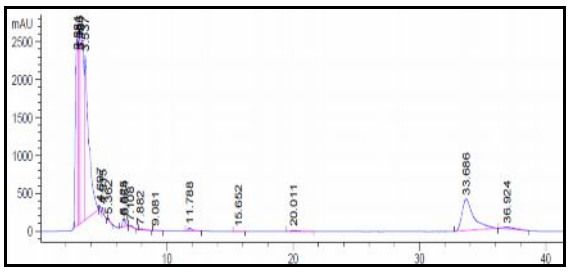
Degradation of Benzene in DAY 7



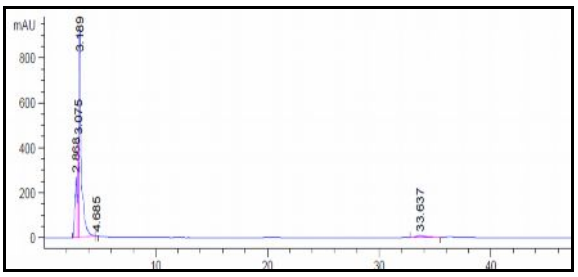
Degradation of Toluene in DAY 1



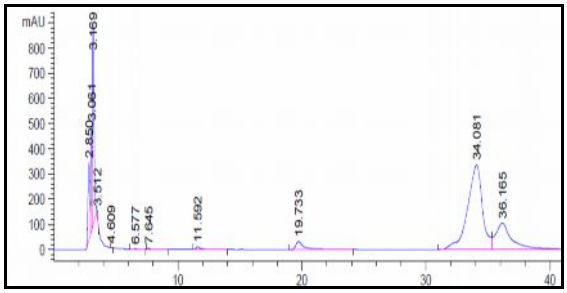
Degradation of Toluene in DAY 7



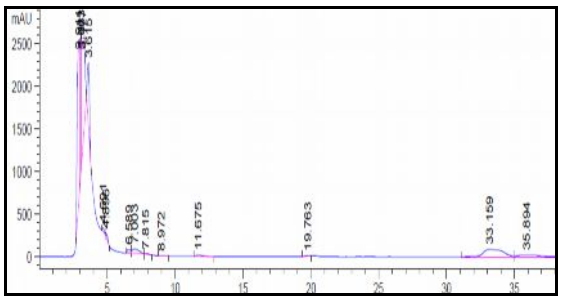
Degradation of Ethyl benzene in DAY 1



Degradation of Ethyl benzene in DAY 7



Degradation of Xylene in DAY 1



Degradation of Xylene in DAY 7

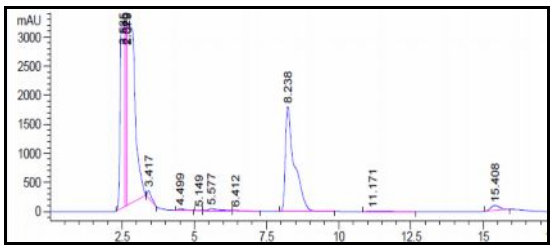
Fig10 : Degradation of single hydrocarbon in day1 and day 7.

Overall Summary Of Degradation In Group I:

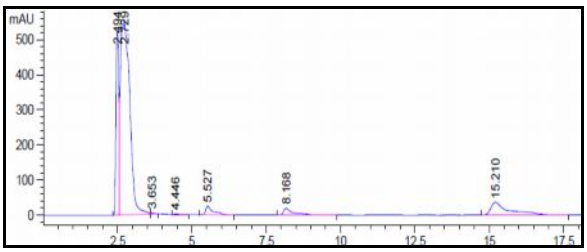
Substrate	Log Phase(hrs)	Stationary Phase(hrs)	Decline Phase(hrs)	Maximum Enzyme Concentration (µg/ml)	Amount of Substrate Degraded
BENZENE	10	55	160	581.9	93.87 %
TOLUENE	8	70	162	623.8	77.78 %
ETHYL BENZENE	10	90	160	593.5	75.79 %
XYLENE	10	70	158	509.5	79.33 %

