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Identification of Microbes in Inoculant for Agarwood (Gaharu) Formation using Molecular Biological Techniques

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The distinctive fragrance and pleasing odor of the agarwood came from the resinous materials impregnated in the phloem of the tree as a response to infection. Artificial inoculants have been developed by "trial and error" methods to boost the resin formation. These artificial inoculants have been used widely in this region despite no strong scientific data and that they contribute to most of the cost for agarwood resin production. In collaboration with an agarwood industry player, a research work was initiated to isolate and identify the microorganisms present in an inoculant that has been proven effective in boosting the agarwood resin formation. Fifteen clones were constructed by extracting and amplifying the 16S rDNA and ITS sequences from the pure cultures isolated from the inoculant. Three genera of microorganisms (*Bacillus, Paenabacillus, Monascus*) have been successfully identified. Two bacteria clones were phylogenetically affiliated with *Paenibacillus* sp. and four clones were associated with *Bacillus* sp. as well as three fungi clones were associated with *Monascus* sp. All the identified species are spore-forming microorganisms. The genera of *Bacillus* and *Paenabacillus* were able to utilize carbohydrate and protein from the tree as their energy sources. These two genera may be crucial to boost resin formation in agarwood.

Key words: Agarwood, Inoculant, 16S rDNA, ITS, phylogeny.

Agarwood (*gaharu*-Malay, *oud*-Arabic, *agar*-Hindi) is one of the most expensive non-timber wood in the world due to its distinctive fragrance. The market price of the highest grade of agarwood may reach as high as USD 300 per kilogram, while agarwood oil may be as high as USD 30000 per kg[1]. Malaysia is one of the main players in the international agarwood trade as Malaysia has abundant agarwood source growing untouched in Malaysian rainforest[2]. There are 19 plant species native to Malaysia that is thought to produce agar wood resin[3]. The pleasing odor of the agarwood came from the resinous materials impregnated in heartwood (phloem) as a natural response to wounds or infection by the forest endotrophic mycorrhizal fungi.[4]The resin formed in agarwood trees are extensively used in incense and perfumes.

The formation of the resin in nature is very slow and takes years. Theoretically, the resin formed as a natural response by the tree to protect its nutrient from the microorganisms. Figure 1 illustrates the theory of resin formation in agarwood tree. A lot of effort has been conducted to boost the resin formation artificially. At an international agarwood conferences in Vietnam, four different

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methods on artificial boosters were highlighted including (i) wounding and application of microbes, (ii) wounding and application of chemicals, (iii) wounding and application of fungi and (iv) shoot culture and application of fungi[5]. In South East Asia particularly in Laos, Cambodia, Thailand and Malaysia, the technique of wounding and application of microbes is widely practiced. A concoction of microbes or known as inoculant in this region is formulated based on "trial and error" method. The inoculant contained unknown microbes that is developed to induce agarwood resin formation artificially. The agarwood tree is wounded by drilling holes on tree. Inoculants in the form of liquid or paste are applied to the holes to induce infection that triggers the tree immune response to convert the nutrient into resin formation. Ironically, the inoculants are available in the market for years despite having little scientific data and that related information is scarce.

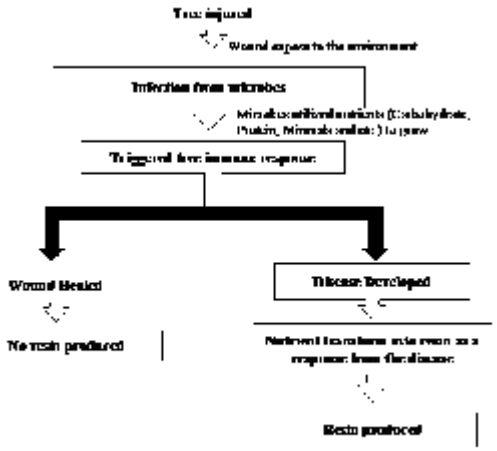

Fig. 1. Theory of agarwood resin formation

In Malaysia, the inoculants are sold at MYR 300 per liter and this amount is usually applied to one to three agarwood trees.

It is widely accepted that microorganisms are the main cause that trigged the formation of resin in agarwood tree[4,5]. An inoculant used by a local company to effectively boost the agarwood resin formation was selected in this study for the identification of microorganisms essential for agarwood resin formation. Knowledge of nutritional requirements of these microorganisms and the effect of environmental factors on agarwood resin formation could be used to model the resin formation as well as to identify optimal growth conditions for the microorganisms. This knowledge is pivotal for effective and cheaper inoculants production. This paper describes the successful cultivation of microorganisms isolated from an effective inoculant sample.

