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Original Article

Screening the Biotransformation Metabolites of α-Pinene by two Bacterial Strains *Paenibacillus popilliae* 1C, and *Streptomyces rochei* AB1

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Abstract

Introduction: Biotransformation can be an effective tool for the structural modification of bioactive natural and synthetic compounds to synthesize novel and more potent compounds. The present study describes the biotransformation of α-Pinene using two bacterial strains *Peanibacillus popilliae* 1C and *Streptomyces rochei* AB1, isolated and identified in our previous study.

Materials and Methods: The inhibitory concentration of the target substance against both strains was evaluated as 15 mg/ml. However, for technical considerations, a concentration of the α-Pinene at 10 mg/ml as Biotransformation Assimilable Concentration (BAC) was used to carry out the biotransformation process. For the biotransformation highlighting, both strains were cultivated on the rich medium (Luria-Bertani (LB) for *Peanibacillus popilliae* 1C and International *Streptomyces* Project 9 (ISP9) for *Streptomyces rochei* AB1) and poor medium (Minimum Medium (MM) for 1C and ISP9 for AB1). The chemical composition and percentage content of each compound were performed by GC/MS and GC/FID, respectively.

Results: The GC/MS analysis revealed that all the biotransformation products were hydrocarbon and oxygenated monoterpenes, which can be divided into two groups. The first group of 11 compounds (Verbenene, Isocarveol, E-2,3-Epoxycarane, γ-Terpinene, Dehydrolinalool, α-Campholene aldehyde, Menthol, Carvacrol, Limonene dioxide, Piperitenone, Ocimenol) is described for the first time in the biotransformation of α-Pinene and a second group bringing together 8 compounds previously reported (Trans Verbenol, p-Cymen-8-ol, α-Terpineol, Myrtenol, Verbenone, Trans Sobrerol, D-Limonene, Pinocarvone).

Conclusions: These bacterial strains possess distinctive biocatalytic capacities toward α-Pinene which leads to the production of other secondary metabolites.

Keywords: Biotransformation, α-Pinene, *Peanibacillus Popilliae* 1C, *Streptomyces Rochei* AB1, Chemical Analysis

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Introduction

Recently, biotechnology has become an emerging discipline of scientific research and is increasingly appliqued in several areas. The biotechnological processes have most notably been employed in the production of secondary metabolites for pharmaceutical purposes.[1] Biotransformation or biocatalysis can be defined as the specific chemical change of metabolites using biological agents including microorganisms like fungi and bacteria,[2] and recently some interest has also been given to archaea.[3] The biological agent (catalyst) can be depicted by an enzyme or a whole dead microorganism containing an enzyme or several enzymes.[4]

Bioconversion is also a term used to describe microbial transformation; however, a subtle difference exists between biotransformation and bioconversion. The bioconversion utilizes the catalytic activity of living organisms and therefore could implicate several chemical/reaction steps. In fact, the bioconversion involves enzymes, which are quite unstable and are produced continuously by a living microorganism. The properties of biotransformation and bioconversion are quite similar and, in many cases, the terms are cited as interchangeable.[5]

The biotransformation process has more advantages over those in chemistry, it operates, often, at ambient temperature, atmospheric pressure, and neutral pH.[4] Thus, the biotransformation provides a potential tool for the selective production of natural flavors and fragrances [6] with high quality

in terms of regioselectivity and stereoselectivity that are not easily obtained through chemical methods.[7] Among these products, many have wide-ranging applications, including flavorings, agrochemicals, antibiotics, antioxidants, and anticancer agents.[8] Biotransformation is also known to respect the rules of green chemistry strategy and comply with the concepts of white biotechnology.[9]

Terpenes and terpenoids (oxygenated terpenes) are the most diverse class of substances in nature. In fact, they are the main compounds of Essential Oils (EOs) which are obtained from several parts of the plants. The (EOs) are a complex mixture of aromatic substances characterized by their intense and distinctive odor; they play an important role in the flavor and fragrance industry and pharmaceutical products.[10]

The unlimited availability of (EOs) has encouraged the development of microbial conversions which conduct to the generation of characteristic flavor compounds[11], especially that flavor compounds derived from microbial or enzymatic processes are authorized to be labeled "natural" according to the mandatory guidelines of the Council of the European Communities (88/388/EWG of June 1988; 91/71/EWG and 91/72/EWG of 16 January 1991). Consequently, the terpenes become the largest group of plant-derived biologically active secondary metabolites submitted to microbial transformations.[12] The literature reports several studies of biotransformation of diterpenes,[13] sesquiterpenes[14], nevertheless, the acyclic, monocyclic, and bicyclic monoterpenes, which are the largest terpenes group, have been the subject of a considerable number of investigations,[15] via bioconversion of monoterpene precursors into their more valuable oxygenated derivatives.[16]

The bicyclic monoterpene α-Pinene is the main compound of turpentine oil and one of the most abundant monoterpenes in several (EOs). Also, it has been the starting reagent of important flavor compounds such as Terpineol, Borneol, Citronellol, Geraniol, Verbenol, and Verbenone,[17] and the precursor chemical of a large number of research studies on biotransformation. A fairly exhaustive review of the investigations performed and reported in the literature is grouped in Table 1-3 and Figure 1. We have considered it useful, for convenience, to group the variously functionalized biotransformation products in Table 1. Tables 2 and 3 include monoterpene alcohols and monoterpenes acids and their derivatives, respectively.

Table 1. Biotransformation Compounds, Variously Functionalized, of α-Pinene by Several Bacteria and Fungi Reported in the Literature

