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Enhanced Production of High-Quality Biomass, δ-Aminolevulinic Acid, Bilipigments, and Antioxidant Capacity of a Food Alga *Nostochopsis lobatus*

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Abstract The growing interest in natural food has raised the global demand for nutraceuticals. We studied enhanced production of biomass, delta-aminolevulinic acid (δ-ALA), bili pigments and antioxidant capacity of a food alga *Nostochopsis lobatus* in a full-factorial (three level) design with supplemental Zn, glutamine, and Zn + glutamine in batch culture. Production of biomass, pigments, and antioxidant capacity all were higher under immobilized cell cultures in comparison to free cell cultures. Maximum biomass (2,390 mg dry wt l^{-1}), δ-ALA (2.715 μg mg^{-1} dry wt h^{-1}), phycocyanin (98.50 mg g^{-1} dry wt), phycoerythrin (158.0 mg g^{-1} dry wt), and antioxidant capacity (140.50 μmoles ascorbic acid equivalent capacity g^{-1} fresh wt) were recorded when Zn and glutamine were supplemented together in the growth medium at pH 7.8. These effects were found to be significantly related to the activities of glutamine synthetase (GS_{max}: 490.2 nmoles mg $protein^{-1}$ min^{-1}), glutamate synthase ($GOGAT_{max}$: 27.0 nmoles mg $protein^{-1}$ min^{-1}), and glutamate dehydrogenase (GDH_{max}: 159.9 nmoles mg $protein^{-1}$ min^{-1}). This study shows that *N. lobatus* could be a promising bioresource for the production of nutritionally rich biomass, δ-ALA, bili pigments, and antioxidants. Use of immobilized cells in batch culture supplemented with Zn and glutamine could be an effective approach for scaling up production for commercial use.

Keywords *Nostochopsis lobatus* · Cyanobacteria · δ-Aminolevulinic acid · Bilipigments · Phycocyanin · Phycoerythrin · Biomass

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Introduction

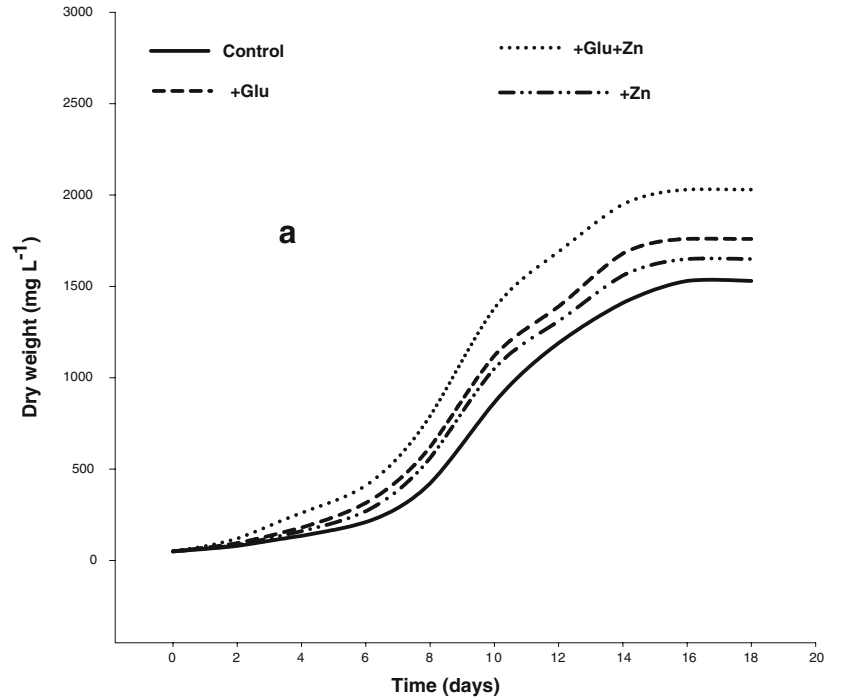
Recent scientific attention has shown growing concern towards cyanobacteria as a source of nutritionally functional food. Apart from being a rich source of proteins, carbohydrates, vitamins, minerals, amino acids, and fatty acids [1–4], some species of cyanobacteria contain new bioactive compounds not found in higher plants and traditional therapeutic sources. Many of these are potential therapeutic drugs [5]. *Nostochopsis lobatus*, a filamentous diazotrophic blue green alga (cyanobacterium) that grows luxuriantly attached to rock surfaces in the form of mucilaginous balls of unialgal population in fresh water lakes and slow moving streams of Indian tropics, is utilized by local tribes as dietary supplement. Recent researches conducted in our laboratory have indicated that *N. lobatus* is a rich source of protein, carbohydrates, and fatty acids [6].

