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Full Length Research Paper

Physico-chemical growth requirements and molecular characterization of indigenous Spirulina

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The present study was designed to explore the indigenous Spirulina and its mass cultivation by optimizing the physico-chemical growth requirements. One hundred and twenty samples were collected from different soils and water from three districts (Sargodha, Lahore and Faisalabad) of Punjab. Collected samples were shifted to laboratory immediately under sterile conditions to avoid contamination and were kept under dim light at 30°C. Then Spirulina was isolated from collected samples and cultivated under different nutrient, temperature and light regimens to get its maximum biomass in our laboratory. Our results showed that maximum growth of indigenous Spirulina was obtained at 30°C and at 1500 lux light intensity. Nitrogen concentrations of 0.625, 1.25 and 1.875 g/L had no effect on the growth, while phosphate concentrations of 0.5, 1.0 and 1.5 g/L had a minimal and gradual effect on growth as the concentrations were increased. For the confirmation and molecular characterization of indigenous Spirulina, DNA was isolated by chloroform-isoamyl alcohol extraction method and its polymerase chain reaction (PCR) was carried out by using specific primer of 16s rDNA gene and PCR products were run on gel giving an amplicon size of 700 bp. Our study shows that Spirulina can be grown in lab conditions by optimizing the physico-chemical growth requirements.

Key words: Amplicon, biomass, physico-chemical, polymerase chain reaction (PCR), Spirulina.

INTRODUCTION

Spirulina is a photosynthetic, filamentous, spiral-shaped, multicellular blue-green alga (cyanobacteria). It is found in waters in which concentrations of carbonate and bicarbonate are high up to pH 11 (Ciferri, 1983). Chemical composition of Spirulina indicates that it has high nutritional value due to its content of a wide range of essential nutrients, such as provitamins, minerals, proteins and polyunsaturated fatty acids such as gamma-linolenic acid (Miranda et al., 1998). Chemically Spirulina is 50 to 70% protein, 5 to 10% lipid and 10 to 20% carbohydrate (reported to dry mass) and it is the world's richest natural source of vitamin B_{12} (Vonshak, 1997). Spirulina has been studied because of its therapeutic, antioxidant and antiviral properties and the presence of compounds such as phenolics (Belay et al., 1993), it also cause regression and inhibition of cancers (Miranda et al., 1998; Estrada et al., 2001). Spirulina has been commercialized due to use in therapeutics and as food additive due to its valuable constituents (Hirahashi et al., 2002). The use of Spirulina as animal food supplement enhances the yellow coloration of skin and eggs yolk in poultry and flamingos; it also enhances growth acceleration, sexual maturation and increase of fertility in cattle. Spirulina was proven in animal experiments to exhibit various biological activities such as lowering plasma cholesterol levels and blood pressure. It is being widely studied for its possible anticancer, antibacterial, and antiparasitic properties, and for several medical conditions such as allergies, ulcers, anemia, heavy metal

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Table 1. District wise prevalence of Spirulina from the collected samples.

District	Total samples		Spirulina present		Percentage	
	Soil	Water	Soil	Water	Soil	Water
Sargodha	20	20	3	5	15	25
Lahore	20	20	3	4	15	20
Faisalabad	20	20	4	8	20	40
Total	120		10 + 17 = 27		22.5	

poisoning, and radiation poisoning (Ross and Dominy, 1990). Many agricultural and industrial materials are being prepared from cyanobacteria. These include: biomass (Thein, 1993), restriction nucleases (Kawamura, et al., 1986), antifungal, antineoplastic (Clardy et al., 1990), antimicrobial (Gerwick et al., 1987), anti-leukemia (Moore et al., 1977) and herbicidal compounds (Entzeroth et al., 1985). The large-scale production of Spirulina biomass depends on many factors, the most important of which are nutrient availability, temperature and light. These factors can influence the growth of Spirulina and the composition of the biomass produced due to change in metabolism (Cornet et al., 1992). The study was undertaken to optimize the physicochemical growth requirements of indigenous Spirulina in laboratory conditions along with its molecular characterization for rapid identification.

