CHAPTER 4
ISOLATION, SCREENING AND IDENTIFICATION OF THE FUNGAL ISOLATES

This chapter described the isolation, screening and identification of fungi capable to degrade benzo(a)pyrene from parts of *Pterocarpus macrocarpus* Kurz. Plant. The fungal isolates were then identified.

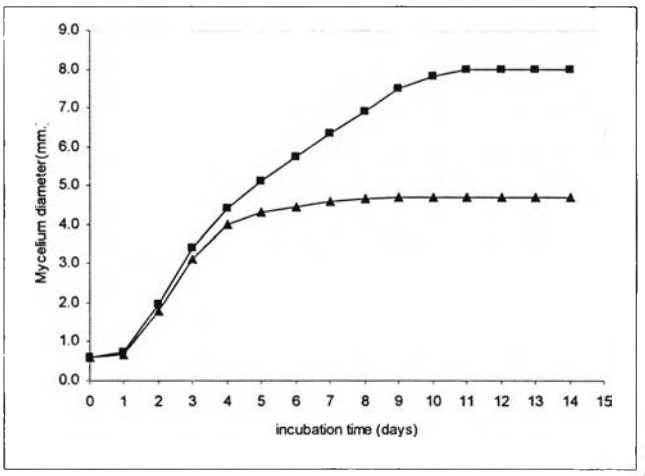
4.1 Isolation, screening of fungi capable of BaP degradation

Fifty three fungal isolates were obtained from the isolation step from leaves (47 isolates) and barks (6 isolates) of *Pterocarpus macrocarpus* Kurz. plant. The isolates from leaves were then classified to epiphytic fungi (40 isolates) and endophytic fungi (7 isolates). All of the fungal isolates were screened for their abilities to tolerate and degrade 100 ppm-BaP using a solid agar. Thirteen fungal isolates were selected based on their greater growth rate in the presence of BaP than that of the control in which BaP was not provided. To confirm the results and to ensure that the disappearance to BaP was not from the adsorption or auto-oxidation, growths in liquid medium of these isolates in the presence of BaP were also performed. The results were the average of 3 independent experiments.

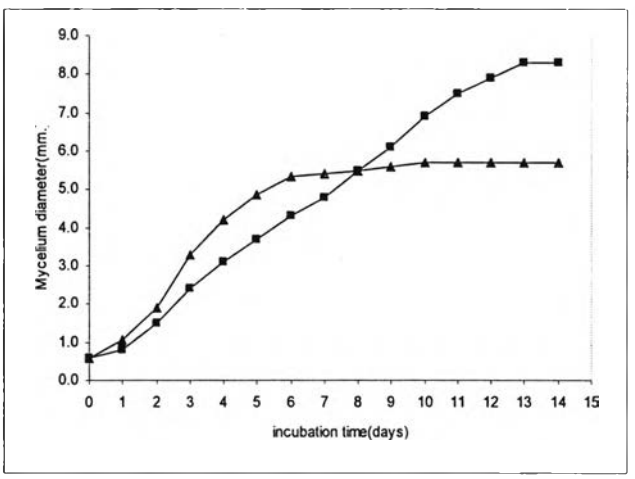
Among 13 fungal isolates tested, there were 3 isolates, designated B002, E033 and N003, which isolated from bark, surface (epiphyte) and inside (endophyte) of leaves, showed greater growth rate on solid agar (Figure 4.1A - 4.1C), as showed in Table 4.1. The characteristic of total fungal isolates were shown in APPENDIX F.

Table 4.1 Summarized steps of experiments

Step of examination	Number of fungal isolates obtained			
	Total	Bark isolation	Leaves isolation	
			Epiphytic fungi	Endophytic fungi
Isolation of tested fungi	53	6	40	7
Screening of fungi with growth on BaP containing solid medium	13	1	7	5
Preliminary screening for selection of fungi which capable for BaP biodegradation	3	1	1	1
Code name designed	-	B002	E033	N003



A



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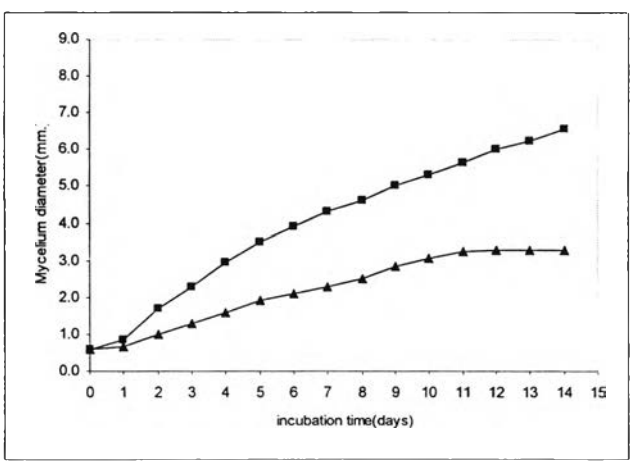