MATERIALS AND METHODS

Isolation of pure culture

Microbial based agarwood inoculant was kindly provided by KayuGaharu (M) Sdn. Bhd (Fig. 2). Fifty microliters of the inoculant was inoculated onto Luria Bertani (LB) agar (Merck, Germany) and Potato Dextrose Agar (PDA) (Merck, Germany) using a 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 the pure cultures were obtained. All the morphologies observed on the pure cultures by microscopic examination techniques were recorded using the methods discussed by Benson *et al*.[6]

DNA extraction and purification

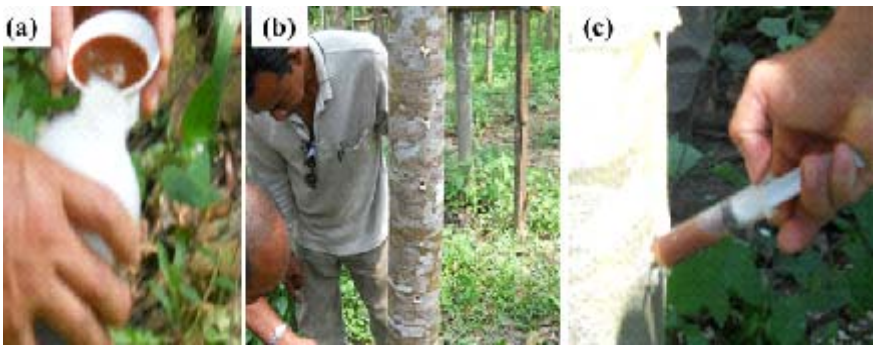

Fig. 2. (a) Microbial based artificial inoculants that proven effectively boost agarwood resin formation. (b) Drilling process and (c) Injection of artificial inoculant into the agarwood tree.

Genomic DNA extraction was carried out from each pure culture sample. Pure culture was subcultured into 10 ml LB broth (for bacteria) and PD broth (for yeast and fungi) in 50 ml Falcon tube 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.

Polymerase chain reaction (PCR) amplification

The bacterial small subunit (16S rDNA) and internal transcribed subunit (ITS) from fungus were PCR-amplified from the total DNA extract 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, 2.5 mg template DNA prepared as described above, 0.5 mM each forward and reverse primer, 200 mM dNTP mix (containing of dATP, dCTP, dGTP, dTTP), (Qiagen, Germany), 1 U of Phusion® High fidelity DNA polymerase (New England Biolabs, USA) and sterile Milli-Q water to a final volume of 50 ml. The PCR was performed in a Mastercycler gradient (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 temperatures depending on the *Tm*of primers) 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.

Construction of gene libraries

Table 1. Summary of primers for PCR amplification (16s rDNA and ITS) and sequencing analysis

Primer*	Sequence (5'- 3')	Reference
fD1	AGAGTTTGATCCTGGCTCAG	7
fD2	AGAGTTTGATCATGGCTCAG	7
rP1	ACGGTTACCTTGTTACGACTT	7
fITS1	TCCGTAGGTGAACCTGCGG	8
rITS4	TCCTCCGCTTATTGATATGC	8
fM13	GTAAAACGACGGCCAG	Zero Blunt Topo primer
rM13	CAGGAAACAGCTATGAC	Zero Blunt Topo primer

Note: * f, forward; r, reverse

To identify the microbial cocktail, 16S rDNA library (for bacteria) and ITS rDNA library (for fungi) were constructed. To do this, the nucleotide sequence of the 16S rDNA region and ITS from all pure cultures 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. The transformants that grew on LB agar containing 100 mg/ml ampicillin were then subjected to colony PCR amplification using the vector specific primers (Table 1) to screen for the presence of recombinant plasmids and the size of the inserts in vector.

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 a biotechnology service company, AIT Biotech (Singapore) either with M13primer, and/or with specific primer listed in Table 1.

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

Initial identification of the bacteria was made using Basic Local Alignment Search Tools (BLAST) available at NCBI (http://blast.ncbi.nlm.nih.gov/). Then, 16SrDNA /ITS

sequences were aligned using the Molecular Evolutionary Genetic Analysis (MEGA) version 5.2, a free online software available at (http://www.megasoftware.net/) and manually corrected for errors. Each new sequence was analyzed against a phylogenetic tree containing all sequences in the MEGA v5.2 using the maximum likelihood method based on Tamura-Nei Model[9] 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[10]. Strains were identified using a consensus based on the neighbor joining, maximum likelihood algorithms and the bacterial nomenclature described in the latest edition of Bergey's Manual.

