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Optimization and Interaction Analysis of Bacterial and Fungal Growth in the Presence of Benzo(A) Pyrene in Wastewater

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Abstract. In wastewater treatment plant, an aerobic approach is adopted in the system which utilize the ability of bacteria in the wastewater to reduce biochemical oxygen demand (BOD) and other organic pollutants. High molecular weight of polycyclic aromatic hydrocarbons (HMW-PAHs) are type of organic persistence and insoluble pollutants which hard to be removed from wastewater by bacteria. Owing to the HMW-PAHs tolerant characteristic and abundancy of fungi in wastewater, the bacteria and fungi interaction is anticipated to degrade the HMW-PAHs in wastewater. Thus, this study is carried out as a preliminary study to understand the interaction by optimized the bacterial and fungal growth in the presence of benzo[a]pyrene (BaP). Bacteria and fungi used in this study were *Sphingobacterium spiritovorum* and *Aspergillus brasiliensis ATCC 16404*. The response surface methodology (RSM) in Design-Expert® Version 7.0.0 software was used to analysed two factors which are pH and temperature on the bacterial and fungal growth. The growth of bacteria and fungi in co-culture and existence of BaP also been studied. The optimization study showed the growth of *S. spiritovorum* and *A. brasiliensis* was optimized at pH 6.2, temperature of 32 ^{o}C, and pH 6.5 and temperature of 33 ^{o}C, respectively. Synergy interaction was observed in the co-culture condition and with the existence of BaP. The present study provides beneficial information about the bacteria and fungi interaction which has the potential to be applied in the wastewater treatment plant.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) is one of the organic pollutants, which became a threat to the environment and mankind. This pollutant became serious threat because of their significant level, found in air, water and soil. These contaminants existed in difference number of benzene rings, which have been associated with causing tumours in animals and cancer in humans [1]. The petroleum process and industrial activities such as processing, combustion and disposal of fossil fuels are some of sources of the PAHs. The PAHs have been classified by their molecular weight as low molecular weights (LMW) or high molecular weights (HMW). LMW-PAHs are those consisting of two or three benzene rings, while HMW-PAHs are those with four or more benzene rings [2]. LMW-PAHs are relatively water soluble, but those containing four or more rings are quite hydrophobic and insoluble, which affect the process of degradation. High benzene rings number much stronger and hard to remove from the media especially in wastewater treatment plant.

Aerobic bacteria are responsible to degrade organic pollutants in wastewater treatment plant. Fungi which also found in wastewater, can transform the pollutants, and detoxified the chemical products [3]. However, due to faster growth of bacteria which rapidly uptake and utilize substrate, bacteria always dominant even fungi are present [4,5]. Thus, influence the performance of the wastewater treatment plant to degrade PAHs. Therefore, it is important to develop a symbiotic interaction between bacteria and fungi for the removal of organic pollutants in wastewater as suggested by Espinosa-Ortiz et al. [6]. This study is carried out to optimize the bacterial and fungal growth. Parameters such as pH and temperature were optimized. It also observed the growth of bacteria and fungus with or without PAH. The PAH used in this study is benzo[a]pyrene, a 5-benzene ring of PAH.

2. Experimental

2.1. Media preparation

Benzo(a)pyrene powder (Sigma-Aldrich (M)) was dissolved in HPLC grade of acetone (Sigma-Aldrich (M)) to prepare a 50 ppm of stock solution. Wastewater was taken from aeration pond of sewage treatment plant in Universiti Teknologi Mara (UiTM) and used to evaluate the growth trend of the bacteria and fungus.