Increasing consciousness about the risks associated with environment-induced human ailments has lead to the growing interest in nutraceuticals, which, in addition to fulfilling nutritional and energy needs, are capable of contributing additional physiological benefits. Antioxidants have gained importance in recent years due to their ability to neutralize free radicals, which are implicated in the etiology of several major human ailments including carcinogenesis-induced tumor promotion [7–10]. Phycobilin pigments of cyanobacteria have been identified as important natural antioxidants and have been biologically tested for preventive roles in free-radical-induced damages [11, 12]. These pigments, which have now gained commercial significance, are exclusive to blue green and red algae. More interestingly, delta-aminolevulinic acid (δ-ALA), which is the common precursor of chlorophyll and bili pigments, has been shown to act as a photosensitizer in the treatment of tumors [13]. Its synthesis requires a number of cofactors, such as Zn for enzyme activities and continuous supply of nitrogen as glutamine. Enzymes of N-assimilation help to remove ammonia from the site of N_2 fixation, an essential step for N_2-fixation to be continued. This helps in maintaining a continuous supply of N for growth and production of metabolites including bili pigments in diazotrophs. In the past, drug researches were focused mostly on fungi, actinomycetes, and higher plant sources. During recent years, there has been an increasing emphasis on large-scale production of cyanobacterial metabolites of therapeutical value by culturing them under improved conditions. In the present study, we investigated enhanced production of δ-ALA, bilipigments, and antioxidant capacity of *N. lobatus* under supplemental Zn and glutamine in batch culture. The possible role of enzymes of N-assimilation was also studied.

Materials and Methods

The cyanobacterium *N. lobatus* (Wood) em; Gietler, a heterocystous diazotroph, was collected from a slow-moving fresh water stream in Udaipur, Rajasthan. A unialgal population was raised using axenic culture in nitrogen-free BG-11 medium. Exponentially grown cells, after centrifugation and repeated washing, were suspended in a 5% solution of sodium alginate. The mixture was pumped drop wise into $CaCl_2$ (0.2 mol l^{-1}), and the beads thus formed (average diameter, 5 mm) were allowed to harden for 2 h. After repeated

Fig. 1 Enhanced production of biomass of *N. lobatus* by supplemental Zn and/or glutamine in **a** free cell ▶ and **b** immobilized cell cultures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+Glu+Zn
+Zn
a
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Time (days)
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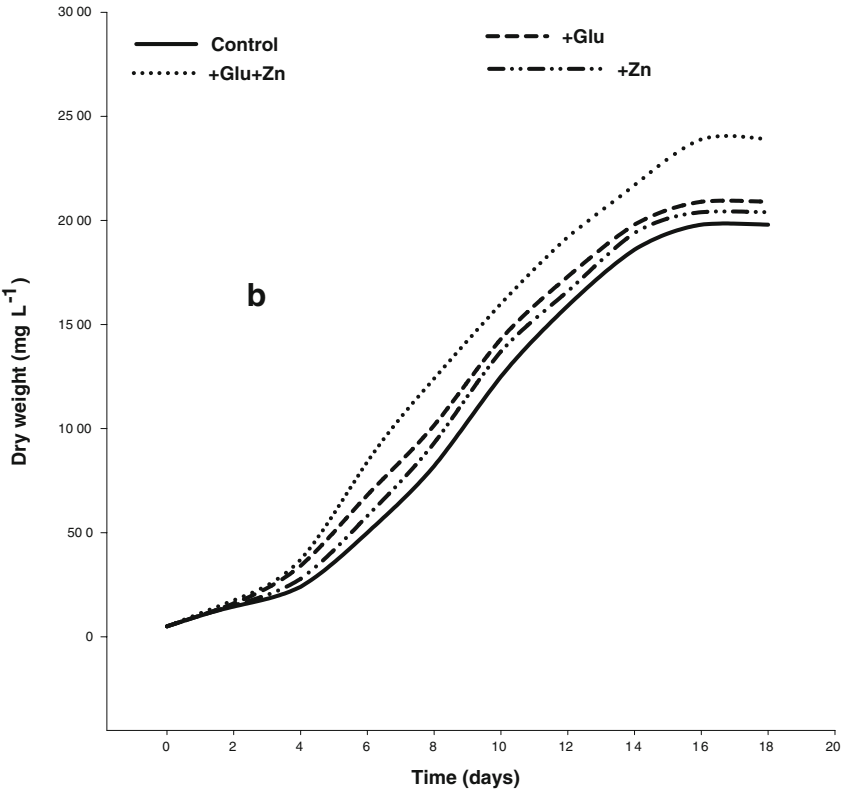
500
1000
1500
2000
2500
3000
0
2
4
6
8
10
12
14
16
18
20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+Glu
+Zn
b
Dry weight (mg L-1)
Time (days)
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50 0
10 00
15 00
20 00
25 00
30 00
0
2
4
6
8
10
12
14
16
18
20

