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# Screening of Microorganisms Capable of Biotransforming Certain Monoterpenes Using Substrate Toxicity Test

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## Abstract

**Monoterpenes, such as Geraniol (G), Geranyl acetate (GA), Citral (CT), Limonene (LN), and Linalool (LL), are the most widely used phytochemicals in the aroma, food, and pharmaceutical industries. Here, we screened several bacteria and fungi to assess their potential to biotransform the selected monoterpenes (G, GA, CT, LN, and LL) through the substrate toxicity test. Three bacteria *Pseudomonas fluorescens* MTCC2421, *Streptococcus mutans* MTCC497, and *Escherichia coli* were found to be resistant to G, GA, and LN while two *P. aeruginosa*, and *S. epidermidis* MTTC 435 to GA and LN. In general, all fungal strains did not show resistance to any of the monoterpenes used, except *Candida albicans* and *Fusarium oxysporum*, which were slightly resistant to lower concentrations (0.05-0.1%) of GA. Interestingly, none of the bacteria and fungi showed any resistance to CT. The maximum concentrations of monoterpenes to which bacteria exhibited resistance ranged from 0.05-0.2%. The growth and biomass profiles of bacteria revealed that *P. fluorescens* and *S. mutans* grew well in the presence of monoterpenes GA and LN. Based on this, *Pseudomonas fluorescens* was capable of biotransforming GA and LN, while *S. mutans* only LN. The biotransformation of GA by *P. fluorescens* produced G and LL on the day 5$^{th}$ and 7$^{th}$ of the incubation. Hence, the study revealed the three potential bacteria, which may be useful in producing new aromatic derivatives from selected monoterpenes through biotransformation.**

**Keywords:** Monoterpenes, Citral, Geraniol, Geranyl Acetate, Limonene, Biotransformation, Substrate Toxicity Test

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## INTRODUCTION

Monoterpenes are $C_{10}$-containing compounds that belong to the isoprenoids family of secondary metabolites. They are the main constituents of the essential oils of aromatic plants. They impart a unique aroma to the essential oils. Several monoterpenes such as geraniol (G), geranyl acetate (GA), citral (CT), limonene (LN), and linalool (LL) are highly popular and widely used in fragrances, cosmetics, hygiene, household products, food, and pharmaceuticals.[1-4] They exhibited a wide range of biological activities such as antibacterial, analgesic, anti-inflammatory, anticancer, antidiabetic, antiobesity, and modulators of the gut microbiota.[1,5,6] Citral and linalool are also involved in the synthesis of vitamins A, E, and ionones.[7-9] At present, many reports are being published on geraniol and citral, demonstrating their potential anticancer effects and their scope in alternative cancer therapy.[10]

With the rapidly increasing importance of monoterpenes in the production of human fragrances and tastes, demand for new monoterpenes on the market will continue to rise. At present, most of the flavouring products available in the market are produced by chemical synthesis. Besides, many such products are also isolated from plants by solvent extraction and hydro-distillation. However, chemical synthesis has several demerits, such as the formation of undesirable chemical mixtures, inappropriate high operating temperatures, and the side or adverse effects of chemically synthesized products. In view of this, consumers now prefer products, which are produced by green synthesis to chemically synthesized products. However, in plants, these products are produced in a very limited quantity, so plants may not be reliable sources for large-scale extraction of such compounds. Therefore, microbial biotransformation relying on microorganisms and their biocatalysts has been proposed as an alternative approach for the production of novel monoterpenoids. This has several advantages over chemical processes. Recently, Mittal *et al.* studied the biotransformation of monoterpenes by microorganisms and plant cell and organ cultures.[11] Considering the increasing and vastly varied significance of monoterpenes viz., geraniol (G), geranyl acetate (GA), citral (CT), limonene (LN), and linalool (LL) in the aroma industry and expanding the field of microbial biotransformation, the present study has been undertaken to screen monoterpene resistant microorganisms from soil samples, which may be used for the production of important monoterpenes through biotechnological approaches. The monoterpene-resistant microbes were screened using the substrate toxicity test.

## MATERIALS AND METHODS

### Chemicals

Authentic geraniol, geranyl acetate, citral, limonene, and linalool were procured from Sigma-Aldrich, India.

