# CHAPTER 7
# BENZO(A)PYRENE BIODEGRADATION BY *FUSARIUM OXYSPORUM* E033



Not only *Aspergillus* strains capable of BaP degradation were isolated in this study, *Fusarium oxysporum* E033 was also isolated and determined for it ability to degrade BaP as described and discussed in this chapter.

## 7.1 Biodegradation kinetic Study

The BaP degradation kinetic of *Fusarium oxysporum* E033 was studied in liquid media. The photo – oxidation, the adsorption by mycelia and biodegradation by this fungus were measured and recorded every 5 days throughout 30 days of incubation. The result was shown in Figure 7.1.

The total loss of BaP from physical adsorption and photo-oxidation were determined as the controls and found to be in the minimum range of approximately 3% and 20% within 30 days of incubation. The total degradation after 30 day-incubation of *Fusarium oxysporum* E033 was approximately 65% (Figure 7.1). The biomass of this promising fungus could be seen in Figure 4.2 C.

The significant BaP biodegradation by this fungal strain could be detected within the first five days of incubation at 32°C in liquid medium supplemented with 5-mM glucose as a carbon source. The degradation rates of *Fusarium oxysporum* E033 was found to be 27.0 μmole BaP/day (calculated from the first five days of incubation).

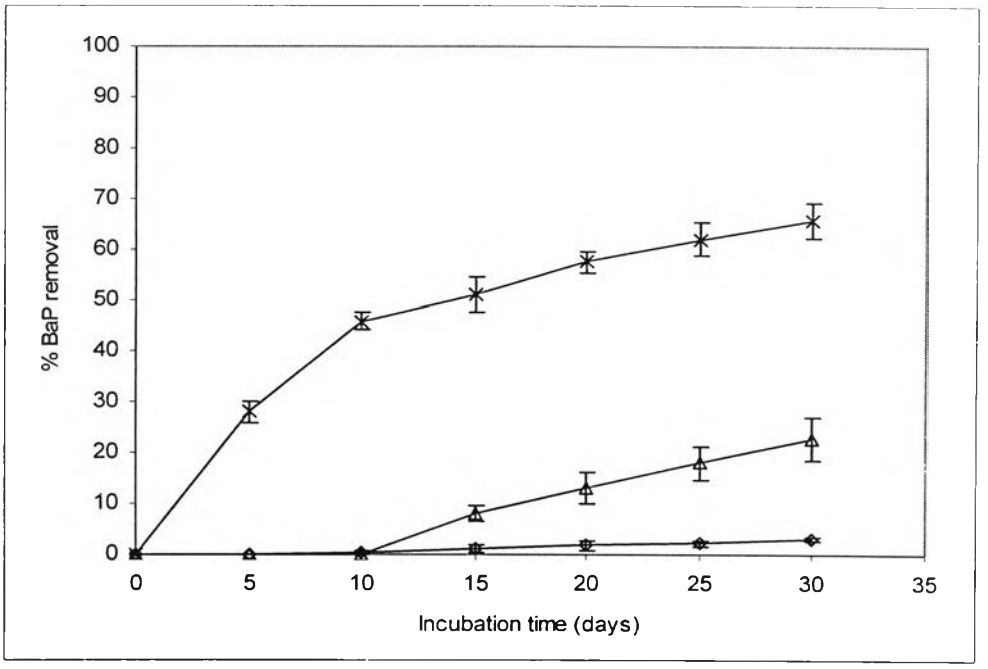


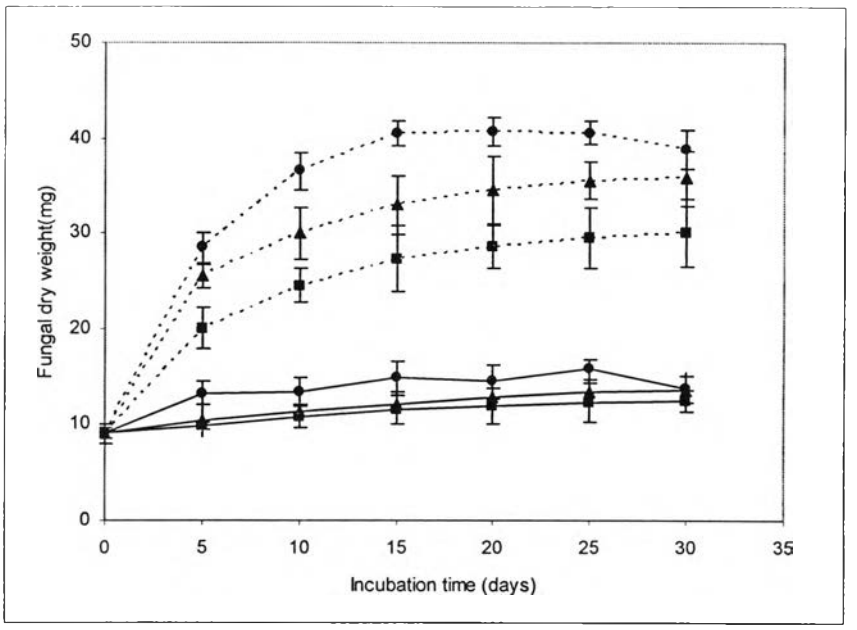

Figure 7.1 Biodegradation of 100-ppm BaP of the fungal isolate in liquid medium. Loss of BaP from auto-degradation (Δ); Loss of BaP from adsorption (□); and Biodegradation (x). (Mean of three replications +/- S.D.)