washing with sterile distilled water, the beads were resuspended in a fresh growth medium in batch culture at 25±1 °C with a light intensity of 80 μmol m^{-2} s^{-1} and a 15:9-h light/dark cycle. Cultures were grown in transparent plastic bags (n=3) with an initial volume of 5 l, air flow rate of 0.36 vvm (LL^{-1} min^{-1}), and initial inoculum of 50 mg dry mass l^{-1}. Similar growth cultures were also prepared for free cells. The effect of supplemental Zn and glutamine were studied in three separate experiments in BG-11 medium supplemented with Zn (0.50 mg l^{-1} as $ZnSO_4$ $2H_2O$), glutamine (1 mM), and a mixture of both.

Growth of the cyanobacterium was measured in terms of dry weight per liter of culture medium. Ten-day-old harvested biomass was analyzed in triplicate for pigments. Chlorophyll was extracted in N, N-dimethyl formamide and absorbance recorded at 664 nm [14]. For carotenoids, acetone extraction procedure was followed. Phycoerythrin and phycocyanin were extracted in 0.05 M phosphate buffer (pH 6.8) and measured following the method of Bennet and Bogorad [15].

For the measurement of δ-ALA, cells were centrifuged at different intervals and resuspended in the growth medium containing 100 mM levulinic acid at pH 7.5. The δ-ALA accumulated in cells was extracted with 5% trichloroacetic acid. One milliliter of this extract was mixed with 0.1 ml of acetyl acetone and 1 ml of acetate buffer (1 M; pH 4.6). The mixture was heated in a water bath, and after cooling, 2 ml of a solution containing 1 g of p-dimethylaminobenzaldehyde and 8 ml of 70% perchloric acid was added. After 15 min of the reaction at room temperature, absorbance was recorded at 553 nm [16].

Glutamine synthetase (GS) activity was assayed following γ-glutamyl hydroxamate method [17]. To 0.5 ml of a twofold assay mixture were added 0.4 ml water and 0.1 ml enzyme and incubated for 10 min at 37 °C. The reaction was stopped by adding 2.0 ml of ferric chloride and OD recorded at 540 nm. γ-Glutamyl hydroxamate was used as standard and activity expressed as n-mole-γ-glutamyl hydroxamate formed per milligram protein per minute. Glutamate synthase (GOGAT) was assayed following the procedure described by Thevanathan [18]. The reaction mixture contained 1.0 ml phosphate buffer (pH 7.0), 0.2 ml 2-oxoglutarate (10.0 mM), 0.2 ml NAD(P)H (1.0 mM), 1.0 ml enzyme, and 0.4 ml water. The reaction was initiated by adding 0.1 ml glutamine (10.0 mM), and the decrease in extinction was recorded at 340 nm. Glutamate dehydrogenase (GDH) was assayed as described in Ahmad and Hellebust [19]. The reaction was initiated by adding 0.1 ml dialyzed crude enzyme preparation in the reaction mixture. Activity was determined following 2-oxoglutarate-dependent oxidation of NAD(P)H and expressed as nanomoles of NAD(P)H oxidized per milligram protein per minute.

