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Research Article

NUTRITIONAL AND THERAPEUTIC EVALUATION OF *SPIRULINA PLATENSIS*

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ABSTRACT

Objective: The present investigation was to isolate and produce Spirulina platensis on high scale for food pharmaceutical and aquaculture due to the presence of high protein content.

Methods: Cultivation of *Spirulina* preparation of culture medium, inoculums build up, growth monitoring, harvesting, drying, procedure for protein estimation, sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and DNA *isolation of Spirulina platensis*.

Results: The study shows that protein content of *S. platensis* was found to be 62% protein profile was studied through SDS-PAGE, the bands were in the range of 35 kDa–44 kDa. *S. platensis* contain high molecular weight DNA. The bands was seen and visualize under the transilluminator it reveals that the alga *S. platensis* contain high molecular weight DNA.

Conclusion: The production of *Spirulina* by simple pH determination method suggests economic production of alga by a simple process. The DNA isolation showing that these algae contain high molecular weight DNA. However, utilization of this biomass for varied end uses, such as food, feed, aquaculture, and pharmaceuticals.

Keywords: *Spirulina*, Harvesting, Sulfate polyacrylamide gel, Protein estimation DNA isolation.

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INTRODUCTION

Population explosion has led in food demand which may not be met by conventional agricultural practices in coming years. Finding new food sources is one of the quests of scientific community. Natural food stuffs with high food values for this purpose should come in focus. Intense efforts have been made in developing countries for alternate new and unconventional protein source. Leaf protein concentrate, fish protein concentrate, and single cell protein (SCP) are major unconventional proteins considered to alleviate protein malnutrition problem [1]. The oldest photosynthetic representatives of formation and accumulation of primary product of photosynthesis are algal masses in water [2]. Algal biomass produced can be used for varied purposes ranging from food to biofuels. There are several natural compounds such as enzyme, pigment, and vitamins which can be obtained from algae. The blue green algae such as *Spirulina* and green algae such as *Chlorella* and *Scenedesmus* have shown advantage of using them as a source of SCP and for wastewater treatment.

SPIRULINA

Spirulina is a kind of aquatic organism that has existed for more than 3 billion years. There are about 30 species of *Spirulina* found in the different parts of the world. The most popular ones reported to be used and experimented are *Spirulina platensis* and *Spirulina* maxima. SCP term coined in 1966 for crude or refined sources of protein, whose origins are unicellular or simple multicellular organism such as bacteria, yeast, fungi, and algae [3,4].

Benefits of *Spirulina*

- Stimulates immune system to destroy invading disease organisms and carcinogens [5,6]
- Potentiates the immune system with its antitumor, antiviral, and interferon inducing effects
- Helps to sanitize the bowel by detoxifying the colon and promoting the growth of bacteria [7,8]
- Inhibition of HIV-1 replication carried by *Spirulina*. These blue-green algae inactivated HIV-1 infectivity directly when preincubated with virus before addition to human T-cell lines [9]
- Promotes tissue repair in wounds and burns and has anti-infectious properties
- Decreases cholesterol levels and helps to lower the risk of cardiovascular disease [10]
- Lactobacillus population in human gastrointestinal tract is increased by *Spirulina* consumption. This means food digestion and absorption improvement, intestinal protection against bacterial infections, and immune system stimulation [11]. Immune system modulation is due to interference on production and NK cytotoxicity [12]
- Works as an anti-inflammatory, helping to reduce the inflammation characteristic of arthritis
- Provides superior nutritional support as one of nature's whole foods for anyone who is weakened by disease, alcohol, or drug abuse
- Helps to balance RNA/DNA (nucleic acids)
- Curbs the appetite and helps to stimulate the metabolism
- Works like an antioxidant to the body of pollutants
- *Spirulina* does not have cellulose in its cell wall, a feature that makes it appropriate and important foodstuffs for people with problems such as intestinal absorption and geriatric patients [13]
- In anemic person hemoglobin count jumps from 6% to 11% after 1½ months intake
- Pain in joints 100% people between 50 years and 70 years got relief after taking *Spirulina* for 6 weeks
- Child growth: Even infants of 6 months can be given *Spirulina* powder (0.5 g–1 g) this helps in general growth and increase resistance to diseases. Best result obtained in between 3 and 6 years of age. Children with retarded growth also showed dramatic improvements
- Diabetes: It showed regulatory effect on both high and low sugar level in the system
- *Spirulina* as the main component, of skin care products, shampoos, dyes, masks, creams, and tonics is marketed internationally
- *Spirulina* has been studied as an animal cell-growth stimulant [14] and in the treatment of residual waters using alginate [15]. Phycocyanin shows activity on vegetable cell cultures with production of secondary metabolites as anthocyanin [16]. This pigment has the ability to inhibit oxidative damage in DNA and hence it may be used as a therapeutic agent [17]

