CHAPTER 8
CONCLUSION AND SUGGESTIONS FOR THE FUTURE WORK

In this chapter, the overall comparison of BaP biodegradation efficiency as well as the biotransformation pathway and products of the three promising fungi isolated from this study, *Aspergillus niger* N003, *Aspergillus niger* B002 and *Fusarium oxysporum* E033, were described. Then, the overall conclusions and the suggestions regarding the useful information produced from this study to develop the remediation strategies for BaP contaminated sites were depicted. The suggestions for further studies were also stated.

8.1 Comparative biodegradation of the three promising fungi

The tolerance and ability of the three promising fungi isolated in this study, *Aspergillus niger* N003, *Aspergillus niger* B002 and *Fusarium oxysporum* E033 to degrade 100-ppm BaP was investigated.

The total degradation after 30 day-incubation of *Aspergillus niger* N003 and *Aspergillus niger* B002 were ranging from 70%-80%, while it was approximately 60% for *Fusarium oxysporum* E033. Only slightly adsorption by cell mycelium of *Aspergillus niger* N003, *Aspergillus niger* B002, and *Fusarium oxysporum* E033 at 3%, 8%, and 3%, respectively, were shown (Figure 8.1).

The significant BaP biodegradation by all three strains 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, B002 and *Fusarium oxysporum* E033 was found to be 38.4, 32.0, and 27.0 μmole BaP/day, respectively (calculated from the first five days of incubation). The BaP depletion kinetic illustrated in this study were 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).

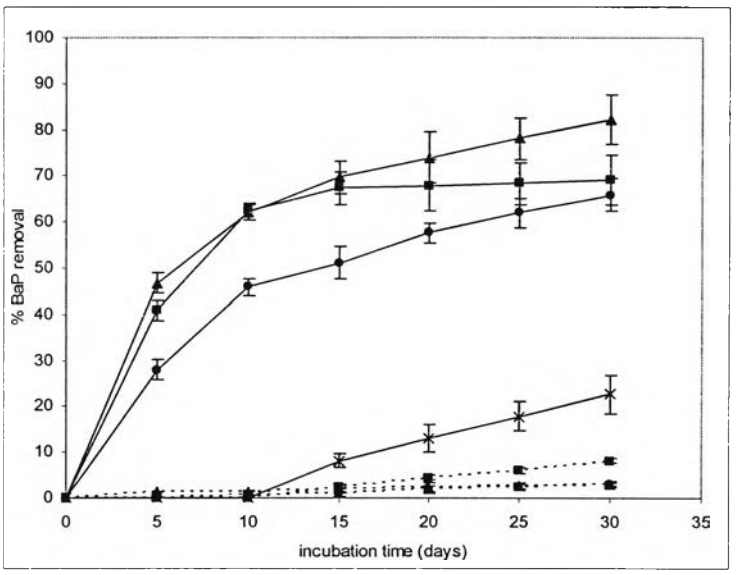


Figure 8.1 Biodegradation of 100-ppm BaP of the fungi isolates in liquid medium. Loss of BaP from auto-degradation (x); Loss of BaP from adsorption (---); and Biodegradation (—). *Aspergillus niger* N003 (▲), *Aspergillus niger* B002 (■), and *Fusarium oxysporum* E033(●). (Mean of three replications +/- S.D.)

According to our results, among the three isolates, the endophytic *Aspergillus* sp. strain N003 is the most efficient strain to tolerate and degrade BaP. This result agrees with the pervious reports showing that of *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).

Two *Aspergillus niger* N003 and B002 isolated in this study exhibited 60-80% BaP biodegradation efficiency within the first 10 days (Figure 8.2.2 A, B). These results 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 abilities of our fungal isolates (*Aspergillus niger* N003, *Aspergillus niger* B002, and *Fusarium oxysporum* E033) when compared to those of other BaP-degrading fungi previously reported.