N°	Conversion products	Bacteria	Fungi
1	Terpinolene	*Pseudomonas maltophilia* [18] *Pseudomonas sp. strain PIN* [19]	
2	Verbenone	*Gluconobacter Japonicus Mtcc 12284* [20] *Serratia marcescens* [21]	*Hormonema sp, Aspergillus niger, Penicillium digitatum; Aspergillus sp; Penicillium sp; Pleurotus sapidus* [17,22-28]
2	α-Pinene epoxide	*Escherichia coli; Pseudomonas aeruginosa; Salmonella typhi; Staphylococcus aureus* [22] *Pseudomonas putida; Pseudomonas fluorescens NCIMB 11671* [29,30]	*Hormonema sp* [22] *Aspergillus sp; Penicillium sp* [25]
4	Carvone		*[Aspergillus niger Penicillium digitatum]* [23]
5	Camphor	*Pseudomonas maltophilia* [18] *Pseudomonas sp* [19]	*Stereum hirsutum* [27]
6	Thujone	*Pseudomonas maltophilia* [18]	
7	Isonovalal	*Pseudomonas rhodesiae CIP 107491*[31] *Pseudomonas rhodesiae*[32]	*Aspergillus sp Penicillium sp* [25]
8	PinoCamphone		*Aspergillus niger, Penicillium digitatum*[23]
9	4-β-hydroxy-α-Pinen-6-one		*Aspergillus niger, Penicillium digitatum*[23] *Botrytis cinerea* [24]
10	9-hydroxy-α-Pinene		*Aspergillus niger, Penicillium digitatum*[23] *Botrytis cinerea* [24]
11	Novalal	*Pseudomonas rhodesiae CIP 107491* [32]	*Aspergillus sp Penicillium sp*[25]
12	Limonene	*Pseudomonas PX1* [28] *Bacillus pallidus BR425*[33] *Pseudomonas maltophilia* [18] *Pseudomonas sp. strain PIN* [19]	*Ceriporia sp* [27]
13	β-Pinene		*Aspergillus niger* [22]
14	p-Cymene	*Pseudomonas sp. strain PIN* [19]	
15	α- Terpinolene	*Pseudomonas sp. strain PIN* [19]	
16	3β-hydroxy-(-)-β-Pinene		*Botrytis cinerea* [24]
17	Pino Carvone	*Bacillus isolates* [34]	
18	Cis Thujone	*Pseudomonas PX1* [28]	
19	Myrtenal	*Pseudomonas fluorescens NCIMB 11671* [35]	
20	8-hydroxy carvotanacetone		*Aspergillus sp* [26]

Table 2. Monoterpenes Alcohols as Biotransformation Products of α-Pinene by Several Bacteria and Fungi Reported in the Literature

N°	Conversion products	Bacteria	Fungi
21	Verbenol	*Gluconobacter Japonicus Mtcc 12284*[20] *Serratia marcescens*[21] [*Escherichia coli*; *Staphylococcus epidermidis*; *Pseudomonas aeruginosa*; *Salmonella typhi*; *Staphylococcus aureus*; *Klebsiella pneumoniae*][30]	[*Hormonema sp* *Aspergillus niger* *Pleurotus sapidus* *Aspergillus niger IOC-3913* *Aspergillus sp, Penicillium sp* *Penicillium digitatum*][22-28]
22	Trans Verbenol	*Serratia marcescens* [21]	
23	Sobrerol		[*Aspergillus niger*; *Penicillium digitatum*][23] [*Aspergillus sp*][25]
24	Carveol	[*Bacillus pallidus BR425*][33]	[*Aspergillus niger*; *Penicillium digitatum*][23]
25	Borneol	[*Pseudomonas sp.*][19]	[*Aspergillus niger, Penicillium digitatum*][23] [*Ceriporia sp.*][27]
26	Trans Sobrerol	[*Gluconobacter Japonicus Mtcc 12284*][20] [*Serratia marcescens*][21]	[*Armillariella mellea* (Honey Fungus][36]
27	α-Terpineol	[*Pseudomonas sp. strain PIN*][19] [*Gluconobacter Japonicus Mtcc 12284*][20]	[*Ceriporia sp.*][27] [*Candida tropicalis*][38] [*Absidia corulea*][38]
28	Terpinen-4-ol	[*Pseudomonas sp.*][19]	
29	p-Cymen-8-ol	[*Pseudomonas sp.*][19]	
30	Pino Carveol	[*Bacillus pallidus BR425*][33] [*Bacillus isolates*][34]	[*Stereum hirsutum*][27]
31	Myrtenol	[*Pseudomonas fluorescens NCIMB 11671*][35] [*Common Cutworm Larvae* (*Spodoptera litura*)][39] [*Human Liver Microsomes*][40]	[*Stereum hirsutum*][27]
32	Fenchol		[*Ceriporia sp.*][27]
33	1-Octen-3-ol		[*Ceriporia sp.*][27]

Table 3. Monoterpene acids and derivatives as biotransformation products of α-Pinene by several bacteria and fungi reported in the literature

N°	Conversion Products	Bacteria	Fungi
34	Terpinolenic acid	[*Pseudomonas maltophilia*][18]	/
35	Isonovalic acid	[*Pseudomonas rhodesiae CIP 107491*][31]	/
36	Novalic acid	[*Pseudomonas rhodesiae CIP 107491*][31]	/

The main objective of this study was to investigate the biotransformation of α-Pinene into other secondary metabolites using two strict aerobic bacteria newly isolated from the ecological niches of Algeria: *Peanibacillus popilliae* 1C and *Streptomyces rochei* AB1. Both strains have already shown the ability to degrade a wide range of complex organic compounds.[41,42]

Materials and Methods

Microorganisms

The two strains naimly *Paenibacillus popilliae* 1C and *Streptomyces rochei* AB1 have been isolated and identified by our research group in collaboration with the laboratory of "Environmental Bioprocesses of the Biotechnology Center" Sfax (Tunisia). The first strain (1C) which has been isolated in May 2008 from soil contaminated with crude oil (petroleum hydrocarbons) from the region of “Hassi-Messaoud” (south of Algeria), is a high degrading hydrocarbons strain.[42] The second strain (AB1) was isolated from moist soil collected from the “Boufarik” region (West of Algiers) in March 2007; and is considered as a protease, peroxidase, and keratinase-producing strain.[43]

Culture Media

Five culture media were used in biotransformation processes incuding:

- Luria-Bertani (LB) Medium: 5g yeast extract, 10 g peptone, and 5g sodium chloride in 1 L distilled water.
- International *Streptomyces* Project 2 (ISP2) Medium: 4 g yeast extract, 10 g malt extract, and 4 g Glucose in 1 L distilled water.
- International *Streptomyces* Project 9 (ISP9) rich Medium: 10 g Glucose, 2.84 g $(NH_4)_2SO_4$, 2.38 g KH_2PO_4, 5.65 g K_2HPO_4 $3H_2O$, 1 g $MgSO_4$ $7H_2O$, 1 ml of the trace element solution (EMT), in 1 L distilled water, for medium (ISP 9) poor is the same as (ISP9) rich except the amount of glucose that differs.
- Minimum Medium (MM): This medium is based on mineral salts, its composition (g/L) is: NH_4Cl (0.4), K_2HPO_4 (0.3), KH_2PO_4 (0.3), NaCl (10), $MgCl_2$ (0.33), $CaCl_2$ (0.05), yeast extract (0.1) and 1 ml of the trace element solution.

