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Biotransformation of Monoterpenes by Whole Cells of Eleven *Praxelis clematidea*-Derived Endophytic Fungi

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***Abstract*—**Monoterpenoids are mainly found in plant essential oils and they are ideal substrates for biotransformation into oxygen-containing derivatives with important commercial value due to their low price and simple structure. In this paper, eleven strains of endophytic fungi from *Praxelis clematidea* were used as test strains to conduct the whole cell biotransformation of the monoterpenoids: (+)-limonene, (-)-limonene and myrcene. The fungi were inoculated in 50 ml Sabouraud medium and incubated at 30 ℃ with the agitation of 150 r/min for 6 d, and then 0.5% (v/v) substrates were added into the medium and biotransformed for further 3 d. Afterwards the cultures were filtered, and extracted using equal volume of ethyl acetate. The metabolites were analyzed by GC-MS technique with NIST database. The Total Ion Chromatogram of the extractions from the eleven strains showed that the main product of (+)- and (-)-limonene biotransformation was limonene-1,2-diol, while it is limonene and linalool oxide for biotransformation of myrcene. This work will help screen the microorganisms to biotransform the monoterpenes.

***Keywords*—**Endophytic fungi, (+)–limonene, (-)–limonene, myrcene.

I. Introduction

THE basic structure of terpenoids is made up of different numbers of isoprene connected end to end. According to the rules of isoprene, two isoprenes are called monoterpenes. Monoterpenes are classified into acyclic monoterpenes, monocyclic monoterpenes, bicyclic monoterpenes, and irregular monoterpenes. According to their structure, monoterpenoids mainly exist in essential oils, most of which have aroma, antibacterial, and flavor-correcting effects, and are widely used in the pharmaceutical industry, perfume industry, insect pheromone and insect repellent, etc. [1]. Among the monoterpenoids, the two monoterpenes, limonene and pinene, derived from lemon essential oil and turpentine oil, have always been ideal biotransformation substrates due to their simple structure and low price. In the reported literature, limonene [2], *α*- and *β*-pinene [3] can all be biotransformed with bacteria, fungi, yeasts and plant cells. Among the reported microbial catalysts, bacteria accounted for 41% and fungi accounted for 33% [4].

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Among the bacteria used for biotransformation of terpenoids, *Pseudomonas* sp. has been reported the most. This genus of bacteria was first isolated from the soil in 1966, and it was discovered that the bacteria can use limonene as the only carbon source for biotransformation [5], [6]. It was subsequently discovered that *P. aeruginosa* can convert tyrosol into hydroxytyrosol [7]-[9], convert myrcene into dihydrolinalool for 1.5 days biotransformation, and 2,6-dimethyloctane for 3 days biotransformation, respectively [10]. *P. rhodesiae* PF1 can generate isonovalal from *α*-pinene oxide [11], *P. putida* converts limonene into perillic acid [12], [13]. In 2013, Molina et al. [14] summarized the study on the biotransformation of terpenoids by *Pseudomonas* sp. In fungi, *Fusarium oxysporum* 152b [15]-[17] was reported to convert R-(+)-limonene and S-(−)-limonene into R-(+)-*α*-terpineol and limonene-1,2-diol, respectively. *Penicillium digitatum* and *Yarrowia lipolytica* can convert (+)-limonene to *α*-terpineol [18]-[22] and perillic acid [23], [24]. Another important mold is *Aspergillus niger*, which has been reported to employ (S)-(+)-Linalool [25], [26], *α*- and *β*-pinene [27], [28], (R)-(+)- and (S)-(−)-citronellol [29] as substrates. In 2014, Parshikov et al. [30] summarized the application of *A. niger* for biotransformation of terpenes.

