STUDY OF REMOVAL BTX BY PSEUDOMONAS PUTIDA IN BIOREACTOR

MOHD ASHRAF BIN ABDUL RAHMAN

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UNIVERSITI MALAYSIA PAHANG

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ABSTRACT

Benzene, toluene and xylene isomers (BTX) are the major components of gasoline which it is used widely as solvents and feedstock by the chemical industry. It became a common environmental contaminant problem that represents a serious threat to groundwater resources and to public health, being both toxic and relatively water soluble compared to other petroleum constituents. The biological treatment that use microorganism such as *Pseudomonas putida* has been selected because the ability to reduce the hazard of BTX. The objective of this research is to remove the BTX from wastewater using *P. putida* in a bioreactor. Before starting the experiment, *P. putida* is prepared by the growth it inside nutrient broth at a suitable temperature and concentration. The bacteria are put in to ratio of benzene, toluene, xylene, benzene-toluene, benzene-xylene, toluene-xylene and BTX solution inside simple conical flask. But the only BTX sample is only running in the fermenter for comparing the growth of bacteria in a conical flask with the growth of bacteria in fermenter. The bacterial growth will be analyzed using UV-Vis Spectrophotometer while concentration BTX after degradation is analyzed by HPLC. From the data obtain, the growth rate of bacteria in toluene sample is higher than other solution. While the growth rate of bacteria in BTX of conical flask is higher than the growth rate of bacteria in BTX of the fermenter. From overall experiment, Toluene is achieved the highest percent of removal compare to percent removal of benzene and xylene. Thus; removal of BTX by bacteria will be a potential biological treatment which it can solve our environmental pollutants in the future.

ABSTRAK

Benzena, toluena dan isomer xilena (BTX) adalah komponen utama petrol mana ia digunakan secara meluas sebagai pelarut dan bahan mentah oleh industri kimia. Ia menjadi masalah pencemaran alam sekitar yang biasa merupakan ancaman serius kepada sumber air bawah tanah dan kesihatan awam, yang kedua-dua toksik dan agak larut dalam air berbanding dengan juzuk petroleum lain. Rawatan biologi yang menggunakan mikroorganisma seperti Pseudomonas putida telah dipilih kerana keupayaannya untuk mengurangkan bahaya BTX. Objektif kajian ini adalah untuk menghapuskan BTX dari air sisa menggunakan P. putida di dalam bioreaktor. Sebelum memulakan eksperimen, P. putida disediakan oleh pertumbuhan ia di dalam sup nutrien pada suhu yang sesuai dan penumpuan. Bakteria dimasukkan ke dalam kepada nisbah benzena, toluena, xilena penyelesaian, benzena-toluena, benzena-xilena, toluena-xilena dan BTX di dalam kelalang kon yang mudah. Tetapi satu-satunya BTX sampel hanya berjalan dalam fermenter untuk membandingkan pertumbuhan bakteria dalam satu kelalang dengan pertumbuhan bakteria dalam fermenter. Pertumbuhan bakteria akan dianalisis dengan menggunakan spektrofotometer UV-Vis manakala BTX tumpuan selepas kemusnahan dianalisis oleh HPLC. Dari data yang diperolehi, kadar pertumbuhan bakteria dalam sampel toluene adalah lebih tinggi daripada penyelesaian lain. Walaupun kadar pertumbuhan bakteria dalam BTX kelalang kon adalah lebih tinggi daripada kadar pertumbuhan bakteria dalam BTX daripada fermenter itu. Dari eksperimen keseluruhan, Toluena dicapai peratus penyingkiran tertinggi berbanding dengan peratus penyingkiran daripada benzena dan xilena. Oleh itu, penyingkiran BTX oleh bakteria akan menjadi rawatan biologi yang berpotensi yang ia boleh menyelesaikan pencemaran alam sekitar kita pada masa hadapan.

.

