CHAPTER 6
BENZO(A)PYRENE BIODEGRADATION BY *ASPERGILLUS NIGER* B002

Beside the ability of endophytic fungus *Aspergillus niger* N003 to degrade BaP as described in Chapter 5, the fungus isolated from bark of *Pterocarpus macrocarpus* Kurz., namely *Aspergillus niger* B002 also illustrated the biodegradability towards BaP. This chapter describes the biodegrability of *Aspergillus niger* B002 towards BaP in liquid medium. The BaP degradation kinetic as well as factors involving its biodegradation are also discussed.

6.1 Biodegradation kinetic Study

The BaP degradation kinetic including the photo–oxidation, the adsorption by mycelia and biodegradation by fungi were determined. The fungal mycelium dry weight was interval collected during the degradation process. The result was shown in Figure 6.1.

The total loss of BaP from physical adsorption and photo-oxidation were determined as the controls and found to be 8% and 20% within 30 days of incubation, respectively. The total degradation after 30 day-incubation of *Aspergillus niger* B002 was approximately 70%.

The significant BaP biodegradation 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 *Aspergillus niger* B002 was found to be 32.0 μmole BaP/day (calculated from the first five days of incubation).

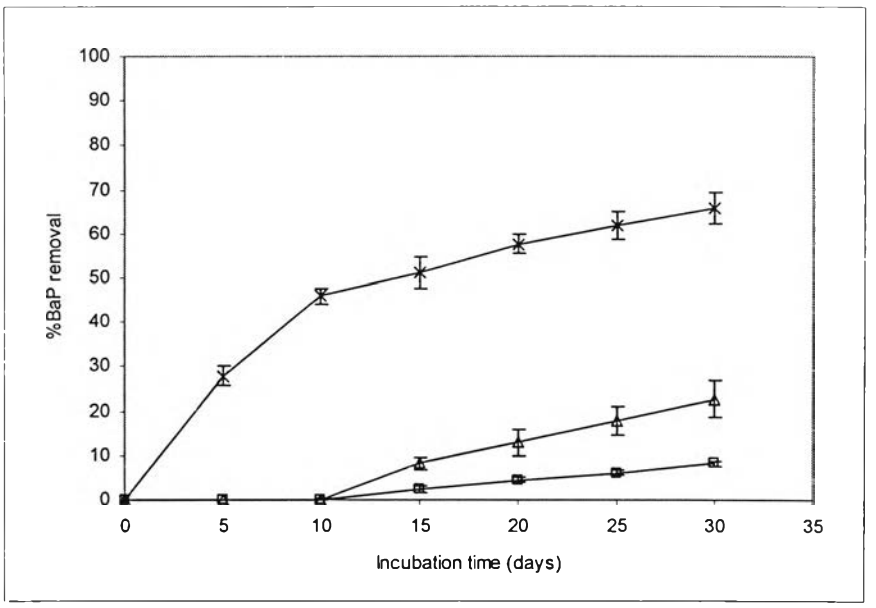


Figure 6.1 Biodegradation of 100-ppm BaP of the fungal isolate in liquid medium. Loss of BaP from photodegradation (Δ); Loss of BaP from adsorption (□); and Biodegradation (x). (Mean of three replications +/- S.D.)

This result agrees with the pervious reports showing that *Aspergillus* sp. has ability to degrade high molecular weight PAHs. The application of *Aspergillus* sp. for BaP degradation has also been reported in which 80 μmole of BaP could be degraded within 18 hours by *Aspergillus ochraceus* (Ghosh et al.,1983) or 0.4 mmole of BaP was eliminated within 48 hours (Datta&Samanta,1988).

The *Aspergillus niger* B002 isolated in this study showed the similar biodegradation efficiency to *Aspergillus terreus* in which approximately 45% BaP was removed within the first 10 days (Figure 6.1). This result expressed the greater biodegradation efficiency than that of *Aspergillus terreus* of which 27.5% of 25 ppm BaP was degraded after 9 days of culture (Capotorti et al., 2004). These results suggested the relatively good BaP degradation ability of our fungal isolate (*Aspergillus niger* B002) when compared to those of other BaP-degrading fungi previously reported.

