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THE RATE OF BIOSYNTHESIS OF ISOLATED ACTIVE PRINCIPLE 4-[(5-CARBOXY-2-HYDROXY)-BENZYL]-1, 10-DIHYDROXY-3, 4, 7, 11, 11-PENTAMETHYLOCTAHYDROCYCLOPENTA<A>NAPHTHALENE FROM AN ANTARCTIC CYANOBACTERIUM *NOSTOC* CCC 537 UNDER DIFFERENT GROWTH REGIMES

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ABSTRACT

The known active principle (AP) 4-[(5-carboxy-2-hydroxy)-benzyl]-1,10-dihydroxy-3,4,7,11,11-pentamethyloctahydrocyclopenta<a>naphthalene has been isolated from an Antarctic cyanobacterium *Nostoc CCC537*, grows well at 20°C and showed antibacterial properties against both Gram (+) and (-) bacteria as well *as Mycobacterium tuberculosis*. The Killing efficiency of AP is high when methanol used as a solvent as compared to water and DMSO. Even the biomass age of 60 day, the target cyanobacterium possess maximum antibacterial potential. To enhance rate of biosynthesis of AP, resuming the nutrient components of growth medium such as nitrate and phosphate. Under 29 µM of phosphate as compared to 116 µM, normal (58 µM), the biomass production were reduced while rate of AP biosynthesis raised. Similarly for nitrate, biomass had increased maximum for 10mM while AP maximum in 5mM concentration. The production rate of AP were analyzed qualitatively by size of inhibition zones (Antibacterial bioassay) and quantitatively by HPLC.

KEYWORDS: Bioactive molecule, *Nostoc CCC 537*, Growth Regimes.

1. INTRODUCTION

Cyanobacteria, a photoautotrophic micro algae produce unparalleled array of secondary metabolites a wide variety of toxins and other bioactive compounds which are species specific [1, 2]. The presence of gene that produce them, has also induced interest to explore the

little explored cyanobacteria especially the extreme habitat colonizing i.e., the Antarctic ones.An active principal 4-[(5-carboxy-2-hydroxy)-benzyl]-1,10-dihydroxy-3,4,7,11,11-pentamethyloctahydrocyclopenta<a>naphthalene from an Antarctic cyanobacterium *Nostoc* CCC 537 having activity against both Gram (+) and (-) bacteria as well as *Mycobacterium* was isolated by Asthana et al. [3] (Fig. 1).The ability to produce bioactive compounds could play an important role in success of *Nostoc* in the tropical and Antarctic region [4]. In spite of pliantly of cyanobacteria are habitat in Antarctica, only Taton *et al*. [5] and Biondi *et al.*[6] reported active isolates from here before Asthana et al. [3].

The increase in biomass yield of cyanobacteria on a commercializationscale via bioprocess intensification for natural products is likely to become the important strategy in near future [1] and give intension towards rate of secondary metabolites production. As secondary metabolites production in cyanobacteria is regulated by environmental regimes[7], the production of bioactive molecule in the Antarctic environment has been simulated by growing cultures at low light irradiance (10-30 µmol photons m^{-2} s^{-1}) compounded with variation in nitrogen and phosphorus levels [5,6] .In the present endeavor we have tried to determine the rate of production under different growth regimes by resuming photoautotrophic growth and bioactivity of isolated active principle 4-[(5-carboxy-2-hydroxy)-benzyl]-1,10-dihydroxy-3,4,7,11,11-pentamethyloctahydrocyclopenta<a>naphthalene.

2. MATERIAL AND METHODS

2.1 Organism and Growth Conditions

The Antarctic strain *Nostoc* CCC 537 (Centre for Conservation and Utilization of Blue Green Algae, Indian Agricultural Research Institute, New Delhi 110012, India) (a kind gift of Prof. P. A. Broady, New Zealand) was grown in Chu-10 medium [8] lacking combined N-source with heterocyst frequency (~ 9%) at 20 °C in culture vessels (1L capacity) under low irradiance (20.1 µmol photons m^{-2} s^{-1}). The vessels were shaken periodically. Purity of cultures was routinely checked by transferring aliquots to 'Luria Broth' medium and incubation in dark (37°C, 24 h). As the cyanobacterium was slow growing and the desired biomass had to be collected at appropriate time, cyanobacterial biomass was harvested after 60 d of growth for elucidation of the biomolecules in reference.Specific growth rate constant (*k*) was calculated as described by Kratz and Myers [9].

2.2 Optimization of Temperature, Biomass Age and Solvent

The optimization of growth temperature was done by incubating culture at 10, 20 and 30°C in culture vessels (1L capacity) under low irradiance (20.1 µmol photons m-2 s-1) (Fig.2). The vessels were shaken periodically. To determine exact age for maximum bioactivity, the culture had been extracted on three different ages i.e., 30 d, 60 d and, 90 d. Selection of proper solvent was done by using three different solvent such as methanol, DMSO and water.

2.3 Protein Estimation

Protein content of the cyanobacterial culture was estimated using the method adopted by Lowry et al. [10] and modified by Herbert et al. [11].

