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# Antarctic cyanobacterium *Nostoc* $_{CCC537}$, a new source of γ-linolenic acid and its antibacterial potential

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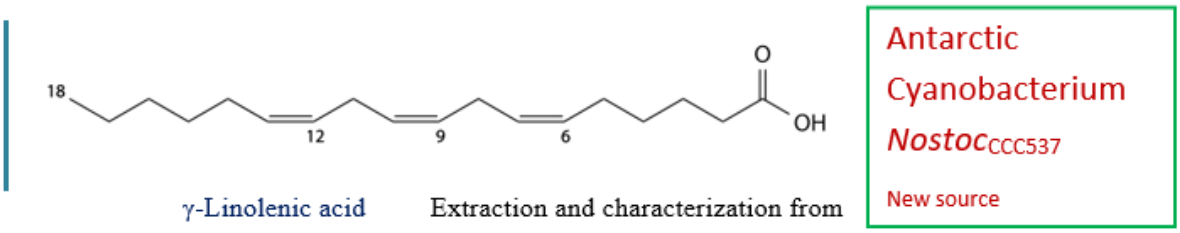
**ABSTRACT**




Unsaturated fatty acids are one of an important component of our healthy diet as needed for various physiological functions as well as it has medicinal significance. The Gamma linolenic acid (GLA), the pharmaceutically important fatty acid, was isolated from the *Antarctic cyanobacetrium*, *Nostoc*$_{CCC537}$. The antibacterial potential of GLA was evaluated against *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosa* ATCC27853, *Salmonella typhi* MTCC3216, *Escherichia coli* ATCC25992 and *Enterobacter aerogenes* MTCC2822. It was observed that GLA production in the cyanobacterium was also regulated by alterations in phosphate or nitrate levels during growth along with temperature. A doubling in the phosphate concentration (116 µM) over that routinely used (58 µM) in the diazotrophic medium, enhanced GLA production 20.5%, while the biomass yield decreased to 7.3% of the control. GLA production, however, decreased to 18% or 23% in 5 mM or 10 mM nitrate, respectively. A downshift in growth temperature to 10°C from 20°C enhanced GLA production. It is suggested that the Antarctic cyanobacterium may also act as a source of an antibacterial agent as well as GLA.

*Keywords: γ- linolenic acid, antimicrobial activity, Antarctic cyanobacterium,* Nostoc *CCC537*

## INTRODUCTION

One of the polyunsaturated fatty acids (PUFA) γ -linolenic acid (GLA) (18:3ω6), not common in human diet, is synthesized by $\Delta^6$-desaturase enzyme from linoleic acid (18:2). PUFA (18-20 C). By now, the compound has gained importance because of its therapeutic potential in clinical practices.[1] GLA is active against rheumatoid arthritis, atopic eczema, asthma, premenstrual syndrome, cardiovascular diseases, ulcerative colitis, cancer, dry eye syndrome, osteoporosis, and diabetic neuropathy.[2] Such long-chain fatty acids are bactericidal to pathogenic strains including Methicillin-resistant *Staphylococcus aureus*[3] and *Helicobacter pylori*.[4] GLA has also been reported for inhibition of *Escherichia coli* and *Pseudomonas aeruginosa* strains *in vitro*.[5,6] Exposure of such strains to GLA also changed their susceptibility towards various antibacterial agents as evident by the increased MICs. The current sources of GLA are, black currant, borage, evening primrose (angiosperms), *Mortierella* and *Mucor* (fungi) and *Spirulina* (cyanobacterium).[2] Higher plants as the GLA resource, mainly suffer from their low yield (borage, 300-600 kg ha$^{-1}$ compared to about 3,048 kg ha$^{-1}$ for rape seed)[7]. Thus, screening of the non-conventional sources of GLA especially cyanobacteria which may be grown at large scale in comparatively smaller space.