MATERIALS AND METHODS

One hundred and twenty samples were collected from Sargodha, Faisalabad and Lahore districts. Twenty samples from pond water and twenty samples from agricultural soil were collected from each district under sterile conditions. For sampling, 200 ml of water and 100 g of soil were taken. 250 ml screwed capped sterile glass bottles were used for water and sterile zipper polythene plastic bags were used for soil sampling. Isolation from soil was carried out by dissolving 50 g of soil in 150 ml of Spirulina medium (Aiba and Ogawa, 1977) in a 500 ml sterile glass flask, mouth of the flask was plugged with cotton and placed it in photoincubator under standard conditions (30°C temperature, 1500 lux light) for 15 days. After 15 days the growth was observed under inverted microscope by placing 2 to 3 drops of growth medium on slide.

Spirulina was isolated and purified (if present) from this mixture. Isolation from water was carried out by placing 2 to 3 drops of sample on slide. The growth was observed under inverted microscope. Identification of Spirulina was made on the basis of its morphological characteristics that is, length, shape, size and diameter of trichome, motility of filament and arrangement of cylindrical trichome. The size in length and diameter of Spirulina was measured with calibrated micrometer (Fott and Karim, 1973). A drop of each sample was taken on a slide and observed under inverted microscope for identification purpose. After identification single filament of Spirulina was picked by using capillary pipette and placed in 96-wells micro-titration plate already filled with 150 µl Spirulina medium (Ayala , 2000). Then micro-titration plate was placed in photo incubator under a 12 hour light/12 hour dark photoperiod at standard conditions (30°C temperature, 1500 lux light) for 15 days. Purified growth of Spirulina was collected aseptically in a sterile test tube and kept at a light intensity of about 1500 lux and a temperature of 30°C (Robert and Masanobu, 2005).

Standard growth curve was made by measuring optical density at 670 nm. In order to produce standard curve known dry weight of Spirulina was dissolved in known volume of Spirulina medium and its OD value was read at 670 nm, then two fold serial dilutions of this solution were made up to 2^{-10} and were read at 670 nm. All readings were taken three times and mean value was taken to construct a standard growth curve relating dry weight of Spirulina biomass (mg/ml) to optical density. This standard curve was subsequently used to calculate the biomass of individual samples based on their optical density. The calculated biomass (the average of three experiments) was used to construct growth curves (Colla et al., 2006). Spirulina growth was optimized using different physical and chemical parameter like temperature, light, nitrogen, phosphorus having three different regimes. For molecular characterization PCR of 16s rDNA was done after isolating the DNA from Spirulina. DNA of Spirulina was extracted as followed by (Fiore et al., 2000). PCR was done by using the cyclic conditions 94°C for 5 min, 94°C for 1 min, 60°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min (Nubel et al., 1997). The following specific oligonucleotide primers were used for amplification of Spirulina 16s rDNA (CYA781-F): 5´-CGGACGGGTGAGTAACGCGTGA-3´ (CYA781-R): 5´-GACTACTGGGGTATCTAATCCCATT-3´

RESULTS

Spirulina was identified on the basis of culture and microscopic characteristics. The spiral shape filaments were solitary, free floating, display gliding motility and the arrangements of the multicellular cylindrical trichome in an open left-hand helix along entire length. The spiral filaments were 0.3 to 1.0 mm in length. The filaments were coiled or helical for the most part though there could be straight forms at times. The filaments were made up of many cells with clear and visible transverse cross walls. The cells making the filaments were shorter than broad. The width varies between 6 to 12 µm. The highest occurrence was observed in soil sample of District Faisalabad 20% followed by as compare to Sargodha 15%, and Lahore 15%. The high occurrence in water samples of District Faisalabad 40% followed by as compare to Sargodha 25% and Lahore 20%. On an average presence of Spirulina was 22.5% in total samples (Table 1). Growth of Spirulina was better when it is grown at temperature of 30°C compared with 25 and 35°C. Minimum growth was observed when it is grown at a temperature of 35°C. Maximum increment of growth 0.375 mg/ml occurs at a temperature at 30°C on day 6 to day 7. Minimum gain of growth 0.01 mg/ml was observed on day 0 to day 1. However, the growth of

Table 2. Cultivation of Spirulina at three different temperature and light intensity regimes.