C

Figure 4.1 Growth of the fungal isolates on malt extract agar illustrated as the extension of mycelium (diameter) on Malt Extract Agar (control) (▲) and the media supplemented with 100-ppm BaP (■). A) Endophyte N003, B) Bark fungi B002 and C) Epiphyte E033

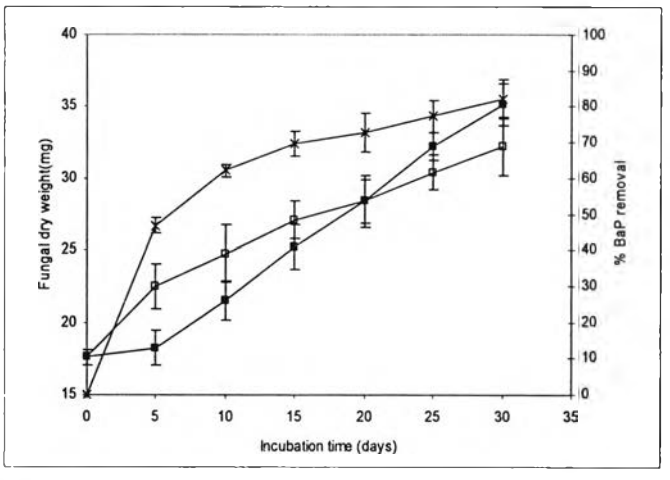
4.2 Preliminary screening for fungal isolates capable of BaP degradation

The biodegradation studies were performed in liquid media. The tolerance and capability of N003, B002 as well as E033 grew in media and degrade 100-ppm BaP were investigated in liquid medium within 30 days of incubation at room temperature (~32°C). Growths of the isolates were determined from the biomass interval examined. The biomass of the selected fungi were determined and shown in Figure 4.2 A-C

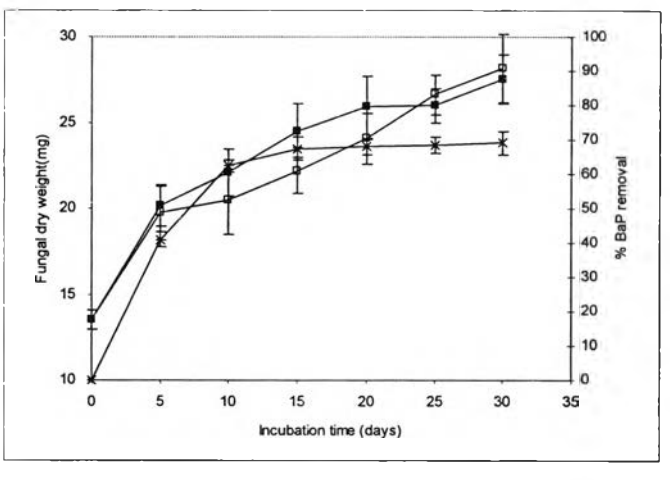
It was found that in the presence of 100-ppm BaP in liquid medium and additional of 5-mM glucose as a carbon source affected on slightly suppressed growth of N003 and B002 by whereas there was a significant suppression of E033. Although the presence of BaP suppressed growth of the fungal isolates, the biodegradation 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).

The three promising fungi were further studied in liquid media to investigate the biodegradation kinetic and determined the factors affecting the BaP degradation. The results and discussions of the biodegradation from these three fungi were shown in the next chapters.



