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Effects of Amino Acids, Carbohydrates and Phosphorus Sources on Growth and Alkaline Phosphatase Activity of the Marine Cyanobacterium *Anabaena* sp. Strain CA

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Alkaline phosphatase (APase) was found to be inducible in *Anabaena* sp. strain CA. Growth was less than control in presence of most amino acids except glycine and serine, but most amino acids enhanced APase activity. Highest APase activity was recorded in tyrosine supplemented culture followed by hydroxyproline, cystein, valine and glutamic acid. Threonine supplemented material showed lowest APase level (1.8 nmol/mg protein/min). Lactose, glucose, sodium pyruvate and succinate stimulated growth but not APase activity. APase activity was high in the presence of sucrose, mellibiose, mannitol, arabinose, maltose and sorbose, even though the growth in these supplements was less than in control. Organic phosphate sources supported good growth of the organism. Best growth occurred in presence of inorganic phosphate, adenosine diphosphate, fructose 1,6-diphosphate or ribulose 1,5-diphosphate, followed by other phosphorus sources tested. APase activity in presence of any of the organic phosphate sources was 3 to 5 fold low as compared to phosphate limited culture. Also, there was no APase activity in cultures grown on inorganic phosphate. These data indicate that most amino acids and a few carbohydrates (sucrose, mellibiose, arabinose and sorbose) are suitable for APase production. Lactose, glucose, pyruvate or succinate may be used as a carbon source during photoheterotrophic growth of the cyanobacterium. Glycine and serine are preferred nitrogen sources for its growth. Phosphate repressible APase activity has been found in *Anabaena* sp. strain CA.

Alkaline phosphatase (APase) ubiquitously occurs in animals, plants and microorganisms. APase activity is regulated differently in different organisms. An interesting effect of phosphate starvation in microorganisms including cyanobacteria is the production of APase. This enzyme enables the cells to obtain orthophosphate from the hydrolysis of organic phosphates. The most detailed study on the regulation and properties of APase has been made in *Anabaena flos-aquae* (3), *A. variabilis* (7), *Anacystis nidulans (2, 9), and Plectonema boryanum* (5). Most of the cyanobacteria have been shown to produce inducible APase (5, 9). However, constitutive production of APase has also been demonstrated in several cyanobacteria (5, 18). The level of APase activity among different species of cyanobacteria varied greatly when cultured at different phosphate levels (18). Besides hydrolysis of organic phosphate, this enzyme has also been suggested to be involved in carbohydrate metabolism (16, 17), transport of substances across membranes, synthesis of fresh organic phosphates (12), and in infection thread formation in rhizobia (8).

In cyanobacteria, the utilization of organic compounds as carbon sources for growth is not general but some of them have been reported to assimilate organic compounds both in light and dark (19). Nitrogen and phosphorus are involved in regulating algal distribution and cyanobacterial bloom formation (14). In the present study, several amino acids, carbon and phosphorus sources were screened for their ability to inhibit or stimulate the growth and APase activity of the marine cyanobacterium *Anabaena* sp. strain CA.

MATERIALS AND METHODS

Organisms and Experimental Conditions

The main test organism was the filamentous marine alga *Anabaena* sp. strain CA (ATCC 3047). This alga was obtained from the laboratory of late Prof. C. Van Baalen, University of Texas, and grown in ASP-2 medium (21) in a shaker (Lab-line Instruments, Inc., U.S.A.)

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at 39±0.5°C under 250 μE $m^{-2}s^{-1}$. *Anabaena ambigua*, *A. variabilis*, *A. doliolum* and *Anabaena* species were isolated from rice fields of Varanasi. *A. cycadeae* was isolated from *Cycas* coralloid roots. All these strains were grown in Chu-10 medium (15) at 25±1°C at a light intensity of 75 μE $m^{-2}s^{-1}$.

All cultures were grown upto mid log phase in low phosphate (LP) medium (100 μM Pi) and then harvested by centrifugation, washed twice with glass distilled water and finally used as inoculum. To determine induced (activity in phosphate-free medium) and basal (activity in 1 mM phosphate medium) levels of APase, cultures were suspended in phosphate-deficient and phosphate-sufficient media separately. The growth medium devoid of phosphate was referred to as phosphate-deficient medium. After 6 days of incubation, APase activity was determined in all *Anabaena* species while in *Anabaena* sp. strain CA this determination was made after 24 h incubation. For kinetics of growth and APase activity, cells were suspended in 500 ml conical flasks containing 100 ml of phosphate-free medium and incubated in light. Samples were withdrawn at regular intervals for the determination of growth and APase activity. At the time indicated in Fig. 1, culture was divided into two equal parts: one was supplied with 300 μM Pi and the other served as phosphate-deficient control.

