J. Indian bot. Soc. ISSN - 0019 - 4468 Vol. 93 (1 & 2) 2014 : 105-110

TECHNOLOGY DEVELOPMENT FOR MASS PRODUCTION OF *SPIRULINA PLATENSIS* IBSD-174 AND ITS UTILIZATION AS NUTRIENT RICH FORMULATIONS

O. N. TIWARI, THINGUJAM INDRAMA, GUNAPATI OINAM, OINAM AVIJEET SINGH AND JERRY J. BRAND

Freshwater Cyanobacterial and Microalgal Repository Microbial Resources Division
Institute of Bioresources and Sustainable Development
(An autonomous Institute under the DBT, Govt. of India) Takyelpat, Imphal-795001, Manipur, India
Email : ontiwari1968@gmail.com
** UTEX The Culture Collection of Algae, The University of Texas at Austin, TX, USA

Spirulina platensis IBSD-174 was isolated from freshwater Loktak lake of Manipur, India during 2005-06 and partial characterization with special attention on morphological and biochemical has been done. The soluble protein content stabilized and enhanced up to 82.7±0.08 µg/ml with the treatment of different level of pH, nitrogen and phosphorus for consecutive three years in laboratory conditions. The strain was deposited and obtained accession number from the fresh water cyanobacterial and microalgal repository of IBSD-DBT, Imphal, Manipur, INDIA a creation of Department of Biotechnology, Govt. of India (Ref. No.: BT/PR11323/PBD/26/171/2008 dated 31st March 2009) for ready reference and commercial exploitation in future.

Keywords: *Spirulina platensis*, Loktak lake, Manipur, Accession number

Microalgae are the organisms capable of producing valuable metabolites such as pigments, proteins and vitamins for feed additive, pharmaceutical and nutraceutical purposes (Gladue and Maxey 1994, Cartens *et al.* 1996, Guerin *et al*. 2003, Hu 2004). *Spirulina platensis* is a planktonic photosynthetic filamentous cyanobacterium that forms massive populations in tropical and subtropical bodies of water which have high levels of carbonate and biocarbonate and alkaline pH of up to 11. *Spirulina platensis is* recognized by one main morphological feature *i.e.* the arrangement of multicellular cylindrical trichomes in an open left-hand helix along the entire length of the filament (Vonshak 1997). *Spirulina platensis* is the most popular in microalgal biotechnology with respect to its being an easy to grow species and having a simple harvest and drying process. *Spirulina platensis* has been produced commercially all over the world due to its high content of protein (up to 70%), pigments (especially the blue pigment, phycocyanin), essential fatty acid, vitamin B_{12} and minerals (Cohen 1997, Boussiba and Richmond 1980, Mahajan and Kamat 1995, Becker 1981). It is also successfully used in aquaculture and poultry industry as well (Belay *et al*. 1996, Wikdors and Ohno 2001).

High pH and temperature are the key factors for the large scale cultivation of *Spirulina* under outdoor cultures. The optimal temperature for the cultivation of *Spirulina* has been reported in the range of 35-38^{o}C. This microalga also requires relatively high pH in the range of 9.5-9.8 (Hu 2004) which inhibits the contamination of most algae in the culture. High amounts of sodium bicarbonate must always be present in the medium to sustain high pH and prevent fluctuations (Zarrouk 1966). Different types of culture systems *e.g.* open ponds (Lee 1997), tubular photobioreactors (Torzillo *et a1*. 1986), inclined glass panels (Hu *et al*. 1996) etc. are used in the mass production of *Spirulina.* On a commercial scale, *Spirulina* can be grown in raceway type open ponds which involve less investment and sun light can be utilized for photosynthesis (Torzillo *et al*. 1986). The objective of the study was to evaluate the influence of pH on the biomass and total soluble proteins of *Spirulina.* The study was also undertaken regarding its mass production under net house out door conditions and possibility of further utilization in the form of value added formulations.

