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Bio Production of Aroma Compounds from Alpha Pinene by Novel Strains

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Abstract
The objective of the present work was to biotransform the terpenic substrate alpha pinene to produce valuable aromatic chemicals. By means of testing the ability of resistance to monoterpenic substrates limonene and alpha pinene, two microbial strains coded as 24B1 (bacterial strain) and 2WASP (fungal strain) were screened from nine microbial isolates isolated from decayed yellow orange citrus fruits (*Citrus reticulata*), collected from distant locations in Navi Mumbai. The ability of these strains to biotransform alpha pinene was examined and the products obtained were identified by GC-MS. The main biotransformation products identified with 24B1isolate were α - terpineol, verbenol, verbenone and sobrerol and the products identified with 2WASP are verbenol, verbenone and myrtenol.

1. Introduction

The flavour and fragrance industries have grown constantly with the growth in the world economy. Recent developments in biotechnology have enabled the production of natural flavourings economically and more resourcefully. Natural flavour compounds obtained by biotransformation tend to substitute the synthetic products due to the high potential offered by the biotransformation in producing new flavours with different applications in industries[1].Biotransformation has significant advantages over chemical reactions. In the last decade the impact of biotransformation is been increasing constantly since biocatalysts drawn consideration to a great extent from the viewpoint of green chemistry [2].Microbial whole cells have shown great potential for biotransformation as there is a large diversity of metabolic processes and enzymes involved. With more than 22,000 individual identified compounds, terpenes represent the largest group of natural flavour products. Terpenes are unsaturated hydrocarbons derived from isoprene unit. Biotransformation of terpenes represents a very attractive alternative for the production of natural aroma compounds [3].The chemical oxidation of terpenes for flavour synthesis tends to be gradually replaced by biotechnological methods and the rising quest for natural sources of aroma compounds is forcing an adaptation of the industrialized methodology.

α-Pinene is the most widely encountered terpenoid in nature extensively used in fragrances, flavourings and medicines. It is found in the essential oils of most coniferous trees and it is released as a byproduct of paper pulping from crude sulphate turpentine.

Alpha pinene is used as raw material for the synthesis of its oxygenated high-value products. Some of the major biotransformation products of alpha pinene are Verbenone, verbenol, sobrerol [4] pinocarveol, pinocarvone, myrtenol and myrtenal [5].

d- Verbenone & verbenol are used as insect repellant, in perfumery, aromatherapy and as food flavouring [6]. Highly pure sobrerol-d is used as a balsamic and a respiratory analeptic [7]. Terpineol has a pleasant odor similar to lilac and is a common ingredient in perfumes, cosmetics, and flavors. Myrtenal has spicy and woody flavours.

Terpenoids may be incorporated in the formulation of foods, cosmetics, herbal remedies, and household products as flavourings [8] and antibacterial agents. Many terpenes have biological activities and are used for the treatment of human diseases [9].

In this perspective, the significance of such compounds is constantly growing. In view of the huge applications of the terpene compounds and the scope to research for novel sources of alpha pinene biotransforming microbial isolates the present study has been carried out to biotransform alphapinene to produce valuable aromatic chemicals. As citrus fruits are rich source of terpenes where it is supposed to have strains more adapted to terpenes-containing environment [10] the decayed yellow orange citrus fruits were used to isolate the novel strains for the production of aromas from alpha pinene. In this prospect, the selected strains might be evaluated to improve their biotransformation competence in producing other valuable aromatic chemicals from terpenes.

Various studies on alpha pinene degradations have been carried out [11, 12, 13].Various efforts have been implemented using diverse microorganisms to produce valuable aromatic chemicals using alpha pinene as substrate and also to scale up the process from laboratory to industrial level [14].

2. Materials and Methods

Potato-dextrose agar, Eosin methylene blue agar, potato-dextrose broth (PDB) and other medium components were purchased from Hi Media Laboratories Pvt Ltd. α-pinene (>98%, Sigma Aldrich) is used as substrate. The reagents were used without any Pre-treatment. All other chemicals or solvents were of analytical grade.

2.1. Screening Experiments

Nine isolated strains from decayed citrus fruits were tested in terms of growth on limonene and α - pinene as sole carbon source. Among the isolated strains, one bacterial strain (24B1) and one fungal strain (2WASP) presented positive results [15] and were submitted to biotransformation reactions in orbital shaker.