The pH of the media was adjusted between 7 and 7.2 by adding either HCl or NaOH (1N) (30%, w/v) solution, prior to sterilization. The medium was divided into 250 ml vials at a rate of 50 ml per vial. The media sterilization was performed by autoclaving at 120 °C for 20 min.

Cultivation and Growth of Microorganisms

The bacterial strains *Paenibacillus popilliae* 1C and *Streptomyces rochei* AB1 have been transplanted on Petri dishes containing solid media LB and ISP2, respectively. These dishes were incubated at 45 °C for 48 h for *Paenibacillus popilliae* 1C

and 30 °C for 72 h for *Streptomyces rochei* AB1.

Preparation of Inocula

After the growth of the two bacterial strains in 48 h and 72 h, they were pre-inoculated by one separated colony in 250 ml flasks containing 50 ml of the different media LB and ISP2 to prepare a bacterial culture and were incubated at 45 °C and 30 °C during 48 h and 72 h for *Paenibacillus popilliae* 1C and *Streptomyces rochei* AB1, respectively.

Biotransformation Assimilable Concentration (BAC)

The optimization of the concentrations was done in order to check the tolerance of the two bacterial strains tested towards the toxic effect of the substance. The diffusion agar method was used for the determination of this concentration. The range of the tested concentration was: 0.05, 0.5, 1, 5, 10, 20, and 30 mg/ml dissolved in methanol for the two strains. The reading of the results was done by measuring the inhibition diameter in mm.

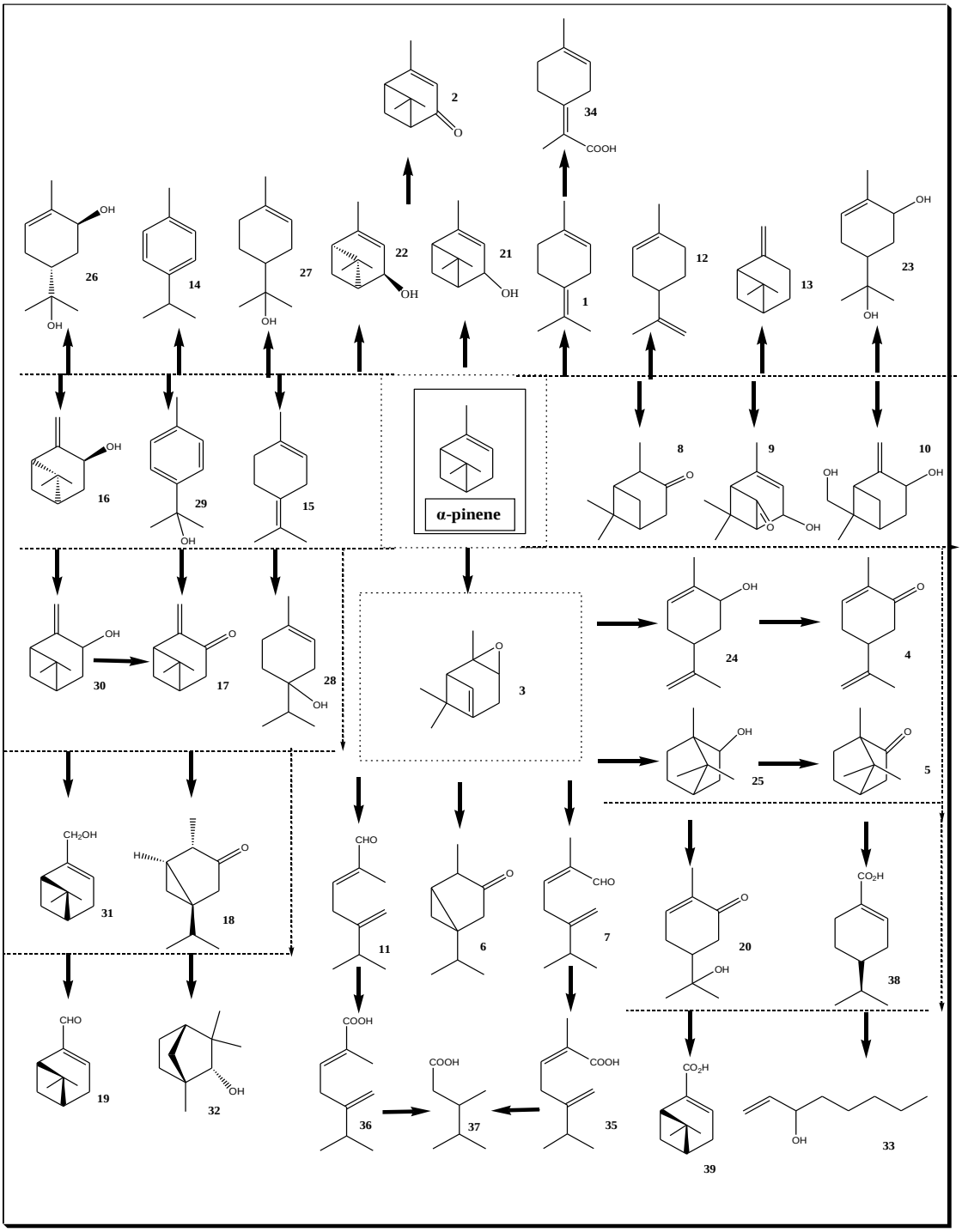

Figure 1. Major Metabolic Biotransformation Pathways of α-Pinene Described in The Literature.

Biotransformation of α-Pinene by Paenibacillus popilliae 1C and Streptomyces rochei AB1

The α-Pinene biotransformations by both bacterial strains were carried out under the same conditions such as: pH = 7.2, incubation temperature = 45 °C for *Paenibacillus popilliae* 1C and 30 °C for *Streptomyces rochei* AB1, incubation period = 7 days and with the same concentration of α-Pinene 10 mg/ml as BAC. The biotransformation could be performed using the rich and poor medium, in this latter case the microbial transformation is often called biodegradation. Thus, the biotransformation of α-Pinene by *Paenibacillus popilliae* 1C was carried out in an LB-rich medium, and in a poor MM medium. Regarding *Streptomyces rochei* AB1 strain, the biotransformation was accomplished in the rich and poor ISP9 medium. The samples were incubated in static mode.[19]

The methodology used consists of three steps: the first one is dedicated to the obtaining of the bacterial culture in the presence of the studied substance, the second step is the centrifugation of the mixture at 6000 rpm for 45 min leading to the bacterial biomass and the supernatant, the last step consists of a liquid-liquid extraction with diethyl ether to recover the produced metabolites which are analyzed and identified by GC/MS.[19]

GC/MS Analysis

The GC/MS analysis was carried out on an HP 6800 gas chromatograph coupled to an HP MSD 5973 mass spectrometer with a fused silica capillary column (HP5-MS 30 m x 0.25 mm x 0.25 µm). The oven temperature of the gas chromatograph was maintained at 100 °C, followed by a gradual increase in temperature at the rate of 5 °C/min till a temperature of up to 220 °C was attained. The injector and the detector temperatures were 250 °C. Helium was used as the carrier gas and was adjusted to a linear flow velocity of 5ml/min, with a split ratio of 1/90. The mass selective detector was operated in the Electron Ionization (EI) mode, with the ion source temperature being 230 °C; the ionization energy, 70 eV; the mass range of *m/z* 40-500. A sample volume of 1ml diluted in ethyl acetate (5 mg/ml) was injected.