MATERIALS AND METHODS

The experimental organism *Spirulina platensis* IBSD-174 was obtained from the freshwater cyanobacterial and microalgal repository (FWCMR) of IBSD-DBT, Imphal, Manipur, INDIA a creation of the Department of Biotechnology, Govt. of India with reference number BT/PR11323/PBD/26/171/2008

Received on April 11, 2012 Accepted on May 16, 2013

Table 1: Influence of pH on total soluble proteins (µg/ml) in *Spirulina platensis* - IBSD 174

pH	Total soluble proteins (µg/ml)
7.0	54.0±0.10
7.5	59.0±0.15
8.0	62.0±0.10
8.5	66.0±0.11
9.0	70.9 ±0.09
9.5	79.3 ±0.07
10.0	**82.7±0.08**
10.5	77.9±0.09

dated 31st March 2009. This strain was originally isolated from Loktak lake of Bishnupur, Manipur, India during 2005-06 through enrichment culture technique. The cyanobacterial strain *Spirulina platensis* IBSD-174 was grown and maintained in chemically defined modified CFTRI medium (Venkataraman *et al*. 1995) under diffused light intensity of 54-67.5 µmole $m^{-2}s^{-1}$ at 28±2°C temperature with 14:10 hrs light and dark phase. The purity and identification was authenticated based upon the keys given by Desikachary (1959). The effect of pH (6.0-10.5) was examined on total soluble proteins (Herbert *et al.* 1971). All the ingredients of CFTRI medium was added accordingly for 1L of medium and the contents were mixed properly with magnetic stirrer and the pH of the medium was adjusted (Thermo Scientific, ORION 2 STAR) using 1N NaOH and 1N HCl. The pure culture of *Spirulina platensis* IBSD-174 was inoculated in 5 litres of modified CFTRI medium (Venkataraman and Becker 1985) in plastic bucket and / or tubs of 15 litres capacity. The containers were kept in a net house covered with polythene sheets. The buckets and tubs were covered with white muslin cloth to prevent the growing cultures from contamination with dust particles and also to protect them from laying of eggs by mosquitoes and other insects. Mass cultivation was carried out under such conditions without providing any additional illumination or temperature regulation for fifteen days. The microalgal suspension was regularly mixed with the help of sterilized stainless steel rods. The algal biomass was harvested on 15th day of growth by filtering through a dense net. The harvested biomass was washed twice with distilled water to remove the adhering salts and dried in an oven at 30-50°C for 30-45 mins.

Isolation of genomic DNA and 16S rRNA analysis

Isolation of genomic DNA was done as per the method described by Tillett and Neilan, 2000 and 16S rRNA was carried out by using PCR mix for 25 µl reaction mixture containing 1X PCR (Tris-EDTA with $MgCl_2$) buffer, dNTP mix (25 mM each of dNTPs), Taq DNA polymerase, 0.3 µM forward primer (536f 5′ -GTGCCAGCAGCCGCGGTRATA -3′), 0.3 µM reverse primer (1488R 5′ -CGGTTACCTTGTTACGACTTCACC-3′), template DNA (50 ng) and sterile double distilled water.

The PCR conditions were set for 28 cycles with initial denaturation at 95°C for 5 mins then 95°C for 1 min, annealing at 55°C for 1 min and final extension at 72°C for 2 mins using thermal cycler (Mastercycler gradient, eppendorf).

The amplified PCR product was loaded with loading buffer in the ratio 5:1 in 2% agarose (Sigma) gel and ran in electrophoresis unit (Elchrom Scientific) using 1X TAE buffer (Promega) for 30 mins at 90V. The size of the PCR product was compared with 200 bp DNA markers (Himedia). The product bands were observed in Gel documentation system (Vilber Lourmet). The quality and quantity of amplified PCR product was checked by nanodrop (Biospectrometer, eppendorf).

Sequencing and analysis of the 16S rRNA gene

The amplified PCR products of the 16S rRNA were sequenced at NCCS-DBT, Pune using the same forward and reverse primers and nucleotide sequences obtained from DNA sequencing were compared with sequence information available in the National Center for Biotechnology Information (NCBI) database using BLAST (Altschul *et al*. 1990, 1997) with nucleotide option by logging onto http://

www.ncbi.nlm.nih.gov. The sequences retrieved were converted to FASTA format.