2.2. Biotransformation with 24 B1 Isolate

A loop full of bacterial culture was transferred aseptically to Erlenmeyer flasks containing 40 ml of PDB and incubated at 30ºC at 275rpm for three days. After reactivation of the culture, 1ml of broth culture was inoculated into 500ml of the culture medium and incubated aerobically in orbital shaker at 30°C and 275 rpm for period of 72 h. After the abundant growth of the culture, the entire culture was centrifuged at 8,000 rpm for 30min and the supernatant was withdrawn. Sterile distilled water was added to a final volume of 300ml. Flasks were then stirred to resuspend the cells and centrifuged again. Supernatant was discarded and precipitated cells of 2.2g were transferred to an Erlenmeyer flasks containing 30ml of mineral medium with composition $(NH4)_2SO_4$: 5.00g/L; KH_2PO_4 : 0.9g/L; NaCl: 0.50g/L; $MgSO_4.7H_2O$: 0.40g/L; $CaCl_2$: 0.60g/L; KCl: 2.15g/L; $FeSO_4.7H_2O$: 0.01g/L; $ZnSO_4$:0.01g/L; $CuSO_4$: 0.01g/L; $NaNO_3$: 3.6g/L) of pH 7 with 150µl of α-pinene. Flasks were kept in orbital shaker at 30°C and 275 rpm for 6 days. The samples were analysed for six days to monitor the period of products formation.

All experiments were carried out in parallel with controls, in the same conditions without the presence of microorganism. The product recovery was performed by liquid – liquid extraction with ethyl acetate. The final solution was dried over anhydrous sodium sulphate. The concentrated sample was analysed using GC-MS.

2.3. Biotransformation with 2WASP Isolate

Methodology is based on Agrawal & Joseph [16]. Culture was grown in PDB, using an active inoculum with an initial optical density of 1 at 660 nm for a period of 18h on a rotary shaker at 30°C and 275 rpm at an initial pH of 7. Biomass was produced by filtration through whatman no 1 filter paper using a Buchner funnel.

Mycelia biomass of 2.2gms wet weight was added to 30ml of phosphate buffer (1M of pH7) with 150µl of alpha Pinene and the mixture was incubated for 8 hrs (16&20) at 30°C. The experiment was carried out in parallel with control, in the same conditions without the presence of microorganism. Products extraction was carried out in separating funnel with dichloromethane. After three extractions the sample was dried over dried anhydrous sodium sulphate. The concentrated sample was analysed using GC-MS.

2.4. Product Analysis

The reaction products were identified by (GC-MS, Agilent 5975C), using a capillary column HP5 (30m×0.32mm). The column temperature was programmed to 50°C for 3min, increased at 5°C/min at 130°C and then increased at 15°C/min at 210°C by 5 min. Helium as the carrier gas, the injection and detector temperatures were at 250°C. The dried solution of 1µl was injected into the GC/MS system. The apparatus operated with a flow rate of 1 ml/min in electronic impact mode of 70eV and in split mode. The identification of the compounds was accomplished by comparing the mass spectra from the NIST library and by additional comparison

of the GC retention time of standard compounds and GC-MS fragmentation pattern.

3. Results & Discussion

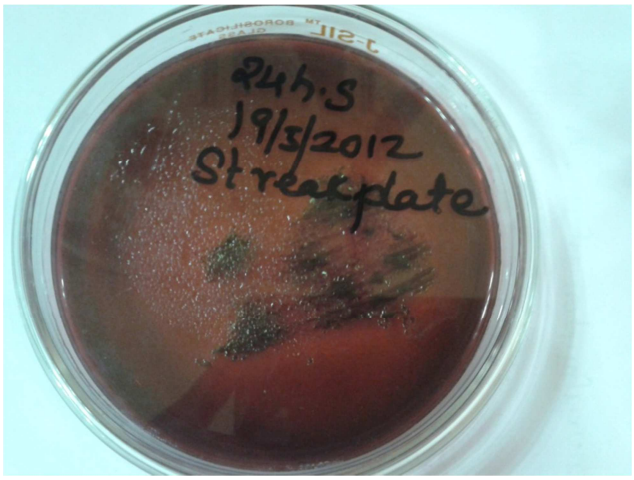

Fig. 1. *Green metallic sheet colonies of 24B1 on Eosin methylene blue agar -*

Fig. 2. *Green velvety growth of 2WASP on Potato dextrose agar*

Among the isolated strains, 24B1 (fig: 1) and 2WASP (fig: 2, identified as *Aspergillus niger* isolates were found to have shown good potential in carbon source study (15) and biotransformation study.

