CHAPTER 5
BENZO(A)PYRENE BIODEGRADATION BY *ASPERGILLUS NIGER* N003

As described in chapter 4, three fungal isolates were obtained as potent BaP degrader. One of them was endophytic fungus, *Aspergillus niger* N003. This chapter describes in details of its biodegradation ability and factors affecting the degradation

5.1 Biodegradation Kinetic study

The BaP degradation kinetic was studied in liquid media. The photo – oxidation, the adsorption by mycelia and biodegradation by fungi were measured and recorded every 5 days until 30 days of incubation. The mycelium dry weights of fungi were collected corresponding to the degradation process. The result was shown in Figure 5.1 and described as following:

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, respectively. While of the total degradation after 30 day-incubation of *Aspergillus niger* N003 was about 80% (Figure 5.1). The biomass of this promising fungus could be seen in Figure 4.2 A.

The significant BaP biodegradation by *Aspergillus niger* N003 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* N003 was found to be 38.4 μmole BaP/day (calculated from the first five days of incubation).

The *Aspergillus niger* N003 isolated in this study showed the similar biodegradation efficiency to *Aspergillus terreus* in which approximately 50% of 100 ppm BaP was removed within the first 10 days (Figure 5.1). 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).

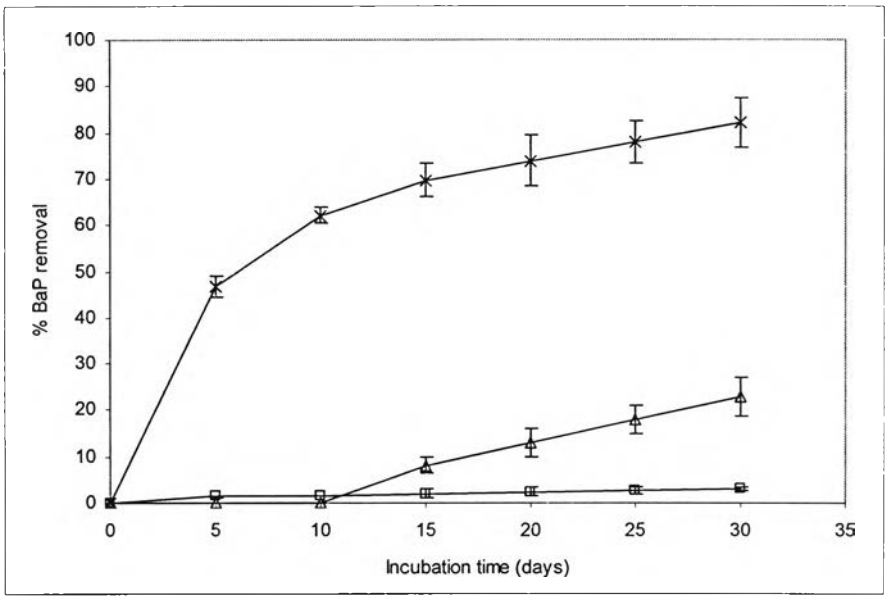


Figure 5.1 Biodegradation of 100-ppm BaP of the fungal isolate in liquid medium. Loss of BaP from auto-degradation (Δ); Loss of BaP from adsorption (□); and BaP Biodegradation (x). (Mean of three replications +/- S.D.)

This result suggested the relatively good BaP degradation ability of our fungal isolate (*Aspergillus niger* N003) 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.

5.2 Factors affecting BaP degradation

5.2.1 Effect of aeration

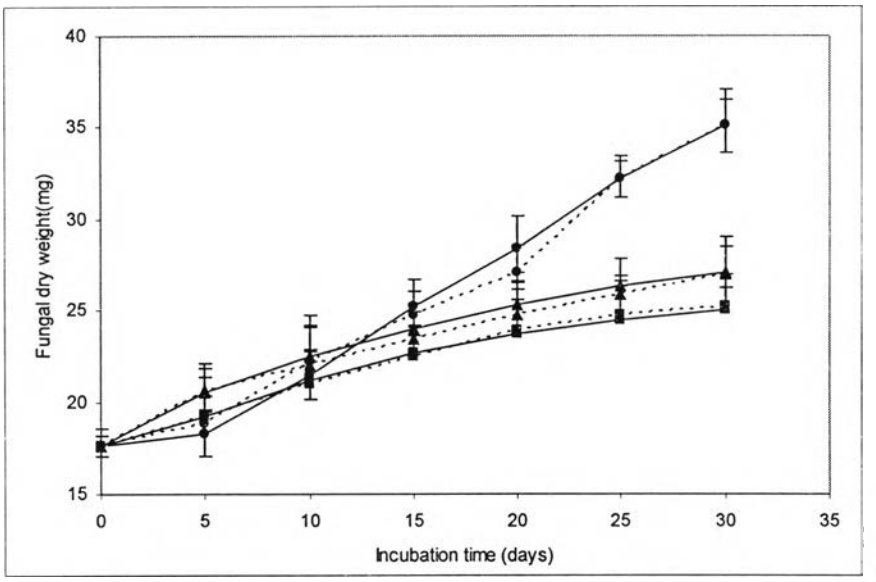
Since degradation of PAH is generally reported to be the oxidation reaction (Cerniglia et al., 1985; Cerniglia, 1992; Cerniglia, 1993), we optimized the aeration rate to demonstrate whether it affected growth of fungi as well as its biodegradation ability of BaP by varying the shaking speed from 60, 120 and 180 rpm. The results were presented in Figure 5.2.1 that aeration was obviously affected the growth of this fungal strains in that the higher the shaking stroke it was, the higher the growth was obtained (Figure 5.2.1 A).