Further studies were conducted to determine the factors affecting the BaP degradation and to investigate the degradation mechanism of these isolates.

## 7.2 Factors affecting BaP degradation

### 7.2.1 Effect of aeration

The aeration rate affected growth of fungi and its biodegradation ability of BaP were performed at the shaking speed 60, 120 and 180 rpm. The result was presented in Figure 7.2.1.

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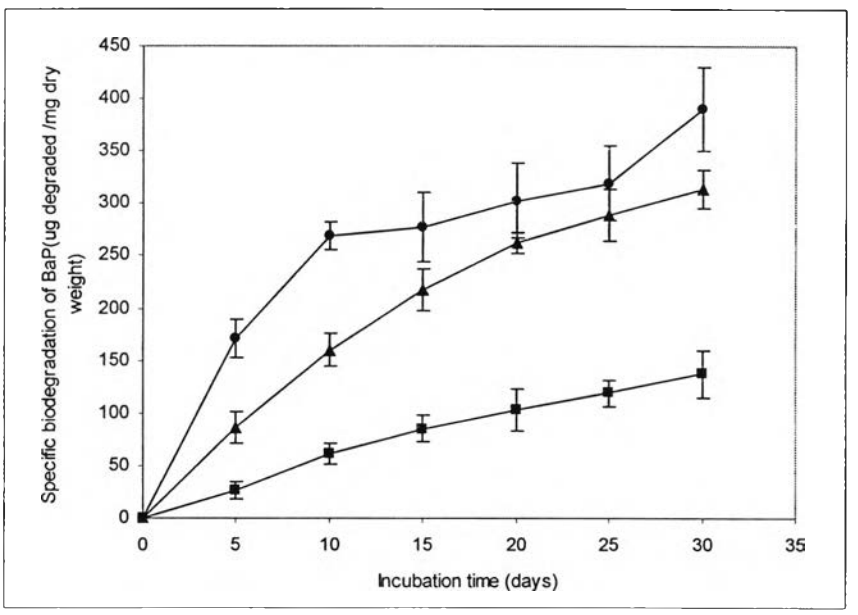



B

Figure 7.2.1 Growth and biodegradation of *Fusarium oxysporum* E033 in glucose-containing medium, in the absence of BaP (---) or in the presence of 100-ppm BaP (—). (**A**) Growth of the fungi was with reciprocal shaking at 60 rpm (■), 120 rpm (▲), and 180 rpm (●). (**B**) Biodegradation of BaP when cells were grown with different reciprocal shaking at 60 rpm (■), 120 rpm (▲), and 180 rpm (●). The biodegradation of BaP was expressed as specific degradation per cell dry weight. (Mean of three replications +/- S.D.)

For the *Fusarium oxysporum* E033, aeration was obviously affected cell growth in that the higher the shaking stroke it was, the higher the growth was obtained (Figure 7.2.1A; without BaP supplementation). However, it was found that, regardless to shaking stroke, growth of *Fusarium oxysporum* E033 was significantly suppressed in the presence of 100-ppm BaP; nonetheless, the biodegradation of BaP was clearly observed.

This phenomena was previously noted by Pineda-Flores and Mesta-Howard (2001) that degradation of PAHs of four or more rings generally did not yield a remarkable increase to the biomass of the employed consortia suggesting that these compounds was not preferentially used as a carbon source, but mostly as an energy source for microorganism. This obtained results were also confirm from Yuan and VanBriesen whose implied the microbial biodegradation that the diversion of energy and electron away, the overall cell yield were decreased (Yuan & VanBriesen, 2002).

Since the fungal growth of each shaking condition tested was different, the comparison of biodegradation of BaP was described as the specific degradation in which the amount of BaP degraded was calculated per weight (mg) of dry fungal mass. The result showed that the faster the shaking stroke, the more the aeration for the fungal growth and the more the biodegradation of BaP (Figure 7.2.1 B). This result agrees with the previous reports that the shaking condition for PAH degradation not only increases the oxygen availability, but it also increases PAH solubility into aqueous phase for the organism uptake (Johnsen et al., 2005).

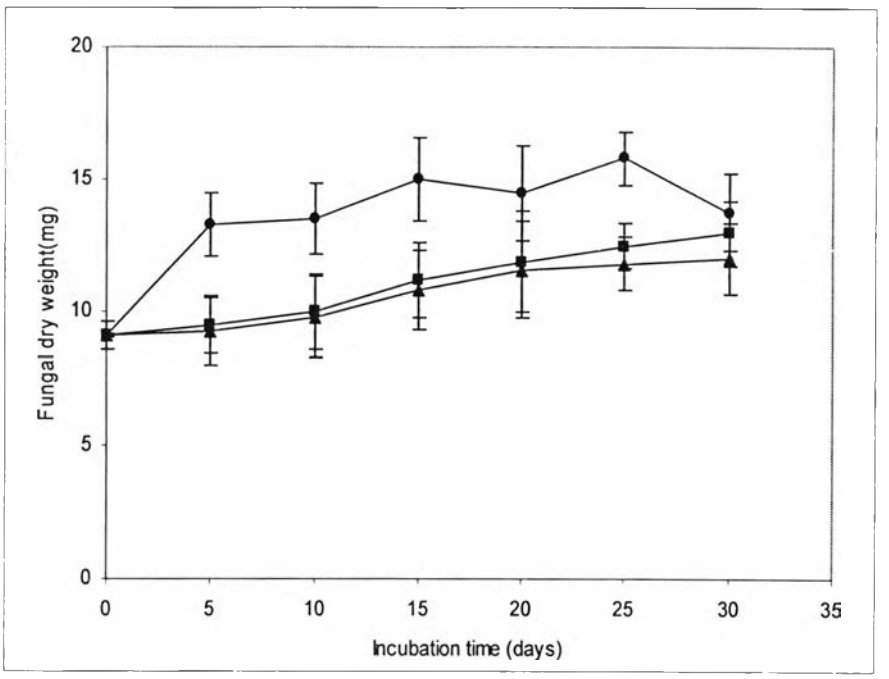
7.2.2 Effect of initial BaP concentration