### Microorganisms

Ten bacteria namely *Escherichia coli* (MTCC901), *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Staphylococcus aureus* (MTCC96), *Streptococcus mutans* MTCC497, *Staphylococcus epidermidis* MTTC 435, *Shigella boydii* MCC 2408, *Acinetobacter baumannii*, *Bacillus mycoides* and the three fungi *Alternaria brassicicola*, *Fusarium oxysporum* and *Candida albicans* were obtained from the CSIR-Institute of Microbial Technology, Chandigarh, India. Bacteria were inoculated on nutrient agar (NAM) and fungal cultures on potato dextrose agar (PDA). Pure colonies were sub-cultured and stored on slant agar at 4°C and 80% glycerol stocks at -20°C.

### Substrate-toxicity test

Substrate toxicity was performed to screen monoterpene-resistant microorganisms in accordance with previous methods.[12,13] The culture plates were prepared by displacing 30 ml sterilized NAM in pre-sterilized Petri dishes. Each 1 ml ($1.0 \times 10^5$ CFU/ml) inoculum is evenly distributed to the agar medium with a sterile glass rod. Wells were bored in agar plates using a sterile cork borer (6 mm). To the wells, 25, 50, 75, and 100 µl of monoterpenes equal to concentrations 0.05-0.2% were added. Bacterial and fungal plates were incubated separately at 37°C, 24 h, and 27°C, 48 h, respectively. Simultaneously, positive and negative control plates were also incubated. The

plates were observed and the mean diameter of the inhibition zone (mm) was measured. Each experiment was performed in triplicate.

**Microbial growth rates**

The cultures were incubated in a rotary shaker at 30°C and 150 rpm for seven days to measure microbial growth. Bacterial growth rates were measured in terms of absorbance at 660 nm. The biomass of fungal strains was filtered and assessed by wet weight. Finally, the microbiological growths were compared to a control without monoterpenes.

**Table 1.** Monoterpene-resistant behavior of the bacterial strains

| Bacterial Strains | Concen. | Zone of inhibition (mm) | | | | |
|---|---|---|---|---|---|---|
| | (%) | Geraniol | Geranyl Acetate | Citral | Linalool | Limonene |
| *Pseudomonas fluorescens* MTCC 2421 | 0.05 | NO | NO | 35 | 15 | NO |
| | 0.1 | NO | NO | 45 | 20 | NO |
| | 0.15 | NO | NO | 55 | 26 | NO |
| | 0.2 | NO | NO | 78 | 32 | NO |
| *P. aeruginosa* | 0.05 | 15 | No | 30 | 13 | NO |
| | 0.1 | 20 | No | 38 | 20 | NO |
| | 0.15 | 28 | No | 45 | 26 | NO |
| | 0.2 | 36 | No | 45 | 26 | NO |
| *Staphylococcus epidermidis* MTTC 435 | 0.05 | No | 15 | 24 | No | 13 |
| | 0.1 | No | 21 | 33 | No | 20 |
| | 0.15 | No | 29 | 42 | No | 25 |
| | 0.2 | No | 35 | 48 | No | 29 |
| *Streptococcus mutans* MTCC497 | 0.05 | No | No | 35 | 12 | No |
| | 0.1 | No | No | 40 | 16 | No |
| | 0.15 | No | No | 40 | 20 | No |
| | 0.2 | No | No | 50 | 20 | No |
| *E. coli* | 0.05 | 11 | No | 40 | No | No |
| | 0.1 | 20 | No | 60 | No | No |
| | 0.15 | 32 | No | 90 | No | No |
| | 0.2 | 32 | No | 90 | No | No |
| *Shigella boydii* MTCC2408 | 0.05 | 11 | No | 18 | 18 | 25 |
| | 0.1 | 15 | 10 | 25 | 28 | 25 |
| | 0.15 | 20 | 16 | 30 | 40 | 25 |
| | 0.2 | 25 | 25 | 30 | 40 | 25 |
| *Acinetobactor baumannii* | 0.05 | 10 | No | 15 | 24 | No |
| | 0.1 | 18 | 15 | 25 | 30 | 1 |
| | 0.15 | 20 | 20 | 25 | 30 | 15 |
| | 0.2 | 28 | 20 | 30 | 35 | 15 |
| *S. aureus* | 0.05 | 23 | No | 20 | 20 | 20 |
| | 0.1 | 15 | No | 20 | 20 | 20 |
| | 0.15 | 15 | 15 | 20 | 24 | 25 |
| | 0.2 | 20 | 15 | 30 | 24 | 25 |
| *P. putida* | 0.05 | 14 | 13 | 30 | 20 | 18 |
| | 0.1 | 20 | 13 | 40 | 20 | 24 |
| | 0.15 | 27 | 19 | 50 | 24 | 30 |
| | 0.2 | 35 | 26 | 60 | 24 | 30 |
| *Bacillus mycoides* | 0.05 | 14 | 12 | 20 | 20 | 20 |
| | 0.1 | 26 | 25 | 20 | 20 | 20 |
| | 0.15 | 35 | 25 | 20 | 24 | 25 |
| | 0.2 | 48 | 25 | 20 | 24 | 25 |