For studying the effect of amino acids, carbon and phosphorus sources on growth and APase production,

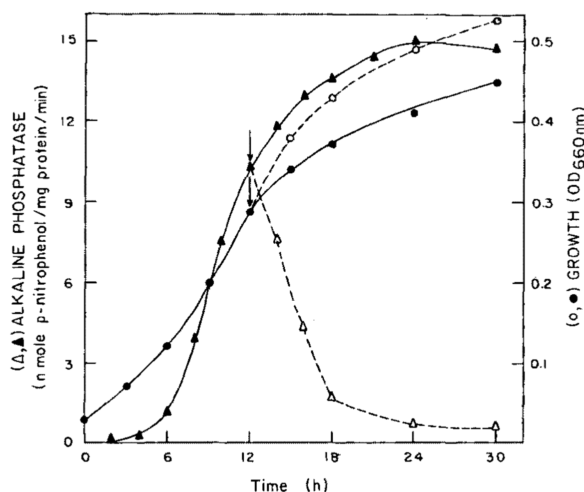

Fig. 1. Effect of inorganic phosphate (Pi) on growth kinetics and alkaline phosphatase activity in *Anabaena* sp. strain CA at ~39°C under shaking conditions.

Cells grown in low phosphate medium were harvested, washed twice with DDW and resuspended in phosphate-deficient medium. At the indicated time cultures were divided into two equal parts: one was supplied with 300 μM Pi and the other served as phosphate-deficient control. At regular intervals samples were withdrawn for the determination of growth and APase activity.

cultures were grown in LP medium. These culutres were then washed and resuspended in phosphate-deficient medium. Amino acids and phosphorus sources were added at the time of resuspension (at zero time) in phosphate-deficient medium. Carbohydrates were added after 12 h incubation of cells in phosphate-deficient medium. Cultures without added amino acids, carbon and phosphorus sources served as control. After 24 h, all the cultures were used for the determination of optical density and APase activity. Amino acids, carbohydrates and other organic compounds were filter sterilized before use.

Growth and APase Activity

Growth was measured in terms of protein (μg/ml) or absorbance (OD at 660 nm). Protein was determined by the method of Lowry *et al.* (11) using lysozyme as the standard. APase assay was performed in 0.2 M Tris-HCl buffer (pH 8.5) by the method of Ihlenfeldt and Gibson (9), using *p*-nitrophenyl phosphate (*p*-NPP) as the substrate. For the assay of enzyme activity, 4 ml cultures were centrifuged, washed with glass distilled water, resuspended in Tris-HCl buffer and treated with 1% toluene (v/v). To this cell suspension, 0.2 ml of 100 mM *p*-NPP was added and incubated at 37°C. The reaction was terminated after 30 min by the addition of 0.2 ml of 1 M K_2HPO_4. Samples were centrifuged and the absorbance of supernatant was read at 420 nm in a Bausch and Lomb Spectronic-20 colorimeter.

Chemicals

Amino acids, carbon and phosphorus sources and other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. and British Drug Houses (BDH, Glaxo), India.

RESULTS AND DISCUSSION

In addition of *Anabaena* sp. strain CA, five other *Anabaena* species were tested for the presence of alkaline phosphatase (APase). Table 1 indicates that all strains except *A. cycadeae* were inducible for APase activity as there was substantial increase in activity upon transfer in phosphate-free medium. Growth and APase activity of the strain CA in the presence of different concentrations of Pi are given in Table 2. Growth increased with increasing concentration of Pi upto 100 μM. One mM and 10 mM Pi concentrations significantly reduced the growth. Little or no APase activity was recorded when the cells were grown in Pi levels exceeding 100 μM. Phosphate-deficient grown cells showed about five-fold higher activity than cells that had been grown in phosphate-containing medium (10 μM Pi). This indicates that the APase production in strain CA may be strictly inducible.

The effect of added Pi (300 μM) on growth and APase activity of strain CA is shown in Fig. 1. Transfer of cells

Table 1. Alkaline phosphatase activity of six *Anabaena* species.

Organism	Alkaline phosphatase (nmol *p*-nitrophenol/mg protein/min) Inorganic phosphate (mM).	
	0	1.0
Anabaena ambigua	12.2	1.5
A. cycadeae	11.7	9.4
A. variabilis	13.6	2.2
A. doliolum	15.3	5.4
Anabaena sp. strain CA	10.2	ND
Anabaena sp.	12.5	3.9

Data are means of three replicates. ND, not detected. Low phosphate grown cells were harvested and washed twice with double distilled water, thereafter resuspended in media with 1 mM Pi and without phosphate. Alkaline phosphatase activity was assayed after 6 days of growth in all strains except *anabaena* sp. strain CA in which enzyme assay was done after 24 h incubation.