The further investigation of biodegradation of BaP by this fungi was carried out with the optimum shaking stoke (180 rpm). The ability of the promising fungi to survive and degrade BaP at various concentrations (100, 200, and 300 ppm) was investigated. The results were showed in Figure 7.2.2 as shown below.

The *Fusarium oxysporum* E033 was able to degrade BaP at various concentrations (100, 200, and 300 ppm). The growth limitation with slower growth rate at higher BaP concentration was revealed when compared to that in 100-ppm BaP (Figure 7.2.2 A). The degradation rate of BaP within the first five days of incubation was decreased from 34 μg BaP/day (with 100 ppm BaP) to 8 μg BaP/day (at 200 ppm and 300 ppm BaP) per mg the fungal dry weight. The biodegradation was suppressed when the concentration of BaP was increased (Figure 7.2.2 B).

This result showed that *Fusarium oxysporum* E033 could tolerate and survive at higher concentration of BaP (200 ppm and 300 ppm), but the growth was significantly limited with slower growth rate when compared to that in 100-ppm BaP (Figure 7.2.2 A). These limited growths at higher concentrations of BaP could result from higher toxicity and lower water solubility, thus the BaP uptake was limited. Consequently, the slower initial specific BaP degradation rate at higher concentration was detected.

In 100-ppm BaP, the degradation rate of BaP was rapid within the first five days of incubation in which 34 μg BaP was degraded per day per mg the fungal dry weight, and then the degradation was continued with a slower rate (Figure 7.2.2 B). The total biodegradation of BaP by *Fusarium oxysporum* E033 after 30 days of incubation was approximately 65% of the initial amount or about 39% degradation per unit biomass was obtained. The growth and substrate depletion kinetic of this fungal strains illustrated in this study was similar to the classical pattern of BaP degradation in that the degradation occurred rapidly only at the early stage previously reported by *Fusarium solani* (Veignie et al., 2004). However, *Fusarium oxysporum* E033 demonstrated the greater biodegradation efficiency than that of *Fusarium solani* of which 6.8% BaP was degraded within 15 days of incubation (Veignie et al., 2004). Moreover, the degradation rate by *Fusarium oxysporum* E033 in this study was greater degradation rate than that of *Fusarium solani* previously reported to have only 17% BaP degradation per unit biomass after 30 days of incubation (Verin et al., 2004b).

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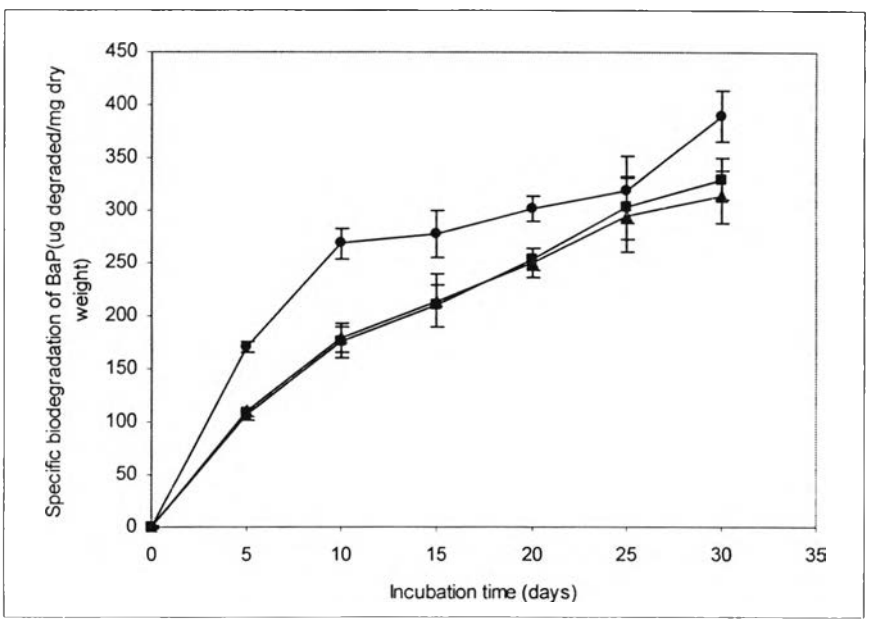