Fatty acid desaturases in cyanobacteria is regulated by temperature[8] and participate in tolerance and acclimation.[9] Cyanobacteria are the only prokaryote to produce PUFA by the

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$O_2$-dependent desaturase.[10] The low temperature induced increase in $O_2$-solubility and the consequent increase in desaturase activity is also reported.[11] Fatty acid profile of *Spirulina platensis* showed changes under fluctuations of temperature as well as nitrate.[12] Howevere, the direct chemical synthesis of GLA is not preferred as it gives both *cis* and *trans* isomer with the equal percentage ratio although only 1/8 of the final yield product has all double bonds in the *cis* configuration.[13] Therefore, screening of microbial source for GLA and its optimization for production led us to select low temperature growing cyanobacteria, *Nostoc* CCC537, an isolate from Antarctica low irradiance and limited availability of phosphorus and nitrogen,[14] with proven antibacterial potential, reported by us.[15] In *S. platensis*, the higher concentration of biomass[16] and algal density[17] were correlated with the GLA level. In the present work, we report the presence of GLA in *Nostoc* CCC537 and its antibacterial potential along with variations in the content at various temperature, nitrogen, phosphorus regimes vis a vis biomass.

## RESULTS

### Identification and estimation of GLA

*Nostoc* CCC537 was slow growing (generation time, 78h) with specific growth rate,0.0128$h^{-1}$ at 20˚C.The sufficient biomass for GLA extraction was obtained after 60 d of growth. The fatty acid methylated ester (FAME) from the test cyanobacterium was subjected to TLC separation along with methyl GLA standard (Figure 1**).**

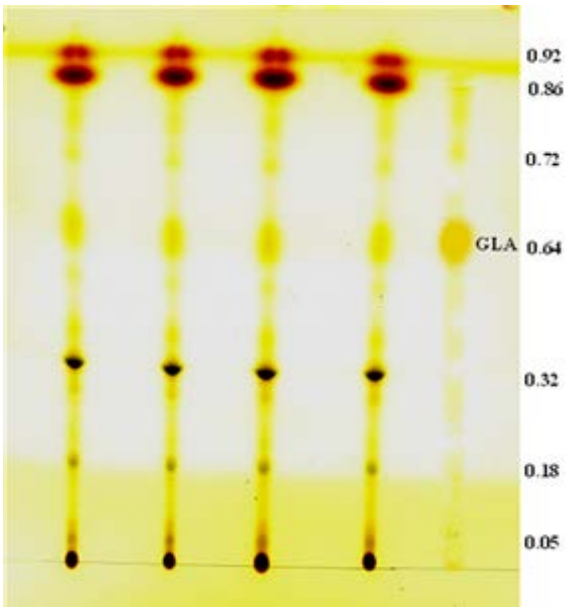


**Figure 1.** TLC profile of fatty acid methyl ester extracted from *Nostoc* CCC537 along with γ-linolenic acid standard.

Out of the extracts separated into 7 bands, one band ($R_f$, 0.64) corresponded to the GLA standard. This band was eluted in methanol and purified by HPLC. The fraction having 4.72 min, retention time corresponded to GLA standard, confirming GLA in test cyanobacterium (Figure 2). The production of GLA in *Nostoc* CCC537 reached 4.68 mg $g^{-1}$ dry wt at the optimum growth temperature ($20^0$C).

### Antibacterial Assay

A number of G (-) and G (+) pathogens were used to document the activity of *Nostoc* CCC537 GLA alongwith the standard (Table 1). It was evident that 16 $\mu$g $mL^{-1}$ *Nostoc* GLA was the MIC for *E. aerogenes* with the minimum of 0.4 $\mu$g $mL^{-1}$ for *S.aureus* while *P. aeruginosa*, *Ecoli*, and *S. typhae* shared a common MIC of 8 µg $mL^{-1}$. For GLA standard, the MIC for *E.aerogenes* was reduced to 8 $\mu$g $mL^{-1}$ followed by *E. coli*, or *S. typhae* (4 $\mu$g $mL^{-1}$) and the least for *S.aureus* (0.2 $\mu$g $mL^{-1}$). As the MIC threshold was limited to 16 $\mu$g $mL^{-1}$ for *Nostoc* GLA, the inhibition zone size in the respective case was compared at a relatively higher concentration (25 $\mu$g $mL^{-1}$) for each GLA type. *Nostoc* GLA (25 µg $mL^{-1}$) revealed maximum inhibition zone size for *S. aureus* (19.0 mm) and minimum for *E. aerogenes* (8.0 mm) with *S. typhi* (14.5 mm), *E. coli* (14.0 mm) and *P. aeruginosa* (12.0 mm) as the intermediates. The inhibition zones for the equivalent dose (25 µg $mL^{-1}$) of pure GLA standard were different for *S. aureus* (23.0 mm), *S. typhi* (19.0 mm), *E. coli* (18.0 mm), *P. aeruginosa* (12.5 mm) and *E. aerogenes* (10.5 mm) in the decreasing order.

