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Isolation and Screening of Terpene Resistant Microorganisms from Terpene Processing Plant

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Abstract: ***The objective of the current study was to isolate and screen strains with potential to tolerate high concentration of terpene substrates. Microorganisms were selectively isolated from soil, liquid and swab samples collected from strategic places (effluent, spillage, and pump body and plant floor) of two different units of terpene processing plant of Privi Organics India Limited, geographically located in India at Mahad, district Raigad, Maharashtra. Thirty seven bacterial and three fungal isolates were obtained from the samples collected from different locations. All the isolates were screened based on their ability to grow in presence of particular terpene substrates like alpha pinene, limonene, dihydromyrcene and citronellol. The capable isolates were further screened for their tolerance towards high concentrations of these substrates. Each isolate was found to be resistant in all terpene substrates at variable terpene concentrations. Subsequently, fourteen strains were selected based on their terpene resistance capacity and growth factor in presence of 10% (v/v) of terpene concentration. This is the highest reported concentration so far in literature.***

Keywords: Isolation, Terpenes, Tolerance, Screening, Biotransformation

1. Introduction

Aroma components in fast moving consumer goods (FMCG) and food products have high demand in the world market [1]. Traditionally, all these fragrance chemicals were obtained from natural sources. Eventually, after elucidation of their structure, synthetic compounds were produced by chemical synthesis. Chemical synthesis often results in formation of undesirable racemic mixtures, leading to overall costly and non-eco-friendly process. Presently, most of these flavour and fragrance compounds are produced via chemical synthesis or extraction from natural materials [2].

The global flavors & fragrances market size was valued at USD 18.6 billion in 2015 and is expected to grow at a compound annual growth rate of 4.5 % for 2016-2025 [3]. Natural flavors and fragrances market is supposed to emerge as the fastest growing product segment. Growing demand for this segment is supported by a rapid shift towards natural products, especially in developed countries. Reserves of these materials have diminished due to economic and conservation factors as well as industrial growth. The use of microbial catalysts may offer a substitute method for producing natural flavour and fragrance [4].

The use of microbial biosynthesis or bioconversion offers significant advantages over traditional extraction of plant materials which include perennial production, increased product specificity, and the use of cheaper raw-materials as substrates for the cultivation of these microbes [5].

With more than 22,000 individual identified compounds, terpenes represent the largest group of natural products [6]. Monoterpenes in plants produced as secondary metabolites are known to have ecological roles as antifungal defenses, pollinator attractants and also restrains herbivore attacks [7].

Terpenes have grabbed market attention because of their activity as natural insecticides and antimicrobial agents, as building blocks for the synthesis of many highly valuable compounds and their supposed roles in prevention and therapy of several diseases. Monoterpenes are phasing out the ozone-depleting chlorofluorocarbons in industries [8]. Terpene-based cleaner can also be used to clean/degrease metal parts without adversely affecting the performance of the plasma-arc coating application [9].

The most abundant bicyclic monoterpene is pinene with the isomers alpha-pinene and beta-pinene ($C_{10}H_{16}$), a main constituent of wood resins (e.g. conifers). Limonene ($C_{10}H_{16}$) is the most abundant monocyclic monoterpene [10]. It represents the main component of essential oils from citrus plants, e.g. lemon and orange. Citronellol, or dihydrogeraniol, is a natural acyclic monoterpenoid. Both enantiomers occur in nature. (+) Citronellol, which is found in citronella oils, e.g. citronella grass (*Cymbopogonnardus*), whereas (−) Citronellol is found in the oils of rose (18–55%) and geranium.

Biotransformation of terpenes represents a very attractive alternative for the production of aromas, because it takes place under mild conditions, is environment friendly and is deemed “natural” and hence can be used as fragrances and flavors in the industry [11]. Some derivatives of limonene are α-terpineol, perillyl alcohol, carveol, carvone and menthol. Some biotransformation products of alpha-pinene are verbenol, alpha-terpineol, pinocarveol and myrtenol. Pinocarveol, pinocamphone and myrtenol are some other compounds obtained by the biotransformation of beta-pinene [12]. Thus, many interesting aroma compounds could be derived from the terpenes. However, many of the pathways and enzymes involved in the biotransformation are not properly elucidated and there are many chemical reactions for which there are no corresponding biological conversions [13].