B

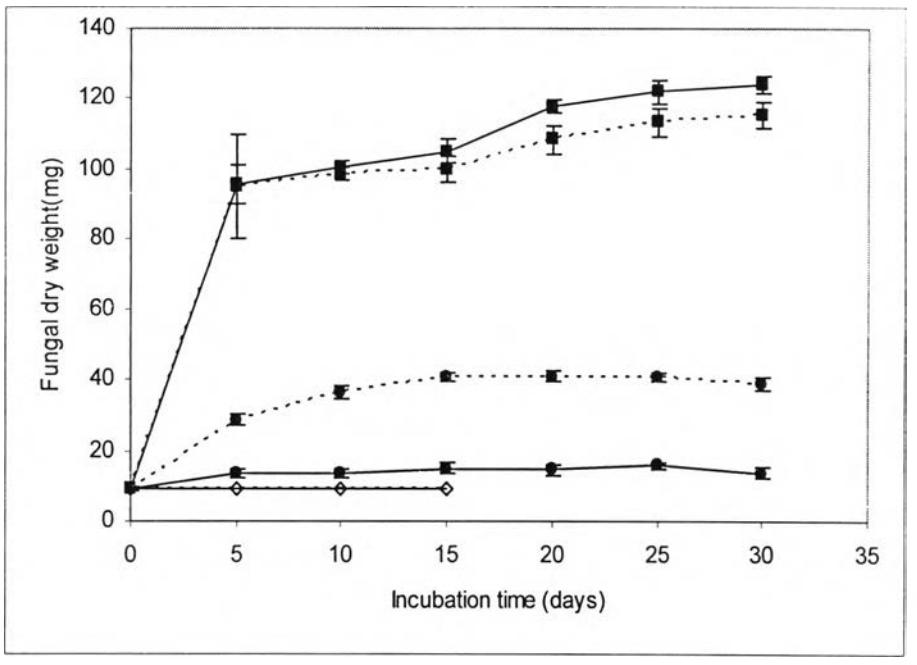
Figure 7.2.2 Growth (**A**) and biodegradation ability (**B**) of *Fusarium oxysporum* E033 in glucose-containing medium at various concentrations of BaP: 100-ppm (●), 200-ppm (■), and 300-ppm (▲). The biodegradation of BaP was expressed as specific degradation per cell dry weight. Loss of BaP by physical adsorption (+) and photo-oxidation (▵) were shown. (Mean of three replications +/- S.D.)

7.2.3 Effect of glucose concentration

To determine the effect of glucose concentration on fungal growth and BaP degradation, the cultures of fungi was grown on minimal media with 0 mM, 5 mM, and 50 mM glucose in the presence or absence of BaP. The results were shown in Figure 7.2.3

The process of co-oxidation has been proposed to be a potentially important mechanism for the dissipation of recalcitrant PAHs from soil (Perry, 1979). Also, Bengtsson and Zerhouni stated that the complementary substrate was needed to promote degradation of PAHs in the soil (Bengtsson & Zerhouni, 2003).

In this study, the considerable effort to induce the biodegradation of BaP by co-metabolism using glucose as a growth substrate was performed with concentration of 5 mM and 50 mM. Growth of fungi was rapidly increased 5 times in 50 mM glucose as a growth substrate than in the presence of 5 mM glucose (Figure 7.2.3A). The biodegradation of BaP was expected to be enhanced. Conversely the results showed that the higher concentration of glucose, the lower biodegradation and for *Fusarium oxysporum* E033, the biodegradation was almost completely repressed (Figure 7.2.3B). The carbon catabolism repression may be responsible for the phenomenon in which the activation of the catabolism of less-preferred carbon sourced was repressed if a more favorable growth substrate was available (Ilyes et al., 2004).

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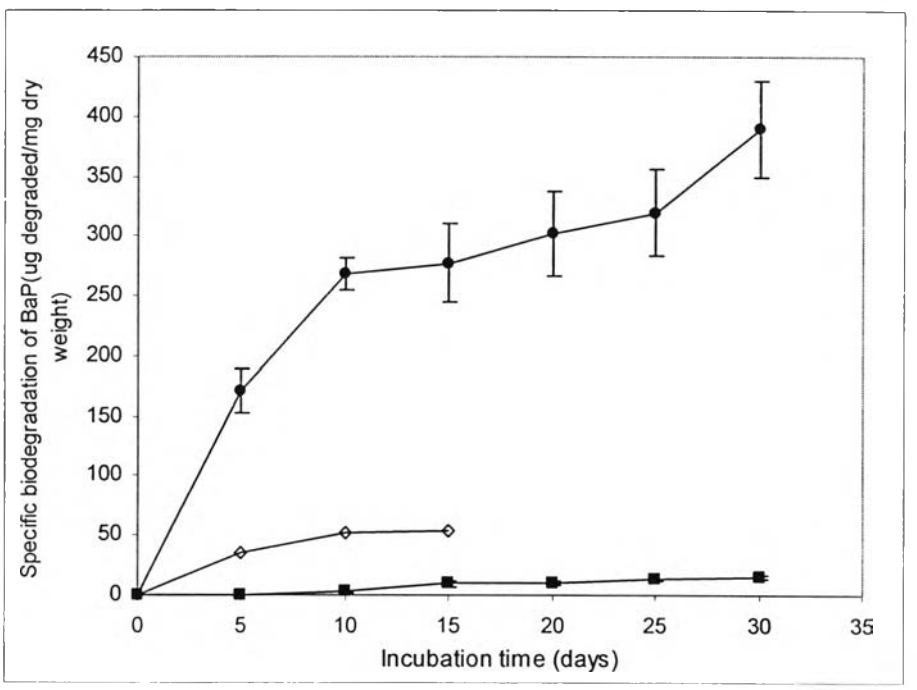



B

Figure 7.2.3 Growth (**A**) and biodegradation ability (**B**) of *Fusarium oxysporum* E033 in the medium with various concentrations of glucose: 0 mM (◊), 5 mM (●), and 50 mM (■). The fungal growth was determined in the absence (---) or in the presence (—) of 100-ppm BaP. (Mean of three replications +/- S.D.)