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

6.2 Factors affecting BaP degradation

6.2.1 Effect of aeration

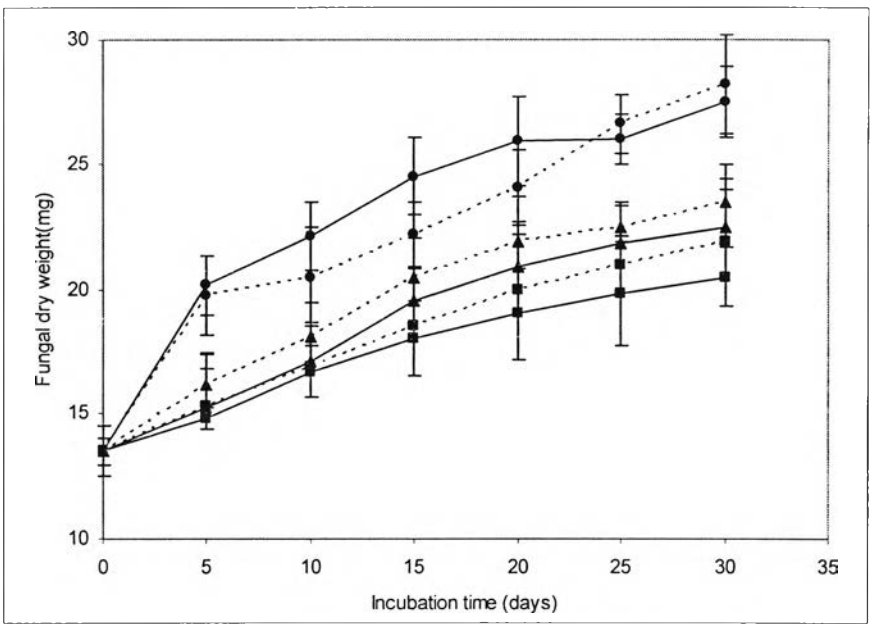
It is well established that the biodegradation of PAHs are generally reported to be the oxidation reaction as previously described in 5.2.1 (Cerniglia et al., 1985; Cerniglia, 1992; Cerniglia, 1993). Therefore, the optimization of the aeration rate was conducted at 60, 120 and 180 rpm. Aeration was obviously affected the growth of fungus in that the higher the shaking stroke it was, the higher the growth was obtained (Figure 6.2.1 A).

For the *Aspergillus niger* B002, when 100 ppm of BaP was applied, the growth of fungi was slightly suppressed. However, the higher shaking rate still gave the higher biomass (Figure 6.2.1A). In addition, similar to that of *Aspergillus niger* N003, the biodegradation of BaP at the higher aeration rate (180 rpm) was greater than those at lower aeration (Figure 6.2.1B).

According to 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 6.2.1 A, 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)

6.2.2 Effect of initial BaP concentration

The ability of fungi to survive and degrade BaP at various concentrations (100, 200, and 300 ppm) was further investigated with the optimum shaking stoke (180 rpm). The results were showed in Figure 6.2.2 as shown below.



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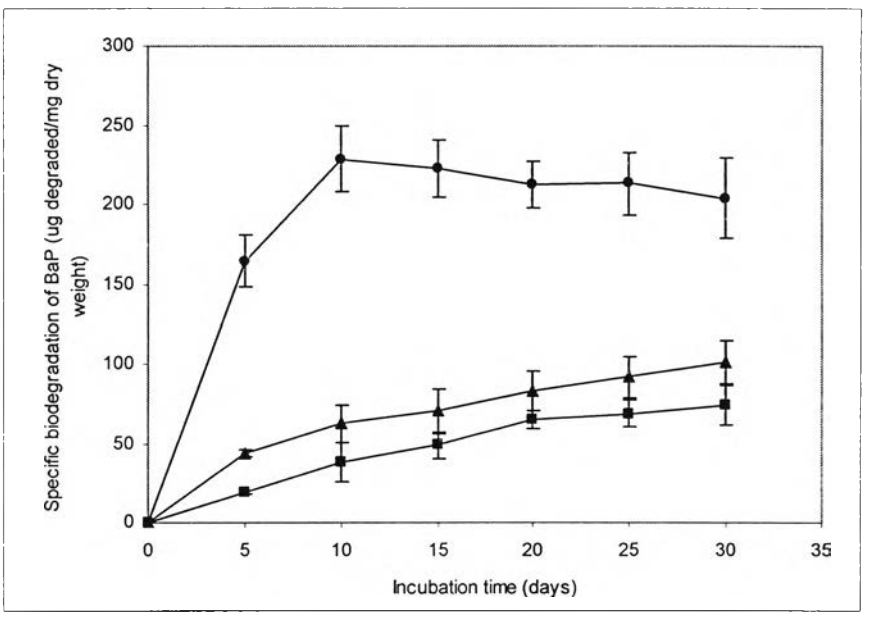

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Figure 6.2.1 Growth and biodegradation of *Aspergillus niger* B002 in glucose-containing medium, in the absence of BaP (---) or in the presence of 100-ppm BaP (—). (A) Growth of the fungi was with reciprocal shaking at 60 rpm (■), 120 rpm (▲), and 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 *Aspergillus niger* B002, growth was relatively limited when the concentration of BaP was increased (Figure 6.2.2A). Consequently, the slower initial specific BaP degradation rate at higher concentration was detected. When compared to the specific degradation at 100 ppm BaP, the specific BaP degradation rate within the first five days at higher concentration was reduced from 35 μg BaP/day to approximately 10-12 μg BaP/day per mg fungal dry weight with both 200 ppm and 300 ppm BaP (Figure 6.2.2 B).

