FORM 2

THE PATENTS ACT 1970
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COMPLETE SPECIFICATION

(See section 10 and rule 13)

TITLE OF THE INVENTION

A METHOD FOR PRODUCTION OF FRAGRANT COMPOUNDS FROM RESINOUS CHIPS OF *AQUILARIA MALACCENSIS* BY FERMENTATION

APPLICANTS

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The following specification particularly describes the invention and the manner in which it is to be performed

A METHOD FOR PRODUCTION OF FRAGRANT COMPOUNDS FROM RESINOUS CHIPS OF *AQUILARIA MALACCENSIS* BY FERMENTATION

FIELD OF THE INVENTION

The present invention in general, relates to a method for production of fragrant compounds, from resinous chips of *Aquilaria malaccensis*.

In particular, the present invention relates to a method for production of fragrant compounds from resinous chips of *Aquilaria malaccensis* by fermentation, which involves novel steps whereby the methodology is simple, cost effective, fully in vitro and environment friendly, can be controlled in lab and scaling up is easily achievable.

More particularly the present invention relates to a method for production of fragrant components of agarwood oil responsible for its distinct aroma from resinous chips of *Aquilaria malaccensis* by fermentation.

The instant application is being filed as a patent of addition of pending patent application numbered 1277/KOL/2014, filed by the same applicants on 10th December 2014 and it is to be understood that by way of this reference, the entire complete specification filed with such application (hereinafter referred to as the parent application) is being incorporated herein in its entirety.

In the main patent application, a method for production of fragrant compounds was done from *Aquilaria malaccensis* cell culture assisted by fermentation with *Fusarium* sp. (closest relative *Fusarium proliferatum*). The present invention applies the same fungus but the starting material comprise resinous chips of *Aquilaria malaccensis* which yield even better results as would be evident from description hereinafter.

BACKGROUND OF THE INVENTION

Artificial *in vivo* induction: The association of fungi with the formation of agarwood has been widely known. The method of mechanical injury coupled with artificial inoculation with fungi to induce agarwood production is well-established. In this method chemical inducers of agarwood are injected into xylem vessels of agarwood trees that lead to resin deposits in the entire tree. By this process, it s believed that 28 times higher yield is possible, as compared to conventional methods of induction such as physical injury and fungal inoculation.

Artificial *in vitro* induction: Production of agarwood compounds from suspension cultures of *A. sinensis* in *in vitro* cultures has been reported. Callus from leaf explants of *A. malaccensis* in MS medium supplemented with 2,4-D (6.0 mg/L) and Kin (2.0 mg/L) was associated with accumulation of furano-monoterpenoids, acids, alcohols and aldehydes. Crude fungal extracts of the fungus *Melanotus flavolivens* elicited the production of 2-(2-phenylethyl) chromones in suspension cultures of *A. sinensis*. Suspension culture from *A. sinensis,* accumulated few sesquiterpenes.

Defects of artificial *in vivo* induction: Cost of production is very high because, (i) mature/old plants (> 10 years old) are required, (ii) land is required to grow, (iii) production of agarwood resin is uncertain and (iv) the quality also varies.

Defects of artificial *vitro* induction: In all the previous reports, fungal elicitors (crude extract), methyl jasmonate, salicylic acid, etc were used for induction. Although agarwood formation is due to plant defense response, in these reports the role of the fungus and a wider gamut of plant microbe interactions have been overlooked.

In general, the prior art technologies of production of agarwood compounds from fungi association involved complicated process and higher cost and is not environment friendly.

Accordingly, there is a need for a process of fungus assisted production of fragrant agarwood oil, which gets rid of the disadvantages as contemplated hereinbefore.

The present invention meets the abovementioned need and other associated needs by providing a method for production of fragrant compounds from resinous chips of *Aquilaria malaccensis*.

