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Production of aquatic feed grade algal powder from turtle breeding wastewater using a locally isolated *Spirulina* sp. JXSC-S1

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***Spirulina* sp. JXSC-S1 was isolated from a local turtle breeding wastewater storage pool in Jiaxing City, Zhejiang Province, China. Morphological studies revealed that the color, shape, spiral pitch, spiral wide and cell size of the strain fits the typical morphological characteristics of *Spirulina platensis*. Growth dynamics of the strain were studied in both Zarrouk medium and turtle breeding wastewater (TBW), and were compared with three *S. platensis* strains S6, S2 and Ns-90020 that are applied in large-scale *Spirulina* culturing in China. All strains grew well in Zarrouk medium. The strain JXSC-S1 demonstrated a growth rate of 1.64 day^{-1}, similar to the growth rates of *S. platensis* S6 and S2 and faster than *S. platensis* Ns-90020. When the culturing medium was changed to TBW, all the strains except the strain JXSC-S1 grew much slower than in Zarrouk meidum. By means of continuous TBW cultivation, the biomass yield of *Spirulina* sp. JXSC-S1 was up to 24±9 g·m^{-2}·day^{-1} in dry weight, with 59.10±3.48% of crude protein in dry mass, meeting the Chinese Spirulina Standard for Animal Feed (GB/T 17423-1998). This study shows that *Spirulina* sp. JXSC-S1 can be used for production of aquatic protein feed by recycling nitrogen and phosphorus from TBW.**

Key words: *Spirulina platensis*, morphological identification, growth kinetics, Zarrouk, turtle breeding wastewater, aquatic protein feed.

INTRODUCTION

Spirulina, an ancient microalga that contains 50-70% of protein is rich in vitamins, amino acids and bioactive substances, has been widely used as food and feed supplements for immune enhancement and growth improvement (Cardoso et al., 2012). In recent years, Spirulina has been large-scale cultivated globally, thanks for its quick growth, high photosynthetic efficiency, and high profits. However, the traditional large-scale cultivation requires provision of a large amount of baking soda, nitrogenous fertilizer and phosphate fertilizer with high cost, almost equivalent to 20-30% of the total spirulina production cost (Madkour et al., 2012).

Turtle breeding wastewater (TBW) contains high concentrations of nutrients (nitrogen, phosphorus and potassium), and is rich in trace elements (such as zinc and magnesium), bioactive substances (such as protein,

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amino acids, sugars, indole acetic acid and ribose) and growth regulators (such as vitamins and growth hormone) (Huo et al., 2013). Use of TBW to cultivate spirulina can greatly reduce the cultivation cost of spirulina while significantly removing nitrogen and phosphorus from TBW. The harvested algae powder could then be applied as aquatic animal (for example, shrimp) feed, given that the quality reaches the national standard. This would solve problem of the short supply of aquatic feed grade spirulina in the market and bring income to the aquaculturists. However, culturing spirulina in wastewater has many technical bottlenecks, the most critical one of which is to find spirulina strains with high pollution resistance. Geng et al. (2004) isolated a *Spirulina* sp. from sewage, in which concentrations of total nitrogen (TN) and total phosphate (TP) were of 4.53-4.96 and 0.39-0.48 mg/L, respectively. The strain grew in sewage with a growth rate of 0.79 day^{-1}, much faster than *Spirulina platensis* FACHB 357 (0.24 day^{-1}) and FACHB 486 (0.36 day^{-1}). Liu et al. (2008) isolated a strain of *Spirulina* sp. from municipal wastewater, whose tolerance concentrations for TN, TP and ammonia were 134, 10 and 9 mg/L, respectively. Growth rate of the strain in the municipal wastewater was 0.52 day^{-1}. Cheunbarn and Peerapornpisal (2010) isolated *S. platensis* from swine wastewater, in which TN, TP and ammonia tolerance concentrations were 108, 8 and 23 mg/L, respectively. Few studies have reported spirulina strains that are able to grow fast in wastewater with ammonia nitrogen of over 50 mg/L (Volkmann et al., 2008). This means that TBW must be diluted before being used to culture spirulina, because TBW always contains high concentrations of ammonia nitrogen, up to 1000 – 3000 mg/L. However, dilution is not preferred in large-scale application, because a large amount of clean water is needed and the other nutrients in the wastewater are remarkably diluted.

