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Morphological and physiological performance of a cyanobacterium *Spirulina platensis* in presence of Ascorbic acid: a growth facilitator

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Abstract

Present study has been undertaken to demonstrate efficacy of ascorbic acid as growth enhancer as well as metabolic promoter of vital pigments in Blue green algae. *Spirulina platensis* has been known as an important nutraceutical which is endowed with balanced proteins, antioxidants, polyunsaturated fatty acids, vitamins and other nutritional components. There is a regular demand for additional biomass availability. Ascorbic acid concentrations ranging from 6-10 mgl^{-1} produced a significant increase in its growth. Axenic cultures of the organism were serially examined in a dose dependent manner. An increase amounting to 61% (on dry weight basis) indicated a significant growth effect, especially from 4-16 days of culture. Area under curve (AUC) for growth of ascorbic acid yielded significant difference across various groups (p-value<0.01). Significant increase of chlorophyll a, phycocyanin, carotenoids and protein content of *Spirulina* was observed. Light microscopic studies indicated that ascorbic acid at $6mgl^{-1}$ increased the frequency of spirally coiled filaments (~41%) in comparison to control cultures (~8 %) . Ultrastructurally not much difference was observed in its morphology. To conclude, our study suggests that ascorbic acid has an important role in boosting the growth of *Spirulina* which needs to be exploited commercially for value added products.

Keywords: Ascorbic acid, Cyanobacteria, Growth facilitator, Nutraceutical, *Spirulina*

Introduction

Arthrospira platensis (*Spirulina*) is a filamentous spirally coiled ubiquitously occurring cyanobacterium that has been produced commercially in various countries Belay (1997). It has a rich nutritional profile with high quality and quantity of proteins making it a robust interventional agent to deal with malnutrition Pinero-Estrada *et al*. (2001). Moreover, it has a huge potential to be designated as an important nutraceutical because of the presence of balanced proteins, coupled with vitamins and minerals Belay *et al*. 1993; Habib *et al*. 2008). It is one of the most promising cyanobacteria in market since the success of its commercial production in 1980's. Although *Spirulina* grows well in sunny, warm alkaline water but the cost reduction at commercial scale remains a major challenge to make it affordable to masses Jensen & Knutsen (1993). In recent years, its productivity has been a matter of concern both in its morphological quality Vonshak & Richmond (1988) and physiological behavior Richmond and Grobbelaar (1986). The present study has been conducted to explore the effect of a biological growth facilitator i.e. ascorbic acid which is easily available, cost effective and is known for enhancing growth of higher plants (Dolatabadian *et al*. 2008; Khan *et al*. 2011).The effect of ascorbic acid as growth promoter in cyanobacteria specifically in *Spirulina* has been unknown. In this backdrop, the study has been carried out to evaluate the effect of varying concentrations of ascorbic acid on the growth and pigment status of *Spirulina* so as to delineate its total productivity on bench scale and make it more profitable.

Materials and Methods

The cyanobacterium, *Spirulina platensis,* belonging to family Oscillatoriaceae, was procured from National Centre for Conservation and Utilization of Blue Green Algae (NCCUBGA), Indian Agricultural Research Institute, New Delhi. All the chemicals used were of analytical grade obtained from the Himedia laboratories Pvt. Ltd., Mumbai, India.

Culture and medium

Spirulina was axenically cultured with 2.5 liters of working volume in Zarrouk medium Zarrouk (1966). All glassware, nutrient media used during experimentation were steam sterilized in an autoclave at 15 lbs pressure $inch^{-2}$ for 15 minutes. The containers were inoculated with the cyanobacteria obtained from a stock culture, maintained at mid log phase of growth. The cultures were maintained at 35 ± 2°C, with pH of 9 – 9.5 exposed to 12 h light-dark cycles in an illuminated chamber at 2.5 kilolux. These were aerated with air pumps of $1watt/m^2$ to provide gentle mixing and CO_2. Due to the acidic nature of ascorbic acid, pH of the culture medium was adjusted following its addition as it is difficult

to maintain axenic cultures of *Spirulina* at lower pH. Wide spectrum of ascorbic acid concentration was initially tested to identify the lowest and highest endpoints of concentrations showing no or inhibitory effect respectively. From this ascorbic acid concentration was finally selected which showed a definite increase in *Spirulina* growth. Prior to sampling, biomass attached on the flask walls was carefully resuspended by swirling the culture contents with magnetic stirrer. At different time intervals, samples (around 200 ml) were taken from each container to monitor biomass in terms of growth, dry weight, protein content, chlorophyll content, amount of carotenoids and phycocyanin.