4.2 GROUP II: Reduction pattern respect to time

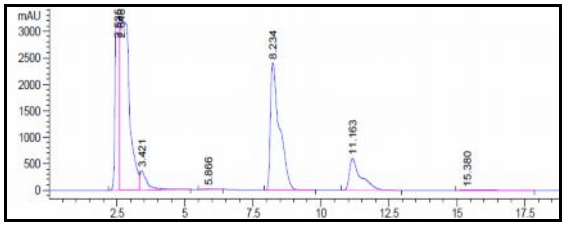
Benzene,Toluene, Ethyl benzene and xylene in combination with one another was observed for degradation pattern in High performance liquid chromatography with the mobile phase of methanol:water in the ratio of 60:40 ,run at a flow rate of 2 ml/min and detected at wavelength of 210nm.The above said hydrocarbons were observed for degradation by comparing the initial concentration in Day1 and the final concentration of substrates in Day7 respectively. The degradation was found to be 93.28%,94.15%, 73.12 %,83.09%,92.8% and 85.2 % for BT,BX,BE,XT,TE and EX respectively (Fig: 11).



Degradation of BT in DAY 1



Degradation BT in DAY 7



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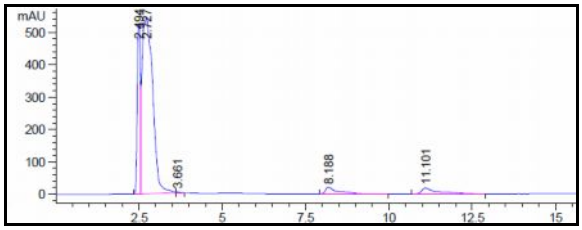
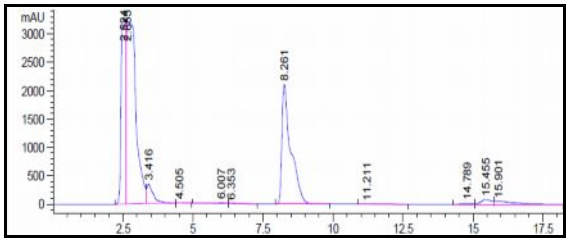
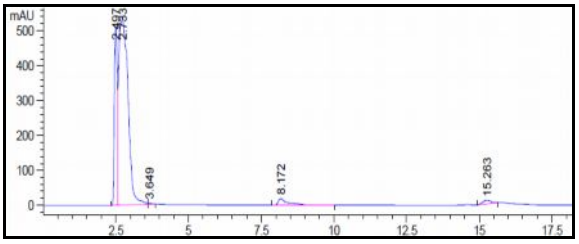