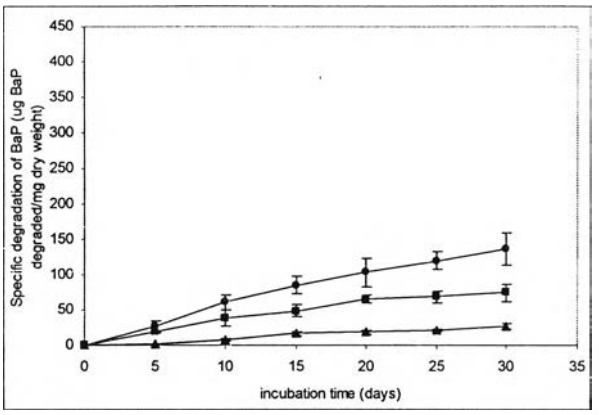
The factors affecting BaP degradation of these fungal isolates were illustrated as followed:

8.2 Factors affecting BaP degradation

8.2.1 Effect of aeration

The results showed that aeration was obviously affected the growth and biodegradation of three fungal isolates in that the faster the aeration, the higher the growth and the higher biodegradation rate as shown in Figure 8.2.1A, B, C.

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 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 8.2.1 A-C). 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).



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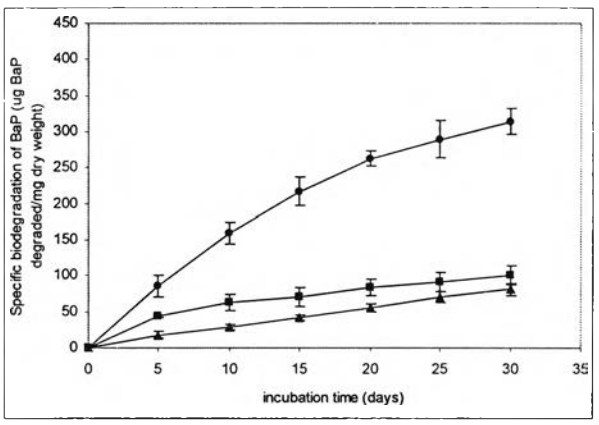

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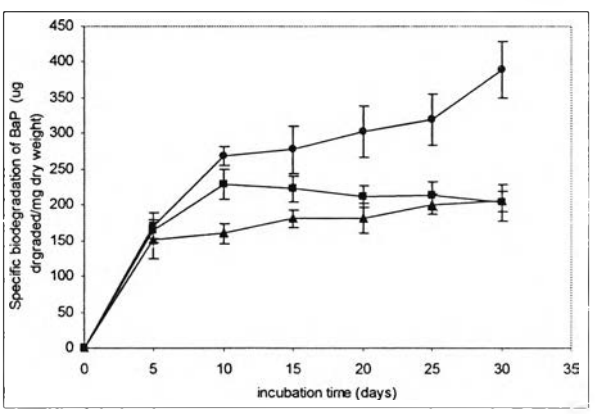


C

Figure 8.2.1 Biodegradation of BaP of the three fungal isolates when grew with different reciprocating shaking at 60 rpm (A), 120 rpm (B), and 180 rpm (C). The biodegradation of BaP was expressed as specific degradation per cell dried weight at the time indicated. *Aspergillus niger* N003 (▲), *Aspergillus niger* B002 (■) and *Fusarium oxysporum* E033(●). (Mean of three replications +/- S.D.)

According to our results, among the three fungi, the *Fusarium oxysporum* E033 was the most efficient strain to degrade BaP, followed by *Aspergillus niger* B002 and *Aspergillus niger* N003, respectively, of which the specific biodegradation per unit biomass at the first 10 days of incubation occurred at 270, 220 and 150 μg BaP per mg biomass, respectively (Figure 8.2.1 C).

8.2.2 Effect of initial BaP concentration

The ability of the three promising fungi to survive and degrade BaP at various concentrations (100, 200, and 300 ppm) were investigated.

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 in our three fungal strains (Figure 8.2.2 A). However, *Fusarium oxysporum* E033 demonstrated the greater biodegradation efficiency than the two *Aspergillus niger* N003 and B002. Also, *Fusarium oxysporum* E033 showed the greater biodegradation 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 having only 17% BaP degradation per unit biomass after 30 days of incubation (Verin et al., 2004b).

The results showed that all three fungal isolates were capable of BaP degradation at even higher BaP concentration. Nonetheless, the biodegradation with 100 ppm BaP was the most efficiently occurred. Among three fungal isolates tested, *Fusarium oxysporum* E033 exhibited relatively highest biodegradability with 350 μg BaP per mg biomass at 30 days of incubation (Figure 8.2.2 A, B, C).