Estimation of growth and biomass

The growth was quantified every 4th day till 24 days. Growth was monitored as an increase in absorbance which was estimated spectrophotometrically. The absorbance was recorded at 550 nm regularly every 4th day interval. The experiments were run in triplicate. The algal biomass was determined gravimetrically and growth was expressed in terms of dry weight (µg ml^{-1}). Homogenous suspension was centrifuged and washed with double distilled water to remove salts. The culture was filtered through preweighed filter paper (Whatman No. 40 of 10 cm diameter). Drying at 70˚C was continued until successive weighing gave a constant reading

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Estimation of protein content

The total protein content of the cultures was analyzed by method of Lowry *et al.* (1951). Cells were harvested and washed by centrifugation, hydrolyzed with 1N NaOH in a boiling water bath for 10 minutes. The contents were cooled, centrifuged and the supernatant was used for estimation of proteins.

Estimation of Chlorophyll a:

Cold extraction method using acetone was employed for chlorophyll *a* estimation. A known volume of culture was centrifuged, washed twice with double distilled water. To the pellet, known volume of acetone was added and the mixture was shaken vigorously and incubated in a refrigerator for 12 hours. The contents were centrifuged at 700 × g, and the absorbance of supernatant was measured at 665nm.The amount of chlorophyll was determined using the specific absorbance coefficient of 13.9 for chlorophyll Mackinney (1941).

Estimation of carotenoids:

Carotenoids were extracted in 90% acetone. A known amount of culture (10 ml) of homogenized algal suspension was taken and centrifuge at 1008 × g for 10 mins. The pellet was washed 3 times and 3 ml acetone was added and the samples were freeze thawed. Absorbance was taken at 450 nm with 85% acetone as blank. The concentration of carotenoids was determined using specific absorbance coefficient (α) of 12 Weber & Wetton (1981)

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Estimation of phycocyanin:

Phycocyanin was estimated by the method given by Kaushik (1987). Homogenous suspension of the culture of known volume was centrifuged at 2800 × g for 10 minutes. The contents were subjected to repeated freeze-thaw cycles till all the pigments were released from the cells and absorbance of supernatant was measured at 565, 615 and 652 nm. The amount of phycocyanin was calculated as mg ml^{-1} using the equation

$$\text{Phycocyanin (PC)} = \frac{A_{615} - 0.474}{5.34} \times \frac{A_{652} \times \text{Volume made}}{\text{Volume used}}$$

Where A_{615} is OD at 615nm, A_{652} is OD at 652 nm

Morphological and ultrastructural studies

Cell morphology was observed for microscopically detectable morphological alterations, occurrence of minor variant (linear form) of *Spirulina*. The culture of *Spirulina* was viewed under Olympus microscope at 400X to count the number of straight and coiled filaments till the development of hormogonia in control cultures. 0.2 ml of diluted culture was taken on slide and pressed firmly to fix the filaments in place. The straight and coiled filaments of *Spirulina* were counted to determine their relative frequency.

Scanning electron microscopy (SEM) was carried out to observe any change in its structure at finer scale. The filaments were fixed with 2.5% gluteraldehyde and dehydrated in increasing grades of ethanol. Critical point dried specimens were loaded on metallic specimen stubs and, thereafter, were conducted with gold in ion sputter unit JOEL JFC -1100 Bozzola & Russell (1992). The conducted specimen was examined under JOEL-JSM 6100.

Statistical analysis

All values were expressed as mean ± standard error mean (SEM) of the three replicates. Serial measurements of growth from baseline to day 24 were stratified by various ascorbic acid groups. They were further analyzed and compared by summary measures of area under concentration time curve (AUC_{0-t} max), time weighted average and maximum percentage difference from baseline using non-parametric Kruskal Wallis test. This was followed by Mann-Whitney- U test for post hoc test for the pair wise comparisons between the various treated groups. Statistical analysis was carried out using Stata 12 IC version and MedCal 3.1 version. Values having p <0.05 were considered as statistically significant.