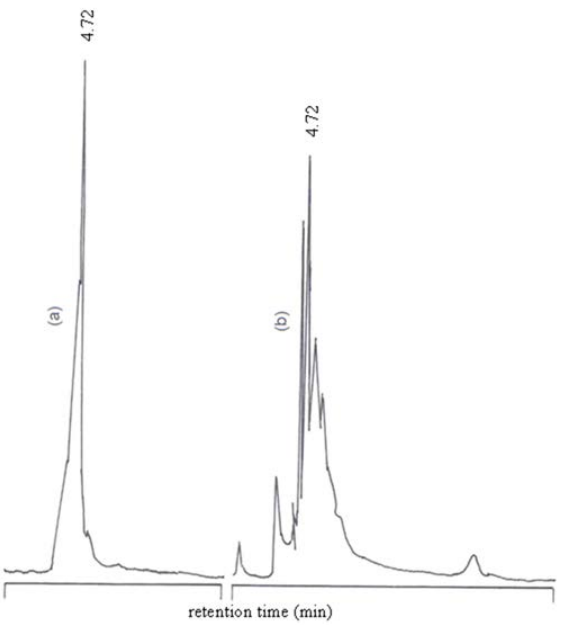


**Figure 2.** HPLC profile of γ-linolenic acid standard (a) and isolated from *Nostoc* CCC537 (b).

The antibacterial potential of GLA was also adjudged by comparing the relative sensitivity of the most resistant bacterium *E. aerogenes* towards higher GLA concentrations (25,30 and 45 $\mu$g $mL^{-1}$) as well as the antibiotics (rifampicin and streptomycin) (Table 1, bottom). MICs of antibiotics for *E. aerogenes* were also determined to compare the overall efficacy of *Nostoc* GLA. The inhibition zone size was higher (1.31-fold) for pure GLA (25 µg $mL^{-1}$) over the equivalent dose of *Nostoc* GLA (8.0 mm). A rise in *Nostoc* GLA concentration to 30 µg $mL^{-1}$ increased the inhibition zone size (12 mm). Interestingly,

rifampcin or streptomycin (30 µg mL$^{-1}$ each) did not produce the inhibition zone indicating need for still higher concentrations of such antibiotics against the test bacterium. Elevating the concentration of antibiotics to 30 $\mu$g mL$^{-1}$ resulted in production of inhibition zone for rifampicin (14mm) and streptomycin (12mm) in close proximity with those produced by *Nostoc* GLA at 30 $\mu$g mL$^{-1}$. However, further increase in concentration of *Nostoc* GLA and also the antibiotics to 45 µgmL$^{-1}$ produced inhibition zone size in the sequence: *Nostoc* GLA (27 mm) > rifamipcin (26mm) > streptomycin (21 mm). It was noteworthy that MICs of antibiotics were also two-fold over that of *Nostoc* GLA (16 $\mu$g mL$^{-1}$). These results collectively proved the antibacterial potential of *Nostoc* GLA.

**Bactericidal property of GLA in liquid culture**

The evaluation of bactericidal activity of any antibacterial agent is complicated by the possible emergence of tolerance in the bacterium. This is clinically important, and tolerance is better evaluated by the 'time-kill assay'. The assay permits interaction of the bacterium with the varying levels of the antibacterial agent as well as the duration of contact to account for the percentage of "persisters" to decipher the bactericidal effect. To document the bactericidal potential of GLA, we examined liquid culture sensitivity of *S. aureus* (most sensitive) against three

