

# Microalgal transformation of progesterone by the terrestrial-isolated cyanobacterium *Microchaete tenera*

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**Abstract** A large number of microorganisms including various microalgal strains are able to convert steroid compounds into useful metabolites. In the present study, the ability of *Microchaete tenera*, a rice paddy field-isolated microalga, was investigated for biotransformation of progesterone. The incubation was carried out at 25°C under continuous illumination in the presence of 0.25 gL<sup>-1</sup> of progesterone. After 5 days incubation of the microalga in BG-11 liquid medium, the broth was extracted and the products were purified by the aid of chromatographic methods. Structure elucidation of the metabolites was performed by spectral data (<sup>13</sup>C NMR, <sup>1</sup>H NMR, FTIR, and MS) and physical constants (melting points and optical rotations). Eventually, four major steroids including 20β-hydroxypregn-4-en-3-one, 20α-hydroxypregn-4-en-3-one, 6β-hydroxypregn-4-en-3,20-dione and 6α-hydroxypregn-4-en-3,20-dione were the results of this biotransformation. The study also showed that the best concentration of starting material, temperature, photoregime, and the influence of CO<sub>2</sub> partial pressure on the production of

bioconverted metabolites were 0.25 gL<sup>-1</sup>, 25°C, continuous light and 2.0±0.1% (v/v), respectively. Highest concentrations of all biotransformed metabolites were obtained in the 5th day.

**Keywords** *Microchaete tenera* · Biotransformation · Progesterone · Steroid · Microalgae

## Introduction

*Microchaete tenera* Thuret ex. Bornet (Microchaetaceae) is a heterocystous cyanobacterium usually found in ponds, rice paddy fields, fresh water lakes, and marine environments (Rouf et al. 2010; Yunes et al. 1994). It is usually attached to submerged plants and other macrophytes and from there it develops radially on the water surface (Ariosa et al. 2005; Desikachary 1959). Although some research on ecological properties of *Microchaete* spp. has been conducted, there are very limited studies on the biological effects of the genus. However, extracellular metabolites of *M. tenera* showed plant growth regulatory effects on regeneration, pigments content and histology of rice callus when added to the medium of plant cell culture (Zaccaro et al. 2002). There are also a few reports on biosorption and detoxification of heavy metals and organic pollutants by the alga (Larsson et al. 2000; Mason et al. 1996). Antifungal activity of *M. tenera* also has been investigated. It was shown that the biomass of *M. tenera* inhibited growth of the fungus *Sphaeropsis sapinea* (Zulpa et al. 2003) and methanol extract of the species exhibited significant antibacterial and antifungal activity against *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Aspergillus niger* (Prashantkumar et al. 2006). Hajimahmoodi et al. (2010) presented antioxidant effects of both cells and extracellular substances of the algae using

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FRAP and DPPH-HPLC assays. Moreover, due to the nitrogen-fixing ability, producing growth-promoting substances as well as soil-conditioning capabilities, some strains of the algae have also been employed as soil biofertilizers (De Caire et al. 2000; Subramanian et al. 1994).

Mostly due to historical reasons, bacteria and fungi are the preferred microorganisms that have usually been used in the biotransformation of exogenous organic compounds. The first application of cyanobacteria in biotransformation of steroids goes back to 1986 when Abul-Hajj and Qian (1986) studied the ability to convert steroids using different algal strains. Since then, many studies on this feature of microalgae have been performed and a variety of products have been obtained (Faramarzi et al. 2008). Currently, steroids and their derivatives have been found extensive applications, such as anabolic, hormone replacement, progestational, antitumor agents, and oral contraceptives as well as sedatives. Research in the field of microbial biotransformation is being continued for developing of newer and more useful steroid analogues that are synthesized with great difficulty through chemical reactions (Al-Awadi et al. 2001; Bigdeli and Ahmadi 1991; Faramarzi et al. 2006).

The aim of the present study was to investigate the ability of *M. tenera* in biotransformation of progesterone, an important and widely used pharmaceutical steroid. Chemical structures of the produced metabolites were elucidated and the influences of steroid concentration, temperature, photoregime and CO<sub>2</sub> concentration on the production of bioconverted metabolites were also investigated.

## Materials and methods

Progesterone was obtained from Abureyhan Pharmaceutical Co. (Tehran, Iran) and was from Roussel (France).