Results

Cyanobacterial growth

Spectrophotometrically, the effect of growth facilitator ascorbic acid on growth pattern of *Spirulina* showed approximately 69% increase with different concentrations of ascorbic acid supplementation compared to control. The growth levels measured serially from day 0-24 exhibited statistically significant differences overall when stratified by different doses of ascorbic acid (F-statistics 163.35; p value < 0.01). Maximum growth enhancement was reported at 6- 10 µg ml^{-1} concentration between 8-16 days, which corresponds to the mid log phase of *Spirulina* growth (Fig 1). Mean absolute deviation (MAD) from baseline to maximum value in growth was the highest at 8 µg ml^{-1} ascorbic acid followed by 10 µg ml^{-1} and 6 µg ml^{-1}. The MAD values were statistically significant among various doses used (P-value <0.01). On posthoc pair-wise analysis, all the doses of ascorbic acid showed statistically significant difference from control.

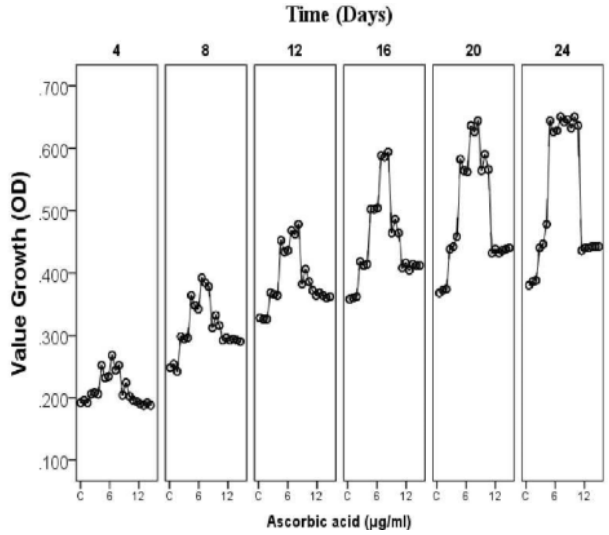

Fig.1: Growth of *Spirulina* growth under different concentrations of ascorbic acid (2-12 µg ml^{-1}) measured spectrophotometrically at 560nm. (F-Statistics 163.35, p-value < 0.01)

Dry weight of Spirulina

Dry weight of *Spirulina* was 61 % higher than the control, the maximum difference was recorded during the mid log phase of growth. (Fig 2*)* The growth levels measured serially from 0-20 days showed overall statistically significant differences when supplemented by different doses of ascorbic acid (F-statistics 10.51; p value < 0.01). Serial measurements of growth from baseline to day 24 stratified by various ascorbic acid concentrations were further analyzed and compared by summary measures of area under curve ($AUC_{0,tmax}$), and maximum percentage difference from baseline using non-parametric Kruskal Wallis test. Differences in $AUC_{0,max}$ values (X^2=19.325; P-value<0.01). Maximum percentage difference (X^2=18.83; P-value<0.01) were statistically significant among for 6-8 µg ml^{-1} of ascorbic acid. From aforementioned results, it is, therefore, inferred that ascorbic acid at 6-8 µg ml^{-1} is the best suited for increased in biomass production. Maximum cell productivity of 0.82 µg $ml^{-1}day^{-1}$ was reported in *Spirulina* cultures in comparison to 0.43 µg ml^{-1} /day in control cultures indicating positive correlation of ascorbic acid and growth of the test organism.

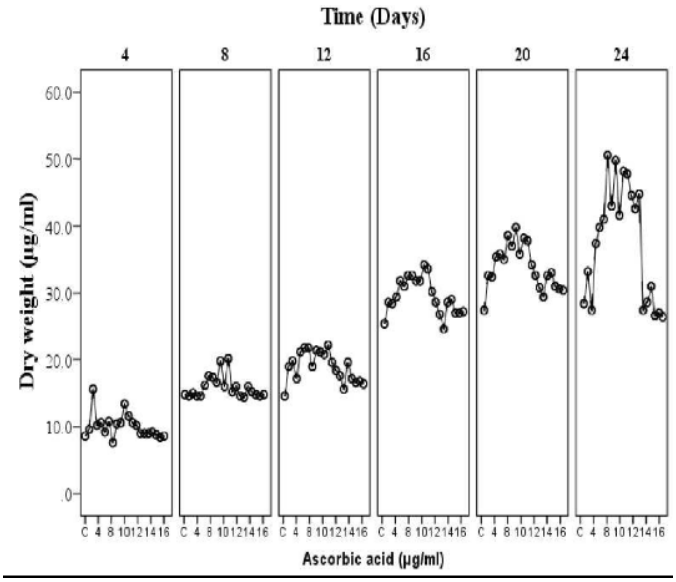

Fig.2: Gravimetric analysis of *Spirulina* growth (µg ml^{-1}) under different concentrations of ascorbic acid (2-12 µg ml^{-1}) measured by dry weight. (F-statistics 10.51, p value < 0.01).