- *Spirulina* can be used in several applications, in the form of tablets, as colorant in pasta or cakes or as novel protein food (meat replacement in ready-to-eat meals). *Spirulina* can be added to pasta, without significantly affecting the original cooking qualities. Up to 4–5 % addition of *Spirulina* is acceptable for consumers regarding taste and color
- Important to note in the area of prevention, *Spirulina* is richly supplied with the blue pigment phycocyanin, a billiprotein, which has been shown to inhibit cancer-colony formation. Predominant blue pigmentation in is rare. The chemical reality of *Spirulina's* blue color is demonstrated by its effects in the brain. Here, phycocyanin helps draw together amino acids for neurotransmitter formation, which increases mental capacity
- *S. platensis* contains about 13.6% carbohydrates some of these are glucose, rhamnose, mannose, xylose, and galactose [18]. A new high molecular weight polysaccharides with immunostimulatory activity has been isolated from *Spirulina* and is called "immulina." This highly water-soluble polysaccharide represents between 0.5% and 2.0% (w/w) of the dry micro algae [19]
- An extract of sulfated polysaccharides called calcium-*Spirulina* (Ca-SP), made up of rhamnose, ribose, mannose fructose, galactose, xylose, glucose, glucuronic acid, galactoronic acid, and calcium sulfate, obtain from *Spirulina*, showed activity against HIV, herpes simplex virus, human cytomegalovirus, influenza A-virus, mumps virus, and measles virus [3]. Current investigation in this field is searching for extracts that inhibit the AIDS virus replication [9] and allows these patients to improve their health
- Lactobacillus population in human gastrointestinal tract is increased by *Spirulina* consumption. This means, food digestion, and absorption improvement, intestinal protection against bacterial infection and immune system stimulation [3]. Immune system modulation is due to interference on production and NK cytotoxic
- *Spirulina* proteins are water extractable and nearly 85% of crude protein can be extracted by three successive extractions. The pH 8.0 was found to be most favorable for extraction the solubility decreases at other pH values [2]. The PER value of *Spirulina* is also higher than that of other vegetables and cereal proteins and soya proteins. Algal protein is often deficient in sulfur containing amino acids methionine and cystine. The pigment composition of the prokaryotic *Cyanobacteria* differs from the one in eukaryotic *Chlorophyceae*. The pigment of *Spirulina*, the only cyanobacterial of commercial importance has been determined as, Chlorophyll-a, B-carotene, echinione, a-cryptoxanine, zeaxanthin, myxoxanthophyll, or myxoxanthophyll-such as myxol-glycoside, oscillaxanthin, or oscillaxanthin-like oscillol-glycoside
- Analysis of *Spirulina* proteins using PAGE reveals the presence of seven bands, of which three are prominent. They are fast moving, low molecular weight fraction [2]. Three bands may be chlorophyll protein complex and biliprotein (C-phycocyanin and allophycocyanin) present in these algae. Characterized two billiprotein, C-Phycocyanin, and allophycocyanin in *S. platensis*. Molecular weights are 44KD for C –phycocyanin and 33KD for allophycocyanin
- The only blue-green alga (cyanobacteria), which has gained importance for mass cultivation, is *Spirulina* [2]. It can be cultivated in either in a liquid or in a culture. When cultivated in aqueous culture the cell growth can be determined by optical density, pH, and chlorophyll measurements. On the other hand, when produced by solid cultivation the growth can be determination of the fermenting solids. pH and protein production (solid or surface culture) or cell content (liquid culture) correlate well, therefore pH determination seems to be a good method to determine cell growth. In general it is produced in open ponds in liquid culture [3] recently; its production in solid-state cultivation systems has been studied.