For resource utilization, the strain spirulina cultured in wastewater should contain crude protein of ≥50% (w/w) in biomass, with all quality parameters meeting the criteria set in the feed grade spirulina powder standards, for example, Quality Requirements of China (State Bureau of Technical Supervision,1998), France (Superior Public Hygiene Council of France, 1984, 1986), Sweden (Ministry of Health, Sweden,1988), Japan (Japan Health Foods Association, 1992) and USA (Earthrise Farms, 1995). Few literatures have succeeded in harvesting spirulina from wastewater with crude protein content of over 50%. Olguín et al. (2003) raised a *Spirulina* sp. strain in a semi-continuous pond containing 2% v/v of pig wastewater. The concentrations of COD, NH_4-N, TN, TP and alkalinity in the culture medium were 54, 24, 29, 3.3 and 94 mg/L, respectively. A biomass yield of 14 - 15 $g{\cdot}m^{-2}{\cdot}d^{-1}$ was obtained, with an average protein content of 48.9%. Phang et al. (Phang et al., 2000) cultured *S. platensis* UMACC 159 in anaerobically digested starch-processing wastewater added with 6 mM of urea and 2.1 mM of K_2HPO_4. A gross biomass productivity of 14.4 $g{\cdot}m^{-2}{\cdot}d^{-1}$ was obtained and the highest crude protein content was 38%. Chaiklahan et al. (2010) isolated a *S. platensis* strain BP from a stabilization pond at a tapioca starch factory in Thailand. The strain was cultured in 20% (v/v) TBW supplemented with 4.5 g/L of $NaHCO_3$ and 0.2 g/L of urea fertilizer. Characteristics of the 20% (v/v) pig wastewater was NH_4-N of 28±2 mg/L, TN of 3.6±0.0 mg/L, TP of 17.6±0.3 mg/L and total alkalinity of 280±28 mg/L. An average biomass yield 12 $g{\cdot}m^{-2}{\cdot}day^{-1}$ was obtained with a crude protein content of 57.9%.

A strain of *Spirulina* sp. was isolated from a TBW storage pool in Jiaxing City, China. The strain was found to be resistant to high ammonia concentrations. In the present study, the isolated species was characterized and the comparison of the isolated species with other three commercial Spirulina species grown in undiluted TBW was done. In addition, the quality of the biomass of the isolated specie was analyzed to assess if it was able to be used as aquatic feed.

MATERIALS AND METHODS

Turtle breeding wastewater

Turtle breeding wastewater was taken from a turtle farm in Jiaxing City, China, which had been pretreated in sequence by sedimentation to remove suspended solids, aerobic biological treatment to remove most of organics and ammonia, microfiltration to remove microorganisms, and ozone oxidation to remove color. Chemical oxygen demand (COD), ammonia nitrogen, TN, TP, total alkalinity, color and pH of the treated TBW was 280±70, 60.5±0.5, 1200±250, 45±10, 8000±2000 mg/L (as $CaCO_3$), 150±50 times and 8.5±0.5, respectively.

Source of microalgae

Strain JXSC-S1 was isolated from a TBW storage pool in Jiaxing City, China. The pool receives TBW from a large turtle farm that is 0.15 km far away from the farm mentioned earlier. *S. platensis* Ns-90020 was provided by Wuhan Botanical Garden, Chinese Academy of Sciences. *S. platensis* S6 and S2 were provided by Institute of Hydrobiology, Chinese Academy of Sciences. *S. platensis* Ns-90020, S6 and S2 are commercialized strains that are commonly used in large-scale *S. platensis* cultivation in China (Hu and Wei, 2006; Hu, 2003; Yin et al., 1997).

Spirulina cultivation experiment

The spirulina cultivation experiment was carried out in 250 ml Erlenmeyer flasks which had been pre-filled with 90 ml of Zarrouk medium (Zarrouk, 2006) or TBW. Algae in the logarithmic growth phase were inoculated into the flasks with a final density of 0.2 in OD_{560}. The flasks were then sealed with film and cultured in an illuminating incubator (model GZP-450, Jing Hong Laboratory Instrument Co., Ltd, Shanghai, China) for ten days. The culturing temperature was maintained at 25 ± 2°C. The photoperiod was 12 h light (6000 ± 2000 lux, 7:00 am - 7:00 pm) and 12 h dark (7:00 pm - 7:00 am). The flasks were hand shaken and OD_{560} was measured once per day. Mixed liquor samples were taken at intervals to observe the algae cell morphology. The algae maximum specific growth rate and grow generation were calculated according

Table 1. Morphological characteristics of JXSC-S1 and typical *Spirulina* strains.