TABLE OF CONTENTS

.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

µm	micrometer
%	percent
^{o}C	degree Celsius
atm	Atmospheric pressure
g/ml	gram per milliliters
g/l	gram per liters
hr	hour
I	liter
mg/l	milligram per liters
pH	potential Hydrogen

LIST OF ABBREVIATIONS

µ	Specific Growth rate
ASTDR	Agency for Toxic Substances and Disease Registry
BTX	Benzene, Toluene and Xylene
DOE	Department of Environment
EPA	Environment Protection Agency
HCI	Hydrochloric Acid
HSDB	Hazardous Substances Data Bank
HPLC	High Performance Liquid Chromatography
MIDA	Malaysian Investment and Development Agency
NaOH	Sodium Hydroxide
OD	Optical Density
ppm	Part per million
rpm	Rotate per minute
US	United States
UV-Vis	Ultraviolet-Visible

1 *INTRODUCTION*

1.1 *Motivation and Statement of Problem*

Benzene, toluene and xylene isomers (BTX) are the major components of gasoline (Cunha, et. al, 2000). They are also used widely as solvents and feedstock by the chemical industry (Reisch, 1992). BTX is classified as priority pollutants by U.S. Environmental Protection Agency (EPA) (Eriksson *et* al., 1998) because of their lower water solubility and their acute toxicity and genotoxicity. BTX becomes the primary source of aquifer contamination due to the sequences of accidental gasoline spills and leakage from service station tanks.

The source of BTX had been found in the wastewater s comes from oil refineries, chemical manufacturing industry, and paint industry. The production BTX in petrochemical sector and industries is very large every year. BTX is chemical compound has highly toxic contaminants, so it is very dangerous to the human and our ecosystem. The production of BTX not only dangerous to the human and ecosystem but it also gives more problem with our equipment. The high level concentration of BTX also can make some of the equipment in the plan easy to damaged and also will corrupt to the line during taking a reading of equipment. The effect of BTX is a crack on active sites insides catalyst pores, leave coke deposits that prevent further reaction at those sites. As more and more active sites are blocked, catalyst will progressively loss activity (Crevier et al, 2007).

Refiners have the need to know exactly how much BTX is in the refinery crude diet to allow blending because running BTX-laden crude’s can produce wastewater and solid waste streams having BTX concentrations that exceed regulatory limits set by EPA. Attaching a certain range to the concentration of BTX in a purchased crude oil is not an easy task and significant errors can be encountered that can produce negative impacts on refinery operations and profitability.

Purification of areas polluted by BTX is difficult, because health threats posed by benzene. To remove the contamination, the physical treatment such as activated carbon and chemical treatment such as acid-gas enrichment has been used. But the cost of treatment is too high or the efficiency to remove in large contaminants is low. There are biological treatment such as bioremediation in the form of bio stimulation with the addition of nutrients and electron acceptors, soil vapor extraction, or reactive barrier can be applied. But this treatment can be successful if the geology and the biogeochemistry of the site must be accurate (Cunnigham et al., 2001).

The other biological treatment that use microorganisms (*Pseudomonas putida*) are being chosen to solve this problem. This is because this treatment can removal aromatic hydrocarbon without any use chemical which cannot bring any negative effect to human health and the environment. But not many people know the ability of this microorganism although many researches were done at last few decades.

1.2 *Objectives*

The objective of this research is to remove the BTX from wastewater using *P. putida* in bioreactor.

1.3 *Scope of this research*

In order to achieve the stated objectives, the following scopes have been identified:

i) To study *P. putida* growth curve

ii) To investigate the effect ratio of BTX on biodegradation of *P. putida*.

iii) To study the performance of *P. putida* in removing BTX in a bioreactor.

1.4 *Main contribution of this work*

The significant of study obtained by researchers to reduce the hazard of BTX. This is because, BTX has high potential risk that can cause health problem to human beings. This hazard causes many people suffer due to exposure to this aromatic hydrocarbon.

It is also reducing the high cost of treatment due to the number of microorganisms that been used to treat a large quantity of contamination is a small quantity without involvement any chemical.

The research also tends to encourage people to understand about this treatment. This is very important because it can become an alternative way to treat BTX in petrochemical waste water. Hence, it will help reduce our pollution problem and save our environment in the future.

1.5 *Organization of this thesis*

The structure of the remainder of the thesis is outlined as follows:

Chapter 2 provides a description the BTX, its properties, world production and effect to human health when exposed. The description of *P. putida*, type of *P. putida* used, and ability to degrade BTX. Moreover, the presence bioreactor will explained about its type, advantage and disadvantage.

Chapter 3 gives a review of the experimental design of this research, beginning from bacteria preparation, followed by preparation of stock solution and sample depending on the ratio of BTX and type of bioreactor used. The sample taken each 2 hour interval for 8 hours. The sample will be analyzed by using HPLC for BTX concentration determination.

