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C-20 KETONE REDUCTION OF HYDROCORTISONE BY RICE FIELD MICROALGA *Chlorella vulgaris* MCCS 013

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A unicellular microalga, Chlorella vulgaris*, was isolated from rice field and applied in the biotransformation experiment of hydrocortisone (**1**). This strain has not been previously tested for hydrocortisone bioconversion. Fermentation was carried out in BG-11 medium supplemented with 0.05% substrate at 25°C for 14 days incubation. The products obtained were chromatographically purified followed by their characterization using spectroscopic methods. 11β,17α,20β,21-Tetrahydroxypregn-4-en-3-one (**2**), 11β,17β-dihydroxyandrost-4-en-3-one (**3**), and 11β-hydroxyandrost-4-ene-3,17-dione (**4**) were the main bioproducts in the hydrocortisone bioconversion. Bioreaction characteristics observed were 20-ketone reduction for accumulation of compound **2** and side chain degradation of the substrate to prepare compounds **3** and **4**. Time course study showed the accumulation of the product **2** from the second day of the fermentation and **3** as well as **4** from the third day. All the metabolites reached their maximum concentration in seven days. Microalgal 18S rRNA gene was also amplified by PCR. PCR products were sequenced to confirm their authenticity as 18S rRNA gene of microalgae. The result of PCR blasted with other sequenced microalgae in NCBI showed 100% homology to the 18S small subunit rRNA of six strains of* Chlorella vulgaris.

Key words: *Chlorella vulgaris*, biotransformation, hydrocortisone.

The potential use of microalgae to modify exogenous steroids has been supported in several of our publications [1–4]. Consequently their culture is simpler and cheaper than that of bacteria, yeasts, or fungi. They are easily and rapidly cultured in an inexpensive medium containing simple salts, which decreases the probability of contamination by other microorganisms. The use of microalgae for steroid bioconversion was firstly reported by Abul-Hajj and Qian [5]. They examined the conversion of 4-androstene-3,17-dione to some hydroxylated derivatives with different strains of microalgae such as *Anabaena cylindrica*, *Scenedesmus quadricauda*, and *Coelastrum proboscideum*. More recently, the ability of green algae for transformation of progesterone, prednisolone, and some other steroids has been reported [5, 6]. Only a few studies have so far been done to use microalgal strains isolated from local habitats for organic compounds bioconversion [4].

Preliminary taxonomical studies show that *Chlorella vulgaris* seems common in the paddy fields of Fars province located in south of Iran, besides microalgae like *Oocystis*, *Scenedesmus,* and some unicellular and filamentous cyanobacteria [7]. Several studies have been done to apply *Chlorella* spp. for biotransformation of organic compounds. In one study, the *Chlorella vulgaris* was screened for its ability to transform progesterone [6]. In other investigations, some steroid estrogens was transformed by *Chlorella vulgaris* [8].

In pursing our works on the bioconversion of steroids by *Nostoc muscorum* [3, 9], *Fischerella ambigua* [4, 10], and *Nostoc ellipsosporum* [2], the biotransformation of hydrocortisone as an exogenous steroid was carried out by a locally isolated strain of a unicellular microalga, *Chlorella vulgaris*. Until today, *Chlorella vulgaris* has not been examined in transformation of hydrocortisone. The aim of this study is to identify the ability of locally isolated *Chlorella vulgaris* to convert hydrocortisone as an exogenous substrate.

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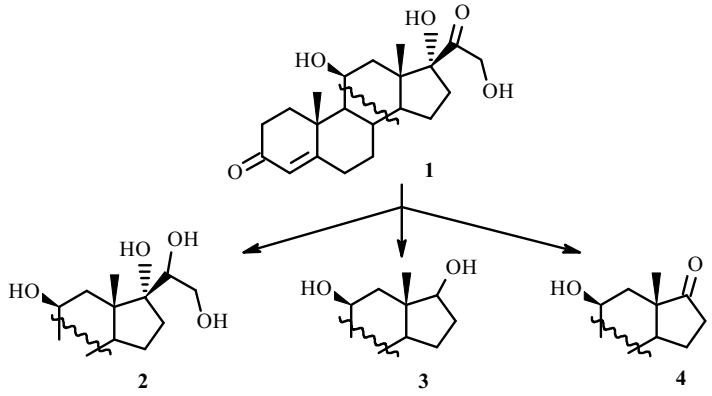
The partial sequence of the 18S rRNA gene of the *Chlorella vulgaris* MCCS 013 is as follows:

5′-tgccaaggatgttttcattaatcaagaacgaaagttgggggctcgaagacgattagataccgtcctagtctcaaccataaacgatgccgactaggga tcggcggatgtttcttcgatgactccgccggcaccttatgagaaatcaaagtttttgggttccgggggggagtatggtcgcaaggctgaaacttaaaggaatt gacggaagggcaccaccaggcgtggagcctgcggcttaatttgactcaacacgggaaaacttaccaggtccagacatagtgaggattgacagattgaga gctctttcttgattctatgggtggtggtgcatggccgttcttagttggtgggttgccttgtcaggttgattccggtaacgaacgagacctcagcctgctaaata gtcacggttggttcgccagccggcggacttcttagagggactattggcgactagccaatggaagcatgaggcaataacaggtctgtgatgcccttagatg ttctgggccgcacgcgcgctacactgatgcattcaacgagcctagccttggccgagaggcccgggtaatcttcgaaactgcatcgtgatggggat agattattgcaattattaat-3′.

The DNA sequence of the 18S rRNA gene of the *Chlorella vulgaris* MCCS 013 was recorded in the NCBI under the accession number EU374171.

The result of PCR blasted with other sequenced microalgae in NCBI showed 100% homology to the 18S small subunit rRNA of six strains of *Chlorella vulgaris*.

The crude extract obtained from 14 days incubation of *Chlorella vulgaris* in the presence of hydrocortisone produced three steroid compounds, 11β,17α,20β,21-tetrahydroxypregn-4-en-3-one (**2**), 11β,17β-dihydroxyandrost-4-en-3-one (**3**), and 11β-hydroxyandrost-4-ene-3,17-dione (**4**), in addition to the substrate **1** as follows:



The mass spectrum of metabolite **2** showed the molecular ion at *m/z* 364, which indicated the addition of two units as compared to that of hydrocortisone (*m/z* 362). It can be imagined that one carboxyl group or double bond in the substrate has been reduced. The IR spectrum showed only one carbonyl group at 1661 cm^{-1}, indicating that the conjugated ketone in C-3 position has not been altered. The elimination of C-20 carbonyl group absorption in the IR spectrum showed that the reduction had taken place at C-20. Additional multiplet resonance at δ 3.70 in the ^{1}H NMR spectrum as compared to the substrate confirmed the metabolite **2**. In addition, the other notable observation obtained was stereospecific reduction at the C-20 ketone group. The configuration of the C-20 hydroxyl group was recognized mainly with comparison of its melting point with the compounds having α-hydroxyl and β-hydroxyl groups at C-20 [9]. Melting point value of the metabolite **2** was similar to the compound with β-hydroxyl group at C-20 position.

The mass spectra of compound **3** and **4** showed the molecular ions at *m/z* 304 and 302, respectively, suggesting the reduction of 58 and 60 units of *m/z* as compared to hydrocortisone (*m/z* 362). The IR spectra indicated the existence of at least one hydroxyl group in compounds **3** and **4**. Furthermore, in compound **3**, the IR spectra showed only one carbonyl group at 1657 cm^{-1}, which was conjugated to C4–C5 double bond. The resonances at δ 3.82 and 4.37 in the ^{1}H NMR spectra clearly showed the existence of two hydroxyl groups. The chemical shift of H-11 was reported for hydrocortisone and other 11-hydroxylated steroids in δ 4.3–4.4 [11], so the resonance in δ 3.82 has been attributed to CH–OH in C-17. These data were supported by ^{13}C NMR, which showed a downfield resonance at δ 84.6 for CH–OH in C-17. In compound **4**, the IR spectra showed two absorptions at 1663 and 1734 cm^{-1}, which confirmed the existence of two carbonyl groups in C-3 and C-17, respectively. These IR data have also been supported by the related ^{13}C NMR spectra. Two signals at δ 199.9 and 219.8 in ^{13}C NMR spectra have been imputed to C-3 and C-17, respectively. 11β,17β-Dihydroxyandrost-4-en-3-one (**3**) and 11β-hydroxyandrost-4-ene-3,17-dione (**4**) both were less polar (R_f 0.6 and 0.7, respectively) than the substrate (R_f 0.45), and the other metabolite, 11β,17α,20β,21-tetrahydroxypregn-4-en-3-one (**2**), was much more polar (R_f 0.18). The mass spectrum of metabolite **2** showed the molecular ion at *m/z* 364. It can be imagined that one carbonyl group or double bond in the substrate has been reduced. The IR spectrum showed that the reduction had taken place at C-20. Additional multiplet resonance at δ 3.70 in the ^{1}H NMR spectrum as compared to the substrate confirmed the metabolite **2** [3, 4].

