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In-vitro glutamine synthetase activity of marine diatom *Nitzschia sigma*

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Abstract

Glutamine synthetase, a central assimilatory enzyme for ammonia produced from nitrogen fixation and thus it is extremely important to understand the role of nitrogen in limiting the biomass and growth rate of *Nitzschia sigma*. Glutamine synthetase activity was determined *in-vitro* using cells permeabilized by freezing/thawing. Assays for glutamine synthetase activity showed that malachite green assay is approximately 30 times more sensitive than molybdate assay for Pi detection**.** Cells permeabilized with N-2- hydroxyethylpiperzine-N'-2-ethanesulfonic acid showed higher enzyme activity than the Tris and Imidazole and at the optimum pH 7.9. Moreover, ammonia uptake along with GS activity was measured at 25˚C and showed that nitrate starved cells of *N. sigma* showed the maximum GS activity (71±5 fmol cell-1 hour-1) along with higher ammonia uptake as compared to nitrate grown cells.

Keywords: microalgae, glutamine synthetase, nitrogen, imidazole, malachite green

Introduction

Nitrogen is a prime nutrient for the microalgae and a major limiting factor in primary productivity. Despite the importance of microalgae in the global nitrogen cycle, all inorganic nitrogen first reduced to ammonium, prior to its assimilation into organic compounds (Seabra *et al*. 2013) [14]. It is the compelling evidence that ammonium is assimilated to glutamine with the help of enzyme Glutamine Synthetase (GS), owing to the distribution of nitrogen groups directly or via glutamate, for virtually all nitrogenous cell compounds (Lea and Miflin 2010) [10]. Being the first enzyme, glutamine synthetase plays a key role in the regulation of nitrogen metabolism and primary productivity (Hirel and Lea 2001) [1]. Information available on GS in microalgae was scarceand the available evidence is consistent with GS being the primary ammonia assimilating enzyme in most marine unicellular algae (Ahmad and Hellebust 1988, Zehr and Falkowski 1988) [1, 18]. The natural population of the microalgae are often influenced by the limited availability of nitrogen (Dugadale 1967).

Glutamine synthetase activities were measured by transferase and synthetase assay (Lee *et al*. 1990; Alwyn *et al*. 1995) which is easy to quantify the amount of end products, glutamylhydroxymate. However, neither the assays uses the true substrate for their GS reaction. The true substrates for the GS reaction include Glutamine determination using 14Cor HPLC and coupled spectrophotometric assays (Lee *et al*. 1990). Since the methods are time consuming and expensive, an alternative method has been developed. It involves the measurement of pi released during the reaction which overcomes these problems and has been used successfully in order to measure the GS activity of different organisms including microalgae (Bressler and Ahmed 1984; Clayton and Ahmed 1987) [4, 6]. The stability and sensitivity of malachite green assay and molybdate assay for the Pi determination has been developed by Gelado-poulos *et al*. (1991) and its use in measuring the enzyme activities involving ATP hydrolysis developed by Rees (1995) [13]. However, the relative sensitivity for these two assays might vary among one another. Most enzyme activities can be determined in cell free extracts and there is a considerable evidence to suggest that the disruption of cells has a deleterious effect on both the activity and characteristics of enzyme (Aragon and Sols 1991; Alwyn *et al*. 1995) [2]. In-situ measurements of enzyme activities using permeabilized cells offers a number of advantage, that includes the prevention of problems associated with the dilution or disruption of protein-protein interaction that may occur during extraction, the ability to determine changes in enzyme activity during relatively short periods (Alwyn *et al*. 1995).

Therefore, a considerable amount of research has been dedicated for GS activity in marine unicellular algae should lead to better understanding of the role of nitrogen in limiting the biomass and growth rate of these organisms in nature. The cells used for this assay were permeabilized by freezing and thawing. The present study was delineated to detect the more sensitive method for Pi accumulation and the use of this assay is described to follow the changes in *in-situ* GS activity during nitrogen starvation and compared the GS activities with the rates of ammonia uptake.