Table 2. Growth and alkaline phosphatase activity of *Anabaena* sp. strain CA cultured in presence of different levels of phosphate.

Phosphate (Pi) concentration (mM)	Growth (μg protein/ml)	Alkaline phosphatase (nmol *p*-nitrophenol/ mg protein/min)
Control (without Pi)	69	10.6
0.01	80	2.0
0.1	88	ND
1.0	60	ND
10.0	43	ND

Data are means of three replicates. Exponentially grown cells were used as inoculum. Other conditions are same as given in Table 1.

grown in LP medium to phosphate-free medium resulted in an increase in alkaline phosphatase activity. APase production started after 6 h and rose ten-fold after 12 h of phosphate deprivation. Addition of Pi (300 μM) to derepressed cells caused the resumption of growth and an immediate cessation of APase activity. APase activity was almost completely lost after about 12 h when Pi was added to Pi-starved cultures. The results show that the APase activity in this strain is repressed by inorganic phosphate in much the same way as reported in bacteria (6, 20) and cyanobacteria (2, 3, 5). Doonan and Jensen (5) mentioned that APase activity of *P. boryanum* is significantly enhanced when cultured in phosphate-deficient as compared to phosphate-sufficient medium.

Anabaena sp. strain CA was grown in the presence of various amino acids and its growth and APase activity were recorded (Table 3). All the amino acids except glycine and serine inhibited growth under nitrogen-fixing condition. Glycine and serine stimulated while hydroxyproline and histidine markedly inhibited the growth of the strain. There was significant increase in APase activity with the supplementation of most amino acids.

Table 3. Effect of amino acids on growth and alkaline phosphatase production by *Anabaena* sp. strain CA.

Amino acids*	Growth (A_{660} nm)	Alkaline phosphatase (nmol *p*-nitrophenol/ mg protein/min)
Control	0.37	10.0
Alanine	0.35	7.7
Arginine	0.28	13.4
Asparagine	0.30	13.7
Aspartic acid	0.26	12.5
Cystein	0.27	16.4
Glutamic acid	0.26	15.4
Glutamine	0.35	8.6
Glycine	0.43	7.9
Histidine	0.19	9.9
Hydroxyproline	0.13	17.8
Isoleucine	0.27	10.7
Leucine	0.32	9.8
Lysine	0.22	8.1
Methionine	0.28	8.6
Phenylalanine	0.32	9.2
Proline	0.33	7.0
Serine	0.47	9.2
Threonine	0.24	1.6
Tryptophan	0.35	7.7
Tyrosine	0.22	22.3
Valine	0.28	15.7

*All amino acids were used at the concentration of 1 mM except aspartic acid and glutamic acid which were used at 0.5 mM. The values are means of three independent observations.

Highest activity was recorded in tyrosine supplemented culture followed by hydroxyproline, cystein and glutamic acid. APase enhancement by aspartic acid, arginine and asparagine was almost the same (12.5 to 13.7 nmole *p*-nitrophenol/mg protein/min). The lowest activity was recorded in threonine-supplemented cultures. Ladha and Kumar (10) reported that glycine and methionine enhanced while histidine slightly inhibited the growth of *Nostoc linckia*. The differential synthesis of APase when the organism is grown on various amino acids seems to suggest that, in addition to Pi, amino acids may also play an important role in the regulation of APase in this alga. The differences in the level of activity may be due to the fact that cyanobacteria variously responded to different amino acids. This may also be due to differences in intracellular amino acid pools during growth on various amino acids. Variation in intracellular amino acid pool level from organism to organism and also in cell types has been reported in cyanobacteria during growth under nitrogen-fixing conditions (4).

The ability of the strain to use the various carbon sources is shown in Table 4. Lactose, glucose, sodium pyruvate and succinate stimulated growth but not APase activity. Highest growth occurred on lactose, followed by succinate and glucose. Sucrose-supplemented cells

Table 4. Effect of carbon sources on growth and alkaline phosphatase activity in *Anabaena* sp. strain CA.