RESULTS AND DISCUSSION

The identification of the *Spirulina platensis* IBSD-174 was authenticated based upon microscopic examination and molecular tools. The microalga *Spirulina platensis* IBSD-174 is a multicellular filamentous cyanobacterium, whose cells (diameter from 1-12 µm) are organized in helicoidal trichomes without ramifications (Richmond 1990; plate 1b). The influence of growth conditions on the chemical composition of *Spirulina* has been studied by many researchers with the purpose of optimizing the production of economically and nutritionally interesting compounds (Cohen *et al*. 1993). The extraction of nutritionally active compounds in pure form is expensive but direct consumption of *Spirulina* as a nutraceutical food is a viable alternative. The pH of

Table 2. Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	
DQ279773.1	*Arthrospira platensis* strain Sp-5 16S ribosomal RNA, tRNA-Ile, and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	1478	1478	90%	0.0	90%	
EF432318.1	*Arthrospira platensis* Sp-16 16S ribosomal RNA, tRNA-Ile, and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	1476	1476	90%	0.0	90%	
EF432314.1	*Arthrospira platensis* Sp-8 16S ribosomal RNA, tRNA-Ile, and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	1476	1476	90%	0.0	90%	
EF432313.1	*Arthrospira platensis* Sp-7 16S ribosomal RNA, tRNA-Ile, and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	1476	1476	90%	0.0	90%	
EU427543.1	*Arthrospira platensis* HZ01 16S ribosomal RNA gene, partial sequence	1476	1476	90%	0.0	90%	
AY672713.1	*Arthrospira platensis* strain AICB 49 16S ribosomal RNA gene, partial sequence; tRNA-Ile and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	1476	1476	90%	0.0	90%	
DQ393282.1	*Arthrospira platensis* strain SAG 257.80 16S ribosomal RNA gene, partial sequence	1476	1476	90%	0.0	90%	
DQ393279.1	*Arthrospira platensis* strain NIES 39 16S ribosomal RNA gene, partial sequence	1476	1476	90%	0.0	90%	
AF329392.1	*Arthrospira* sp. FACHB438 16S ribosomal RNA gene, complete sequence; tRNA-Ile and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence	1476	1476	90%	0.0	90%	
EF432315.1	*Arthrospira platensis* Sp-12 16S ribosomal RNA, tRNA-Ile, and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene	1474	1474	85%	0.0	91%	

