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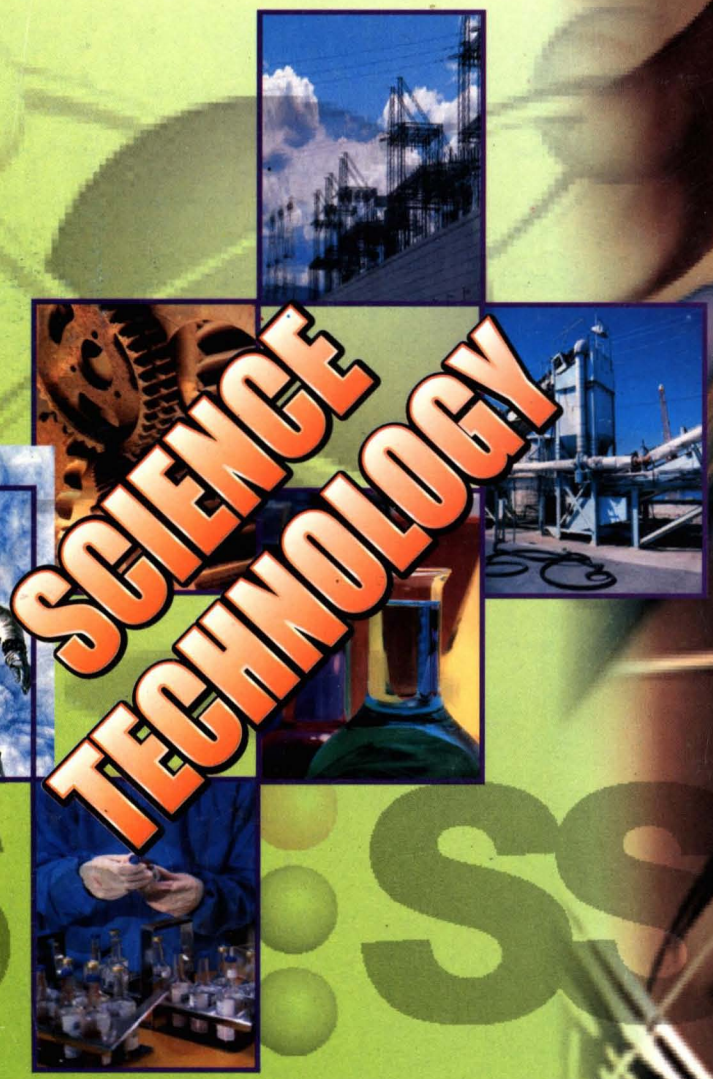


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# Antimicrobial Activities of *Piper betle* Endophytes

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ABSTRACT

*Evidence had shown that microbial endophytes are capable of producing secondary metabolite(s) similarly to that of their host plants (Strobel & Daisy, 2003). Therefore, this study was performed to compare the antimicrobial activities produced by Piper betle endophytes with the bioactive substances of their host plant, which had previously been studied. Plant samples were collected from three different locations. The inner soft tissues were cultured onto nutrient agar and potato dextrose agar to isolate bacterial and fungal endophytes. 14 endophytes were successfully isolated, which were later fermented for two weeks. The fermentation broth was subjected to bioassay tests using disc diffusion agar method every two days of the fermentation process. The tests were against eight ATCC strains. Two isolates were found moderately active against Aspergillus niger ATCC 16404 after six fermentation days. All other isolates did not show any activity against tested microorganisms. The findings showed that P. betle endophytes produced different bioactive metabolites from its host plant which was found to be strongly active against Bacillus subtilis but no activity against A. niger.*

**Keywords:** *Piper betle, endophytes, bioassay tests*

## Introduction

Endophytes are organisms living within the tissues of host plants. Typically, endophytes coexist with their hosts without any pathogenic symptoms. Some of these endophytes may be producing bioactive substances that may involve in the host-endophyte relationship. They promote plant growth by one or more of three factors: the production and secretion of plant growth regulators, antagonistic activity against phytopathogens and the supply of biologically fixed nitrogen (Van Buren et al. 1993; Bashan & Holguin 1997). As a direct result of the roles that these secondary metabolites may play in nature, they may ultimately be shown to have applicability in medicine, agriculture and industry.

Endophytes are consistently reported present in the root, stem, fruit and tuber tissues of a wide range of agricultural, horticultural and forest species. The extensive and frequent occurrence of such populations seems to indicate that healthy plants carry populations of endophytic microorganisms, and the Plant Kingdom represents a vast and relatively unexplored ecological niche for these organisms (Chanway 1995).

It now seems that endophytes are attracting more attention than epiphytic microbes. This may be due to closer biological associations developed between these organisms in their respective hosts than the epiphytes or soil-related organisms. Hence, the result of this may be the production of a greater number and diversity of classes of biologically derived molecules possessing a range of biological activities. In fact, a recent comprehensive study has indicated that 51% of biologically active substances isolated from endophytic fungi were previously unknown (Schutz 2001). This compares with only 38% novel substances from soil microflora. Sometimes extremely unusual and valuable organic substances are produced by these endophytes. An example is the taxol compound, the world's first billion dollar anticancer drug.

The opportunity to find new and interesting endophytes among the myriad of plants is great. It would appear that the study of plant associated microorganisms, in general, may offer opportunities for discoveries of unique and promising compounds or novel biological systems. In this study, *Piper betle* was chosen due to the plant being known to have certain medicinal value and antimicrobial activities.