Day	Growth (mg/ml)					
	25°C	30°C	35°C	500 lux	1000 lux	1500 lux
0	0.382	0.387	0.380	0.301	0.303	0.301
1	0.390	0.392	0.390	0.306	0.313	0.316
2	0.405	0.408	0.403	0.316	0.323	0.334
3	0.418	0.426	0.420	0.326	0.339	0.354
4	0.431	0.443	0.436	0.341	0.357	0.390
5	0.561	0.801	0.538	0.377	0.502	0.724
6	0.691	1.163	0.668	0.397	0.637	1.094
7	0.760	1.538	0.734	0.428	0.783	1.410

Table 3. Cultivation of Spirulina in Media with Different $NaNO_3$ and K_2HPO_4 Concentrations.

Day	Growth (mg/ml)					
	$NaNO_3$ (0.625 g/L)	$NaNO_3$ (1.25 g/L)	$NaNO_3$ (1.875 g/L)	K_2HPO_4 (0.5 g/L)	K_2HPO_4 (1.0 g/L)	K_2HPO_4 (1.5 g/L)
0	0.436	0.477	0.509	0.326	0.329	0.323
1	0.441	0.479	0.507	0.334	0.344	0.349
2	0.456	0.499	0.533	0.341	0.357	0.377
3	0.471	0.507	0.540	0.354	0.372	0.403
4	0.484	0.522	0.553	0.372	0.387	0.418
5	0.841	0.882	0.920	0.581	0.607	0.653
6	1.198	1.239	1.267	0.933	0.964	0.997
7	1.570	1.607	1.642	1.285	1.341	1.390

Spirulina increases when light intensity increases. Highest growth 1.41 mg/ml of Spirulina was observed at 1500 lux on day 7. Likewise as in temperature highest gain of growth of Spirulina was observed from day 6 to day 7 (0.316 mg/ml) (Table 2). The highest growth of Spirulina (1.642 and 1.39 mg/ml) was observed at 1.875 g/L $NaNO_3$ and 1.5 g/L K_2HPO_4, respectively. However, it was observed that growth of Spirulina is better in $NaNO_3$ compared with K_2HPO_4. More than 1 mg/ml growth of Spirulina was observed on day 6 in sodium nitrate salt while more than 1 mg/ml growth of Spirulina was observed on day 7 in dipotassium hydrogen phosphate salt (Table 3). The precipitated DNA was used for Polymerase Chain Reaction (PCR). Yielded PCR amplicon product size was of 700 bp (Figure 1).

DISCUSSION

The study evaluated the influence of temperature, light, phosphate and nitrogen concentration in the medium for biomass production of indigenous Spirulina. Samples were collected randomly from the three districts upon analysis it was revealed that the presence of Spirulina in our sampling areas was not uniform. Spirulina was identified on the basis of culture and microscopic characteristics. These morphological observed characteristics of Spirulina correspond to the description of Baldia et al. (1991).

Our results showed that the highest occurrence was observed in soil samples of Distt Faisalabad 20% followed by as compare to Sargodha 15%, and Lahore 15%. The sample collected from water showed the highest occurrence of Spirulina of Distt Faisalabad 40% followed by as compare to Sargodha 25% and Lahore 20%. This difference is due to the presence of carbonates and bicarbonates and high pH in soil and water of the sampling districts (Baldia et al., 1991).

The growth of Spirulina was recorded highest at temperature 30°C and lowest growth at 25 and 35°C. Values were 1.538 mg/ml and 0.760 and 0.734 mg/ml respectively. It was recorded that optimum temperature for the growth of Spirulina was best at 30°C, but within 5°C higher and lower did not alter its growth. The same results were observed by (Baldia et al., 1991; Colla et al., 2006; Rafiqul et al., 2003). It has been shown by previous workers (Danesi et al., 2001; Vonshak, 1997) that the optimal growth temperature for Spirulina was 30°C. In respect to increase in biomass, the best responses were obtained at 30°C. Results also showed that till seven days its metabolites did not affect its increased growth as no media was added from outside during the week. It was