**Biotransformation assay**

Biotransformation of GA by *P. fluorescens* was performed in 150-mL Erlen Mayer flasks containing 50 ml nutrient broth medium (yeast extract 2 $gL^{-1}$, beef extract $gL^{-1}$, peptone 5 $gL^{-1}$, sodium chloride 5 $gL^{-1}$, pH 7). An inoculum of *P. fluorescens* and GA (each 25µl) was added to the nutrient medium and the bacteria were allowed to grow on an orbital shaker at 150 rpm and 37°C. Samples (5 ml) were aseptically taken from the cultures at regular intervals (24 h) for 8 days. Two controls were used: a media-only (without the inoculums and substrate), and bacterial control without substrate.

**Biotransformation products of GA**

The biotransformation products from the samples were extracted after removing the bacterial cells by centrifugation. The supernatant was extracted thrice with 25 ml of diethyl ether. Thus, the pooled extract was washed three times with distilled water (10 ml), dried over anhydrous sodium sulfate, and filtered through Whatman paper No. 1. The sample was evaporated to dryness and subjected to thin-layer chromatographic separation (TLC).[14] Samples and standards were loaded directly onto silica gel-G plates. The plates were developed in a solvent system consisting of toluene: ethyl acetate (96:4 v/v) at 4°C. The plates were then removed and dried at room temperature. Spots were visualized by exposing plates to iodine vapour. Identification of spots was done by comparing relative frontal (Rf) values of the standards used. Analysis of the biotransformation products was also performed by gas chromatography-mass spectrometry (GC-MS).

**Statistical analysis**

The mean and standard deviation of minimum inhibition zone (MIZ) diameter (mm) were calculated based on percent zone reduction in comparison to the control plate.

## RESULTS

**Biotransformation potential of microbes**

The results of the substrate toxicity tests are presented in Tables 1 and 2. Five of the ten bacterial strains *P. fluorescens* MTCC2421, *P. aeruginosa, S. mutans* MTCC497, S. *epidermidis* MTTC435P, and *E. coli* were found to be resistant while the remaining five *Shigella boydii* MTCC2408, *P. putida, Acinetobactor baumannii*, *Bacillus mycoides*, and *S. aureus* highly sensitive to all monoterpenes used. Four bacteria *P. fluorescens* MTCC2421, *P. aeruginosa, S. mutans* MTCC497, and *E. coli*, showed resistance to GA and LN at all concentrations 0.05-0.2%. Three bacteria including *P. fluorescens* MTCC2421 and *E. coli* and *S. epidermidis* MTTC435P showed resistance to both G and LL, whereas the other three *P. aeruginosa, S. mutans* MTCC497, and *E. coli* were found sensitive to G. Among all, only *S. epidermidis* MTTC435P was susceptible to GA

