International Journal of Research in Engineering and Science (IJRES)
ISSN (Online): 2320-9364, ISSN (Print): 2320-9356
www.ijres.org Volume 4 Issue 9 ǁ September. 2016 ǁ PP. 06-20

Characterization of Enzyme Produced From *Pseudomonas Putida*for BTX (Benzene, Toluene & Xylene) Treatment in Petrochemical Industry Wastewater System

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Abstract: *One of the big challenges in petrochemical industries is waste management. Currently, huge money was spending on the disposal of the waste. Industries are trying hard to find an alternative method to reduce the cost and improve the effectiveness of current waste management including treatment efficiency. Most of petrochemical wastes are containing benzene, toluene and xylene (BTX) which are very harmful to environment and living organisms. Common method used to separate the BTX from the waste are by using liquid-liquid extraction and stripping process. One of the alternative to treat BTX is biological treatment method that used the natural capability of microorganisms to degrade to less harmful product is been applied. Some of examples are Pseudomonas Putida. (P. putida), Rhizobium, and Agrobacterium. P. putida is selected in this study for the biological treatment of BTX in petrochemical wastewater because it can produce an enzyme that has the capability of breakdown the aromatic hydrocarbon to carbon dioxide (CO_2) and water (H_2O). The main objective of this study is to produce and extract the enzymes produce, characterised the enzymes. This study also to investigate the effect of different concentration on the treatment as well as the growth of the bacteria. The enzyme is purified using salt precipitation and analysed using SDS_PAGE technique. UV-Vis is used to study the growth of the bacteria in the culture stock by measuring its optical density. The concentration of BTX was varied to determine the effect of the concentration on the percentage removal and the growth of P. putida. Enzymes detected or purified in this study was benzene reductase. Other expected enzymes were not able to be purified or analysed. It was found that in this experimental study, the removal of benzene is at 74% to 80%. The removal of toluene is at 62% to 75%. The removal of xylene is at 23% to 42%. Increasing the concentration of contaminants will reduce the removal capabilities.*

Keywords: *Pseudomonas Putida, enzyme production, SDS-PAGE, purification, BTX treatment*

I. INTRODUCTION

Nowadays, benzene, toluene, xylene (BTX) are among the frequent hazardous chemical presence in petrochemical wastewater. Based on their toxicity and carcinogenic potential that will harm human, animal and environment a proper treatment is needed to reduce the hazard of BTX before discharging petrochemical wastewater into the water body. The standards of discharge of petrochemical wastewater need to follows the guide and regulation that by Department of Environment (DOE) and Occupational, Safety and Health Administration (OSHA).

The waste water treatment process is the process of removing harmful materials or contaminants form the water or effluent discharge form certain process in order to make it safe to discharge the water to the environment. There are many methods that can be used to remove BTX in the petrochemical waste. Usually physical treatment is needed as the primary treatment in wastewater to remove most of the solid materials before it proceeds to the biological and chemical treatment. Physical treatment may apply electrical attraction, gravity and Van der Waal forces as well as using physical barrier [1].

Chemical treatment utilised chemical reaction or reactions to improve the water quality. A commonly used chemical process in many industrial wastewater treatment operations is neutralization. Neutralization consists of the addition of acid or base to adjust the pH levels back to neutral [2]. Biological treatment methods use microorganisms, mostly bacteria, in the biochemical decomposition of wastewater to stable the end products [3]. Some natural microorganisms have the capabilities of degrading BTX. They are extensively presence in the activated sludge of wastewater treatment plants as well as contaminated soil [4]. Examples of microorganisms that can be used in biological treatment are *Pseudomonas putida*, *Rhizobium*, and *Agrobacterium*.

The enzyme from *P. putida* is needed to be purified. There are several method involved in enzyme purification such as affinity chromatography, immunoaffinity chromatography, chromatographic methods and salt precipitation. Affinity chromatography is separation of the enzyme based on specific biological interaction such as its substrate recognition [5]. Other method is salt precipitation. Salt precipitation is one of useful and ideal method to be used in a laboratory scale. This is because it increases the ionic strength of the solution cause a reduction in the repulsive effect between identical molecules of a protein. It's also reducing the forces holding the solvation shell around the protein molecules. Hence, when these forces are sufficiently reduced, the protein will precipitate. Ammonium sulphate is effective in salt precipitation because of its high solubility, lack of toxicity to most enzymes and its stabilizing effect on some enzymes [6].