**Table 1.** Inhibition zone size and MIC of *Nostoc* GLA along with the standard against different bacteria (top) and a comparison of effectiveness of GLA and antibiotics (rifampicin and streptomycin) against *E. aerogenes* only (bottom)

| Target | Inhibition zone (mm) | | MIC (µg mL$^{-1}$) | |
|---|---|---|---|---|
| | GLA (25 µg mL$^{-1}$) | | | |
| | Nostoc | Standard | Nostoc | Standard |
| *S. aureus* ATCC 25923 | 19.0 | 23.0 | 0.4 | 0.2 |
| *P. aeruginosa* ATCC 27853 | 12.0 | 12.5 | 8.0 | 8.0 |
| *E. coli* ATCC 25992 | 14.0 | 18.0 | 8.0 | 4.0 |
| *S. typhi* MTCC 3216 | 14.5 | 19.0 | 8.0 | 4.0 |
| *E. aerogenes* MTTCC 2822 | 08.0 | 10.5 | 16.0 | 8.0 |

| Antibacterialagents against *E. aerogenes* | (µg mL$^{-1}$) | | | MIC (µg mL$^{-1}$) |
|---|---|---|---|---|
| | 25 | 30 | 45 | |
| *Nostoc* GLA | 08 | 12 | 27 | 16 |
| Rifampicin | n.d. | 14 | 26 | 32 |
| Streptomycin | n.d. | 12 | 21 | 32 |

n.d. – not detected

concentrations of *Nostoc* GLA (10, 20 or 30 µg mL$^{-1}$) during a common contact duration (0-120 min) along with the control (lacking GLA) (Figure 3). The general trend reflected that 30 min contact was least effective (relative to control) as the maximum decline in viable cell count was only 1.2-fold even for the highest dose (30 µg mL$^{-1}$). The extension of contact to 60 min reflected an increase in bacterial sensitivity towards 20 µg, and also 30 µg mL$^{-1}$ GLA as the viable cell count decreased 1.52 and 2.1-fold, respectively. For the highest contact duration (120 min), even the lowest GLA dose (10 µg mL$^{-1}$) proved effective in lowering the viable cell count 1.83-fold. GLA concentration doubled to 20 µg mL$^{-1}$ brought down the cell count 3.1-fold. The highest GLA dose adopted (30 µg mL$^{-1}$) was also on top of the rest as evident from a ~5-fold decline in the bacterial cell count. We can infer that even 30 $\mu$g mL$^{-1}$ GLA dose was not sufficient to eliminate the entire bacterial population in the present set of conditions. It was also obvious that bactericidal property depended on the contact duration and concentration of the antibacterial agent as well. To find the significant difference among level of different doses of GLA (10, 20 or 30 µg mL$^{-1}$) on *S. aureus*, one way ANOVA was used which is statistically significant ($F_{4,40}$ = 27.96; $p$<0.001). S-N-K test as applied for pair-wise group comparison with respect to growth inhibition of *S. aureus* against different GLA concentrations was highly significant except the control and 30 µg mL$^{-1}$ after 30 min contact (Table 2).

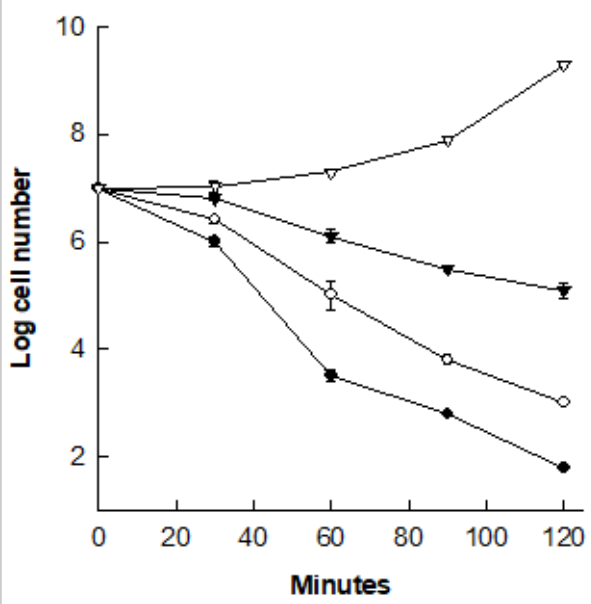


**Figure 3.** Liquid growth response of *S. aureus* as control (∇-∇), and in 10 µg mL$^{-1}$ (▼-▼), 20 µg mL$^{-1}$ (o-o) or 30 µg mL$^{-1}$ (●-●) of GLA from *Nostoc* CCC537. One way ANOVA ($F_{4,40}$ = 27.96; $p$<0.001) Error bar denotes standard deviation.