Strains	Width of trichome (μm)	Shapes of the helix end and the end cell	Calyptra on end cells	Gas vesicles	Cell length(μm)
Spirulina sp. JXSC-S1	5.5-6.0	Both ends rounded	Present	Present	4-5
S. platensis Ns-90020	5.3-5.7	Both ends rounded	Present	Present	5
S. platensis S6	5.5-5.8	Both ends rounded	Present	Present	4
S. platensis S2	5.8-6.0	Both ends rounded	Present	Present	4
S. platensis essential features	5-6	Both ends rounded	Present	Present	2-6
S. maxima essential features	6-8	Slightly diminished at one end	Absent	Absent	8-12
S. subsalsa essential features	1.5-2.5	Slightly diminished at both ends	Absent	Absent	2-5

to literature (Vonshak and Richmon, 1988). Each cultivation was conducted with three replicates.

Spirulina sp. morphological identification

After sampling, microalgae mixed liquor was washed with Milli-Q ultrapure water and then re-suspended in Milli-Q ultrapure water. 0.1 ml of the sample was put on the centre of a microslide, and then observed under a microscope (400× magnification; Olympus, Tokyo, Japan) for the morphological features including width of trichome, cell size, shape of the helix, gas vesicles and calyptra on the end of cells. Each sample was observed three times.

Statistical analysis was performed by paired t-test for two sample means using statistical software Origin (version 8.5). p Value <0.05 was set as cut off to assess statistical significance.

RESULTS AND DISCUSSION

Morphological study

Spirulina is a type of microalgae characterized with a corkscrew-shape filament. The common spirulina species include *S. platensis*, *Spirulina* subsalsa and *Spirulina* maxima. *S. platensis* as a spirulina species with a trichome width of 5 - 6 μm, a cell length of 2 - 6 μm, both shape of helix end and end cell round, evident calyptra on end cells, and granular cytoplasm containing gas vacuoles. *S. platensis* is different from *S. maxima* and *S. subsalsa* which do not have calyptra on end cells and gas vesicles.

Moreover, *S. subsalsa* has slimmer trichome (1.5 -2.5 μm) and *S. maxima* has wider trichome (6 - 8 μm) and longer cells (8 - 12 μm), slightly diminished at one end of nature and shape end and end cell (Hu and Wei, 2006). The morphology analysis results of the four species are given in Table 1. The isolated strain JXSC-S1 was observed to be blue-green corkscrew-shape filaments with morphological characteristics similar to the typical *S. platensis* strains of S6, S2 and Ns-90020, fitting with the taxonomy definition of *S. platensis* while different from *S. maxima* and *S. subsalsa*.

Culturing of the four species in Zarrouk medium and in TBW

Growth curves of S. platensis JXSC-S1, Ns-90020, S6 and S2 are shown in Fig. 1. The indexes describing the growth kinetics were calculated according to the growth curves and are listed in Table 2. Besides the four S. platensis strains, growth indexes of three other S. platensis strains cultured with Zarrouk culturing medium (Hu, 2003) are also listed in Table 2 for comparison. The three strains: Africa Chad, FACHB439 and S1 are used in large-scale microalgae culturing (Hu, 2003).

In Zarrouk medium, all the four strains underwent the exponential growth phase after culturing for two days, and then reached the logarithmic phase on day 9. The growth rate of *S. platensis* JXSC-S1 was similar to those of S6 (p = 0.255) and S2 (p = 0.184), much faster than Ns-90020 (p = 0.018). *S. platensis* JXSC-S1 had a maximum specific growth rate of 1.64 day^{-1} and growth generation of 13.96 h. These data were similar to those of *S. platensis* S1 (p = 0.193) and S6 (p = 0.235), slightly slower and longer than those of *S. platensis* S2 (p = 0.342), respectively, while much faster and shorter than those of *S. platensis* Ns-90020 (p = 0.017), Africa Chad (p: 0.029) and FACHB439 (p: 0.011). The air flotation capacity and flotation rate of *S. platensis* JXSC-S1 were similar to those of *S. platensis* Ns-90020 (p = 0.296), S6 (p = 0.187), S2 (p = 0.259) and FACHB439 (Hu, 2003) (p: 0.376).