Two standard assays were used for determining antioxidant capacity of *N. lobatus*. These included: (1) free radical scavenging by oxygen radical absorption capacity (ORAC) and (2) free radical formation by ferric reducing/antioxidant power (FRAP). For preparing aqueous extract, algal balls were crushed and stirred in distilled water for 1 h. The ORAC was measured in terms of 2,2-azobis (2-amidinopropane) dihydrochloride-induced damage to β-phycoerythrin. The declining fluorescence was recorded every 5 min. ORAC was expressed as micromolar trolox equivalent per gram of fresh weight [20]. For FRAP assay, ferric chloride was mixed with 2,4,6-tripyridyl-s-triazine (TPTZ) to form fe^{3+}-TPTZ complex. In the presence of an antioxidant, this complex is reduced to Fe^{2+}-TPTZ with an intense blue color with peak absorbance at 595 nm [21]. The values were expressed as micromolar ascorbic acid equivalent antioxidant capacity (AEAC). The data presented here are the means of three independent experiments with three replicates in each. Means are supported by standard errors. Treatment effects were evaluated by analysis of variance. Data were log-transformed when necessary to equalize variances.

Table 1 Enhanced production of pigments in *N. lobatus* by supplemental Zn and/or glutamine in free and immobilized cell cultures.

Pigments	Free cells				Immobilized cells			
	Control	+Glu +Zn	+Glu	+Zn	Control	+Glu +Zn	+Glu	+Zn
Chlorophyll	$9.56^{a}\pm0.93$	$13.68^{b}\pm1.12$	$11.90^{b}\pm0.88$	$10.50^{b}\pm0.90$	$15.30^{a}\pm1.35$	$26.80^{bc}\pm2.14$	$21.80^{b}\pm1.80$	$19.00^{b}\pm1.35$
Carotenoids	$2.08^{a}\pm0.18$	$2.80^{b}\pm0.33$	$2.50^{a}\pm0.27$	$2.25^{a}\pm1.62$	$3.40^{a}\pm2.87$	$4.56^{b}\pm0.51$	$4.25^{b}\pm0.32$	$4.20^{b}\pm0.36$
Phycocyanin	$46.62^{a}\pm4.45$	$60.80^{b}\pm6.12$	$55.60^{b}\pm4.66$	$53.58^{b}\pm4.71$	$66.35^{a}\pm5.90$	$98.50^{bc}\pm9.22$	$79.86^{b}\pm6.67$	$76.75^{b}\pm6.53$
Phycoerythrin	$79.70^{a}\pm7.32$	$95.00^{b}\pm9.05$	$89.56^{b}\pm7.65$	$88.86^{b}\pm6.92$	$109.25^{a}\pm10.35$	$158.0^{bc}\pm13.08$	$135.5^{b}\pm10.76$	$129.85^{b}\pm10.8$

Values (mg g^{-1} dry wt) are mean ± 1 standard error. Mean values in the same row that contain different letters are significant at $p<0.01$ (analysis of variance)

Results

The data on bimass accumulation in *N. lobatus* are presented in Fig. 1. The alga grew faster under immobilized cultures, and both Zn and glutamine supplements fastened the biomass production significantly. Maximum biomass was observed in the medium supplemented with Zn and glutamine together, the values being 2,030 and 2,390 mg dry wt l^{-1} for free and immobilized cells, respectively (Fig. 1). When added separately, glutamine showed superiority over zinc for enhanced production of biomass. Immobilized cells contained higher amounts of chlorophyll, carotenoids, and bilipigments (Table 1), which increased substantially when cultures were supplemented with Zn and glutamine. The promotive effects were significantly greater when both supplements were considered together ($p<$ 0.01). Under immobilized culture, addition of Zn and glutamine together enhanced the production of chlorophyll, carotenoids, phycocyanin, and phycoerythrin by 75.20%, 34.11%, 48.46%, and 44.62%, respectively. In immobilized cell cultures, the increases in pigments were significantly higher than those recorded in free cell cultures ($p<0.01$).