2.2. Bacterial growth preparation

The bacteria strain, *Sphingobacterium* s*piritovorum*, was isolated from municipal sludge by Othman et al. (2009) [7]. This strain was previously studied and is proven to degrade LMW-PAHs [8]. The bacteria strains, which were preserved at -80^{o}C in microbeads, revived by aseptically transferring the beads into a nutrient broth (NB, OXOID Thermo Fisher Scientific) and left to grow at 30°C in an incubator. A series of dilution streaking was performed, and the strain was sub-cultured three times to attain the active bacterium before it was used in further experiment. After the sub- culturing process, a loop full of a single bacterial colony from new nutrient agar plate was taken and put into centrifuge tube with 25 ml of NB. It was left to grow for 2 days at 30^{o}C (150 rpm). The plate count method was used to establish the growth curve after the bacteria was inoculated in wastewater. The colonies formed on the agar plates were enumerated and expressed as colony-forming unit per mL of culture (cfu/ml).

2.3. Fungal growth preparation

The fungus used in this study is *Aspergillus brasiliensis* ATCC 16404 which was purchased from BioFocus (M) Sdn Bhd. The presence of this strain in wastewater and the ability of this fungus to degrade PAHs make this strain is suitable for this study [9]. From previous studies, *A. brasiliensis* surface is found to increase the adhesion of *Pseudomonas putida* KT 2242 and able to be modified in the presence of surfactants [10,11]. The versatile of the fungal surface is an advantage in the degradation of PAHs. From a single test tube of sterile distilled water (5 to 6 ml), a 1.0 ml was withdrew using a sterile pipette, applied directly to the fungal pellet, and stirred to form suspension. The suspension was mixed well and evenly spread on freshly prepared of potato dextrose agar (PDA, OXOID Thermo Fisher Scientific) medium, in the 9 cm petri dish. The fungal spore was noticeable after 3 days of incubation in 30^{o}C. A loopful of 7 days fungal spores were scraped, suspended with potato dextrose broth (PDB) and adjusted the optical density, OD_{600} to 0.9 to 1 to get 1 x 10^{6} spores/ml. A 5 µl of spore suspension was inoculated in 50 ml tube contain 15 ml of potato dextrose broth (PDB, OXOID Thermo Fisher Scientific) and incubated at 30^{o}C for 3 days to form fungal mycelium. The growth trend of fungus was identified by filtering and weighting the mycelium of fungus.

2.4. Inhibition study of fungus and bacteria

In this experiment, the procedures of Hamzah et al. [11] are followed. Briefly, a 5 µl of *A. brasiliensis* spore suspension was dropped in the middle of a sterilised PDA plate petri dish. Subsequently, a 10 µl of *S.* s*piritovorum* suspension was inoculated at four different points around the fungal inoculum at 1 cm. Then the plate was incubated at 30°C, and the mutual growth inhibition was visually analysed by comparing the growth pattern of individual colony plate daily for 5 days.

2.5. Design experiment for optimization study

This optimization showed which factors would give huge effect to the responses (removal of BaP). In this experiment, the influence of two significant variables, which are temperature and pH assessed through a full central composite design (CCD) in response surface methodology (RSM) using Design-Expert® Version 7.0.0 software. The 3-D response surface plots were constructed to analyze the interactions between the two factors to identify the best bacteria and fungi growth conditions. The range limit set in the experimental design was based on the previous research with two variables, pH (4.0-8.0) and temperature (28ºC - 35ºC), on fungi and bacterial growth. Experiments were conducted in 50 ml sterile wastewater with specific value of the variables. The flasks were incubated at 150 rpm for 7 days.

2.6. Bacterial and fungal growth trend

Based on the optimization data, the growth trends were observed. There were 3 different conditions of the experiment which were (1) *S.* s*piritovorum* with and without BaP, (2) *A.brasiliensis* with and without BaP, (3) mixture of *S.* s*piritovorum* and *A.brasiliensis* with and without BaP. The concentration of BaP used in this study was 2 ppm which above its solubitiy limit. All experiments were conducted by inoculated 1 x 10^6 cfu/ml of inital bacteria suspension and 3 days age of fungal mycelium into 50 ml of steriled wastewater in the 250 ml of Enlenmeyer flask. The pH of the wastewater was adjusted according to the optimum value. The flask was capped by cotton ball and covered the whole flask with aluminum foil before incubated in incubator shaker for 7 days. Each flask was sacrified everyday to obtain the growth.