For *Aspergillus niger* N003, we found that, the presence of BaP did not affect or reduce the biomass of fungi. The higher shaking rate, the higher biomass obtained (Figure 5.2.1A). The biodegradation of BaP was expressed as specific degradation per cell dry weight were increased when the aeration rate increased, and the results showed that at the shaking stroke at 180 rpm, the biodegradation of BaP was much more higher than those at 60 rpm or 120 rpm (Figure 5.2.1B).

Since the fungal growth of each shaking conditions tested were different, the comparison of biodegradation of BaP was described as the specific degradation in which the amount of BaP degraded was calculated as dry weight fungal biomass. 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 5.2.1 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).

5.2.2 Effect of initial BaP concentration

Biodegradation of BaP carried out with the optimum shaking stoke (180 rpm) was further investigated. The ability of fungi to survive and degrade BaP at various concentrations (100, 200, and 300 ppm) was investigated. The results were showed in Figure 5.2.2 as shown below.



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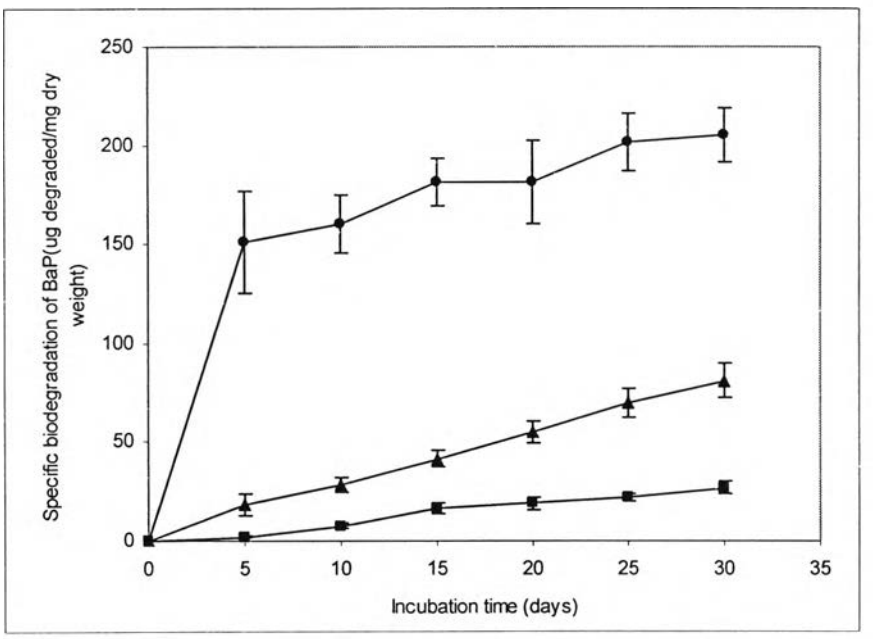

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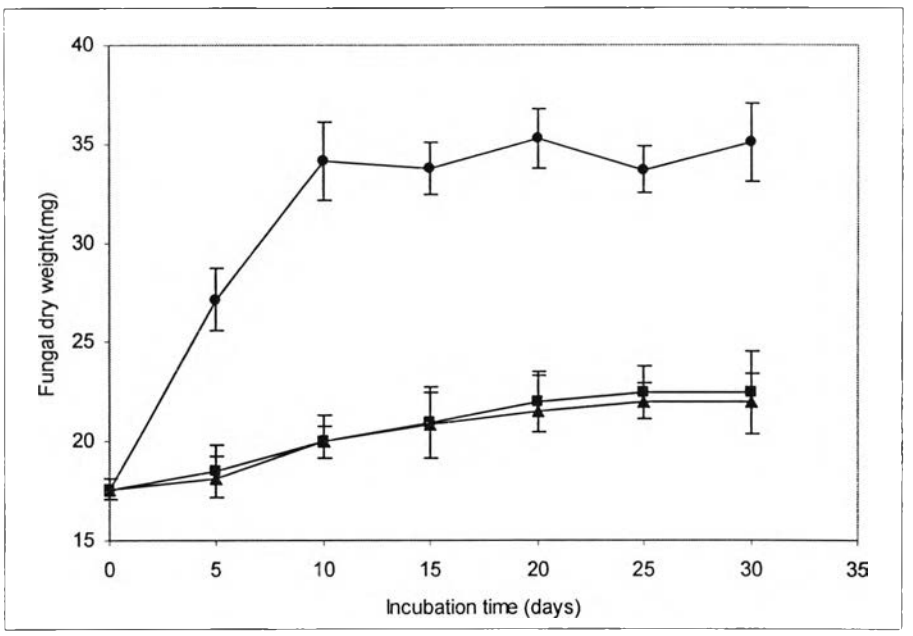
Figure 5.2.1 Growth and biodegradation of *Aspergillus niger* N003 in glucose-containing medium, in the absence of BaP (---) or in the presence of 100-ppm BaP (—). (A) Growth of the fungi was with reciprocal shaking at 60 rpm (■), 120 rpm (▲), and 180 rpm (●). (B) Biodegradation of BaP when cells were 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.)