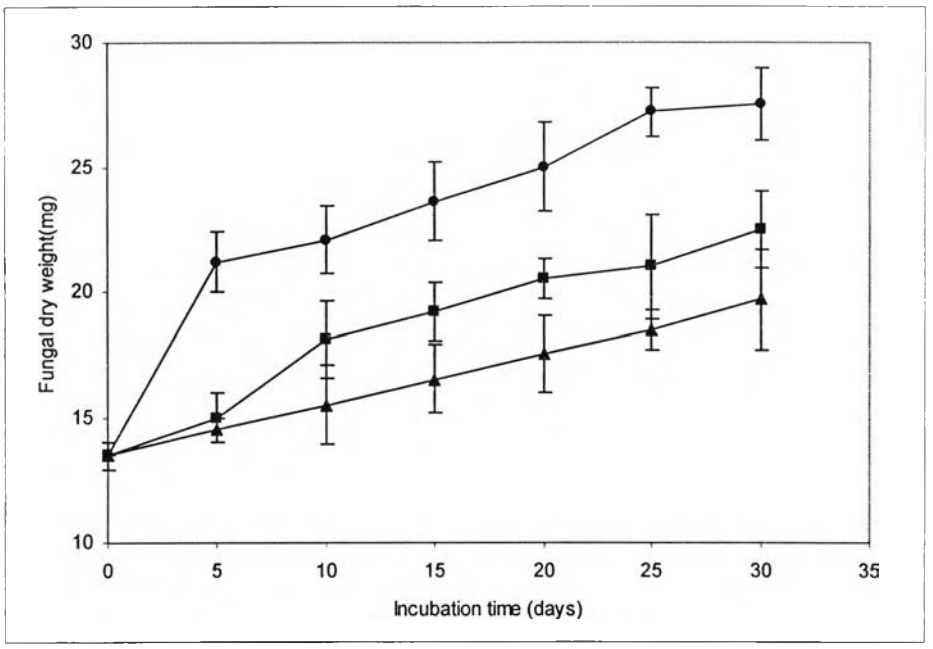
In 100-ppm BaP, the degradation rate of BaP was rapid within the first five days of incubation in which 34 μg BaP was degraded per day per mg the fungal dry weight, and then the degradation was continued with a slower rate (Figure 6.2.2 B).

6.2.3 Effect of glucose concentration

As the same experimental done with *Aspergillus niger* N003, effect of glucose concentration on fungal growth and BaP degradation were determined. The cultures of three promising fungi were 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 6.2.3.

The *Aspergillus niger* B002 showed the similar results which obtained with the *Aspergillus niger* N003. The biomass in rich media was 5 times higher than grew in 5 mM glucose at the first 5 days of incubation (Figure 6.2.3A). The biodegradation of BaP was less (figure 6.2.3B).

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).



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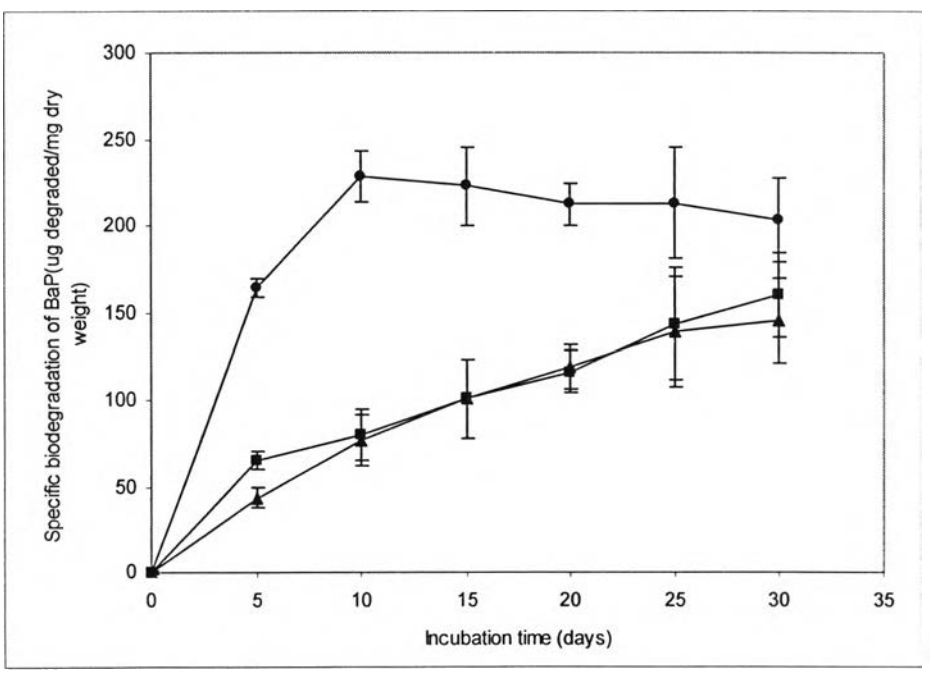

