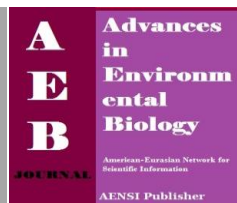




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Microbial Biotransformation of (R)-(+)-Limonene by Using two Novel *Pseudomonas* sp.

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ABSTRACT

Microbial biotransformation is one of the most suitable technologies to obtain highly added value natural compounds under environmentally friendly conditions. In this research work, the biotransformation of (R)-(+)-limonene by two novel *Pseudomonas* species under different pH and temperature conditions was investigated. The four substrate obtained by biotransformation, α -terpineol, cyclohexanemethanol, 4-hydroxy- α , α , 4-trimethyl- and linalool 1 synthesized by *Pseudomonas* sp. Ma.A toneka and *Pseudomonas* sp. Sh.A toneka; and carveol made by *Pseudomonas* sp. Ma.A toneka have a significant potential for industrial applications, such as: perfume manufactory.

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INTRODUCTION

Terpenes, hydrocarbons derived from isoprene units, are the largest class of plant secondary metabolites. Up until now, over 30 000 natural terpenes have been identified [1,3,4]. Among various terpenes, *d*-limonene (4-isopropenyl-1-methylcyclohexene) is the most abundant, which is widely available as monoterpene hydrocarbon, and particularly as a major component in oils from citrus peel [2,5]. In citric essential oils, it represents 70% of the lemon oils and 93% of the sweet orange oils [6,7]. The characteristic organoleptic properties of limonene and its usage in food and other applications encouraged the scientists and industries to work extensively on its synthesis and microbial conversions [8-12]. Because of its availability and the low price, it is frequently used as substrate for the chemical synthesis of nature-identical odorants [6,13]. The biotransformation of (+)-limonene using different microorganisms (i.e. bacteria, yeasts and fungi) has been extensively studied, in order to find different ways of producing compounds with a higher value.

However, most studies dealing with microbial conversions of limonene have reported low yields of products due to volatility of substrate and the toxicity of limonene to most of the microorganisms [5,14]. Different oxygenated terpenes have been reported as biosynthetic products (e.g. α -terpineol, perillyl aldehyde, carveol, carvone, piperitone, etc.) [6,7,11,12,14-18]. These compounds have shown antimicrobial, antioxidant, and anti-inflammatory properties. In addition, they possess fat reduction and arterial pressure regulation capabilities [19]. These characteristics greatly enhance industrial interest in such compounds. Biotransformation of *d*-limonene represents a very attractive alternative for the production of aromas [20], because it takes place under mild conditions, does not generate toxic wastes, and allows production of "natural" aromas that can be used as fragrances and flavors in the industries [21,22].

In this study, the biotransformation of (R)-(+)-limonene by *Pseudomonas* sp. Ma.A toneka and *Pseudomonas* sp. Sh.A toneka, and also, the influence of changes in the pH and temperature values on the process were investigated.

MATERIALS AND METHODS

Isolation and identification of strains:

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Pseudomonas sp. Ma.A toneka and *Pseudomonas* sp. Sh.A toneka were isolated from an enrichment culture containing 10 ml wastewater sample, and the *d*-limonene at different concentration values (i.e. 5, 10, 15, 20, 25, 30, 35, 50, 60, 70%) obtained from Citrus Processing Plant of Kosar – Ramsar, Iran, by incubation in a rotary shaker at 30°C/150 rpm. After 24h a loop of each flask was transferred to petri dishes containing YM medium (1.5% agar), and the streak plate method was conducted. The Petri dishes were incubated at 30°C until complete colonies' growth. Then, every single different colony was transferred to a sterile petri dish containing YM medium. Afterward, 2ml of each bacterial suspension equivalent to 1 McFarland was inoculated in a 50 mL Erlenmeyer flask containing 20 mL of mineral medium (the composition of the medium in g/L: $(\text{NH}_4)_2\text{SO}_4 = 5.00$; $(\text{NH}_4)_2\text{HPO}_4 = 1.42$; $\text{NaCl} = 0.50$; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O} = 0.40$; $\text{CaCl}_2 = 0.60$; $\text{KCl} = 2.15$; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O} = 0.01$; $\text{ZnSO}_4 = 0.01$; $\text{CuSO}_4 = 0.01$; pH not adjusted) and 1 ml (5%, v/v) of *d*-limonene. During 7 days of incubation in a rotary shaker at 30°C/150 rpm, every 24h, a loop of each flask was transferred to petri dishes containing YM medium, as already described. All the colonies that presented a satisfactory growth (>30 CFU) after 48h at 30°C they were considered the possible users of *d*-limonene as sole carbon source [23].