Chapter 4 is devoted to effect removal of BTX of bacteria based on the parameter. The peak area of the sample recorded from HPLC converted into the product concentration via the standard curve of BTX prepared. In this chapter also discuss about the effect of the parameter used in the degradation of BTX by bacteria.

Chapter 5 draws together a summary of the thesis and outlines the future work which might be derived from removal BTX by *P. putida*.

2.0 *LITERATURE REVIEW*

2.1 *Overview*

This chapter discussed about the BTX, *Pseudomonas putida* and bioreactor. This is included the main properties and applications. In addition, the type of bioreactor included advantage and disadvantage is explained properly to make the reader understanding the difference of each type.

2.2 *BTX*

The aromatic compounds benzene, toluene and the xylenes (meta, para and ortho) are often grouped together as BTX (US DOE, 2000 and Anne, 2013). They are part of the six major platform chemicals (along with ethylene, propylene and butadiene), and form the basis for the production of a whole array of bulk chemicals, as shown in figure 2.1 (Blaauw, 2008).

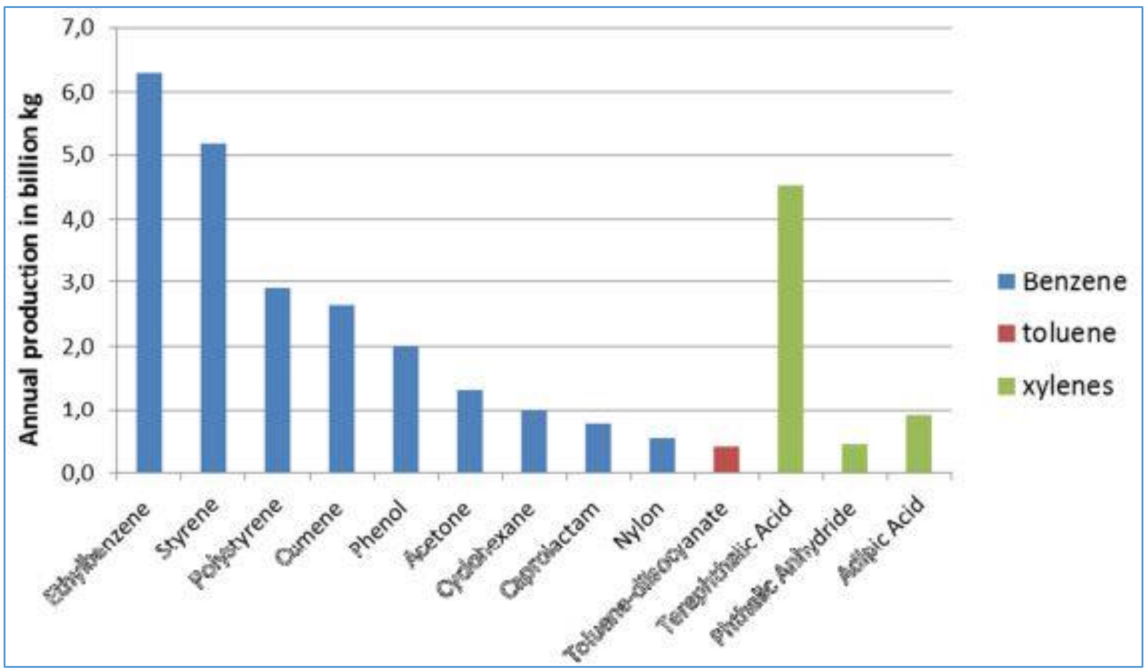

Figure 2.1: Major products derived from BTX (US DOE, 2012 and Anne, 2013).

In the Malaysia alone, BTX production is about 775 thousand metric tons annually which it is produced by Titan Petchem (M) Sdn Bhd and Aromatics Malaysia Sdn Bhd (MIDA, 2009). BTX is currently most often produced by the catalytic cracking of the naphtha fraction of crude oil, although production from pyrolysis gas and from coal is significant (Sweeney, 2008). The demand for the different chemicals within the BTX group is 67:5:28 respectively, although no process directly gives this ratio. Therefore, toluene is often converted into benzene and xylene to adjust the ratios (Anne, 2013).