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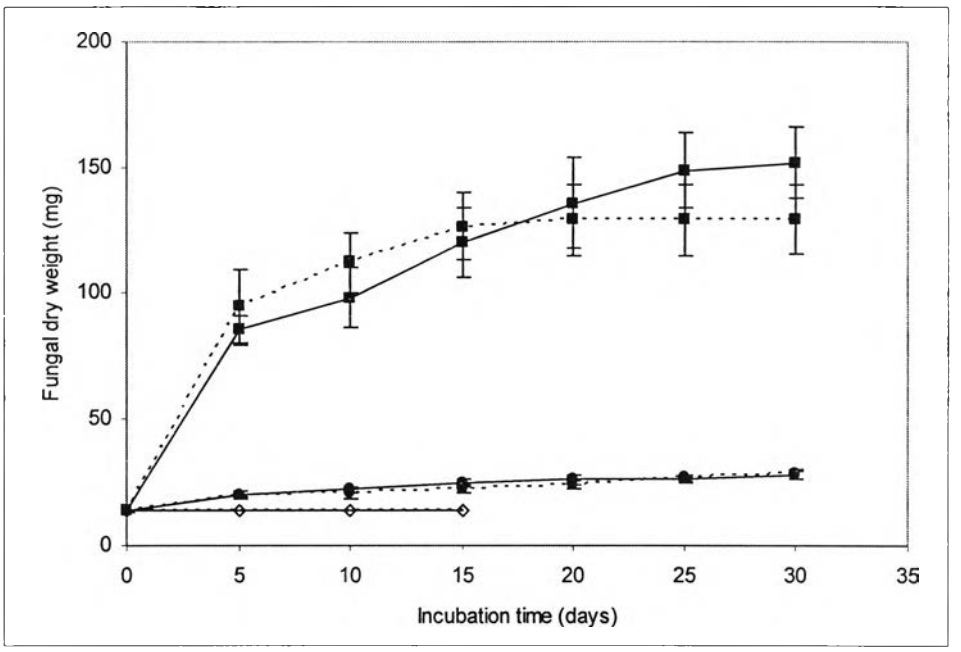
Figure 6.2.2 Growth (**A**) and biodegradation ability (**B**) of *Aspergillus niger* B002 in glucose-containing medium at various concentrations of BaP: 100-ppm (●), 200-ppm (■), and 300-ppm (▲). The biodegradation of BaP was expressed as specific degradation per cell dry weight. (Mean of three replications +/- S.D.)

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 6.2.3 A). The biodegradation of BaP was expected to be enhanced. Conversely the results showed that the higher concentration of glucose, the lower biodegradation (Figure 6.2.3 B). The carbon catabolite repression in all of these organisms 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).

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 6.2.3B). The comparison in our results of this study, the 5 mM glucose as growth substrate was relatively suitable for this fungi by giving higher specific biodegradation of BaP (Figure 6.2.3 B).

6.2.4 Effect of bioavailability of BaP

According to previous studies on BaP biodegradation, the other limiting factors besides the growth substrate and oxygen availability, the substrate bioavailability to the fungus is also one of the limiting factors. 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 fungi.



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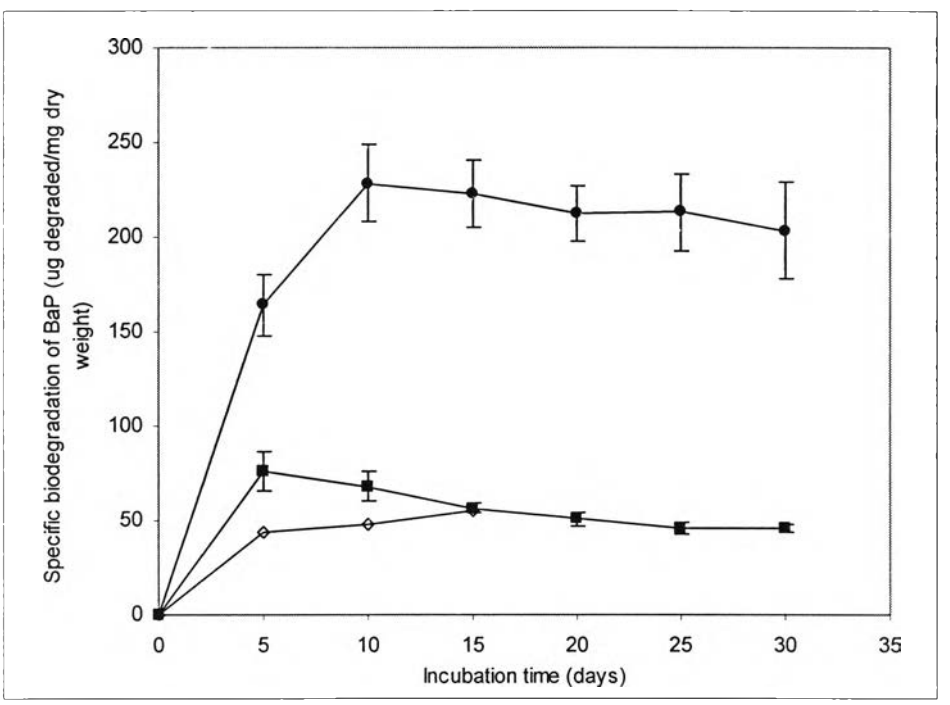