Optical rotations were measured in the solvents specified for each product in 10-cm cells on a Perkin-Elmer 142 automatic spectropolarimeter. Melting points were determined on a melting point apparatus (Gallenkamp, UK) and were uncorrected. Mass spectra (MS) were obtained with a Finnigan MAT TSQ-70 instrument (Bremer, Germany) by electron impact at 70 eV. Infrared (IR) spectra were recorded using KBr disks on a Magna-IR 550 Nicolet FTIR spectrometer. The <sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance (NMR) spectra were obtained using a Bruker DRX (Avance 500) spectrometer at 500 and 125 MHz, respectively, with tetramethylsilane (TMS) as internal standard in CDCl<sub>3</sub>. Chemical shifts ( $\delta$ ) are given in parts per million (ppm) relative to TMS. The coupling constant (J) is given in hertz (Hz).

HPLC apparatus consisted of a Smartline HPLC pump 1,000, a PDA detector 2,800, and a degasser 5,000, all from Knauer (Germany). The data were acquired and processed

by means of ChromGate software (version 3.3.1) from Knauer. Samples (20  $\mu$ L) were injected using autosampler 3950 with a sample loop of 100  $\mu$ L. Chromatographic separation was achieved on a Lichrospher 100 RP & EC C8 reverse phase column (C8, 25 $\times$ 0.46 cm i.d., 5  $\mu$ m particle size) from Teknokroma (Spain).

The cyanobacterial strain, *M. tenera*, was isolated from soil in a recent study (Hajimahmoodi et al. 2010). DNA sequence analysis of phylogeny with that in the National Center for Biotechnology Information (NCBI) database further confirmed the species. Ribosomal RNA gene of the axenic strain was sequenced and deposited in NCBI under the accession no. of JF290484. It was grown and maintained in sterile BG-11 medium (Borowitzka 1988) agar slant and subcultured freshly before use in the biotransformation process. For short storage, the cyanobacterium was maintained at 4°C on BG-11. It was transferred to fresh medium every 2 months.

## Biotransformation conditions

The cells of *M. tenera* were inoculated into 20 500-mL Erlenmeyer flasks containing 100 mL of sterile BG-11 liquid medium enclosed with cotton plugs. pH was adjusted to 7.0 with 1 M HCl and/or NaOH prior to autoclaving at 121°C for 20 min. Flasks were illuminated at about 405  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup> supplied by three fluorescent tubes under continuous light photoregime from all sides at 25°C and bubbled with sterile air without shaking for 5 days. After this period, progesterone (dissolved in ethanol) was added at a final concentration of 0.25 gL<sup>-1</sup> under sterile conditions and incubation was prolonged for five more days. Control media without the algal cells were also incubated under the same condition.

## Extraction and purification of transformed products

After incubation, the broth was separated from the microalgal cells, washed with distilled water and extracted three times with equal volume of chloroform. The organic phase was separated, filtered, and the extract was evaporated under reduced pressure. The residue was spotted on F1500/LS 245 thin layer chromatography (TLC) sheets (Schleicher & Schuell, Germany) and fractionated using the solvent system n-hexane/ethyl acetate (6:4, v/v) and visualized by spraying the plates with a mixture of phosphoric acid (85%)/distilled water (1:1, v/v) and heating in an oven at 110°C for 10 min until the colors developed. Residue was dissolved, loaded and chromatographed on preparative TLC using a solvent system of acetone/chloroform/ethyl acetate (3.5:5.5:1, v/v/v). The purified metabolites were crystallized in appropriate solvents and then identified using spectral data (<sup>13</sup>C NMR, <sup>1</sup>H NMR, FTIR and MS) and their physical constants (melting points and optical rotations).

Time course study and influences of temperature, photoregime, and the steroid concentration

For a time course study, a 5-day growth cell of *M. tenera* was transferred into a 500-mL Erlenmeyer flask containing 100 mL of BG-11 medium supplemented with  $0.25 \text{ gL}^{-1}$  of progesterone and then the incubation continued for 12 days under continuous light. Sampling was carried out every day. Control was similarly processed without the microalga. The optimum substrate concentration was determined using concentrations of 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, and  $0.5 \text{ gL}^{-1}$ . The temperature was varied from  $20^\circ\text{C}$  to  $40^\circ\text{C}$  with an interval of  $5^\circ\text{C}$ . In order to evaluate the impact of the photoregime on the biotransformation yield, three programs including continuous illumination at about  $405 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , 16/8-h light/dark periods, and complete dark were used. For each experiment, only one parameter was changed at a time. The procedure was carried out in triplicate for each analytical determination.