The characterization of different compounds or metabolites outcome from biotransformation was realized based on the following databases: W11N17 (DB1) (Wiley11-Nist17, Wiley, Hoboken, USA); NBS 75k (DB2) and Mass Finder 3 (DB3) (D.H. Hochmuth, www.massfinder.com). The identification was performed applying two filters, namely spectral similarity match over 85% and linear retention index match calculated using a C_7-C_{30} saturated *n*-alkane homolog series (1000 g/ml) supplied by Merck KGaA (Darmstadt, Germany) with a filter window of ±10 LRI units. The values of the linear retention index calculated (LRI_{cal}) were compared to the one provided by literature (LRI_{Lit}). Further identification was achieved based on mass spectra reported by specialized literature.[44]

Results and Discussion

Determination of the Biotransformation Assimilable Concentration (BAC)

In this experiment, series of diluted solutions were prepared with the following decreasing concentrations (mg/ml): 10, 8, 5, 1, 0.5, and 0.05. The six substrate solutions including the stock solution were tested against both strains *Peanibacillus popilliae* 1C and *Streptomyces rochei* AB1. The analysis revealed the total absence of inhibition zones, which express the non-toxicity of the substrate against both strains. For technical convenience considerations related to the reliability of the experimental results, we set a concentration of 10 mg/ml as BAC of the α-Pinene to carry out the biotransformation process.

Biotransformation of α-Pinene by Peanibacillus popilliae 1C and Streptomyces rochei AB1

Biotransformation of α-Pinene by Peanibacillus popilliae 1C

As noticed previously, the α-Pinene substance was submitted to microbial transformation by *Peanibacillus popilliae* 1C using LB-rich medium, and MM poor medium. The GC/MS chromatographic profile obtained using the LB-rich medium revealed the presence of nine compounds as shown in Table 4.

According to our exhaustive literature review (Table 1-3), a few number of studies were carried out on biotransformation using a bacterial strain of the genus *Bacillus*, the only strains used were *Bacillus pallidus* and *Bacillus isolates*.

Table 4. Biotransformation Products of α-Pinene by *Peanibacillus popilliae* 1C

N°	Tr	Nom	LRI_{lit}	LRI_{Calc}	(%)	Data Base
1	3.14	Verbenene	967	960	0.62	DB1
2	5.42	Trans Verbenol	1140	1135	59.47	DB1, DB2, DB3
3	6.08	p-Cymen-8-ol	1179	1174	t	DB1
4	6.17	α-Terpineol	1186	1180	4.03	DB1, DB2, DB3
5	6.3	Myrtenol	1194	1190	4.54	DB1, DB2, DB3
6	6.54	Verbenone	1204	1200	6.94	DB1, DB2, DB3
7	6.68	Isocarveol	1212	1206	t	DB1, DB2, DB3
8	7.33	E-2,3-Epoxycarane	1231	1228	1.34	DB1
9	10.12	Trans Sobrerol	1374	1370	19.24	DB1
Total					96.18	

t: trace (< 0.1%)

Table 5. Biotransformation Products of α-Pinene by *Streptomyces rochei* AB1

N°	Tr	Nom	LRI_{lit}	LRI_{Calc}	(%)	Data Base
1	3.82	D-Limonene	1024	1024	0.2	DB1, DB2, DB3
2	4.14	γ-Terpinene	1054	1050	t	DB1, DB2, DB3
3	4.88	Dehydrolinalool	1088	1080	1.9	DB1, DB2
4	5.1	α-Campholene aldehyde	1108	1105	0.3	DB1, DB2
5	5.43	Trans Verbenol	1140	1135	40.4	DB1, DB2, DB3
6	5.73	PinoCarvone	1164	1160	1.1	DB1, DB2, DB3
7	5.86	Menthol	1165	1165	2.2	DB1, DB2, DB3
8	6.18	α-Terpineol	1186	1180	4.6	DB1, DB2, DB3
9	6.3	Myrtenol	1194	1190	4.6	DB1, DB2, DB3
10	6.55	Verbenone	1204	1200	4.1	DB1, DB2, DB3
11	6.68	IsoCarveol	1212	1206	0.7	DB1, DB3
12	7.34	E-2,3-Epoxycarane	1231	1228	1.8	DB1
13	7.73	Unknown 1		1256	1.5	
14	8.15	Carvacrol	1289	1285	0.8	DB1, DB3
15	8.31	Limonene dioxide	1294	1290	12.9	DB1
16	8.51	Piperitenone	1340	1335	0.9	DB1
17	8.58	Unknown 2		1340	3.4	
18	10.08	Trans Sobrerol	1374	1370	12.6	DB1
Total					89.4	

Unknown 1: 41 (39), 43 (69), 71 (30), 79 (14), 81 (13), 82 (25), 83 (100), 97 (28), 109 (18), 125 (5), 135 (4).
Unknown 2: 41 (77), 43 (51), 51 (20), 53 (34), 55 (37), 65 (28), 67 (44), 69 (69), 77 (51), 79 (57), 81 (67), 91 (100), 93 (56), 94 (29), 95 (41), 105 (26), 107 (42), 109 (54), 119 (33), 123 (31), 135 (16), 150 (6), 151 (4).