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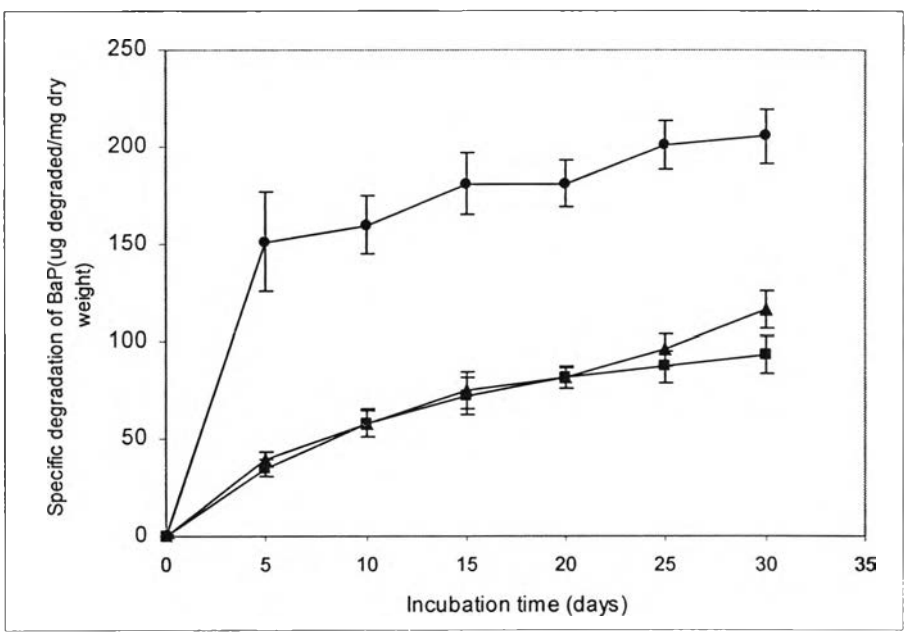

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Figure 5.2.2 Growth (A) and BaP biodegradation ability (B) of *Aspergillus niger* N003 in glucose-containing medium at various concentrations of BaP: 100-ppm (●), 200-ppm (■), and 300-ppm (▲). The biodegradation of BaP was expressed as specific degradation per cell dry weight. (Mean of three replications +/- S.D.)

The study with *Aspergillus niger* N003 showed that at the higher concentration of BaP supplied, the growth of fungal isolate was significantly limited (Figure 5.2.2A) and the biodegradation of BaP was reduced when compared to that with 100 ppm BaP. When *Aspergillus niger* N003 grown in the presence of 100 ppm BaP could uptake approximately 200 μg BaP per mg dry weight within 30 days of incubation, it could only uptake 50% of the BaP when grown with 200 ppm and 300 ppm BaP (Figure 5.2.2 B).

In 100-ppm BaP, the degradation rate of BaP was rapid within the first five days of incubation in which 30 μg BaP at 100 ppm BaP was decreased to 8 μg BaP per mg dry weight within 30 days of incubation, and then the degradation was continued with a slower rate (Figure 5.2.2B). The total biodegradation of BaP after 30 days of incubation was approximately 80% of the initial amount supplied (Figure 5.1).

5.2.3 Effect of glucose concentration

To determine the effect of glucose concentration on fungal growth and BaP degradation, 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 5.2.3.

The fungal growth of *Aspergillus niger* N003 grown in the 50 mM glucose was rapidly increased 5 times within the first 5 days of incubation compared to that in the presence of 5 mM glucose (Figure 5.2.3A). However, the specific degradation was decreased 3 times (Figure 5.2.3B). In the absence of glucose, the fungal growth was obviously limited. It is interesting, however, that the biodegradation of BaP was detected at approximately 50 μg BaP/ mg dry weight within 15 days of investigation (Figure 5.2.3 A, B).

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,

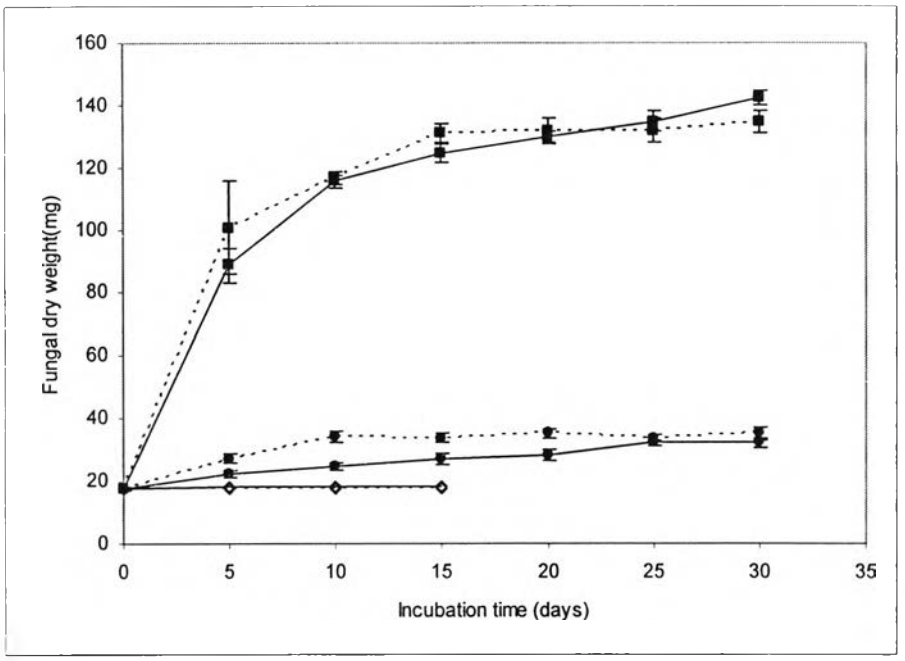
Bengtsson and Zerhouni (2003) stated that the complementary substrate was needed to promote degradation of PAHs in the soil (Bengtsson and Zerhouni, 2003).