Chlorophyll A content

There was approximately 49% increase in chlorophyll *a* content with 8 µg ml^{-1} ascorbic acid on 20th day. After 20th day the content declined in the control but increased further (51%) in ascorbic acid supplemented medium on 24th day. The chlorophyll *a* content measured serially from 0-24 day, showed significant difference when stratified with different ascorbic acid regimes (F-statistics 24.22; p value < 0.05). MAD values in chlorophyll *a* concentration were the highest for ascorbic acid dose of 8 µg ml^{-1} (3.07±0.008) followed by 6 µg ml^{-1} (2.93±0.02). MAD values showed significant difference among various doses of the ascorbic acid regimes used (P-value <0.01). Hence, the stimulatory effect of ascorbic acid on chlorophyll content is maximised at 8 µg ml^{-1} dose (Fig 3).

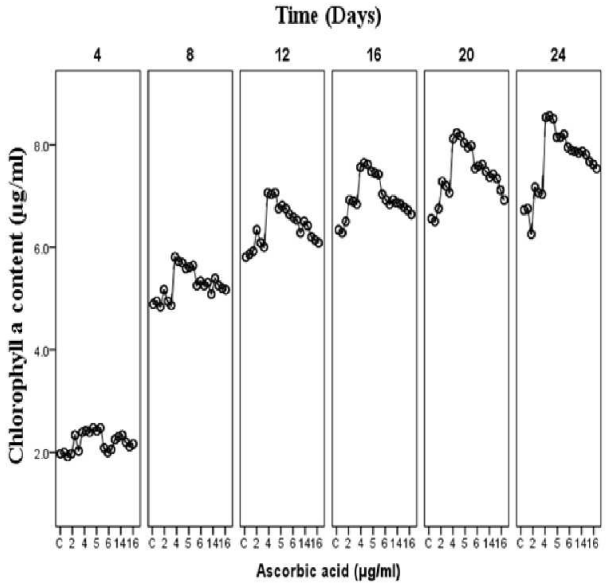

Fig.3: Chlorophyll *a* content (µg ml^{-1}) at different concentrations of ascorbic acid (2-12 µg ml^{-1}) on every 4th day of growth. (Values are expressed as Mean ± SE; n = 3. F-statistics 24.22; p value < 0.05).

Carotenoid content

Significant enhancement content of carotenoids (~42%) was recorded with nutrient medium supplemented with ascorbic acid. Maximum increase was noted between 8-12 days of growth. Decline in carotenoid production was recorded with concentrations above 12 µg ml^{-1} (Fig 4). The carotenoid content measured serially from 0-24 day was significantly different when stratified with different doses of ascorbic acid (F-statistics 7.69; p value < 0.01). MAD value from baseline to maximum value for carotenoids was highest for 8 µg ml^{-1} ascorbic acid dose (2.48±0.03) followed by 6 µg ml^{-1} (2.30±0.03). Posthoc analysis depicts that 8 µg ml^{-1} ascorbic acid group showed significantly greater MAD as compared to control. $AUC_{0,max}$ values showed significant increase among various ascorbic acid groups (X^2=11.282; P-value<0.05) was statistically significant among various ascorbic acid groups.

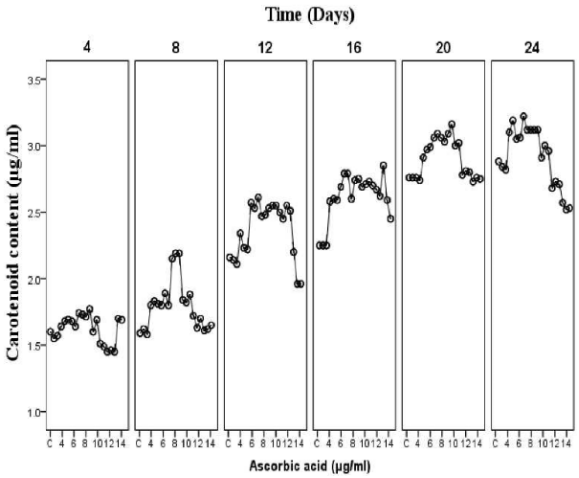

Fig.4: Carotenoid content (µg ml^{-1}) at different concentrations of ascorbic acid on every 4th day of growth. (Values are expressed as Mean ± SE; n = 3. F-statistics 7.69; p value < 0.01.