BTX has an environmental impact due to the fact that it is produced from fossil resources in an energy intensive process (Anne, 2013). Moreover, it actually includes into volatile compounds which has been classified as the major pollutants in Chinese households by different investigators (Zhang et al., 2004). The presence of this substance brings a lot of problems to the environment especially in water area when it is not handled carefully.

2.2.1 *Benzene*

It is commonly known as phene. Benzene is the first member of a series of aromatic hydrocarbons recovered from refinery streams during catalytic reformation and other petroleum processes (ATSDR, 2006). It is a clear, colorless, aromatic hydrocarbon which has a characteristic sickly and sweet odor. It is both volatile and flammable. Selected chemical and physical properties of benzene are presented in Table 2.2.

Table 2.2: Chemical and Physical Properties of Benzene (Fischer Scientific, 2008).

Property	**Value**
Molecular weight	78.11 g/mole
Melting point	5.5^{o}C
Boiling point	80.1^{o}C
Density at 20^{o}C	0.879 g/ml
Vapor pressure at 25^{o}C	0.13 atm.
Flash point (closed cup)	-11.1^{o}C
Solubility in water at 25^{o}C	1.8 g/l

Benzene contains 92.3 percent carbon and 7.7 percent hydrogen with the chemical formula C_6H_6. The benzene molecule is represented by a hexagon formed by the six sets of carbon and hydrogen atoms bonded with alternating single and double bonds. The benzene molecule is the cornerstone for aromatic compounds, most of which contain one or more benzene rings (US EPA, 1988). The arrangement of hydrogen and carbon atoms in benzene is presented in figure 2.3.

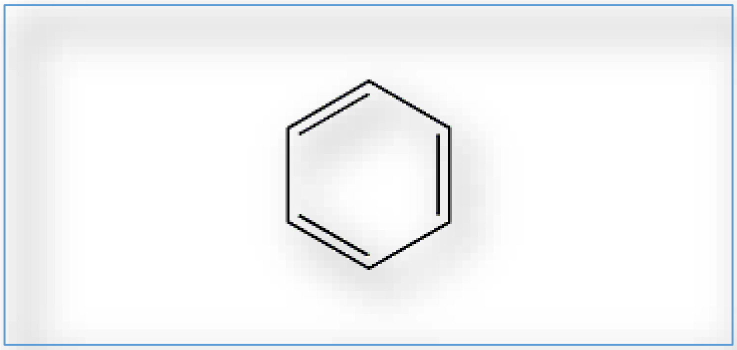

Figure 2.3: Benzene Structure (EPA, 1988)

Benzene is one of the world's commodity chemicals. Its primary use (85% of production) is as intermediate in the production of other chemicals, predominantly styrene (for styrofoam and other plastics), cumene (for various resins), and cyclohexane (for nylon and other synthetic fibers). It is an important raw material for the manufacture of synthetic rubber, gums, lubricants, dyes, pharmaceuticals, and agricultural chemicals (ATSDR, 2006).

Benzene is widespread in the environment because of its use in many industrial processes and its presence in gasoline. It is also a component of both indoor and outdoor air pollution. Benzene levels measured in ambient outdoor air have a global average of 6 micrograms per cubic meter ($\mu g/m^3$) (range 2-9 $\mu g/m^3$). People living around hazardous waste sites, petroleum-refining operations, petrochemical manufacturing sites, or gas stations may be exposed to higher levels of benzene in air (ATSDR, 2006). Most people thought that the level of exposure to benzene through food, beverages, or drinking is not as high as their exposure through the air.

The benzene contamination of groundwater is caused by leakage from underground storage tanks and seepage from landfills or improper disposal of hazardous wastes. Effluent from industries is also a source of groundwater contamination. In addition, benzene in water can also be absorbed through wet skin and inhaled as it volatilizes during showering, laundering, or cooking. The typical drinking water should be contains less 1 ppm of benzene (EPA, 2003).

Several agencies in a round world classify benzene as a confirmed human carcinogen. It estimates that 1 of 10000 people had exposure to 0.004 ppm benzene in the air will get cancer in their lifetime. It is also estimated that 1 of 10000 people had exposure to 0.1 ppm in drinking water would get the same problem (Dosemeci et al., 1994). Some study shows that benzene can cause dangerous hematologic toxicity such as anemia, thrombocytopenia, or pancytopenia (lack of erythrocytes, leukocytes or platelets in the blood) after chronic exposure. These effects are believed to be caused by the metabolites of benzene, which most likely damage the DNA of the pluripotential stem cells (Ward et al., 1996). Based on the available information, it appears that benzene can cause serious health problem to human by inhalation or oral exposure.