The average yield of *S. platensis* JXSC-S1 was 42 g·m^{-2}·d^{-1} in Zarrouk medium, significantly higher than that of *S. platensis* Ns-90020 (p = 0.014), S6 (p = 0.026), S2 (p = 0.012), Chad (Hu, 2003) (p: 0.014), FACHB439 (Hu, 2003) (p:0.036) and S1 (Hu, 2003) (p:0.029). The crude protein content of S. platensis JXSC-S1 was 62.8 ± 6% in dry biomass, similar to that of *S. platensis* Africa Chad (p = 0.581), FACHB439 (p = 0.274) and S1 (p = 0.191), slightly higher than that of *S. platensis* S2 (p = 0.123) and S6 (p = 0.135), and lower than that of *S. platensis* Ns-90020 (p = 0.041). The protein content and growth

Table 2. Growth indexes of *S. platensis* JXSC-S1 and commercially used *S. platensis* strains in Zarrouk medium and TBW.

Strains	Medium	Maximum specific growth rate (d^{-1})[a]	Grow generations (h)[a]	Floating rate (%)	Biomass yield ($g \cdot m^{-2} \cdot d^{-1}$)[b]	Crude protein content (%)
JXSC-S1	Zarrouk	1.64	13.96	91.6	29±13	62.8±6
Ns-90020		1.02	23.26	91.84	15±8	70.3±5
S6		1.57	11.77	92.6	15±9	61.7±10
S2		1.78	10.51	98.2	18±6	59.4±6
Chad (Hu,2003)		1.24	11	—	12±6	55-65
FACHB439 (Hu ,2003)		1.13	19.51	88.89	20±4	50-70
S1 (Hu, 2003)		1.52	12.05	—	17±7	55-65
JXSC-S1	TBW	1.55	14.15	92.4	24±9	59.10±3.48
Ns-90020		0.07	—	—	—	—
S6		0.33	49.56	51.4	8±3	45.1±5
S2		Death	death	—	—	—

[a]: The maximum specific growth rate and the grow generation are calculated based on the OD_{50} values. [b]: The biomass yield is based on the biomass values.

rate of the local isolated spirulina strain were consistent with literature results. It has been reported that the protein assimilation capacity of a spirulina was negatively influenced by its cell division rate. A slow cell division rate and a long generation time tended to result in a high assimilation capacity of proteins and thereby a great protein accumulation amount (Yin et al., 1997).

Once the culturing medium was changed to the TBW, only *S. platensis* JXSC-S1 remained fast growing. *S. platensis* S6 slightly proliferated during the culturing for ten days, while *S. platensis* Ns-90020 and S2 did not proliferate at all. Growth rates of the above three *S. platensis* strains in the TBW were significantly lower than in the Zarrouk medium (Ns-90020, $p = 0.028$; S6, $p = 0.042$; S2, $p = 0.034$). By comparison, *S. platensis* JXSC-S1 grew fast in TBW, the OD_{560} value on day 10 was over 4.0. With a maximum specific growth rate of 1.55 day^{-1} and a growth generation time of 14.15 h, *S. platensis* JXSC-S1 showed little difference when growing in TBW, in comparison with when growing in Zarrouk ($p = 0.396$).

The fast grow of *S. platensis* JXSC-S1 in TBW might be attributed to its tolerance to high concentrations of ammonia and nitrogen. High concentrations of ammonium ions inhibit nitrate reductase in *S. platensis* (Liu et al., 2008). Ammonia nitrogen of 40 mg/L has been reported as the highest tolerable concentration of *S. platensis* (Lin et al., 2009). Round (1984) reported that ammonia nitrogen of above 45 mg/L would inhibit growth of *S. platensis*. Isolated from TBW storage pond and adapted to the TBW environment, *S. platensis* JXSC-S1 remained its high growth rate even under a ammonia nitrogen concentration of 60 mg/L.