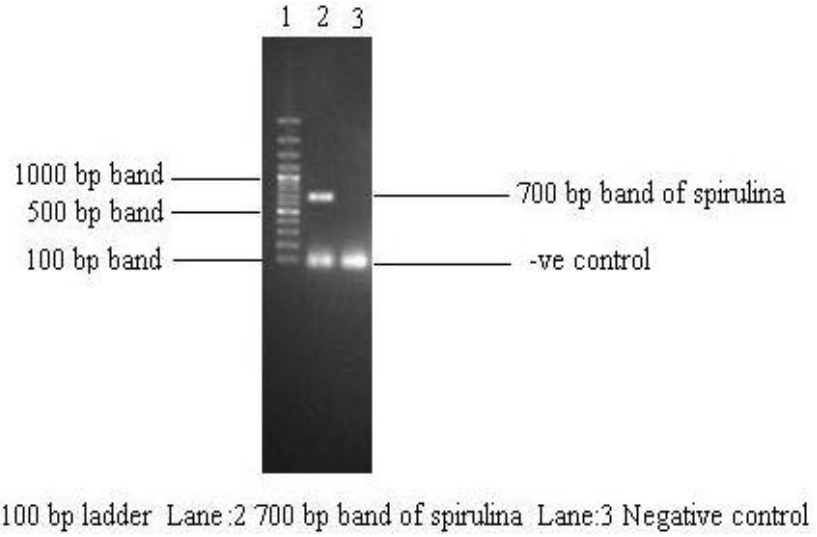

Figure 1. Agarose gel electrophoresis of Spirulina having amplicon Size of 700 bp.

observed that in initial four days the growth was not remarkable may be due its lag phase. In this period of lag phase after 4th days growth increased sharply meaning the organism enters into exponential phase. The highest growth was observed during the interval between 5th and 6th days. All the values for temperature effect were significant ($P < 0.05$) which also agrees with Colla et al. (2006). The highest biomass values were obtained at 30°C due to the fact that the partial pressure of CO_2 in the medium was higher at 30°C than at 35 and 25°C, leading to a higher concentration of bicarbonate and consequently an increased rate of photosynthesis. At higher temperatures (that is, 35°C) there was an increase in dark cycle respiratory activity in which the cells use reserve material (for example, carbohydrates) for respiration and a concomitant decrease in cell weight (Vonshak et al., 1982).

Results showed that maximum growth was recorded on 1500 lux, 50% decrease in growth at 1000 lux and lowest at 500 lux. The values were 1.410, 0.783 and 0.428 mg/ml, respectively. It showed that optimum light intensity for the growth of Spirulina was 1500 lux, but at 1000 lux and 500 lux lower growth recorded. Baldia et al. (1991) also studied the effect of light intensities on the growth response of Spirulina and observed that at 1500 lux Spirulina grow at its maximum, 50% decrease in growth at 1000 lux and lowest at 500 lux which also agrees with the results of Reichert et al. (2006). Lag phase of 4 days was also observed in this parameter. All the values for light effect were significant ($P < 0.05$) which also agrees with Baldia et al. (1991). Due to this reason when light intensity was high photosynthesis rate increased that is directly proportional to growth. It was observed that lower the population density, higher the specific growth rate. But when the growth increased the availability of light to each cell decreased. So at higher light intensity there was increased availability of light to each cell which intensified the growth rate (Vonshak et al., 1982; Qiang et al., 1998).