The method employs a new technology which involves *Fusarium* sp. (closest relative *Fusarium proliferatum*) assisted fermentation of resinous agarwood chips to produce aroma compounds. Details of the fungus *Fusarium* sp. (closest relative *Fusarium proliferatum*) and its capability to induce aroma compounds *in vitro* when fermented along with calli of agarwood has been already established in the main Patent application no. 1277/KOL/2014.

The fermentation process is simple, fast, cost effective and fully *in vitro,* developed under controlled laboratory conditions using the tools of basic fermentation and microbial technology. Conditions of fermentation, solvent extraction have been suitably optimised but can be modified, manipulated and defined as per need without changing any of the basic parameters. It is also possible to scale up the system to bioreactors where conditions for production can be further controlled. In such conditions, extraction and other downstream operation can be optimized conveniently and even automated.

THE OBJECTS OF THE INVENTION

It is the principal object of the present invention to provide a methodology for *Fusarium* sp. (closest relative *Fusarium proliferatum*) assisted fermentation of agarwood chips to produce aroma compounds whereby the method is simple, quick, cost effective, fully *in vitro,* developed under controlled laboratory conditions using the tools of basic fermentation and microbial technology.

It is another object of the present invention to provide a methodology for *Fusarium* sp. (closest relative *Fusarium proliferatum*) assisted fermentation of agarwood chips to produce aroma compounds, whereby the method can be optimized, modified, manipulated and defined as per need without changing any of the basic parameters and it is also possible to scale up the system to bioreactors where conditions for production can be further controlled. In such

conditions, extraction and other downstream operation can be optimized conveniently and even automated.

It is another object of the present invention to provide as many aroma compounds as possible, by applying the above methodology.

It is another important object of the present invention to provide a method for production of fragrant components of agarwood oil, responsible for its distinct aroma from resinous chips of *Aquilaria malaccensis* by fermentation.

How the foregoing objects are achieved and the other aspects of the present invention will be clear from the following description which is purely by way of understanding and not by way of any sort of limitation.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides a method for production of fragrant compounds from resinous chips of *Aquilaria malaccensis* by fermentation process comprising:

i) subjecting collected resinous agarwood chips to pulverization and surface sterilization;

ii) co-culture of fungal candidate isolate identified as *Fusarium* sp. (closest relative *Fusarium proliferatum*) with pulverized and sterilized resinous agarwood chips for a suitable duration;

iii) solvent extraction of the tissues followed by analysis by GC-MS and data interpretation by Venn analysis to differentially determine compounds formed due to fermentation, among the compounds identified by GC-MS profiling.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

The nature and scope of the present invention will be better understood from the accompanying drawings, which are by way of illustration of some preferred

embodiments and not by way of any sort of limitation. In the accompanying drawings:-

Figure 1 is a view of chips of resinous agarwood, at a distillation unit which are applied as source material in the present invention.

Figure 2 is a view of *In vitro* fermentation of resinous agarwood chips with a *Fusarium* associate (H15).

Figure 3 is Venn diagram showing distribution of a total of 353 compounds detected by GC-MS in the 5 metabolic phenotypes, appropriately labeled in the figure.

DETAILED DESCRIPTION OF THE INVENTION

The following describes some preferred embodiments by way of understanding and not by way of any limitation. Furthermore, the results provided here in after are all exemplary and should not be construed as any sort of restriction.

1. Methodology

1.1. Agarwood samples

Agarwood (*Aquilaria malaccensis*) samples were collected from four major agarwood production sites of Assam in the northeast of India *viz.* Janji (26°52'N; 94°27'E), Hojai (26° 0'18"N; 92°51'E), Namti (26°51'N; 94°37'E) and Nahorani (26°13'N; 93°52'E) that are known globally for high quality agarwood. Locally available superior *Boha* agarwood oil (Bh), collected from a reputed distillation unit in Namti was included as the reference profile. The resinous agarwood chips (Fig. 1) were further pulverised and subjected to surface sterilisation [$HgCl_2$ (0.1%) and ethanol (70.0 %) for 1.0 min. each].