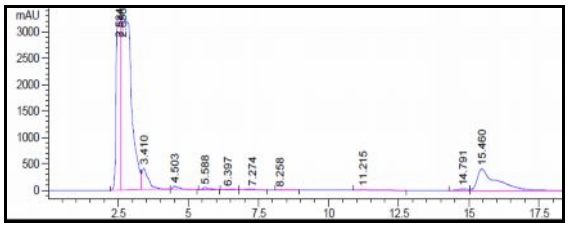
Degradation of BX in DAY 7



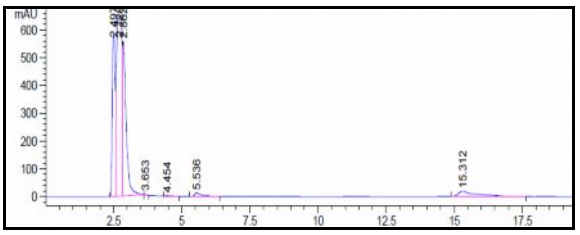
Degradation of BE in DAY 1



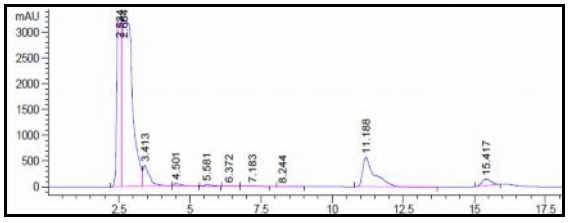
Degradation of BE in DAY 7



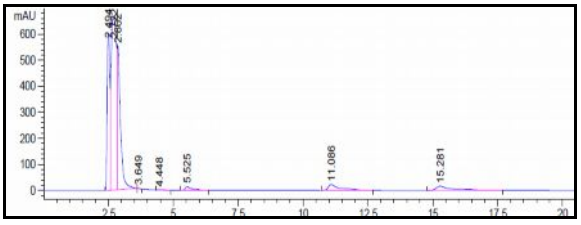
Degradation of XT in DAY 1



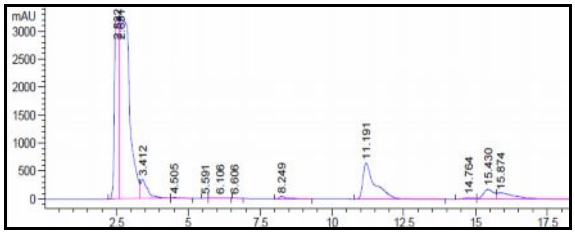
Degradation of XT in DAY 7

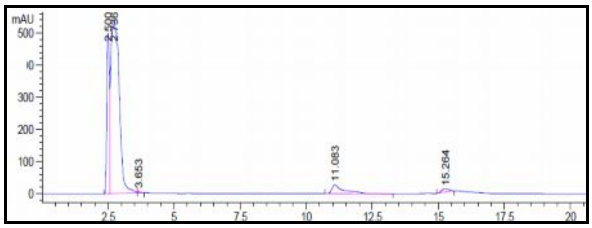


Degradation of TE in DAY 1



Degradation of TE in DAY 7





Degradation of EX in DAY 1 **Degradation of EX in DAY 7**

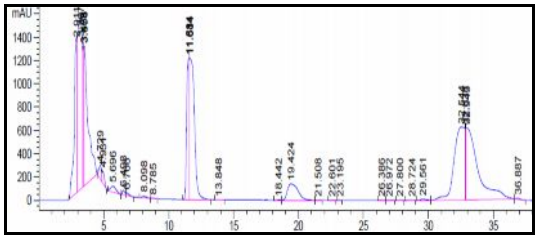
Fig11 :Degradation of dual combination of hydrocarbon in day1 and day 7

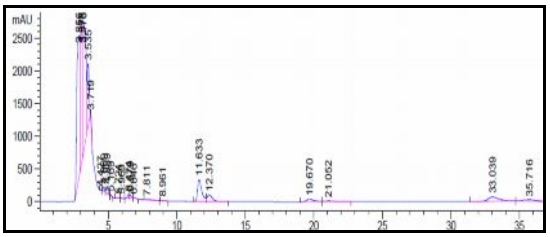
Overall Summary of Degradation In Group II:

Substrate	Log Phase(hrs)	Stationary Phase(hrs)	Decline Phase(hrs)	Maximum Enzyme Concentration (µg/ml)	Amount Of Substrate Degraded
BT	8	80	180	631.1	93.28
BX	10	80	170	586.7	94.15
BE	15	60	170	461.6	73.12
XT	20	80	160	521.4	82.095
TE	8	80	170	588.3	92.84
EX	10	80	170	489.4	85.25

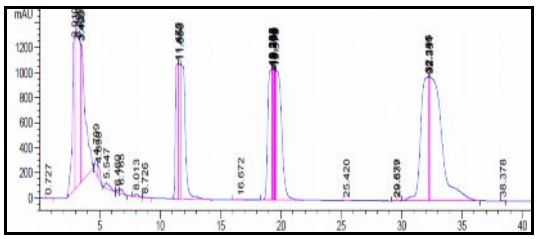
4.3 GROUP III: Reduction pattern respect to time