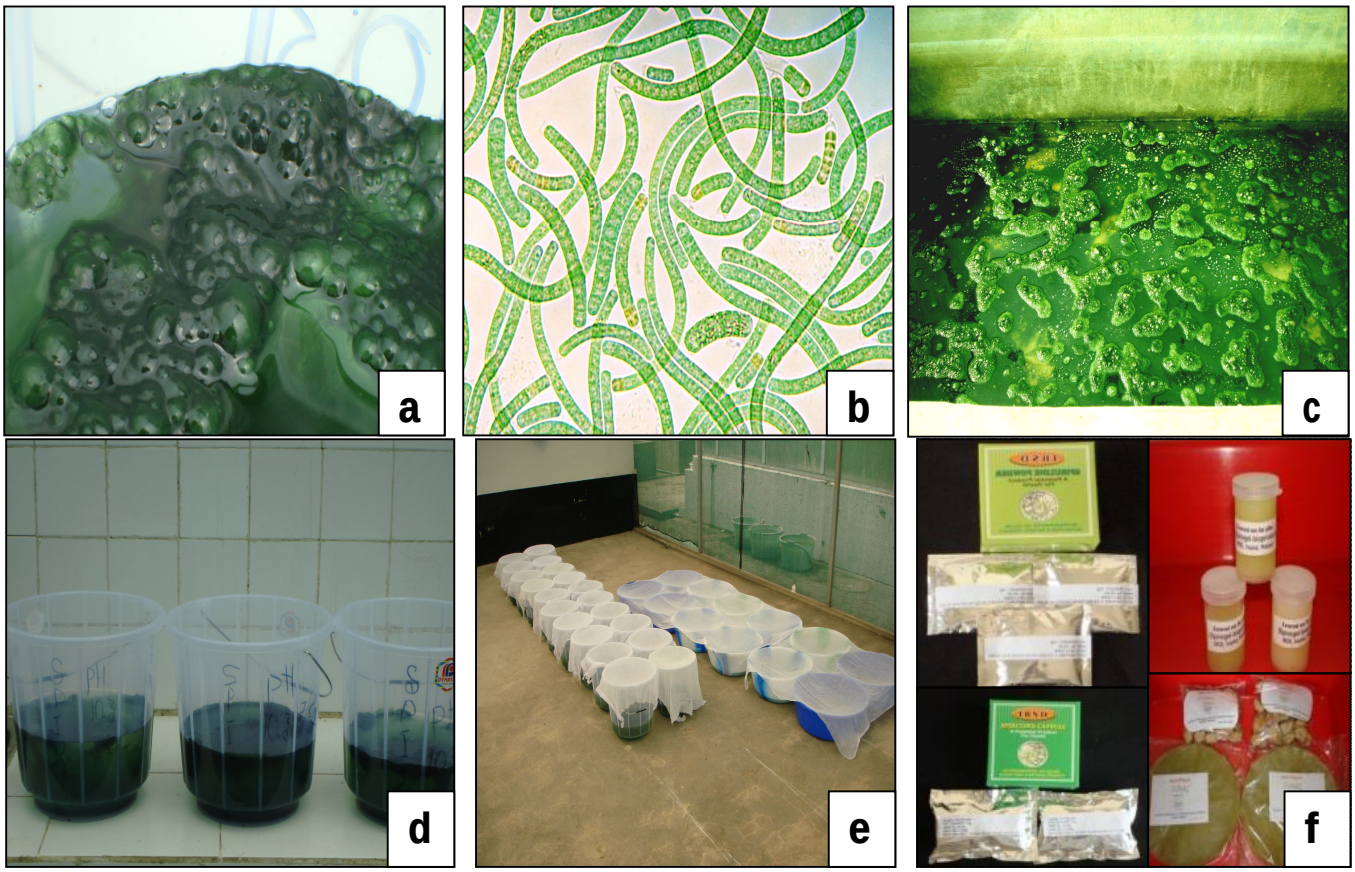

Plate 1(a-f):Growth of *Spirulina platensis* and its mass production for possible utilization as nutritionally rich formulations (a) *Spirulina* biomass in liquid culture (b) Photomicrograph showing *Spirulina* filaments (c) Growing *Spirulina* in tanks (d) Buckets with *Spirulina* (e) Growth units of *Spirulina* covered with muslin cloth (f) *Spirulina* formulations: Spiro-capsule, Spiro-powder, Spiro-gel, Spiro-bori and Spiro-papad developed with dried *Spirulina* biomass

the growing medium has been reported to be a very important parameter which influences the growth of the organism. The enhancement in the pH from 7.0 to 10.0 enhanced the level of total soluble proteins and further increase in pH decreased its content at 15th day of incubation (Table 1). These results are in accordance with the reports of other laboratories where an increase in pH above 10.5 was also shown to be harmful for the growth of this alkaline pH loving organism (Richmond *et al*. 1980, Goksan *et al*. 2007). The optimal growth temperature for *Spirulina platensis* was shown to be between 30^{o}C to 35^{o}C and higher temperature than this may certainly be deleterious for its growth (Danesi *et al*. 2001, Vonshak 1997). The *Spirulina* cultures grew efficiently as liquid culture and in plastic tubs or buckets (Plate-1 a, c, d) and muslin cloth cover prevented the contamination of *Spirulina* biomass (Plate-1 e). Detailed chemical composition of the harvested biomass indicated that the product had immense biotechnological potential (crude protein- 67.00%; crude lipids-3.1%; carbohydrate- 14.5%; nucleic acid- 4.5%; essential minerals-7.6% and vitamins-1.2%). Growth conditions optimized for biomass production and productivity were usually used in the commercial production of *Spirulina* without considering chemical composition, but higher concentrations of potentially useful compounds such as polyunsaturated fatty acids, proteins and phenolics can be obtained by manipulating growth conditions. According to Torzillo and Bernardini (1991), for outdoor cultures of *Spirulina*, upto 34% of the biomass produced during the daylight period may be lost through respiration at night such chemically analyzed biomass was utilized for the development of different formulations (Plate-1 f). Our studied strain showed maximum similarity with *Ar-*

throspira platensis strain Sp-5 16S ribosomal RNA, partial sequence bearing accession no. DQ279773.1 having 90% similarity revealing that morphological identification is similar with the molecular characterization.