3.1. Biotransformation Products of 24B1 Isolate

In order to study the period of products formation with 24B1 culture which is an unknown strain, the results were analysed for six days.The aroma compounds detected on biotransformation of α-pinene with 24B1 culture were of great significance. From Fig:3 the biotransformation products detected with their respective retention times were verbenol (14.5), isoborneol (15.05), P- menthe - 5 diene-8-ol (15.25),α-terpineol(15.987), verbenone (16.6), and trans sobrerol (21.032). It is worth noting that verbenone, verbenol and sobrerol were detected in 24hrs of incubation. It was observed that there is a reduction in α- pinene concentration in 24hrs and the products still remained even after six days of incubation. The microbe free control experiments also accumulated verbenone, verbenol and sobrerol with undegraded α-pinene. Biotransformation of α - pinene is reported in some studies with varying microorganisms isolated from different sources. Wright *et al*. [17] used a bacterial strain *Serratia marcescens* isolated from sewage sludge, which oxidized the terpene hydrocarbon α-pinene to produce trans-verbenol as the major product with Verbenone and trans-sobrerol as minor products in 24hrs. The bioproduction of α-terpineol as main product has also been reported using α and β-pinenes as substrate [18, 19].

3.2. Biotransformation Products of 2WASP (*Aspergillus niger*) Isolate

From (fig:4) on biotransformation of α - pinene with 2WASP, the products obtained with respective retention times were, Verbenol (13.690), myrtenol (15.592), verbenone, (16.094) and 1,2-Ethanediol,1,2-dimyrtenyl (22.120) in eight hours of incubation at 30°C. No auto oxidation products were detected in control conducted without organism.The experiment is set for 8hrs incubation to obtain the comparative results with the work done by Prema and Bhattacharya [20] in which *Aspergillus niger* strain isolated from agarwood metabolised α-pinene in eight hours of incubation produced-verbenone, cis-verbenol, and trans-sobrerol at 28°C and Agrawal & Joseph (16) where resting cells of a locally isolated strain of *Aspergillus niger* was used to produce verbenone from α - pinene in 6hrs of incubation at 30°C.

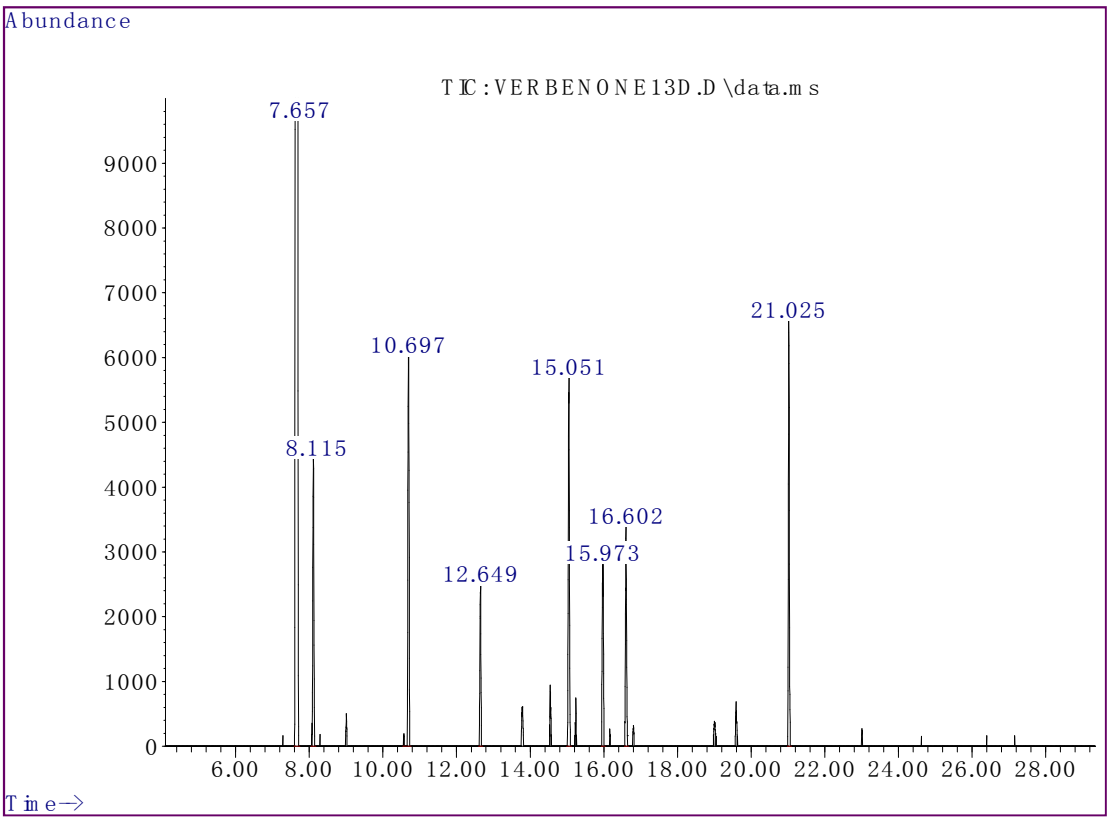

Fig. 3. *GC-MS generated Chromatogram of extracted sample of 24 B1 biotransformation broth*