The mass spectra of compounds **3** and **4** showed the molecular ions at *m/z* 304 and 302, respectively. The IR spectra of compound **3** showed only one carbonyl group at 1657 cm^{-1}, which was conjugated to C4-C5 double bond. The resonances at δ 3.82 and 4.37 in the ^{1}H NMR spectra clearly showed the existence of two hydroxyl groups. These data were supported by ^{13}C NMR, which showed a downfield resonance at δ 84.6 for CH–OH in C-17. In compound **4**, the IR spectra confirmed the existence of two carbonyl groups in C-3 and C-17. These IR data have also been supported by the related ^{13}C NMR spectra. Two signals at δ 199.9 and 219.8 in the ^{13}C NMR spectra have been imputed to C-3 and C-17, respectively [4]. As these results show, the isolated alga may be considered useful biocatalysts for some kinds of biotransformation. It has a potential for site- and regioselective bioconversion of hydrocortisone and probably other pregnane-like steroids. *Chlorella vulgaris* was also shown to convert hydrocortisone at different concentrations between 0.5 to 2.5 mg/mL. Based on TLC profile, the best substrate concentration was 1 mg/mL, and in a concentration above 2.5 mg/mL, hydrocortisone was not converted to any metabolite.

To sum up, as far as we know, *Chlorella vulgaris* transformation of hydrocortisone has never been reported before. A few studies have been done to apply unicellular microalgae isolated from local habitats for biotransformation of organic compounds. In one study, a *Chlamydomonas* species was able to transform naphthalene to 1-naphthol [12]. The reduction of the carbonyl group in 3-acetylisoxazole by *Synechococcus elongatus* PCC 7942 gave the corresponding (*S*)-alcohols with high enantioselectivity [13]. Bacteria and fungi are the microorganisms usually employed in steroid biotransformation, while microalgae have been less investigated up to now [4]. There are also some limited reports on the conversion of steroid substances using strains belonging to the microalgae [5, 6, 14]. The use of microalgae to produce biologically active compounds is an interesting research subject because of the simple nutritional demands of these microorganisms.

Hydrocortisone is one of the most useful intermediates for the production of some valuable pharmaceutical steroid compounds and has been used in many studies as a substrate in biotransformation experiments [15].

Although some of the bioconversions on steroid compounds are well established, efforts are ongoing to identify new microorganisms capable of performing useful bioconversions. Earlier studies have clearly demonstrated that bacteria and fungi metabolize hydrocortisone. 1,2-Double bond formation using *Cylindrocarpon radicicola*, *Streptomyces lavendulae*, *Fusarium caucasicum*, *Fusarium solani*, and *Septomyxa affinis* and 1-dehydrogenation of hydrocortisone to prednisolone by *Arthrobacter simplex*, *Bacillus sphaericus*, and *Bacterium cyclooxydans* have already been applied in industrial production [4].

In our previous papers on hydrocortisone bioconversion using microalgal strains, it was reported that *Nostoc muscorum* [3], *Fischerella ambigua* [4], and *Nostoc ellipsosporum* [2] converted the substrate into some pregnane- and androstane-derived products. The main characteristics observed were hydrocortisone side chain cleavage and C-20 ketone reduction. Here, we also found that the isolate strain of *Chlorella vulgaris* transformed hydrocortisone at the same characteristics to obtain some androstane- and pregnane-like steroids.