B

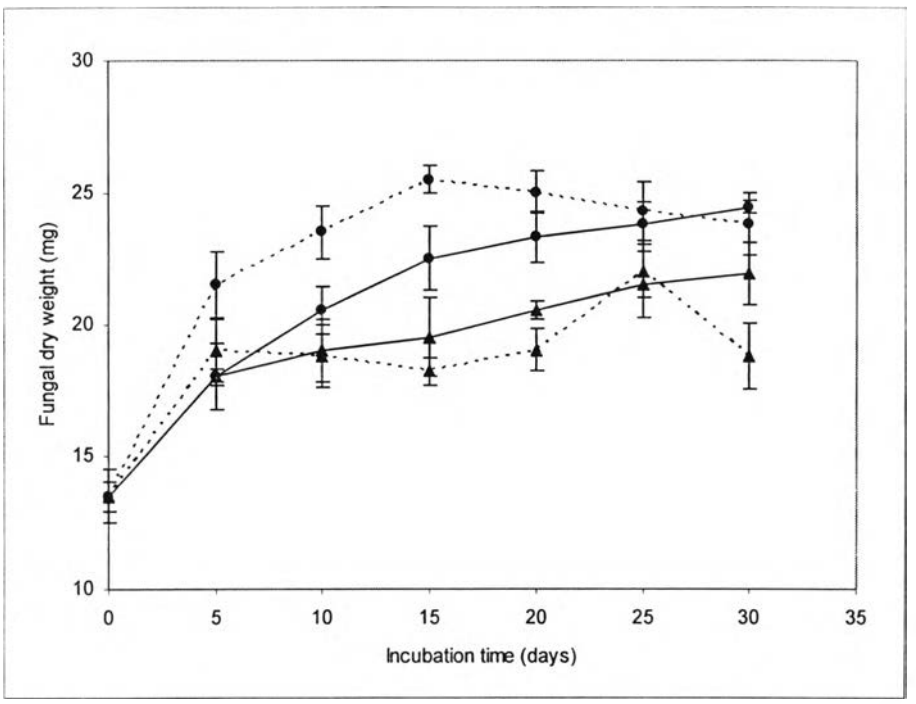
Figure 6.2.3 Growth (**A**) and biodegradation ability (**B**) of *Aspergillus niger* B002 in the medium with various concentrations of glucose: 0 mM (◊), 5 mM (●), and 50 mM (■). The fungal growth was determined in the absence (---) or in the presence (—) of 100-ppm BaP. (Mean of three replications +/- S.D.)

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 6.2.4

The *Aspergillus niger* B002 showed the suppression of biomass when grew with ethanol supplemented with BaP (Figure 6.2.4A). Although, the growth was depressed, the biodegradability of this fungal strain was greater in ethanol than in methanol (Figure 6.2.4 B).

The comparison of cell growth in each solubility-enhancing agent was investigated in the presence and absence of 100-ppm BaP. It was found that growth of *Aspergillus niger* B002 showed a slightly growth suppression when grown in ethanol or methanol in present of BaP (Figure 6.2.4 A), both the biodegradation and biomass in absence and presence of BaP grown in ethanol was greater than in methanol. This indicated that methanol was more toxic to this fungal strain than ethanol. The biodegradation supplemented with ethanol was significantly expressed equally as growing in 5 mM glucose at 30 days of incubation (Figure 6.2.3 B and Figure 6.2.4B). However, the biodegradation of BaP in 5 mM ethanol was rapidly increased after 10 days of incubation (Figure 6.2.4B), while the biodegradation of BaP when grown in 5 mM glucose was immediately increased after inoculation (Figure 6.2.3 B).

The results obtained for growth in the presence of ethanol and methanol were different from previous reports as described and discussed in Chapter 5.