1.2. Fungus (Fusarium sp.)

A promising agarwood associated fungus isolate (*Fusarium* sp.; H15) had been previously identified (closest relative *Fusarium proliferatum*) in the main patent application numbered 1277/KOL/2014 which has a detailed description of the screening of fungus and co-culture fermentation process that led to the identification of the *Fusarium* isolate, H15. Its role in inducing aroma compounds

in vitro when fermented along with calli of agarwood has been already established in the Patent application no. 1277/KOL/2014. The fungus has also been submitted to International Depository Authority(IMTECH, Chandigarh) as Patent Deposit bearing Accession No MTCC–5997, on 10.3.2015 (date of accession) and accepted on 1.5.2015 for deposit under Budapest Treaty.

The sequence listing of the *Fusarium sp*. is as follows:

>H15 (419 bp)

CCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTC

TGTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAATC

AAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGA

AGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAAT

TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCC

AGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACC

CTCAAGCCCCCGGGTTTGGTGTTGGGGATCGGCGAGCCCTT

GCGGCAAGCCGGCCCCGAAATCTAGTGGCGGTCTCGCTGCA

GCTTCCATTGCGTAGTAGTAAAACCCTCGCAACTGGTACGC

GGCGCGGCCAAGCCGTTAAACCCCCAACTTCTGAATGTTGA

CCTCGGATC

In the present invention, the same fungus of the main patent application was used to ferment agarwood chips *in vitro* during the new experiments. For the purpose, fresh fungal culture was grown in Potato dextrose agar plates using routine spread plate techniques.

1.3. Experimental set up

The experimental set up included the following 5 samples/ treatments:

(a) Boha oil (**Bh**)

(b) Un-soaked resinous wood (**USR**)

(c) Soaked resinous wood (**SR**)

(d) Resinous wood fermented with fungus (**RH15**), and

(e) Pure culture of fungus (**PH15**) fermented without resinous wood

Into 1.0 g pulverised resinous wood in a 50 ml tube, 10.0 ml sterile water was added. For RH15, fresh culture of *Fusarium* (H15 block scooped by cork borer from PDA plates) was added into the tubes and fermented under ambient conditions for 45 days (Figure 2). From figure 2, the development of fungal mat on the surface of the broth may be noted.

Similarly, the pure culture of *Fusarium* (PH15) and resinous wood without *Fusarium* (SR) were subjected to fermentation. Bh and USR as per description were not fermented. On 20th day, 50 µl of the liquid from each tube under fermentation was spread onto fresh PDA plates and incubated at 25°C for 3-7 days to confirm the viability of the fungus by re-isolation.

1.4. Solvent extraction

At the end of 45 days of fermentation, the entire contents of RH15, SR and PH15 were homogenised and then extracted with 10X n-hexane (Lichrosolv, Merck). Extraction of unfermented Bh and USR was also performed similarly (eg. 250 mg *Boha* oil extracted with 2.5 ml n-hexane). All samples were extracted thrice with equal volume of n-hexane every 24 hr for a total of 72 hrs on a rocker (Rockymax, Tarsons, India) at 40 RPM. Extractions were performed by transferring each n-hexane fraction into glass vials that were tightly sealed and stored at 4°C till analysis.

1.5. Gas Chromatography and Mass Spectroscopy (GC-MS)

Samples were filtered through 0.45 µm filters (MiniSart, Sartorius, Germany) and subjected to GC- MS analysis performed on a Shimadzu GC2010 Plus - triple quadrupole (TP-8030) GC-MS/MS system fitted with EB-5MS column (length-30 m, thickness -0.25 µm, ID-0.25mm). The oven programme started at 70°C for 2 min and ramped at 20°C/min up to 140°C and without holding, again ramped at 5°C/min to 290°C and then held for 5 min. A 1.0 µl sample was injected at 280°C using He as carrier gas (1ml/min) with a split ratio was 10:1. The mass spectrometer was operated in the electron ionization (EI) mode at 70 eV with a source temperature of 200°C with a continuous scan from 50 to 600

m/z. The peaks were identified by matching the mass spectra with the National Institute of Standards and Technology (NIST, USA) library.