Table 6. Biodegradation Products of α-Pinene by *Streptomyces rochei* AB1

N°	Tr	Nom	LRI_{lit}	LRI_{Calc}	(%)	Data base
1	5.43	Trans Verbenol	1140	1135	62.0	DB1, DB2, DB3
2	6.19	Ocimenol	1155	1150	4.0	DB1, DB3
3	6.30	Myrtenol	1194	1190	4.4	DB1, DB2, DB3
4	6.53	Verbenone	1204	1200	9.1	DB1, DB2, DB3
5	10.08	Trans Sobrerol	1374	1370	10.1	DB1
Total					89.6 %	

In the present study, the biotransformation of α-Pinene by *Peanibacillus popilliae* 1C using an LB-rich medium made it possible to obtain eight oxygenated monoterpenes and only one hydrocarbon monoterpene, from where the Verbenene and E-2,3-Epoxycarane are detected for the first time and which have never been reported in the literature. Also, the biotransformation leads to Isocarveol, while the literature reports their isomers, PinoCarveol,[27,33,34] and Carveol.[23,33] The other obtained metabolites were Trans Verbenol, p-Cymen-8-ol, α-Terpineol, Myrtenol, Verbenone, and Trans Sobrerol. These later are all described as products of biotransformation of α-Pinene by several strains.[19-21] According to the literature, the biotransformation of α-Pinene by *Bacillus pallidus* and *Bacillus isolates* conducts to Carveol,[33] PinoCarveol,[33,34] PinoCarvone,[34] and Limonene.[33] Based on the obtained results, it seems that the strain using the rich medium advantages the oxidation pathway of the substrate. Thus, among the nine identified compounds, eight were oxygenated monoterpenes (seven alcohols and a ketone) and only one monoterpene hydrocarbon. The oxygenated monoterpenes were obtained via two steps, the Wagner-Meerwein rearrangement followed by β-oxidation pathways. The monoterpene hydrocarbon, namely the Verbenene, was reached via the first step. The β-oxidation pathway is commonly involved in the biotransformation processes of α-Pinene as reported in previous studies[27,44] and has been conducted to diverse oxygenated compounds (Table1).

As to the biotransformation of α-Pinene using the poor MM medium, which corresponds more to the biodegradation process, we observed a total absence of the bacterial growth of *Paenibacillus popilliae* 1C. This is probably due to the unavailability of a carbon source compared to the rich medium but also because the strain did not use α-pinene as a carbon and energy source. This was actually confirmed by the chromatographic profile which shows only the α-pinene peak at 2.8 min.

Biotransformation of α-Pinene by Streptomyces rochei AB1

To the best of our knowledge, the strain of *Streptomyces* genus is used for the first time in our case of α-Pinene biotransformation. Thus, the α-Pinene biotransformation by *Streptomyces rochei* AB1 strain was carried out also in the rich and poor ISP9 medium. As regards to the biotransformation in rich ISP9 medium, the chemical analysis reports the presence of 18 compounds, whereabouts 16 were correctly identified and two remained unidentified. We have noticed the identification of eight compounds described for the first time in the biotransformation of α-Pinene by this strain compared to the other bacteria and fungi strains. These are the following compounds: γ-Terpinene, Dehydrolinalool, α-Campholene aldehyde, Menthol, IsoCarveol, Carvacrol, Limonene dioxide, Piperitenone. The other compounds have already been described previously (Table 5). Among the identified compounds, we recorded two monoterpene hydrocarbons which are D-Limonene and γ-Terpinene, and 14

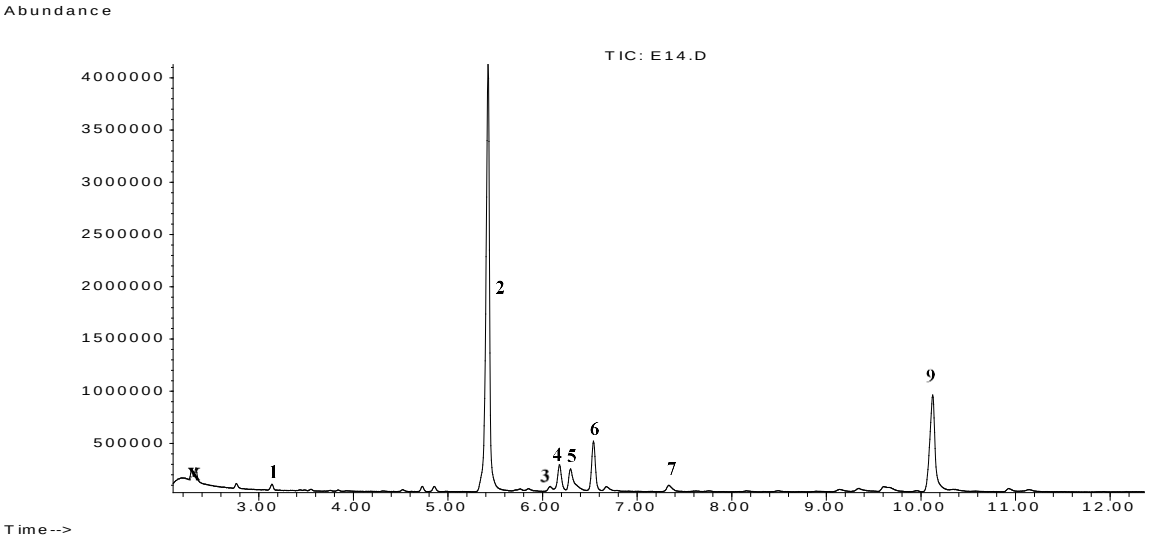

Figure 2. Chromatogram Profile of Biotransformation Products of α-Pinene by *Peanibacillus popilliae 1C*.