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 5.2.3A). The biodegradation of BaP was expected to be enhanced. Conversely, the results showed that the higher concentration of glucose, the lower biodegradation for this fungal strain (Figure 5.2.3B). The carbon catabolite repression in this organism 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 *Aspergillus niger* N003 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 5.2.3A, B). The comparison in our results of this study, the 5 mM glucose as growth substrate was relatively suitable for this fungus by giving higher specific biodegradation of BaP (Figure 5.2.3B).

5.2.4 Effect of bioavailability of BaP

According to previous studies on BaP biodegradation, there are several limiting factors influencing the rate and extent of BaP degradation, such as nutrient, oxygen content, and etc. Besides the growth substrate and oxygen availability, the substrate bioavailability to the fungus is also one of the limiting factors. Since BaP has low water solubility (0.0038 mg/L, at 25°C) (Mackay et al., 2000), addition of surfactants as well as organic solvents may enhance the solubility and increase bioavailability of BaP to the organism. However, there have been reports that addition of surfactants has inhibitory and toxicity effects to the organisms (Volkering et al., 1995) and results in a significant



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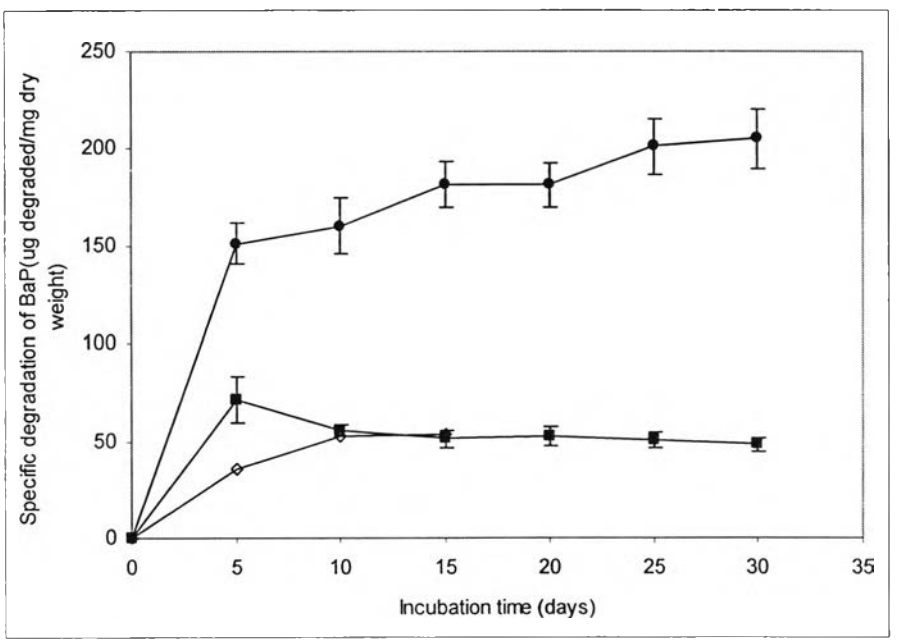