Biological treatment is a modern alternative in wastewater treatment with a low operational cost. It is basically the same biological activities that would occur naturally in the receiving water except that it is practiced under controlled condition. Most biological treatment use bacteria as primary microorganisms and degradation of organic compound. The treatment work when waste is utilised as food by microorganism during their growth process to produce protoplasm for new cell [7]. The study choose *P. putida*the biological treatment of BTX in petrochemical wastewater due to its capability to breakdown the aromatic hydrocarbon to carbon dioxide (CO_2) and water (H_2O) since *P. putida* is aerobic metabolisms. Besides, *P. putida*is non-pathogenic which is not causing any dieses while the reaction occur [8].

An enzyme produced from *P. putida* reacts with BTX as a catalyst that will break down the bond between carbon-carbon atoms. Benzene dioxygenase enzyme mediated the meta-cleavage of benzene-like metabolism from wide range of aromatic compounds. The specificity of an enzyme for its substrate is generally a function of the enzyme's "active site" or binding site. The improvement of the techniques in the enzyme production, purification and characterization will affect the reaction with BTX. As a consequence, enzymes are highly specific and are able to discriminate between slightly different substrate molecules. In addition, enzymes exhibit optimal catalytic activity over a narrow range of temperature, ionic strength and pH. They have no ability to adapt to changing conditions or substrate sources. Their level of activity is a function of these conditions. If they are not in optimal conditions, their activity decreases or stops. Thus, the characterization of enzyme is needed to identify the maximum condition of an enzyme react with BTX.

II. MATERIALS AND METHODS

2.1 Chemicals

Benzene, toluene, xylene have 99% purity. Phosphate buffer solutions use were disodium hydrogen phosphate, sodium dihydrogenphosphate and sodium chloride. Lysis buffer used was tris base, 15% glycerol. Nutrient agar and broth was used as the bacteria growth medium

2.2 Organism and culture condition.

24 g of nutrient agar powder was weighted and mixed with 1000mL of distillate water inside 1L of sample bottle. All the apparatus used in this experiment are sterilized to avoid contamination. After that, the agar medium was prepared by transferring the nutrient agar inside laminar flow chamber to avoid contamination and then was let to cool for a few minutes. Preparation of *P. putida*by inoculum it into petri dish that contain agar medium inside a laminar air flow chamber by streaking method. Then, nutrient agars with inoculum of *P. putida* were put in incubator for 24 hours. [7].

Nutrient broths were prepared by weighted nutrient food for about 8g and mixed with 1000mL of distilled water inside 1L sample bottle. After 24 hours, three loops of *P. putida* colonies from nutrient agar were transferred into the sample bottle contained 20 ml of nutrient broth solution using inoculating loops. It was then placed in incubator oven at 37°C for 24 hours. The appearance turns into cloudy after 24 hours. The nutrient food is stored inside incubator oven at temperature 37^{o}C in 24 hour to allow it to grow to form colonies [7].

20ml of bacteria colony was added into conical flask containing 180ml of nutrient broth. Then the conical flask was placed in incubator shaker at 37^{o}C at 180 rpm. An amount of sample will be taken at certain hours and placed in the cuvette that will immediately analyse using UV-VIS with wave length 600nm. The procedure of cell dry weight is carried out simultaneously with bacteria growth. 1.5ml of sample was transferred in the microbiological centrifuge tube. The 1.5ml sample in tube was centrifuged at 12000 rpm for 20 minutes before placed in the oven at 70^{o}C for the sample to be dry overnight. Weights of tube with samples were recorded before and after the sample were dried [9].

2.3 Preparation of cell free extract

Harvested cell were suspended in Phosphate buffer solution (PBS) (NA_2HPO_4,$NA_2H_2PO_4$,NaCl,H_2O), at pH 7.3 and then centrifuged at 12000 × g for 10 minutes at 4^{o}C. Next, the cells were re-suspended with the same buffer and washed three times with PBS buffer [10].

2.4 Enzyme Purification

After that, the pallet was re-suspended with lysis buffer (Tris base, glycerol) and maintain at 0^{o}C for sonification at a peak amplitude (10 microns) for 30 second. The unbroken cells and cell wall materials were removed by centrifugation at 12000 × g for 10 minutes at 4°C to remove cell debris and the supernatant was decanted and kept at 4°C [10]. Further enzyme purification was conducted on at 4°C using SDS-PAGE for 1 hour [11].