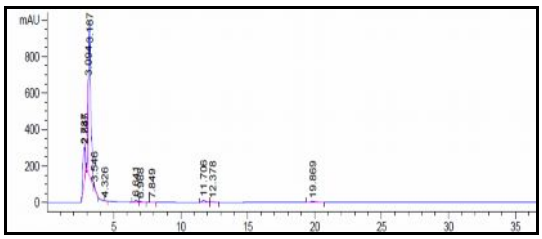
Degradation of BET,BTX,TEX and BEX was observed in High performance liquid chromatography with the mobile phase of methanol: water in the ratio of 60:40 ,run at a flow rate of 2 ml/min and detected at wavelength of 210nm.The above said hydrocarbons were observed for degradation by comparing the initial concentration in Day1 and the final concentration of substrates in Day7 .The maximum degradation is seen in the flask containing BET and was as high as 94.516 %. The flask containing BTX had 85.53 % degradation of hydrocarbons while the flask having BEX showed 83.5% consumption of the hydrocarbons. The flask containing TEX had 81.22% of it's hydrocarbons degraded (Fig :12).



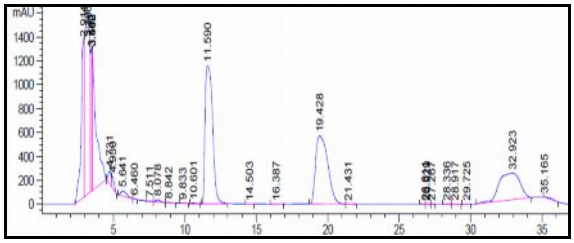


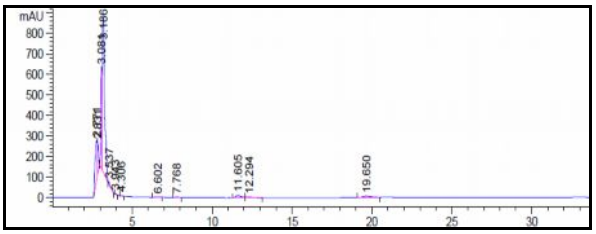
Degradation of BET in DAY 1 **Degradation of BET in DAY 7**



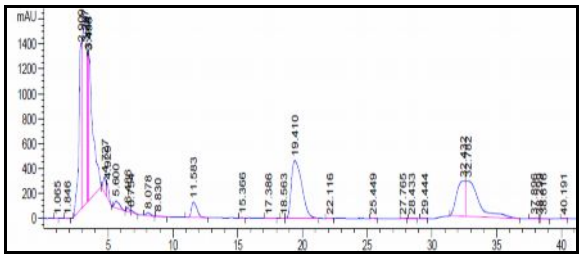


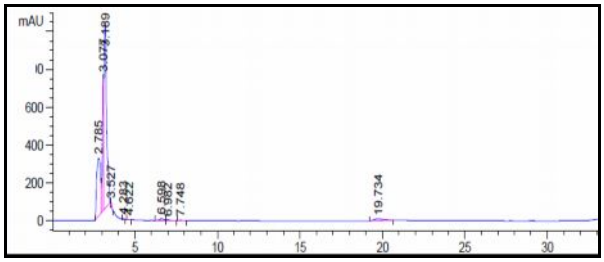
Degradation of BTX in DAY 1 **Degradation of BTX in DAY 7**