The main setbacks in the biotransformation processes are the chemical instability, high volatility and low solubility

of the precursor and product, high cytotoxicity of the compounds towards microbial cells and low biotransformation rates [14].

The interest in terpene-tolerant microorganism screening is increasing in the last years. A focused and systematic terpene resistant strain screening might surpass the cytotoxicity problems [15]. Also, it will enable innovations in the field of fermentation and might bring several benefits to industries [16].

Researchers have tried to isolate such resistant strains from habitat that are exposed to high terpene concentrations at regular basis such as waste of citrus processing plants [17], parts of pine trees [18], decayed citrus fruit and vegetable from storage facilities and markets or fruit juice factories. [19], [20]. In this paper we describe the isolation and screening of terpene resistant microorganisms from the surroundings of aroma chemical terpene processing plant.

Privi Organics India Limited (hereafter referred as POIL) is equipped with two state-of-the-art manufacturing units (Unit-I & Unit-II) that are located in the MIDC area of Mahad, Raigad district in Maharashtra. These units together have a total production capacity of 9,000 TPA, which is the highest among any aroma chemical producer in India for last 20 years. Significantly, both the plants have dedicated facilities for continuous production and processing of all the key terpene products. The environment around the plants is continuously exposed to higher concentrations of aroma components especially terpenes.

Microorganisms were selectively isolated from soil, liquid and swab samples collected from strategic places such as effluent collection pump, spillages in and around plant, material transfer pump and floors of terpene processing plant at Privi production facility. The selected strains will be evaluated for their tolerance capacity against different terpene chemicals and will be used to check the biotransformation capability with different terpene substrates.

2. Materials and methods

2.1 Materials

D-limonene is procured from Loba Chemie while alpha pinene, citronellol and dihydromercene as standards of analytical grade were obtained from POIL factory. Dehydrated media such as nutrient broth, potato dextrose broth and agar were bought from Himedia Laboratories Pvt. Ltd. Tween 80 of biological grade was also bought from Himedia Laboratories Pvt. Ltd. Water used for experiments was of high purity grade from a Millipore ultrapure water system. Membrane filters of 0.2 micron size were procured from Sartorius Stedium.

2.2. Methods

2.2.1. Microbial Sample collection

Samples were collected from various locations of POIL factory situated in Mahad region of Maharashtra, India. Sample collection areas were selected based on the probability of obtaining potential microorganisms, such as process liquid spillage area, liquid transfer pump body, floor of different terpene processing plants such as alpha-terpineol plant, crude sulfated turpentine oil processing (CST) plant, limonene pump from CST plant, dihydromercinol (DHMOL) plant and citral plant. All the defined locations are constantly exposed to higher concentration of different terpenes for years and hence the microorganisms isolated from such areas are more likely adapted to survive under high concentration of terpene conditions. Such isolates may have promising potential in the field of terpene fermentation and/or biotransformation. Samples of soil and liquid were collected from plant surroundings and pumps in Ziploc bags and plastic tubes respectively. Samples in form of swab were also collected in plastic tubes by swabbing the surface of pumps where soil was not available. All the samples were stored in refrigerator until further processing.

2.2.2. Enrichment of soil samples

All the soil samples were enriched for microbial load by inoculating 1 gm of soil in 100 ml of nutrient broth containing respective terpenes at 2% (v/v) concentration and incubated at 30˚C and 200 RPM for 4 days. Table 1 shows types of samples collected from various plant area and specific terpenes which were used during enrichment.