> ibsd
1246bpCTTCCGGGGGCGTGGGTAACTCCCCCC
GTGTGACGGGGGGTGTGTACAAGGCCCGGGA
ACGGATTCACCGCGGTATGCTGACCCGCGAT
TACTAGCGATTCCACCTTCATGCACGCGATTT
GCAGACTGCAATCTAAACTGAAGAAAGTTTT
AGGGAATGGCTCCAGACCCCCGTCTTGGCTC
CCCTTGTACCCCCCCATTGAAAAAGGGGTGA
ACCCCAGGACAAAGGGCGCATGATACTTTGA
CTTCTCCCCCCCTTTCCCCCGGTTTGTCCCCG
GCATTCCCCCTAAAGTGCCCAATTGAATGATG
GCAACTAAGGACGAGGGTTGCGCTCGTTGCG
GGACTTAACCCAACATCTCACGACACGAGCT
GACGACAGCCATGCACCACCTGTGTTCCCGC
TCCCGAAGGCAATCACGGCTTTCACCAAGAT
TCCAGACATGTCAAGCCCTGGTAAGGTTCTTC
CCGTTGCATCCAATTAAACCACATACTCCACC
GATTGGGCGGGCCCCCGTCAATTCCTTTGAGT
TTCAGACTTGCGTGCGTACTCCCCAGGGGGG
AAACTTAACTCGTTAGTTTCGGCACAGCCCGG
GTCGATACAGGATACACCTAGTTTCCATTGTT
TACGGCTAGGACTACTGGGGTATCTAATCCC
ATTCGCTCCCCTAGCTTTCGTCCCTCAGTGTC
AGTTACGGCCCAGCAGAGCGCTTTCGCCACA
CGGTGTTATTCCCGATATCTACGCATTTCACC
GCTACACCGGGAATTCCCTCCGCCCCCACCGT
ACTCTAGGGGTTCAGTTTCCACTGCCTTTCAG
TAGTTGAGCTACTGTCTTTGACAGCAGATTTG
AACTACCACCTACACCCTCTGTACGCCCAATC
ATTCTGGCAAACAGTTACCTGGTGCGTATTAC
CGCGGTGCCGGGCACGGAGTTAGCCGAGGCT
TATTCCTCAAGTACCGTCATTGTGTTCTTCCTT
GAGAAAAGGGGTTTACAACCCAAGAGCCTTC
TTCCCCCTCGCGGTCTTGCTCCGTCAGGCTTG
CGCCCATTGCGGAAAATTCCCCGGAGTGCCT
CCCGTAGTCAGTCCCCGTGGTGTCAGTCATCA
GTGCAGACCTCATCCTGTCAGTCCCCTTGTGA
TCGTCGCCTCCCCTTGTATATCTCGCACACTC
ATCAAATAGGAGAGGGCCTCCAACATCTTCT
CCCGTAGGGCTCA

Authors are very much thankful to the DBT, Govt. of India for financial assistance and we express our sincere gratitude to the Director, IBSD-DBT, Imphal, Manipur for all kinds of support and help.

REFERENCES

Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ 1990 Basic local alignment search tool. J. Mol. Biol. **215**(3) 403-410.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ 1997 Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. **25** (17) 3389-3402.

Becker EW 1981 Algae mass cultivation, production and utilization. *Process Biochem.* **16** 10-14.

Belay A, Kato T and Ota Y 1996 *Spirulina* (*Arthrospira*): potential application as an animal feed supplement. *J Appl Phycol.* **8** 303-311.

Boussiba S and Richmond A 1980 C-Phycocyanin as a storage protein in blue-green algae. *Arch. Microbiol*. **125** 143-147.

Cartens M, Molina E and Robles A 1996 Eicosapentaenoic acid (20:4n-3) from the marine microalga *Phaeodactylum tricornutum*. *Journal of the American Oil Chemists Society* **73** 1025-1031.

Cohen Z, Reungjitchachawali M., Angdung W and Tanticharoen M 1993 Production and partial purification of gamma-linolenic acid and some pigments from *Spirulina platensis. J Appl. Phycol.* **5** 109–115.