Biotransformation of progesterone in a two-tier culture vessel

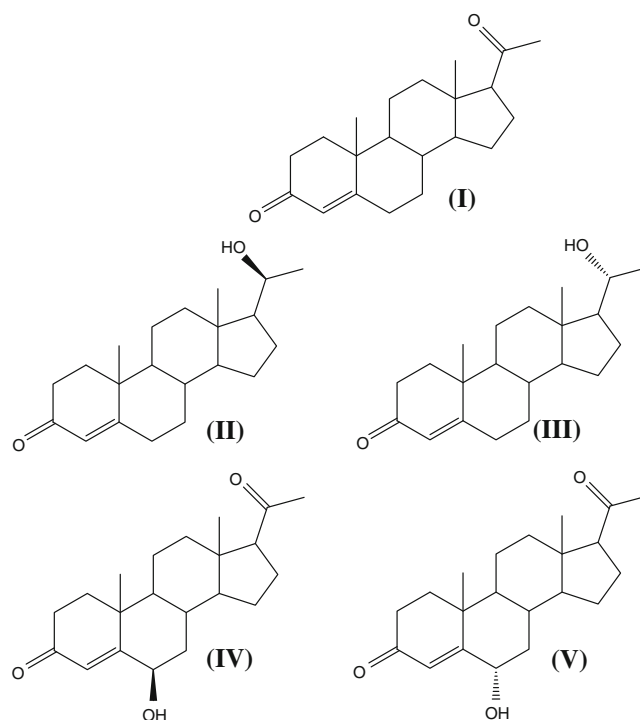
Biotransformation of progesterone was also examined in a two-tier flask consisting of two 1-L small-neck Erlenmeyer flasks containing 200 mL of BG-11 liquid medium (broth) in upper compartment and 200 mL  $\text{KHCO}_3/\text{K}_2\text{CO}_3$  buffer mixture in lower compartment at ratios of 50/50, 62/38, 73/73, and 73/27 from stock solution of 3.0, 3.0, 3.0, and 4.0 moles  $\text{L}^{-1}$  which generated  $\text{CO}_2$  partial pressure of  $0.5 \pm 0.1$ ,  $1.0 \pm 0.1$ ,  $2.0 \pm 0.1$ , and  $3.0 \pm 0.1\%$  (v/v), respectively (Tripathi et al. 2001). After 5 days of the algal growth, the steroid ( $0.25 \text{ gL}^{-1}$ ) was added to the upper flask. Harvesting was the same as described previously. The results were compared with those presented earlier.

HPLC analyses

Quantitative studies were performed by HPLC. Detection was done by a Knauer PDA detector 2,800 at 254 nm. The applied isocratic solvent was methanol/water (63:37, v/v) with a flow rate of  $1.0 \text{ mL min}^{-1}$  at  $25^\circ\text{C}$ .

## Results and discussion

After 5 days incubation of *M. tenera* in the presence of progesterone (I), the starting material was transformed into four major monohydroxylated derivatives (II to V; Fig. 1). The produced metabolites were isolated and purified by chromatographic methods. According to TLC profile, it was found that all the resulting compounds were more polar



**Fig. 1** Chemical structures of progesterone (I) and its metabolites produced by *M. tenera*:  $20\beta$ -hydroxyprogesterone-4-en-3-one (II),  $20\alpha$ -hydroxyprogesterone-4-en-3-one (III),  $6\beta$ -hydroxyprogesterone-4-en-3,20-dione (IV), and  $6\alpha$ -hydroxyprogesterone-4-en-3,20-dione (V)

than progesterone itself. No biotransformation occurred in control medium. Steroid products were then identified using spectral data ( $^{13}\text{C}$  NMR,  $^1\text{H}$  NMR, FTIR, and MS), melting points and optical rotations. HPLC profile of the extract presented well resolved peaks (chromatogram not shown). There were also other minor metabolites found in the transformed mixture, which was not purified for characterization due to trace quantity.