In recent years, the role of plant endophytic fungi in the field of biocatalyst has begun to receive attention. Plant endophytic fungi need to co-evolve with host plants to adapt to changes in the environment, showing that they may produce abundant enzymes to adapt to the host [31]. Based on the above assumptions, the plant endophytic fungi should be applied to biotransformation, and some reports confirm this hypothesis. For example, the endophytic fungus *Botryosphaeria* sp., isolated from the seaweed *Bostrychia radicans*, can convert racemic camphor into 6-endo-hydroxy camphor, 6-exo-hydroxy camphor, 5-exo-hydroxy camphor, 5-endo-hydroxy camphor, 3-exo-hydroxy camphor and 8-hydroxy camphor [32]. *Phomopsis* sp., isolated from *Pinus taeda*, can biotransform limonene into carvone (0.536 g L^{-1}) and limonene-1,2-diol (2.08 g L^{-1}), more interesting is that the only main product, limonene-1,2-diol (2.10 g L^{-1}) is produced if citrus peel extract is used as the substrate [33]. Therefore, it is feasible to use plant endophytic fungi to biotransform monoterpenoids.

In this experiment, myrcene, which is an acyclic monoterpene, (+)- and (-)-limonene, which is monocyclic monoterpene, were selected as the substrate for biotransformation. Eleven endophytic fungi, isolated from

Praxelis clematidea, were used as test strains. The products of the extraction of culture broth were analyzed by GC-MS. Through this experiment, our goal is to screen some strains suitable for biotransformation of monoterpenes to produce compounds with important commercial value.

II. Materials and Methods

A. Materials and Apparatus

(+)-Limonene, (-)-Limonene, Myrcene, AR grade, were purchased from Tokyo Chemical Industry Co., Ltd., Japan; absolute Ethanol, Ethyl Acetate, Petroleum Ether, AR grade, were purchased from Sinopharm Reagent Co., Ltd., Shanghai, China. Thin layer chromatography silica gel plate (GF_{254}), whose size is 50 mm×200 mm×(0.20~0.25) mm, was purchased from Qingdao Ocean Chemical Plant Branch, Shangdong, China.

The apparatus used in this experiment were listed as: Shaking incubator (Shanghai Zhichu Instrument Co., Ltd., Shanghai, China), XH-C Vortex Mixer (intan Baita Xinbao Instrument Factory, Zhejiang, China), Clean Bench SW-CJ-1FD (Suzhou Antai Air Technology Co., Ltd., Zhejiang, China), Medical Centrifuge Machine H1650 (Hunan Xiangyi Laboratory Instrument Development Co., Ltd., Hunan, China).

B. Separation and Identification of Plants Endophytic Fungi

Fresh and healthy specimens of *Praxelis clematidea* were collected from the Xiamen Campus of Huaqiao University and identified by Dr. Qizhi Wang from the College of Chemical Engineering, Huaqiao University. The plant specimen certificate code is 431127 and stored in the specimen room of Huaqiao University.

The endophytic fungi were separated from the leaves of *Praxelis clematidea*. The separation protocol was recorded in [34]. All the strains were stored in the Natural Products Laboratory, College of Chemical Engineering, Huaqiao University.

The endophytic fungi were identified by molecular biology identification. First, The fungal ITS sequence was amplified with the primer: ITS1 (5′-TTCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), then the sequences were assembled and uploaded to National Center for Biotechnology Information (NCBI) searching for BLAST sequence similarity.

C. Whole-Cell Biotransformation of Monoterpenoids

The experiment was done according to [27] with some modifications: a loopful of each strain was inoculated in 250 mL Erlenmeyer flasks containing 50 mL of sterile Sabouraud medium (g L^{-1}: peptone 10, glucose 40, 1000 mL distilled water, pH 4.0-6.0) and incubated aerobically in orbital shaker at 30 °C and 200 rpm for 6 days. After the microorganisms' growth, 0.5 mL of substrate solution (substrate:absolute ethanol = 1:1, v/v, the final concentration of substrates is 0.5%) was added and biotransformed for 3 days under the same conditions. The experiments were carried out in parallel with controls, in the same conditions without the presence of microorganism.

At the end of the experiment, the cultures were filtered, and the product recovery was performed by liquid–liquid extraction with an equal volume of ethyl acetate. The final solution was dried over anhydrous sodium sulfate and stored at 4 °C for TLC and GC/MS analysis. In the TLC analysis, the developer is 5% vanillin sulfuric acid developer (5 g vanillin is dissolved in 100 mL of 10% sulfuric acid ethanol solution).