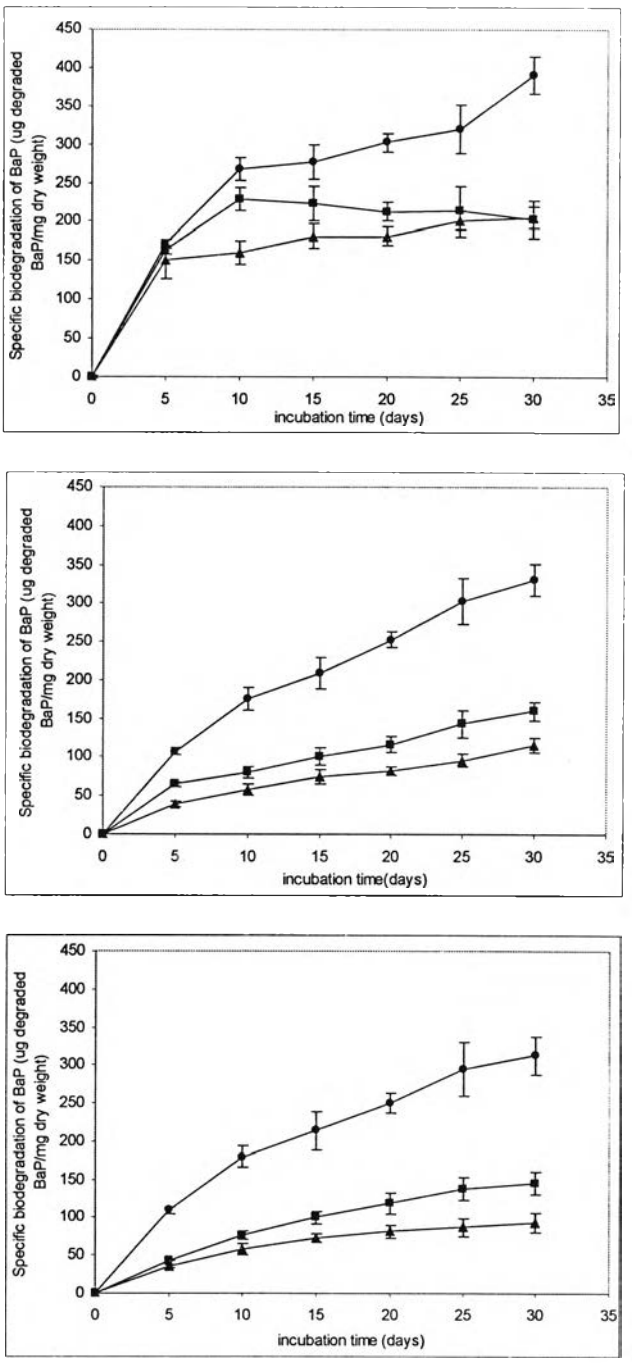


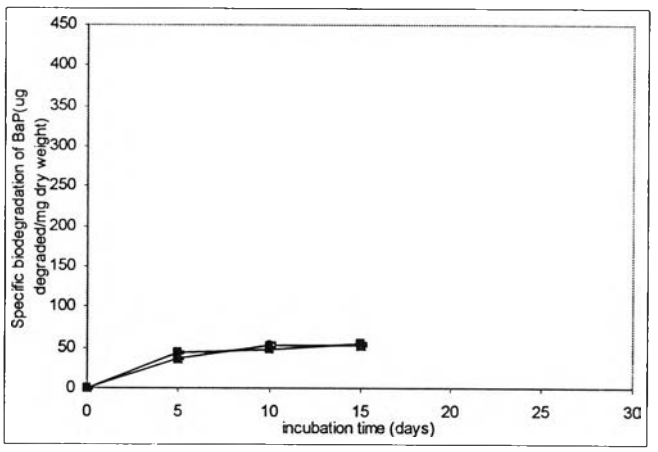
Figure 8.2.2 The biodegradation of BaP of the three fungal isolates expressed as specific degradation per cell dried weight at the time indicated in glucose-containing medium in various concentrations of BaP: 100-ppm (A), 200-ppm (B), and 300-ppm (C). *Aspergillus niger* N003 (▲), *Aspergillus niger* B002 (■) and *Fusarium oxysporum* E033(●).(Mean of three replications +/- S.D.)

8.2.3 Effect of glucose concentration

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 & 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. The biodegradation of BaP was expected to be enhanced as growth of the fungi was approximately 5-time increased. Contrarily, the results showed that the higher concentration of glucose, the lower biodegradation for all fungal strains (Figure 8.2.3 C), especially, for *Fusarium oxysporum* E033 that its biodegradation was almost completely repressed (Figure 8.2.3 C).