Materials and Methods

Organism and culture conditions

The marine diatom *N. sigma* were isolated from the Parangipettai coastal waters and was grown at 25˚ C in a medium containing 10Mm sodium nitrate or 10mM

Ammonium chloride as a sole nitrogen source. Nitrogen starved cells were routinely obtained by aseptically transferring nitrate grown cells to nitrogen deficient medium as previously described (Larson and Rees 1994).

Cell permeabilization

Cells were concentrated by centrifugation at 1500 g for 10 min and re-suspended in a buffer containing 0.2M HEPES-KOH (pH - 7.9), 50mM MgCl2, 5mM Na2 EDTA and 1mM DTT to give a cell density of 5 x 107 cells/ml. Cell suspensions (1-2.5 ml) were frozen immediately in liquid nitrogen for 10-20 sec and stored at -20˚C prior to start enzyme activity.

Effect of different substrates on pi accumulation in nitrate starved cells

Different substrates such as Glutamate+Ammonia+ATP, Ammonia + ATP, Glutamate + ATP, Glutamate + ATP, Glutamate + Ammonia, ATP, Glutamine + ATP, were used to determine the concentration of Pi accumulation of nitrate starved cells during GS activity. Glutamine was added to give a final concentration of 0.5 mM. Absorbance of the sample was measured at 660 nm against the blank containing ultrapure water. The data were expressed in terms of mean ± SD.

Determination of ammonia uptake

Cells were harvested by centrifugation at 1500g for 10 min and the pellets were washed three times and resuspended in nitrogen free ASP2 medium (pH - 8.0) (Larson and Rees 1994). Cell supensions were incubated in light (150 µmol m-2 s-1) at 20˚C in the presence of 200 mM NaHCo3. To determine the ammonia uptake, ammonium chloride was added to the 2.5 ml of cell suspension to give an initial concentration of 400 µM. Most of the report showed that this concentration of ammonia was sufficient to give maximum uptake rates. Sample (1ml) was taken immediately after the addition of ammonia and after 1 min of incubation. The cells were removed by vaccum filtration through whatmann GF/C filter discs.

The filtrate containing ammonia was determined by the alkaline hypochlorite/ Phenol nitroprusside method.

Glutamine synthetase assay and Pi accumulation

The GS activity was measured based on the procedure of Slawyk and Rodier (1988). The reaction mixture contained 0.2 M Hepes-KOH (pH-7.9), 50mM MgCl2, 5mM Na2-EDTA, 100mM Potassium glutamate, 50 mM ammonium acetate, 8mM ATP. To 0.1 ml of thawed cell suspension, the reaction mixture was added and made a final volume to 0.75ml. Complete mixture devoid of glutamate can be considered as blank. The reaction was started by adding ATP and the mixture was incubated at 30˚C for 15 min. After incubation, the reaction was stopped by adding 0.25 ml of 1N H2SO4. The acidified reaction containing ATP is stable for at least 1 hour. The mixture was centrifuged at 14,000 g for 1 min and the supernatant was collected. To 50 µl of supernatant 0.95 ml of double distilled water was added and used for Pi determination.

Absorbance of the samples were read against blank containing distilled water and the values were converted to Pi concentrations from the standard curves (0 - 25µM K2HPo4) with 25 µM Pi giving an absorbance of about 1.72. ATP hydrolysis caused by the acidic colour reagent may overestimate the Pi concentration and can be prevented by the addition of 50 µl 1.3M trisodium citrate, 2 min after the addition of color reagent (Lanzetta *et al*. 1979). Enzyme activities can be calculated by substracting the blank (-glutamate) values from those of the complete reaction mixture and can be expressed as fmol Pi cell-1 h-1.

Other methods

Permeabilization was determined by using Evan's blue dye (Crippen and Perrier, 1974). All experiments were carried out in and statistical analysis was performed using Origin. Version.9.