2.4 Extraction and Isolation of Bioactive Molecule

Cellular extraction was done according to Asthana et al. [12]. Cyanobacterial biomass (10 g, fresh wt) was pelleted, washed with deionised water and lyophilised (Christ Alpha 1-2, Germany). The biomass (1g dry wt) was extracted twice with 100 mL methanol, centrifuged (20,000 x g, 30 min), the supernatant evaporated to dryness and residue redissolved in methanol (5 mL). This extract was processed for TLC (Merck Silica Gel-60) with the solvent CCl_4 : methanol (9:1, v:v). UV-transillumination of the plates revealed 9 spot, and these were eluted individually with minimum amount of methanol (1 mL). The eluates were bioassayed for their antibacterial potential against non-pathogenic *E. aerogenes* MTCC 2822 (IMTECH, India) using slightly modified Kirby Bauer disc diffusion susceptibility method [13] on 3.8% Mueller-Hinton [14] agar (HiMedia, India). The spot corresponding to maximum inhibition zone was eluted with ethanol and rechromatographed with a different solvent (hexane : ethyl acetate, 1:1, v:v). Resulting resolution in 8 spots, all these spots were eluted in methanol individually and antibacterial potential tested as above. The most effective spot was eluted in ethanol (1 mL), and subjected to reverse phase HPLC (Waters, USA) equipped with a C_{18}column (300 x 3.9 mm) and a solvent gradient of 60-100% methanol (1 mL min^{-1}, 20 min) followed by methanol (100%) for 10 min. Fraction (1 mL) collected every minute was bioassayed to select the most effective one.

2.5 Production of Bioactive Molecules in Varying Levels of Phosphate, Nitrate Regimes

'Active Principle' levels in the target cyanobacterium cells were optimized by growing biomass in varying concentration of phosphate and nitrate in the growth medium. Thecells were grown in a medium supplemented with the prescribed level of phosphate (58 µM) of

Chu-10 (control), half (29 µM), or double (116 µM) over the control. The nitrate sets had 5 mM or 10 mM nitrate added to the growth medium and the medium lacking nitrate served as control.

2.6 Statistical Analysis

Statistical analysis was carried out by one-way ANOVA and Student- Newman- Kuels (S-N-K) test using the SPSS software (SPSS Inc., version 12.0). ANOVA test has been used to test the significance difference among the mean level of bacterial count at different time interval. If this resulted as significant then multiple comparison (S-N-K test) has been applied to find out the pair-wise significant difference. All the experiments were carried out in triplicates with standard deviation (SD) represented in bars.

3. RESULTS

3.1. Optimization of Temperature for Photoautotrophic Growth

It was necessary to determine the optimum growth temperature of the cyanobacterium *Nostoc* CCC 537 for desired biomass. Photoautotrophic growth was compared at three different temperatures (10, 20 and 30°C) (Fig. 2). The initial concentration (100 µg mL^{-1})of the starter culture was common to all the three sets. The test organism was grown at 20°C, showed slow initial trend of growth at least for the first 48 h, followed by the subsequent rise in biomass up to 4 d i.e., ~3-fold (304 µg mL^{-1}) over the initial inoculum size (100 µg mL^{-1}). This followed a slow pace of the growth to attain 346 µg mL^{-1} on 10^{th} d thus amounting to the generation time of 78 h (*k*, 0.0128). The organism was slow-growing at 10°C for the first 6 d as evident from a 26% increment in the biomass (135 µg mL^{-1}, *k*, 0.0033). Interestingly, the biomass reflected a rising trend from 6 d onwards ending up with slow pace of growth, this phase may be referred as decreasing log growth phase. In contrast, the cyanobacterium proved sensitive to 30°C as evident from decrease in the biomass level during 0-6 d, and the ultimate cell death thereafter. Therefore, the target cyanobacterium was invariably grown at 20°C.

3.2. Selection of Biomass Age

Subsequent to identification of antibacterial activity of *Nostoc* CCC 537, it was imperative to determine the exact cell age as active principle production, is a function of age. Bioassay of lipophilic (methanolic) extract of cyanobacterial biomass had been measured in terms of inhibition zones formed taking *E. aerogenes* as the target (Table 1). The from 30 d old

biomass produced 6 mm inhibition zone on the bacterial lawn. For 60th d sets, extract had maximum impact (13 mm zone) compared to the cells grown until 90th d (11 mm). Therefore, 60 d culture age was selected for optimal intracellular antimicrobial activity.

3.3. Solvent vs. Bioactivity

Antibacterial property of crude extract of *Nostoc* CCC 537 was also examined using other solvents such as water, and DMSO in addition to methanol (15 µL each) against non-pathogenic bacterium *E. aerogenes* using slightly modified Kirby Bauer Disc Diffusion Susceptibility Method (Table 1). The diameter range of inhibition zone as achieved by various extracts was in the sequence: 7 mm (methanol) > 3 mm (DMSO) while no inhibition zone in case of water. Therefore, only methanolic extract of *Nostoc* CCC 537 was adopted for further purification and identification of the bioactive molecule.

3.4. 'Active Principle' Production under Different Nutrient Regimes

3.4.1 Phosphate

3.4.1.1 Photoautotrophic Growth of *Nostoc* CCC 537 in Phosphate

The test organism was grown in selected phosphate concentrations (29, 58, 116 µM) along with a parallel set containing the prescribed level (58 µM, K_2HPO_4, top curve); half of this concentration (29 µM, 3rd from the top); the level doubled to 116 µM is represented in the middle curve (Fig. 3). Cyanobacterial growth in the routinely used P level (58 µM) picked up subsequent to 2 d of incubation, and the biomass increased 3-fold during 4 d. This followed a slow pace of increment during 4-10 d and the ultimate biomass level reached 346 µg mL^{-1}. For phosphate concentration limited to29 µM, cyanobacterial growth was slowest with some sign of improvement only after 4 d but ending up with the ultimate lowest biomass yield (222 µg mL^{-1}). For doubling the phosphate concentration to 116 µM over the routinely used level (58 µM), there was no increase in cyanobacterial growth as expected because of the apparent improvement of only 46-unit in biomass yield (268 µg mL^{-1}) over the lowest phosphate level (29 µM). Also, the ultimate biomass yield was lowered by 78-unit from the value in control. The inset based on the growth response clearly indicated highest specific growth (0.0128) in the routine growth medium (58 µM phosphate) and also the lowest generation time (78 h). This is in contrast to the extended average doubling time of 153 h for 29 µM and 116 µM phosphate. Table 2 incorporating ANOVA value indicates highly significant differences between groups of cyanobacterium grown in different phosphate concentrations *vs.* time. S-N-K test as applied for pair-wise group comparison with regard to photoautotrophic growth