1.6. Venn analysis

Multiple dataset analysis feature of Vennture free software (http://www.irp.nia.nih.gov/branches/lci /nia_bioinformatics_software.html) was used to study the distribution of compounds from the GC-MS profiles by Venn analysis that classifies the compounds based upon their shared representation in the profiles.

2. Result

A total of 353 compounds were identified in the above 5 profiles. Venn analysis (Figure 3), performed to determine the distribution of the compounds, revealed the highest number of unique compounds were present in Bh (87), followed by RH15 (64).

78 unique compounds were formed in SR that were absent in USR and 91 compounds occurred in RH15 which were absent in SR. However, most interestingly, among the 10 compounds common between profiles of reference agarwood oil (Bh) and resinous agarwood fermented with fungus (RH15), important aroma components of agarwood oil that included γ-eudesmol, agarospirol, (-)-aristolene were formed only during fermentation of the resinous agarwood chips in presence of associated *Fusarium* (H15). These compounds were absent in the rest of the profiles which confirms the role of fungus during fermentation.

Formation of agarwood compounds

Among the compounds formed during fermentation of resinous agarwood with fungus (RH15), 10 compounds were shared with the profile of agarwood oil (Bh):

1/ Aristolene epoxide,
2/ γ–Eudesmol,
3/ Agarospirol,
4/ (-)-Aristolene,

5/ (-)-Globulol,
6/ 6-(1-Hydroxymethylvinyl)-4, 8a-dimethyl-3, 5, 6, 7, 8, 8a-hexahydro-1H-naphthalen-2-one,
7/ Isophytol,
8/ Cyclotetradecane, 1, 7, 11-trimethyl-4-(1-methylethyl)-,
9/ Cyclohexane, 1, 4-didecyl-,
10/ Propanoic acid, 3, 3'-thiobis-, didodecyl ester

Among them, agarospirol, γ- eudesmol, aristolene are important fragrant components of agarwood oil responsible for its distinct aroma. Apart from that several other aroma compounds were detected in the profile of RH15. Thus, it would be clear that a novel fermentation system using a promising *Fusarium* fungus associated with agarwood (H15) is advocated by the present invention for the first time.

No prior art exists on *Fusarium* fungus assisted fermentation of agarwood chips to produce aroma compounds.

The fermentation process is simple, very fast, cost effective and fully *in vitro,* developed under controlled laboratory conditions using the tools of basic fermentation and microbial technology.

Conditions of fermentation, solvent extraction have been suitably optimised but can be modified, manipulated and defined as per need without changing any of the basic parameters. It is also possible to scale up the system to bioreactors where conditions for production can be further controlled. In such conditions, extraction and other downstream operation can be optimized conveniently and even automated.

The most important factors are the fungus and the substrate (resinous wood) and therefore require careful selection prior to fermentation.

Amount of variation in the product is likely to be significantly minimized, owing to the limited scope for diverse interactions in controlled environment. This is a

suitable trait for commercial production of natural compounds as precision is desirable. The process is environment friendly, production and downstream processing is much easier, can be controlled in laboratory and scaling up is easily achievable. The methodology yields a large number of aroma compounds.

The present invention has been described with reference to some drawings, test results and preferred embodiments, purely for the sake of understanding and not by way of any limitation and the present invention includes all legitimate developments within the scope of what has been described hereinbefore and claimed in the appended claims.