Table 1: Summery of Samples and terpenes used for study

Sr. no.	Sample collection location	Type of samples	Terpenes exposed (2%)
1	CST plant	Soil and swab	Alpha pinene (AP)
2	Citral plant	Soil, liquid, swab	Citronellol (CN)
3	DHMOL plant	Soil and liquid	Dihydromercene (DHM)
4	CST plant (Limonene pump)	Soil, liquid, swab	Limonene (LM)
5	Alpha terpineol plant	soil	Limonene (LM)

2.2.3. Isolation of microorganisms from sample

Terpene resistant microorganisms were isolated from various samples by serial dilution method.

Soil samples: 1 ml enriched broth from each flask was diluted in 9 ml sterile saline and further serially diluted up to 10^{10} dilution. Last 3 dilutions were plated on sterile nutrient agar plates and potato dextrose agar plates.
Liquid samples: 1 ml liquid sample from each tube was serially diluted up to 10^{10} dilution. Last 3 dilutions were plated on sterile nutrient agar plates and potato dextrose agar plates.
Swab samples: Cotton swabs were directly spread on sterile nutrient agar plates and potato dextrose agar plates.
All the plates were incubated at 30˚C for 72 hours. Morphologically distinct & isolated colonies of bacteria and fungi were selected from plates and streaked on sterile nutrient agar plates and potato dextrose agar plates respectively so as to obtain pure colonies. These pure colonies were stored at 4°C for further use. All the isolates were coded to facilitate their future identification. They were further assessed for their morphology and gram nature and also evaluated for terpene tolerance.

2.2.4. Screening of terpene resistant microorganisms

Microbial strains isolated from different locations were tested for their resistance against terpenes. All the test

isolates were separately grown in presence of different terpenes at variable concentration ranging from low (2%) to high (10%). Various terpene substrates used for screening are as listed in table 1. Each bacterial isolate was grown in 50 ml of sterile nutrient broth containing specific terpene substrates starting with 2% concentration. Isolates which could grow in presence of 2% terpene concentration were further tested at 4% terpene substrate concentration using similar procedure and the study was carried forward with all such resistant isolates till 10% of maximum terpene substrate concentration. For fungal isolates, potato dextrose broth was used as growth medium instead of nutrient broth. Tween 80 was added in the broth as an emulsifying agent [21] so that microbial cells will be uniformly exposed to the given concentration of terpenes. All these flasks were incubated on rotary orbital shaker, at 30°C and 200 RPM for 4 days. The tolerance of microbial cells in given experimental terpene concentration was checked by spread plate method. 1 ml of broth sample from each flask was spread separately on sterile nutrient agar or potato dextrose agar plate by spread plate technique and incubated at 30˚C for 48-72 hours.

2.2.5. Morphological characterization

Isolates were selected on the basis of their capability to grow in presence of up to 10% terpene concentration and further studied for their morphological characteristics. The selected isolates were grown on nutrient agar plates and colony characteristics such as size, color, shape, margin, consistency, opacity were recorded after 48 hours of incubation. Gram staining and motility tests were also done for all the isolates.

3. Results and Discussion

3.1. Enrichment and isolation of microorganism from different samples

Soil is considered as a dynamic system as it inhabits several diverse groups of microorganisms. A large population of soil micro flora is in dormant state due to unfavorable conditions in the soil environment. Enrichment of soil samples (collected from terpene exposed locations in processing plant) into the broth containing respective terpene substrate at 2% concentration allows only desired soil micro flora i.e. terpene tolerant microorganism to flourish. Liquid and swab samples were not enriched as there are less chances of common micro flora to inhabit in such terpene enriched locations. In this study, a total of 37 bacterial and 3 fungal strains were isolated from soil, liquid and swab samples. All the isolates were coded according to the plant location from which they were isolated. (Refer Table 2a-2d).