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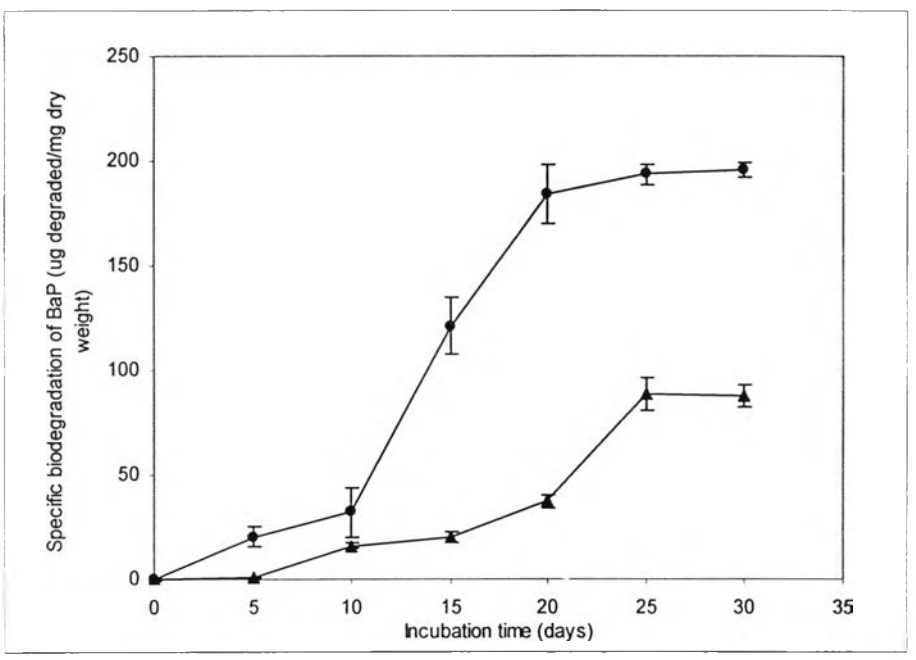

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Figure 6.2.4 Growth (**A**) and biodegradation ability (**B**) of *Aspergillus niger* B002 in the medium supplemented with 5 mM methanol (▲) or 5 mM ethanol (●). The fungal growth was determined in the absence (---) or in the presence (—) of 100-ppm BaP. (Mean of three replications +/- S.D.)

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

The spent liquid media and fungal cell culture were collected at every 5 days during the incubation and extracted with dichloromethane to recover the remaining BaP and its metabolites. The analysis of BaP and BaP metabolites were performed by LC-MS. 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+ 285, suggesting the formation of trans-dihydroxy-dihydrodiol of BaP (m/z= 284) (Figure 6.3.1). While a main molecular ion at MH+ 279 was observed in the intracellular fraction (Figure 6.3.2).

This could be explained that there were intermediates occurred during the biodegradation process from the extra-cellular fractions and the intracellular fractions, while there was no detectable metabolite was detected from liquid media. This result probably indicated that no sufficient level of water-soluble compound was released during the BaP degradation (Viegnei et al., 2002).

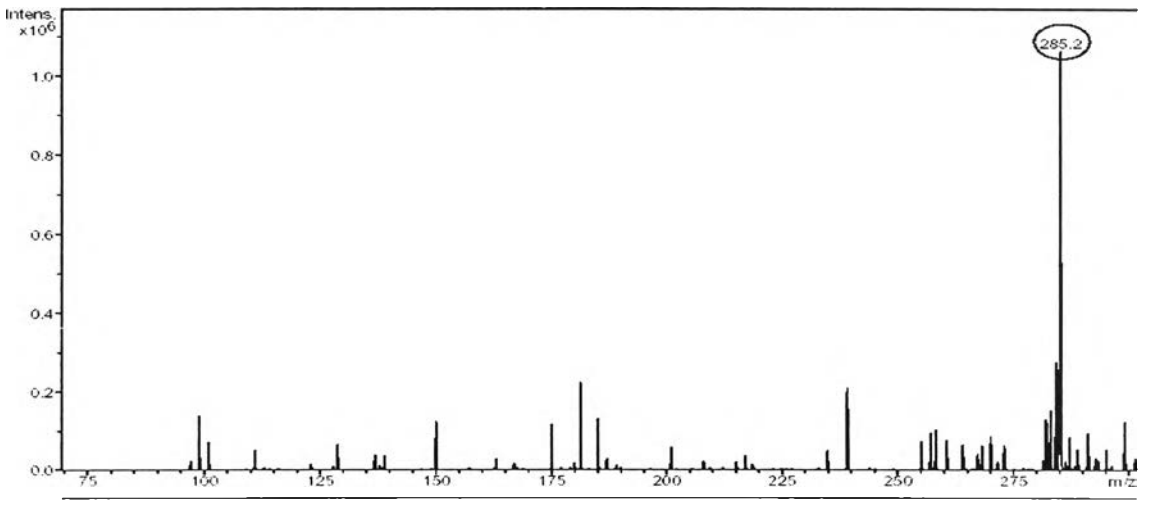

Figure 6.3.1 Mass spectrum of the metabolite intermediate obtained from the extra-cellular fraction of the *Aspergillus niger* B002