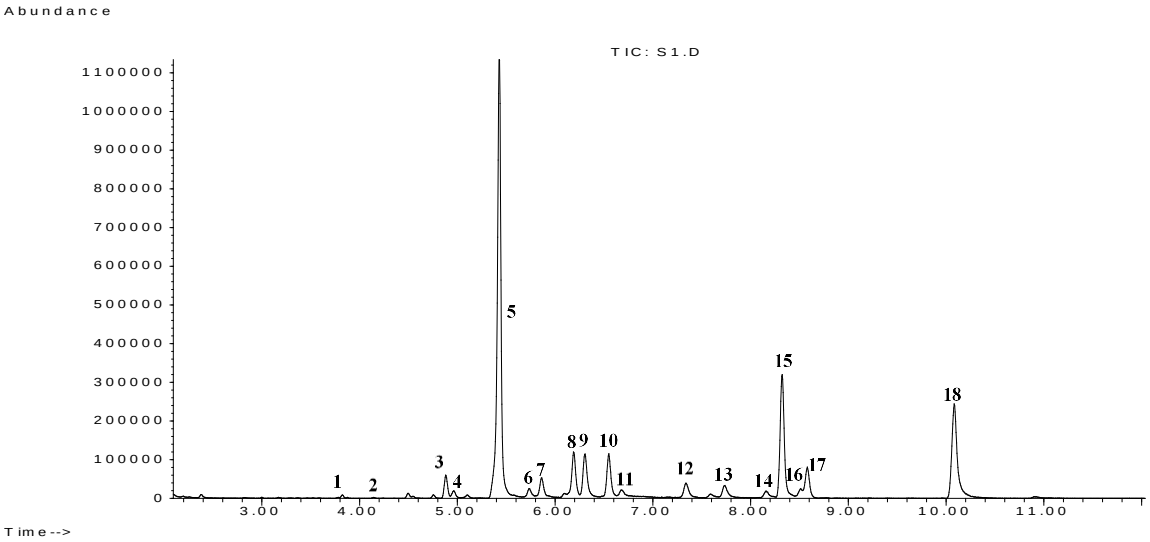

Figure 3. Chromatogram Profile of Biotransformation Products of α-Pinene by *Streptomyces rochei AB1*.

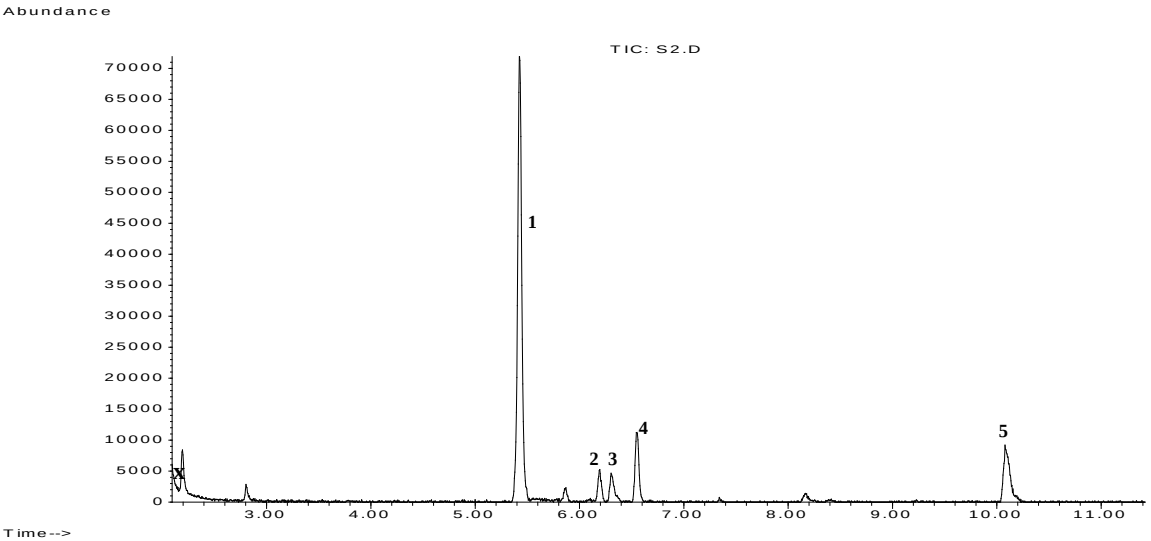

Figure 4. Chromatogram Profile of Biodegradation Products of α-Pinene by *Streptomyces rochei AB1*.

oxygenated monoterpenes, variously functionalized, characterized by the presence of alcohol, carbonyl, and epoxide. By using the *Streptomyces* strain, the biotransformation caused on one hand, the cleavage of only the Cyclobutane ring and both Cyclobutane and Cyclohexane rings leading to monocyclic and acyclic monoterpenes, respectively. On the other hand, the Wagner-Meerwein rearrangement leads to mono- and bicyclic oxygenated monoterpenes. The obtained monoterpenes especially belong to three monoterpenes skeletons: 2,6-Dimethyloctane, p-Menthane, and Pinane. Among the new obtained metabolites, we noticed the presence of Carvacrol which is characterized by an aromatic ring quite rarely encountered in biotransformation. This result reveals the notable performance of the actinobacteria strain used in the present study. Two compounds were recorded that could not be identified based on spectral databases and retention indices. In view of the mass spectra of the two compounds and the percentages of the different fragments, it would appear that they are oxygenated monoterpenes.

Indeed, the mass spectrum of unknown compound 1, shows a base peak at m/z = 83 and a mass peak at m/z = 135 which corresponds to M-H_2O. Therefore, the mass of the compound would be 150/152 which most probably corresponds to a monoterpene alcohol. The base peak at m/z = 83 corresponds to a fragment of the C_5H_7O type commonly encountered in the fragmentation of terpenes.[45] The mass spectrum of the second unknown compound reveals the presence of a base peak at m/z = 91 which corresponds to Tropylium ion. This agrees with the existence of a Methylbenzene fragment in the structure of a more likely cyclic oxygenated monoterpene with a mass peak at m/z = 150. In addition, the mass spectrum displays an important fragment at m/z = 69, 93 corresponding to the isoprene unit and the rearrangement of the Tropylium ion, respectively.

As regards to the biotransformation in poor ISP9 medium, the biotransformation of α-Pinene by *Streptomyces rochei* AB1 corresponding more to the biodegradation process, generated five oxygenated monoterpenes (Table 6). It must be noticed the identification of Ocimenol, which is an acyclic monoterpene alcohol, for the first time. The biodegradation led to obtaining four other monoterpenes previously described in the α-Pinene biotransformation. Thus, Trans Verbenol, Verbenone, and Myrtenol are obtained *via* a rearrangement reaction of α-Pinene. As to Trans Sobrerol, the biotransformation provokes the cleavage of the Cyclobutane ring of α-Pinene monocyclic monoterpene alcohol which led to monocyclic monoterpene alcohol. However, the new acyclic alcohol, described Ocimenol is reached via the cleavage of Cyclobutene and Cyclohexane rings of α-Pinene. The obtention of Ocimenol correlates more with a biodegradation process rather than bioconversion.

