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Isolation, Selection and Fermentation of D-Limonene Biotransformed Microorganisms

KEYWORDS: Limonene, perillyl alchol, biotransformation, flavor

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ABSTRACT *D-limonene biotransformation emerges as an attractive compared to traditional chemical methods, they proceed under mild conditions, have a prominent regio and enantio selectivity, and do not produce toxic wastes and the products obtained might be labeled as natural. The present studies involved in the isolation of microorganisms that are resistant to environment containing limonene and are able to biotransform limonene to perillyl alchol as one of reaction products. Perillyl alcohol has anticancer property against lung, liver and mammary carcinogenesis. For the isolation and screening, samples collected from specific areas of citrus processing plant (citrus fruit processing tank, machine traps, plant floor, soils at waste dumping area) and samples collected from petrol, diesel contaminated area. The samples were incubated at 30^{0}C at 150 rpm for 48h or 7 days in YM media containing 0.1% limonene for isolation and screening purpose. Two strains were isolated from petroleum soil NPII and processing tank (ST) respectively. Thin layer chromatographic analysis of reaction products from two samples (ST, S-NPII) showed that d-limonene was biotransformed in to various reaction products may be further analyzed by techniques such as GC-MS and NMR studies to detect the products.*

INTRODUCTION:

Bioactive monoterpenoid is the most abundant in nature and represent more than 90% orange peel oil [1].D-limonene is cheap starting substrate available in citrus processing plants as a waste stream [2].D-limonene is used in flavor industry as precursor in synthesis of aroma compounds [3]. Including their aromatic property its oxygenated derivative such as perillyl alchol,carveol,carvone,geranioland menthol have shown anticancer activity[4] .Most of these terpenoids are considered as Generally recognized as safe, obtained from natural source, can be added to foods[5]. Problems encountered in monoterpene bio transformations are i).chemical instability of monoterpenes.ii). Low solubility of monoterpenes iii).volatility of monoterpenes.iv).Toxicity of monoterpenes v).Low product concentrations and product yield.vi).long incubation times. In the last years the solvent resistant microorganism screening is increasing. Isolation of solvent resist resistant microorganisms might bring several benefits to the industries [6]. The present study involves the isolation, screening of microorganisms resistant to d-limonene and its use as sole carbon source which is the characteristic feature of biotransforming agent.

MATERIALS AND METHODS:

Materials: Limonene procured from Hi-media labs, Perillyl alcohol from Sigma Aldrich Ltd&Organic solvents from Sd-Fine chemicals of analytical grade.

Samples were collected in a citrus processing plant (Cifal herbal private limited, gudur, A.P, INDIA), where it is assumed to have strains more adapted to limonene-containing environment. Samples were collected at different locations i.e from processing tank (ST), soil collected at waste dumping area(S, SM), leakage areas of the tank(S-TL), and petroleum soil(S-NPI, II) collected at Nizamabad, telangana, India.

Composition of YM medium: gm/100ml distilled water (glucose-1,peptone-0.5,malt extract-0.3, yeast extract-0.3, pH not adjusted).

Composition of mineral salt media-(in g/L: (NH4)2SO4 = 5.00; (NH4)2HPO4 = 1.42; NaCl = 0.50; MgSO4.7H2O = 0.40; CaCl2 = 0.60; KCl = 2.15; FeSO4.7H2O = 0.01; ZnSO4 = 0.01; CuSO4 = 0.01; pH not adjusted).

Methods:

Isolation:

Each sample collected above was transferred to 150 ml Erlenmeyer flasks containing 50 ml of sterile distilled water and kept for incubation for 24 hrs.After that a loopful of inoculums transferred into 150 ml of Erlenmeyer flasks containing 50 ml YM medium and 50 µL (0.1%, v/v) of *d*-limonene. After 48h or 7 days of cultivation in a rotary shaker at 30ºC/150 rpm, a loop of each flask was transferred to Petri dishes containing YM medium(2gm/100ml agar), following the streak plate method. The Petri dishes were incubated at 30ºC until complete colonies growth, limited to 7 days. Each different colony appeared, these were maintained on 18ml tubes with YM slant medium.

Selection:

One loop of each isolated strain was transferred to a150 ml Erlenmeyer flask containing 10 ml of sterile YM medium and200 µl (2%, v/v) of *d*-limonene. The flasks were inoculated and shaken on a rotary shaker (30ºC/150 rpm) for 48h. After this period, a 0.1µl sample of the culture broth was transferred to a Petri dish (YM medium) and homogenized with 'L' shaped rod .The culture growth was evaluated after 48h at 30ºC and all the strains that presented a satisfactory growth were considered resistant to 2ml of limonene in 100 ml (v/v) of medium.

Screening process: In the screening process all the isolated colonies considered resistant were evaluated as utilization of limonene as sole carbon source. Thus, one 100 µl aliquot of the culture broth from the last test was inoculated in a 150 ml Erlenmeyer flask containing 10 ml of mineral salt medium and 0.1 ml of (1%) d-limonene. After 48h incubation at 30ºC and 150 rpm, a 100 µl sample of the culture medium was homogenized in a Petri dish (YM medium) as already described. All the colonies that presented a satisfactory growth after 48h at 30ºC were con-

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sidered as probable users of *d*-limonene as sole carbon source.