Among all of the microalgae studied for 20-ketone reduction of hydrocortisone biotransformation, it seems that *Chlorella vulgaris* is being reported for the first time. The biotransformation observed in this work may be a research subject for further studies in using this microorganism and other related unicellular microalgae.

EXPERIMENTAL

Instrumental Analyses. Melting points (mp) were determined on a Reichert-Jung hot stage melting point apparatus. Optical rotations were measured in 1 dm cells on a Perkin–Elmer 142 automatic spectropolarimeter. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker (DRX-500 Avance) NMR spectrometer in $CDCl_3$ with tetramethylsilane (TMS) as internal standard. Chemical shifts (δ) are given in parts per million (ppm) relative to TMS. Coupling constants (J) are given in hertz (Hz). Infrared (IR) spectra were recorded on a Magna-IR 550 Nicolet FTIR spectrometer. Mass spectra (MS) were obtained with a Hewlett-Packard 6890 instrument by electron impact (EI) at 70 eV. Thin layer chromatography (TLC) and preparative TLC were performed, respectively, on 0.25 and 0.5 mm thick layers of silica gel G (Kieselgel 60 $HF_{254+366}$, Merck). Layers were prepared on glass plates and activated at 105°C 1 h before use. Chromatography was performed with acetone–hexane (1:1, v/v) and visualized by spraying the plates with a mixture of methanol–sulfuric acid (6:1, v/v) and heating in an oven at 100°C for 3 min until the colors developed. The compounds were also visualized under a UV lamp (Strstedt-Gruppe HP-UVIS) at 254 nm.

Chemicals. Hydrocortisone was kindly donated by Aburaihan Pharmaceutical Co. (Tehran, Iran), which had been purchased from Pharmacia & Upjohn S.A. (Guyancourt, USA). Other reagents and solvents were from Merck.

Collection, Preservation, and Identification of the Alga. The microalga was isolated during a screening program from soil samples collected from paddy fields of Shiraz located in the southern part of Iran (Fars province) from April to December, 2004. Primary culturing was done in BG-11 medium [1]. After colonization, pure cultures of living specimens were prepared using subculturing with agar plate in BG-11 medium [16]. Preserved specimens were prepared and the living specimens were incubated in 50 mL conical flasks under unlimited carbon dioxide condition (using CO_2 enrichment condition). Constant illumination was used at 60 uE/m·s intensity with white fluorescent lamps. Temperature was 25±2°C. The identification was done using famous manuals [17, 18].

Identification of the Algal Strain. The strain was identified by morphological characterization and assigned according to 18S rRNA gene sequence. The classification of the isolate alga was performed by Micoralgal Culture Collection of Shiraz University of Medical Sciences, Faculty of Pharmacy, Shiraz, Iran, as a strain of *Chlorella vulgaris* MCCS 013.

Incubation Condition. The fermentation experiments were conducted in twenty 500 mL conical flasks, each containing 100 mL of BG-11 liquid medium. Inocula from the fresh culture of *Chlorella vulgaris* was used at a final cell density of approximately 2.6–3 × 10^6 cells mL^{-1} and illuminated continuously with fluorescent lamps at 60 uE/m·s intensity, and incubated at a temperature of 25±2°C with shaking at 80 rpm for seven days. Hydrocortisone (1g) was dissolved separately in 20 mL of ethanol. One milliliter of the ethanol solution was added to each 500 mL conical flask (final concentration of the substrate was 0.05% in each flask). Incubation was continued for another 14 days at the same conditions and the control was similarly processed without the microorganism. We also examined the optimum substrate concentration. The amount of the substrate varied from 0.025 to 0.2 g 100 mL^{-1} with a step of 0.25. The results were obtained according to TLC analyses.

Cell density (number of cell mL^{-1}) was determined by both turbidity (optical density) and direct counting, using a light microscope with a 0.1 mm deep counting chamber (Neubauer haemocytomer). Correlation between these two methods was analyzed up to certainty [19].