In the absence of glucose, it was noticeably that BaP was degraded at approximately 50 μg BaP/mg dried weight within 15 days of investigation (Figure 8.2.3 A). According to our results, glucose at 5 mM served as a good growth substrate and it was relatively suitable for these three fungi giving higher specific biodegradation of BaP (Figure 8.2.3 A, B, C) than those when glucose at higher concentration, i.e. 50 mM, was provided and the inhibition of biodegradation was observed.



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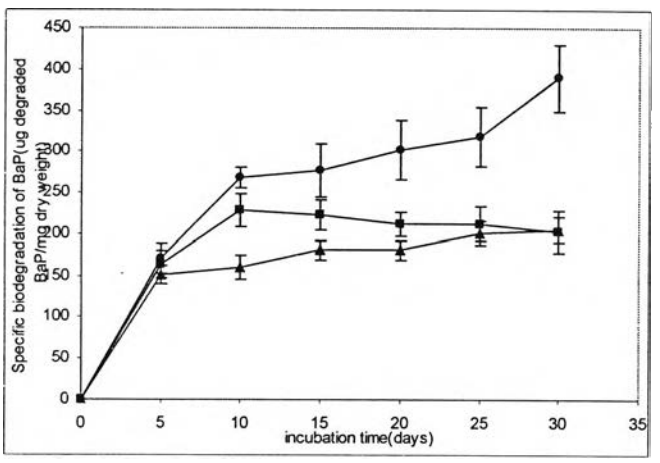

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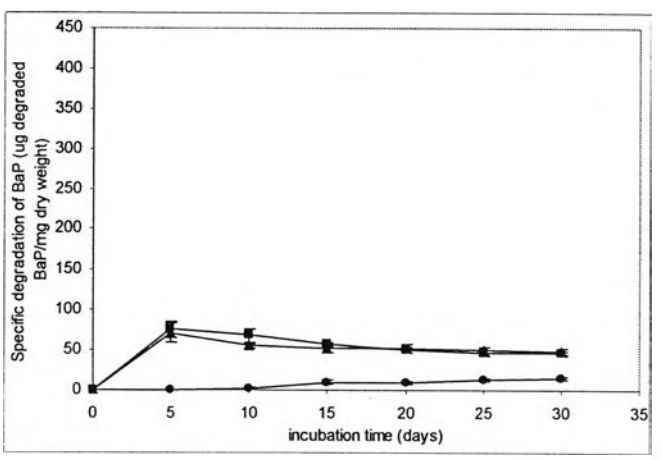


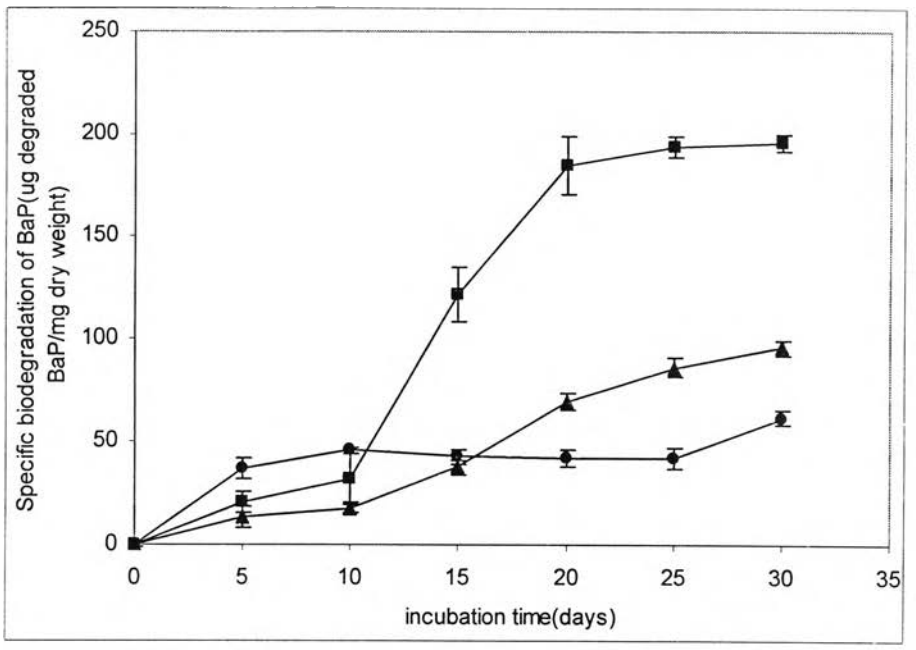
C

Figure 8.2.3 Biodegradation ability of the three promising fungi in the medium with various concentrations of Glucose: 0 mM (A), 5 mM (B), and 50 mM (C). *Aspergillus niger* N003 (▲), *Aspergillus niger* B002 (■) and *Fusarium oxysporum* E033(●). (Mean of three replications +/- S.D.)