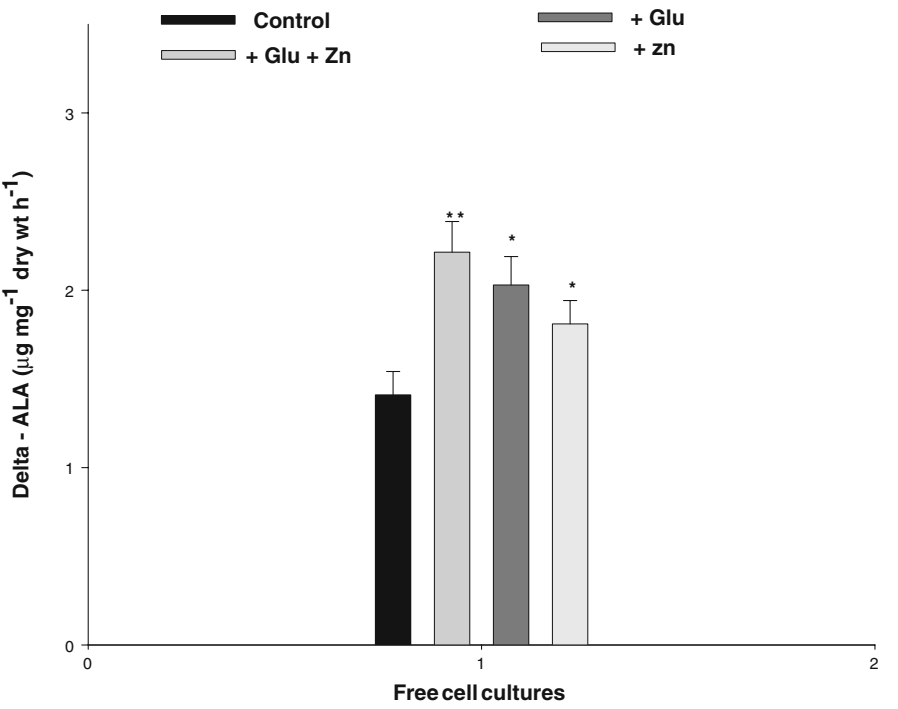
Similar to pigments, immobilized cells showed higher rates of δ-ALA biosynthesis, which enhanced maximally in Zn- and glutamine-supplemented cultures (Fig. 2). In free and immobilized cell cultures, Zn and glutamine addition enhanced δ-ALA biosynthesis by 69.12% and 57.10%, respectively. When both supplements were considered together, δ-ALA synthesis enhanced by 86.2%. Enzyme activity also remained at maximum under immobilized cell culture. The activities of GS (490.2 nmoles mg protein^{-1} min^{-1}) and GDH (159.9 nmoles mg protein^{-1} min^{-1}) were at maximum under Zn supplement, whereas that of GOGAT was 27.0 nmoles mg protein^{-1} min^{-1} in Zn and glutamine supplemented growth medium (Fig. 3).