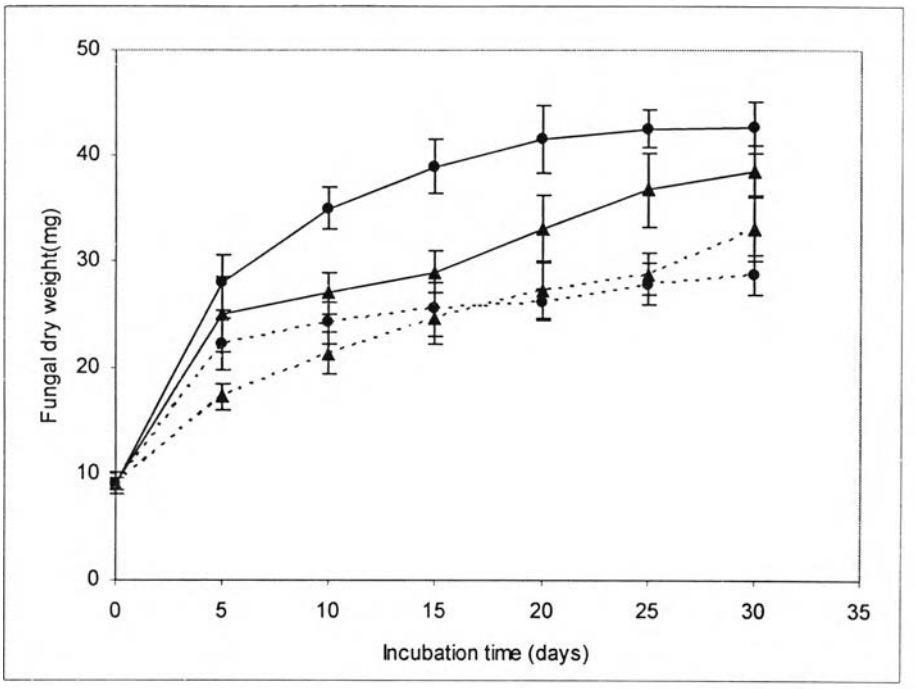
Growth of the fungal strain and BaP degradation were also examined in the absence of glucose. While there was no increase of cell growth, it was noticeably that BaP was degraded at approximately 50 μg BaP/mg dry weight within 15 days of investigation (Figure 7.2.3 A)

Similar to growth of the two *Aspergillus* sp., in absence of BaP, growth of *Fusarium oxysporum* E033 with 50 mM glucose was rapidly increased as the fungal dry weight was increased 5 times within the first five days of growth compared to that in the presence of 5 mM glucose (Figure 7.2.3 A). The presence of 100-ppm BaP obviously inhibited growth of *Fusarium oxysporum* E033 in the medium supplemented with 5 mM glucose. Contrarily, the growth inhibition was insignificantly observed when higher concentration of glucose (50 mM) was provided. BaP biodegradation of *Fusarium oxysporum* E033 at 50 mM glucose was almost completely repressed (Figure 7.2.3 B)

7.2.4 Effect of bioavailability of BaP

The substrate bioavailability to the fungus is also one of the limiting factors. Since the organic solvent-enhancing PAH biodegradations have been successfully reported either in laboratory test or in soil remediation (Jimenez and Bartha, 1996; Kilbane, 1997; Lee et al., 2001). In this investigation, two common organic solvents used in solubilization of hydrophobic contaminants, methanol and ethanol were chosen to enhance the solubility of BaP in the liquid medium. Methanol has been stated to be one of the effective PAH-extracting agents (Bergknut et al., 2004; Chen et al., 2005), whereas ethanol has been demonstrated to not only enhance PAH solubility (Chen et al., 2005), but also to increase the degradation rate of anthracene in aqueous medium (Field et al., 1995). Methanol and ethanol provided in the liquid medium at 5 mM served as a BaP solubility-enhancer as well as a carbon source for fungal strain.

The ethanol and Methanol at 5 mM were used in order to enhance the bioavailability of BaP for fungi in liquid media. The results obtained from the experiments was expressed in Figure 7.2.4

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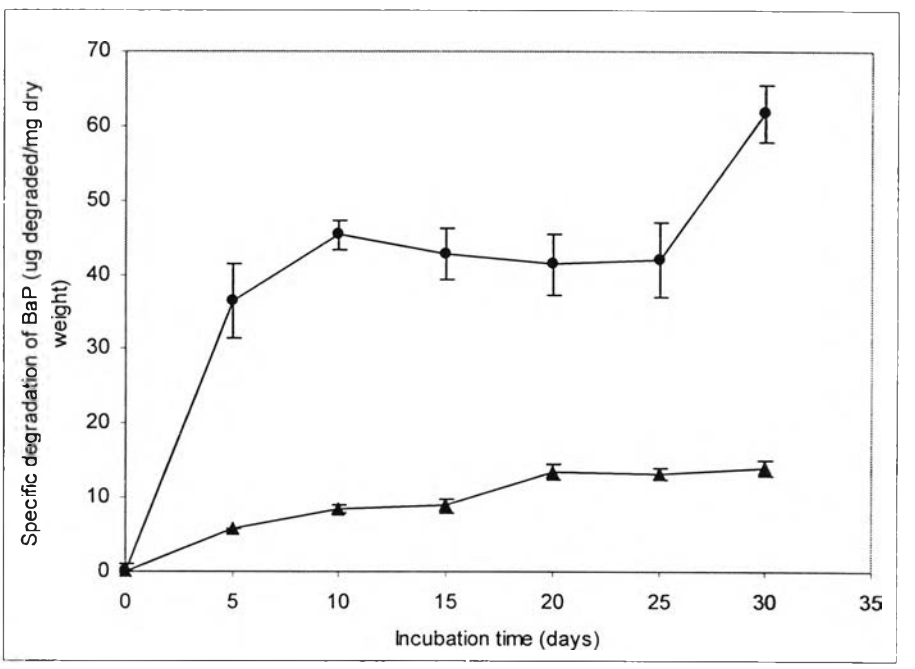