Different nitrogen ($NaNO_3$) concentrations (0.625, 1.25 and 1.875 g/L) in Spirulina medium showed that they have no significant effect on growth of Spirulina. The biomass concentration at $NaNO_3$ 0.625 g/L was 1.570 mg/ml, at the $NaNO_3$ 1.25 g/L growth was 1.607 mg/ml and at $NaNO_3$ 1.875 g/L it was 1.642 mg/ml. Colla et al. (2006) studied that nitrogen concentrations in medium has no influence on the production of Spirulina biomass but it has effect on the chemical composition of Spirulina which also agrees with the studies by Piorreck et al. (1984) and Hanaa et al. (2009). Results also showed that till seven days its metabolites did not affect its increased growth because no media was added from outside during the week. Lag phase of 4 days was also observed in this parameter experiments. The highest growth was observed during the interval between 5th and 6th days. All the values for nitrogen effect were significant ($P < 0.05$) which also agrees with Colla et al. (2006). The fact was that Nitrogen required for the synthesis of amino acids, which make up proteins and other cellular components such as phycocyanin but has no significant effect on biomass (Colla et al., 2006). This indicates that the concentration of sodium nitrate in standard Spirulina medium (2.50 g/L) can be reduced to 0.625 g/L without loss of biomass productivity and leads to decrease production cost in large-scale cultivation.

Growth on three different concentrations of phosphate indicated that there was gradual increase in growth as the phosphate concentrations increased. The biomass concentrations achieved at K_2HPO_4 1.5 g/L were gradually higher then the K_2HPO_4 1.0 g/L and at K_2HPO_4 0.5 g/L. Growth recorded was 1.390, 1.341 and 1.285 mg/ml, respectively. The same results were observed by

Stevens et al. (1981) which also agrees with Ernst et al. (2005). All the values for phosphate effect were significant ($P < 0.05$) which also agrees with Ernst et al. (2005).

In the present study confirmation of Spirulina was done by polymerase chain reaction using primer pair CYA106 F and CYA781 R which was specific for the amplification of 16S rDNA gene segment from the genus Spirulina. Similar study was conducted by Nubel et al. (1997); Boutte et al. (2006) their results were also similar to the present work, as they used same set of primers and similar amplification conditions.

Now-a-days in the world people are competing for food supplementation. Spirulina can act as a source of nutraceuticals. This study helps in optimizing the growth of indigenous Spirulina for large scale industrial production. Its extensive study should be done like physiology, growth, reproduction etc. This will pave an avenue for further nutraceuticals.

REFERENCES

Aiba S, Ogawa T (1977). Assessment of growth yield of a blue-green alga, *Spirulina platensis*, in axenic and continuous culture. J. Gen. Microbiol., 102: 179-182.

Ayala F (2000). Solarium Biotechnology, La Huayca, I Region, Chile. Communication Personal, pp. 3-20.

Baldia SF, Nishijima T, Hata Y (1991). Effect of physico-chemical factors and nutrients on the growth of *Spirulina platensis* isolated from lake Kojima, Japan. Nippon Suosan Gakkaishi, 57: 481-490.

Belay A, Ota Y, Miyakawa K, Shimamatsu H (1993). Current knowledge on potential health benefits of Spirulina. J. Appl. Phycol., 5: 235–241.

Boutte C, Grubisic S, Balthasart P, Wilmotte A (2006). Testing of primers for the study of cyanobacterial molecular diversity by DGGE. J. Microbiol. Meth., 65: 542-550.

Ciferri O (1983). Spirulina, the edible organism. Microbiol. Rev., 47: 551-578.

Clardy J, Kato Y, Brinen L, Moore B, Chen J, Patterson G, Moore R (1990). Paracyclophanes from blue-green algae. J. Am. Chem. Soc., 112: 4061-4063.

Colla LM, Reichert COC, Costa JAV (2006). Production of biomass and nutraceutical compounds by *Spirulina platensis* under different temperature and nitrogen regimes. Biores. Technol., 98(7): 1489-1493.

Cornet JF, Dussap CG, Dubertret G (1992). A structured model for simulation of cultures of the cyanobacterium *Spirulina platensis* in photobioreactors: Coupling between light transfer and growth kinetics. Biotechnol. Bioeng., 40: 817-825.

Danesi EDG, Rangel CO, Pelizer, LH, Carvalho JCM, Sato S, Moraes IO (2001). Production of *Spirulina platensis* under different temperatures and urea feeding regimes for chlorophyll attainment. In: Proceed. 8th Intl. Congress Engin. Food. 2: 1978–1982.

Entzeroth M, Mead D, Atterson GP, Moore R (1985). A herbicidal fatty acid produced by Lyngbya aestuarii. Phytochemical, 24: 2875-2876.