Comparative advantage of *Spirulina*

- 5000% more iron than spinach
- 500% more calcium than whole milk
- 1000% more β-carotene than carrots
- 2000% more β-carotene than papaya
- 300% more protein than fish, meat, or poultry and above all without cholesterol.

MATERIALS AND METHODS

Cultivation of *Spirulina*

Preparation of culture medium

Zarrouk's medium [20,21] is used throughout the operation of *Spirulina*. The following chemicals are to be weighed separately and mixed with good, contamination free tap water (Table 1).

Test alga: Mother culture of *S. platensis.*

The mother culture was obtained through the courtesy of Dr. Soham Pandya and Mrs. Usha Dhage (E.S.T.D.C.S.V.'Dattapur) [22].

Inoculum build up

The mother culture (25 ml) is then transferred to plastic tub containing 5 l of media. The pH and O.D. are taken after transfer of culture. After 12 days of incubation the well-developed culture is then transferred to the tub containing 10 l media. The O.D. and pH are measured during incubation period. After 12 days of incubation, the 9 l of culture is transferred to the cement tub containing 100 l media and 1 l is maintained as a stock culture. The O.D. and pH are measured during incubation period.

Growth monitoring

This step includes online measurements of culture depth temperature, pH, and agitation regular agitation by hand stirring for keeping alga in healthy condition.

- Microscopy
  The alga is observed daily with the help of microscope for the bacterial contamination.
- pH measurements
  The pH was measured firstly by pH paper method for approximate reading and after that by pH meter for accurate reading.
- O.D. measurements
  O.D. is measured by photoelectric colorimeter at 560 nm during the period of incubation from the day of addition of inoculum to the medium.

Harvesting

Spirulina can be harvested when optical density of culture reaches 0.8–1 at 560 nm. The nutrient medium employed helps to float the trichome in early hours of the day. Harvesting can be done by pouring the algal suspension on cloth filter supported by bamboo frames. The harvested slurry is then washed with tap water 5–6 times to remove salts and to bring the pH to 7.5–8.0, and weight of wet *Spirulina* was measured. The wet *Spirulina* produced from liquid culture was calculated.

Drying

The washed algal concentrate is dried under sunlight on the polythene 5 h.

- Dry weight measurement

The weight of dry powder was measured and calculated w/w with wet *Spirulina*.

Procedure for protein estimation

Sample which was given after harvesting from that 1 g of sample was taken homogenized, freeze and thawed. Then distilled water was added. Extract was then centrifuged for 10 min at 15000 rpm. Supernatant was collected and used for protein estimation.

Protein was estimated quantitatively by Bradford method.

- Requirements
  1. 0.5 ul/ug BSA

2. 0.15 molar NaCl
3. Coomassie brilliant blue (CBB) – G

- Composition of CBB – G:
  - CBB-G-250 – 10 mg
  - 95% ethanol – 5 ml
  - 85% phosphoric acid – 10 ml
  - Distilled water – 85 ml
- Composition of CBB – G:
  - CBB-G-250 – 10 mg
  - 95% ethanol – 5 ml
  - 85% phosphoric acid – 10 ml
  - Distilled water – 85 ml

Procedure

1 ml of supernatant was taken in the test tube. Then, volume was made up to 10 ml by addition of 0.15M NaCl to the tube. Then, 1 ml freshly prepared CBB solution was added to the tube. Allow the tube to stand for 5 min at room temperature. The faint blue color was developed. The absorbance was recorded at 595 nm, and concentration was measured with respect to standard graph (Table 2).