Products Isolation and Analyses. At the end of incubation, the content of the flasks was extracted with three volumes of chloroform. The extract was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was loaded on preparative TLC and fractionated with a chloroform–acetone (1:1, v/v) solvent system and then the metabolite was crystallized in ethanol. Purified metabolite was identified by melting point and spectral data (^{13}C NMR, ^{1}H NMR, FTIR, and MS).

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 18S rRNA gene. The applied PCR condition has been described by Nubel et al. (1997). 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.

Metabolite 2 was crystallized from methanol; mp 132–134°C, $[\alpha]_D$ +91° (MeOH); lit. [4]: mp 133–135°C, $[\alpha]_D$ +85°; IR (ν_{max}, KBr, cm^{-1}): 3536, 2910, 1661; MS (EI) *m/z* (%): 364 (18) (M^+,$C_{21}H_{32}O_5$), 346 (19), 331 (8), 315 (56), 303 (46), 285 (100), 267 (31), 227 (64), 148 (38), 124 (40), 91 (82), 79 (55); ^{1}H NMR ($CDCl_3$, δ, ppm, J/Hz): 1.1 (3H, s, H-18), 1.43 (3H, s, H-19), 3.63 (2H, dd, J = 18.2, 4.9, H-21), 3.70 (1H, m, H-20), 4.37 (1H, s, H-11), 5.66 (1H, s, H-4); ^{13}C NMR ($CDCl_3$, δ, ppm): 199.8 (C-3), 172.7 (C-5), 122.1 (C-4), 84.2 (C-17), 74.3 (C-20), 68.2 (C-11), 64.2 (C-21), 55.3 (C-9), 50.5 (C-14), 46.6 (C-13), 41.3 (C-10), 39.4 (C-12), 33.7 (C-1), 33.2 (C-2), 32.8 (C-16), 32.1 (C-6), 29.7 (C-7), 29.3 (C-8), 23.6 (C-15), 20.8 (C-19), 17.7 (C-18); R_f 0.18 (chloroform–acetone; 1:1, v/v).

Compound 3 was crystallized from methanol; mp 240–242°C, $[\alpha]_D$ +164° (MeOH); lit. [3]: mp 241–243°C, $[\alpha]_D$ +142°; IR (ν_{max}, KBr, cm^{-1}): 3433, 2976, 1657; MS (EI) *m/z* (%): 304 (78) (M^+, $C_{19}H_{28}O_3$), 303 (20), 261 (100), 235 (55), 188 (20), 123 (60), 109 (50), 82 (85); ^{1}H NMR ($CDCl_3$, δ, ppm): 1.11 (3H, s, H-18), 1.47 (3H, s, H-19), 3.82 (1H, m, H-17), 4.37 (1H, m, H-11), 5.77 (1H, s, H-4); ^{13}C NMR ($CDCl_3$, δ, ppm): 199.9 (C-3), 171.4 (C-5), 122.7 (C-4), 84.6 (C-17), 67.1 (C-11), 55.4 (C-9), 49.4 (C-14), 46.2 (C-12), 43.2 (C-13), 38.4 (C-1), 35.6 (C-10), 35.5 (C-16), 34.3 (C-2), 32.6 (C-6), 32.2 (C-7), 32.6 (C-6), 31.1 (C-8), 25.8 (C-15), 21.3 (C-19), 15.3 (C-18); R_f 0.6 (chloroform–acetone; 1:1, v/v).

Metabolite 4 was also crystallized from methanol; mp 196–199°C, $[\alpha]_D$ +226° (MeOH); lit. [4]: mp 197–198°C, $[\alpha]_D$ +225° ($CHCl_3$); IR (ν_{max}, KBr, cm^{-1}): 3522, 1734, 1663; MS (EI) *m/z* (%): 302 (100) (M^+, $C_{19}H_{26}O_3$), 286 (32), 227 (41), 189 (64), 149 (40), 123 (80), 91 (80), 75 (60); ^{1}H NMR ($CDCl_3$, δ, ppm): 1.20 (3H, s, H-18), 1.52 (3H, s, H-19) , 4.52 (1H, s, H-11), 5.74 (1H, m, H-4); ^{13}C NMR ($CDCl_3$, δ, ppm): 219.8 (C-17), 199.9 (C-3), 171.7 (C-5), 122.8 (C-4), 68.6 (C-11), 57.1 (C-9), 52.8 (C-14), 47.1 (C-13), 39.7 (C-10), 41.4 (C-12), 31.4 (C-8), 35.7 (C-1), 35.4 (C-16), 34.2 (C-2), 32.2 (C-6), 31.9 (C-7), 22.1 (C-15), 21.5 (C-19), 16.3 (C-18); R_f 0.7 (chloroform–acetone; 1:1, v/v).