**Table 2.** Monoterpene-resistant behavior of the fungal strains

| Fungal Strains | Concen. (%) | Zone of inhibition (mm) | | | | |
|---|---|---|---|---|---|---|
| | | Geraniol | Geranyl Acetate | Citral | Linalool | Limonene |
| *Candida albicans* | 0.05 | 14 | No | 40 | No | 18 |
| | 0.1 | 20 | No | 50 | 15 | 25 |
| | 0.15 | 24 | 15 | 65 | 20 | 32 |
| | 0.2 | 30 | 15 | 75 | 20 | 38 |
| *Alternaria brassicicola* | 0.05 | 25 | 45 | 65 | 65 | 65 |
| | 0.1 | 33 | 54 | 70 | 76 | 78 |
| | 0.15 | 45 | 69 | 90 | 90 | 90 |
| | 0.2 | 60 | 80 | 90 | 90 | 90 |
| *Fusarium oxysporum* | 0.05 | 15 | No | 35 | 30 | 25 |
| | 0.1 | 20 | No | 45 | 42 | 25 |
| | 0.15 | 20 | 14 | 60 | 50 | 36 |
| | 0.2 | 20 | 14 | 80 | 80 | 42 |

and LN. Three other *P. fluorescens* MTCC2421, *P. aeruginosa, S. mutans* MTCC497 were highly sensitive to linalool. Interestingly, all bacterial strains were susceptible to CT at all concentrations from 0.05-0.2% but three of them namely *P. fluorescens* MTCC2421, *E. coli,* and *P. putida* were highly susceptible with the zone of inhibition values of 30-90 mm (Table 1). The toxicity assay revealed that all the fungal strains were very sensitive to all monoterpenes used; however, *C. albicans* and *F. oxysporum* showed little resistance to GA (0.05%) (Table 2).

**Analysis of biomass profiles**

Biomass of *P. fluorescens, P. aeruginosa,* and *S. mutans* accumulated in the media in the presence of G, GA, and LN was measured recording the absorbance at 660 nm and compared with the