Fig: 1.Isolate S-T in YM-medium

Fig: 2.Isolate S-NP II in YM medium

Fermentation process*:* A loopful culture of each strain S-NPII, S-T were transferred into 250ml Erlenmeyer flasks containing each 100ml of YM-media &cultured on a shaker for 24-48hrs at 150rpm.After 24 hrs of the growth 0.2 %(v/v) limonene was added to the broth as substrate under sterile conditions. The flasks were shaken at 30^{0}C for 5 days. The biotransformed products are collected by centrifugation at 10,000 rpm at 4^{0}C for 8 min.The biomass were discarded and supernatant was extracted with ethyl acetate The organic extract were combined and washed with distilled water for (3x10ml)dried over anhydrous sodium sulfate filtered using what man No.1 filter paper. The solvent was concentrated under reduced pressure to get crude reaction products [7].

Analysis of products: Preliminary analysis of the reaction products was carried out by TLC on pre coated aluminum plates. Plates were placed in the solvent system N-Hexane: Diethyl ether (50:50, v/v) in the developing chamber. The plates were dried and visualized by iodine absorption [7]

RESULTS: The two incubation periods were maintained for the isolation, 48 hrs and 7 days for favorable growth and recovery of bacterial and fungal strains respectively in YM agar medium containing the substrate limonene (0.1%). Two bacterial strains (Gram positive and gram negative bacteria)and two fungal strains were observed after 48hrs and 7 days of incubation. These isolated colonies were inoculated into YM medium containing 2% of limonene. These four strains were not showed satisfactory number colony forming units when plated on YM agar plate. This study revealed that the isolated strains were not able to biotransform d-limonene which were not used for further study. Two strains were isolated from petroleum soil (NPII) and processing tank (ST) respectively (Fig 1&Fig 2). These strains showed satisfactory number colony forming units when plated on YM agar plate after their growth in YM Medium containing 0.1%, 2% limonene and mineral salt medium containing 1% d-limonene. This revealed that the isolated strains were able to biotransform D-limonene which were used for further study.

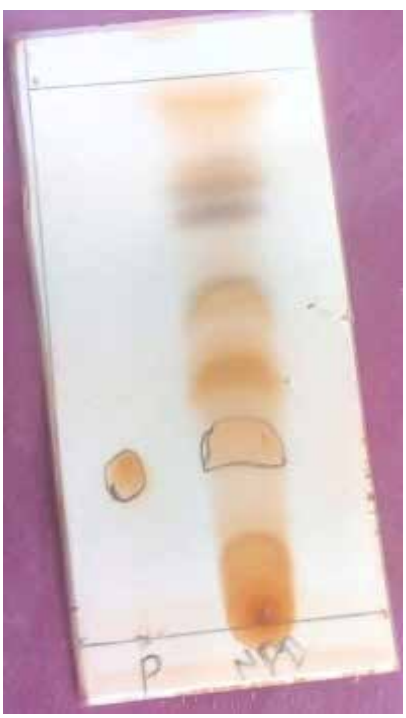

Fig 3. TLC of S-NP II

Thin layer chromatography: TLC studies once again proved that the strains(S-NPII and S-T) were able to transform d-limonene to mixture of biotranformed products. Among which one of product shown to be perillyl alcohol, i.e. an anticancer compound compared with the reference standard (Fig 3).

DISCUSSION:

For the isolation of bacteria 48h incubation and for fungi 7 days incubation period, were chosen to favorable growth and recovery. The more number of days might favor the growth of stressed and sporulated microorganisms or those with a low growth rate. But some microorganisms might enter in the death phase, which might difficult their isolation. The taxonomic position of a strain is related to its enzymatic activity on terpenoid hydroxylation reported by Abrhamin his study [1].Substrate resistance does not promise a high biotransformation activity, but it is a necessary feature to a biotransforming agent. Generally, the minimum inhibitory concentration (MIC) of limonene towards some bacteria and yeasts are lower than 2% [8], but some fungi, e.g. Penicillium digitatum, presents only a small reduction on its bioconversion activity in concentrations of 4-8% limonene [9].Some bacteria, e.g. Pseudomonas sp., might have no growth inhibition in concentrations of up to 10% α- or β-pinene [10].In biotransformation processes limonene concentration used vary

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from 0.2 to 1% however 0.2% limonene is the optimum concentration for its biotransformation to perillyl alcohol and p-ment-1-ene-6,8-diol using Pseudomonas putida [7] and is toxic to Bacillus stearothermophilus [11].In this study the isolated strains are belongs to Gram positive, gram negative and fungi were observed.

CONCLUSION: Thin layer chromatographic analysis of reaction products from two samples (ST, S-NPII) showed that d-limonene was biotransformed in to various reaction products further to be analyzed by techniques such as GC-MS and NMR studies to detect the products.

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Conflict of Interest: There is no conflict of interest by authors

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