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BIOTRANSFORMATION OF SOME MONOTERPENOID KETONES BY *Chlorella vulgaris* MCCS 012

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Biotransformation of several monoterpene ketones, including carvone, pulegone, piperitone, menthone, and fenchone, was carried out by the locally isolated unicellular microalgae Chlorella vulgaris*. The microalgal strain was isolated during a screening program from soil samples collected from paddy-fields of Fars Province, in the south of Iran.* Chlorella vulgaris *was cultured in 250 mL conical flasks, each containing 50 mL of BG-11 liquid medium and 20 μL levels of terpene substrates, incubated at a temperature of 28±2°C and illuminated continuously with fluorescent lamps with shaking at 80 rpm. The metabolites were identified by thin-layer chromatography and GC-MS.* Chlorella vulgaris *has the ability to reduce the C=C double bond of carvone to yield* trans*-dihydrocarvone and* cis*-dihydrocarvone. The cell line reduced menthone and pulegone to the same product and gave menthol. Study of* Chlorella vulgaris *with substrates of piperitone and fenchone showed no reaction in these substrates.* Chlorella vulgaris *MCCS 012 was assigned according to the 18S rRNA gene sequence. The DNA sequence of the 18S rRNA gene of* Chlorella vulgaris *MCCS 012 was recorded in the NCBI under the accession number EU374170.*

Keywords: biotransformation, monoterpene, microalgae, *Chlorella vulgaris*.

Biotransformation has been extensively applied in the fermentation industry, for example, in the production of steroids. In the course of our work related to the biotransformation of exogenous steroid microalgae, we investigated the bioconversion of some monoterpenoid ketones by *Chlorella vulgaris* MCCS 012. [1–4]. Monoterpenes are natural substances that present a small carbon pool with a high turnover rate in the annual global carbon cycle [5]. The amount of volatile monoterpenes emitted from trees is estimated to be 127×10^{14} g of carbon/year [6]. The low price and simple structure of monoterpenes such as carvone, pulegone, and menthone make them ideal targets for bioconversion by microorganisms to yield commercially important products [7]. Commercially useful chemical building blocks and pharmaceutical stereoisomers can also be produced by bioconversion of monoterpenes. Products produced by bioconversion processes may be considered as natural [8]. Only a few studies have been reported on the biotransformation of monoterpenes by *Chlorella* species. One study cites the biotransformation of carvone and menthone by the microalga *Chlorella minutissim* [9]. Preliminary taxonomical studies show that *Chlorella vulgaris* is common in the paddy-fields of Fars Province located in the south of Iran, beside microalgae like *Oocystis*, *Scenedesmus*, and some unicellular and filamentous cyanobacteria [1]. In this study, the biotransformation of carvone, pulegone, piperitone, menthone, and fenchone as an exogenous substrate was carried out by a locally isolated strain of a unicellular microalga *Chlorella vulgaris*. Until today, *Chlorella vulgaris* has not been examined in the transformation of monoterpenes. The aim of this study is to identify the ability of locally isolated *Chlorella vulgaris* to convert monoterpenoid ketones as an exogenous substrate. The strain was recognized by morphological characterization and assigned according to the 18S rRNA gene sequence. The isolated alga was classified by the Microalgal Culture Collection of Shiraz University of Medical Sciences, Faculty of Pharmacy, Shiraz, Iran, as a strain of *Chlorella vulgaris* MCCS 012. The partial sequence of the 18S rRNA gene of *Chlorella vulgaris* MCCS 012 is as follows:

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1	gcatttgcca	aggatgtttt	cattaatcaa	gaacgaaagt	tgggggctcg	aagacgatta
61	gataccgtcc	tagtctcaac	cataaacgat	gccgactagg	gatcggcgga	tgtttcttcg
121	atgactccgc	cggcacctta	tgagaaatca	aagtttttgg	gttccggggg	gagtatggtc
181	gcaaggctga	aacttaaagg	aattgacgga	agggcaccac	caggcgtgga	gcctgcggct
241	taatttgact	caacacggga	aaacttacca	ggtccagaca	tagtgaggat	tgacagattg
301	agagctcttt	cttgattcta	tgggtggtgg	tgcatggccg	ttcttagttg	gtgggttgcc
361	ttgtcaggtt	gattccggta	acgaacgaga	cctcagcctg	ctaaatagtc	acggttggtt
421	cgccagccgg	cggacttctt	agagggacta	ttggcgacta	gccaatggaa	gcatgaggca
481	ataacaggtc	tgtgatgccc	ttagatgttc	tgggccgcac	gcgcgctaca	ctgatgcatt
541	caacgagcct	agccttggcc	gagaggcccg	ggtaatcttc	gaaactgcat	cgtgatgggg
601	atagattatt	gcaattattaa				

The DNA sequence of 18S rRNA gene of *Chlorella vulgaris* MCCS 012 was recorded in the NCBI under the accession number EU374170. The result of PCR blasted with other sequenced microalgae in NCBI showed 100% homology to the 18S small subunit rRNA of other strains of *Chlorella vulgaris*.