2.7. Statistic analysis

The growth trend was analysed by plotting a growth with respect to days of incubation using Excel software.

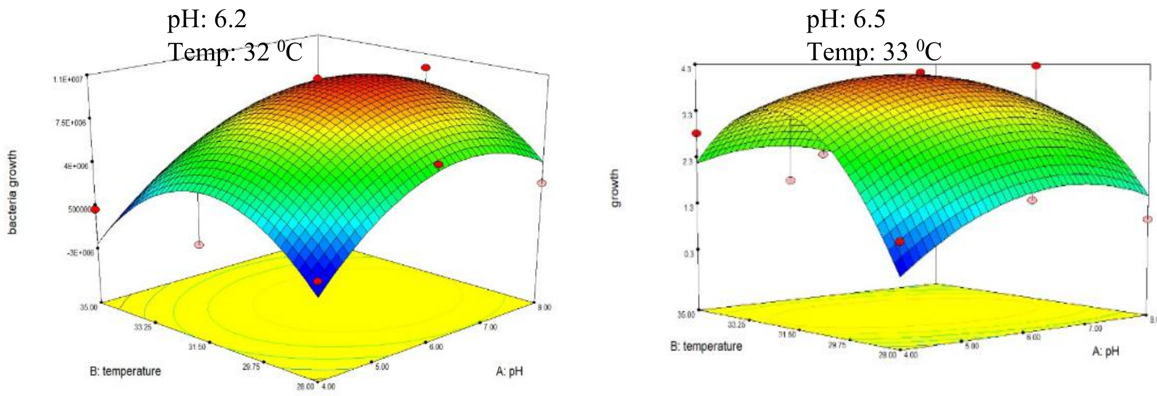
3. Results and Discussion

3.1. Inhibition between fungi and bacteria

The inhibition between bacteria and fungi used was observed in order to identify their compatibility throughout the experiment and also the potential used to work together to degrade PAHs in future. So, the inhibition between *S. spiritovorum* and *A. brasiliensis* in this study has been observed, to see the relevant in using this strains. Their inhibition were identified by looking at their growth pattern everyday. As a result, *S. spiritovorum* and *A. brasiliensis* interaction shows synergy interaction with no inhibition zone was observed after 5 days.

3.2. Optimization of pH and temperature

Two parameters which are pH and temperature were tested for investigating their effects on the bacteria and fungi growth. The data in optimization study was analyzed using response surface methodology (RSM) using Design-Expert® Version 7.0.0 software. The study on the optimal growth conditions of bacteria and fungi could increase the removal rate of PAHs from water, soil and other matrices. The 3D response surface plots were drawn to illustrate the interaction between the factors involved which are pH and temperature with the response of the study, growth of bacteria or fungi. The optimum values of the selected variables were obtained by analyzing the response surface 3D plot.



(a) (b)

Figure 1. The 3D response surface plots for (a) *S. spiritovorum* (b) *A. brasiliensis.*

Effect of pH on bacteria and fungi growth was evaluated based on the range limit in RSM. The figure 1 show 3D response surface plots for the pH and temperature optimization on the *S. spiritovorum* and *A. brasiliensis.* At lower pH, both bacteria and fungi growth are low in growth compare to the growth in neutral and alkaline condition. Apart from that, the lowest and highest temperature show weak growth of bacteria and fungus, indicates that they could not grew well in too low and too high temperature. Based on the figure, the growth of *S. spiritovorum* was optimized at pH 6.2 and temperature of 32^{o}C. Meanwhile, the growth of *A. brasiliensis* was optimized at pH 6.5 and temperature 33^{o}C. This result is supported by Ibrahim et al. [12] which the temperature that less than 30^{o}C and more than 40^{o}C do not support bacterial growth.