Result

Cell permeabilization by freezing/thawing of microalgae is a simple and convenient method, showed 99% or greater of *N. sigma* cells was permeable to Evan's blue dye. It is one of the appropriate methods for permeabilizing the microalgae (Syrett 1973) [16] and showed no effect on enzyme activity, if the cells were thawed rapidly at 30˚C. The standard curves of Pi were generated to determine the relative sensitivity of malachite green assay and molybdate assays. The linear regression equation was fitted to the standard curve data and was summarized in Table 1. The resulting ratio of the regression coefficient (b1/b2) showed that the malachite green assay is approximately 30 times more sensitive than molybdate assay for Pi detection. The high correlation coefficient in malachite green assay indicates that this Pi measurement procedure can be performed with high degree of precision and hence, the malachite green assay was carried out to determine the Pi accumulation during the GS activity for *N. sigma* cells.

The linear regression equation has the form Y = b(x) + a, where y is the blank corrected absorbance units and x is 10-9 moles Pi assayed. Absorbance reading was taken at 660 nm for malachite green assay and 850 for molybdate assays respectively. n - number of points used for a regression analysis; r - correlation coefficient for the fitted regression line; b1/b2: ratio of regression coefficients for malachite green to molybdate assays.

GS reaction was measured in terms of Pi accumulation in both nitrate grown and nitrate starved cells. Owing to the presence of nitrate in the medium, the nitrogen grown cells undergoes metabolism by normal biosynthetic pathway, whereas in case of nitrate starved cells, it differs and hence, the effect of different combinations of substrates on Pi accumulation during GS activity were determined in nitrogen starved cells (Table. 2). The reaction occurredin nitrate starved cells due to the presence of glutamate, ammonia and ATP and the enzyme reaction was found to be more in nitrate starved cells. In the absence of either glutamate or ammonia or both glutamate+ammonia observed with decreased enzyme activity. The blank consisted of complete reaction mixture devoid of glutamate. Glutamine synthetase activities in the nitrate starved cells were determined with five different combinations of substrates and the GS activity was found to be 493-249 = 244 fmol cell-1 hour-1.Pi accumulation during GS activity in respect to time interval for both nitrate grown and nitrate starved cells were studied. The accumulation of Pi in nitrogen grown and starved cells were measured from 15 min interval to 60 min. Pi accumulation in the nitrate grown cells was increased and showed linear curve for the whole 60 min interval (Fig. 1A). In case of nitrate starved cells, the

curve was linear for first 45 min (Fig. 1B). The most commonly used buffer for determining the GS activity was Tris. However, in the present study the maximum activity was recorded for the cells assayed in Hepes buffer. Cells frozen and assayed in tris buffer showed maximum of 74% of the activity, whereas 93% activity were obtained with Hepes buffer and there was much decreased activity with imidazole (57%) as compared with tries and Hepes buffer (Fig. 2.).The pH in the range from7.3 to 8.2 was recorded during GS activity and the maximum activity was recorded at the pH 7.9. The activity was decreased at the pH below 7.6 and above 8 with the negligible result being detected at the pH 7.1 (12%). The earlier reports for the diatom *P. tricornutum* showed the pH range from 7.7 to 8.4. From the present study it clear that different species have their different kind of adaptation for their metabolic activity, when cultured under *in-vitro* conditions.

Ammonia uptake along with GS activity was measured at 25˚C, which is the temperature at which the cultures were grown. Nitrate starved cells of *N. sigma* showed the maximum GS activity (71±5 fmol cell-1 hour-1) along with higher ammonia uptake as compared to nitrate grown cells (Table. 3). This is due to the presence of nitrate in the medium provided sufficient energy for the cells to divide and hence showed lesser GS activity.

Table 1: Linear regression for phosphate standard curve using malachite green and molybdate assays.