## Materials and Methods

### Plant samples

Healthy *Piper betle* plants were collected from three different states, Negeri Sembilan, Pahang and Johor. The samples were kept at 4°C until further use.

**Isolation of microbial endophytes**

Several young plant stems were cut into approximately two centimeters in size. The materials were thoroughly surface treated with 70% ethanol. They were then dried under a laminar flow hood. Using a sterile technique, outer tissues were removed and the inner tissues were carefully excised and placed on agar plates. Two type of agar plates were used, nutrient agar supplemented with cyclohexamide and potato dextrose agar supplemented with chloramphenicol. The plates were incubated at room temperature for one week to the growth of endophytes. The grown endophytes were subcultured to fresh agar plates. Pure cultures were transferred to slant agar and kept as stock cultures.

**Identification of the endophytes**

All isolates were examined macro and microscopically.

**Fermentation of isolated endophytes**

A single colony from bacterial-like isolates was inoculated into ten mililitres of fermentation broth. The broth was shaken at 220 rpm and 37°C for 14 days. The antimicrobial assays of the broth were performed every two days.

**Tester strains**

All strains were purchased from American Type Culture Collection (ATCC) and kept at the Department of Microbiology, Faculty of Applied Sciences, Universiti Teknologi MARA, Shah Alam. The following cultures were used: *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *Streptococcus pyogenes* ATCC 19615, *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404. All bacteria were cultured overnight with shaking at room temperature in nutrient broth. For yeast and mould, potato dextrose broth was used.

**Bioassay test**

a) Preparation of bioassay plate

100 mililiter of molten nutrient agar (NA) was inoculated with one ml of $10^6$ cfu/ ml bacterial broth culture, mixed well and poured into sterile Petri dishes. They were allowed to set at room temperature. The same procedure was carried out using yeast broth culture in potato dextrose agar (PDA).

Mould assay medium was prepared using spores harvested from 3 to 5 days cultures. 0.85% of sterile saline was used to harvest the spores. The turbidity of the spore suspension was adjusted to 75 – 80% transmission at 530 nm to obtain $10^6$ spores/ ml. One ml of the suspension was added to 100 ml of sterile molten PDA. The medium was poured into Petri dishes and were allowed to solidify at room temperature.

b) Antimicrobial activity tests

The standard Kirby-Baeur method was followed to qualitatively determine the growth inhibition of tester strains used by microbial endophytes fermentation broth. 100 ml of the broth was loaded onto a sterile 13 mm nitrocellulose paper disc. The discs were allowed to dry under laminar flow hood. The discs were then positioned on bioassay plates and incubated at 37°C for overnight. Fresh fermentation broth was used as negative control. As positive controls, Streptomycin and cyclohexamide were used.

c) Collection of data

All plates were observed for zones of inhibition, and the diameter of these zones was measured in milimetres. Results obtained in this study are presented in Table 2. Each test was carried out in duplicates.

## Results

14 isolates were isolated from nutrient and potato dextrose agar selective media. Their identifications are tabulated in Table 1. For the antimicrobial tests, the broth of PH1 and J2 were found active against *Aspergillus niger* ATCC16404 at day-six of fermentation.

Table 1: Characteristics of endophytic isolates

| Sources | Designation | Macroscopic observation | Microscopic observation |
|---|---|---|---|
| Negeri Sembilan | NS1 | White, round, flat, regular | Gram positive, short rod |
| | NS2 | White, round, regular | Gram positive, long rod |
| | NS3 | Creamy white, round, flat | Gram negative, short rod |
| | NS4 | Creamy, mucoid, small colonies | Gram positive, short rod |
| | NS5 | Mould-like colony, greenish, cottony | Filamentous, septate |
| Pahang | PH1 | Yellowish, irregular, dry | Gram positive, long rod |
| | PH2 | Creamy, round, dry | Gram negative, long rod |
| | PH3 | Creamy, regular, small | Gram negative, long rod |
| Johor | J1 | Creamy, regular, very small colonies | Gram positive, long rod |
| | J2 | White, regular, dry | Gram positive, short rod |
| | J3 | Mould-like colony, greenish, cottony | Filamentous, septate |
| | J4 | Mould-like colony, dark green | Filamentous, septate |
| | J5 | Mould-like colony, green but grey at center, white aerial hyphae, cottony | Filamentous, aseptate |
| | J6 | Mould-like colony, white, small colonies | Filamentous, aseptate |

Table 2: Diameter of inhibition zones against *A. niger* ATCC 16404

| **Isolates** | **Size of inhibition zones (mm)** |
|---|---|
| PH1 | 12 |
| J2 | 10 |
| Cyclohexamide | 30 |
| Fermentation broth | nil |

## Discussion and Conclusion

The results supported the finding by earlier researchers (Bacon & White 2000) that different individuals of the same plant species might harbour different species of endophytes. Diversity of the endophytes in plants is influenced by many factors, for example, the climate, humidity and age of the plant. Different parts of the plant itself can also have different types of endophytes. Usually, they can be easily isolated from roots, shoots or young tissues of plants. In the study, the internal tissues of the stems were used so as to avoid contaminations.

The antimicrobial properties of the two positive isolates were moderately active against *A. niger* ATCC 16404. However, if the fermentation parameters can be optimized, it is hoped that metabolite production from these endophytes can be further enhanced.

Compared to the study by Zaidah et al. (2003), preliminary results showed that the endophytes of the *P.betle* produced different metabolites from its host. *P.betle* was found to be strongly active against *Bacillus subtilis* and was non-active against *A.niger*.

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