Phycocyanin production:

Phycocyanin, one of the major protein components of *Spirulina,* showed significant increase (~38%) with ascorbic acid supplementation. The maximum increase was recorded during 12th -15th day of growth. There was decrease in phycocyanin content after 16th day of culture. Ascorbic acid at 6-8 µg ml^{-1} had significant effect on phycocyanin production (Fig 5). The phycocyanin content measured serially from 0-16 day showed significant difference when stratified with different ascorbic acid regimes (F-statistics 21.91; p-value< 0.01). MAD value from baseline to maximum value in phycocyanin was the highest for 8 µg ml^{-1}. Differences in $AUC_{0,\ max}$ values (X^2=18.99; P-value< 0.01) was statistically significant among various ascorbic acid groups.

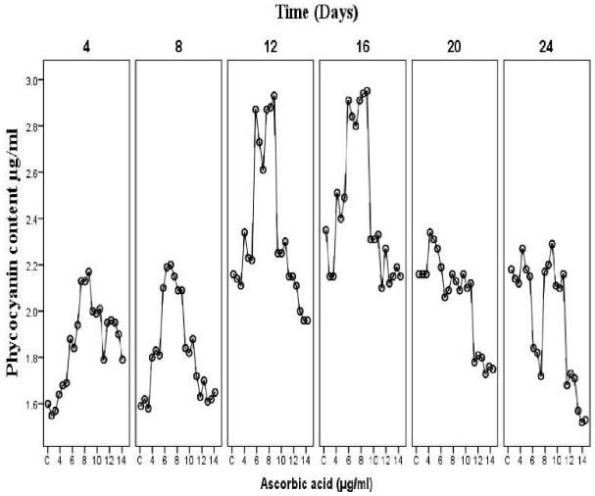

Fig.5: Phycocyanin content (µg ml^{-1}) at different days of growth. F-statistics 21.91; p value < 0.01.

Protein Content

Ascorbic acid significantly increased protein content in *Spirulina* cultures. There was about 48% increase in the content with 6-10 µg ml^{-1} concentration. The protein content increased throughout the growth phase till 24th day (Fig 6). Further, the content measured serially from 0-24 day showed significant difference when stratified with different ascorbic acid regimes (F-Statistics 2.97, p-value < 0.01). Protein content from 4th to 24 day at various ascorbic acid concentrations was further analyzed and compared by summary measures of $AUC_{0,tmax}$, and MAD from baseline using non-parametric Kruskal Wallis test. MAD value from baseline to maximum was the highest for 8 µg ml-1 ascorbic acid dose (0.68±0.04). Differences in $AUC_{0,tmax}$ values (X^2=19.30; P-value 0.04) was statistically significant among various ascorbic acid groups.

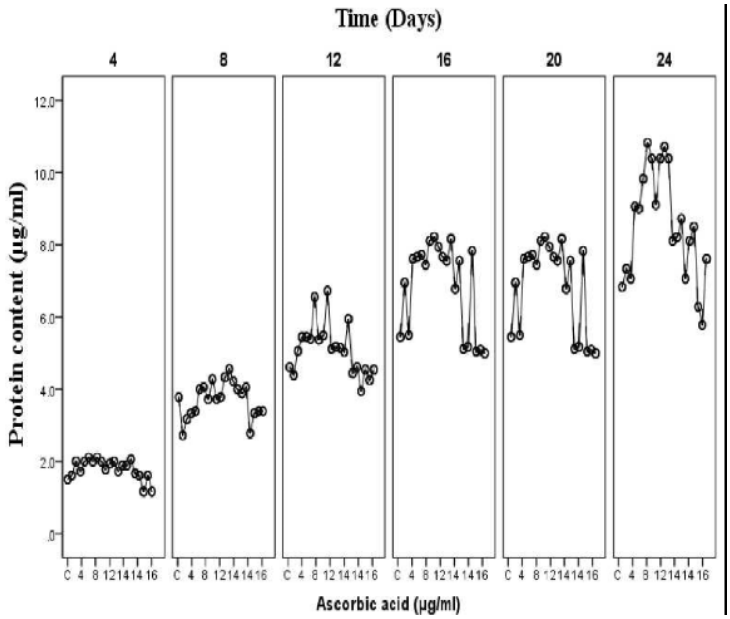

Fig.6: Protein content (µg ml^{-1}) at different days of growth. F-statistics 2.97; p value < 0.01.