Sulfate polyacrylamide gel electrophoresis (SDS – PAGE) sodium dodecyl sulfate poly-acrylamide gel electrophoresis (Tables 3-5)
#SDS-PAGE was carried out using extracts prepared from *S. platensis*.

#Materials

1. Electrophoresis apparatus
2. Micropipette.
   - Chemicals

1. Acrylamide, bisacrylamide
2. Ammonium persulfate
3. TRIS
4. N,N,N',N' Tetramethylene diamine
5. Glycine
6. Sodium dodecyl sulfate
7. Mercaptoethanol
8. Glacial acetic acid
9. Isobutanol
   - Lysing buffer
     - SDS - 0.2 g
     - 1.5 m TRIS (pH-6.8) - 500 ul
     - Glycerol - 1 m
     - Mercaptoethanol - 50 ul.

Protocol for SDS - PAGE

1. The glass plates were washed; rinsed with distilled water and then cleaned with tissue paper to remove traces of water. Glass plates were assembled as per requirement. Following (Tables 4 and 5)15 ml of 10% resolving gel was prepared. Polymerization began as soon as the TEMED was added. Without any Bromophenol blue (5 mg) was dissolved in 5ml of distilled water
2. Delay, the mixture was rapidly swirl and proceeded to the next step
3. Poured the acryl amide resolving solution into the gap between the glass plates. Sufficient space for the stacking gel (approx. 1.5 cm) was left. Carefully over layer the acrylamide solution with isobutanol. Gel was placed in vertical position at room temperature
4. After polymerization was completed, poured off the overlay and washed the top of the gel several times with deionized water to remove any unpolymerized acrylamide. Remaining water was removed with the edge of a paper towel
5. The stacking gel was poured directly into the surface of the polymerized resolving gel, containing a clean Teflon comb for making well, with special care to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces of the comb completely
6. The samples were prepared by adding a protein sample and the gel loading dye
7. After polymerization was completed, Teflon comb was removed carefully

Table 1: Composition of Zarrouk's media

Chemicals	Formula	g/l
Sodium bicarbonate	$NaHCO_3$	16.0
Dipotassium hydrogen phosphate	K_2HPO_4	0.5
Sodium nitrate	$NaNO_3$	2.5
Potassium sulfate	K_2SO_4	1.0
Sodium chloride	NaCl	1.0
Magnesium sulfate	$MgSO_4$	0.2
Calcium chloride	$CaCl_2$	0.04
Ferrous sulfate	$FeSO_4$	0.01
EDTA	EDTA	0.08
Trace elements (A-5 solution)		5.0 ml

Table 2: A-5 M solution preparation (g/l)

Chemicals	g/l
Manganous chloride	1.81
Molybdic acid	0.017
Zinc sulfate	0.222
Copper sulfate	0.079
Boric acid	2.86
pH	9.5

Table 3: Acrylamide resolving gel (10%)

Distilled water	5.9 ml
30% acrylamide mix	5 ml
1.5M Tris (pH-8.8)	3.8 ml
10% sodium dodecyl sulfate	150 ul
10% ammonium persulfate (0.1 g in 1 ml) (freshly prepared)	150 ul
TEMED	9 ul

*Loading dye (0.1%)

Table 4: Acrylamide stacking gel (5%)

Distilled water	3.4 ml
30% acrylamide mix	830 ul
1.5 m Tris ()Ph-6.8	630 ul
10% sodium dodecyl sulfate	50 ul
10% ammonium persulfate	50 ul
TEMED	5 ul

Bromophenol blue (5 mg) was dissolved in 5 ml of distilled water

Table 5: Tris-glycine electrophoresis buffer (×5)

25Mm Tris base	1.1 g
250 Mm glycine (pH-8.3)	9.4 g
Distilled water	100 ml
10% sodium dodecyl sulfate	50 ml

*During gel electrophoresis ×1 of Tris-glycine electrophoresis buffer should be used