3.2. Screening of terpene resistant microorganisms

Screening of terpene resistant strains from total of 40 isolates was done by gradually exposing the strains to increasing concentration (2%-10%) of specific terpene based on the location of samples they were isolated from. During this study, uniform distribution and availability of terpene substrates to microbial cells was insured by using emulsifying agent Tween 80 as it gives complete homogenous mixture of two insoluble compounds. Degree of emulsion formed with different substrates varied according to the nature of terpene substrates. Proper homogeneous emulsion was formed in broth containing citronellol whereas this was not observed in other substrates (alpha pinene, DHM and limonene). This effect was due to relatively higher hydrophilic nature of citronellol. Due to this effect substrate loss in flasks containing citronellol, during fermentation, is expected to be lower than that of the other substrates. Terpene tolerance of all the isolates with respect to the substrates is described in tables below. Tolerance was evaluated on the basis of growth of isolates on solid medium after exposing them to variable terpene concentration.

Table 2a: Primary screening of isolates for limonene tolerance

Sr no.	Isolates	Growth in increasing concentration of limonene				
		2%	4%	6%	8%	10%
1	LM/L/1.1	+	+	+	+	+
2	LM/L/1.2	+	-	-	-	-
3	LM/SW/1.1	+	+	+	+	+
4	LM/SW/1.2	+	+	+	+	+
5	LM/SW/1.3	+	+	+	+	+
6	LM/SW/1.4	+	+	+	+	+
7	LM/SW/1.5	-	-	-	-	-
8	LM/SW/1.6	-	-	-	-	-

Keys: +: Growth; -: No growth; L: Liquid; SW: Swab

Table 2a shows the effect of various concentrations of limonene on isolates. Limonene is considered to accumulate in the microbial plasma membrane and thus cause a loss of membrane integrity and dissipation of proton motive force. This fact makes it very difficult for microorganisms to survive in presence of limonene. In this study, out of 8 strains isolated from limonene plant sample, five bacterial strains were found to have tolerance for up to 10% limonene concentration. Since these strains were isolated from limonene exposed areas, they tend to have adapted to limonene by some mechanism such as alterations in membrane structure. Two Fungal isolates could not grow when limonene was added in the medium during inoculation. But these strains could survive only up to 2% limonene concentration when added after 48 hours of growth. Pigmentation property of isolate LM/SW/1.4 was lost after exposing it to more than 8% limonene concentration. Color of colonies changed from yellow to off-white.

Table 2b: Primary screening of isolates for citronellol tolerance

Sr no.	Isolates	Growth in increasing concentration of citronellol				
		2%	4%	6%	8%	10%
1	CN/S/1.1	+	-	-	-	-
2	CN/S/1.2	+	+	+	+	+
3	CN/S/1.3	+	+	-	-	-
4	CN/S/2.1	+	-	-	-	-
5	CN/S/2.2	+	-	-	-	-
6	CN/L/1.1	+	+	-	-	-
7	CN/L/1.2	+	+	-	-	-
8	CN/L/2.1	-	-	-	-	-
9	CN/L/2.2	+	+	+	+	+

Keys: +: Growth; -: No growth; S: Solid; L: Liquid

Table 2b shows the effect of concentrations of citronellol on isolates. *Domenico Trombetta* [22] states that some terpene alcohol penetrates the cell membrane resulting in the ion

leakage and interference with metabolic activities which ultimately leads to cell death. In this study, a total of 9 isolates were exposed to higher concentration of citronellol and out of those only 2 were able to survive at 10% concentration. From five terpenes; citronellol has higher hydrophilicity than other molecules which facilitates the exposure of this molecule to cells.

Table 2c: Primary screening of isolates for alpha pinene tolerance

Sr no.	Isolates	Growth in increasing concentration of alpha pinene				
		2%	4%	6%	8%	10%
1	AP/S/1.1	+	+	+	+	+
2	AP/S/1.2	+	+	+	+	+
3	AP/S/1.3	+	+	+	+	+
4	AP/S/2.1	+	-	-	-	-
5	AP/S/2.2	+	+	+	-	-
6	AP/S/3.1	+	+	+	+	+
7	AP/S/3.2	+	+	+	+	+
8	AP/SW/1	+	-	-	-	-
9	AP/SW/2	+	-	-	-	-

Keys: +: Growth; -: No growth; S: Solid; SW: Swab

Table 2c shows the effect of various concentrations of alpha pinene on the isolates isolated from the pinene rich environment. As the concentration increased, the survival rate of isolates reduced. Only five out of nine isolates were able to survive at high alpha pinene concentration. There were no phenotypic changes seen with respect to any isolate.