Initially, the optimal growth patterns of *Chlorella vulgaris* were established. The growth was determined by direct counting and absorbance at 680 nm. Monoterpene substrates were then added to the cell cultures in the logarithmic phase of growth ($2.8–3.0 \times 10^6$ cells mL^{-1}). After 24 hours incubation, *Chlorella vulgaris* MCCS 012 reduced the C=C double bond of (+)-carvone (**1**) to yield *trans*-dihydrocarvone (**6**) and *cis*-dihydrocarvone (**7**). The microalga reduced both (–)-menthone (**2**) and (+)-pulegone (**3**) to dl-menthol (**8**). In this study *Chlorella vulgaris* did not react with (–)-fenchone (**4**) and (–)-piperitone (**5**). The chemical structures of the substrates and the products that underwent biotransformation by *Chlorella vulgaris* are shown in Fig. 1.

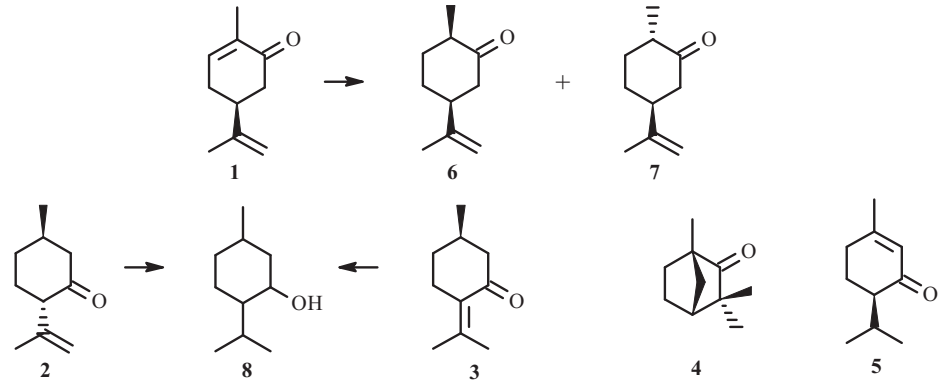

Fig. 1. Chemical structures of the substrates and the products biotransformed by *Chlorella vulgaris*.

Microalgae are photoautotrophic and, therefore, do not require an organic substrate for energy. Consequently, the culture of microalgae is simpler and cheaper than that of bacteria, yeasts, or fungi. They are easily and quickly cultured in an inexpensive medium containing simple salts, which decreases the probability of contamination by other microorganisms. This is an important advantage when considering the potential of microalgae in the biotransformation of readily available chemicals such as carvone and menthone into compounds with a higher economic value in the food and perfume industries.

In this study, various monoterpenes, including carvone, pulegone, piperitone, menthone, and fenchone, were chosen for biotransformation evaluation. Using these compounds, we examined the biotransformation of monoterpenoids in a cell culture of *Chlorella vulgaris* to elucidate the metabolic behavior of the green algal cells at a concentration of ca. 100 ppm. Both carvone and piperitone are unsaturated ketones. (+)-Carvone was reduced to *trans*-dihydrocarvone and *cis*-dihydrocarvone. No biotransformation of (–)-piperitone was observed. It is possible that the position of the methyl and isopropyl groups in carvone and piperitone relative to the carbonyls determines the orientation and the fitness of the molecules in the catalytic sites of the reductase involved. In fungi, enoate reductases are responsible for the reduction of activated C=C bonds. The enzymes responsible for the reduction of carvone are apparently present in many yeasts, fungi, plants, and algae [10]. Methylethenyl group reduction and hydroxylated derivatives were not found in the metabolism of carvone by *Chlorella vulgaris*.

Dihydrocarvone and neodihydrocarveol were found in the biotransformation of (–)-carvone by four species of microalgae, *Nannochloris*, *Dunaliella*, *Porphyridium*, and *Isochrysis* [9]. They found an initial reduction of the α,β-unsaturated double bond, followed by the carbonyl group in carvone, leaving the exocyclic C=C bond of the 1-methylethenyl group unaffected [9]. It is interesting to note that *Chlorella vulgaris* reduced the carbonyl group and the exocyclic C=C bond of the 1-methylethenyl group in (+)-pulegone to give menthol. Van Dyk′s group [10] showed that the black yeast *Hormonema* sp. could transform (+)-pulegone to neomenthol. Alcohol dehydrogenases are involved in the reduction of carbonyl groups and are responsible for the formation of *S*-alcohols from (–)-menthone and (+)-pulegone following Prelog′s rule. Dehydrogenases following Prelog′s rule are apparently more abundant [10]. Reduction of the unconjugated carbonyl group of (–)-menthone by *Chlorella vulgaris* gave menthol. This result is similar to findings observed in the biotransformation of (–)-menthone by *Chlorella minutissim* [9]. During the biotransformation of foreign substrates by *Chlorella vulgaris*, the cells showed similar metabolic behaviors, resulting in reduction, on the structure of the substrate added. In recent years, bioconversion yields and production technology have both improved, which suggests that the production of many economically viable terpenoid compounds will be possible in the future [8]. To the best of our knowledge, no research has been conducted on the biotransformation of monoterpenes by *Chlorella vulgaris*. As these results show, this microalga might be considered as a useful biocatalyst for some types of monoterpenes.