Carbon source*	Growth (A_{660} nm)	Alkaline phosphatase (nmol *p*-nitrophenol/ mg protein/min)
Control	0.39	11.2
Arabinose	0.33	12.0
Cellobiose	0.22	7.4
Fructose	0.36	10.0
Galactose	0.25	5.8
Glucose	0.40	3.9
Glycerol	0.24	8.8
Lactose	0.43	7.1
Maltose	0.28	11.9
Mannose	0.33	9.5
Mellibiose	0.33	13.7
Mannitol	0.24	12.6
Raffinose	0.30	7.3
Ribose	0.25	7.3
Sorbose	0.30	11.9
Sucrose	0.18	14.2
Sodium acetate	0.28	3.8
Sodium pyruvate	0.40	11.5
Sodium succinate	0.42	10.6

*All carbon source were used at the final concentration of 0.25% (w/v). Glycerol was used v/v. Values are means of three replicates.

showed least growth but supported highest APase activity. APase activity was high in the presence of sucrose, mellibiose, mannitol, arabinose, maltose and sorbose even though the growth in these supplements was less than that of control. APase activity did not correlate with the growth of the cells cultured on various carbon sources. Different levels of APase on growth in various carbohydrates were observed in strain CA. This seems to suggest that the carbohydrates, in addition to Pi, may also be involved in the regulation of APase production in this cyanobacterium. Mishra *et al.* (13) reported that glucose significantly stimulated the growth under both photoheterotrophic and chemoheterotrophic conditions; fructose, sucrose, maltose, α-ketoglutarate, pyruvate, ribose, acetate and succinate either showed a slight stimulatory effect or did not affect growth at all. The failure of ribose, acetate and formate to stimulate growth and nitrogenase activity of another cyanobacterium *Aulosira fertilissima* may be due to the lack of requisite enzymes to metabolize these compounds (1).

The APase activity varied according to the nature of the carbon sources supplemented in phosphate-free medium. Similar results have been reported in *Escherichia coli* (16) and *Agrobacterium tumefaciens* (17). The differential synthesis of APase observed when *Anabaena* sp. strain CA is grown on different carbohydrates may be due to a change in the lipid component of the cell envelope as reported in gamma-irradiation resistant

Table 5. Effect of different phosphate sources on growth and alkaline phosphatase formation in *Anabaena* sp. strain CA.

Carbon source*	Growth (A 660 nm)	Alkaline phosphatase (n mol p-nitrophenol/ mg protein/min)
Control	0.39	9.9
Adenosine diphosphate	0.63	2.5
Adenosine triphosphate	0.56	2.3
Fructose 6-phosphate	0.60	2.1
Fructose 1,6-diphosphate	0.62	3.3
Glucose 6-phosphate	0.58	2.2
Phosphoenol pyruvate	0.50	1.9
Ribulose 1,5-diphosphate	0.62	2.2
Sodium β-glycerophosphate	0.58	2.8
KH_2PO_4	0.65	0.0

*All phosphate sources were used at final concentration of 50 μM. Means of triplicate samples are given.

mutants of *E. coli* (16). It was suggested that the amount of phospholipids in the cell envelope increases when *E. coli* is cultured on acetate instead of glucose. It is also possible that the different carbohydrates may affect the nucleotide pool of the cells either directly or indirectly during growth. The role of nucleotides in the regulation of APase synthesis has been demostrated in *E. coli* (22).

The effect of various phosphorus sources on growth and APase activity is given in Table 5. All the phosphorus sources supported good growth of the organism. Best growth occurred in the presence of orthophosphate, adenosine diphosphate, fructose 1,6-diphosphate or ribulose 1,5-diphosphase followed by other phosphorus sources tested. Fructose 1,6-diphosphate and ribulose 1,5-diphosphate supplemented cultures gave equal growth (0.62 O.D. at 660 nm). APase activity detected in cultures supplemented with any of the phosphate esters tested was intermediate between that of phosphate saturated and limited cultures (Table 5). No activity was detected in inorganic phosphate grown cultures. Growth was less than control in the presence of fructose (Table 4) but addition of fructose 1,6-diphosphate or fructose 6-phosphate as a phosphorus source stimulated growth more than 1.5 fold in comparison to control cultures. This indicates that *Anabaena* sp. strain CA preferred phosphorylated sugars more as a phosphorus source than as a carbon source for its growth. The ability to utilize a range of organic phosphates appears widespread among cyanobacteria. Our results are in good agreement with the findings of Ihlenfeldt and Gibson (9) who reported that *Anacystis nidulans* can utilize a number of phosphate esters in place of inorganic phosphate as phosphorus source. However, APase activity of *Anacystis nidulans* in the presence of organic phosphate was low as compared to that in phosphate limited cultures (9).

This study suggests that most amino acids and a few

carbohydrates such as sucrose, mellibiose, arabinose, and sorbose are suitable for APase production. Lactose, glucose, pyruvate or succinate may be used as a carbon source during photoheterotrophic growth of the cyanobacterium. Glycine and serine are preferred nitrogen sources for its growth. Phosphorylated sugars were preferentially used by the alga as a phosphorus source rather than a carbon source. Phosphate repressible APase activity has been found in *Anadaena* sp. strain CA.

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