We claim:

1. A method for production of fragrant compounds from resinous chips of *Aquilaria malaccensis* by fermentation process comprising:

i) subjecting collected resinous agarwood chips to pulverization and surface sterilization;

ii) co-culture of fungal candidate isolate identified as *Fusarium* sp. (closest relative *Fusarium proliferatum*) with pulverized and sterilized resinous agarwood chips for a suitable duration;

iii) solvent extraction of the tissues followed by analysis by GC-MS and data interpreting by Venn analysis to differentially determine compounds formed due to fermentation, among the compounds identified by GC-MS profiling.

2. The method as claimed in claim 1 wherein steps i) and ii) comprise adding 10.0 ml sterile water into 1.0 gm pulverised resinous wood in 50 ml tubes, adding fresh culture of *Fusarium* sp. (closest relative *Fusarium proliferatum*) into the tubes and fermenting under ambient conditions for 45 days.

3. The method as claimed in claim 1 wherein surface sterilization is done by subjecting the puliverized wood into 0.1% $Hgcl_2$ and 70.0% ethanol for I minute each.

4. The method as claimed in claim 2 wherein the *Fusarium* sp has a sequence listing as follows:

>H15 (419 bp)

CCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTC

TGTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAATC

AAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGA

AGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAAT

TCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCC

AGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACC

CTCAAGCCCCCGGGTTTGGTGTTGGGGATCGGCGAGCCCTT

GCGGCAAGCCGGCCCCGAAATCTAGTGGCGGTCTCGCTGCA

GCTTCCATTGCGTAGTAGTAAAACCCTCGCAACTGGTACGC

GGCGCGGCCAAGCCGTTAAACCCCCAACTTCTGAATGTTGA

CCTCGGATC

5. A method for production of fragrant compounds from resinous chips of *Aquilaria malaccensis* by fermentation process as claimed in claim 1 wherein the compounds shared with profile of agarwood oil comprise Aristolene epoxide, γ–Eudesmol, Agarospirol, (-)-Aristolene, (-)-Globulol, 6-(1-Hydroxymethylvinyl)-4, 8a-dimethyl-3, 5, 6, 7, 8, 8a-hexahydro-1H-naphthalen-2-one, Isophytol, Cyclotetradecane, 1, 7, 11-trimethyl-4-(1-methylethyl)-, Cyclohexane, 1, 4-didecyl- and Propanoic acid, 3, 3'-thiobis-, didodecyl ester.

Dated this 9th day of May 2016.

(S. GHOSH)
for seenergi IPR
Applicants' Agent

ABSTRACT

A METHOD FOR PRODUCTION OF FRAGRANT COMPOUNDS FROM RESINOUS CHIPS OF *AQUILARIA MALACCENSIS* BY FERMENTATION

A method for production of fragrant compounds from resinous chips of *Aquilaria malaccensis* by fermentation process comprising:

i) subjecting collected resinous agarwood chips to pulverization and surface sterilization;

ii) co-culture of fungal candidate isolate identified as *Fusarium* sp. (closest relative *Fusarium proliferatum*) with pulverized and sterilized resinous agarwood chips for a suitable duration;

iii) solvent extraction of the tissues followed by analysis by GC-MS and data interpreting by Venn analysis to differentially determine compounds formed due to fermentation, among the compounds identified by GC-MS profiling.



FIG 1

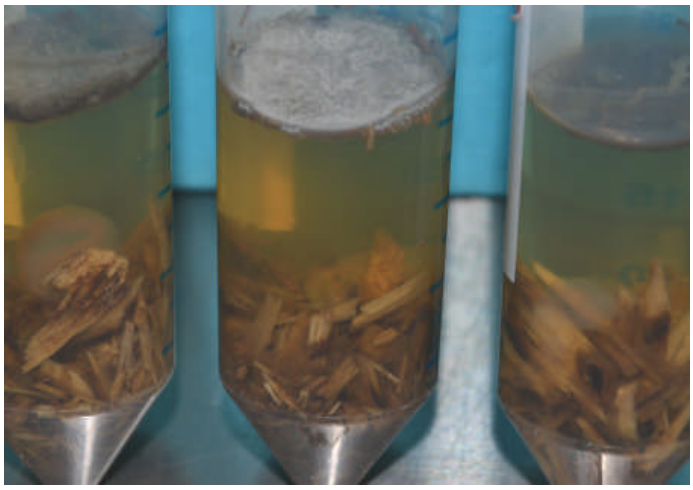

FIG 2

Dated this 9th day of May, 2016.