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Figure 7.2.4 Growth (**A**) and biodegradation ability (**B**) of *Fusarium oxysporum* E033 in the medium supplemented with 5 mM methanol (▲) or 5 mM ethanol (●). The fungal growth was determined in the absence (---) or in the presence (—) of 100-ppm BaP. (Mean of three replications +/- S.D.)

The determination of growth of *Fusarium oxysporum* E033 grown in either methanol or ethanol was remarkable increased in presence of BaP. In addition, the biomass in ethanol supplemented with BaP was slightly greater than growth in methanol (Figure 7.2.4 A). Also, the specific degradation in media supplemented with ethanol was significantly higher than in methanol supplemented media (Figure 7.2.4 B).

For *Fusarium oxysporum* E033, cell growth was significantly decreased in the presence of 100-ppm BaP in glucose-containing medium, cell growth was markedly increased in the medium supplemented with either methanol or ethanol indicating that more BaP was solubilized into the aqueous phase and became more available for cell growth (Figure 7.2.4 A). Normally, most micro-organism including fungi were inhibited by 1-15% of ethanol (Ingram and Buttke, 1984). However, when the biodegradation of BaP was taken into consideration, it was found that methanol and ethanol, although provided at low concentration (5 mM, i.e. <1%, v/v) showed an adversely effect in that the biodegradation was almost completely inhibited (Figure 7.2.4 B).

Ethanol was shown to be a better solubility enhancing agent than methanol to support the fungal growth and increase biodegradation (Figure 7.2.4 A,B).

Since the concentration of methanol or ethanol used in this study was low and it did not harmful to cells, the alcohol toxicity to organism would not be the reason for the inhibition of BaP biodegradation.

## 7.3 Identification of benzo(a)pyrene metabolites and the proposed degradation pathways

The analysis of BaP and BaP metabolites were performed by LC-MS as described in 3.4.3. The LC-MS results showed that after 30 days of incubation, there were peaks of interest in both extra-cellular fraction and intracellular fraction. The extra-cellular fraction showed a main molecular ion at MH+ 283, suggesting the formation of BaP

quinone (m/z= 282) (Figure 7.3.1). While a main molecular ion at MH+ 285 and MH+ 301 were observed in the intracellular fraction (Figure 7.3.2).

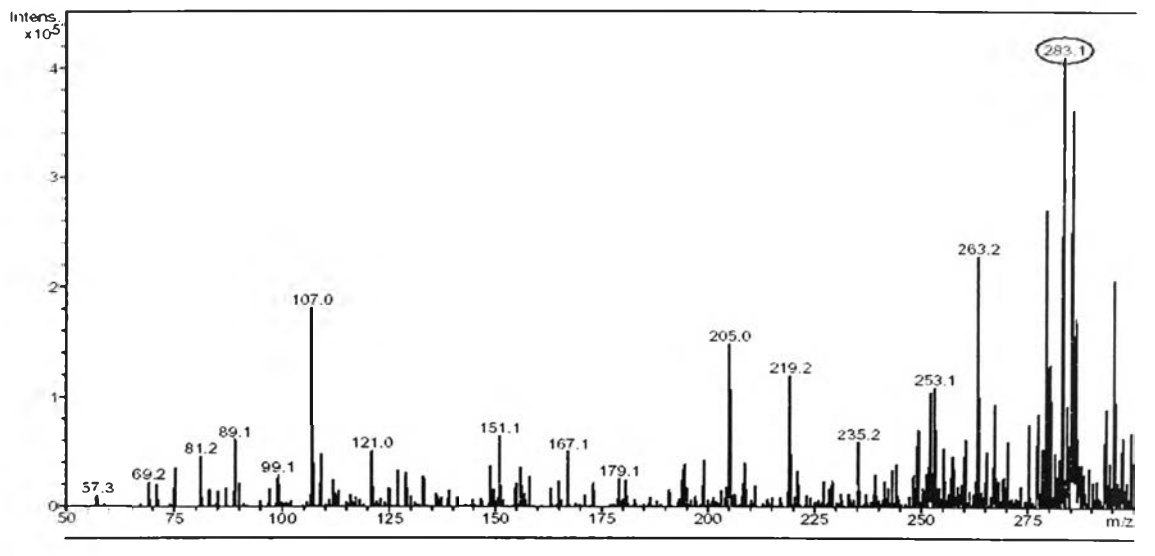


Figure 7.3.1 Mass spectrum of the metabolite intermediate obtained from the extra-cellular fraction of the *Fusarium oxysporum* E033