8. The wells were washed immediately with deionized water to remove unpolymerized acrylamide. The gel was mounted in electrophoresis apparatus. Tris buffer was added to the top and bottom reservoirs. Air bubbles, trapped at the bottom of the gel between the glass plates, were removed.
   - Loading of protein samples

   Sufficient amount of protein from the samples was loaded in predetermined order into the bottom of the wells. This was done with a microliter syringe, which was precleaned with Tris-buffer from the bottom of the reservoir after each sample was loaded. In a well of the same gel, protein molecular weight marker was also loaded.
   - Staining
     a. Staining solution was prepared and filtered through the Whattman filter paper No. 1

b. The gel was immersed in staining solution for 4 h. Subsequently, the gel was destained in distaining solution for 2 h (Table 6 and 7).
c. In destained gel, minute quantity of stained protein bands could be detected
d. Detected band were photographed for permanent record.

DNA isolation of *S. platensis*

Buffers and solution
Ethanol
70% Ethanol
Phenol: Chloroform (1:1,v/v)
Sodium acetate (3 M, pH- 5.2)
STES buffer
0.2 M Tris Cl (pH-5.2)
0.5M NaCl
0.1% (W/V) SDS
0.01 M EDTA
(store it at room temperature for 15 min)
TE buffer (pH-7.6)
100 mM Tris Cl (pH-7.6)
10 mM EDTA (pH-8.0)
TAE Buffer
×50
242 g of Tris base
57.1 ml of glacial acetic acid
100 ml of 0.5 M EDTA (pH-8.0)
×1 6 ml ×50 TAE were dissolved in 300 ml distilled water and it is used as an electrophoretic buffer.
Agarose gel (1%) (electrophoretic gel)

Dissolve 0.5 g Agarose powder in 50 ml TAE buffer and heat to make a gel and add 5 ul ethidium bromide to the gel carefully.

Procedure for DNA isolation

- Take approximately 1 mg of *Spirulina* powder in the 1.5 ml vial
- Add 50 ul STES buffer to the vial
- Then add 20 ul TE buffer to the vial
- Add 60 ul phenol:chloroform to the vial, cap the tube and mix the organic and aqueous phases by vertexing for 1 min
- Centrifuge the tube at 15000 rpm for 15 min
- Transfer the upper aqueous phase to a fresh vial then add 50 ul ethanol and maintain for 15 min at 0°C.
- Centrifuge it at 15000 rpm for 10 min at 4°C.
- Remove the supernatant by aspiration and rinse the pallets with 100 ul of 70% ethanol in water, centrifuge the tube at 15000 rpm for 10 min

Table 6: Staining solution (100 ml)

Coomassie brilliant blue	0.25 g
Methanol	45 ml
Glacial acetic acid	10 ml
Distilled water	45 ml

Table 7: Destaining solution (200 ml)

Glacial acetic acid (10%)	20 ml
Methanol (10%)	90 ml
Distilled water (45%)	90 ml

- Set the electrophoresis apparatus adjust the comb and pour the gel to make a well
- Remove the supernatant by aspiration and allow the pallets to dry in air for 20 min
- Redissolve the pallets in 40 ul TE (pH-7.6)
- Add the gel loading dye (Bromophenol blue) 5ul to the vial containing TE and DNA pallets and load to the well of the gel
- In another well molecular weight marker was loaded
- Started the electrophoresis, carried out first at 50 V and after 1 h it was carried out at 100 volt, 3 h and band, was shown under the transilluminator.

RESULTS

The test alga *S. platensis* grown and there growth was monitored through regular measurement of pH and O. D. at 560nm at regular time interval. On the 1st day of inoculation of mother culture to 5 l media, 5 l–10 l tub and 10 l tub–100 l cement tub, O.D. and pH were 0.45 and 8.5, respectively.

During the growth, the O. D. rises from 1st day of inoculation to the harvesting stage rapidly, but pH not rises rapidly. Therefore, at the time of harvesting O. D. and pH was 1.15 and 10.5. The O. D. and pH increase during growth are mention in the Table 8.