into different phosphate concentrations (29, 58, 116 μM) revealed highly significant differences in growth (Table 3).

3.4.1.2. Production of 'Active Principle'

Qualitative Determination

The lyophilized biomass (1g) of cyanobacterium derived from varying phosphate concentrations (as above) was processed for the estimates of AP production (Fig. 4). To start with, equal biomass recovered from the three phosphate level sets was subjected to methanol extraction for TLC. The respective 'H' spots (asthana *et al.* 2009) from the three sets also varied in intensity when UV-illuminated thus indicating the possible difference in levels of the AP. 'H' spot (extracted in MeOH) was tested for its relative inhibitory potential against *S. aureus.* The inhibition zone (27 mm) and phosphate depletion (29 μM) were negatively correlated in the sense that lesser biomass in sets facing P-limitation, synthesized AP in the highest amount. Next in order (22 mm) was the efficacy of biomass grown in normal P-level (58 μM) or control. However, excessive phosphate (116 μM) supply not only retarded cyanobacterial growth but also the AP synthesis as evident from the just 10 mm inhibition zone.

The data in inset reflect that biomass yield in different phosphate levels also varied as applicable to general growth (ref. Fig. 4). An almost 18.2% (0.90 g dry wt) reduction in biomass was evident for phosphate level reduced to half (29 μM) from the control of 58 μM (1.10 g dry wt). The cyanobacterial response to double the phosphate concentration (116 μM) was not positive as apparent from an almost 7.2% reduction in biomass (1.02 g dry wt). The overall observations indicated a rise in production of AP with decrease in phosphate concentration.

Quantitative Determination

The qualitative determinations of AP in the cyanobacterium from different phosphate level history prompted to go for the quantitative assessment. This also involved equivolume of the 1st TLC extract as used in previous experiments for further purification (as described in methodology). The HPLC data revealed that AP output was maximum (1.84 mg g^{-1} dry wt) for 29 μM phosphate sets, followed by 1.70 mg g^{-1} (58 μM phosphate) and minimum (1.44 mg g^{-1}) for 116 μM phosphate sets (Fig. 5 a,b,c).

3.5.1. Nitrate

3.5.1.1 Photoautotrophic Growth of *Nostoc* CCC 537 in Nitrate

Photoautotrophic growth of the diazotrophic *Nostoc* CCC 537 was monitored in nitrate-supplemented media (5 and 10 mM) along with the one lacking combined nitrogen source as control (common duration of 6-10 d as applicable to previous comparisons). It was only beyond 3 d that a distinction could be made in the cyanobacterial response to nitrate additions. As expected, the highest nitrate concentration (10 mM) sets also recorded maximum growth rate (*k* 0.019) and yield (687 μg mL^{-1}) followed by 5 mM (*k*, 0.017; yield 533 μg mL^{-1}) and the least in nitrate-lacking sets (*k*, 0.0128; yield 346 μg mL^{-1}) (Fig. 6). The data in overall indicated that doubling time of the cyanobacterium was inversely proportional to nitrate concentrations used. The ANOVA indicates highly significant differences between groups of cyanobacterium grown in different nitrate concentrations (Table 4). S-N-K test as applied for pair-wise group comparisons with respect to photoautotrophic growth in different nitrate levels (0, 5, 10 mM) revealed highly significant difference (Table 5).

3.5.1.2. Production of 'Active Principle'

Qualitative Determination

Although nitrate additions (5 or 10 mM) stimulated general cyanobacterial growth, it was not the case with regard to production of the AP (expressed as inhibition zones) (Fig. 7). It was evident that nitrate-lacking (control) cells permitted maximum synthesis of the AP as the inhibition zone size attained its maximum (24 mm). The lowest nitrate concentration (5 mM) though stimulatory to general cyanobacterial growth, the inhibition zone size was lowered 3-fold (8 mm). For a doubling in nitrate concentration to 10 mM which on one hand promoted cyanobacterial growth to its maximum, on the other lowered inhibition zone size to the minimum most (5 mm).

Quantitative Determination

In this case also, equivolume of the 1st TLC extract from samples with different nitrate background was subjected to further purification (as in methodology) and processed for HPLC (Fig. 8 a,b).Quantity wise, the AP level turned out to be much higher (1.20 mg g^{-1} dry wt) for low nitrate level (5 mM) relative to a drastic decline to 0.73 mg $g^{-1}$for the elevated nitrate dose (10 mM). It may be added that the AP yield (1.70 mg g^{-1}) for nitrate-lacking sets still remained superior to both the nitrate levels (ref. Fig. 5c).