Plausible Metabolic Pathways of the Newly Biotransformation Metabolites

In Figures 2-7, possible pathways of the new metabolites (E-2,3-Epoxycarane, α-Campholene aldehyde, Dehydrolinalool, Menthol, Piperitenone, Verbenene, γ-Terpinene, Carvacrol, Limonene dioxide, Isocarveol, and Ocimenol) obtained during this study are proposed. The biocatalytic reactions encompassed oxidation, including hydroxylation and ketonization, which occurred at different positions on α-Pinene, as well as hydrogenation, hydrolyzation, esterification, rearrangement, and cyclic cleavage.[22,44,46,47]

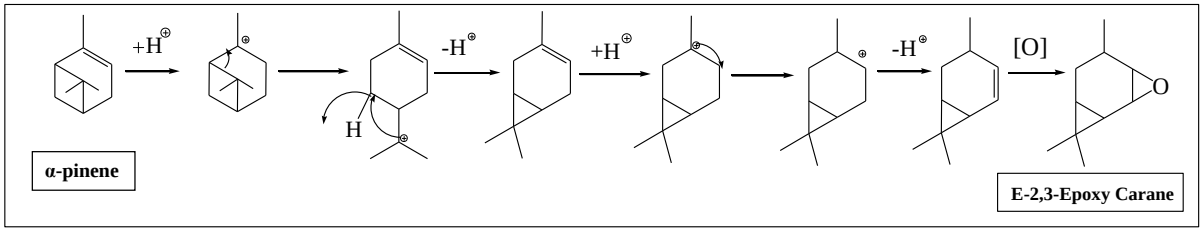

Figure 5. Plausible Biotransformation Pathway of α-Pinene to E-2,3-Epoxycarane.

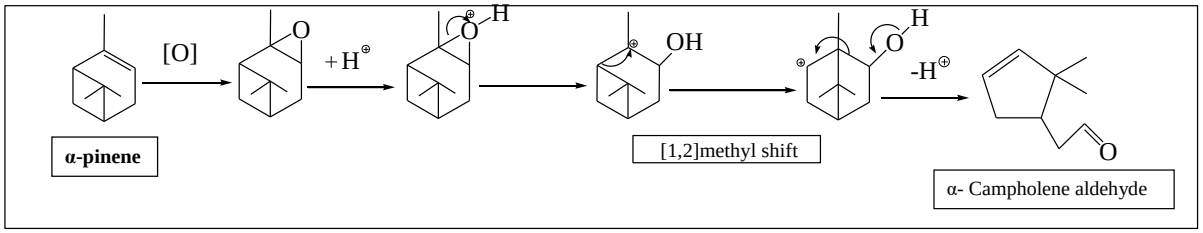

Figure 6. Plausible Biotransformation Pathway of α-Pinene to α-Campholene Aldehyde.

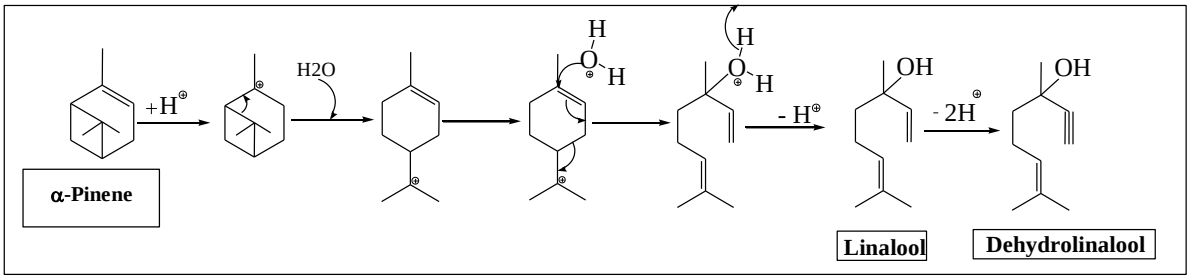

Figure 7. Plausible Biotransformation Pathway of α-Pinene to Dehydrolinalool.

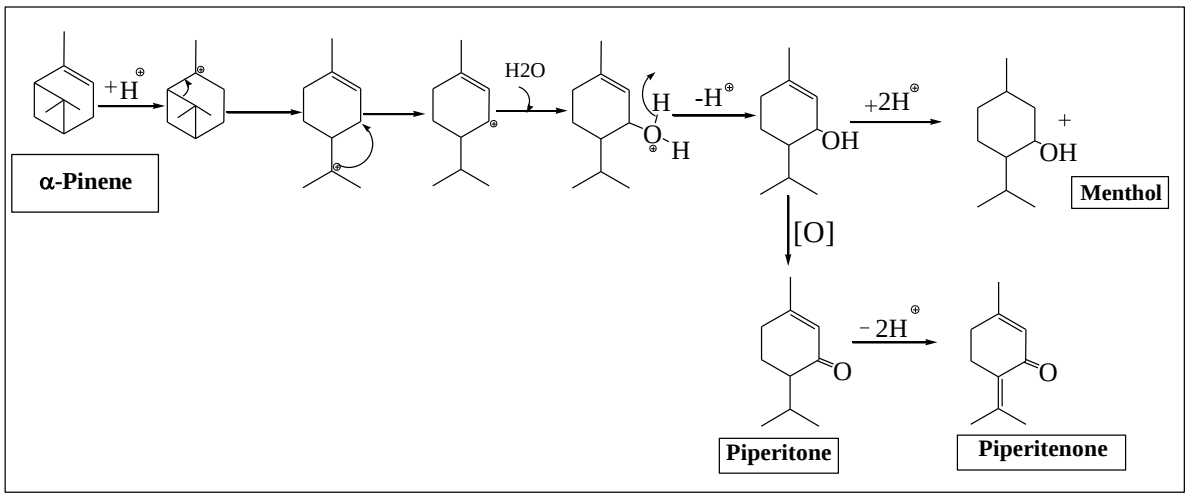

Figure 8. Plausible Biotransformation Pathway of α-Pinene to Menthol and Piperitenone.

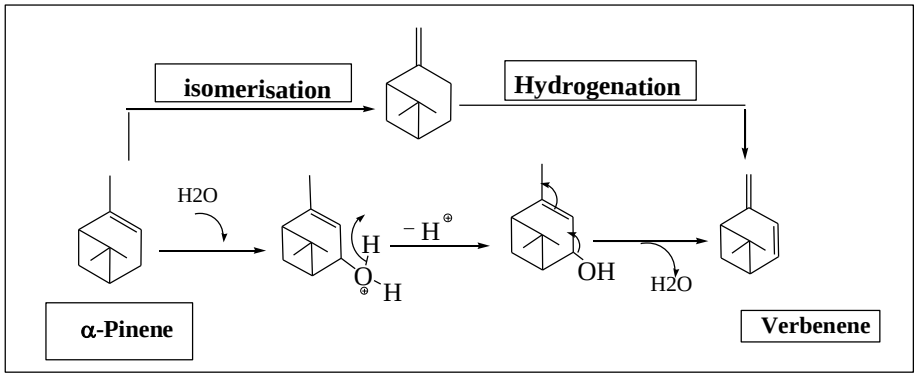

Figure 9. Plausible Biotransformation Pathway of α-Pinene to Verbenene.