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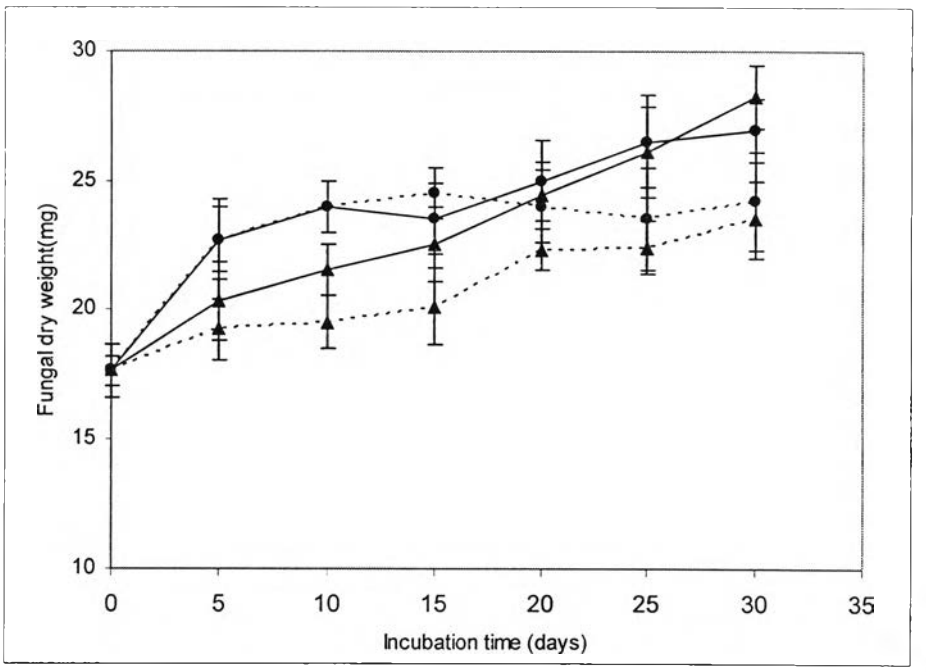
Figure 5.2.3 Growth (**A**) and biodegradation ability (**B**) of *Aspergillus niger* N003 in the medium with various concentrations of glucose: 0 mM (◊), 5 mM (●), and 50 mM (■). The fungal growth was determined in the absence (---) or in the presence (—) of 100-ppm BaP. (Mean of three replications +/- S.D.)

decrease in contaminant degradation due to extensive micellation (Graves and Leavitt, 1991; Smith et al., 1997). On the other hand, works on 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.

From Figure 5.2.4 A, it was found that the biomass of the *Aspergillus niger* N003, either solubilized by ethanol or methanol, was greater in the presence of BaP than in the absence of BaP. BaP was degraded at the same rate in the presence of either ethanol or methanol in that 100 μg BaP was reduced per mg dry weight of fungi within 30 days (Figure 5.2.4 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* N003 grown on either methanol or ethanol were slightly lower than that grown in 5 mM glucose (Figure 5.2.1A and Figure 5.2.4A). This indicated that either methanol or ethanol was non toxic to this fungal strain. 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 lower than that of only 50% obtained by using glucose supplemented as carbon source at 30 days (Figure5.2.1B and Figure5.2.4B).



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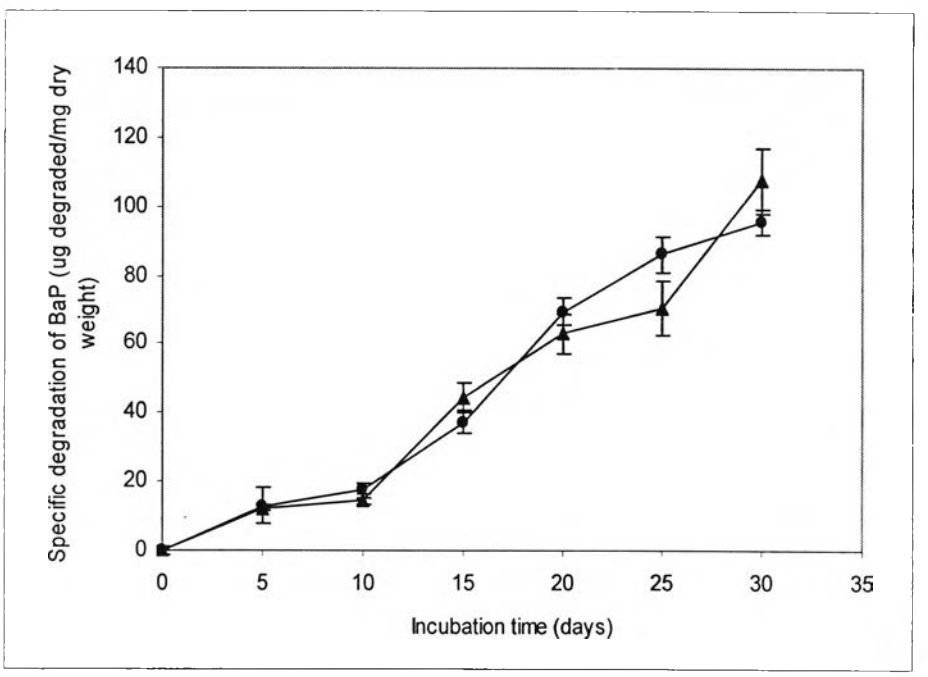

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Figure 5.2.4 Growth (**A**) and biodegradation ability (**B**) of *Aspergillus niger* N003 in the medium supplemented with 5 mM methanol (▲) or 5 mM ethanol (●). The fungal growth was determined in the absence (---) or in the presence (—) of 100-ppm BaP. (Mean of three replications +/- S.D.)

Addition of solvent such as alcohols to the fungal culture could yield either positive or negative effect to the fungal growth as well as its biodegradability. Previous reports showed that addition of ethanol or acetone could enhance 2-6 times of PAH biodegradation in soil (Schafran, 1999; Lee et al., 2001). However, the addition of ethanol was reported to be harmful to *Aspergillus nidulans* of which growth was failed on ethanol. Moreover, acetaldehyde, produced from ethanol utilization pathway, although might act as a physiological inducer of the enzyme responsible for its xenobiotics biodegradability (Flipphi et al., 2002), its accumulation in cells might have an adverse effect to the biodegradation mechanism of the fungi as occurred in our result. Therefore, the investigation of factors involving the biodegradation mechanism will be necessary.