Ernst A, Manfred D, Peter MJH, Ute IAW (2005). Nitrate and Phosphate Affect Cultivability of Cyanobacteria from Environments with Low Nutrient Levels. App. Environ. Microbiol., 3379–3383.

Estrada JE, Bescos P, Fresno, AMVD (2001). Antioxidant activity of different fractions of *Spirulina platensis.* Protein Extract Farmaco., 56: 497–500.

Fiore MF, David HM, Siu MT, Hung, L, Jack, TT (2000). Miniprep DNA isolation from unicellular and filamentous cyanobacteria. J. Microbiol. Meth., 39: 159–169.

Fott B, Karim AGA (1973). Spirulina plankton community in a lake in Jebel Marra, Sudan. Arch. Protistenk. Bd., 115: 408-418.

Gerwick W, Reyes S, Alvarado B (1987). Two malyngamides from the Caribbean cyanobacterium Lyngbya majuscula. Phytochemical., 26: 1701-1704.

Hanaa HAE, Farouk KE, Gamal SE (2009). Characterization of nutraceutical compounds in Blue green alga *Spirulina maxima*. Electronic J. Environ. Agric. Food Chem., 11: 1113-1126.

Hirahashi T, Hazeki MK, Saeki Y, Ui M, Seya T (2002). Activation of the human innate immune system by Spirulina augmentation of interferon production and NK cytotoxicity by oral administration of hot water of *Spirulina platensis*. Intl. Immunopharmacol., 2: 423-34.

Kawamura M, Sakakibara M, Watanabe T, Kita K, Hiraoka N, Abayashi A (1986). A new restriction endonuclease from *Spirulina platensis*. Nucleic Acids Res., 14: 1985-1990.

Miranda MS, Cintra RG, Barros SBM, Filho JM (1998). Antioxidant activity of the microalga *Spirulina maxima*. Brazilian. J. Med. Biol. Res., 31: 1075–1079.

Moore R, Mynderse J, Kashiwagi M, Norton T (1977). Antileukemia activity in the oscillatoriaceae: isolation of debromoaplysiatoxin from Lyngbya. Science, 196: 538-539.

Nubel U, Garcia-Pichel F, Muyzer G (1997). PCR primers to amplify 16s rRNA genes from cyanobacteria. Appl. Environ. Microbiol., 63: 3327-3332.

Piorreck M, Baasch KH, Pohl P (1984). Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. Phytochemistry, 23: 207–216.

Qiang H, Zarmi Y, Richmond A (1998). Combined effects of light intensity, light-path and culture density on output rate of *Spirulina platensis* (Cyanobacteria). Eur. J. Phycol., 33: 165- 171.

Rafiqul IM, Hassan A, Sulebele G, Orosco C, Roustaian P (2003). Influence of temperature on growth and biochemical composition of *Spirulina platensis* and *S. fusiformis*. Iran. Intl. J. Sci., 4: 97-106.

Reichert CC, Reinehr CO, Costa JAV (2006). Semicontinuous cultivation of the cyanobacterium *Spirulina platensis* in a closed photobioreactor. Braz. J. Chem. Eng., 23: 23-28.

Robert, Masanobu (2005). Traditional microalgae isolation techniques. Algal culturing techniques. Elsevier Academic Press. 83-100.

Ross E, Dominy W (1990). The Nutritional Value of Dehydrated, Blue-Green Algae (*Spirulina Platensis*) for Poultry. Poult. Sci., 69(5): 794-800.

Thein M (1993). Production of Spirulina in Myanmar. (In. Doumengue, F., Durand-Chastel, H., Toulemont, A.,) Eds. Spiruline algue de vie. Musee Oceanographique. Bulletin de l Institut Oceanographique Monaco. Numero. Special, 12: 175-178.

Vonshak A (1997). *Spirulina platensis* (Arthrospira). Physiology, Cellbiology and Biotechnology. Taylor & Francis, London. ISBN 0-2035-8670-0, pp. 233.

Vonshak A, Abeliovich A, Boussiba S, Arad S, Richmond A (1982). Production of Spirulina biomass: affects of environmental factors and population density. Biomass, 2: 175–185.