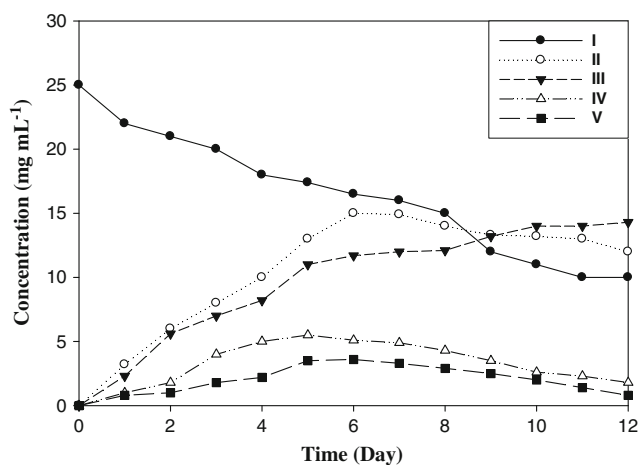
***20β-Hydroxyprogesterone-4-en-3-one (II)*** This compound was crystallized from chloroform; yield, 18.2%; m.p.,  $167\text{--}169^\circ\text{C}$ ,  $[\alpha]_{\text{D}} +86^\circ$  ( $\text{CHCl}_3$ ); lit (Hill et al. 1991); m.p.,  $171\text{--}172^\circ\text{C}$ ,  $[\alpha]_{\text{D}} +88^\circ$  ( $\text{CHCl}_3$ ); *Rf* (hexane/ethyl acetate, 6:4 (v/v)): 0.53; retention time (methanol/water, 63:37 (v/v)): 40.83 min. Chemical structure of compound II was confirmed according to the spectral analyses (data not shown) and comparison with those published by Farooq et al. (1994).

***20α-Hydroxyprogesterone-4-en-3-one (III)*** This compound was crystallized from methanol; yield, 15.7%; m.p.,  $154\text{--}157^\circ\text{C}$ ,  $[\alpha]_{\text{D}} +101^\circ$  ( $\text{CHCl}_3$ ); lit (Hill et al. 1991); m.p.,  $160.5\text{--}161^\circ\text{C}$ ,  $[\alpha]_{\text{D}} +102^\circ$  ( $\text{CHCl}_3$ ); *Rf* (hexane/ethyl acetate, 6:4 (v/v)): 0.48; retention time (methanol/water, 63:37 (v/v)): 28.2 min. Chemical structure of compound III was elucidated based on comparing the obtained spectral analyses (not shown) with those of published data (Al-Awadi et al. 2001).

**6 $\beta$ -Hydroxypregn-4-en-3,20-dione (IV)** This compound was crystallized from chloroform; yield, 8.6%; m.p., 169–172°C,  $[a]_D +103^\circ$  (CHCl<sub>3</sub>); lit (Hill et al. 1991); m.p., 174–177°C,  $[a]_D +105^\circ$  (CHCl<sub>3</sub>); *R<sub>f</sub>* (hexane/ethyl acetate, 6:4 (v/v)): 0.33; retention time (methanol/water, 63:37 (v/v)): 11.5 min. The structure of compound IV was proved depend on its spectral data (not shown) with those of the literature (Al-Awadi et al. 2001).

**6 $\alpha$ -Hydroxypregn-4-en-3,20-dione (V)** This compound was crystallized from chloroform; yield, 4.2%; m.p., 208–210°C,  $[a]_D +176^\circ$  (CHCl<sub>3</sub>); lit (Hill et al. 1991); m.p., 211–213°C,  $[a]_D +179.6^\circ$  (CHCl<sub>3</sub>); *R<sub>f</sub>* (hexane/ethyl acetate, 6:4 (v/v)): 0.22; retention time (methanol/water, 63:37 (v/v)): 8.98 min. Chemical structure of compound V was confirmed according the spectral analyses (data not shown) and comparison with those published by Al-Awadi et al. (2001).