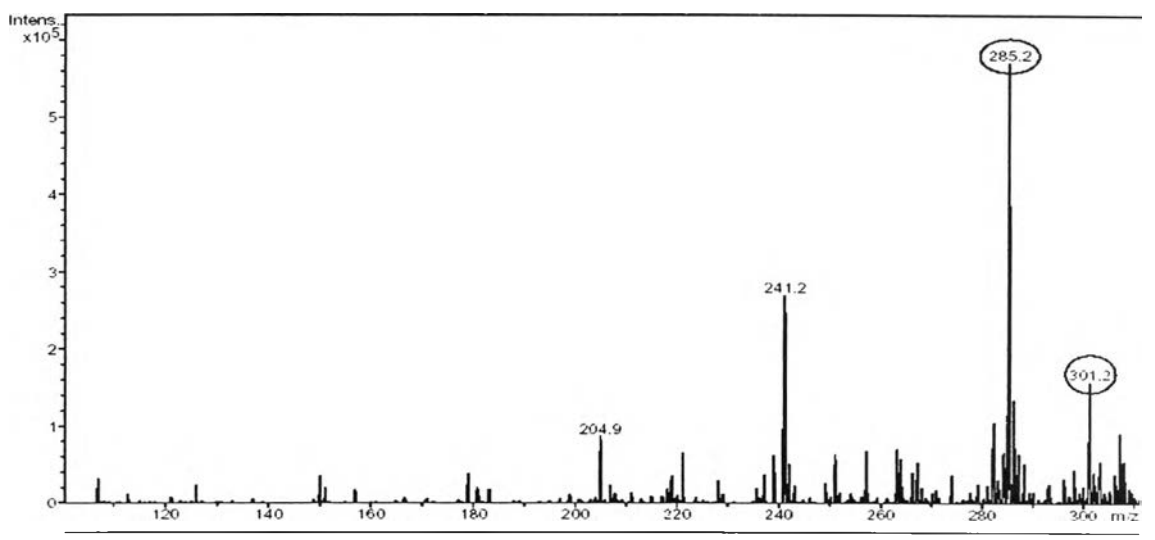


Figure 7.3.2 Mass spectrum of the metabolite intermediate obtained from the intracellular fraction of the *Fusarium oxysporum* E033

This indicated that the intermediates occurred during the biodegradation process from the extra-cellular fractions and the intracellular fractions. The main ions of BaP metabolites analyzed by electro-spray mass spectrometer were conclusively summarized in Table 7.1

Table 7.1 List of the possible BaP metabolites from *Fusarium oxysporum* E033 analyzed by the electro-spray mass spectrometer. The base peak of each fraction was shown and the suggested possible compounds were illustrated.

| Fungal isolates | Location of extractable fraction | Main ions (MH+) | Suggested the possible BaP metabolite (stereochemistry not implied) |
|---|---|---|---|
| *Fusarium oxysporum* E033 | extra-cellular | 283 | BaP 1,6 quinone (m/z=282) |
| | intracellular | 285 | 7,9-dihydroxy 7,9 dihydrodiol BaP (m/z=284) |
| | | 301 | BaP diol epoxide (m/z=300) |

## 7.4 Analysis of the metabolites from mass spectrum obtained from *Fusarium oxysporum* E033

For the extra-cellular fraction, a mass spectrum at MH+ 283 was revealed which corresponds to the molecular mass of 282 suggesting the formation of BaP quinone.

On the other hand, the products formed from intracellular fraction of *Fusarium oxysporum* E033 gave a positive molecular ion at MH+ 285 and MH+ 301. Mass spectral analysis indicated that 2 major compounds from the biodegradation of *Fusarium oxysporum* E033 were corresponding to 7,9-dihydroxy 7,9 dihydrodiol BaP or 8,10-dihydroxy 8,10 dihydrodiol BaP having its molecular mass 284, while the other important compound at MH+ 301 was considered to be the BaP dihydrodiol epoxide (BPDE) (m/z 300).

Although 2 major compounds from the BaP biodegradation of *Fusarium oxysporum* E033 in this study were described, this result was shown to be different from previous study of *Fusarium solani*. During the biodegradation of *Fusarium solani*, only BaP quinone product type, i.e. either 1,6 BaP quinine or 3,6 BaP quinone was detected from the extracted cell mycelium at the end of the incubation (256 hrs.) (Viegnei et al., 2002). BaP quinone (1,6- or 3,6-, or 6,12- BaP quinone) was also reported to be a degradation metabolite by many fungus, such as by *Phanerochaete chrysosporium* (Haemmerili et al., 1986); *Strophoria coronilla* (Steffen et al., 2003); *Pycnoporus cinnabarinus* (Rama et al., 1998); *Trametes vesicolor* (Majcherczyk et al., 1998).

These results phenomenon suggested that the *Fusarium oxysporum* E033 has 2 BaP degradation pathways. For the extra-cellular degradation, the intermediate was shown to be BaP quinone which previously described as the dead end product. For the intracellular pathway, BaP-dihydrodiols epoxides was detected as the dead end metabolite which also could be observed in the biodegradation byother higher organisms (Cerniglia&Gibson, 1979, 1980)

The formation of several metabolites obtained from the biodegradation of *Fusarium oxysporum* E033 was similar to the report of *Cunninghamella elegans* which metabolized BaP to 7,8 dihydroxy 7,8 dihydrodiol BaP or 9,10dihydroxy 9,10 dihydrodiol BaP derivative and also 1,6 BaP quinone and 3,6 BaP quinone products (Cerniglia&Gibson, 1980).