The reaction products were identified by GC/MS (Angilent 8860 GC System-5977B GC/MS with G4513A autosampler) using a capillary column HP-5ms (30 m×0.25 mm×0.25 μm). The column temperature was programmed to 60 °C for 1 min, increased at 20 °C/min at 300 °C for 13 min. Helium was the carrier gas, and the injection and detector temperatures were 250 °C. 1 μL of the solution was injected into the GC/MS system. The apparatus operated with a flow rate of 1.5 mL/min and in split mode (split ratio 1:5).

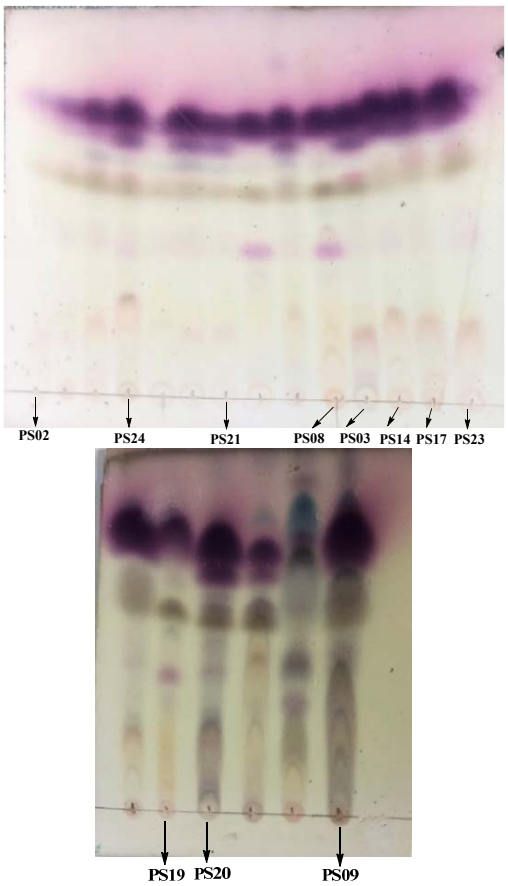


Fig. 1 Result of TLC analysis for extraction. The developing system is chloroform: methanol = 10:1 with 5% vanillin sulfuric acid developer

The identification of the compounds was accomplished by comparing the mass spectra with those from the National Institute of Standard and Technology (NIST) 11.0 database.

III. Results and Discussion

A. Strain Identification

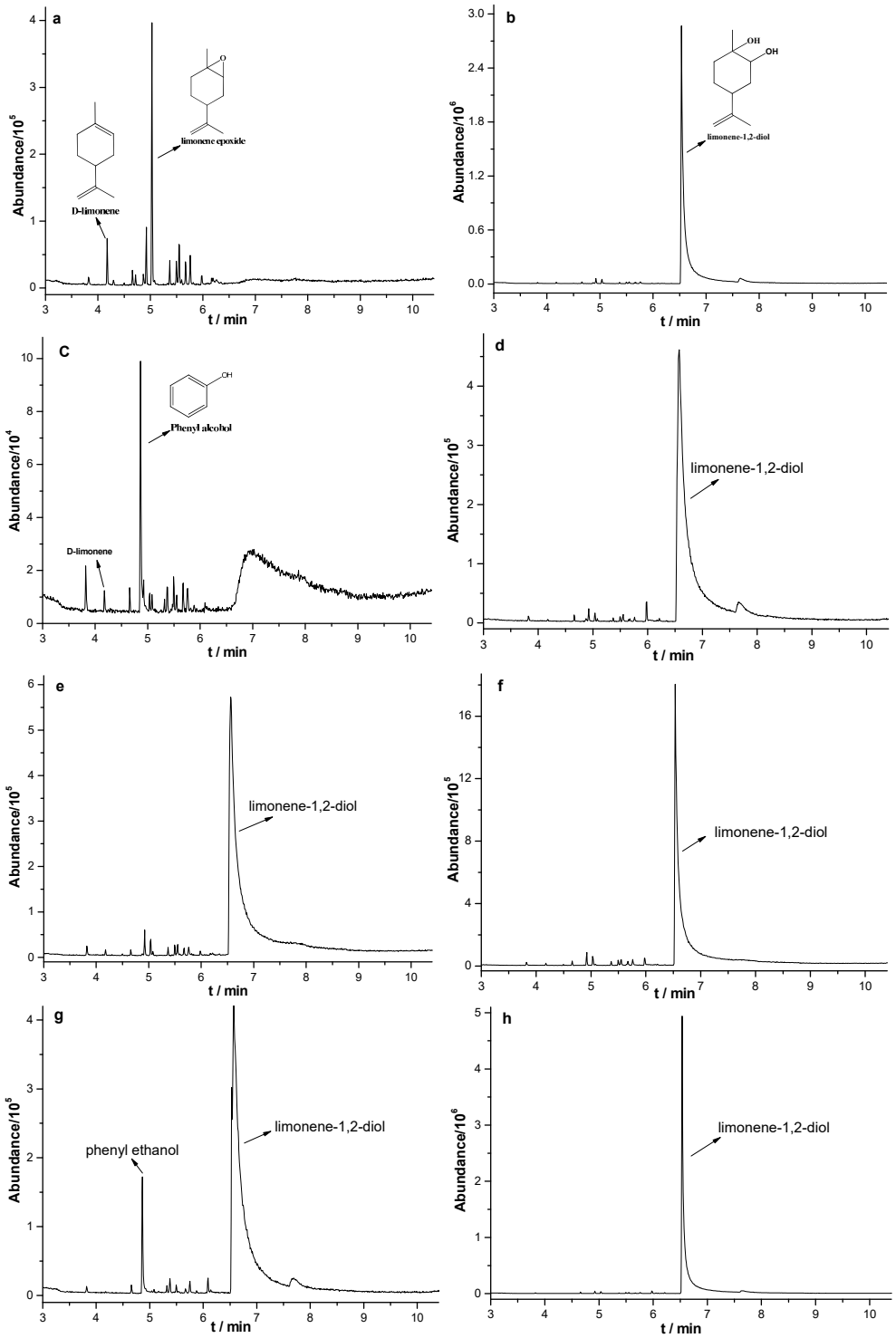
After comparison of ITS sequence similarity, it was found that these endophytic fungi belonged to *Alternaria Nees* in the *Ascomycota* (Berk.) *Caval.*-Sm., except *Diaporthe Nitschke* for