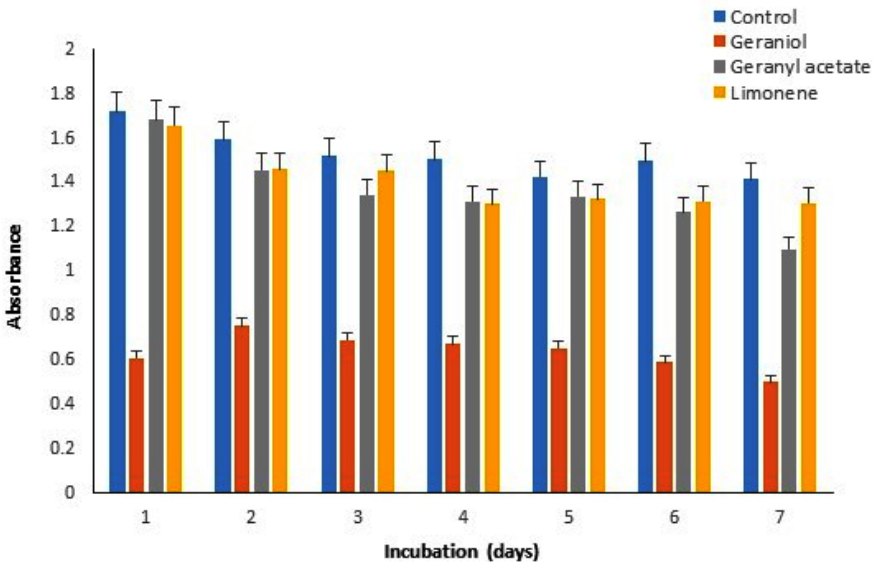


**Figure 1**. Effect of monoterpenes (0.05%) on the growth of *P. fluorescens*

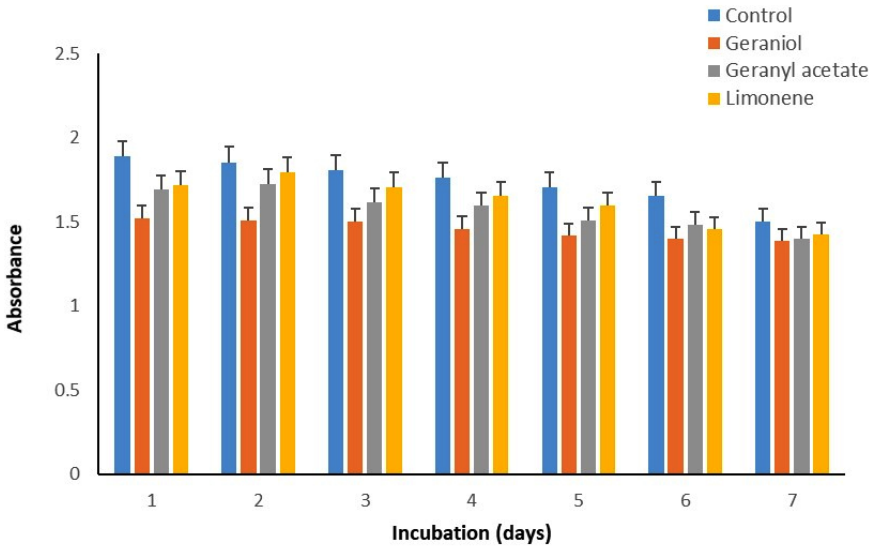


**Figure 2.** Effect of monoterpenes (0.05%) on the growth of *P. aeruginosa*

control (Figure 1-3). For *P. fluorescens* MTCC242, the biomass accumulated was highest at ~100% during 1-4 days in the presence of GA and LN, which slightly decreased by 4% in GA but increased by 13% in LN on day 7 as compared to the control (Table 3). However, the biomass decreased significantly from 35 to 56% in the presence of G during the incubation period of 1-7 days. We did not observe any biomass accumulation for *P. aeruginosa* during the incubation period in the presence of G (Table 2). Overall, the biomass of bacteria declined significantly by 50-100% in the presence of GA and decreased by a comparatively very low margin of 10% in the presence of LN. In

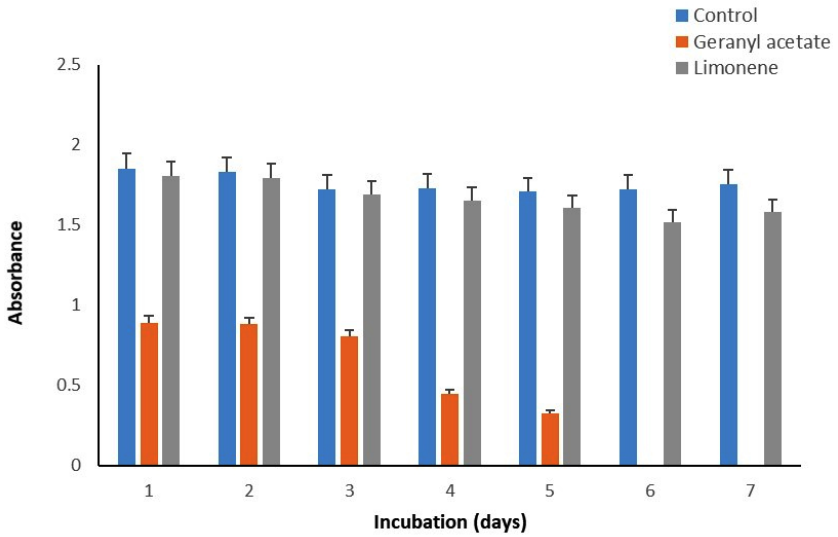


**Figure 3.** Effect of monoterpenes (0.05%) on the growth of *S. mutans*

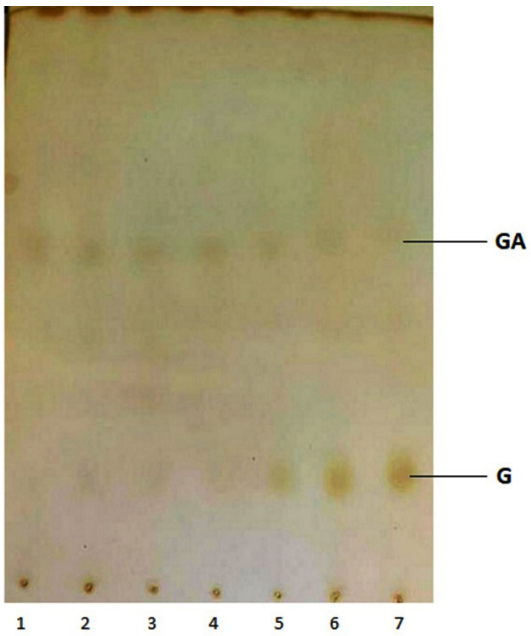


**Figure 4.** A thin-layer chromatogram of the biotransformation products of geranyl acetate. Lines 1-7 represent the incubation period from 1-7 days

the case of *S. mutans*, the biomass was highest at ~118% in the presence of GA and LN on day 7 compared to the control. The biomass, however, decreased from 10 to 21% in the presence of G and LN from 1-4 days of incubation and again increased by 100% and 118% at day 7 (Table 3). Thus, these results revealed that *P. fluorescens* MTCC242 and *S. mutans* are suitable for the biotransformation of GA and LN and *P. aeruginosa* for LN.