A



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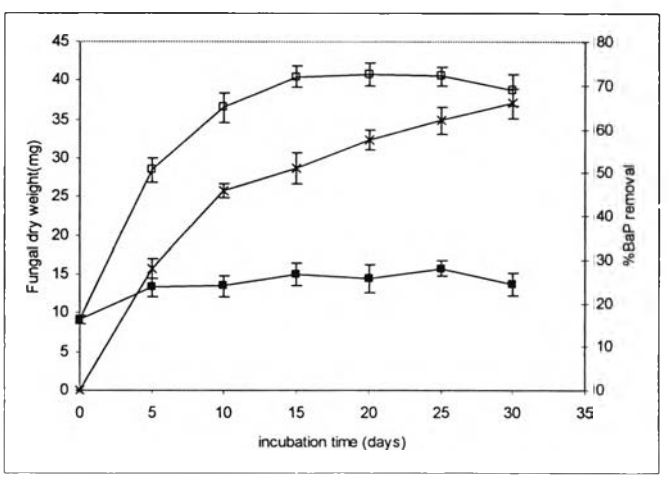

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Figure 4.2 Growth of the fungal isolates illustrated as biomass grown in liquid medium, in the absence (□) and in the presence of 100-ppm BaP (■) and biodegradation (x). A) Endophyte N003, B) Bark fungi B002 and C) Epiphyte E033

4.3 Identification of fungal isolates

The 3 promising fungi having potential ability to degrade BaP were further identified morphologically and genetically. The results were illustrated as below:

4.3.1 The morphology observation

The colonial characteristic of three isolated fungal strains (A) N003, (B) B002 and (C) E033 grew on Malt Extract Medium and in Malt Extract Broth (10 mg/l glucose) were shown in Figure 4.3.1 A)

The spore morphology of the three fungal strains were identified by cultured slide and stained with the phenylalanine solution illustrated in Figure 4.3.2 A, B, C. The results showed that N003 and B002 were *Aspergillus* sp. N003 and *Aspergillus* sp. B002, and fungal isolate E033 was *Fusarium* sp.

4.3.2 The morphology observation by Scanning Electron Microscope (SEM)

Scanning electron micrograph of the selected fungi were illustrated in Figure 4.3.2 A-4.3.2C

4.3.3 Molecular identification of selected fungi by using 18s rDNA sequences comparison

The *Aspergillus* sp. N003 and B002, and *Fusarium* sp. E033 were then identified genetically analysis using a comparison of the 18s rDNA sequence to other 18s rDNA sequences available in NIH genetic sequence database (GenBank). The alignment results showed that the isolates N003 and B002 have 96% and 99%, respectively, similarity to *Aspergillus niger*, while the analysis of E033 exhibited 98% similarity to *Fusarium oxysporum*.

The alignment of the 18s rDNA sequences of the 3 promising fungi were shown in APPENDIX G.