For a time course study, the production of II–V, as a function of time, were detected by HPLC (Fig. 2). The starting material, progesterone at 0.25 gL<sup>-1</sup>, was transformed into four metabolites within 5 days. Compounds II and III appeared in the broth from the first day while compounds IV and V were produced from the second day. Compounds II and V were reached their highest concentrations at the sixth day whereas the highest level of compounds IV were on day 5. Compound III increased throughout the experiment. The HPLC profile followed up to 12 days showed that after the 8th day, IV and V disappeared in the broth while the rest of the compounds remained in the fermentation liquid. The highest yield of bioconversion of progesterone was obtained at 25°C for producing of all the metabolites. Increasing temperature over 30°C, production of the metabolites reduced and



**Fig. 2** Time course profile for the biotransformation of progesterone (I) by *M. tenera*: 20 $\beta$ -hydroxypregn-4-en-3-one (II), 20 $\alpha$ -hydroxypregn-4-en-3-one (III), 6 $\beta$ -hydroxypregn-4-en-3,20-dione (IV), and 6 $\alpha$ -hydroxypregn-4-en-3,20-dione (V)

eventually at 40°C, the substrate remained unconverted in the broth and no bioconversion occurred. The effect of the substrate concentration in the range of 0.05 to 0.4 gL<sup>-1</sup> on progesterone biotransformation by *M. tenera* was also studied. Increasing concentration of progesterone from 0.05 to 0.4 gL<sup>-1</sup> showed that optimum concentration of the substrate, which gave maximum bioconversion efficiency, was 0.25 gL<sup>-1</sup> and higher concentrations of the substrate ( $\geq 0.4$  gL<sup>-1</sup>) inhibited the bioconversion completely. The influence of photoregime on the bioconversion of progesterone by *M. tenera* was examined under continuous illumination, continuous dark, 16/8 light/dark photoperiods and it was found that continuous illumination gave better results. The effect of aeration on the production of steroids using two-tier culture vessel was also evaluated, it was found that CO<sub>2</sub> partial pressure of 2.0 $\pm$ 0.1% (v/v) produced by a 3-molar solution of KHCO<sub>3</sub>/K<sub>2</sub>CO<sub>3</sub> (73:27) buffer mixture, had the highest effect on metabolites production.

Bioconversion of progesterone with *M. tenera* in appropriate condition resulted in formation of steroid metabolites II to V (Fig. 1). It has already been shown that some bacterial, fungal and algal species are capable of performing various transformations on pregnane-based compounds (Faramarzi et al. 2008; Mahoto and Mukherjee 1984). The present work shows that *M. tenera* is able to convert progesterone into four steroid metabolites. The biotransformation characteristics observed were included C-6 $\alpha$ - and C-6 $\beta$ -hydroxylations and 20-carbonyl reduction. The 20 $\alpha$ - and 20 $\beta$ -hydroxy derivatives were produced in higher amounts compared with C-6 $\alpha$ - and C-6 $\beta$ -hydroxy steroids which accumulated in the algal medium in moderate amounts. 20-Ketone reduction naturally occurs during the metabolism of progesterone by liver microsomes, and subsequently 20 $\alpha$ - and 20 $\beta$ -progesterone are produced. These metabolites have progestational activity lower than progesterone itself, but 20 $\alpha$ -hydroxyprogesterone shows in vitro stimulatory effects on GnRH secretion similar to progesterone (Rasmussen and Yen 1983). 6 $\alpha$ -Hydroxyprogesterone is another natural occurring metabolite which is produced during the metabolism of progesterone, especially by fetal liver and placental enzymes during pregnancy (Lisboa and Gustafsson 1969). In the study of Afzal et al. (2010), it was found that progesterone transformation products including 20 $\alpha$ -, 6 $\alpha$ -, and 6 $\beta$ -hydroxyprogesterone exhibit antioxidant activity more than progesterone alone. 6 $\beta$ -Hydroxylation has been reported by the isolated cytochrome P-450 enzyme and various *Bacillus thermoglucosidasius* (Sideso et al. 1998; Wang et al. 2000). By contrast, 6 $\alpha$ -hydroxylation is relatively rare being confined to a few filamentous fungi, e.g., *Absidia orchidis*, *Rhizopus nigricans*, *Aspergillus flavus*, *Calonectria decora*, *Diaporthe celastrina*, and *Rhizopus*



*arrhizus* and a few *Bacillus* species (Sideso et al. 1998; Walaa et al. 2009). Biotransformation of progesterone by microalgal cultures has been already studied by some research groups, among them, Pollio et al. (1994, 1996) reported the reduction of 20-ketone group with some species of *Galdieria* and *Cyanidium* and 6 $\beta$ -hydroxylation with species of *Chlorella*, *Muriella*, and *Selenastrum*. The present study shows that *M. tenera* possesses 6 $\alpha$ - and 6 $\beta$ -hydroxylases active for progesterone. However, this is the first report of the production of 6 $\alpha$ -hydroxyprogesterone by an algal species.

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