Cohen Z 1997 The chemicals of *Spirulina platensis* (*Arthrospira*). *Physiology, Cell-Biology and Biotechnology.* Taylor and Francis, London. Pp 175-204.

Danesi E D G, Rangel CO, Pelizer LH, Carvalho JCM, Sato S and Moraes IO 2001 Production of *Spirulina platensis* under diverent temperatures and urea feeding regimes for chlorophyll attainment. *In: Proceedings of the Eighth International Congress on Engineering and Food* **2** 1978–1982.

Desikachary TV 1959 *Cyanophyta*. Indian Council of Agricultural Research. New Delhi, India 686.

Gladue RM and Maxey JE 1994 Microalgal feeds for aquaculture. *J Appl Phycol*. **6** 131- 141.

Goksan TA and Zekeriyaoglu IAK 2007 The growth of *Spirulina platensis* in different culture systems under greenhouse condition. *Turk J Biol*. **34** 47-52.

Guérin G, Ler CE, Kohler E and Laigr 2003 Development of SCAR marker linked to dominant gene conferring resistance *Colletotrichum acutatum* in strawberry. *Acta Horticulturae*. **626** 85–91.

Herbert D, Phipps PJ and Strange RE 1971 Chemical analysis of microbial cells. In: *Methods in microbiology* Vol.(v) B (ed) J.R. Morris and D.W. Ribbons. Academic Press, New York. 209-234.

Hu Q 2004 Industrial of Microalgal Cell-mass and Secondary Products-major Industrial Species: *Arthrospira* (*Spirulina*) *platensis*. In: Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Richmond, A. (Ed) Oxford, Blackwell. 264-272.

Hu Q, Guterman H and Richmond A 1996 A flat inclined modular photobioreactor (FIMP) for outdoor mass cultivation of photoautotrophs. *Biotechnol. Bioeng*. **51** 51-60.

Lee YK 1997 Commercial production of microalgae in the Asia-Pacific rim. *J Appl Phycol*. **9** 403-411.

Mahajan G and Kamat M 1995 Gamma-linolenic acid production from *Spirulina platensis*. *Appl Microbiol Biotechnol*. **43** 466-469.

Richmond A 1990 Large scale microalgal culture and applications. In: Round F.E., Chapman D.J., Eds, Progress in phycological Research, vol. **7**, *Biopress, Brislol, UK*.

Richmond A, Vonshak A and Arad SM 1980 Environmental limitations in outdoor production of algal biomass. In: Shelef G, Soeder CJ. eds. Algae Biomass, Amsterdam, Elsevier/North Holland Biomedical Press. Pp 65-72.

Tillett D and Neilan BA 2000 Xanthogenate nucleic acid isolation from cultured and environmental cyanobacteria, *J Phycol*. **36** 251-258.

Torzillo G and Bernardini P 1991 On-line monitoring of chlorophyll fluorescence to assess the extent of photoinhibition of photosynthesis induced by high oxygen concentration and low temperature and its effect on the productivity of outdoor cultures of *Spirulina platensis* (cyanobacteria). *J Appl Phycol*. **34** 504–510.

Torzillo G, Pushparaj B and Bocci F 1986 Production of *Spirulina* biomass in closed photobioreactors. *Biomass* **11** 61-74.

Venkataraman LV and Becker EW 1985 Biotechnology and Utilization of Algae–the Indian Experience. *Department of Science and Technology*, New Delhi, India. 257.

Venkataraman LV, Bhygyalashmi N and Ravishankar AG 1995 Commercial production of micro and macro algae problems and potentials. *Indian Journal of Microbiology* **35** 1-19.

Vonshak A 1997 *Spirulina*: growth, physiology and biochemistry. In: Vonshak, A. (ed.) *Spirulina platensis* (*Arthrospira*). *Physiology, cell-biology and biotechnology*. Taylor and Francis, London. 43-65.

Wikdors GH and Ohno M 2001 Impact of algal research in aquaculture. *J Phycol*. **37** 968-974.

Zarrouk C 1966 Contribution a l'etude d, une cyanophycee. Influence de divers *facteurs* physiques et chimiques sur la croissance et la photosynthese de *Spiru-*