RESULTS AND DISCUSSION

Microscopic examination of microbes isolated from the inoculants

Fifteen species of pure culture of microorganisms were successfully isolated from the inoculant sample. The morphological characteristics of the pure cultures indicate that the inoculant sample comprise of prokaryotic and eukaryotic microorganisms (Table 2). Three red-colored filamentous fungi were successfully isolated from the inoculant (Fig. 3). The tip splitting colonies were also observed on the plates (Fig. 3). The gram's staining analysis on the bacteria shows that all samples isolated were gram-positive bacteria. Under microscope examinations, most of the bacteria were rod-shape (data not shown).

Table 2.Colony morphology of the pure culture isolated from the inoculants

Sample	Configuration	Color	Size	Elevation	Margin	Media
1	Filamentous	Whitish Brownish	1.1 cm	Flat	Ciliate	PDA
2	Filamentous	Grayish Reddish	1.3 cm	Flat	Ciliate	PDA
3	Filamentous	Whitish Brownish	1.5 cm	Flat	Ciliate	PDA
4	Irregular Spreading	Yellowish	6.5 cm	Hilly	Lobate	LB
5	Round Scalloped Margin	Yellowish	0.3 cm	Umbonate	Wavy(undulate)	LB
6	Round	Yellowish	0.2 cm	Flat	Smooth(Entire)	LB
7	Round Raised Margin	Yellowish	0.1 cm	Umbonate	Smooth(entire)	LB
8	Round Scalloped margin	Yellowish	0.1 cm	Convex	Lobate	LB
9	Irregular Spreading	Whitish	3 cm	Hilly	Lobate	LB
10	Round	Yellowish	0.05 cm	Flat	Smooth(Entire)	LB
11	Round with scalloped margin	Yellowish	1.7 cm	Umbonate	Irregular(erose)	LB
12	Round with scalloped margin	Yellowish	0.7 cm	Flat	Lobate	LB
13	Concentric	Yellowish	0.4 cm	Convex	Wavy(Undulate)	LB
14	Round	Yellowish	0.2 cm	Flat	Smooth(Entire)	LB
15	Round	Whitish	1.4 cm	umbonate	Smooth	LB

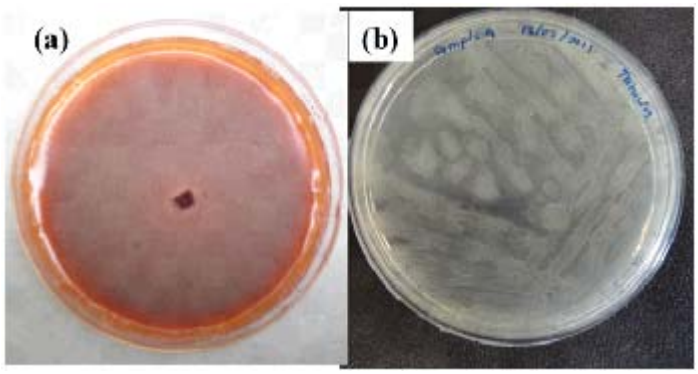

Fig. 3. Morphologies of (a) *Monascus* sp.(after 2 weeks of culture) and (b) *Paenabacillus* sp. isolated from inoculant sample.

Identification of microbes isolated from the inoculant

A total of 15 clones were selected for sequencing. The full sequencing results for 9 clones were good while the other 6 showed poor nucleotide sequencing results even after repeated sequencing analyses. Table 3 shows the sequencing results for the clones harboring the amplicons of 16S rDNA from pure bacteria samples and ITS from fungi samples.