After the SDS-PAGE was completed, the gel was stained using blue staining of SDS-PAGE. The gel was placed in orbital shaker for 1 hour at suitable speed, to ensure the gel will not damage. Next, the gel was de-stained by pouring off stain and de-staining with distilled water. Distilled water was replaced for several times.

2.5 BTX treatment

The procedure of BTX treatment using ratio bacteria to solvent solution effect is performed by added each stock solution of BTX in conical flask with ratio 1:1:1 to obtain 1000ml sample solution. Then the sample solution will be added with bacteria solution with variable bacteria ratio. Then, gas chromograpy (FID) is used to identify the removal BTX. At each concentration of BTX, SDS-PAGE was done, to identify whether the enzyme can be produce or not [7].

2.6 Sample Extraction and Dilution

Before going through the extraction procedure, the sample obtained was centrifuged at 10000×g for 10 minutes and filtered using the 0.25 µm of nylon filter to remove the biomass retain in the sample solution. Each sample prepared was extracted with 50ml of dichloromethane (DCM) using separator funnel to separate water from the BTX solvent. After that the sample obtained was diluted with DCM in 10ml of volumetric flask. Next, 1.5ml sample prepared in the volumetric flask was transferred to GC vial for the analysis procedure [9].

III. RESULTS AND DISCUSSION

3.1 *P. putida* Growth Rate at Difference Concentration

From Figure 1, growth of *P. putida*at 15 ppm of BTX slightly lower compare to at 5ppm and 10 ppm. Growth at 5ppm and 10ppm does not have any significance different. Bacteria at high concentration of BTX might have difficulty to growth. The percentage removal is lowest at high concentration because the amount of bacteria is not compatible with the amount of substrate that provided in the solvent solution [12].

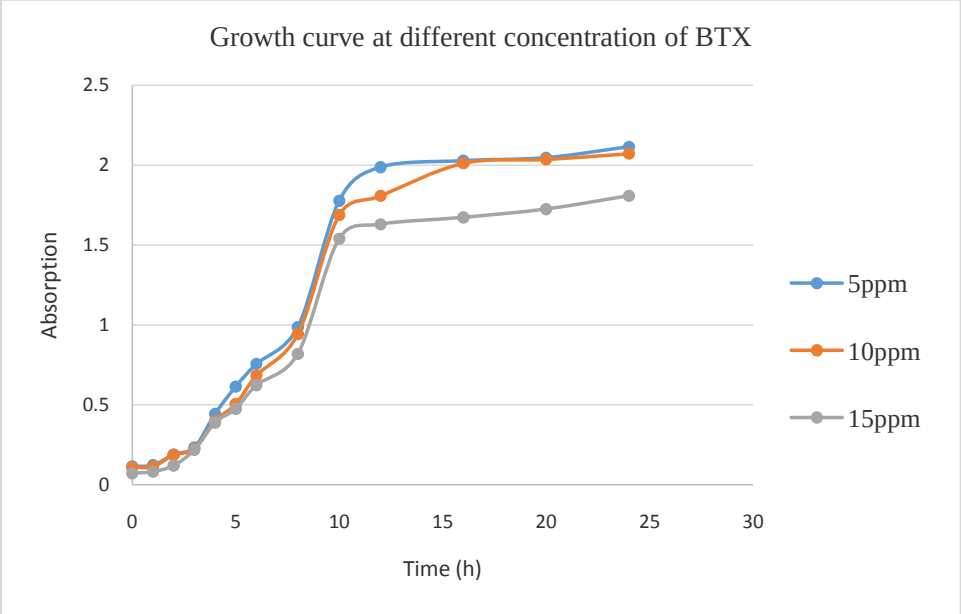

Figure 1: Growth rate of *P. putida* at Difference Concentration

3.2 BTX Treatment in Petrochemical Industry

For the variable of concentration of BTX (benzene, toluene and xylene), the bacteria to the solvent ratio is 1:9 and in were put in incubator shaker with the speed of 180 rpm at 37^{o}C. The percentage removals are analyzedusing gas chromatography (FID) as shown in table below.

