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Biodegradation of Benzene Gas by Candida Tropicalis in Batch Experiment

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Abstract*: Three sets of batch experiments with different concentration of feed were conducted to observe the biodegradability of benzene gas by yeast strain Candida tropicalis. During the first phase of this study, C. tropicalis was grown in a nutrient solution containing only toluene and benzene as its sole carbon source in an acclimation experiment. There was a delay in the rate of the microbial growth following the nutrient changes, however, the optical density of C. tropicalis reached over 1.200 OD_{600nm} within 9 days of incubation. There was also a positive result of protein and sugar test that proved there were microbial activities happening during the incubation of C. tropicalis with benzene gas as its sole carbon source. The acclimated culture was further studied in a batch experiment of biodegradation. It was found that C. tropicalis managed to remove over 90% of benzene fed within 100 hours of incubation during the batch study.

***Keywords*: Batch experiment, Benzene gas, Biodegradation, Candida tropicalis**

I. INTRODUCTION

Biological method or biodegradation is considered as the best option for treating volatile organic compounds (VOCs) contaminated air at low concentrations [1]. Biodegradation usually exploits the capability of a microorganism to utilize the pollutant as their carbon source. It can be carried out by using a single microbe, microbial consortium or activated sludge.Although bacteria are extensively studied for its potential in the environmental application, this microorganism does pose some drawbacks when it comes to the application for extreme condition. Bacteria are unlikely able to withstand the variable change in pH, temperature and moisture change. As for fungi, even though it seems robust for broader application in the environment, its potential for production of biohazards is always a big obstacle to control. Yeast, on the contrary, robust and adaptable to environmental changes and very easy to cultivate. Moreover, its ability to grow quickly and its ability to withstand unfavourable environmental conditions are another advantage of using yeast for bioremediation [2]. Some cold-adapted yeast strains also possess higher metabolic versatility compared to bacteria on the task of degrading petroleum hydrocarbon compounds [3]. These qualities own by yeast makes it as excellent candidate for bioremediation of phenol contaminated water and soils in cold climatic zones. One of yeast strains that widely studied for their ability to degrade environmental contaminants is *C. tropicalis*. Several studied have been carried out to investigate the ability of *C. tropicalis* to biodegrade hydrocarbon compound such as toluene [4,5], phenol [6,7], polycyclic aromatic hydrocarbon (PAH) [8] and petroleum oil [9-12]. In this study, benzene has been chosen as the model of VOC to be treated with a biological method by using yeast strain *C.* tropicalis. Benzene is the most noxious compound in the benzene, toluene, ethylbenzene and xylene (BTEX) group of VOCs, and it is the lesser being reported. This study was begun with the adaptation of the yeast strain to grow with the presence of VOC, followed by the determination of the strain's ability to uptake carbon from the benzene as its exclusive carbon source, which is the principal pathway of biodegradation by a microorganism.

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II. MATERIALS AND METHODS

A. Acclimation of yeast *Candida tropicalis* to grow with VOC as primary carbon source

To grow the yeast, carbon-free nutrient solution consisting of a hydrocarbon minimal medium (HCMM) and a trace metal solution was used. The HCMM contains 1.55 g/L KH_2PO_4, 0.85 g/L $Na_2H_2PO_4.2H_2O$, 2.0 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgCl_2.6H_2O$. The trace metal solution made up of 10.0 mg/L EDTA, 2.0 mg/L $ZnSO_4.2H_2O$, 1.0 mg/L $CaCl_2.2H_2O$, 5.0 mg/L $FeSO_4.7H_2O$, 1.0 mg/L $NaMoO_4.2H_2O$, 0.2 mg/L $CuSO_4.5H_2O$, 0.4 mg/L $CoCl_2.6H_2O$, 1.0 mg/L $MnCl_2.4H_2O$. For the acclimation process, the growth media was initially supplied with toluene as its sole carbon source. After that, toluene was gradually replaced with benzene, the target compound in this study. The glassware setup is shown in Fig. 1. 250 mL conical flask was used in this experiment. 100 mL of the nutrient solution was added in the conical flask. The nutrient solution was inoculated with 100 µL of concentrated yeast *C.* tropicalis.