5.3 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 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 mass analysis operated in the positive ion mode using 0.1% formic acid scanned from m/z 50-320 of 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 5.3.1). While a main molecular ion at MH+ 287 was observed in the intracellular fraction (Figure 5.3.2).

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

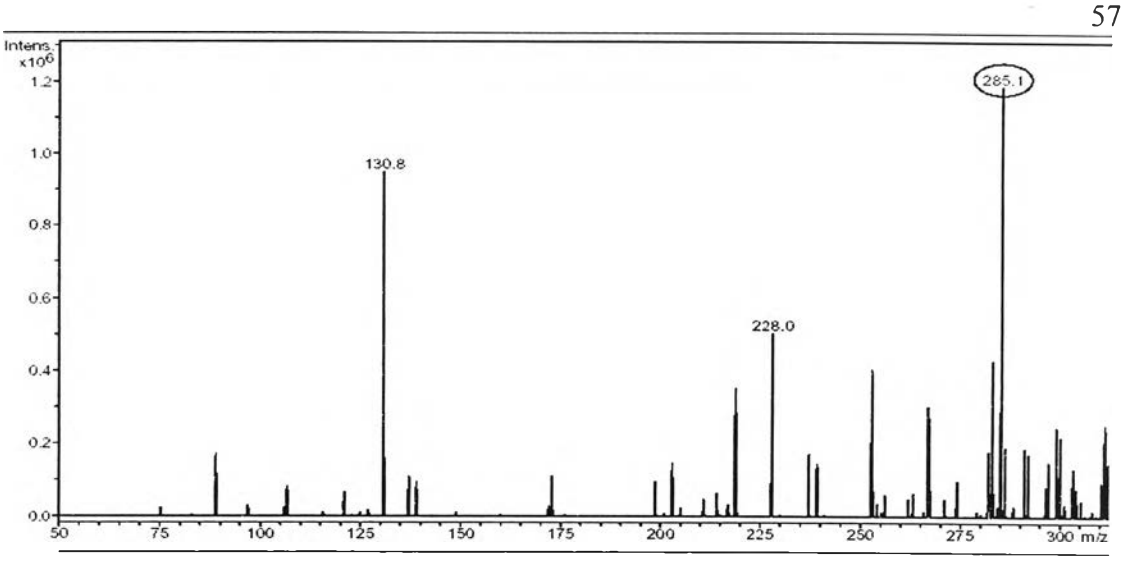

Figure 5.3.1 Mass spectrum of the BaP intermediate obtained at the end of incubation period (30days) from the extra-cellular fraction of *Aspergillus niger* N003 grown in 100 ppm BaP. A molecular ion at MH+ 285 indicated in circle represents dihydroxy-dihydrodiol of BaP.

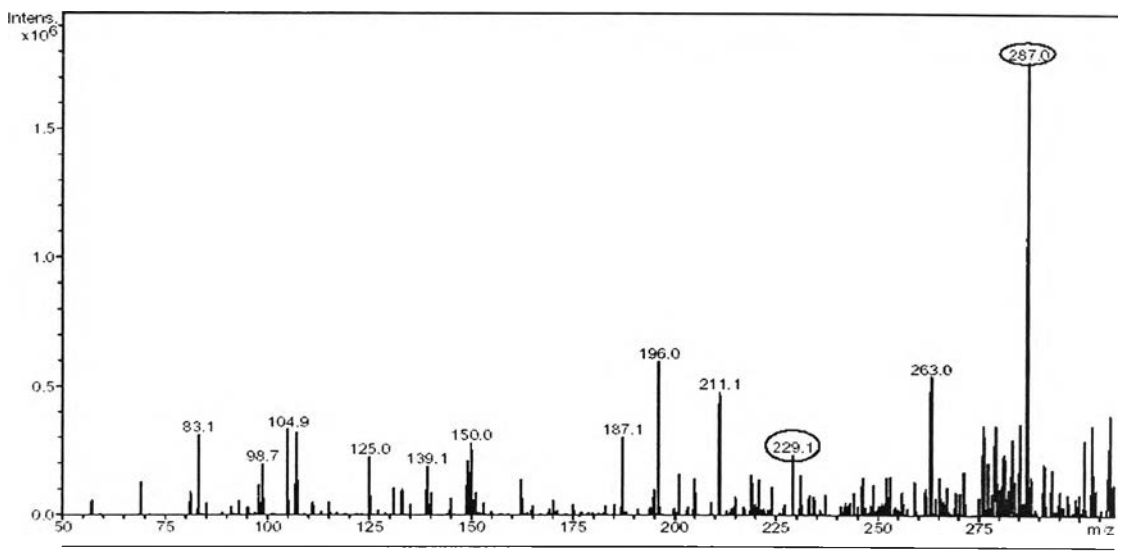

Figure 5.3.2 Mass spectrum of the BaP intermediate obtained at the end of incubation period (30days) from the intracellular fraction of the *Aspergillus niger* N003 grown in 100 ppm BaP.