**Biotransformation of GA**

The main product of the biotransformation of GA by *P. fluorescens* was G (Figure 4). Geraniol was first detected on the day 5$^{th}$ of incubation, which was transformed into LL on the 7$^{th}$ day (Figure 4). In addition, some other products were produced, which could not be resolved on TLC, most likely they were hydrocarbons. The rate of biotransformation of GA varied with the incubation time. On the day 5$^{th}$, the biotransformation of GA using *P. fluorescens* produced 50% geraniol. The presence of GA and G was further confirmed by GC-MS (Figure 5).

## DISCUSSION

Microbial biotransformation is a fast-growing alternative method of chemical synthesis for the production of many human products such as flavors, fragrances, food additives, and more. This method relies on microbes (bacteria, fungi, and yeast) and their enzymes, which are capable of transforming selected compounds into desirable products with multiple benefits. Since the availability of potential microbes is the first essential requirement for any biotransformation, in the present study, we screened a few bacteria that can transform the monoterpenes G, GA, LL, and LN through a substrate toxicity assay.

Results of the toxicity assay revealed that microbes had varying degrees of tolerance to the selected monoterpenes used at concentrations

**Table 3.** Biomass (%) of monoterpene-resistant bacteria

| Treatment | Days | Biomass accumulation (%) | | |
|---|---|---|---|---|
| | | *P. fluorescens* MTCC 2421 | *P. aeruginosa* | *S. mutans* MTCC497 |
| Control | 1st | 100 | 100 | 100 |
| | 4th | 88 | 94 | 95 |
| | 7th | 82 | 94 | 79 |
| Geraniol | 1st | 64 | 00 | 79 |
| | 4th | 53 | 00 | 83 |
| | 7th | 44 | 00 | 100 |
| Geranyl acetate | 1st | 100 | 49 | 100 |
| | 4th | 100 | 28 | 99 |
| | 7th | 96 | 00 | 118 |
| Limonene | 1st | 100 | 89 | 89 |
| | 4th | 87 | 89 | 87 |
| | 7th | 113 | 100% | 118 |

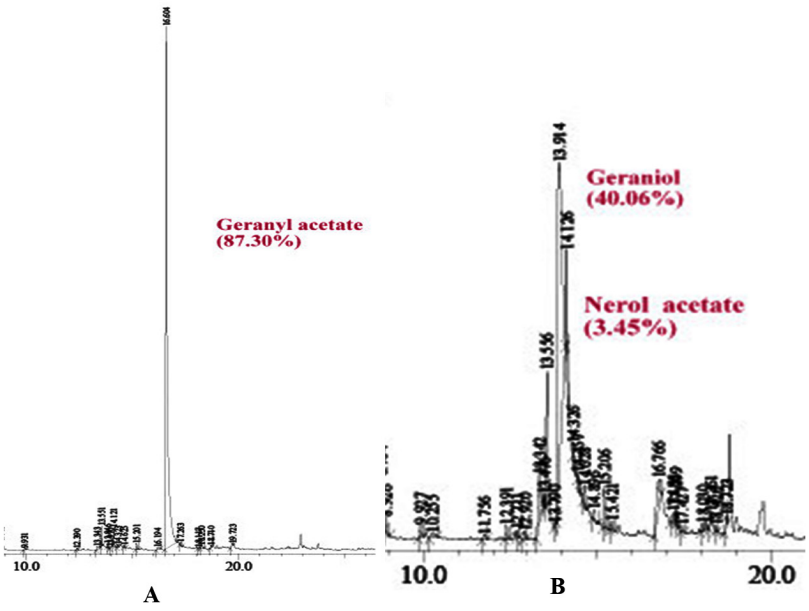


**Figure 5.** The GC-MS shows the biotransformation products of geranyl acetate on the day 1 (A) and day 5 (B) of incubation