Degradation of TEX in DAY 1 **Degradation of TEX in DAY 7**





Degradation of BEX in DAY 1 **Degradation of BEX in DAY 7**

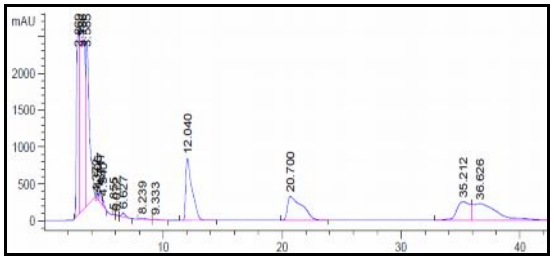
Fig12:Degradation in combination of three hydrocarbon in day1 and day 7

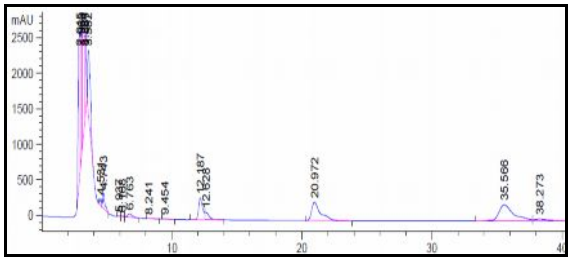
Overall Summary of Degradation in Group III:

Substrate	Log Phase(hrs)	Stationary Phase(hrs)	Decline Phase(hrs)	Maximum Enzyme Concentration (µg/ml)	Amount of Substrate Degraded
BET	6	110	170	582.6	94.516
BTX	8	90	170	524.7	85.53
TEX	8	90	170	535.1	81.22
BEX	8	110	170	457.2	83.5

4.4 GROUP IV: Reduction pattern respect to time:

Degradation of BTEX was observed in High performance liquid chromatography with the mobile phase of methanol: water in the ratio of 60:40 ,run at a flow rate of 2 ml/min and detected at wavelength of 210nm.The above said hydrocarbons were observed for degradation by comparing the initial concentration in Day1 and the final concentration of substrates in Day7 .When we look at the degradation of the hydrocarbons there was 89.85 % reduction of the hydrocarbons at the end of 7 days (Fig:13).





Degradation of BTEX in DAY 1 **Degradation of BTEX in DAY 7**

Fig13 :Degradation of all four hydrocarbon in day1 and day 7

5. Discussion:

Pseudomonas species has a greater adaptability to its environment as they a vivid in growth in the presence of hydrocarbons[25,27,40]. Co -cultures have shown to be better in the process of bioremediation than individual cultures.When phenantherene was degraded by bacteria, they achieved around 20% removal while fungi achieved 35-50 % removal. When they were co- cultured they achieved 73.61± 6.38 % of degradation[45].Here on using the co culture we were able to achieve 89.85% of BTEX degradation in 7 days. The reason for performing the degradation in different groups is to study the interaction of one component on another. By performing the growth in LB Broth, we were able to establish a base line of the normal growth of the co culture. When a Minimal Salt Medium (MSM) with the hydrocarbons Benzene,Toluene,Ethyl benzene and Xylene as their sole source of carbon and energy theacclimatising phase on a averagearound 10 hours for all the samples. This was depicting that BTEX were not it's priority source of carbon and energy,they would rather prefer Glucose (present in nutrient broth) to these hydrocarbons, showing a diauxic behaviour[13,14].Once there was a deprival of glucose, there was a stress condition to use the alternativesource of energy, the co-culture was forced to uptake these hydrocarbons BTEX for their survival. The mode of metabolism of these hydrocarbons has been studied well and it was found that there were two major pathways involved in this process, TOD and TOL pathway[35]. In the TOD pathway Xylene and Ethyl benzene are metabolised to their respective catechol derivatives. Amongst all the groups BX had the least bacterial growth, which was closely followed by EX. The maximum growth was seen in the group three flask containing BET which was almost half of the growth of the co- culture on nutrient medium. This data suggested that Xylene is the least preferred source for the bacterial co culture. As to why this happens is for further study.