The Small test tube was inserted into the conical flask to place toluene or benzene liquid so that it served as a gas form of carbon source for the culture inside the flask. The syringe was used to take out the liquid sample to check the optical density on a daily basis. The acclimation process was carried out in three batches. For the first batch, 100 µL of liquid toluene was added into the test tube. For the second batch, 300 µL was added into the test tube along with 100 µL of liquid toluene directly into the nutrient solution. For the third batch, 5 mL of liquid toluene was added into the test tube. The conical flasks were incubated at 28℃, and the growth of the yeast was monitored by measuring the optical density of the liquid culture daily. When the strain had adapted to toluene as its sole carbon source to grow, the toluene was replaced with benzene. 100 µL of benzene was added into the test tube instead of toluene, and the growth curve of yeast *C. tropicalis* was plotted against time. This acclimated culture was further used for the batch experiment of the removal of benzene by C. tropicalis.

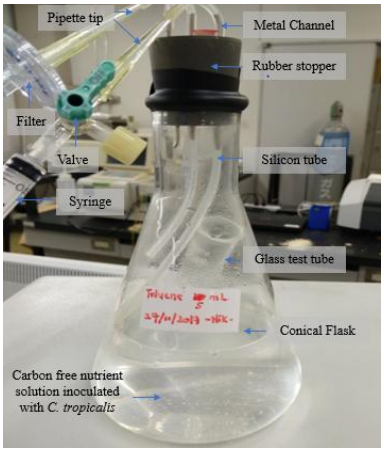

Fig. 1 Glassware setup for the adaptation process

B. Batch experiment for biodegradation of benzene gas with yeast Candida tropicalis

A Batch experiment was carried out according to Rahul's experiment [13]. 250 mL serum bottle with septa fitted cap was used as shown in Fig 2. The serum bottle was filled with 50 mL carbon-free nutrient solution followed by 100 µL of acclimated yeast *C. tropicalis* using a micropipette (100 µL, Eppendorf,Germany).Similar to the acclimation experiment, the small test tube was inserted into the serum bottles to place a benzene solution. There were three sets of bottles in this experiment where each set contained a different amount of benzene solution in the test tube. By using a micropipette (100 µL, Eppendorf, Germany), 20 µL, 50 µL and 70 µL of benzene were added into the test tube of set A, B and C respectively. The bottles were incubated at 28 ˚C. The amount of benzene gas in the air space inside the bottle was recorded daily by collecting gas from the airspace inside the serum bottles using 50 µL gastight syringe (1705N, Hamilton, USA) to check the removal of benzene gas. This experiment was carried out duplicates. A bottle without *C. tropicalis* inoculation served as the control. Protein test was conducted to confirm the protein production by the yeast using Lowry Method [14].Meanwhile, phenol sulfuric acid method [15] was used to detect the presence of sugar.

Fig. 2 Glassware set up for batch experiment

III. RESULTS AND DISCUSSION

A. Acclimation of yeast *Candida tropicalis* in different growth media

Microbial acclimation or also known as adaptation is one of the keys to a success biodegradation process [16,17]. Previous studies have either used the targeted compound [18,19] or compound from the same group [20] for the adaptation process. In this study, toluene was selected for the adaptation because it has the most similar physical structure with benzene but less stable due to the presence of a methyl group on it. Furthermore, toluene had been successfully degraded by *C. tropicalis* in a few previous studies [4,21].

The approach of using toluene for the acclimation process was adopted from previous studies which have shown better biodegradation when the microbe was acclimated to toluene. Acikgoz and Ozcan [22], in their study, demonstrated that phenol degraded by toluene-acclimated cultures significantly better than phenol-acclimated culture. Phenol removal by cross-toluene acclimation in the medium has increased by 14 percent. Babaarslan [23] also reported that the toluene-acclimated microorganisms completed the biodegradation of benzene and toluene in only 2 days, while benzene was fully degraded in 4 days with benzene-acclimated microorganisms. This result substantiates that the strain that was acclimated to grow with toluene as its sole carbon source has a better capacity to degrade single-substrate compared to the one without acclimation or benzene-acclimated. Fig. 3 shows the growth curve of C. tropicalis when supplemented with toluene and benzene as its only source of carbon to grow. A standard life cycle of a microbe will demonstrates four different phases of growth. The microbial growth curve of microbial population against time consists of the lag phase, exponential phase, stationary phase and death phase. When the adaptation process started by growing *C. tropicalis* with 100 µL toluene of gas form, there was a long lag phase where there was almost no growth until day 7. However, on day 8, the OD_{600nm} reading was increased from 0.113 to 0.187 indicating that the culture had entered the exponential phase where the yeast grows at its highest rate, and it has started to adapt to the surrounding and consumed the nutrient in the growth medium. The number remained almost the same on day 9, showing the growth phase entering the stationary phase.