The main ions of BaP metabolites analyzed by electro-spray mass spectrometer were conclusively summarized in Table 5.1

Table 5.1 List of the possible BaP metabolites from *Aspergillus niger* N003 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)
Aspergillus niger N003	extra-cellular	285	7,9-dihydroxy 7,9 dihydrodiol BaP (m/z=284)
	intracellular	287	dihydroxy- dihydrodiol BaP derivatives (m/z=286)

5.4 Analysis of the metabolites from mass spectrum obtained from *Aspergillus niger* N003

The mass spectrum of the extra-cellular fraction of BaP degradation by *Aspergillus niger* N003 (Figure 5.3.1 and Table 5.1) revealed the main peak at MH+ 285 which corresponds to molecular mass of m/z= 284. 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.

On the contrary, for the intracellular fraction (Figure 5.3.2 and Table 5.1), the positive mass spectrum of MH+ 287 correspond to 7,8-dihydroxy 7,8 dihydrodiol BaP or 9,10-dihydroxy 9,10 dihydrodiol BaP, or trans 4,5-dihydroxy 4,5 dihydrodiol BaP having a molecular mass of m/z 286 (Figure 5.4.1).

The detection of possible BaP biodegradation metabolites from both extra-cellular and intracellular fraction suggests that *Aspergillus niger* N003 has two degradation pathways for BaP. The detection of dihydroxy dihydrodiol BaP as the BaP metabolite in BaP degradation by *Aspergillus niger* N003 is similar to those previously reported by *Cunninghamella elegans* (Cerniglia&Gibson, 1980), and *Aspergillus ochareceus* in which 7,8-dihydroxy 7,8 dihydrodiol BaP were reported (Datta&Samanta, 1988).

In addition, there was an interesting mass peak at MH+ 229 which probably suggested the formation of chrysene (m/z 228), one of the smaller four fused ring PAHs. The formation of chrysene from BaP was proposed to transform via the cis 4,5-dihydroxy 4,5 dihydrodiol BaP to 4,5 chrysene dicarboxylic acid (Schneider et al., 1996) and finally be oxidized to chrysene. The evident of chrysene formation from the BaP degradation was reported by the ring cleavage product via cis 4,5-dihydroxy 4,5 dihydrodiol BaP and dicarboxylic acid chrysene by *Mycobacterium* sp. strain RJGII-135 grown on a mixtures of yeast extract, peptone and soluble starch (Schneider et al., 1996). However, chrysene formation from BaP degradation by fungi has never been reported.

Although this ring cleavage pathway is normally found in bacteria oxidation (Schneider et al., 1996), and according to our results, it is possible that this pathway might exist in *Aspergillus niger* N003 (Figure 5.4.2).

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

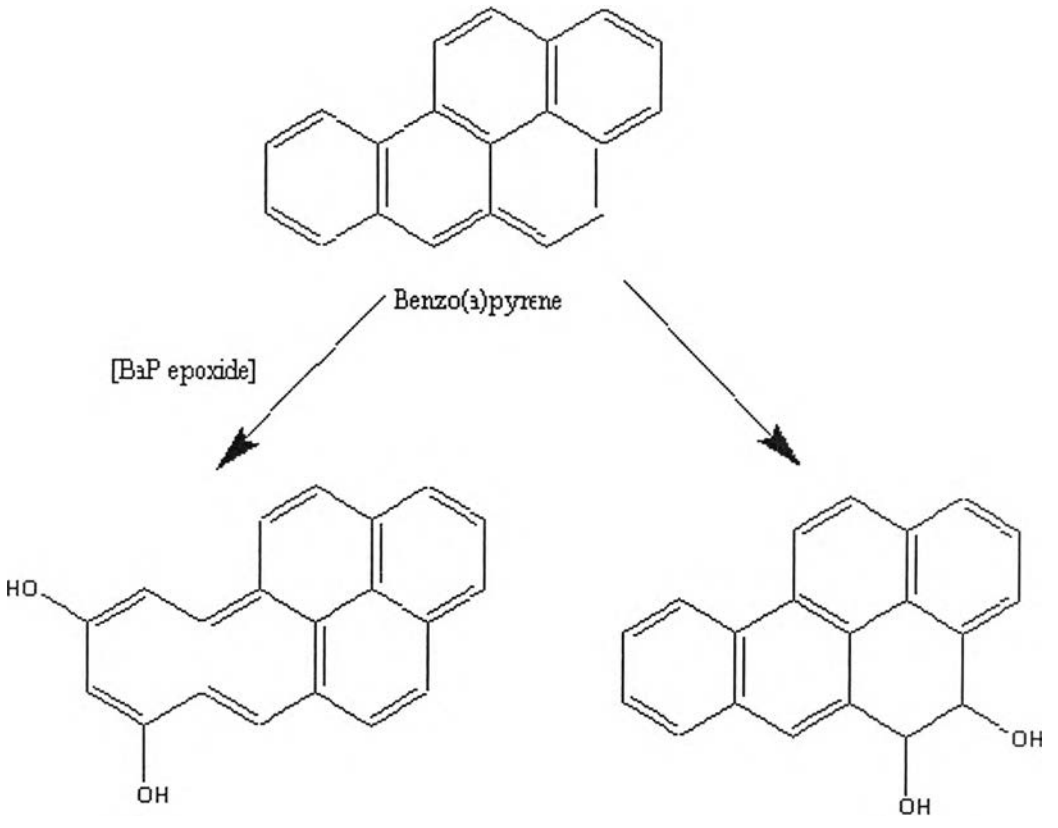

Figure 5.4.1 The proposed BaP oxidative pathways of the extra-cellular and intracellular of *Aspergillus niger* N003. Compound in bracket was not detected. (Absolutely stereochemistry was not implied)