Assays	N	r2	a	B	b1/b2
Malachite green assay	12	0.99	0.416	0.0248	
Molybdate assay	12	0.97	0.035	0.00081	30.61

Table 2: Effect of different substrates on Pi accumulation in nitrogen starved cells

Treatment	Absorbance	Pi accumulation (fmol cell-1
Glutamate + Ammonia + ATP	2.14±0.09	493±15
Ammonia + ATP	1.50±0.04	249±12
Glutamate + ATP	1.56±0.06	260±9
Glutamate + Ammonia	0.12±0.03	38±4
ATP	1.45±0.04	235±12
Glutamine + ATP	1.53±0.02	253±11

Table 3: Comparison of GS activity with ammonia uptake in nitrate grown and nitrate starved cells of N. sigma

Culture Conditions	GS activity (fmol cell-1 hour-1)	Ammonia Uptake (fmol cell-1 hour-1)
Nitrate grown	32±3	25±4
Nitrogen starved	71±5	79±4

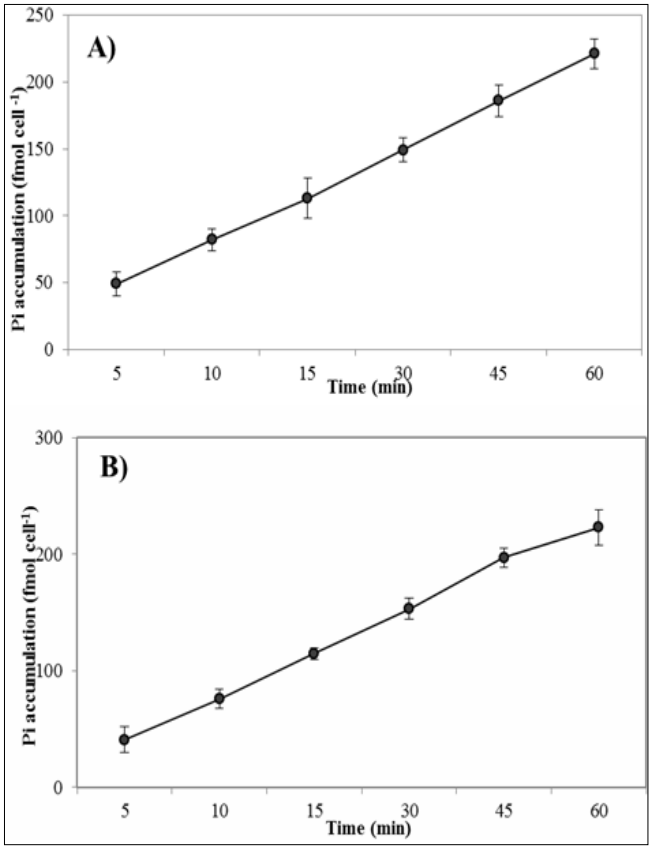

Fig 1: Time course and effect of Pi accumulation on Nitrate grown (A) and Nitrate starved cells (B)

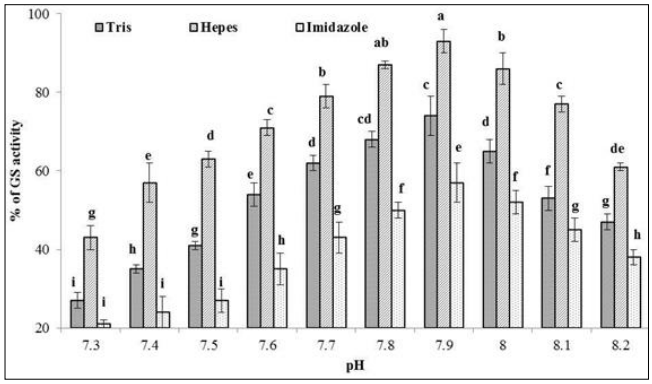

Fig 2: Influence of different buffers on Glutamine synthetase activity (Bars sharing the alphabets are highly significant; P <0.05)

Discussion

The use of malachite green assay for the determination of Pi release in the *N. sigma* culture offers numerous advantages for the routine measurement of GS activity. The assay is simple and more sensitive than the molybdate assay and has the abilty to measure enzyme activity with the true substrate (Baykov *et al.* 1988) [3]. The GS sensitivity was measured for diatom *S. coastatum* showed 28.4 times more sensitive (Clayton and Ahmed, 1987) [6]. However, the standard curve of Pi in *N. sigma* cells confirmed that malachite green assay is 30.6 times more sensitive than the molybdate assays and showed that the sensitivity of GS activity might be depends on the species and their habitat where they grow. The enhanced sensitivity is accompanied by a high degree of

precision for triplicate measurements and this allows the detection and quantification of GS activity in the laboratory cultures of phytoplankton. In addition to that, it has also offers practical significance for the direct measurements of GS activity from the field sample. After the addition of acidic dye reagent to the sample, there will be a stable increase in Pi due to ATP hydrolysis, which showed a potential disadvantage in the study.