The strains that showed the greatest resistance to *d*-limonene and capable of using limonene as sole carbon source were identified by 16s rRNA gene sequencing technique.

Biotransformation experiments:

2ml of each bacterial suspension equivalent to 1 McFarland from the previous test was inoculated in a 50 mL Erlenmeyer flask containing 20 mL of mineral medium and 1 ml (5%, v/v) of *d*-limonene. They were incubated in a rotary shaker at 30°C/150 rpm for 10 days. The culture medium with the highest specificity and bioconversion values was selected to evaluate additional biotransformation parameters.

Extraction of products:

After 10 days, the flasks were removed from the rotary shaker. 1 ml of each product was transferred in a micro centrifuge tube, and adding 1 ml ethyl acetate terminated the reaction. The micro centrifuge tubes were vigorously shaken to accomplish quantitative extraction of the terpenes. The mixture was pipetted in a microcentrifuge tube and centrifuged (3 min, 13,000×g) to separate the two layers. Subsequently, 1 µl of the ethyl acetate layer was analyzed by gas chromatography - mass spectroscopy (GC-MS) [24].

Analytical conditions:

The products obtained were analyzed in an Agilent Technology GC 7890 gas chromatograph with Mass model 5975 (GC-MS). A capillary column of 30 m × 0.25mm × 0.25µm id was employed. Nitrogen was the carrier gas, with a constant pressure in the head of the column of 0.8 bar and split ratio of 1:10. The column temperature was programmed at 60 °C for 4 minutes, increased by 3 °C/minute to 100 °C, and then increased by 4 °C/minute to 210 °C for 1 minute. The temperature in both the injector and the detector was 250°C. The isomers obtained (i.e. Carveol and Terpineol) were identified on the basis of their retention times and mass spectra.

Effect of pH:

The effect of pH on limonene biotransformation was studied by dissolving YM culture medium in a 0.1 M HCl at pH values of 2.5, 4 and 5.5 and also a 0.1 M NaOH at pH values of 7.5, 10 and 12.5. The bioassays were carried out in 50 mL Erlenmeyer flask by adding 20 ml of the sterile YM medium with adjusted pH and *d*-limonene (2%, v/v). Culture media were inoculated with 50µl of strains and incubated at 30°C for 72 h, with an agitation of 150 rpm using an orbital shaker. The microbial transformation was monitored every 24h, after adding the substrate.

Effect of temperature:

The effect of temperature on limonene biotransformation was studied by incubation of strains inoculated in YM medium containing *d*-limonene in a rotary shaker with 150 rpm at 4,25,30, 37, 42, 50 °C for 72 h.

RESULTS AND DISCUSSION

Characterization of the complex substrates:

Orange peel oil is a cheap and wide spread terpene source and could be used in bioconversion processes, either as an inducing agent for biocatalyst production or as precursor for the reaction itself. As for any other agro-industrial product, its composition may vary depending on seasonal conditions. The composition of orange peel oil obtained from Citrus Processing Plant of Kosar in Ramsar-Iran (Table 1) was determined by GC-MS method. The concentration of the main compound in this mixture was consistent with already reported values in the other studies.