Bactericidal potential of GLA was also evaluated against the target bacteria adopted for the antibacterial assay earlier. The viable cell count in the form of cfu (% control) during contact (0-60 min) in the liquid culture of the five bacterial strains challenged with *Nostoc* GLA (at a common dose of 25 µg mL$^{-1}$) is shown in Figure 4. The topmost curve reflects high resistance of *E. aerogenes* as cfu decreased

**Table 2.** Multiple comparison (*Student Newman*-Keuls test, q-value) for growth of *S. aureus* in *Nostoc* GLA (10-30 µg mL$^{-1}$) (based on Figure 3).

| Min | GLA (µg mL$^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | 0 vs 10 | 0 vs 20 | 0 vs 30 | 10 vs 20 | 10 vs 30 | 20 vs 30 |
| 30 | 3.46 | 9.53 *** | 15.56 *** | 6.02 ** | 12.09 *** | 6.07 *** |
| 60 | 9.23 *** | 19.93 *** | 30.42 *** | 10.49 *** | 21.19 *** | 10.70 *** |
| 90 | 50.77 *** | 86.67 *** | 107.20 *** | 20.53 *** | 56.43 *** | 35.89 *** |
| 120 | 35.89 *** | 56.43 *** | 50.77 *** | 100.52 *** | 67.37 *** | 52.13 *** |

* significance level (**p < 0.01; ***p < 0.001)

to ~55% at 60 min. Based on the overall pattern, *E. aerogenes* could be grouped as the most resistant bacterium followed by *P. aeruginosa* (71.74% decline), *E. coli* (~83%), *S. typhi* (89%) to fall in the second group while the third group comprising the sole member *S. aureus*, represented the most sensitive counterpart as evident from an almost 98% drop in the viable cell count. To find the significant difference among colony forming unit of target bacteria against 25µg mL$^{-1}$, one way ANOVA was used which is statistically significant ($F_{6,98}$ = 76.14; $p$<0.001). S-N-K test as applied for the pair-wise group comparison with respect to growth inhibition of different bacteria against GLA (25 µg mL$^{-1}$) revealed highly significant pattern with respect to time interval (10 min) (Table 3).

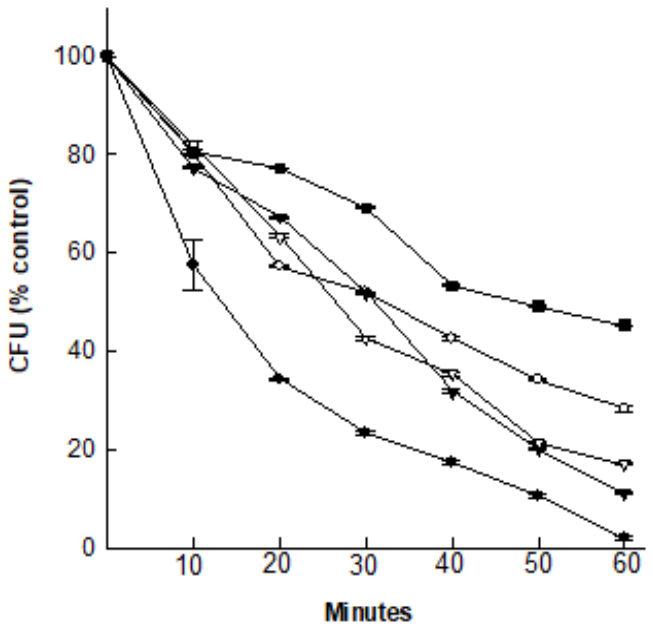


**Figure 4.** GLA (25 µg mL-1)-sensitivity of *S. aureus* (●-●), *P. aeruginosa* (o-o), *S. typhi* (▼ -▼), *E. coli* (∇-∇) and *E. aerogenes* (■--■) as a function of contact duration (0-60 min) (starter inoculum 107 cfu mL-1). One way ANOVA (F6,98 = 76.14; p<0.001).

**Biomass yield and GLA production with respect to phosphate, nitrate and temperature**

The present study evaluated the influence of phosphate and nitrogen concentration in the medium along with temperature on the production of biomass and GLA by *Nostoc* CCC537. Phosphate concentration was reduced to half (29 µM) and even doubled (116 µM) relative to the prescribed one (58 µM) for BG11 medium following 60 d of cyanobacterial growth (Figure 5a and 5b). The biomass yield decreased by 18.2% in 29 µM phosphate, and by 7.3% in 116 µM set relative to the prescribed concentration (58 µM). For 58 µM phosphate, GLA production reached 4.68 mg g$^{-1}$ dry wt but decreased to 3.72 mg g$^{-1}$ in 29 µM phosphate.