The cells finally reached the dead phase on day 10 as the OD_{600nm} reading started to decrease and remains the same until day 12.When the toluene gas was increased to 300 µL with the addition of 100 µL of toluene added directly into the growth media, the culture has shown a fluctuating growth. The exponential growth phase was observed from day 1 until day 4, before it reached steady growth. The culture growth showed a slight rise on day 7 before it fluctuated until day 14. Then the growth increased from 0.32 to its maximum OD_{600nm} reading of 0.449 that was achieved on day 19. In case of this batch, the presence of toluene both in gas and liquid form could have interfered the uptake of carbon source by the yeast. It was well understood that a high concentration of specific compound could be toxic to microorganism. According to Darracq *et al.* [24], the substrate concentration in the aqueous phase that exceeding the inhibitory threshold will jeopardizing the microorganisms. 100 µL of toluene directly into the growth media gave a relatively high concentration when it became soluble in the growth media and could be fatal to the yeast. At this point, some cell might be growing, and some others were dead, thus gave a fluctuate growth curve. Further addition of toluene to 5 mL on the next batch shown slight improvement. Its immediate exponential phase went on just until day 2, then the growth became stationary temporarily until day 4 before it increased again until day 9 with maximum OD_{600nm} reading of 0.436. There was no further increased in OD_{600nm} reading until day 20, indicating the culture remained on stationary phase even under a longer incubation period. The yeast has grown rapidly at the beginning because there was ample supply of toluene as its carbon and energy source. This culture has entered the stationary phase earlier because the high concentration of toluene could have become unfavourable for growth and the rate of cell growth is about the same with the rate of cell death. When the number of cells growing exceeds the dead cell, the graph showed an increasing curve. Finally, when the carbon source switched to benzene gas, the acclimated culture surprisingly showed a rapid growth in which its exponential phase started almost without lag phase and lasted until day 8. It entered the stationary phase until day 11 before it started its dead phase on day 12. During the acclimation process, there was a delay in the rate of the microbial growth following the nutrient changes. This is because when a microorganism is introduced to a new medium, it takes some time to adjust to the new environment. That is the reason why the yeast had grown very slowly when toluene was given, even though there was always an ample supply of carbon available. However, a rapid increase in microbial growth was noticed when the yeast supplied with benzene afterwards, indicated that it has adapted to the new carbon source. Prior research on BTEX suggest that microbe that has undergone an acclimation process gives a significant effects upon its biodegradation pathway and performance on the default compounds and its co-contaminants from the environment [25].

B. Protein and Sugar Tests

Table 4.1 shows the sugar and protein amount produced during the adaptation process of yeast *C. tropicalis* to volatile organic carbons. The positive result of sugar and protein test was showing that there were metabolic activities happened during the incubation. This result indicated that *C. tropicalis* has successfully utilized toluene and benzene for its sole carbon source because carbon is the most crucial element needed for metabolic activity to happen. The results have shown more sugar were produced by the strain when they were feed with 300 µL of toluene liquid with 100 µL of toluene gas and 100 µL of benzene gas, followed by 5 mL of toluene gas. Theoretically, more protein should be presented compared to sugar as sugar is supposed to be the simpler glucose available to be utilized by the strain. No further experiment was carried out to explain this condition for this study. However, there was a report by Rabinowitz and White [26] which stated that yeast cells might develop a survival process called Autophagy, a mechanism of self-cannibalism where it will degrade its organelles and protein during stress or starvation to promote survival. This phenomenon could explain the lower amount of protein during the adaptation process of *C. tropicalis* to degrade benzene.