strain PS19,The serial ITS accession number for PS02, PS03, PS08, PS09, PS14, PS17, PS19, PS20, PS21, PS23, PS24 is MK640567, MK640571, MK640575, MK640579, MK640582, MK640584(PS19), MK640585, MK640586, MK640588, MK640589, respectively. PS08 failed to find a comparison result for the low similarity, so its species is not yet determined.

B. Analysis of (+)-Limonene Bioconversion Products

The Total Ion Chromatography (TIC) of the extract is shown in Fig. 1.

In Fig. 1, it can be seen the purple spot (R_f = 0.73 and 0.66) and grey spot (R_f = 0.54) commonly appeared in most of samples. Compared to the strain PS08 and PS19 to other strains, one red spot (R_f = 0.39) appeared in both of two strains. Based on the TLC analysis results, each extract was subsequently subjected to GC-MS to analyze its specific chemical components.



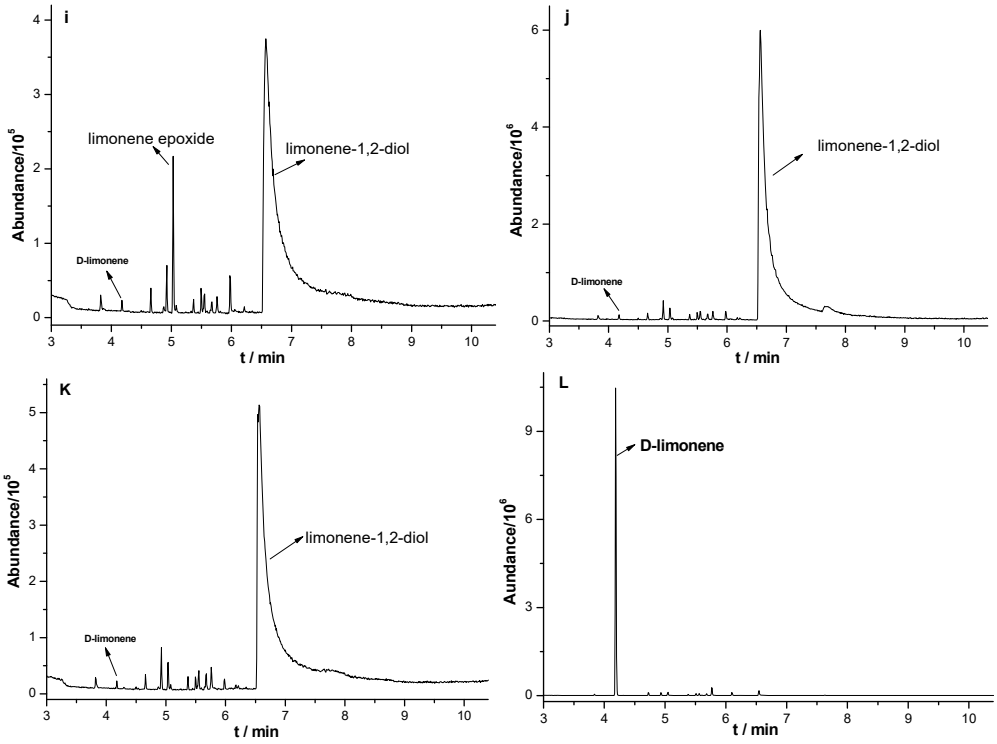


Fig. 2 TIC of the extraction for (+)-limonene biotransformation. a.PS02, b.PS03, c.PS08, d.PS09, e.PS14, f.PS17, g.PS19, h.PS20, i.PS21, j.PS23, k.PS24, L.limonene control

It can be seen from Fig. 2 that limonene-1,2-diol (t_R = 6.64 min) is the main signal in the extraction except PS08. The report of biotransformation of (+)-limonene to limonene-1,2-diol by plant endophytic fungi is consistent with the literature reported by [33] and [35]. The authors used the endophytic fungus *Phomopsis* sp. and *Cladosporium* sp.to biotransform (+)-limonene, and its main product is also limonene-1,2-diol (2.08 g L^{-1}, 1.5 g L^{-1}, respectively).