EXPERIMENTAL

Chemical Compounds. (+)-Carvone (**1**), (–)-menthone (**2**), and (+)-pulegone (**3**) were purchased from Merck. (–)-Fenchone (**4**) and other reagents were from Sigma and Fluka. (–)-Piperitone (**5**) was kindly provided by Dr. A. R. Shahverdi (Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Tehran University of Medical Sciences).

Collection, Preservation, and Identification of the Alga. The microalga was isolated, during a screening program, from soil samples collected from the paddy-fields of Shiraz located in southern Iran (Fars Province) from April to December 2004. Primary culturing was carried out in BG-11 medium [3]. After colonization, pure cultures of living specimens were prepared using subculturing and the agar plate method in BG-11 medium [11]. Preserved specimens were prepared and the living specimens were incubated in 50 mL-conical flasks, under constant illumination. Identification of the alga was carried out using appropriate manuals [12, 13].

18*S* Ribosomal RNA sequencing: for this purpose, DNA content was first extracted from the *Chlorella vulgaris* and then PCR was applied using two set primers. The sequences were amplified using the primers 5′-GTCAGAGGTGAAATTCTTGGATTTA-3′ as forward and 5′-AGGGCAGGGACGTAATCAACG-3′ as reverse, which amplify a ~600-bp region of the 18*S* rRNA gene. PCR products were electrophoresed in a 1% (w/v) agarose gel using TBE buffer containing 1 μg/mL ethidium bromide. A single ~600-bp band of DNA was cut and extracted from the gel using the Core Bio Gel Extraction Kit. The sequence was determined by the CinnaGen Company with the primers. Sequence similarity searches were done with BLAST through the databases of NCBI and the software GeneDoc.

Biotransformation. The fermentation experiments were conducted in 250 mL conical flasks, each containing 50 mL of BG-11 liquid media. A fresh culture of *Chlorella vulgaris* was used as inocula at a final cell density of approximately $2.8–3.0 \times 10^6$ cells mL^{-1}. Cell cultures were fed with 5 μL of monoterpene substrates in triplicate and incubated as described earlier. After 24 hours (analysis time), the cultures were sampled and the samples extracted with dichloromethane for 2 min and centrifuged. The dichloromethane extract for each analysis was reduced under nitrogen gas to 100 μL prior to TLC and GC/MS-analysis. For each substrate, control experiments were also carried out, where a sterilized conical flask containing 50 mL of medium was fed with standard samples, and after incubation the cells were analyzed by GC/MS. Furthermore, the dichloromethane extracts containing the cells were examined by GC/MS to determine the presence of monoterpenes in non-transformed cells.

From (+)-carvone we obtained *trans*-dihydrocarvone and *cis*-dihydrocarvone with 68 and 15% yield, and from (–)-menthone and (+)-pulegone we obtained menthol with 43 and 38% yield, respectively.

Growth Determination. Cell density (number of cells mL^{-1}) was determined by linear absorbance at 680 nm and direct counting using a light microscope with a 0.1 mm deep counting chamber (Neubauer haemocytomer).

Thin Layer Chromatography. Qualitative analysis of the reaction products was carried out by TLC on a 0.25 mm thick layer of silica gel G (Kieselgel 60 $HF_{254+366}$, Merck). The TLC solvent system was *n*-hexane–diethyl ether (1:1, v/v), and the spots were visualized by iodine vapor and spraying of the plates with a mixture of vanillin–sulfuric acid (6:1, v/v) and heating in an oven at 100°C for 3 min until color development. The compounds were also visualized under a UV lamp at 254 nm.

The qualitative identification was based on the R_f values of the investigated compounds and the color of the detected compounds during TLC. Control flasks were also extracted using the same procedure as described above and analyzed by TLC [8].

GC/MS Analysis. The GC/MS analyses were carried out using a Hewlett-Packard 6890. The gas chromatograph was equipped with an HP-5MS capillary column (phenyl methyl siloxane, 25 m × 0.25 mm i.d.). The oven temperature was increased from 60°C to 200°C at the rate of 3°C/min and finally maintained at 200°C for 5 min. The carrier gas was helium at a flow rate of 1 mL/min, and the volume injected was 1 μL of the concentrated samples. The mass spectrometer was operated in EI mode at 70 eV. The interface temperature was 250°C, and the mass range was 30–600 *m/z*. The compounds were identified by comparing the retention indices of the peaks on the HP-5MS column with literature values, computer matching to the Wiley 275 Library, mass spectral database, and comparing the fragmentation patterns of the mass spectra with those reported in the literature [14]. Relative percentage amounts of the separated compounds were calculated from total ion chromatograms by the computerized integrator [15].

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