Quality of the harvest spirulina

Color and morphology are two main indicators for spirulina quality. Green, long spiral filaments are preferred because this kind of filaments are found to contain high contents of active components. Spirulina cultured for 10 days in TBW are shown in Figure 1. Blue-green and long filaments with mean spirals of ≥ 10, *S. platensis* JXSC-S1 cultured in TBW demonstrated almost the same color and algal morphology as cultured in Zarrouk medium (p:0.295). However, the other three spirulina strains behaved quite different. The color was green for *S. platensis* S6, palegreen for *S. platensis* N_S-90020 and yellowish for *S. platensis* S6. *S. platensis* S6 demonstrated short filaments with few spirals. Some filaments of *S. platensis* Ns-90020 were straightened and lost the spiral characteristics. Filaments of *S. platensis* S2 even broken into pieces. The loose, straight and broken filaments of S. platensis S2, S6 and Ns-90020 might be attributed to the high stress caused by TBW which contained high concentrations of nitrogen and other contaminants. Wang and Zhao (2005) and Lin et al. (2009) have found that the spirulina filaments generally shaped with regular spirals, but the spirals tended to curl or become straight when the environment conditions were changed or spirulina species suffered from unpleasant conditions. The changes in the shape of algal filaments would directly affect their physiological-biochemical characteristics. The change into a linear shape would cause a decay of algal species in the culturing system and a reduced efficiency of nutrient uptake, and further result in serious problems such as a sharp decline in algal yields, deterioration in algal (nutrition) quality, difficulties in collection and susceptibility to the Oscillatoria pollution.

Spirulina powder manufactured from harvested *S. platensis* TBW biomass grown in the TBW contained a crude protein content of approximately 60%, and heavy metals (Pb, As, Cd and Hg) of 0.073±0.019, 0.87±0.04,

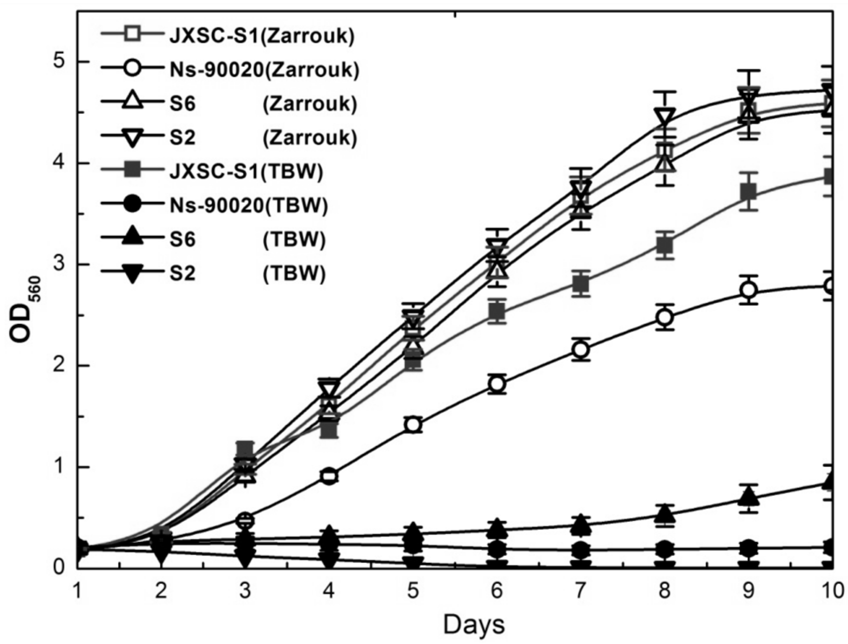

Figure 1. Growth curves of *S. platensis* JXSC-S1 and S6, S2, Ns-90020 in Zarrouk medium and TBW (error bar is standard deviation of three parallel samples).

Table 3. Quality of *S. platensis* JXSC-S1 powder cultivated in TBW.

Properties	Spirulina powder cultivated in TBW	Feed grade standard[a]
Moisture (%)	6.27±0.23	≤7
Crude protein (%)	59.10±3.48	≥50
Ash (%)	7.15±0.89	≤10
Pb (mg/kg)	0.073±0.019	6.0
As (mg/kg)	0.87±0.04	1.0
Cd (mg/kg)	0.090±0.011	0.5
Hg (mg/kg)	0.070±0.005	0.1
Total plate count (cells/g)	$1.7\times10^3\pm0.2\times10^3$	5×10^4

[a]: the Chinese Spirulina Standard for Feed Grade (GB/T 17423-1998).

0.090±0.011 and 0.070±0.005 mg/kg, respectively, meeting the Chinese Spirulina Standard for Feed Grade (GB/T 17423-1998) (Table 3). This suggests that the new strain could be used for production of high quality aquatic feeds spirulina protein from TBW.