4. DISCUSSION

4.1. Optimization of Growth Temperature

Cyanobacteria represent a potentially rich source of secondary metabolites such as antibacterial, antifungal, antialgal and toxins [15,16,17,18], which are species specific [1,4,12]. As the search for biomolecules has been extended to stromatolites of extreme environments [19], the Antarctica harboring rich cyanobacterial diversity [20], seem to have a promising future. Therefore, examination of *Nostoc* CCC 537 an Antarctic strain was based on the assumption that the existence of antibacterial metabolites might indicate adaptation specificity to different habitats [21] in order to sustain their niche against microbial invasions. The present endeavour therefore, was to select and process the Antarctic strain *Nostoc* CCC 537 in anticipation to isolate and characterize new/unique biomolecules in such an unique environment. The Antarctica has characteristic fluctuations in temperature year around, whereas cyanobacteria therein could have evolved adaptive strategies to withstand and grow under the sub-optimal temperature regimes. According to Tang and Vincent [22] cyanobacteria of Antarctican origin have optimal growth temperature usually much higher than the range common to their place of origin. Therefore, it may be expected that such an organism retain inherent qualities irrespective of the habitat. During laboratory trials, *Nostoc* CCC 537 had its optimal photoautotrophic growth at 20°C relative to 10°or 30°C (ref.Fig. 2). Tang et al.[23] reported that among the 27 isolates of polar cyanobacteria, the temperature optimum was highly variable as *Phormidium subfuscum* grew in the 5-25°C range with the optimum at 15°C; growth rate of *Phormidium tenue*, followed the increase in 5-35°C range. In a recent report, Biondi et al.[6], observed that cyanobacteria grew better around 20°C than at lower and higher temperatures in a way confirming the usual psychrotolerant nature of Antarctic cyanobacterial community. These workers also observed that almost 51 cyanobacterial genera from benthic mats of Antarctica were slow growers (0.02-0.44 d^{-1}). The present observation also fall in the same line as evident from the specific growth (*k*) of 0.0128 h (0.30 d^{-1}) at 20°C (ref. Fig. 2). Such a growth parameter when tested for other polar cyanobacteria was also in the 0.02-0.4 d^{-1} range [22, 23, 24]. The subsequent report on 30 Antarctic Oscillatorians from shallow ponds on the Mc-Murdo Ice Shelf revealed most of the isolates had only minimal or no growth below 5°C but grew well above 20°C [25].Thus, growing target strain at 20°C under low irradiance (20.1 µmol photons m^{-2} s^{-1}) seems justified in adoption of *Nostoc* CCC 537 for optimum biomass and so also bioactive component in reference.

4.2. Biomass Age

There are reports of cyanobacterial production of biomolecules extracellularly by mesophilic *Nostoc* sp. [26], *N. spongiaeforme* [27], *N. commune* [28] and intracellularly by *N. commune* [29], *N. linckia*[30], *N. muscorum* [31] and *Nostoc* ATCC 53789 [32]. In batch cultures, *N. insulare* had its metabolism dependent on the cell age [33]. Cyanobacterial metabolites with antimicrobial activity, may either be produced constitutively throughout the exponential and linear phase, or specific to pre-stationary or stationary phase [34]. In the present context, also cyanobacterial biomass at 30 d, 60 d and 90 d was processed for antibacterial bioassay of the intracellular as well as extracellular samples (ref. Table 1).It is apparent from the growth curve of *Nostoc* CCC 537 that it entered the decreasing log growth phase 6th d onward. Since secondary metabolites are produced in the pre-stationary phase/stationary phase, it was imperative to harvest the cyanobacterial biomass for intracellular and the spent medium for extracellular levels of the biomolecule at selected time intervals as above. The only Volk [33] reported on exometabolite production in *N. insularae* as a function of cell age (40-152 d). The present organism was routinely grown at low irradiance (20.1 µmol photons m^{-2} s^{-1}); the level generally used for growing Antarctic strains [5, 6]. Extracts from *Nostoc* CCC 537 at 60 d, produced larger inhibition zones, reflecting that the duration was optimal for procuring the intracellular metabolites. This is because the inhibition zones (6 mm) for 30 d sets were reduced to more than 50% relative to 60 d sets. For extension of growth to 90 d, the values of zone size with regard to intracellular fractions did not show any improvement and rather the effectivity got restricted to only 11 mm. Coming to extracellular sets, there was no inhibition of the bacterial growth under comparable conditions. However, the effectivity of extracellular sets improved for spent medium collected at 60 d as evident from the inhibition zone (7 mm). Similar fractions collected afterward (90 d) rank the efficacy on top of the rest as evident from the maximum inhibition zone size (13 mm). In other words, the extracellular metabolite level at 90 d was in close proximity with that of the intracellular metabolite level on 60 d. Therefore, concentrating on intracellular metabolite in the present endeavour seem to be justified.

4.3. 'Active Principle' Production under Different Nutrient Regimes

4.3.1. Phosphate Regulation

Subsequent to evaluation of the AP, the prospect of its production was explored by supplementing the growth medium with varying levels of phosphate. This is because it was the most important limiting nutrient in addition to nitrogen for diazotrophs [35].