2.2.2 *Toluene*

It is commonly known as toluol or methyl benzene. It is a colorless, highly flammable liquid with a sweet, aromatic odor, moderately soluble in water and has a moderate vapor pressure. Because of its small molecular weight and other physical/chemical properties, toluene is rapidly absorbed from the respiratory tract (Reyna, 2008). The main chemical and physical properties of toluene are summarized in Table 5.3.

Table 2.4: Chemical and Physical Properties of Toluene (HSDB, 2008)

Property	**Value**
Molecular weight	92.14 g/mole
Melting point	-94.9^{o}C
Boiling point	110.6^{o}C
Density at 20^{o}C	0.879 g/ml
Vapor pressure at 25^{o}C	0.04 atm.
Flash point (closed cup)	6^{o}C
Solubility in water at 25^{o}C	0.5 g/l

Toluene contains 91.3 percent carbon and 8.7 percent hydrogen with the chemical formula C_7H_8. It is a mono-substituted benzene derivative or one in which a single hydrogen atom from a group of six atoms from the benzene molecule has been replaced by a univalent group such as CH_3. The arrangement of hydrogen and carbon atoms in toluene is presented in Figure 5.4.

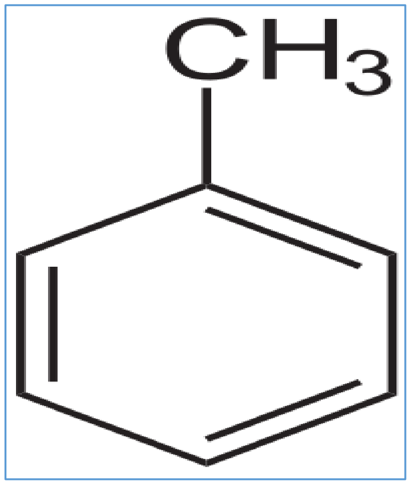

Figure 2.5: Toluene Structure (HSDB, 2008)

Toluene is used as a component is gasoline. It is also an important chemical intermediate that can be reacted to make other chemical products to produce products such as nylon, soda bottle and polyurethanes, which are used in a variety of consumer products like furniture, bleeding, footwear and clothing. Toluene may be reacted to form other industrially important intermediates such as benzene, styrene and xylene (fetter, 2012).

Toluene can be enters surface water and groundwater from spills of solvents and petroleum products as well as from leaking underground storage tanks at gasoline stations and other facilities. Leaking underground storage tanks also contaminate the soil with toluene and other petroleum product components. Toluene dissolved in water does not break down quickly while the water is under the ground because there are few microorganism in underground water. When the water is brought to the surface, the toluene will evaporate into the air which it will combine with oxygen and form benzaldehyde and cresol (U.S. Environmental Protection Agency, 1999). These compounds can be harmful to humans.

There have been numerous toxicity studies conducted in animals and in humans after exposure to toluene. Based on researches had done, exposure to toluene directly can cause tiredness, confusion, weakness, drunken-type actions, memory loss, nausea, and loss of appetite which based on how much amount take in (ASTDR, 2000). But researchers do not know if the low levels of toluene people breathe at work will cause any permanent effects on their brain or body after many years. . Some epidemiological studies did not detect a statistically significant of any increased risk of cancer due to inhalation exposure to toluene. However, these studies were limited due to the size of the study population and lack of historical monitoring data. So, some experts have classified toluene as a Group D, not classifiable as to human carcinogenicity (U.S. Department of Health and Human Services, 1993).

2.2.3 Xylene

It is also called xylol or common name is dimethyl benzene. Xylene is usually found as a mixture of three isomers: m-xylene, o-xylene, and p-xylene, with the m- isomer predominating. The main properties of xylene are a clear, sweetish smell and highly flammable but some chemical and physical properties differ from isomer to isomer which are shown in Table 2.6. For this research, xylene that consist all of three isomers will be used.

