

## IDENTIFICATION OF MICROBES IN INOCULANTS FOR AGARWOOD (GAHARU) FORMATION USING MOLECULAR BIOLOGICAL TECHNIQUES

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### Abstract

Agarwood (Gaharu) is the most expensive non-timber wood in the world due to its distinctive fragrance. The resin formed in agarwood trees are extensively used in incense and perfumes. However, the formation of resin in nature, is a natural response to wounds or infection to fungi, takes around 20-24 years. Hence, the development of microbial based inoculants that boost the formation of resin is at high demand. In this study, reverse engineering techniques was deployed to identify the microbial cocktail in agarwood inoculants. Phylogenetics study showed that the microbial cocktail comprised of the species of *Monascus*, *Bacillus* and *Paenibacillus*. The microbe consortium may be responsible to boost the resin formation in agarwood.

**Keywords:** Agarwood (Gaharu), Inoculants, Fungi, Bacteria

### 10. Introduction

Agarwood (Gaharu) is the most expensive non-timber wood in the world due to its distinctive fragrance. The resin formed in agarwood trees are extensively used in incense and perfumes. However, the formation of the resin in nature took 20-24 years; as a natural response to wounds or infection by the forest endotrophic mycorrhizal fungi (Tabin et al., 2009). Hence, the development of inoculants that boost the formation of resin is at high demand particularly for commercialization of agarwood based products. In South East Asia particularly in Laos, Cambodia, Thailand and Malaysia, the inoculant formulations are developed based on "trial and error" method. To date, very few scientific studies on inoculants have been recorded and related information is scarce.

### 11. Materials and Methods

#### 2.1 Isolation of pure culture

Microbial based agarwood inoculants were kindly provided by Kayu Gaharu (M) Sdn. Bhd. Fifty microliters of inoculants were inoculated onto Luria Bertani (LB) agar (Merck, Germany) and Potato Dextrose Agar (PDA) (Merck, Germany) using micropipette (Eppendorf, Germany). The cultures were then incubated at 37°C for LB agar (to isolate bacteria) and 32°C for PDA (to isolate yeast and fungi). The procedure was repeated until pure cultures were obtained.

#### 2.2 DNA extraction and purification

Genomic DNA extraction was carried out to obtain genomic DNA samples from each pure culture sample. Then, pure culture was subcultured into 10 ml LB broth (for bacteria) and PD broth (for yeast and fungi) in 50 ml Falcon tubes and cultured in an incubator shaker overnight at 37°C (for bacteria) and 30°C (for

yeast and fungi). The genomic DNA was extracted using i-genomic BYF DNA Extraction Mini Kit (iNtRON Biotechnology, South Korea). The final flow-through containing plasmids was collected and kept at -20°C until further usage.

### 2.3 Polymerase chain reaction (PCR) amplification

The bacterial small subunit (SSU) rDNA (16S rDNA) and internal transcribed subunit (ITS) from fungus were PCR-amplified from the extracted total DNA using oligonucleotide primers, which target bacteria, yeast and fungi. The forward and reverse primers used are listed in Table 1. All primers were supplied by AIT Biotech, Singapore. The reaction cocktail for the PCR contained (final concentration); 1X PCR buffer (containing), 2.5 µg template DNA prepared as described above, 0.5 µM each forward and reverse primer, 200 µM dNTP mix (containing dATP, dCTP, dGTP, dTTP), (Qiagen, Germany), 1 U of Phusion® high fidelity DNA polymerase (New England Biolabs, USA) and sterile Milli-Q water to final volume of 50 µl. The PCR was performed on a gradient Mastercycler (Eppendorf, Germany). The following PCR procedure was used: initial denaturation for 30 sec at 98°C and 30 cycles consisting of denaturation at 98°C for 10 sec, annealing (various temperature depending on the  $T_m$  primer) for 30 sec and extension at 72°C for 30 sec. Final extension was carried out at 72°C for 10 min followed by cooling at 4°C. All PCR products were purified before further use using QiaQuick gel extraction kit (Qiagen, Germany) according to the manufacturer's protocol.

### 2.4 Construction of gene libraries

To identify the microbial cocktail, 16S rDNA library (bacteria and yeast) and ITS rDNA library (fungi) were constructed. To do this, the nucleotide sequence of the 16S rDNA region (bacteria) and ITS (fungi) from all pure culture of microbes were first isolated. The Zero Blunt®Topo® cloning vector (Invitrogen, USA) was used to clone the ITS and 16S rDNA. After ligation, the mixtures were transformed into competent *Escherichia coli* DH5α cells.

Transformants that grew on LB agar containing 100 µg/ml ampicillin were then subjected to colony PCR amplification using the vector specific primers (Table 1) to screen the presence of recombinant plasmids and the size of the inserts in the vector.

### 2.5 Nucleotide sequence determination of 16S /ITS rDNA inserts

Plasmid DNA was extracted from each of the correct clones using a QIAprep Spin Miniprep Kit (Qiagen, Germany) according to the manufacturer's protocol. The plasmid inserts were sequenced by the biotechnology company AIT Biotech (Singapore) either with primer M13, and/or with specific primer listed in Table 1.