Cyanobacterial growth and secondary metabolite(s) production are likely to be affected by nutrient condition(s) [7]. It is also a fact that the Antarctican habitat suffers from limitations of phosphorus and nitrogen availability [20, 36, 37]. In another report, *Prymnesiumparvum* (a haptophyte alga) grown under nutrient sufficient-conditions, did not produce allelopathic substances in contrast to nutrient-deficient cultures [38]. The correlation between growth rate and external nutrient concentration is well-established and controlled by the internal concentration(s) of the limiting nutrient(s)[39]. Accordingly, phosphate nutrition is as vital for photoautotrophic growth in cyanobacteria as carbon and nitrogen. Experiments with *O. agardhii* and *A. variabilis* suggest that growth rate limiting concentration of phosphate, the crucial nutrient for cyanobacteria, can be expected to be at or below the chemical detection level. The latter investigator also reported that P-deficiency altered the cellular N/P ratio, resulting in a drop in the protein to carbohydrate ratio, nucleic acid and also ATP. Cyanobacteria show average phosphorus tolerance in the 0.05 and 20 mg L^{-1} range, and its level for optimal growth varies from species to species[40]. However, the role of nutrients as control on algal productivity still remains little explored in polar aquatic environments [41]. In the present investigation also, 116 µM (20 mg L^{-1}) the highest phosphate concentration was used in order to assess its possible role in cyanobacterial biomass/bioactive molecule production. The lowest phosphate concentration (29 µM) was not favourable to cyanobacterial growth (ref. Fig. 3), but stimulated AP production as evident from the maximum inhibition zone size (27 mm) (ref. Fig. 4). The highest phosphate level (116 µM) used though stimulated the biomass yield to some extent (1.02-fold), contributed towards the least amount of AP (10 mm inhibition zone). The corresponding values of the AP present in target biomass grown in varying phosphate levels could be arranged as: 1.84 mg g^{-1} dry wt (29 µM phosphate) > 1.70 (58 µM) > 1.44 mg (116 µM) as ascertained by HPLC (ref. Fig. 5). It is just possible that *Nostoc* CCC 537 also behaved in line with the cyanobacterium adopted by Ray and Bagchi [7] in increasing secondary metabolite production under conditions of P-deficiency.

4.3.1. Nitrate Regulation

In continuation with phosphate, the role of combined nitrogen (nitrate) manipulation was also observed albeit, the cyanobacterium is a diazotroph. Growth of the cyanobacteria and role of different nitrogen sources is well established [42, 43]. Cyanobacteria in general, perform better growthwise in nitrate nitrogen than in NH_4^+, although the latter is energetically more favourable [44, 45]. The source-dependent variation in growth of *N. muscorum*; being optimal

in nitrate rather than in NH_4^+/N_2 is also reported [46]. This perhaps justifies the selection of nitrate in the present endeavour in an attempt to optimize AP production. The addition of nitrate (5 or 10 mM) in the growth medium decreased the generation time of *Nostoc* CCC 537 (ref. Fig. 6). The maximum biomass yield (1.21 g dry wt) in the highest nitrate level (10 mM) led to the least amount of AP as adjudged from the inhibition zone size (5 mm) (ref. Fig. 7). The nitrate level lowered to 5 mM though favoured biomass production to a lesser extent (1.16 g), the inhibition zone size got increased 1.6-fold (8 mm) thus indicating that high biomass yield may not accompany high production of the AP. This was evident as cyanobacterium facing nitrogen limitation, produced the highest amount of the bioactive molecule (24 mm inhibition zone); but the least biomass (1 g dry wt). The HPLC-based quantification also arranged the AP amount as: 1.70 mg g^{-1} dry wt (-nitrate) > 1.20 (5 mM) > 0.73 (10 mM nitrate) (ref. Fig. 8).

AP/biomolecule is a secondary metabolite produced in the stationary phase of microbes. There are a few reports on the production of allelopathic compounds, toxins and bioactive peptides under nutrient stress. However, nutrient stress is reported to enhance production of allelopathic compound in various algae [7, 47]. Toxin production by freshwater cyanobacteria is negatively correlated with N and positively with P concentration [48]. Therefore, higher production of AP by *Nostoc* CCC 537 at half the phosphate concentration than the prescribed one (58 μM), seems to be in tune with other workers albeit in a variety of algal forms. Repka et al.[49] reported highest peptide concentrations in *Anabaena* strain 90 at 2.6 mg L^{-1} phosphate the level prescribed for Z_8 medium. The present data on maximum AP production by *Nostoc* CCC 537 at 29 μM (5 mg L^{-1}) is in agreement with those reported in *Anabaena* strain 90 by Repka et al.[49]. The difference in optimum production by the two cyanobacteria might be correlated with the strains originating from different niches.

Table 1. Relative antibacterial potential of the AP in different solvents as on different ages aginst E. aerogenes

Strain Inhibition zone (mm)

Different Solvent			
	Water	DMSO	Methanol
N. CCC 537	n.d.	3.0	7.0
Biomass Age			
N. CCC 537		60 day	90 day
	6.0	13.0	11.0

*n.d. not detected

Table-2. ANOVA (F) value and significance level between different phosphate concentration groups (based on Fig. 2).

Days	F	Significance
1	203.94	0.000
2	53.59	0.000
3	7946.41	0.000
4	26488.86	0.000
5	7855.95	0.000
6	3266.55	0.000
7	3008.32	0.000
8	17049.18	0.000
9	911.21	0.000
10	1251.66	0.000

Table 3. Multiple comparison (S-N-K test, q-values) regarding photoautotrophic growth of *Nostoc* CCC 537 in phosphate (29, 58, 116 µM)-containing medium (based on Fig 2).

	Phosphate (µM)		
Days	58 vs 116	58 vs 29	116 vs 29
1	2.21	18.49***	16.28***
2	2.08	9.82***	7.74**
3	102.49***	114.81***	12.31***
4	182.63***	212.63***	30.00***
5	98.46***	116.41***	17.94***
6	64.02***	70.35***	6.27**
7	56.42***	74.31***	17.88***
8	121.95***	181.06***	59.11***
9	27.29***	42.07***	14.78***
10	31.24***	49.46***	18.22***

*significant level (**p < 0.01, ***p < 0.001)

Table 4. ANOVA indicating the role of nitrate levels in supporting growth of *Nostoc* CCC 537 (based on Fig. 5).