Light microscopy

Spirulina is a non heterocystous filamentous cyanobacterium which showed rapid growth after 4th day of culture. The helical trichomes of varying sizes showing different degree of coiling and even occurrence of straight uncoiled filaments were observed (Fig 7). Ascorbic acid had a protective effect on maintaining its helical morphology. The culture supplemented with ascorbic acid at 6 µg ml^{-1} showed decrease in the percentage of straight filaments (Fig 7b) as compared to control (Fig 7a, Fig 8). The development of hormogonia was observed on 40th day in control (Fig 7c) whereas, ascorbic acid supplemented group showed further growth (Fig 7d). The growth span of *Spirulina* increased and resulted in delayed formation of hormogonia. Further Scanning electron microscopy illustrated no structural alterations in coiled and straight filaments when supplemented with ascorbic acid Fig.7 (e, f).

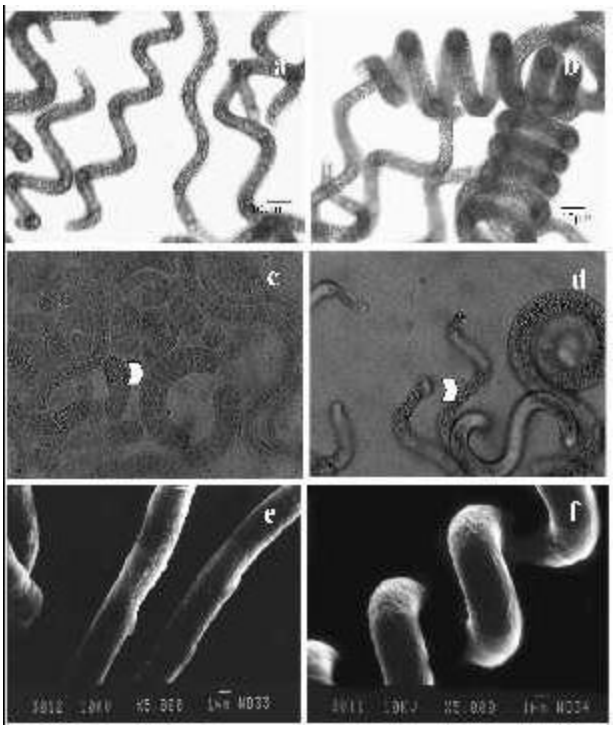

Fig. 7 (a-d) Light microscopic study showing comparative morphological changes of *Spirulina platensis* when cultured with ascorbic acid supplementation. (a) Higher no. of spirally coiled *Spirulina* in control (b) Higher number of helically coiled *Spirulina* seen with ascorbic acid indicating conserved morphology (c) *Spirulina* filaments showing formation of hormogonia on 40th day of growth in control (d) Ascorbic acid supplemented group showing growth on 40th day. (e-f) Electron micrograph of *Spirulina* showing no structural alteration in straight and coiled filaments of *Spirulina* when supplemented with ascorbic acid.

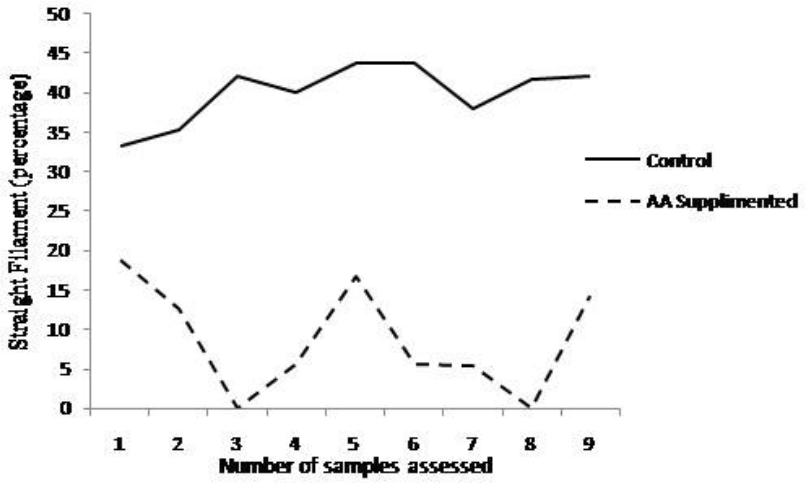

Fig. 8: Light microscopic study showing of effect of ascorbic acid supplementation (6μg ml^{-1})) on morphology of *Spirulina.* Ascorbic acid supplementation significantly conserved the coiled morphology of *Spirulina.*