Table 1:

Summary of primers for PCR amplification

Primer*	Sequence (5'-3')	Reference
rD1	AGAATTTGATCCTGGCTCAO	Weisburg et al., 1991
rD2	AGAATTTGATCATGGCTCAO	Weisburg et al., 1991
rP1	ACGGTTAOCCTGTTACOA CT	Weisburg et al., 1991
rITS1	TCCGTAGGTGAACCTGCGO	Bomeman and Hartin, 2000
rITS4	TCTCCGCTTATTGATATOC	Bomeman and Hartin, 2000
rM13	GTA AAAACGACCGCCAO	Zero Blunt Topo primer
rM13	CAAGAAACAGCTATGAC	Zero Blunt Topo primer

(16S rDNA and ITS) and sequencing analysis.

Note: \* f, forward; r, reverse.

a. Identification of microbes by comparative sequence analysis of 16S /ITS rDNA

Initial identification of the bacteria was made using Basic Local Alignment Search Tools (BLAST) available at NCBI (<http://blast.ncbi.nlm.nih.gov/>). Then, 16S/ITS rDNA sequences were aligned using the ARB software package, free online software available at (<http://www.arb-home.de/home.html>) and manually corrected for errors. Each new sequence was analyzed against a phylogenetic tree containing all sequences in the ARB database using the maximum parsimony “quick add” tool to get a first estimate of the affiliation of new bacterial/fungus specimen. The family of each strain was identified and a new tree was reconstructed using sequences from all species within the corresponding family as described in Bergey’s Manual. Strains were identified using a consensus based on the neighbour joining, maximum parsimony and FASTDNA maximum likelihood algorithms and the bacterial nomenclature described in the latest edition of Bergey’s Manual (Krieg et al., 2010). A base frequency filter was generated based on the selected sequences excluding all positions different in more than 70% of the strains to enable a comparison of homologous positions.

12. Results and Discussion

a. Microscopic examination of microbes isolated from the inoculants

Fifteen species of microbes were successfully isolated from the inoculants.

b. Identification of microbes isolated from the inoculants

Bulk DNA was extracted from each culture, and after amplification of the 16S rDNA and ITS rDNA regions, two libraries were constructed. Several clones identified through sequencing are shown in Table 2.

Table 2: Similarity in partial 16S / ITS rDNA sequences clones to sequence of their closest relatives available in the Genbank nucleotide sequence databases (<http://blast.ncbi.nlm.nih.gov/>).

Clone name	Sequencing primers	Number of sequenced bases	Phylogenetic affiliation (GenBank Accession number)	Sequence identity (%)
Filamentous				
Fungi				
pGubr2	M13, ITS1, ITS4	1105	<i>Alopecurus ruber</i> (AJ0477256.1)	100
Bacteria /				
Yeast				
pGubr4	M13, rD1, rD2, rP1	1468	<i>Bacillus licheniformis</i> (J030926.1)	99
pGubr6	M13, rD1, rD2, rP1	1472	<i>Pantothricus</i> sp. (J030926.1)	99
pGubr7	M13, rD1, rD2, rP1	1401	<i>Bacillus licheniformis</i> (J030926.1)	99

c. Phylogenetic affiliation of the microbes in inoculants.

*Aquilaria agallocha* Roxb. Seedlings, *Tropical Ecology*, 50, 243-248.

The phylogenetic analysis revealed a large diversity affiliation. Overall, there was a good agreement between the different algorithms (distance, matrix, maximum parsimony and maximum likelihood) used for the phylogenetic analysis. Phylogenetically, the inoculant microbial cocktail comprised of the species of *Monascus*, *Bacillus* and *Paenibacillus*.

### 13. Conclusions

As a conclusion, a consortium of bacteria and fungi was successfully isolated from the inoculants. Three species of fungi and four species of bacteria were identified from the inoculants. The identification of the microbes in the inoculants is crucial for the production of effective inoculants with low cost production.

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### References

Tabin, T., Arunachalan, A., Shirivastava, K., & Arunachalam, K. (2009). Effect of arbuscular mycorrhizal fungi on damping off disease in

Weisburg, W. G., Barus, S. M., Pelletier, D.A., & Lane, D.J. (1990). 16S ribosomal DNA amplification for phylogenetic study, *Journal of Bacteriology*, 173 (2), 697-703.

Borneman, J. & Hartin, R.J. (2000). PCR primers that amplify fungal rRNA genes from environmental samples, *Applied and Environmental Microbiology*, 66(10), 4356-4360.

Krieg, N.R., Ludwig, W., Whitman, W.B., Hedlund, B.P., Paster, B.J., Staley, J.T., Ward, N., Brown, D., & Parte, A. (2010). *Bergey's Manual of Systematic Bacteriology Volume 4* (2<sup>nd</sup> Edition). USA: Williams & Wilkins.