After O. D. reaches to the range of 1.0–1.15 and pH was constant at 10.5 for 2–3 days then liquid culture of *Spirulina* was harvested with the help of cloth filter after 12 days of incubation. From 100 l of liquid *Spirulina* culture, 550 g of biomass was obtained. Then that wet *Spirulina* was sun dried, 70 g of *Spirulina* powder was obtained from 550 g of wet *Spirulina*. Thus, 70 g *Spirulina* powder was obtained from 100 l liquid *Spirulina*.

The protein content of *S. platensis* was found to be 62% (w/w dry weight) when the extract obtained after centrifugation diluted with 2000 ml distilled water. Protein profile was studied through sodium dodecyl SDS-PAGE the bands were in the range of 35 kDa–44 kDa.

The alga *S. platensis* contains chlorophyll and also highly organized structure. Due to these structures it may contain high molecular weight DNA. The bands were seen and visualize under the transilluminator and compared with the molecular weight marker (ladder) and it was be in the range of 20 kbp–25 kbp. Thus, it reveals that the alga *S. platensis* contains high molecular weight DNA.

DISCUSSION

Spirulina is the common name for human and animal food supplements produced primarily from species of cyanobacteria: Platensis, it is microscopic blue-green alga in the shape of a spiral coil, living both in sea and fresh water. It is a rich source of many important nutrients such as proteins, complex carbohydrates, iron, Vitamins A, K, B complexes, minerals, lipids, and essential fatty acids [23,24]. It is edible, filamentous, alkalphilic, photoautotrophic cyanobacterium belonging to the class Cyanophyta.

Growth was measured as an increase in optical density and pH of *S. platensis*. Thus, pH increases during growth and show maximum at the time of harvesting.

Literature reveals that pH determination can be used as an indicator of microbial growth. If an standard experiment is done to have a calibration curve for each process condition, the cell growth can be rapidly estimated by culture pH [25].

Table 8: O.D. and pH during 12 days of growth

Day	1	2	3	4	5	6	7	8	9	10	11	12
O.D.	0.45	0.49	0.55	0.61	0.67	0.73	0.79	0.86	0.91	9.7	1.00	1.12
pH	8.5	8.5	8.7	8.9	9.0	9.3	9.6	9.9	10.2	10.5	10.5	10.5

The higher growth indicated through pH and optical density during 10–12 days of growth periods led to biomass production of 75 g/100 l, which is in the line with earlier studies on *S. platensis*.

The literature also explored the use of test species as a rich source of protein, it contains 60–71% of protein and comparable with the literature. Thus, it is use as a SCP worldwide. Protein profile of *S. platensis*, using SDS-PAGE showed bands, which could be compared with C- phycocyanin (mw 44 kDa) and Allophycocyanin (mw 38 kDa) [26].

The *Spirulina* contains high content of DNA was isolated by gel electrophoresis and its molecular weight was in the range of 20 kbp–25 kbp compared with molecular weight marker (ladder) [27].

The present study also show nearly same result that the *S. platensis* contains 62% protein and protein profile of *S. platensis*, using SDS-PAGE show band and were in the range of 35 kDa–44 kDa. DNA isolation by electrophoresis shows molecular weight in the range of 20 kbp–25 kbp.

CONCLUSION

The present study indicated potential of the test species, *S. platensis* which grow in Zarrouk's media were harvested when pH not more than 10.5 constant for 2–3 days, show protein content of 62% of dry weight.

The biomass dry weight production of 75 g/100 l of *Spirulina* liquid by simple pH determination method suggests economic production of alga by a simple process. The DNA isolation showing that these algae contain high molecular weight DNA.

However, utilization of this biomass for varied end uses, such as food, feed, aquaculture, and pharmaceuticals needs to be established. Furthermore, there is need of obtaining qualitative and quantitative information about *Spirulina* protein. Toxicological studies should also be done to know any adverse effects if it is used as SCP.

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AUTHORS CONTRIBUTIONS

Both authors have contributed to the preparation of this review and editing of the manuscript.

CONFLICTS OF INTEREST

We declare that we have no conflicts of interest between us.

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