Cell permeabilzation by Evans blue dye is a simple and convenient method for permeability which offers numerous leads for measuring enzyme activities (Aragon and Sols, 1991) [2]. It enables reliable result which helps to determine the comparison of cell in various activities such as uptake rate, accumulation rate etc. This aspect might be useful to study the control of cell metabolic activity using inhibitors or reductants. It should be studied in *P. tricornutum* and *C. kessleri,* where the former possessed both cytosolic and chloroplastic isoforms and the later showed the ratio of these two isoforms differs in nitrate starved and nitrate grown cells (Sumar *et al*. 1984; Casselton *et al*. 1986) [15, 5]. However, the study was not carried out for *N. sigma* cells and the present study on GS activity will favour to regulate the GS isoforms of *N. sigma* grown under *in-vitro* condition.

In the present study, Glutamine Synthetase (GS) activity was measured in terms of Pi accumulation and can be expressed in terms of fmol cell-1 hour-1. Different combination of substrates influenced the rate of Pi accumulation in the nitrate starved cells grown under *in-vitro* condition. In the combination of glutamate, ammonia and ATP, GS activity was found to be more, whereas the cells devoid of either glutamate or ammonia or both glutamate and ammonia possessed lower activity with lower accumulation. This is due to the lack of ATP hydrolysis or reduced hydrolysis, when compared to the complete reaction mixture. The accumulation of Pi was very low in the absence of ATP. The present study result was collated with the GS activity of *P. tricornutum* which showed the Pi accumulation in response to different substrate combinations (Rees *et al*. 1995) [13]. But, the rate of accumulation was found to be lower for *P. tricornutum* and was found to be higher in *N. sigma*. In the absence of glutamate, ammonia and ATP present in these tubes showed some of the activity which could be attributable to carbomoyl-phosphate synthetase (ammonia activity) activity. The amount of ATP hydrolysis was identical with the complete reaction mixture devoid of glutamate and ammonia. Moreover, glutamine substituted for glutamate and ammonia showed the similar activity to the complete reaction mixture devoid of glutamate and ammonia indicating that there was no detectable carbomoyl phosphate synthetase activity or any other reaction requiring glutamine and ATP. Pi accumulation in respect to time interval (5 – 30 min) for nitrate grown cells showed a linear curve upto 60 min, whereas the curve was linear upto 45 min in nitrate starved cells. The present study was quite comparable to the earlier reports in *p. tricornutum*. *P.tricornutum* in nitrate grown medium showed linear curve for 60 min, whereas the cells in nitrate starved medium was linear up to 30 min interval which was varied to the present report. This variation might be due to the physical parameter such as adaptation of microalgal species grown under *in-vitro* conditions and may vary among the different species. No such studies have been carried out on the GS activity in *N. sigma* cells.

GS activities were compared with maximum rates of ammonia uptake in nitrate grown and starved N. sigma cells are studied and indicated that the enzyme activities were close to the rate of uptake (Table. 3.) Because ammonia uptake rate are greater than those of any other nitrogen source (Syrett *et al*. 1986) [17] and hence the assay could be useful for the measurement of maximum uptake rate. In the present study, the nitrate starved cells noted that there is a relative increase in the rate of ammonia uptake which is 3 folds greater than the nitrate grown cells. This is due to that the ammonia uptake is passive for nitrogen grown cells and is found to be low.

Conclusion

From the present study it is clear that modified GS assay showed the enhanced sensitivity for the *in-vitro* enzyme studies that might even detectable in the field study which are within the realm of possibility and practicality. However, laborious interpretation of such data derived from the natural microalgae populations demands a wider knowledge to study the GS characteristics and their isoforms.

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Conflict of Interest

All authors disclose no Conflict of Interest

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