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REFRENCES

1. Y. Ghasemi, M. Tabatabaei Yazdi, A. Shafiee, M. Amini, Sh. Shokravi, and G. Zarrini, *Pharm. Biol.,* **42**, 318 (2004).
2. Y. Ghasemi, M. Tabatabaei Yazdi, A. Dehshahri, H. Niknahad, Sh. Shokravi, M. Amini, A. Ghasemian, and M. A. Faramarzi, *Chem. Nat. Comp*., **42**, 702 (2006).
3. M. Tabatabaei Yazdi, H. Arabi, M. A. Faramarzi, Y. Ghasemi, M. Amini, Sh. Shokravi, and F. Aziz Mohseni, *Phytochemistry*, **65**, 2205 (2004).
4. M. Tabatabaei Yazdi, Y. Ghasemi, A. Ghasemian, Sh. Shokravi, H. Niknahad, M. Amini, A. Dehshahri, and M. A. Faramarzi, *World. J. Microbiol. Biotechnol*., **21**, 811 (2005).
5. A. Pollio, G. Pinto, M. Della Greca, A. D. Maio, A. Fiorentino, and L. Previtera, *Phytochemistry*, **37**, 1269 (1994).
6. A. Pollio, G. Pinto, M. Della Greca, A. Fiorentino, and L. Previtera, *Phytochemistry*, **42**, 685 (1996).
7. Y. Ghasemi, S. Rasoul-Amini, M. H. Morowvat, S. B. Mosavi Azam, S. Shokravi, A. Mohagheghzadeh, M. B. Ghoshoon, and M. J. Raee, *Biotechnology*, **7** (2), 293 (2008).
8. K. M. Lai, M. D. Scrimshaw, and J. N. Lester, *Appl. Environ. Microbiol.*, **68**, 859 (2002).
9. M. A. Faramarzi, M. Tabatabaei Yazdi, A. Shafiee, and G. Zarrini, *Steroids*, **67**, 869 (2002).
10. Z. Moradpour, M. Torshabi, M. A. Faramarzi, M. Tabatabaei Yazdi, Y. Ghasemi, H. Jahandar, N. Zolfaghary, and G. Zarrini, *Res. J. Microbiol.,* **1**, 289 (2006).
11. D. N. Kirk, H. C. Toms, C. Douglas, and K. A. White, *J. Chem. Soc., Perkin Trans. II*, **9**, 1567 (1990).
12. S. J. Todd, R. B. Cain, and S. Schmidt, *Biodegradation*, **13**, 229 (2002).
13. K. Itoh, H. Sakamaki, K. Nakamura, and A. Horiuchi, *Tetrahedron Asymmetry*, **16**, 1403 (2005).
14. Y. J. Abul-Hajj and X. Qian, *J. Nat. Prod*., **48**, 244 (1986).
15. L. L. Smith, *Steroids*, in: H. J. Rehm and G. Reed, eds., *Biotechnology*, Vol. **6a**, Weinheim, Verlag Chemic GmbH, 1984, pp. 31–78.
16. M. M. Allen, *J. Appl. Phycol*., **4**, 1 (1968).
17. G. W. Prescott, *Algae of the Western Great Lake Areas*, W.M.C. Brown Company Publisher, Dubuque, Iowa, 1962.
18. D. M. John, B. A. Whitton, and A. J. Brook, *The Freshwater Algal Flora of the British Isles*, *an Identification Guide to Freshwater and Terrestrial Algae*, Cambridge, Cambridge University Press, 2003, pp. 39–43.
19. R. A. Andersen, *Algal Culturing Techniques*, Elsevier Academic Press, 2005, pp. 239–287.