Table 3. Similarity in partial 16S rDNA / ITS 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 (Gen Bank Accession number)	Sequence identity (%)
Filamentous Fungi				
pGahr1	M13, fITS1, ITS4	1112	*Monascus pilosus* (JN942665.1)	99
pGahr2	M13, fITS1, ITS4	1105	*Monascus ruber* (AB477256.1)	100
pGahr3	M13, fITS1, ITS4	1076	*Monascus fumeus* (GQ121015.1)	99
Bacteria / Yeast				
pGahr4	M13, fD1, fD2, rP1	1468	*Bacillus licheniformis* (HM006898.1)	99
pGahr5	M13, fD1, fD2, rP1	1474	*Paenibacillus xylanilyticus* (HQ258920.1)	99
pGahr6	M13, fD1, fD2, rP1	1472	*Paenibacillus* rhizophaerae (NR043166.1)	99
pGahr7	M13, fD1, fD2, rP1	1401	*Bacillus licheniformis* (HM006898.1)	99
pGahr8	M13, fD1, fD2, rP1	1473	*Bacillus licheniformis* (HM006898.1)	99
pGahr9	M13, fD1, fD2, rP1	1476	*Bacillus amyloliquefaciens* (AY620954.1)	99

Phylogenetic affiliation of the microbes in the inoculant

The phylogenetic analysis revealed three distinct families of microbes successfully isolated from the inoculant. Overall, there was a good agreement between the different algorithms (distance, matrix, maximum parsimony and maximum likelihood) used for the phylogenetic analysis as described in details by Schmidt and Von Haeseler[11]. The inoculant comprised of *Bacillaceae* and *Paenibacillaceae* bacteria family as well as *Monascaceae* fungi family. Detail information on the environmental sequences are available in Table 3.

Bacillus

Four clones (pGahr4, pGahr7 and pGahr8) clustered in the *Bacillus* genus form a distinct branch together with *Bacillus licheniformis* (Fig. 4), a gram-positive spore forming bacterium widely distributed as a saprophytic organism in the environment[12]. *B.licheniformis* encodes numerous secreted enzymes that hydrolyze polysaccharides, proteins, lipids and other nutrients. As a saprophytic organism, *B.licheniformis* must utilize a variety of nitrogenous compounds as nutrients for growth and metabolism such as protein, ammonia, nitrate and amino acids like arginine, and asparagine via arginine deaminase, and asparaginase activities[13]. As an endospore forming bacterium, the organism can survive under unfavorable conditions such as dryness and high temperature. In contrast, the clone pGahr9 form a deep branch in the *Bacillaceae* family (Fig. 4), which indicates the pGahr9 might constitutes a new genus[14]. When the nucleotide sequence of the pGhar9 is compared to the 16S rDNA sequence of *Bacillus amyloliquefaciens*, the differences are uniformly distributed along the DNA, suggesting that the large evolutionary distance is not due to the chimera formation or faulty DNA amplification. *Bacillus amyloliquefaciens* is a gram-positive rod that has been isolated from wide range of environments including foods and soil[15]. This species is closely related to *Bacillus subtilis* and responsible for the world production of α-amylase and protease production. *B.amyloliquefaciens* degraded casein, elastin, gelatin and starch at optimal temperature between 30 to 40°C[16].

Paenibacillus

Clone pGahr5 is closely related to the *Paenibacillus xylanilyticus* (Figure 4), a gram-positive, facultative anaerobe and a spore forming

bacterium that was isolated from soil and plant related environment[17]. *P. xylanilyticus* possesses enzymes that are involve in the metabolism of plant carbohydrate polymers including amylase, cellulase, β-glucanase, pectinase and xylanase[18]. The xylanase produced by this species can hydrolyze xylan (a heterogenous polymer that constitutes a major components of hemicelluloses of cell walls) to xylanoligosaacharides and xylose[19]. The optimal growth for this species is at 37°C and formed oval subterminal spores during the environmental stress conditions like dryness, high temperatures or heavy rainfalls[17]. On the other side of the branches, pGahr6 formed distinct affiliation with plant growth promoter bacterium, *Paenabacillus rhizosphaerae* (Fig. 4). The gram positive bacterium was isolated from the rhizosphere of the legume,*Cicerarietinum* in Argentina and is strictly aerobic[20]. This species also produces spores during unfavorable conditions. Not much scientific information is available on this species.