3.3. Bacterial and fungal growth

In this experiment, the bacterial and fungal growth have been studied with and without BaP, to determine if the existence of BaP will affect the growth of bacteria and fungus. Figure 2 shows the growth of A. brasiliensis in individual culture and mixed culture without the presence of BaP in the medium. From the figure, the growth of *A.brasiliensis* in individual culture is slightly increases and sustain the growth until day 7. Meanwhile in mixed culture, the fungus growth is enhanced by the present of *S. spiritovorum* without the present of BaP. However, in the present of BaP, similar pattern of the fungal growth is observed as in the individual culture. From the graph, it can conclude that *S. spiritovorum* facilitate the growth of *A. brasiliensis* but BaP do affect each growth but they still able to sustain their growth. Studies have shown that fungi are competent enough to degrade organic pollutants in wastewater as bacteria [8,9].

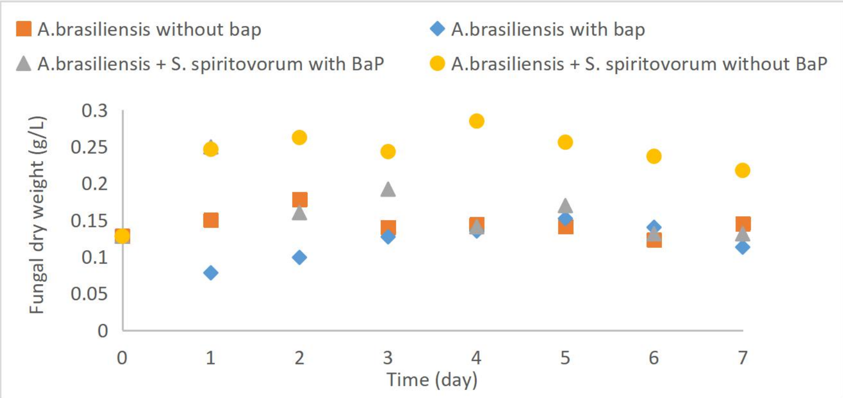


Figure 2. The growth of *A.brasiliensis* individually and co-culture with *S. spiritovorum* with and without BaP.

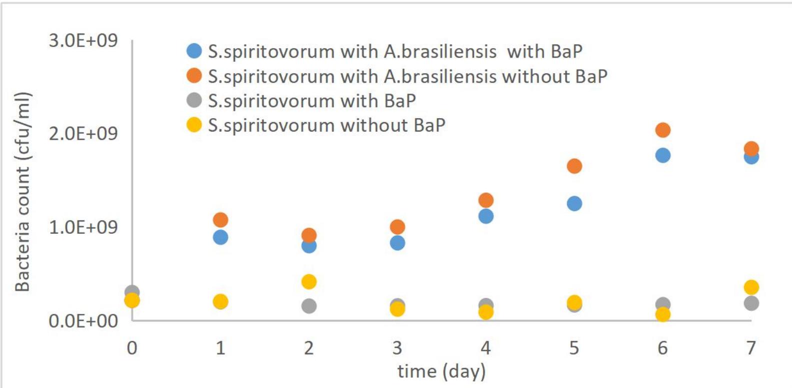


Figure 3. The growth *S. spiritovorum* individually and mixed with *A.brasiliensis* with and without BaP.

Figure 3 shows the individual bacteria growth and co-culture in the presence of BaP which illustrate similar pattern of individual bacterial growth, which is grow constantly in both condition (with and without BaP). However, in the presence of *A.brasiliensis*, the *S. spiritovorum's* growth is increases and sustain the growth after day 6, with and without BaP. This explain that both bacteria and fungus can survive even BaP is present and indicates positive toleration with BaP.

4. Conclusion

The optimization study showed that the growth of *S. spiritovorum* was successfully optimized at pH 6.2 and temperature of 32^{o}C and the growth of *A. brasiliensis* was at pH 6.5 and temperature of 33^{o}C. The *S. spiritovorum* and *A. brasiliensis* grew positively in mixed culture and with the existence of the benzo[a]pyrene. The synergy interaction between bacteria-fungi-BaP indicates the potential of the interaction to be applied in treating organic compound in wastewater treatment plant.

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