from 0.05-0.2% (Tables 1 and 2). It was found that *P. fluorescens* and *S. epidermidis* MTTC 43 effectively tolerated G (0.2%), while others were unable to tolerate >0.05% G. The toxicity test indicated that *P. aeruginosa*, *P. fluorescens*, *S. mutans* MTCC497, and *E. coli* MTCC901 tolerated 0.05-0.2% GA while *S. boydii* MCC2408, *A. baumannii,* and *S. aureus* could survive only in the presence of 0.05% GA. Two bacterial strains *S. epidermidis* MTTC 435 and *E. coli* MTCC901 grew well in the presence of 0.05-0.2% LL, whereas the other four *P. aeruginosa, P. fluorescens, S. mutans* MTCC497, and *E. coli* MTCC901 in 0.05-0.2% LN. Fungal strains *C. albicans* and *F. oxysporum* were more sensitive and could survive only in 0.05-0.1% GA (Table 2). These results revealed a correlation between microbial growth and monoterpene concentrations. In general, microbes exhibited resistance to varying concentrations (0.05 to 0.2%) of monoterpenes. Hence, any of these concentrations may be used to perform the microbial biotransformation of the monoterpenes. Several previous studies have also suggested a concentration range of monoterpenes from 0.1 to 0.2% most suitable for their biotransformation by *Pseudomonas, Saccharomyces* spp. and *Penicillium, Aspergillus spp*.[13,15-17]

Although simple microbial resistance to monoterpenes with added carbon sources does not guarantee high biotransformation activity, it is an essential property of a biotransformation agent. Therefore, we performed initial physiological studies to characterize microbial growth behaviour in the presence of monoterpenes. Two bacteria *P. fluorescens* and *S. mutans* showed the best growth profiles in the presence of GA. The biomass content of these bacteria was almost equal to the control throughout the incubation of 1-7 days. However, the biomass of *P. aeruginosa* significantly declined by 51-100%. These results suggest the rapid consumption of GA in *P. fluorescens, S. mutans*, and *P. aeruginosa*, which are most likely to have substrate-degrading metabolic pathways.[15] The biomass is directly proportional to the growth rates, therefore, the higher the biomass the higher will be the growth. Here, the maximum microbial growth was recorded within the first two days of incubation compared to the control. Fungal growth was reduced only at the lower concentrations (0.05%) of GA on day 1 of incubation (Table 2).

Thus, the substrate toxicity test and biomass accumulation profiles suggest that *P. fluorescens* and *S. mutans* MTCC497, *P. aeruginosa* have the potential for biotransformation of GA and LN. Previous studies have reported that *P. fluorescens* and *P. putida* biotransformed limonene into limonene-1,2-oxide and perillyl alcohol.[18,19] In contrast, in the present study, *P. putida* was found to be sensitive to limonene. Besides the biotransformation of GA and LN, the biotransformation of geraniol can be carried out by *S. mutans* MTCC497 and *P. fluorescens*. Earlier, we reported an enzyme, geranyl acetate esterase (GAE) from lemongrass leaves that catalyzes the biotransformation of GA into G.[20] However, in the literature, no report is available on the biotransformation of GA by microbial enzymes. Here, we carried out the biotransformation of GA by *P. fluorescens* producing G, LL, and other products (Figures 4 and 5). This action of *P. fluorescens* can be attributed to homologous esterase and synthase enzymes. In accordance with a previous study, optimization of several parameters like the catalyst, reaction medium, stirring rate, molar ratio, and temperature is being carried out to improve the efficiency of the microbial biotransformation system.[21] Certainly, the outcomes of this study may be used to carry out the biotransformation of geranyl acetate, geraniol, and other monoterpenes to produce newer commercial aromatic derivatives.

## CONCLUSION

The present study revealed three potential bacteria *P. fluorescens, S. mutans*, and *P. aeruginosa* with an ability to biotransform GA, G, and LN. However, none of the fungi was found capable of biotransforming the selected monoterpenes. The biotransformation with various monoterpenes can be carried out utilizing particular bacteria in order to choose the finest strains beneficial for industrial applications. Thus, the current work underlines the importance of the screening of microorganisms as the first step in the biotransformation processes.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

## FUNDING

None.

## DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

## ETHICS STATEMENT

Not applicable.

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