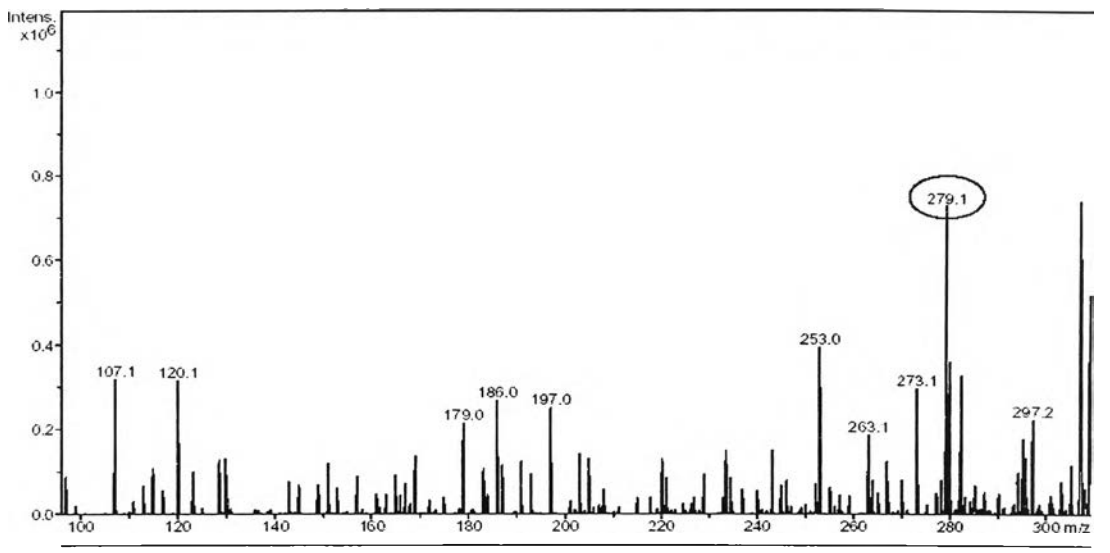

Figure 6.3.2 Mass spectrum of the metabolite intermediate obtained from the intracellular fraction of the *Aspergillus niger* B002

The main ions of BaP metabolites analyzed by electro-spray mass spectrometer were conclusively summarized in Table 6.1

Table 6.1 List of the possible BaP metabolites from *Aspergillus niger* B002 analyzed by the electro-spray mass spectrometer.

Fungal isolates	Location of extractable fraction	Main ions (MH+)	Suggested the possible BaP metabolite (stereochemistry not implied)
Aspergillus niger B002	extra-cellular	285	7,9-dihydroxy 7,9 dihydrodiol BaP (m/z=284)
	intracellular	279	unidentified product (m/z =278)

6.4 Analysis of the metabolites from mass spectrum obtained from *Aspergillus niger* B002

The mass spectrum of the extra-cellular fraction of BaP degradation by *Aspergillus niger* B002 (Figure 6.3.1 and Table 6.1) clearly revealed the main peak at MH+ 285. This result indicated that there was a major compound having a mass of 284 corresponding to 7,9-dihydroxy 7,9 dihydrodiol BaP or 8,10-dihydroxy 8,10 dihydrodiol BaP. The result was similar to that of *Aspergillus niger* N003 (Figure 5.3.1). According to the BaP degradation pathway proposed by *Aspergillus ochareceus* (Datta & Samanta, 1988), this intermediate could be 7,9-dihydroxy 7,9 dihydrodiol BaP or 8,10-dihydroxy 8,10 dihydrodiol BaP. This degradation pathway could be illustrated in Figure 6.4.1.

Contrary to that described for *Aspergillus niger* N003 BaP biodegradation, from the intracellular fraction of *Aspergillus niger* B002, the mass spectra were found at MH+ 279 and 297. According to the previous reports, there was no BaP intermediate with these molecular mass proposed in any degradation pathway. To identify these intermediates, further studied will be necessary.

The detection of possible BaP biodegradation metabolites from both extra-cellular and intracellular fraction suggests that *Aspergillus niger* B002 has two degradation pathways for BaP. The detection of dihydroxy dihydrodiol BaP as the BaP metabolite in BaP degradation by *Aspergillus niger* B002 is similar to those previously reported by *Cunninghamella elegans* (Cerniglia&Gibson, 1980), and *Aspergillus ochareceus* in which 7,8-dihydroxy 7,8 dihydrodiol BaP was detected (Datta & Samanta, 1988).