It also can be seen in Fig. 2i (PS21) two main products, limonene epoxide (t_R = 5.03 min) and limonene-1,2-diol. This metabolic pathway has been clearly studied, that is the C-1, 2 position of (+)-limonene is first epoxidized and then hydroxylated to form diol compound [36]-[38]. The question is limonene epoxide is not common in these test fungi. The reason may be explained limonene epoxide is converted to limonene diol in different level in Fig. 1, which the peak (t_R = 5.03 min) is weak or undetectable. The ratio of limonene diol to limonene epoxide depended on the epoxide hydrolase activity level.

In Fig. 2 c and Fig. 2g, the signal of phenyl ethanol can be seen. Combined with Fig. 1, it is speculated that the red spot (R_f = 0.39) is phenyl ethanol. Normally, 2-Phenethyl alcohol (2-PE) is produced by biotransformation of *L*-phenylalanine as substrate [39], [40], but there are few reports on biotransformation of limonene into 2-PE, so its bioconversion mechanism needs to be further clarified.

C. Analysis of (-)-Limonene Bioconversion Products

The TIC of the extract after the biotransformation of (-)-limonene by endophytic fungi is shown in Fig. 3.

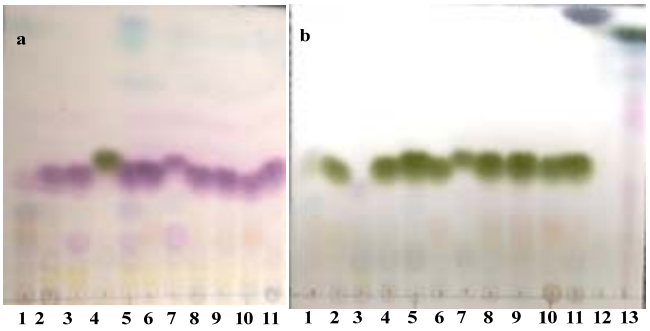


Fig. 3 Result of TLC analysis from the extraction of (-)-limonene and myrcene biotransformation by endophytic fungi: The developing system is petroleum ether: ethyl acetate =14:6 with 5% vanillin sulfuric acid developer. a. substrate-(-)-limonene, b. substrate-myrcene. 1.PS02, 2.PS03, 3.PS08, 4.PS09, 5.PS14, 6.PS17, 7.PS19, 8.PS20, 9.PS21, 10.PS23, 11.PS24, 12. myrcene standard, 13.(-)-limonene standard

In Fig. 3 a, it can be seen that the main spot is the same except the column **4** (PS09) for (-)-limonene biotransformation, so it was sampling for GC-MS analysis from column **3** and **4**. In Fig. 3 b, the main spot is the same to the nine column except column **1** (PS02) and **3** (PS08), so the column **4** was also sampling for myrcene biotransformation. The result of GC/MS was shown in Fig. 4.