Table 2.6: Chemical and Physical Properties of m-xylene, o-xylene, and p-xylene (Reynas, 2009)

Parameter	**m-xylene**	**o-xylene**	**p-xylene**
Molecular weight	106.17 g/mole	106.16 g/mole	106.16 g/mole
Melting point	-47.4 ^{o}C	-25 ^{o}C	13.2 ^{o}C
Boiling point	139.3 ^{o}C	144.4 ^{o}C	138.3 ^{o}C
Density at 20^{o}C	0.8684 g/ml	0.8801 g/ml	0.8610 g/ml
Vapor pressure at 25^{o}C	0.01 atm.	0.008 atm.	0.011 atm.
Flash point (closed cup)	17 ^{o}C	25 ^{o}C	25 ^{o}C
Solubility in water at 25^{o}C	0.162 g/l	0.178 g/l	0.198 g/l

Xylene contains 90.6 percent carbon and 9.4 percent hydrogen with the chemical formula C_8H_{10}. But the arrangement of carbon atoms and hydrogen atoms is different according isomers. This is because the o-, m- and p- designations specify to which carbon atoms (of the benzene ring) the two methyl groups are attached. Counting the carbon atoms from one of the ring carbons bonded to a methyl group, and counting towards the second ring carbon bonded to a methyl group, the o-isomer has the IUPAC name of 1,2-dimethylbenzene, the m- isomer has the IUPAC name of 1,3-dimethylbenzene, and the p- isomer has the IUPAC name of 1,4-dimethylbenzene (Wikipedia, 2012). The molecular structure of xylene isomers is shown in figure 2.7.

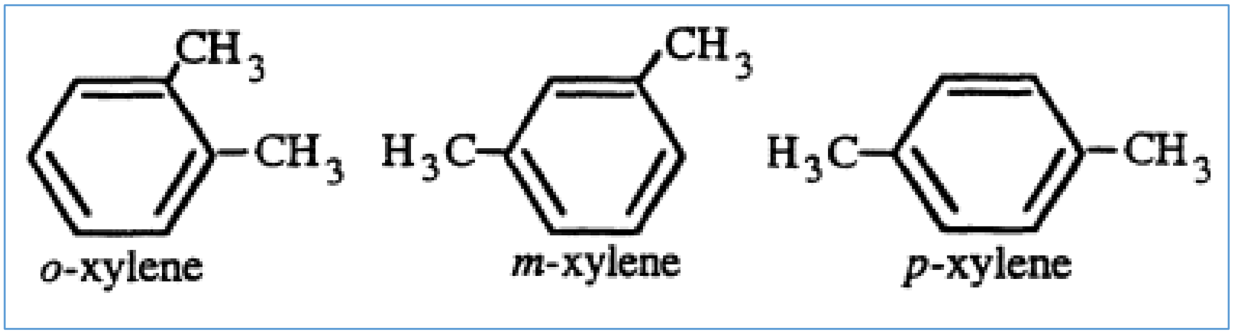

Figure 2.7: o-xylene, m-xylene and p-xylene structure (HSDB, 2009)

Xylenes are primarily manufactured by the petroleum refining industry. Xylene isomers are used as intermediate feedstock in the production of resins, which are used to produce molded plastic, films, and beverage bottles. Mixtures of xylene isomers are used as solvent for paints and coatings, and are added to gasoline to increase its octane rating (Reyna, 2009).

Xylenes are released to the atmosphere primarily as fugitive emissions from industrial sources, in automobile exhaust, and through volatilization from their use as solvents. Discharges into waterways and spills on land result primarily from use, storage, and transport of petroleum products and waste disposal. When xylene is released to soil or surface water, it is expected to volatilize into the atmosphere where it is quickly degraded. Any xylene in soil or surface water that does not volatilize quickly will undergo biodegradation (Shim et al., 2002).

The exposure to xylene in large amount can irritate our lungs, causing chest pain and shortness of breath. Extreme overexposure can cause pulmonary edema, a potentially life-breathing condition in which the lungs fill with fluid. However, no definitive evidence for carcinogenic effects of xylene in humans. Epidemiological studies looking for associations with xylene exposure and specific cancers either reported no cases or a limited number of cases exposed reported concurrent exposure to multiple solvents (Uchida et al., 1993). Some experts have determined that xylene is not classifiable as to its carcinogenicity in humans, due to inadequate evidence for the carcinogenicity of xylenes in humans and animals

2.3 Pseudomonas putida

Pseudomonas putida is a ubiquitous, aerobic, Gram-negative bacterium that shows great metabolic versatility (Timmis, 2002; Dos et al, 2004). It was officially discovered in the mid 1900’s. It is a very common bug that lives in soil and freshwater environment all over the world where it moves about by way of one or more flagella close to the surface (Hamilton, 2007). There are many functions of *P. putida* based on table 2.1 but *P. putida* ATCC 49128 will use in this research because its ability to remove BTX.