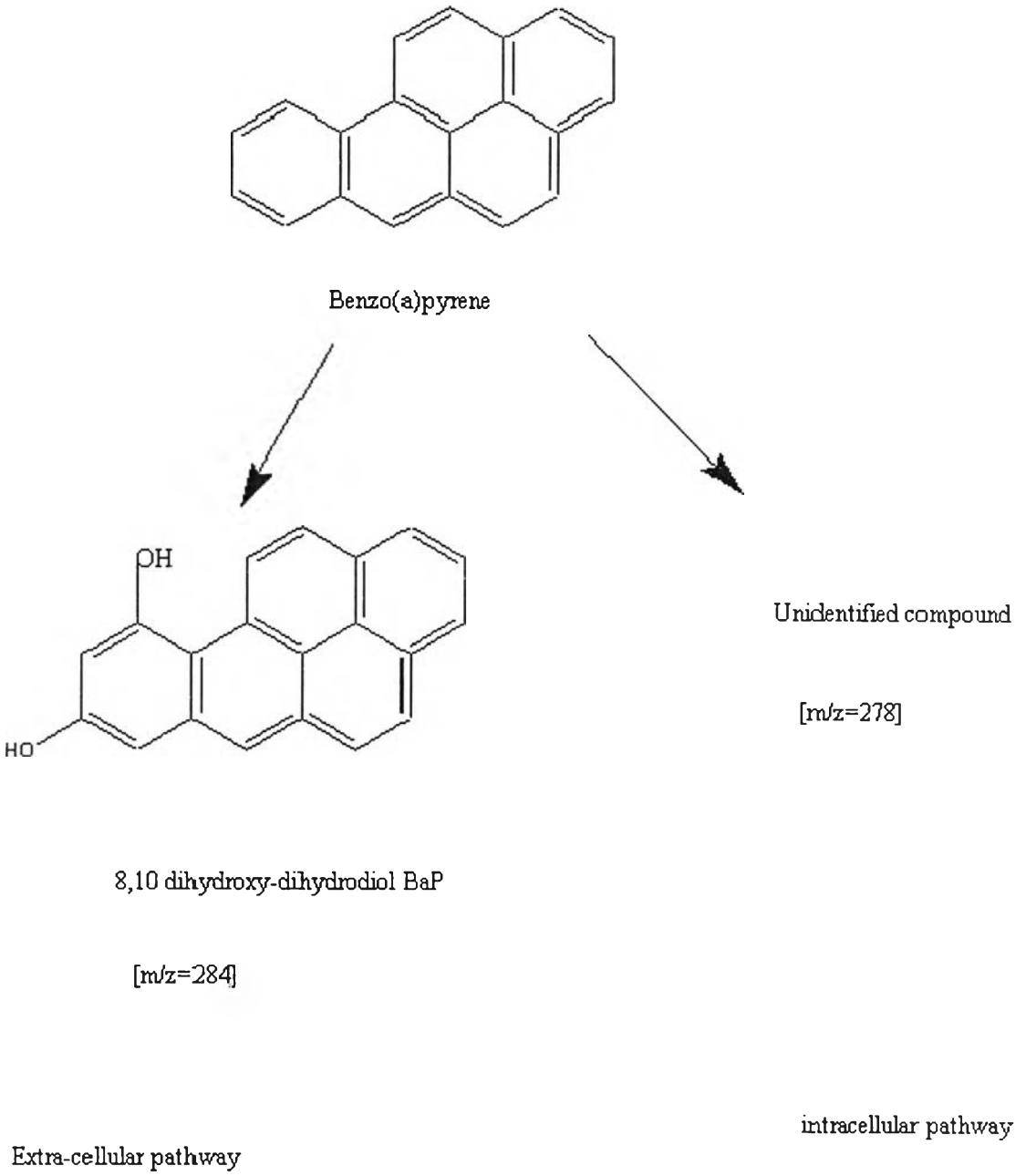

Figure 6.4 The proposed BaP oxidative pathways of the extra-cellular and intracellular of *Aspergillus niger* B002. (Absolutely stereochemistry was not implied).

6.5 Conclusion

The *Aspergillus niger* B002 isolated from bark of *Pterocarpus macrocarpus* Kurz. exposed to the traffic smoke was able to degrade the initial amount of 100 ppm BaP. The degradation efficiency of approximately 70% after 30 days of incubation at 32°C was reported. The metabolite produced from the biodegradation was detected and suggested to be dihydroxy-dihydrodiol-BaP. The higher aeration rate provided to the fungus was found to increase the fungal biomass as well as the biodegradation. A high concentration of glucose (50 mM) only promoted the fungal biomass but repressed the degradation. The high concentration of BaP (more than 100 ppm BaP and up to 300 ppm) suppressed both the fungal biomass and the biodegradation. The increase of BaP bioavailability through solvent addition, i.e. ethanol or methanol, did not promote the biodegradation ability of the fungal isolate.