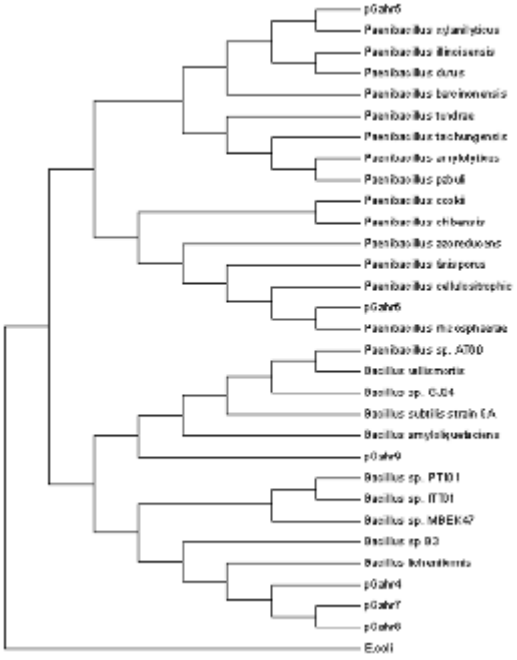

Fig. 4. Phylogenetic analysis of 16S rDNA gene sequence from clones pGahr4, pGahr5, pGahr6, pGahr7, pGahr8, pGahr9 with close relative using neighbor-joining method. The *E.coli* sequence was included as an out-group. The scale bar, 5 nt substitutions per 1000 nt

Monascus

pGahr1, pGahr2 and pGahr3 clones appeared to be distant from known *Monosacus*genera (Fig. 5). They might constitute new genus. When the nucleotide sequences of the clones were compared to the ITS sequence of the 8 *Monascus* species, the differences were uniformly distributed along the DNA, suggesting that the evolutionary path is not due to faulty DNA amplification. The genus *Monascus* belongs to the *Ascomycetes* class, and is characterized by non-ostiolateascoma arising singly at the tips of stalk-like hyphae and scattered throughout the mycelium[21]. The *Monascus* species has gained more attention from the scientists worldwide for the production of biologically active compound such as monacolin K, dimerumic acid and γ-aminobutyric acid (GABA)[22].

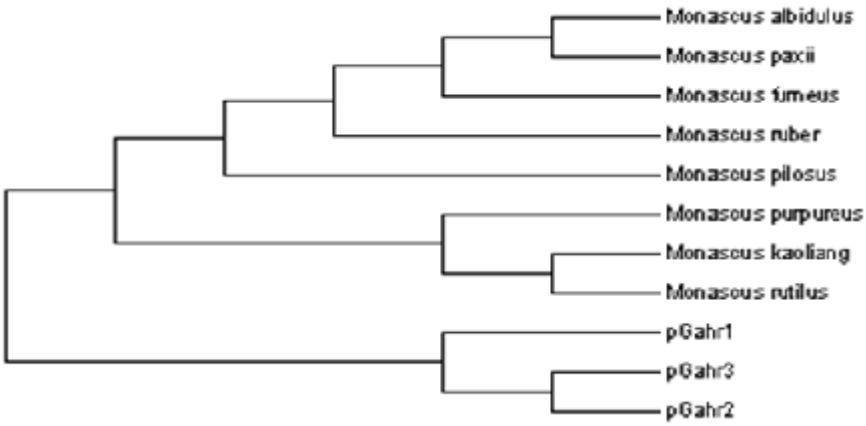

Fig. 5. Phylogenetic analysis of ITS region sequence from clones pGahr1, pGahr2, pGahr3 with close relative using neighbor-joining method. The scale bar, 5 nt substitutions per 1000 nt

The successful cultivation and phylogenetic identification of the possible microorganisms present in the inoculant sample has provided us information of their optimal growth conditions and physiology. Both genera of *Bacillus* and *Paenabacillus* perhaps utilized carbohydrates and proteins from the tree host for growth and metabolism. This lends support to the hypothesis concerning the agarwood resin formation. The agarwood tree converts the nutrients into resinous form in order to protect itself against the pathogens. No plausible explanation to the presence of the *Monascus* genus in the inoculant can be suggested at this point of time. It would be interesting to identify and confirm the identity of microorganisms present on the agarwood resin harvested from the agarwood tree that has been treated with the same inoculant used in this study. The knowledge from this study can be used in future attempts to produce a cost effective inoculants that can help local farmer to reduce the levy cost that mainly come from the inoculants.

CONCLUSION

The present work is an attempt to study artificial inoculant that has been reported to be effective for resin formation in agarwood tree. The 16S rDNA and ITS identification showed that three genera of microorganisms are present in the inoculant sample. It is noteworthy that the closest relatives to most identified species are known to hydrolyze carbohydrates and proteins except *Monascus* sp. Most of the species isolated are spore forming microorganisms. This characteristic makes them suitable candidates to develop a low cost inoculants since no nutrient are required to preserve the microbes as they are in the spore or latent form during unfavorable conditions. Further confirmation of the present of this genera on the agarwood resin harvested from the three injected with the same inoculants used in this study, is ongoing.

ACKNOWLEDGEMENTS

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