Table 2.7: Type of *P. putida* strain and its Function

Type of *P. putida* strain	**Journal/Source**	**Functions**
RB 1353	-Dorn, Frye and Maier.2003, Appl. Environ. Microbiol, 69 (4)	-Decompose the Naphthalene and Salicylate Catabolism
KT 2440	-Fonseca, Moreno and Rojo.2011, Environmental Microbiology Report.	-As experimental model for Agriculture, biocatalysis and bioplastic production.
	-Molina *et*. al., 2000. Soil Biology Biochemistry (32) 315.	-Stimulants the germination of seeds Herbaceous plants and plants Growth.
	-Espinosa-Urgel *et*. al., 2002. Microbiology, 148, 341	-Defend or resist from the plant-Produced antimicrobial compounds
		-Produces microcolonies (biofilm) to Cover the root system of plants.
	-Duque *et*. al.,2007b. En: *Pseudomonas* vol. V. Editor J.L Ramos. Kluwer. London. Chap. 8, 227.	-Detect the presence of Chemo-Attracts produced by plants.
	-Jimenez *et*. al., 2002. Environ.	-Degrade the aromatic compounds

	Microbiol. 4:848	
DSM 548	-Monteiro, Boaventura and Rodrigues. 2000. Phenol Biodegradation by *P. putida* DSM 548 in a Batch Reactor. Elsevier Science S.A.	-Degrade the phenol waste
NBRI 0987	-Srivastava *et*. al., 2008. Effect Of High Temperature on *Pseudomonas putida* NBRI 0987 Biofilm Formation and Expression of Stress Sigma Factor Rpos. Curr. Microbiol. 56,453-457	-Promote the growth of rhizobacteria and enhance plant health. -Enhance biofilm formation and Protecting Pseudomonas from high temperature.
CCMI 852	-Otenio *et*. al.,2005. Benzene, Toluene And Xylene Biodegradation By *Pseudomonas Putida* CCMI 852. Brazilian Journal of Microbiology. 36,258-261	-biodegrade the Benzene, Toluene and Xylene.

Figure 2.8: *Pseudomonas aeruginosa* (Dr. Howard Porter, 2012)

Pseudomonas putida is very similar to strains of *Pseudomonas aeruginosa* as shown in figure 2.8, an opportunistic human pathogen in generic term it seems to be missing the key virulent segments that *P. aeroginosa* has. Being a non-pathogenic bacteria, there has been only a handful of episodes where P. putida has infected humans. For the most part, it has been with immune compromised patients, causing septicemia, pneumonia, urinary tract infections, nosocomial bacteremia, septic arthritis, or peritonitis. It is also closely related to *Pseudomonas syringae*, an abundant plant pathogen, but again it lacks the gene that causes such disease (Dervisoglue *et* al., 2007).

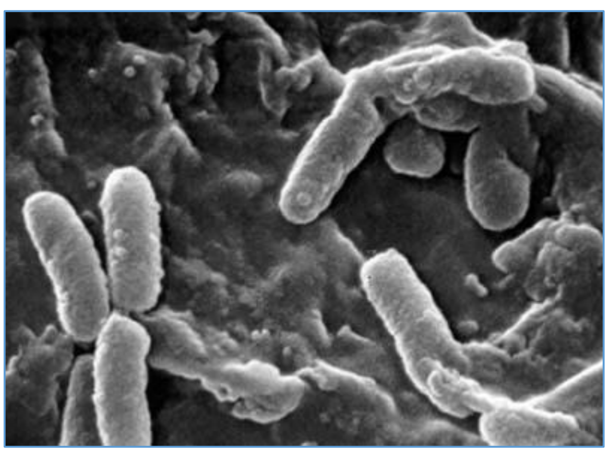

Figure 2.9: *Pseudomonas putida* (Fekete et al., 2009)

There are several cases of disease caused by *P. putida* have been investigated, being that the bacterium rarely colonizes mucosal surfaces or skin. One case was a 43-year-old female who was receiving nightly peritoneal dialysis treatments following a laparoscopic ovarian cyst operation.