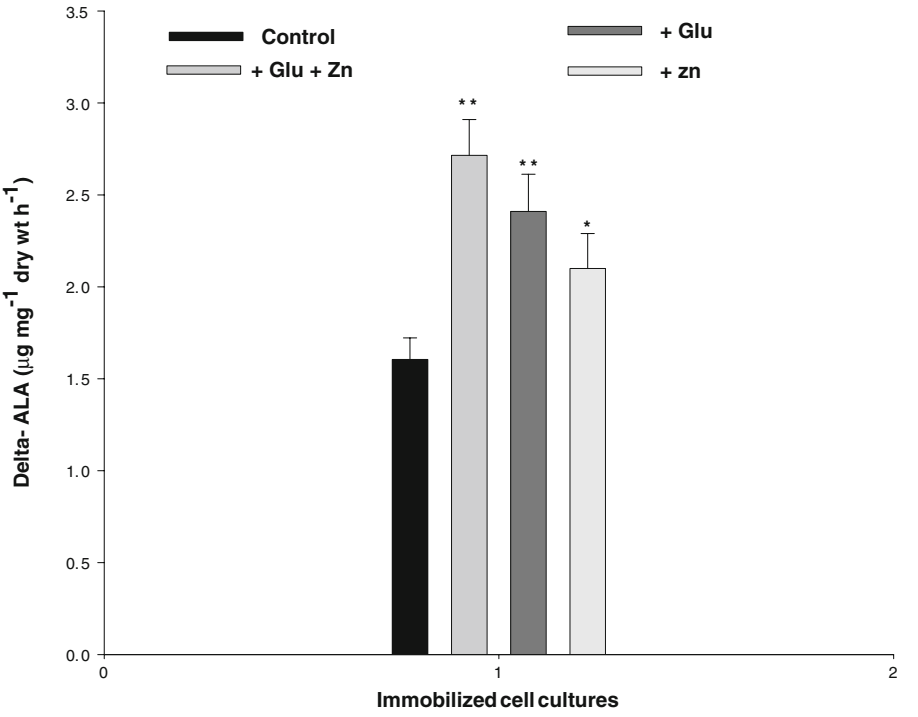
Similar to the biomass and pigments, antioxidant capacity of aqueous extract of *N. lobatus* remained high in immobilized cell cultures (Table 2). The antioxidant capacity of aqueous extracts of free and immobilized control cells were 45.95 and 86.55 μM AEAC (FRAP assay). Similar was the trend for ORAC assay (Table 2). The effect of treatment on antioxidant activity was highly significant ($p<0.01$). Maximum antioxidant capacity (140.50 μM of ascorbic acid equivalent and 65.20 μM trolox equivalent per gram of fresh wt) was observed in immobilized cultures supplemented with zinc and glutamine together. When considered alone, glutamine showed superiority over Zn in this respect (Table 2).

Discussion

Cyanobacteria are a comparatively less explored biodiversity resource for their nutraceutical use [4, 22]. The reason for considering *N. lobatus* for investigation was that this strain was collected from a slow-moving stream of Udaipur, India, where, due to its natural growth in the form of mucilaginous balls of unialgal population, it is utilized by local tribes as a dietary supplement. Recent researches conducted in our laboratory have indicated that *N. lobatus* is a nutritionally rich cyanobacterium [6]. In the present study, *N. lobatus* appeared superior to the well known food alga *Spirulina platensis* with respect to the production of biomass and pigments, except phycocyanin [4, 23]. Substantially enhanced production of nutritionally rich

Fig. 2 Enhanced production of δ-ALA in *N. lobatus* by supplemental Zn and/or glutamine in free and immobilized cell cultures. Values are mean ($n=9$) ± 1 standard error. *Single asterisks*, $p<0.05$; *double asterisks*, $p<0.01$ (analysis of variance)


Control
+ Glu + Zn
+ Glu
+ zn
Delta - ALA (μg mg-1 dry wt h-1)
3
2
1
0
0
1
2
**
*
*
Free cell cultures


Control
+ Glu + Zn
+ Glu
+ zn
Delta- ALA (μg mg-1 dry wt h-1)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
**
**
*
Immobilized cell cultures