As can be seen from Fig. 4, the metabolites are complex and diverse. In Fig. 4 a, the main signal is limonene-1,2-diol. Combing the data in Figs. 4 a and 2, plant endophytic fungi can transform both (+)- and (-) limonene into limonene-1,2-diol, indicating that the corresponding oxidase has no stereospecific

requirements for the substrate. The assumption does not apply to strain PS09. The products of PS09 are myrcene, linalool oxide, 2,4-tert-butylphenol and m-camphorene.

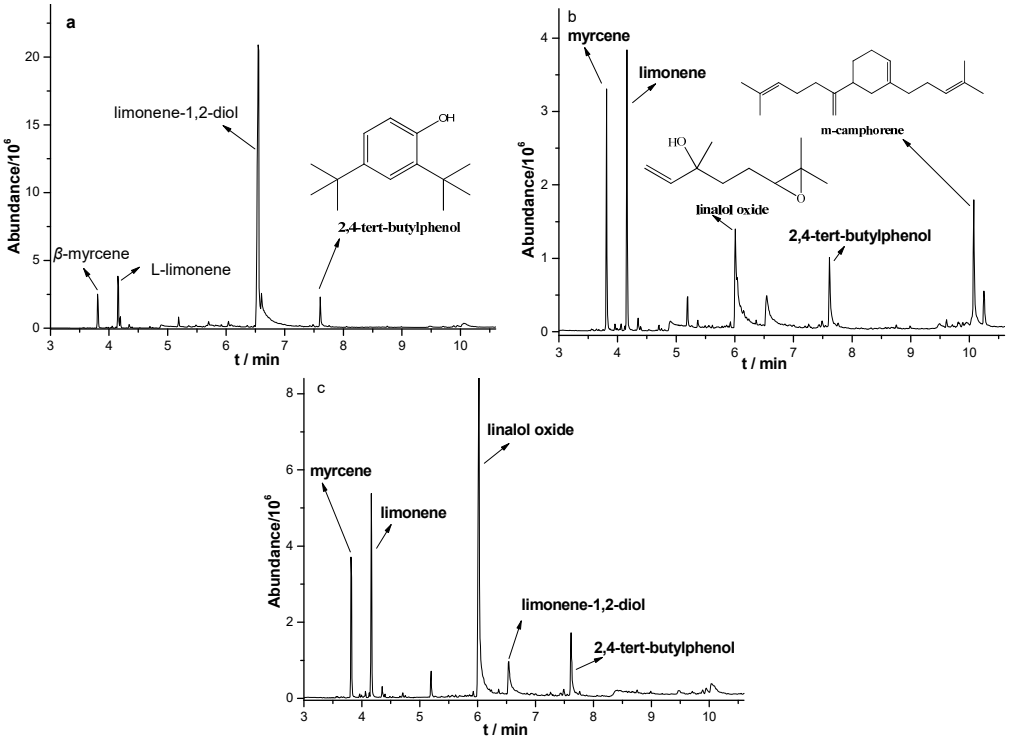


Fig. 4 TIC of the extraction: a. PS08 for (-)-limonene, b. PS09 for (-)-limonene, c. PS09 for myrcene

In Fig. 4 c, the signal showed the signals are myrcene, limonene, and linalool oxide, This result is consistent with the [10]. The authors reported that *Pseudomonas aeruginosa* can biotransform myrcene into dihydrolinalool and 2,6-dimethyloctane for 1.5 days, the products are belonging to ring-opening compound, so the linalool oxide is explainable. In this paper, the bacteria can also biotransform myrcene into 2,6-dimethyloctane and α-terpineol for 3 days, the formation of the latter is aided by the intermediate product limonene, so the closed-loop monoterpenoids, limonene and limonene-1,2-diol, can be formed by myrcene biotransformation.

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