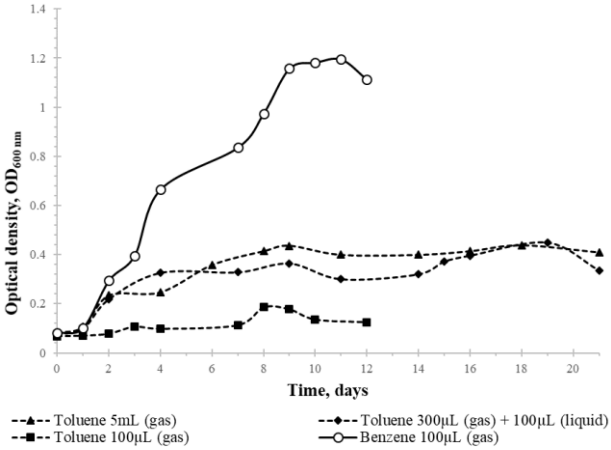

Fig. 3. Growth curve of C. tropicalis supplemented with a different form of VOC as its sole carbon source

Table-1: Protein and sugar content in broth culture

	1st Batch	2nd Batch	3rd Batch
Sample age (day)	21	21	21
Protein test	18.1	27.5	22.2
Sugar test	17.1	31.1	38.5

C. Batch Biodegradation of VOC Benzene with Candida Tropicalis

The graph demonstrated that Batch A with 20 µL of benzene begins with slow removal after 24 hours incubation with only 19% removal of benzene. However, it reached 88% of benzene removal within 48 hours of incubation and reached 100% removal after 100 hours. Batch B started with a higher removal where it reached 39% removal after 24 hours and steadily increased until it reached 94% benzene removal after 100 hours. Batch C with the highest benzene of 70 µL started with the slowest removal of only 10% removal after 24 hours incubation.

Nevertheless, it did show a progressive removal and manage to reach 91% removal within 100 hours of incubation.Meanwhile, the negative control without *C. tropicalis* showed less than 10% removal throughout the experiment. Since benzene is slightly soluble in water, the 5% to 9% loss should dissolve into the nutrient solution. Previous studies have reported that the aqueous solubility of benzene is 1770 to 1780 g m^{-3} [27,28] which suggests that some of the benzene vapour will dissolve in the liquid phase. The optical density of each batch also has increased from 0.06 OD_{600nm} to 0.142, 0.206, 0.224 OD_{600nm} in batch A, B and C respectively. This indicated that strain *C. tropicalis* has not only utilized benzene gas for its energy source, but also for its cellular synthesis. The results showed confirming the biodegrading activity made by *C. tropicalis* and its capability to use benzene as a carbon source.

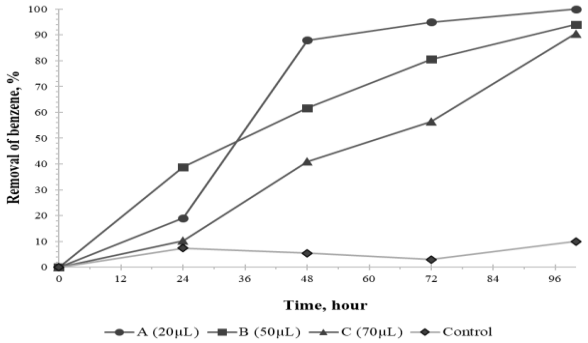

Fig. 4. The removal of benzene gas in a batch experiment

IV. CONCLUSION

Yeast strain *C. tropicalis* was successfully acclimated to grow with the presence of benzene gas. The yeast strain showed an excellent performance in consuming benzene as its sole carbon source in a batch experiment. There were also positive result of protein and sugar test that proved there were microbial activities happened during the incubation, which further confirm that the *C. tropicalis* has utilized benzene gas as its sole carbon source to grow. The findings of this study indicated that this yeast strain is highly potential to biodegrade benzene gas release into the environment. The future of this study may be focused on the bio-treatment of benzene gas by this strain using bioreactor to highlight its capability to degrade volatile organic compounds.

V. ACKNOWLEDGMENT

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