Table 1: Percentage of BTX removal at different concentration

Sample	Concentration (ppm)	% Removal
BTX=5 ppm		
Benzene	25	94.075
Toluene	12.5	68.759
Xylene	12.5	36.907
BTX=10 ppm		
Benzene	50	92.185
Toluene	25	66.076
Xylene	25	33.495
BTX=15 ppm		
Benzene	75	91.924
Toluene	37.5	62.543
Xylene	37.5	28.065

The data show that benzene has the highest percentage removal from at any given concentration. Benzene has percentage removal of more than 70%. Toluene percentage removal is between 60% to 75% while xylene percentage removal is only between 23% to 42%. Mixing the three main contaminant forming BTX mixture shows the significant reduction however the same pattern occurs in which benzene will have the highest removal percentage compare to toluene and xylene. Increasing the concentration of BTX mixture from 50 ppm to 150 ppm does not give huge percentage removal different yet there is sight reduction of the removal. The degradation of benzene, toluene and xylene suggests in the present of TOL pathway [12].

3.3 Production and Purification of Enzyme

The purified enzymes are analysis through SDS-PAGE. After staining, the gel showed a protein band, which indicates the molecular mass of the protein.

3.3.1 Characterization of enzyme from *P. putida*in Benzene, toluene and Xylene (BTX) Treatment

Different concentrations of BTX are analysis using SDS-PAGE to analysis the appearance of enzyme product by *P. putida* from the treatment of the BTX.From Figure 2 below, it is estimated that the molecular mass of the protein is 39kDa which indicate the presence of benzene dioxygenase. The benzene 1,2-dioxygenase from *P. putida* catalysed the dihydroxylation of benzene to (1R,2S)-cis-cyclohexa-3,5-diene-1,2-diol (benzene cis-dihydrodiol). The enzyme system has three components which are a flavoproteinreductase and a ferredoxin, which transfer electrons from NADH, and a catalytic iron-sulfur protein (ISPBED). ISPBED contains a Rieske-type [2Fe-2S] cluster, a mononuclear iron oxygen activation centre, and a substrate-binding site [13].

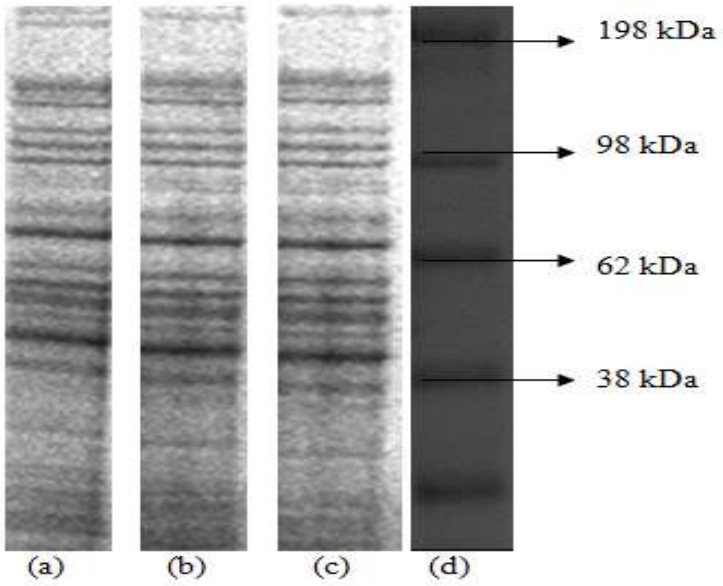

Figure 2: (a) 5ppm (b) 10ppm (c) 15ppm (d) indicator

The presence of benzene dioxygenase in this mix of BTX samples determined based on protein molecular value of 39 kDa. This might happened because the ratio of benzene is higher compare to toluene and xylene. *P. putida* react with BTX as a catalyst which will break down the bonding between carbon-carbon bonds

such as benzene dioxygenase enzyme mediated the meta-cleavage of benzene-like metabolism from wide range of aromatic compounds. The specificity of an enzyme for its substrate is generally a function of the enzyme's "active site" or binding site. The improvement of the techniques in the enzyme production, purification and characterization will affect the reaction with BTX.

IV. CONCLUSION

As conclusion, from this research it can be conclude as BTX treatment from petrochemical wastewater using *P. putida* is successfully conducted with high percent of removal. The growth curve of *P. putida*has complete the bacteria life cycle in period 64 hours. The lag phase occur in 0 to 1 hours and continuous with exponential phase at 1 until 7 hours. Then proceed to stationary phase at 7 hours until 50 hours and last phase is death phase that occur at 54 hours until 68 hours. The cell dry weight obtain is proportional to the time of experimental. The enzyme that is produce is benzene dioxygenase and able to removal of benzene, toluene and xylene because this enzyme has the ability to degrade aromatic ring.

V. ACKNOWLEDGEMENTS

The authors would like to acknowledge the financial support from University Malaysia Pahang (Grant RDU141308 for supporting this research.

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