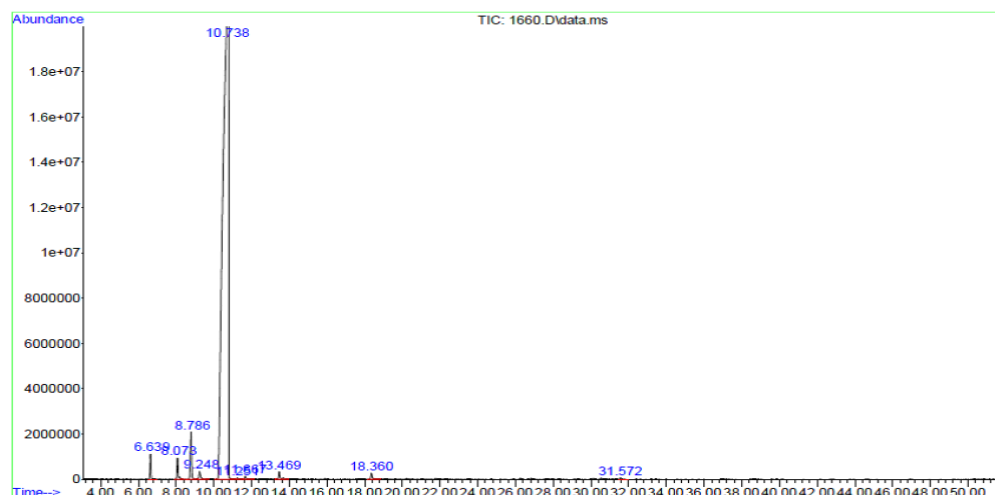


Fig. 1: Chromatogram of citrus peel oil

GC-MS analysis of orange peel oil showed that it contains %95.11 *d*-limonene (Table 1).

Table 1: Main constituents of orange peel oil used in this study

Compounds	Area%
alpha-Pinene	0.74
Sabinene	0.82
beta-Myrcene	2.04
Octanal	0.4
D-Limonene	95.11
gamma-Terpinene	0.11
Linalool L	0.11
Decanal	0.29

Isolation and identification of strains:

Pseudomonas sp. Ma.A toneka (70W1) and *Pseudomonas* sp. Sh.A toneka (76W1) were isolated from a wastewater sample from Citrus Processing Plant of Kosar in Ramsar, using *d*-limonene as sole source of carbon and energy. They were identified by using 16s rRNA gene sequencing technique, as a newly strains that were subsequently submitted in GenBank of NCBI as *Pseudomonas* sp. Ma.A toneka (GenBank ID: KF548196) and *Pseudomonas* sp. Sh.A toneka (GenBank ID: KF548197). The short bacilli produced milky, slightly slimy colonies on YM agar. The Gram stain was negative, and they were catalase & oxidase positive. The strains utilized argentine and tryptophan as sole source of carbon and energy for growth. D-glucose, arabinose, amygdalin, sucrose, sorbitol, inositol, mannitol, and lysine were not utilized. The bacilli strains were resistant to limonene concentrations up to 70% in the YM broth. The optimum growth temperature was 30°C.

Utilization of the monoterpene substrate for bacterial growth:

The two microorganisms were first tested for their ability to grow on *d*-limonene as the sole carbon and energy source. The growth was observed after 24 h, because the biomass obtained at this time was a good indicator of the ability of the substrate to support bacterial growth. The utilization of the terpene indicated that the substrate is actively involved in the metabolic pathway of the corresponding bacterial strain.

Effect of pH and temperature:

The limonene-biotransformation ability of *Pseudomonas* sp. Ma.A toneka and *Pseudomonas* sp. Sh.A toneka was evaluated at different pH and temperature levels on the YM medium. Their biodegradation activity decreased at pH 2.5 and 12.5; and the optimum pH range was from 4 to 10. The limonene-biotransformation capability of the isolated strains decreased at 4 and 50 °C and at the optimum temperature of 25 to 37 °C.

Biocatalytic activity of *Pseudomonas* sp. Ma.A toneka and *Pseudomonas* sp. Sh.A toneka:

In the biotransformation experiments, *d*-limonene almost disappeared and new neutral metabolites were accumulated corresponding to the decline of *d*-limonene (Fig. 1). A comparison of the mass spectrum obtained by GC-MS (Fig. 4 and Fig. 5) with published spectra [25] identified the metabolites (Fig. 2 and Fig. 3).

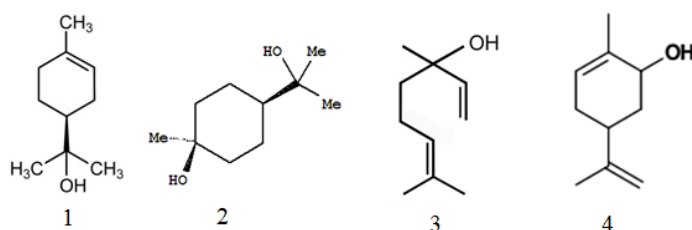


Fig. 2: Products of the biotransformation of *R*-(+)-limonene by *Pseudomonas* sp. Ma.A toneka and *Pseudomonas* sp. Sh.A toneka. 1. α -terpineol, 2. Cyclohexane methanol, 4-hydroxy- α , α , 4-trimethyl, 3. linalool L, 4. Carveol.