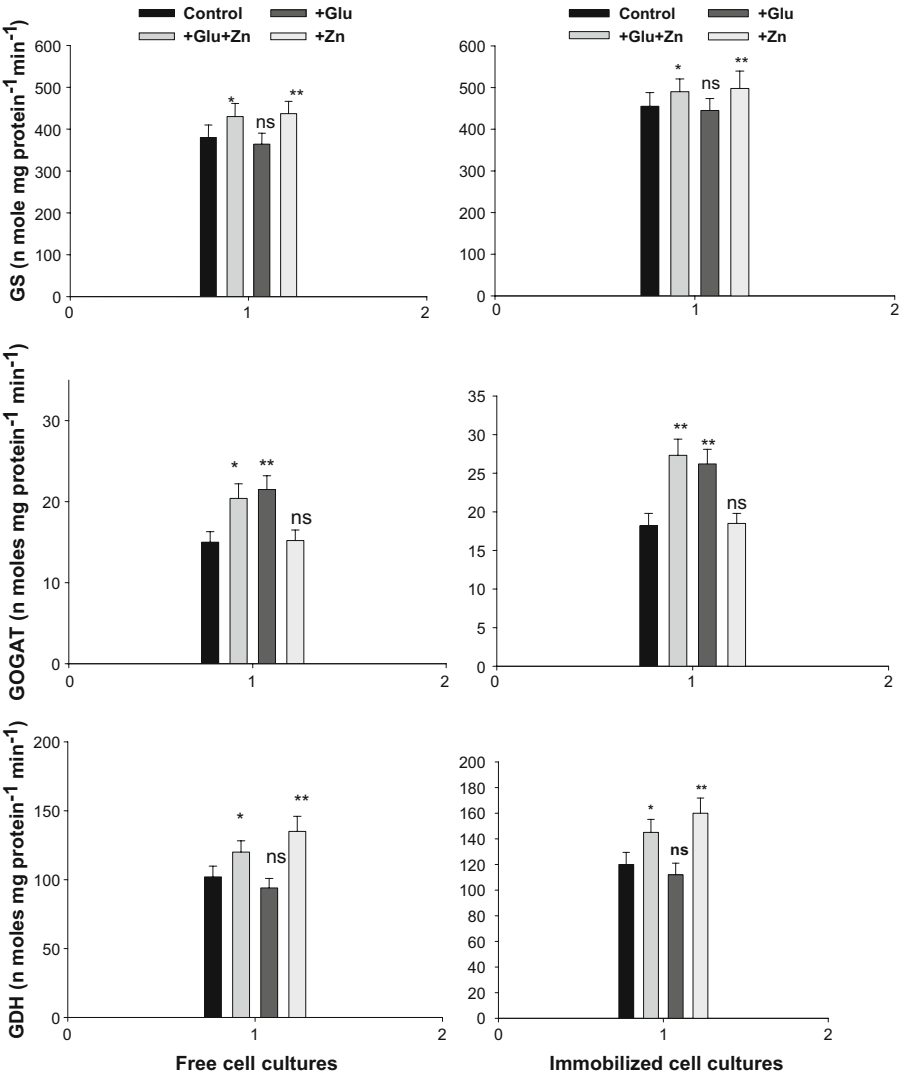


Fig. 3 Changes in glutamine synthetase (*GS*), glutamate synthase (*GOGAT*) and glutamate dehydrogenase (*GDH*) activities in *N. lobatus* as influenced by supplemental Zn and/or glutamine in free and immobilized cell cultures

biomass by *N. lobatus* under immobilized cultures supplemented with Zn and glutamine has merit for human consumption on the commercial scale.

Potentially high concentrations of phycobilins and carotenoids recorded in *N. lobatus* further increase its consumptive use. Both of the pigments have antioxidant activity and are used as natural food additives [12, 24]. The amount of phycoerythrin was exceptionally high in *N. lobatus* (10.9% of dry wt in normal growth medium), a value greater than that reported in *Anabaena* sp. (8.3%) [25], which increased substantially (15.8% of dry wt) by supplemental Zn and glutamine. It indicates great possibility of combined use of Zn and glutamine for scaling up the production and has an added advantage because phycoerythrin

Table 2 Enhancement of antioxidant capacity of *N. lobatus* by supplemental Zn and/ or glutamine in free and immobilized cell cultures.

Antioxidant capacity	Free cells				Immobilized cells			
	Control	+Glu +Zn	+Glu	+Zn	Control	+Glu +Zn	+Glu	+Zn
FRAP Assay	$45.95^{a}\pm4.76$	$66.05^{b}\pm5.90$	$58.25^{b}\pm6.11$	$56.86^{b}\pm5.18$	$86.55^{a}\pm6.75$	$140.50^{bc}\pm11.66$	$126.52^{b}\pm9.78$	$120.28^{cd}\pm10.20$
ORAC Assay	$26.80^{a}\pm2.70$	$45.26^{b}\pm4.15$	$38.35^{b}\pm3.25$	$34.00^{b}\pm2.95$	$38.62^{a}\pm3.07$	$65.20^{bc}\pm5.83$	$56.80^{b}\pm4.67$	$52.53^{cd}\pm4.73$