(S. GHOSH)
for seenergi IPR
Applicants' Agent

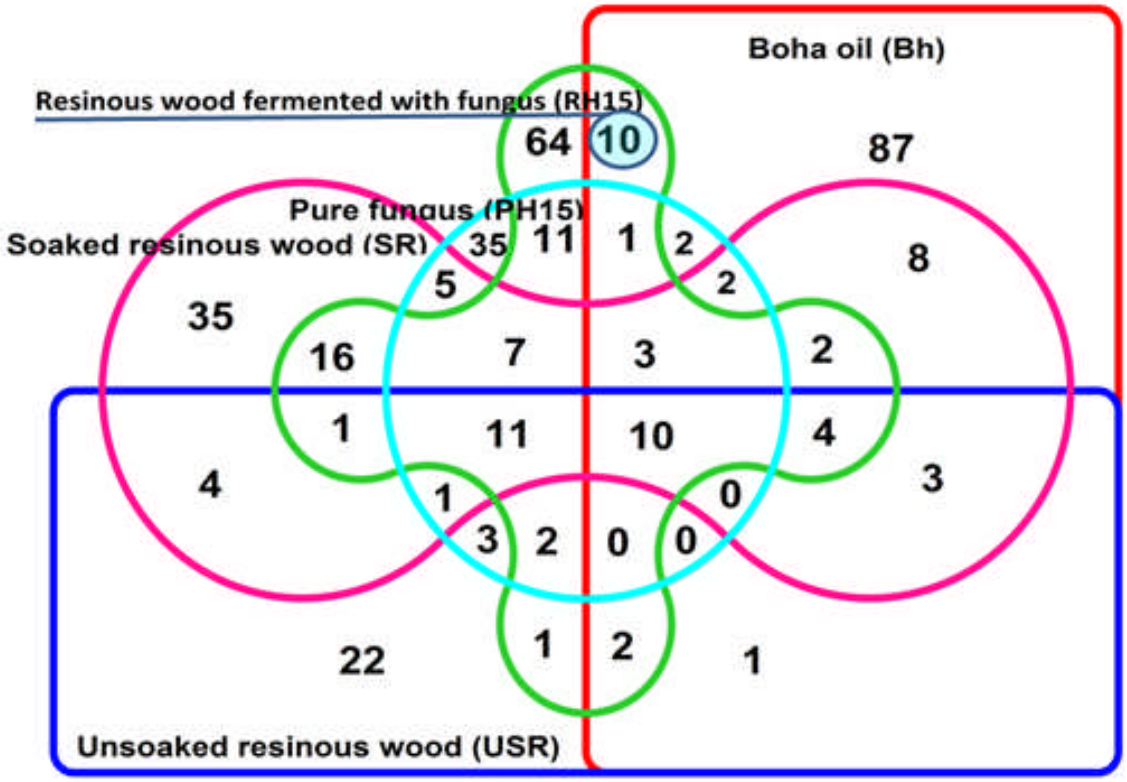

FIG 3

Dated this 9th day of May, 2016.