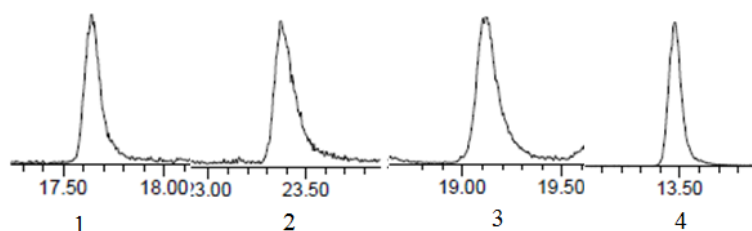


Fig. 3: Retention time of products. 1. α -terpineol, 2. cyclohexanemethanol, 4-hydroxy- α , α , 4-trimethyl, 3. linalool L, 4. Carveol.

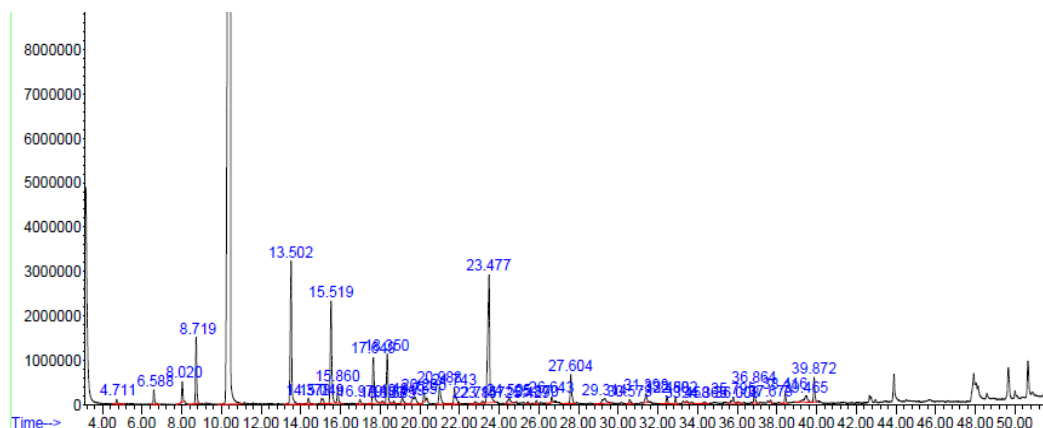


Fig. 4: Chromatogram obtained on the *(R)*-(+) limonene biotransformation by *Pseudomonas* sp. Ma.A toneka

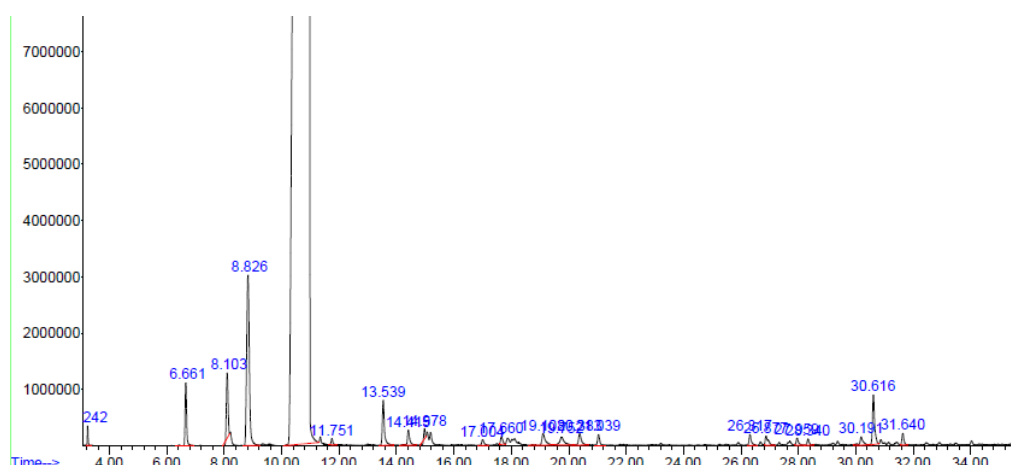


Fig. 5: Chromatogram obtained on the *(R)*-(+) limonene biotransformation by *Pseudomonas* sp. Sh.A toneka.