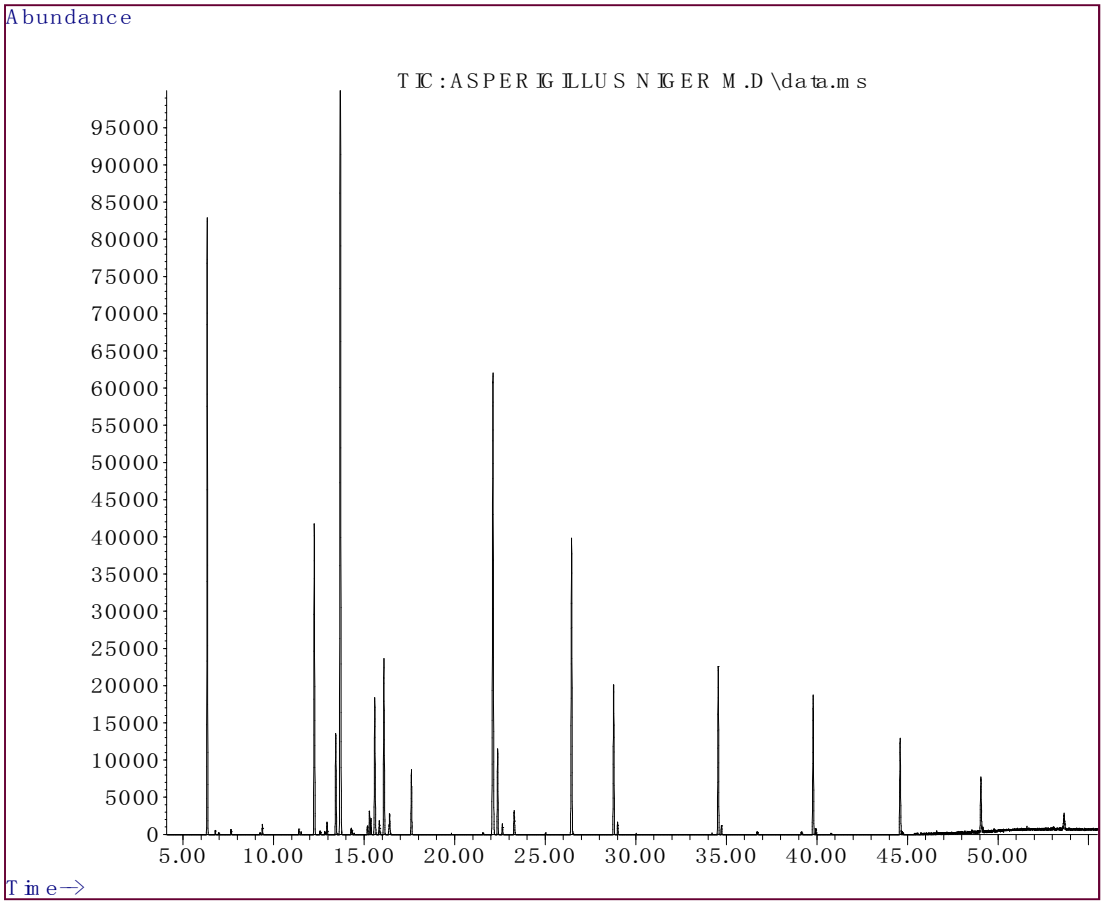

Fig. 4.*GC-MS generated Chromatogram of extracted sample of 2WASP biotransformation broth*

4. Conclusion

The isolated strains 24B1 and 2WASP showed good potential in biotransformation study. Interesting compounds such as α- terpineol, verbenol, verbenone and sobrerol, were produced from 24B1 isolate, verbenol, verbenone and myrtenol were produced from 2WASP. This work presents a very promising step towards a future industrial application of microorganisms for the biotransformation of the abundantly available natural monoterpene α- pinene to produce valuable aromatic products. The selected best isolates were further subjected to biochemical tests and RNA sequencing for characterisation and perform the optimization studies for the better yield and to scaleup the process.

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