Days	F	Significance
1	0.614	0.527
2	43.88	0.000
3	5744.34	0.000
4	2153.47	0.000
5	7621.95	0.000
6	2363.39	0.000
7	20304.97	0.000
8	51231.58	0.000
9	13201.80	0.000
10	18401.67	0.000

Table. 5. Multiple comparisons (S-N-K test, q-values) for growth of *Nostoc* CCC 537 in nitrate (0, 5, 10 mM) containing medium (Fig. 5).

	Nitrate (mM)		
Days	**0 vs 5**	**0 vs 10**	**5 vs 10**
1	0.50	0.60	1.11
2	8.86***	7.05**	1.81
3	56.74***	107.12***	47.00***
4	46.98***	63.17***	16.19***
5	86.74***	119.48***	32.69***
6	39.02***	68.53***	29.51***
7	112.24***	201.03***	88.79***
8	177.06***	319.38***	142.32***
9	89.18***	162.24***	73.05***
10	104.89***	191.59***	86.70***

* Significant level (**p < 0.01, ***p < 0.001)

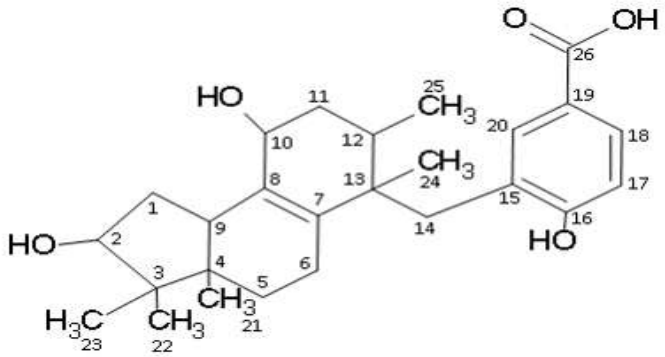

Fig.1.Known isolated active principle 4-[(5-carboxy-2-hydroxy)-benzyl]-1,10-dihydroxy-3,4,7,11,11-pentamethyloctahydrocyclopenta<a>naphthalene from Antarctic strain *Nostoc CCC 537* (Asthana *et al.* 2009).

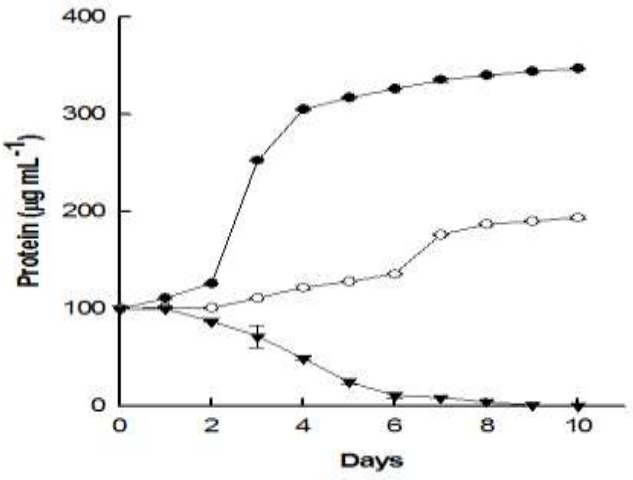

Fig. 2. Photoautotrophic growth of *Nostoc* CCC 537 at 10°C (o-o), 20°C (•-•), or 30°C (▼-▼) and specific growth (*k*) *vs.* temperature (inset).

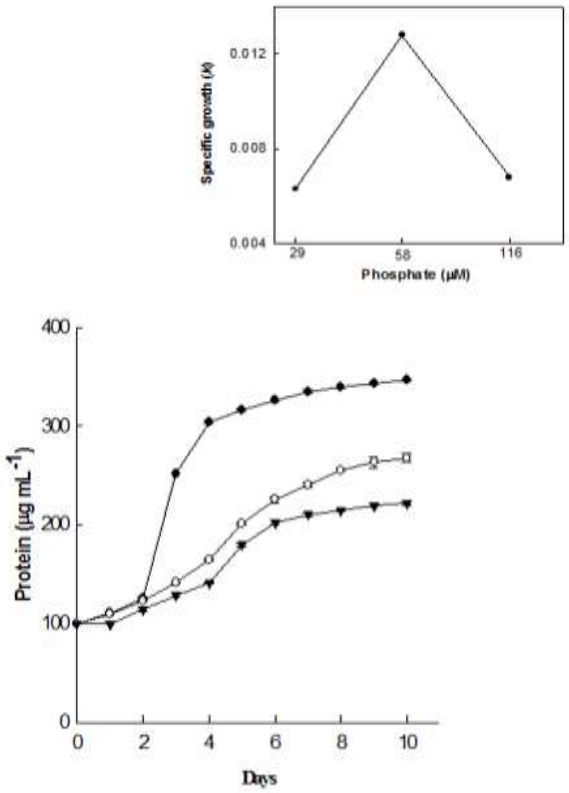

Fig. 3. Photoautotrophic growth *Nostoc* CCC 537 in 29 µM (▼-▼), 58 µM (●-●) or 116 µM (o-o) phosphate; specific growth (*k*) *vs.* phosphate concentrations in the inset