Conclusion

The aim of this research was to study the pathways involved in the microbial transformation of α-Pinene using two strict aerobic bacteria newly isolated from the ecological niches of Algeria: *Peanibacillus popilliae* 1C and *Streptomyces rochei* AB1. Initial results revealed that these bacterial strains have distinct biocatalytic abilities towards α-Pinene. In order to determine the concentration of the biotransformation reaction, we performed a series of tests that allowed us to estimate the inhibition concentration at 15 mg/ml. For technical convenience related to the reliability of the experimental results, a concentration of 10 mg/ml was set as the BAC of α-Pinene to perform the biotransformation process. GC/MS analysis (Supplementary data) revealed that all biotransformation products were hydrocarbon and oxygenated monoterpenes, which can be divided into two groups. The first group of 11 monoterpenes includes Verbene, Isocarveol, E-2,3-Epoxycarane, γ-Terpinene, Dehydrolinalool, α-Campholene aldehyde, Menthol, Carvacrol, Limonene dioxide, Piperitenone, Ocimenol. To our knowledge, these compounds are described for the first time in the biotransformation of α-pinene. The second group comprises 8 compounds which are Trans Verbenol, p-Cymen-8-ol, α-

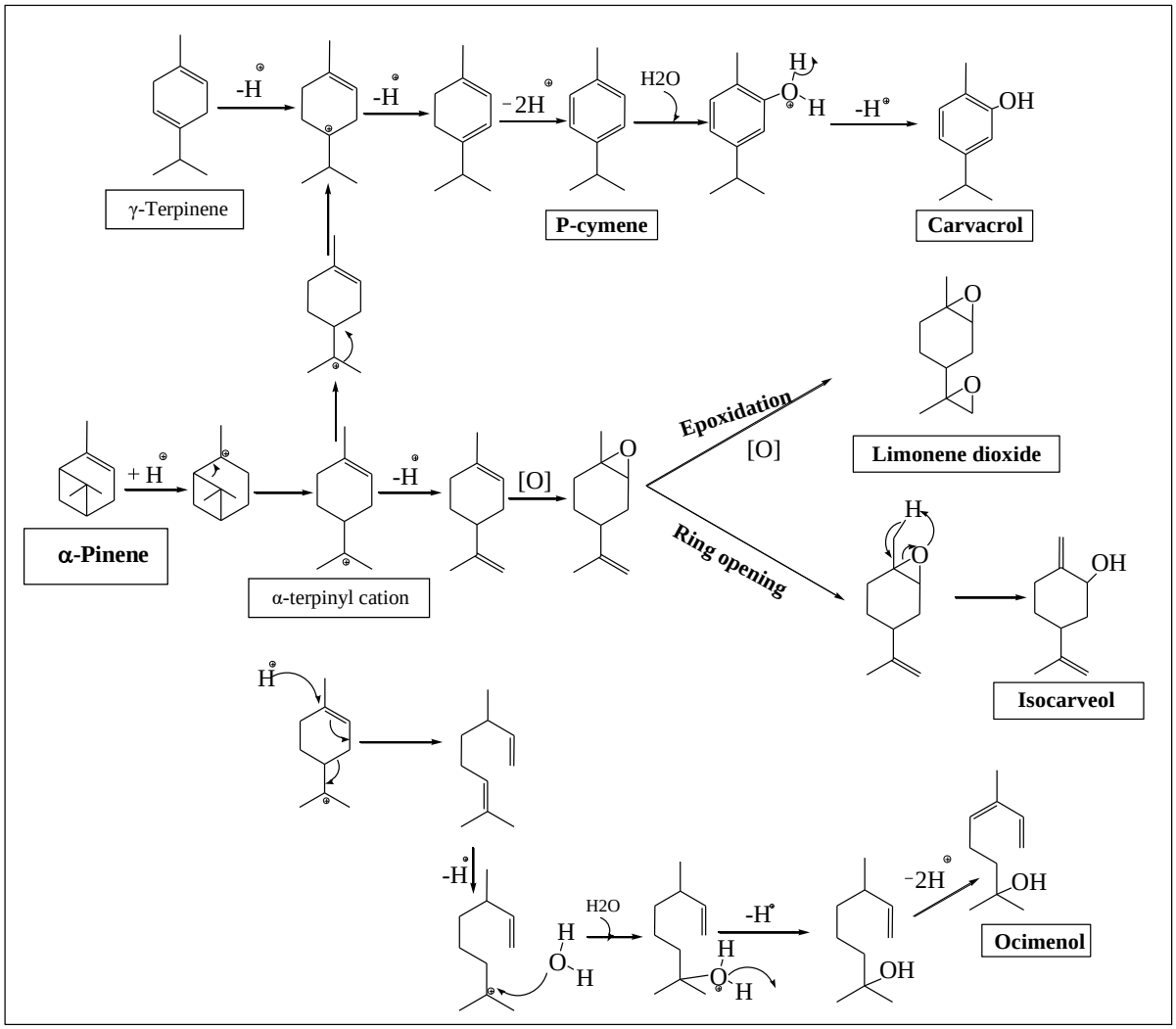

Figure 10. Plausible Biotransformation Pathways of α-Pinene to Carvacrol, γ-Terpinene, Limonene dioxide, Isocarveol and Ocimenol.

Terpineol, Myrtenol, Verbenone, Trans Sobrerol, D-Limonene, Pinocarvone have been previously reported in the biotransformation of α-pinene. The strains used in the present study showed very interesting biotransformation abilities. The metabolic pathway involved in the biotransformation seems to be different from that of the strains used in the previous work as reported in literature. The metabolic pathways involved in obtaining new products revealed the involvement of epoxidation reactions and in particular Wagner-Meerwein type rearrangements. It would be very interesting to test the strains used in the biotransformation of other monocyclic and bicyclic monoterpenes to clearly elucidate the preferred metabolic pathway.

Authors' Contributions

FS and MB were involved in experimental part of biotransformation of a pinene, NR was involved in GC/MS analysis, MRZ was involved in processing results. KE, F-Z F and F-Z M were involved in strain purification, NB participated in writing and processing results, AB contributed in supervising of microbiology experiments, MEH accomplished GC/MS processing and writing of the article.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

Acknowledgment

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