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FIG 1

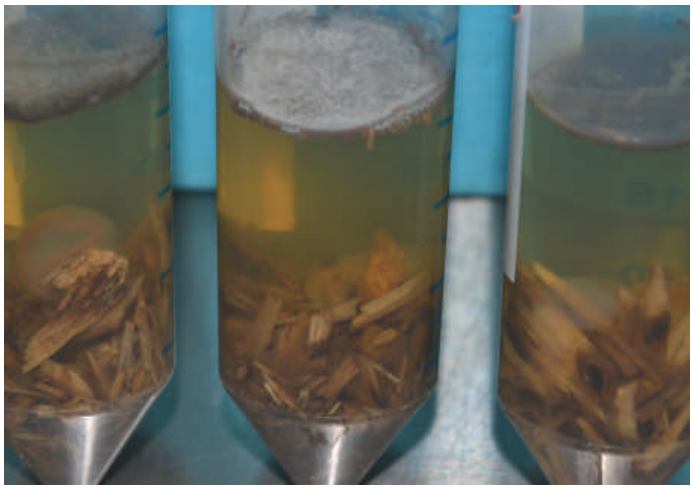

FIG 2

Dated this 9th day of May, 2016.

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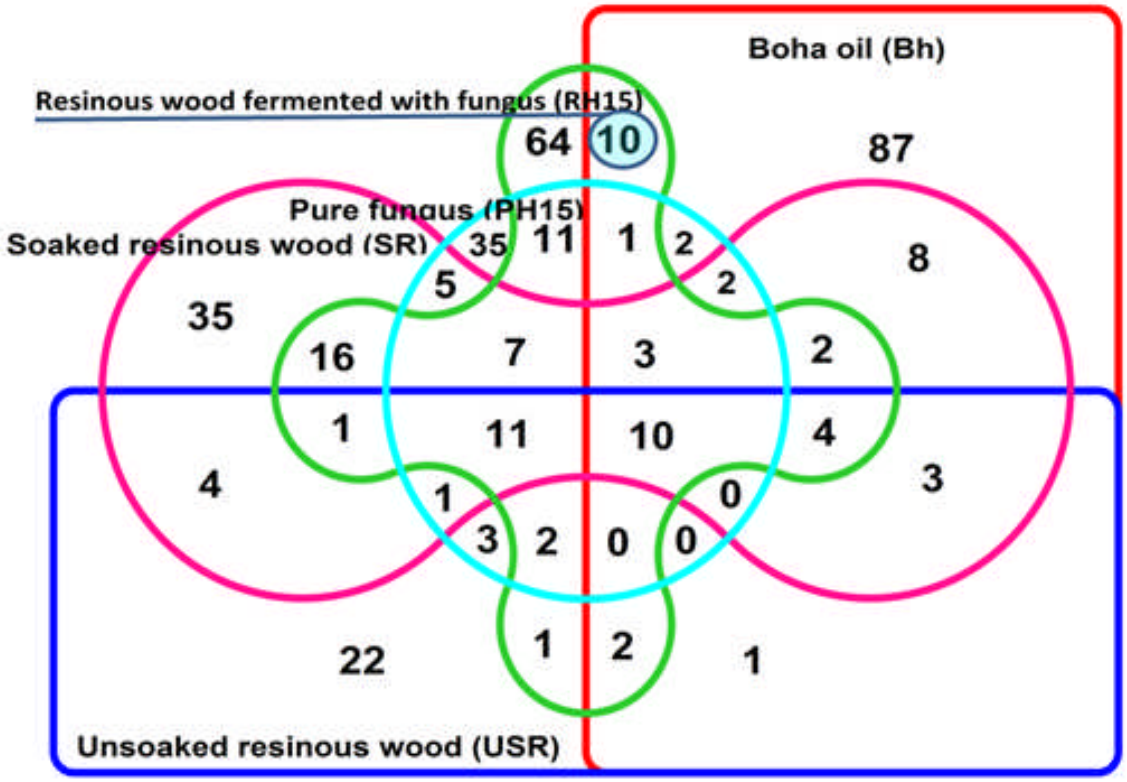

FIG 3

Dated this 9th day of May, 2016.

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