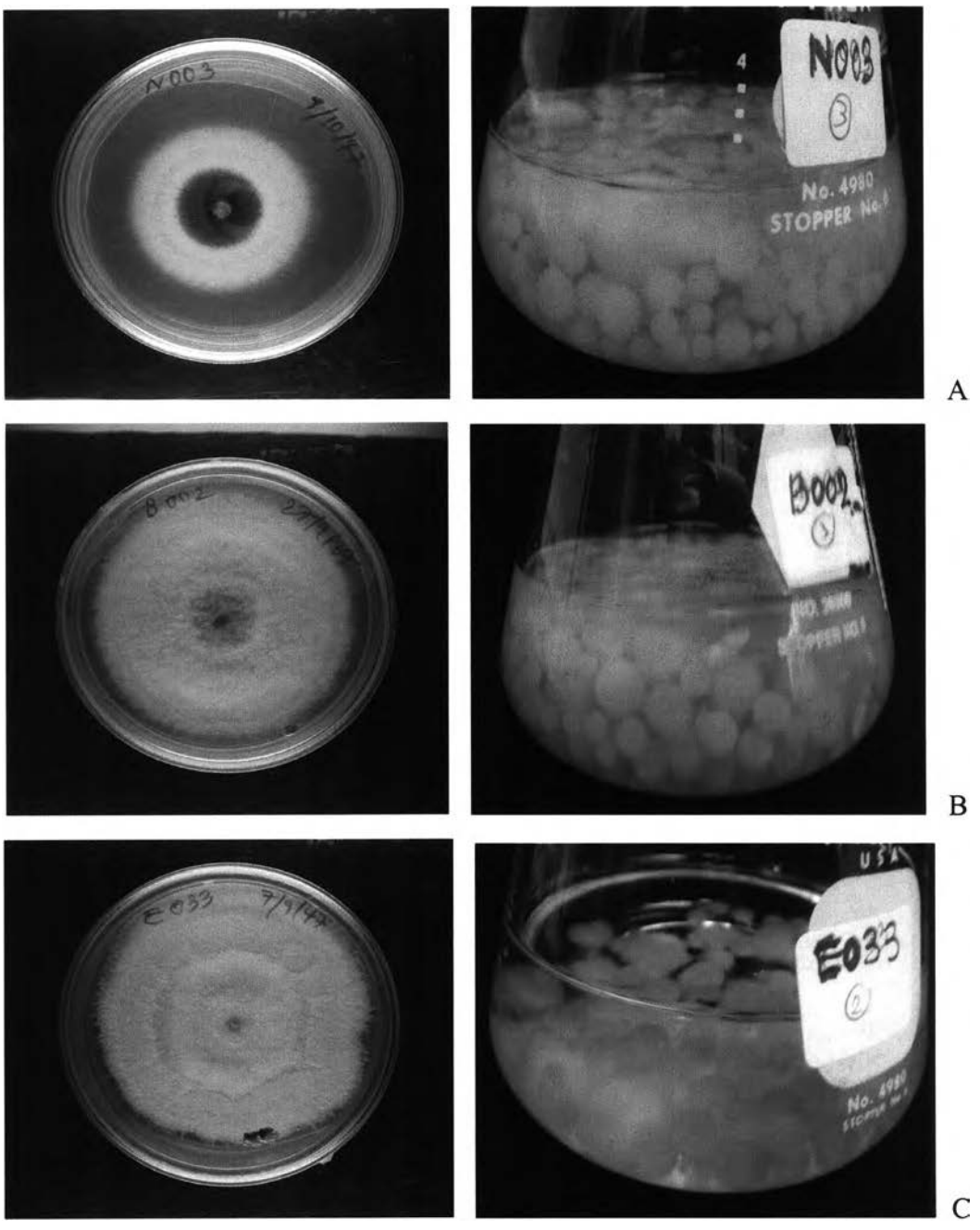

Figure 4.3.1 Colony characteristic of (A) N003, (B) B002), (C) E033 were grown on Malt Extract Agar (MEA) (left) or in liquid medium (10 g/l glucose) formed the fungal pellet (right).

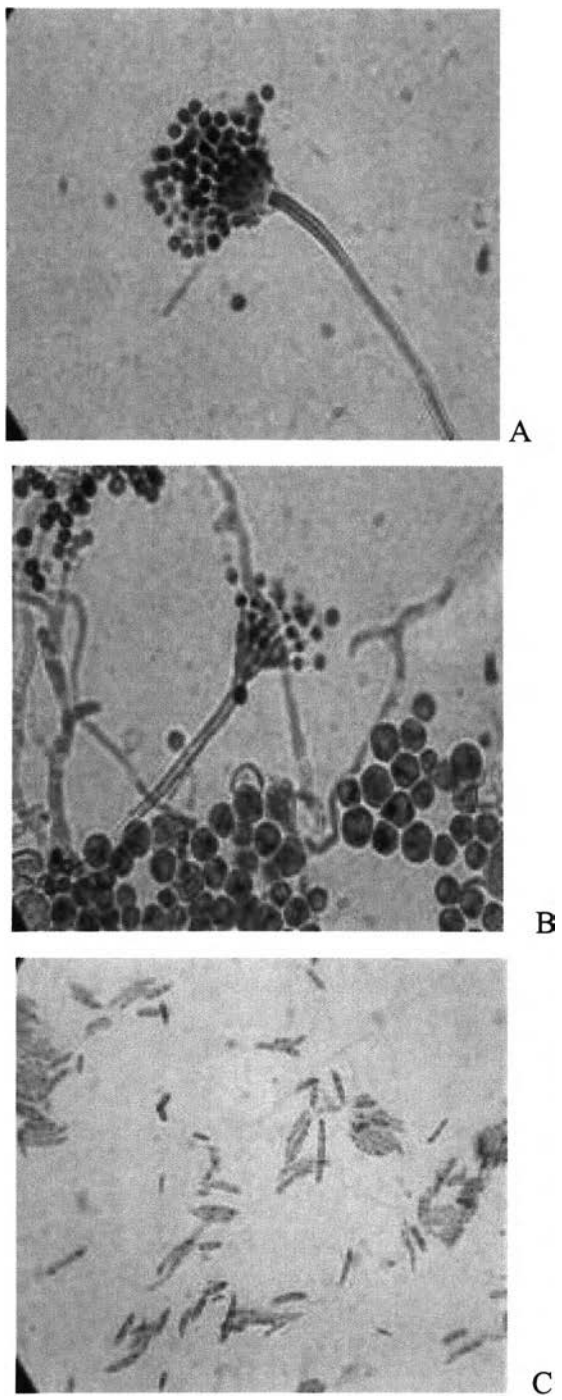

Figure 4.3.2 Light micrographs of structure and conidia of (A) *Aspergillus* sp. N003, (B) *Aspergillus* sp. B002, (C) *Fusarium* sp. E033. The specimen were prepared by culture and strained with phenyl-alanine