Table 2d: Primary screening of isolates for DHM tolerance

Sr no.	Isolates	Growth in increasing concentration of alpha pinene				
		2%	4%	6%	8%	10%
1	DHM/S/1.1	+	+	-	-	-
2	DHM/S/1.2	-	-	-	-	-
3	DHM/S/2.1	+	+	+	+	-
4	DHM/S/2.2	+	+	+	+	-
5	DHM/S/3.1	-	-	-	-	-
6	DHM/S/3.2	+	-	-	-	-
7	DHM/L/1	+	+	+	-	-
8	DHM/L/2	+	+	+	-	-
9	DHM/L/3	+	+	+	-	-

Keys: +: Growth; -: No growth; S: Solid; L: Liquid

In the above table, it was seen that the survival rate in high DHM concentration has decreased rapidly from 2%. Only two isolates were able to survive at 8% of DHM concentration. These two isolates have ability to synthesize greenish-blue colored pigment.

Literature study shows that few organisms can tolerate high concentration of terpenes. *Penicillium digitatum* can tolerate 4-8% limonene but with reduced bioconversion activity. *Pseudomonas* spp. has no growth inhibition in 10% alpha and beta pinene [23]. Based on the data presented in above tables, 14 out of 40 isolates were found to have potential for terpene resistance. These 14 isolates were able to tolerate up to 10% of terpene concentration given to them and were selected in primary screening for further study purpose.

3.3. Morphological characterization

Colony characteristics of selected 14 isolates are tabulated in Table no. 3.

Table 3: Colony characteristics of selected isolates

Sr. No	Isolates	Size	Color	Opacity
Gram positive, motile coccobacilli				
1	AP/S/1.1	Small	White	Opaque
2	AP/S/1.2	Small	Colorless	Translucent
3	AP/S/3.1	Medium	Off- white	Opaque
4	CN/L/2.2	Small	Off- white	Translucent
5	LM/L/1.2	Small	Off- white	Opaque
6	LM/SW/1.5	Small	Colorless	Transparent
Gram negative, motile coccobacilli				
7	LM/L/1.1	Small	Off white	Opaque
8	LM/SW/1.1	Small	Off white	Translucent
9	AP/S/1.3	Small	White	Opaque
Gram positive, motile short rods				
10	LM/SW/1.3	Medium	Off- white	Translucent
11	AP/S/3.2	Small	Colorless	Transparent
12	CN/S/1.2	Small	Off white	Opaque
Gram negative, motile short rods				
13	LM/SW/1.2	Medium	Off white	Translucent
Gram positive, motile cocci				
14	LM/SW/1.4	Small	Yellow	Translucent

Colonies of all isolates were circular in shape and have even margin. Most of the colonies were of butyrous consistency except for colonies of isolates LM/L/1.1 and AP/1.3 which were mucoid. Some of the isolates were coccobacilli and short rods, except for cocci isolate LM/SW/1.4.

4. Conclusion

A total of 40 microbial strains were isolated from various locations of POIL factory situated in Mahad. Out of these 40 isolates, 14 were found to be capable of tolerating up to 10% terpene concentration. These 14 strains can be further explored for their potential metabolic pathways to produce range of natural terpene based products. This study can be used to have insight of biotransformation ability using a repertoire of terpene substrates and their product profiles. The selected best isolates can further be subjected to biochemical and molecular identification. Optimization studies of potential isolate with respect to other physiological parameters for their possible exploration will be very much influential for high scale production.

This study will help to overcome current trends of chemical synthesis and its racemic byproduct formation which is not eco-friendly. This work may thus mark a very promising step towards the biotechnological production of valuable natural flavour and fragrance compounds from abundantly available natural terpenoids.

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