Moreover, a similar report by *Aspergillus ochraceus* (Datta & Samanta in 1988) was stated that the metabolites found in its BaP degradation were 7,8 dihydroxy 7,8 dihydrodiol BaP or 9,10dihydroxy 9,10 dihydrodiol BaP derivative and 1,6 BaP quinone , 3,6 BaP quinone (Datta&Samanta, 1988).

The results demonstrated that a *Fusarium oxysporum* E033 has the ability to oxidize BaP by two different pathways as illustrated in Figure 7.4.

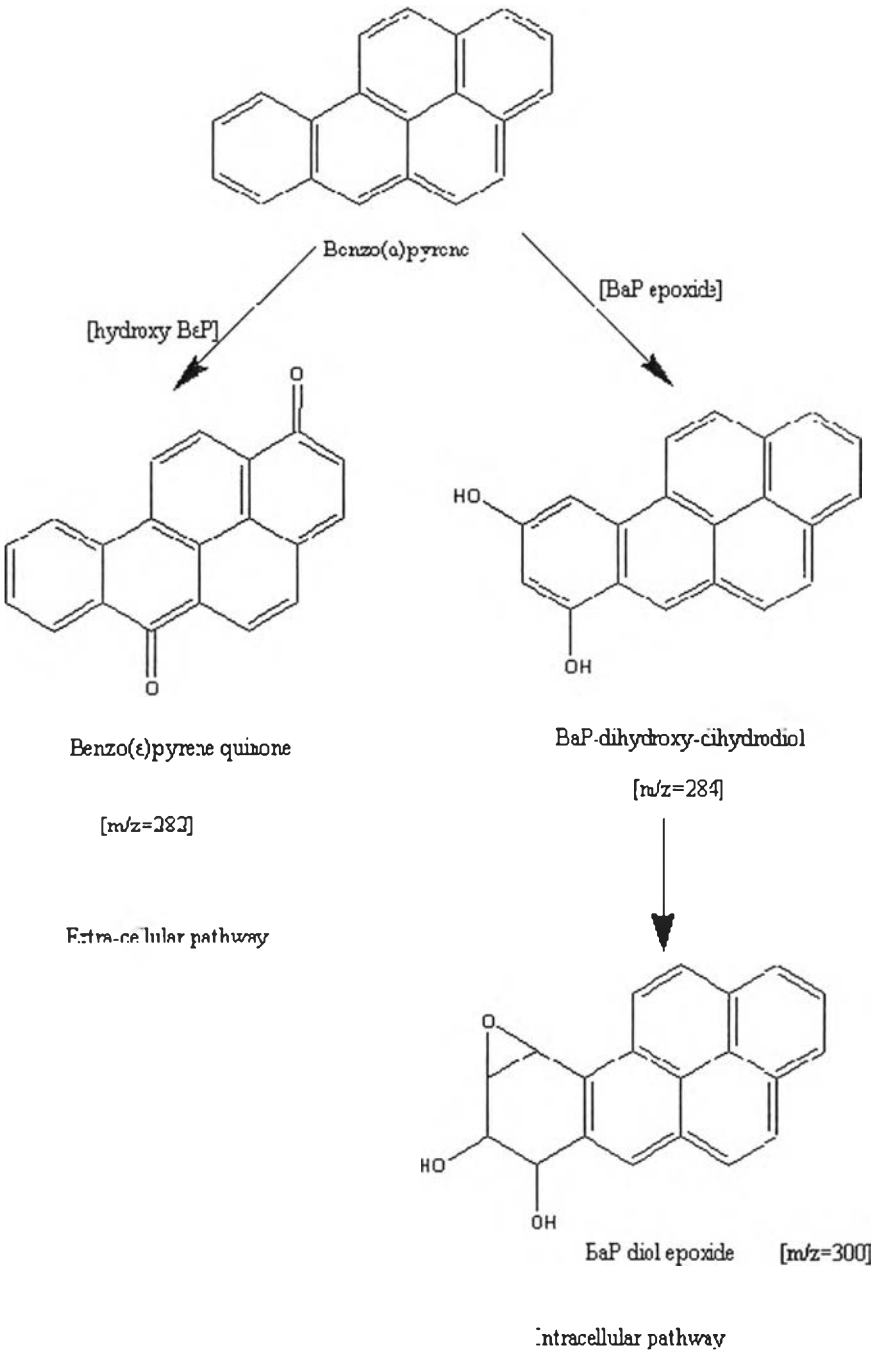


Figure 7.4 BaP oxidative pathways of the extra-cellular and the intracellular of *Fusarium oxysporum* E033 *via* BaP quinone and *via* the dihydrodiol BaP and BaP dihydroxy-epoxide. A compound in bracket was not detected. (Absolutely stereochemistry was not implied)

## 7.5 Conclusion

A *Fusarium* oxysporum E033 had the ability to degrade BaP with a degradation efficiency up to 65% of the initial amount supplied and formed the two transformation products, a trans-dihydroxy-dihydrodiol BaP and BaP quinone at the end of incubation. A relatively higher aeration increased fungal biomass as well as the biodegradation. While the high glucose concentration (50mM) increased fungal biomass, it repressed the degradation of BaP. At higher concentration of BaP, the fungal growth, i.e. biomass, as well as its BaP biodegradation was suppressed. The increase of BaP bioavailability by the addition of solvent, such as ethanol or methanol was successfully increased the fungal biomass, but the BaP biodegradation ability of fungus was decreased.