Discussion

The present study, for the first time, demonstrated the efficacy of ascorbic acid as growth enhancer as well as metabolic promoter for a cyanobacterium *Spirulina.* Ascorbic acid has been shown to increase the heterocyst frequency three times as compared to control in *Anabaena ambigua* Wahal (1973). However, various studies on higher plants have attributed various functions to ascorbic acid including regulation of plant cellular mechanism

against environmental stress (Khan *et al*. 2011), regulation of cell cycle Kerk & Feldman (1995) and protective effect against UV-B radiation He & Haeder (2002). Growth enhancement by ascorbic acid supplementation can be explained in number of ways. Earlier studies reported that ascorbic acid can significantly accelerate the growth of higher plants, regulate cell wall expansion (Ohkawal *et al*. 1989; Esaka *et al*. 1990; Veljovic-Jovanovic *et al.* 2001) but the same may not be applicable to *Spirulina* as it is a prokaryotic organism and possesses peptidoglycan cell wall instead of cellulosic cell wall present in higher plants.

Ascorbic acid has been suggested to increase growth rate by stimulating cell expansion and solute uptake which is likely to be applicable to *Spirulina* as well (Gonzalez-Reyes *et al*. 1995). Lin & Varner (1991) had further suggested that ascorbate oxygen generates Docosahexaenoic acid (DHA) which reacts with the side chain of Lysine and Arginine residues in cell walls and prevents cross linking of structural proteins with hemicelluloses resulting in more extensible cell wall. *Spirulina* too, possess DHA and hence this may be one of its modes of action of ascorbic acid.

Besides positive effect of ascorbic acid on cyanobacterial growth, it also causes protective and enhancing effect on photosynthetic pigments in higher plants (Nahed *et al*. 2006). Present findings show that exogenous supplementation of ascorbic acid significantly enhanced chlorophyll *a*, carotenoids, and phycocyanin contents. Growth enhancing effect of ascorbic acid on *Spirulina* is in consonance with the observation of Wong (2000). This indicates that phytohormones have promotory effect on algal pigments. Significant increase in the pigment contents may be due to the formation of zeaxanthin which acts as a photoprotectant thereby protecting the photosynthetic pigments from damage. Our study also suggests an increase (~48%) of protein content in cells on supplementation of ascorbic acid. The protective role of ascorbic acid on protein degradation is in accordance with the effect reported by Dolatabadian *et al*. (2008) in Sunflower. The results signify that increased pigments of *Spirulina* due to ascorbic acid may be enhancing the rate of photosynthesis and thus promoted other pigments of *Spirulina.* Further, ascorbic acid is commercially available and cost effective product which can promote biomass production in such an important organism.

Spirulina showed conserved morphology when supplemented with ascorbic acid. Various environmental factors mainly temperature, light intensity Van Eykelenburg (1980), physical and chemical factors affect the morphology of *Spirulina* (Jeeji Bai and Seshadri 1980; Jeeji Bai 1985). Solar UV radiation (280-400nm) is another important environmental factor that affects its morphology in natural environment and in commercial production. The conserved morphology may be due to protective role of ascorbic acid under stress conditions. It also provides first line defense against UV radiation thereby protecting *Spirulina* from morphological alterations. Thus this could be considered as another reason for using ascorbic acid in commercial production as minor variants (straight filaments) are known to reduce the efficiency of harvesting and production compared to normal coiled ones (Vonshak & Richmond 1988; Belay 1997).

Conclusion

The study demonstrates efficacy of ascorbic acid as a growth enhancer of *Spirulina* as well as metabolic promoter of its vital pigments. The compound has potential to be used as the growth facilitator as it not only enhance the growth of *Spirulina* positively but also concomitantly enhance the protein content, novel pigments and maintained coiled nature of *Spirulina*. This can be of immense applied value for lowering the cost of biomass production of *Spirulina* on commercial scale. However, the mechanistic pathway through which this triggers the growth needs to be further investigated.

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