Values are mean of three replicates ± 1 standard error. Data expressed as micromoles of AEAC for FRAP assay and as micromoles of trolox equivalent per gram of fresh wt for ORAC assay. Mean values in the same row that contain different letters are significant at $p<0.01$ (analysis of variance)

is now increasingly being used in diagnostics and biomedical research including fluorescence immunoassays [12]. Recent researches have indicated that phycobilins of cyanobacteria have antioxidant, anti-inflammatory, and neuroprotective properties [1, 12]. The synergy of bilipigments and δ-ALA with antioxidant capacity of *N. lobatus* under Zn and glutamine supplements has scientific and commercial significance. δ-ALA, the common precursor of chlorophyll and bili pigments, has been shown to act as a photosensitizer for photodyanamic therapy in the treatment of various tumors [13]. The N-assimilating enzymes play key roles in the enhanced production of bili pigments probably via glutamine-mediated enhancement of δ-ALA synthesis. Among phototrophs, purple and green bacteria synthesize δ-ALA from glycine-catalyzed ALA synthase, in a reaction identical to those that occur in mitochondria of animals, yeasts, and fungi. In cyanobacteria and chloroplasts, δ-ALA is formed exclusively from the intact carbon skeleton of glutamate in a series of steps in which activation of α-carboxyl group of glutamate by glutamyl-t RNA Glu formation is the first step. Glutamate-1-semialdehyde aminotranferase is the terminal enzyme to convert glutamate to δ-ALA [26]. Formation and regulation of glutamate, the exclusive precursor of δ-ALA in chloroplast and cyanobacteria, involves at least three enzymes. GS regulates the synthesis of glutamine. The functions of GDH and GOGAT are more or less similar in that these two enzymes generate glutamate required for continued synthesis of δ-ALA. The enzymes GDH and GOGAT use 2-oxoglutarate and glutamine, respectively, for the production of glutamate, the former being more active during the dark and the latter during the light periods [18]. Zinc, being the cofactor of GDH, is essential for maintaining the enzyme activity and, hence, for continuous supply of glutamine.

Antioxidants form an integral part of today's nutraceutical market. Although antioxidant properties have been reported from a wide variety of organisms, including microbes [27], most natural antioxidant-based nutraceutical preparations, including soft drinks and food additives, use higher plant sources. Blue green algae are probably the least explored biodiversity resource with respect to antioxidant capacity [11, 28]. Our data indicate that the antioxidant capacity of aqueous extract of immobilized cells of *N. lobatus* grown in normal BG-11 medium (38.62 μM of TE g^{-1} fresh wt) was greater than those reported for the commonly used plant sources such as kokam (*Garcinia indica*; 29.3) [10], turmeric (*Curcuma longa*; 13.5) [29] and tea, fruits, and common vegetables [30]. Addition of a mixture of Zn and glutamine in the medium substantially enhanced the antioxidant capacity. This has relevance for enhancing antioxidant activity for commercial use. The antioxidant properties of Zn can involve direct binding of Zn to sulfhydryl group or induction of some other substance, such as metallothioneins, that serves as the ultimate antioxidant [31]. Zinc deficiency has also been shown to reduce GS activity [31]. In addition, in the presence of Fe^{2+}, the antioxidant activity of Zn depends directly on GS [32]. Furthermore, because bili pigments of cyanobacteria have been shown to have strong antioxidant properties [12], Zn-mediated enhanced production of these pigments indicates the potential role of this element towards enhanced production of antioxidants from cyanobacteria. High biomass, pigments, and antioxidant capacity under immobilized cultures indicate that a growth culture simulating the in situ growth conditions of the alga would be an effective approach for scaling up its production. Cell density and irradiance often regulate the synthesis of biliproteins in cyanobacteria. Low irradiance due to mutual shading of cells (high density), as occurs in the case of unialgal balls and under immobilized cell cultures of *N. lobatus*, could enhance the synthesis of bili pigments, especially of phycoerythrin [25].

The present study indicated that *N. lobatus* could be a promising bioresource for commercial scale production of nutraceuticals. Efficient production of nutritionally rich

biomass coupled with substantially high amount of bilipigments, δ-ALA, and high antioxidant capacity of this cyanobacterium indicate its significance for nutraceutical industries. Use of immobilized cells in batch culture supplemented with Zn and glutamine could be an effective approach for scaling up production for commercial use.

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