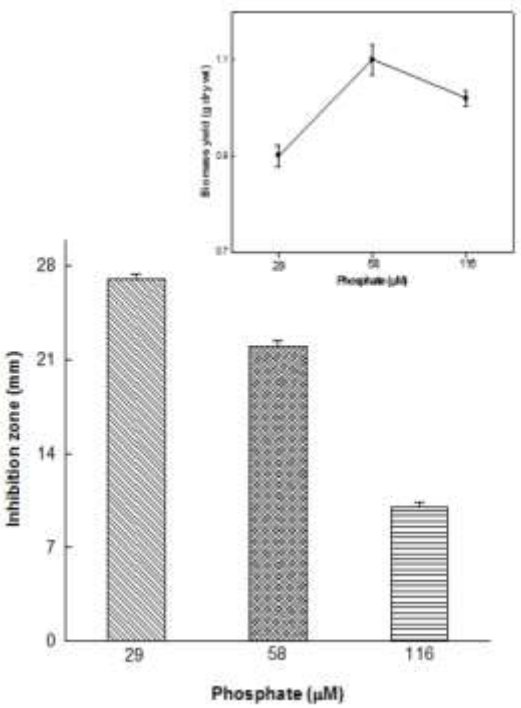

Fig. 4. Biomass yield of *Nostoc* CCC 537 in 29, 58 and 116 µM phosphate (inset) and the corresponding inhibition zone size against *S. aureus* by the 'active principle' (common dose, 15 µL).

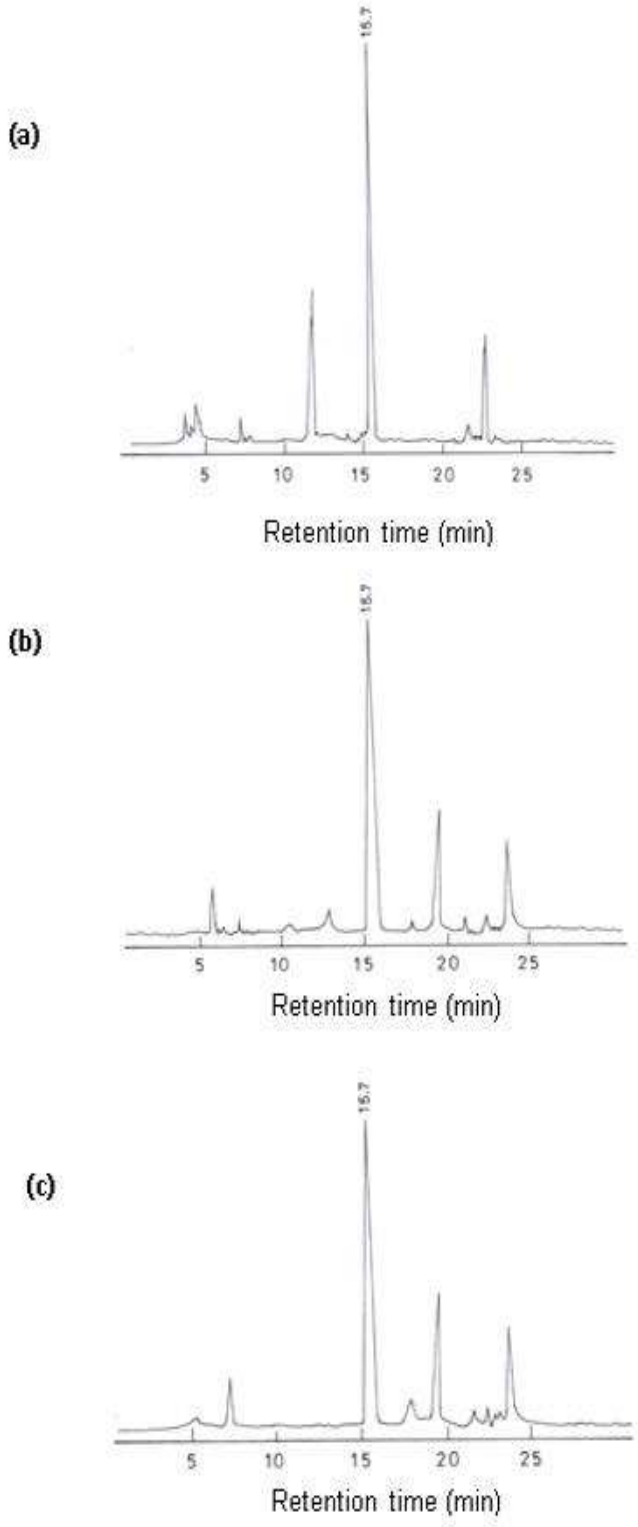

Fig. 5. HPLC chromatogram of 'active principle' from *Nostoc* CCC 537 at retention time 15.7, grown in 29 µM (a) and 116 µM phosphate (b); reproduction of Fig. 9 for comparison (c)