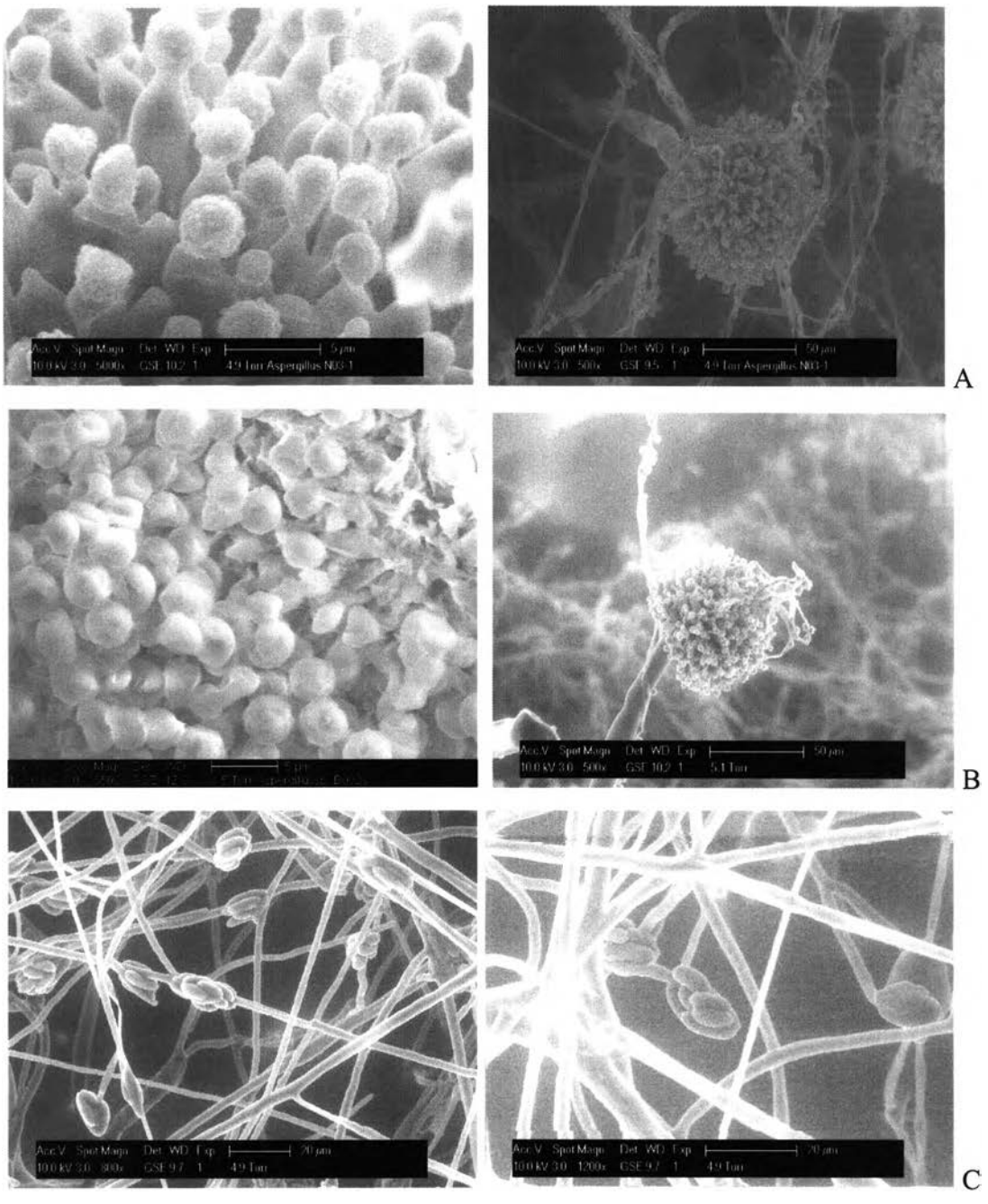

Figure 4.3.3 Scanning Electron Micrographs of (A) *Aspergillus* sp. N003 showing high magnification of a group of conidial head (left), a conidial head (right), and (B) *Aspergillus* sp. B002, a group of conidia head (left) and a conidial head (right), and (C) conidia of *Fusarium* sp. E033