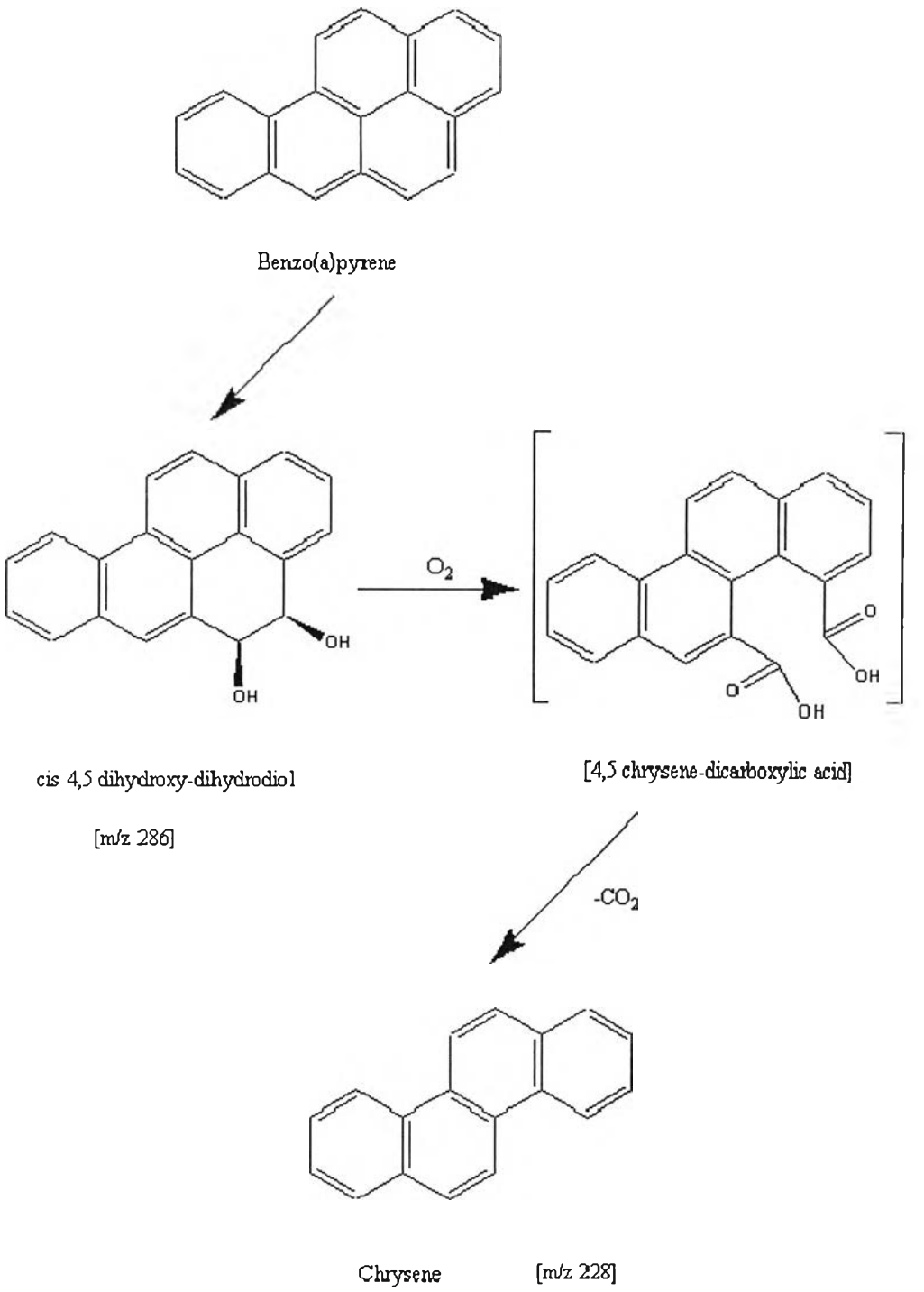

Figure 5.4.2 The oxidation of BaP to chrysene via cis 4, 5-dihydroxy 4,5 dihydrodiol BaP and dicarboxylic acid chrysene generally found in bacteria *Mycobacterium* sp. RJGII-135. Compound in bracket was not detected.

5.5 Conclusion

The fungal isolate, *Aspergillus niger* N003 was able to degrade 100 ppm benzo(a)pyrene with an approximately 80% degradation efficiency. The higher aeration rate promoted the fungal biomass as well as the biodegradation. The higher concentration of glucose increased fungal biomass but depressed the BaP degradation.

The higher concentration of BaP than 100-ppm was found to be toxic to the fungus in which its growth was suppressed and reduced it biodegradability to BaP. The attempt to increase the BaP bioavailability using solvent, i.e. ethanol and methanol, did not promote the biodegradation ability of the fungus. The biotransformation products were detected and suggested to be dihydroxy-dihydrodiol-BaP and also probably be chrysene, one of the smaller four-fused rings of PAHs.