8.2.4 Effect of bioavailability of BaP

There have been reports of the utilization of surfactant(s) to increase the bioavailability of PAHs of fungi (Garon et al., 2002; Zheng & Obbard, 2002). However, the toxicity of high concentration of surfactant to fungi has been concerned as well.

The comparison results of the ability to degrade BaP of three promising fungi grown in media supplemented with either ethanol or methanol is shown in Figure 8.2.4 A, B. The biodegradation ability of fungi in ethanol was greater than in methanol. The *Aspergillus niger* B002 gave the relatively highest specific biodegradation in ethanol (Figure 8.2.4 A), while the biodegradability of *Fusarium oxysporum* E033 was almost completely inhibited when grown in methanol containing media (Figure 8.2.4 B).



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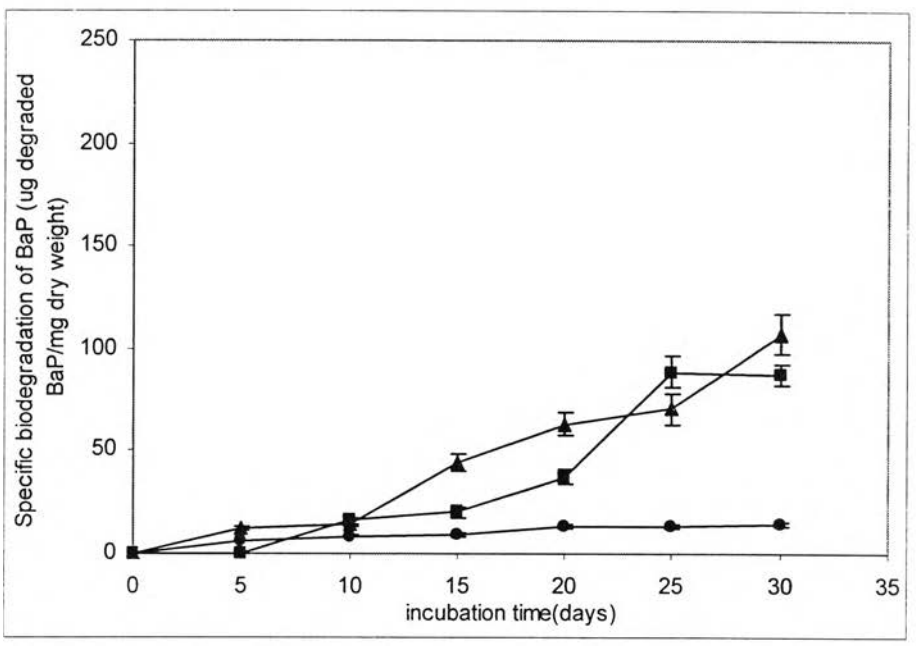

B

Figure 8.2.4 Biodegradation ability of the three promising fungi in the medium supplemented with 5 mM ethanol (A) or 5 mM methanol (B). *Aspergillus niger* N003 (▲), *Aspergillus niger* B002 (■) and *Fusarium oxysporum* E033(●). (Mean of three replications +/- S.D.)

8.3 Benzo(a)pyrene metabolites

The analysis of BaP degradation and BaP metabolites were performed by LC-MS. The LC-MS results showed that after 30 days of incubation, the intermediates produced during the biodegradation process were from the extra-cellular fractions and the intracellular fractions. There was no detectable metabolite was detected from liquid media. The main ions of BaP metabolites analyzed by electro-spray mass spectrometer were conclusively summarized in Table 8.1

The results of the present investigation demonstrated that our three promising fungi (*Aspergillus niger* N003 and B002, and *Fusarium oxysporum* E033) can oxidize BaP by different pathways depending on type of the fungi. Several types of metabolites were produced and can be qualitatively identified and compared to those previously reported.

The mass spectrum of metabolites revealed from *Aspergillus niger* N003 and B002 indicated these fungi oxidized BaP to dihydroxy-dihydrodiol-BaP, while *Fusarium oxysporum* E033 biotransformed BaP to BaP quinone and BaP diol epoxide.