As the organisms can only metabolise the fatty acid and they are not evolved to consume all the complex PAH's as a source of their energy. Also it is important to understand the role lipases, as the PAH's are too big to pass into the cell and hence be acted upon by intracellular lipase. The maximum concentration was found to be 631.1µg/ml, in the flask containing Benzene and Toluene alone. It was closely followed by the flask containing Toluene alone, which had 623.8µg/ml of lipase. The least lipase concentration of 461.6µg/ml was found in the flask containing Benzene and Xylene, followed by 489.4µg/ml in the Ethylbenzene and Xylene flask. This data, also corresponds to the data from growth curve, suggests that in the presence of Xylene, there is very less amount of lipase produced. This might suggest that the lipase produced by the co culture of *Pseudomonas fluroscence*(MTCC-8127) and *Pseudomonas putida*(MTCC-1192) is not susceptible to degradation by xylene. It is also non-tolerant to the rest of the hydrocarbons under study, namely Benzene, Toluene and Ethylbenzene. The reason for the lesser activity of the lipase produced by the co culture of *Pseudomonas fluroscence*(MTCC-8127) and *Pseudomonas putida*(MTCC-1192) in the presence of xylene also requires a close look in further studies.

When we look at the process of degradation closely, we will find that when benzene alone was taken as the substrate for growth, there was a 88.87% degradation of benzene. In group 2, when benzene was taken in combination with toluene, there was 96.57 % degradation in the total benzene present in the system. In group 3, when benzene was taken with toluene and ethylbenzene, there was 94.16% reduction of benzene. This clearly suggested that the presence of toluene is having a positive effect on the degradation of benzene. There was a 7% increase in the consumption of benzene, when toluene was also present, when compared to the system when benzene alone. When we look at Benzene and ethylbenzene, they both are degraded in a very similar manner. In group 1, ethyl benzene was degraded to almost 86% which itself very close to the amount benzene was degraded. In group 2, the flask where B and E are taken, we can see this more clearly. The rate of degradation of benzene and ethylbenzene is very similar.Ethylbenzenegets degraded to 96.13%, when compared to benzene which was degraded by 93%, which suggests that they both are preferred equally. Toluene had the upregulating effect on the degradation of ethylbenzene also. When T and E were present, there was 92.01% of degradation of E which was a 6% rise in the consumption of Ethylbnezene. Toluene was also degraded more in the presence of both B and E. In group 1, there was only 77.87% consumption of toluene, which was very less when compared to TE flask where T was degraded by 93.67% or in BT flask where it was degraded to 90.02%. In group 3, the flask containing BET all three components were degraded very extensively and closely. The percentage degradation were as follows, B - 94.16%, E – 94.52 %, T – 94.87%. This confirmed that the presence of toluene and toluence itself was degrading faster and enhancing the degradation regime of other hydrocarbons.

Xylene showed a negative feedback as there was a reduction in degradation of benzene in the presence of xylene. When B and X were present, benzene was degraded only 77.27%. In the EX flask, there was 87.33%

degradation of ethylbenzene which was very close to the original degraded amount of ethylbenzene in group 1 (85.59%). In the BEX flask, the consumption was not affected significantly. The consumption of xylene was unaffected by the presence of benzene. In X flask there was 79.33%, and in BX flask there was 75% reduction of xylene. In EX flask, there was 83% degradation of xylene which was 4% higher. Thus there was an increase in the consumption of xylene in the presence of ethylbenzene. Thus we conclude that the consumption of xylene was not affected by the presence of toluene. In TX flask, there was 80% reduction of xylene and 83.3% of toluene. This suggested that even toluene was not affected by the presence of xylene. These results were reflected in other group 3 flasks. In BTX flask, Toluene increased the consumption of Benzene and the consumption of xylene was unaffected. These observation hold good even in TEX flask and BEX flasks.

6. Conclusion

Our findings clearly indicate that presence of toluene in any combination of hydrocarbons achieved a maximum degradation and presence of xylene was deteorating the percentage degradation comparatively. The reason for these interaction was unclear and needs further investigation.

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