Conclusions

A *Spirulina* strain, JXSC-S1, was isolated from a TBW storage pool. The strain was identified as *S. platensis* based on its morphological characteristics. In comparison with other three commercially used Spirulina, JXSC-S1 had similar growth rate, biomass yield and protein content when cultured in TBW and in Zarrouk medium; while the other three strains can only be cultured in Zarrouk medium. Using TBW as the culturing medium, *S. platensis* JXSC-S1 biomass yield averaged 24±9 $g\cdot m^{-2}\cdot day^{-1}$ in dry weight, and the crude protein content in dry mass was 59.10±3.48%. The quality of harvested biomass met the Chinese Spirulina Standard for Animal Feed (GB/T 17423-1998). This study shows that *S. platensis* JXSC-S1 is able to be used for production of aquatic animal protein feed by recycling nitrogen and phosphorus from turtle breeding wastewater.

Conflict of interests

The author(s) did not declare any conflict of interest.

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REFERENCES

Cardoso NF, Limaa EC, Royera,B., Bacha MV, Dottob, G.L., Pintob LAA, Calvetec T(2012).Comparison of Spirulina platensis microalgae and commercial activated carbon as adsorbents for the removal of Reactive Red 120 dye from aqueous effluents. J. Hazard. Mater. 7(241–242):146–153.

Chaiklahan R, Chirasuwan N, Siangdung W (2010). Cultivation of Spirulina platensis using pig wastewater in a semi-continuous process. J. Microbiol. Biotechnol. 20:609-614.

Cheunbarn S, Peerapornpisal Y (2010).Cultivation of Spirulina platensis using anaerobically swine wastewater treatment effluent. Int. J. Agric. Biol. 12:586-590.

Geng YH, Li YG, Hu HJ, Li XL, Miao FP(2004).Separation and cultivation of new strain of Spirulina sp. from sewage. J. Wuhan Bot. Res. 22: 329-333.

Hu HJ (2003). The biology and biotecnnology of Spirulina. Beijing: Academic Press Inc. pp. 145–150.

Hu HJ, Wei YX (2006).The freshwater algae of China (Systematics, Taxonomy and Ecology). Beijing: Acade. Press Inc. 142–144.

Huo SL, Xi BD, Zan FY, Zeng F, Zhang JT, Su J (2013). Dissolved organic matter in turtle breeding wastewater from combined treatment process. Desalination Water Treat. 51: 2351-2361.

Lin CY. Shen XW, Zhao ZK, Wu HZ, Xu H (2009). Photosynthesis and differentially expressed proteins in two morphologic filaments of Spirulina platensis. Chem. J. Chin. Univ. 30:1152-1157.

Liu YH, Ruan R, Kong QX, Liu CM, Luo J, Yu F(2008). Mass culture of high oil contentm icroalgae on wastewater and power plant flue gases. Chin. J. Bioprocess. Eng. 6:29–33.

Madkour FF, Kamil AEW, Nasr HS (2012). Production and nutritive value of Spirulina platensis in reduced cost media. Egypt. J. Aquat. Res. 38:51-57.

Olguín EJ, Galicia S, Mercado G, Pérez T (2003). Annual productivity of Spirulina (Arthrospira) and nutrient removal in a pig wastewater recycling process under tropical conditions. J. Appl. Phycol. 15:249-257.

Phang SM, Miah MS, Yeoh BG, Hashim MA (2000). Spirulina cultivation in digested sago starch factory wastewater. J. Appl. Phycol. 12:395–400.

Volkmann H, Imianovsky U, Oliveira JLB, Sant'Anna ES (2008).Cultivation of Arthrospira (Spirulina) platensis in desalinator wastewater and salinated synthetic medium: protein content and amino-acid profile. Brazil.J.Microbiol.**39**:98–101.

Vonshak A, Richmon A (1988). Mass production of the blue-green alga Spirulina:an overview. Biomass 15:233–247.

Yin CT, Hu HJ, Li YG, Gong XM, Shi DJ (1997). Studies on middle temperature strains selection of spirulina platensis. J. Wuhan Bot. Res. 15:250–254.

Zarrouk C (1966). Contribution a letude du cyanophycee. Influence dedivers facteurs physiques et chimiques sur la croissance et laphotosynthese de Spirulina maxima (Setch et Gardner) Geitl. Paris.