Two metabolites obtained from the biodegradation of *Fusarium oxysporum* E033 was similar to those reported in *Cunninghamella elegans* which metabolized BaP to 7,8 dihydroxy 7,8 dihydrodiol BaP or 9,10dihydroxy 9,10 dihydrodiol BaP derivatives 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) 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).

Table 8.1 List of the possible BaP metabolites from the three promising fungi 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 N003	extra-cellular	285	7,9-dihydroxy 7,9 dihydrodiol BaP (m/z=284)
	intracellular	287	dihydroxy- dihydrodiol BaP derivatives (m/z=286)
Aspergillus niger B002	extra-cellular	285	7,9-dihydroxy 7,9 dihydrodiol BaP (m/z=284)
	intracellular	279	unidentified product (m/z=278)
Fusarium oxysporum E033	extra-cellular	283	BaP 1,6 quinone (m/z=282)
	intracellular	285 301	7,9-dihydroxy 7,9 dihydrodiol BaP (m/z=284) BaP diol epoxide (m/z=300)

8.4 Overall conclusion

The fungi capable of BaP biodegradation were screened and isolated from leaves and barks of *Pterocarpus macrocarpus* Kurz. plant located along the traffic road of Bangkok, Thailand. Three efficient strains were identified morphologically and genetically as *Aspergillus niger* N003, *Aspergillus niger* B002 and *Fusarium oxysporum* E033. Their abilities to degrade 100-ppm BaP are in the range of 65%-80% within the 30-day period. *A. niger* strains are the most efficient fungal isolates among the three fungal strains to degrade BaP with highest total degradation. However, when compared the biodegradation as the specific biodegradation (μg BaP/mg dry weight), *Fusarium*

oxysporum E033 is the most effective and tolerant strain among the three isolates having the relatively highest specific BaP biodegradation.

The factors affecting the degradation of the three fungal isolates were established. The fungal biomass as well as the biodegradation efficiency for all of the three fungal strains was increased at the relatively high aeration. At high concentration of glucose, the biomass productions were significantly increased, but the opposite results were obtained in their biodegradation. The attempt to enhance the bioavailability of BaP to fungi by increasing the solubility of substrate using alcohols was successful to increase the biomass production; however, the biodegradation ability of fungi was repressed.

The *Aspergillus niger* N003 and *Aspergillus niger* B002 expressed the ability to transform BaP to BaP dihydrodiol derivatives, while the *Fusarium oxysporum* E033 was able to form BaP quinone and the diol epoxide via two different pathways.

The new pathway was suggested from the intracellular biodegradation by *Aspergillus niger* N003 that BaP was degraded to the smaller PAHs compound, chrysene. However, to get the better understanding of this fungal biodegradation pathway and mechanism, the enzymatic system involving the biodegradation would be necessary.

8.5 Suggestions for future work

It is necessary to address some issues for future research to expand our knowledge on the practical application of BaP and other PAHs remediation. A number of approaches may be applied to bioremediation to improve microbial BaP degradation. The suggestions on further research on BaP degradation are as followed:

8.5.1 Co-metabolism

As BaP might not be utilized as a carbon and energy source for some microorganisms, a suitable growth substrate must be supplied to initiate growth of the organism in order to induce the production of catabolic enzymes. The type of growth substrate can markedly influence the extent of BaP degradation. The application of

analogous substrates, such as the lower molecular weight PAH can be supplied to stimulate the induction of enzyme(s) responsible for the biodegradation of high molecular weight PAHs. The full understandings of the degradation potential of microorganisms as well as the mechanism by which they metabolize are worthy.

8.5.2 Limiting factors for BaP degradation

A number of physical, chemical, biological or environmental factors including temperature or pH may influence the rate and extent of BaP degradation.

8.5.3 Bioavailability

The bioavailability of BaP may be increased by the application of surfactants. However, some inhibitory effects and toxic to micro-organisms have been observed during their application. Therefore, it is important to choose an appropriate surfactant and concentration for the bioremediation strategy.

8.5.4 The enzymatic and degradation mechanism knowledge

The understanding of biodegradation pathway, the mechanism of PAH biotransformation, the profile of metabolites formation as well as the toxicity of the oxidation products which might be accumulated are necessary. The factors involving the regulation of the biodegradation are awaited to be revealed.