Gas chromatography (Fig. 4 and Fig. 5) indicated that the biotransformation products are ' α -terpineol', 'cyclohexane methanol, 4-hydroxy- α , α , 4-trimethyl' and 'linalool L' by *Pseudomonas* sp. Ma.A toneka and *Pseudomonas* sp. Sh.A toneka; and 'carveol' by *Pseudomonas* sp. Ma.A toneka. However, in the control

sample, the absence of the substrates indicates that the bacteria is directly responsible for their formation. The first biodegradation study was performed at the Indian National Chemical Laboratory in Poona in the 1960s. The authors isolated a *Pseudomonas* strain that was able to grow with *d*-limonene as the sole carbon and energy source. Compounds accumulating during growth on *d*-limonene were extracted from the culture medium, and fractionated using different solvents and pH values, in which one of them was carveol [10].

Cadwallader *et al.*, [8] obtained a *d*-limonene-degrading *Pseudomonas gladioli* strain by selective enrichment medium, and observed the transient accumulation of perillic acid (probably as a pathway intermediate), and the formation of α -terpineol and an unidentified compound as dead-end products. A number of *d*-limonene-degrading *Bacillus* strains was studied by the group of Patrick Oriel from Michigan. *Bacillus stearothermophilus* BR388 was the isolated strain in this study that converts *d*-limonene to perillyl alcohol, α -terpineol, and perillaaldehyde [26]. The same metabolites were accumulated during growth of an *E. coli* construct carrying a 9.6-kb chromosomal fragment from *B. stearothermophilus* BR388, which allowed the construct to grow with *d*-limonene as the sole carbon source [27]. In a later study, a 3.6-kb sub-fragment was found to encode a limonene hydroxylase that hydroxylated limonene in the 6 or 7 position, giving rise to a mixture of (mainly) perillyl alcohol and carveol (the latter formed from carveol by means of a nonspecific dehydrogenase from the host strain) in a 3:1 ratio [28].

These results strongly suggest that the occurrence of multiple products is due to incomplete enzyme region-specificity (rather than the simultaneous expression of multiple enzymes with different region-specificities). In contrast to the wild-type *Bacillus* strain and the construct carrying the 9.6-kb fragment, α -terpineol did not accumulate [28]. Apparently, the formation of this compound was due to another enzyme encoded on the 9.6-kb fragment, but not on the 3.6-kb fragment. However, many strains capable of growth on *d*-limonene can utilize a wide range of hydroxylated derivatives [9, 29]. The wide substrate range can be related to the observation that such oxidized limonene derivatives are generally co-produced by the limonene-producing plants or trees [30] or generated by auto-oxidation of limonene [31].

One of the products that was studied was α -terpineol, which is a monoterpenoid that has a significantly higher added value than limonene (about 60 to 100 times more), and a market value of 13,000 tons commercialized per year. Moreover, α -terpineol is considered to be a safe additive (GRAS 3045), which is commonly used as fragrance in the industry of perfumes, fragrances, cosmetics and toiletries [32, 33]. It is also used in the pharmaceutical industry as an antifungal and disinfectant product [33, 34], and in the food industry as a preservative due to its antimicrobial and antioxidant properties [35-37].

Limonene can be transformed into α -terpineol by different bacterial strains, such as: *Pseudomonas gladioli* [8,38], *Escherichia coli*, [39] and *Sphingobium spp.* [40]. In all cases, the process is highly enantiospecific [11, 12,40-42], but the concentration of the products depends on the reaction conditions, such as pH and temperature [43].

Conclusions:

In this study, the biotransformation of (*R*)- (+)-limonene by *Pseudomonas* sp. Ma.A toneka and *Pseudomonas* sp. Sh.A toneka was evaluated. The stoichiometric formation of α -terpineol, cyclohexanemethanol, 4-hydroxy- α , α , 4-trimethyl- and linalool L by *Pseudomonas* sp. Ma.A toneka and *Pseudomonas* sp. Sh.A toneka; and carveol by *Pseudomonas* sp. Ma.A toneka was observed. Variation in pH (ranged from 4 to 10) and temperature (from 25 to 37 °C) had no effect on the growth and the *d*-limonene-biotransformation ability of *Pseudomonas* sp. Ma.A toneka and *Pseudomonas* sp. Sh.A toneka. But, the limonene-biotransformation potential of the isolated strains decreased at 4 and at 50 °C, pH levels of 2.5 and also 12.5. Therefore, *Pseudomonas* sp. Ma.A toneka and *Pseudomonas* sp. Sh.A toneka described in this paper have a significant potential for industrial applications, such as: perfume, food and the pharmaceutical industry.

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