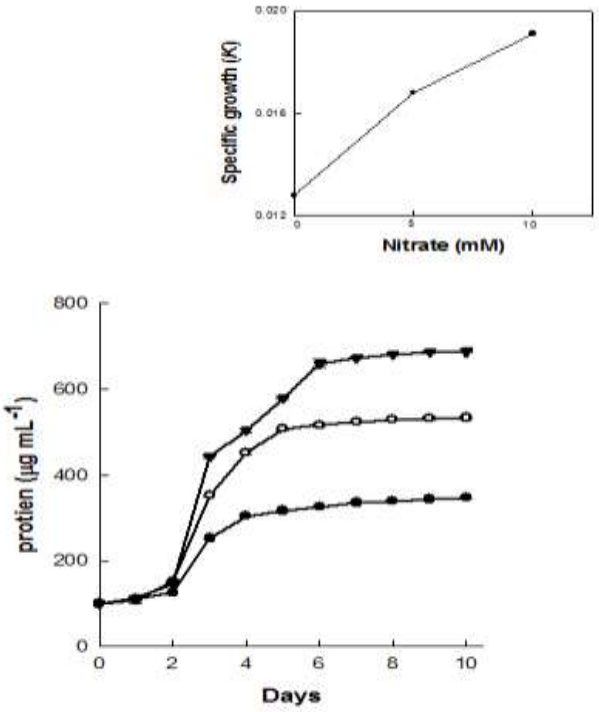

Fig. 6. Photoautotrophic growth of *Nostoc* CCC 537 in 5 mM (o-o) and 10 mM (▼-▼) nitrate along with nitrate lacking control (●-●); specipic growth (*k*) *vs.* nitrate concentration (inset).

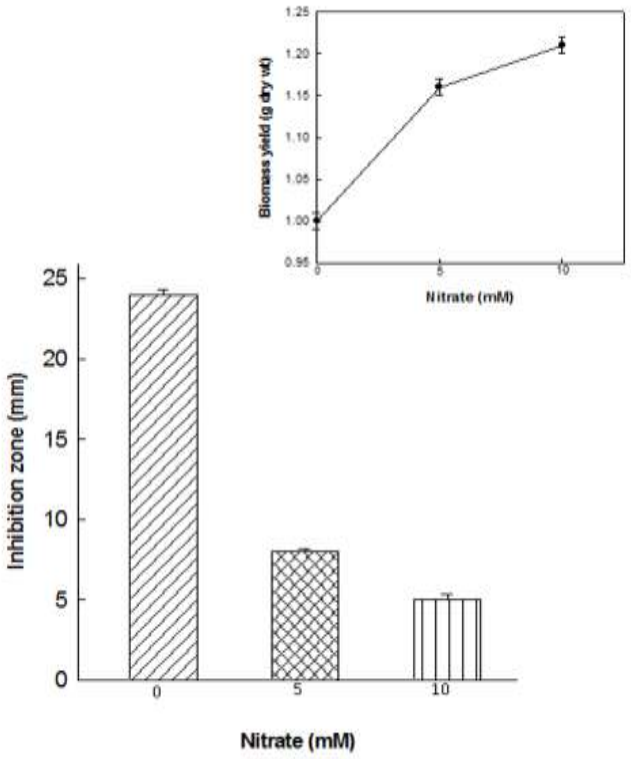

Fig. 7. Biomass yield of the *Nostoc* CCC 537 (inset) and inhibition zone size by bioactive molecule from 5 mM or 10 mM nitrate set along with those from control (nitrate-lacking) at a common dose of 15 µL

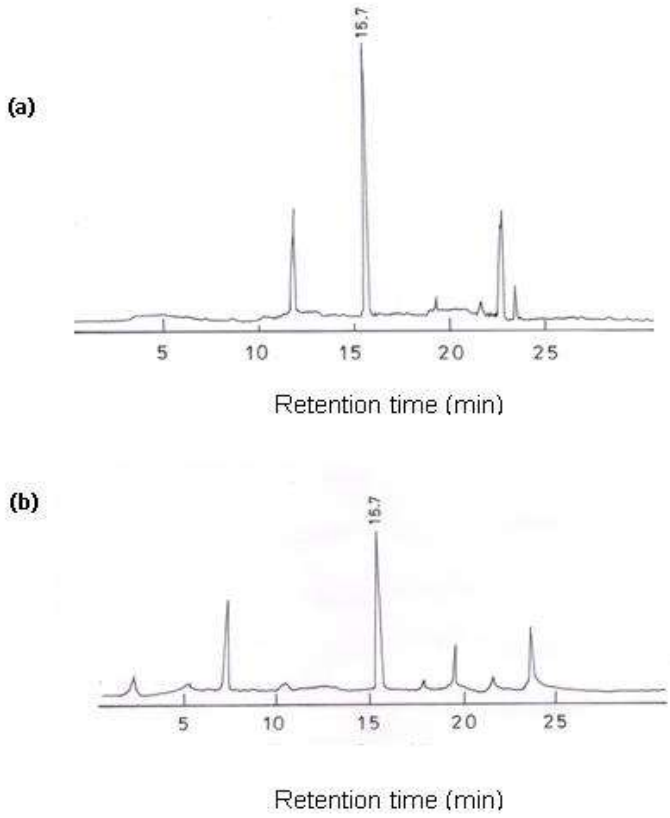

Fig. 8**. HPLC chromatogram of 'active principle' from *Nostoc* CCC 537 (retention time, 15.7 min), derived from different cultural history i.e., 5 mM (a) and 10 mM (b) nitrate.**

CONCLUSIONS

In controlled laboratory conditions the production rate of isolated active principle as well as biomassof*Nostoc CCC 537*wereenhanced. The rate of production of AP in target biomass grown in varying phosphate levels arranged as: 1.84 mg g^{-1} dry wt (29 µM phosphate) > 1.70 (58 µM) > 1.44 mg (116 µM). Under different nitrate regimes AP amount as: 1.70 mg g^{-1} dry wt (-nitrate) > 1.20 (5 mM) > 0.73 (10 mM nitrate). The consequence of present endeavor is that, use mixed combination of nitrate and phosphate in different concentrations will be favourbetter rate of an AP production.

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