**Table 3.** Group comparison (*Student Newman*-Keuls test, q-values) for growth of target bacteria in *Nostoc* GLA (25 µg mL$^{-1}$) (based on Figure 4)

| TARGET STRAINS | 10 | 20 | 30 | 40 | 50 | 60 |
|---|---|---|---|---|---|---|
| *S. aureus vs. P. aeruginosa* | 11.6 *** | 97.8 *** | 110.7 *** | 104.3 *** | 90.1 *** | 71.4 *** |
| *S. aureus vs.S. typhi* | 10.2 *** | 140.7 *** | 110.0 *** | 59.3 *** | 36.7 *** | 24.6 *** |
| *S. aureus vs. E. coli* | 12.6 *** | 124.1 *** | 74.1 *** | 74.2 *** | 41.5 *** | 41.1 *** |
| *S. aureus vs. E. aerogenes* | 11.8 *** | 182.9 *** | 176.9 *** | 148.8 *** | 146.5 *** | 117.4 *** |
| *P. aeruginosa vs. S. typhi* | 1.41 | 42.9 *** | 0.64 | 45.0 *** | 53.2 *** | 46.8 *** |
| *P. aeruginosa vs. E.coli* | 1.05 | 26.3 *** | 36.5 *** | 30.1 *** | 48.3 *** | 30.3 *** |
| *P.aeruginosa vs. E. aerogenes* | 0.22 | 85.1 *** | 66.3 *** | 44.5 *** | 56.4 *** | 45.9 *** |
| *S. typhi vs. E coli* | 2.46 | 16.6 *** | 35.9 *** | 14.9 *** | 4.8 ** | 16.5 *** |
| *S. typhi vs .E. aerogenes* | 1. 63 | 42.2 *** | 66.9 *** | 89.5 *** | 109.8 *** | 92.8 *** |
| *E. coli vs. E. aerogenes* | 0.82 | 58.8 *** | 102.8 *** | 74.6 *** | 104.9 *** | 76.3 *** |

* significance level (*p < 0.05; **p < 0.01; ***p < 0.001)

However, GLA production enhanced to 5.64 mg g$^{-1}$ for 116 µM phosphate contray to biomass yield as stated above. Nitrate addition (5 or 10 mM) though stimulatory to cyanobacterial growth (16 or 21%) during 0-60 days, retarded GLA production more in 10 mM ((3.60 mg g$^{-1}$ dry wt) than in 5 mM nitrate (3.84

mg $g^{-1}$) (Figure 6a and 6b)). The data in overall indicated that combined N supply may favour only biomass production and not the GLA. Although the optimum temperature for *Nostoc* CCC537 is 20 °C but its isolation from "restricted environment" having low temperature led us to reduce it to 10 or 15 °C to monitor the biomass. Interestingly, decrease in growth temperature reduced the biomass yield by 47.8% and 17.4% respectively in contrast to 20°C (Table 4). The variations in inhibition zone size on lawn of *E. coli* resulting from 10 µl of *Nostoc* GLA from 10, 15 or 20 °C (control) sets also suggested the possible difference in the extent of GLA synthesis although the assessment is just qualitative (Table 4). The range of inhibition zone size was 17 mm, 14 mm and 4 mm for 10°, 15° and 20 °C set, respectively.

## DISCUSSION

Microalgae including cyanobacteria produce a broad spectrum of bioactive agents, and constitute the valuable resource of a wide array of lipids with varied potential applications.[18] The filamentous strains of cyanobacteria produce PUFA.[19] GLA from *Spirulina* is more expensive (4-6 times) than GLA from evening primrose.[20] However, GLA production in *Spirulina* could be manipulated up to 1.4% [21] 2.4% [22] and 2.0% [23]. In the present study, we identified another cyanobacterium, *Nostoc* CCC537 from the extreme environment (the Antarctica) as the source of GLA, and also monitored the role of N, P and temperature regimes on its production. The *Nostoc* GLA was effective against various

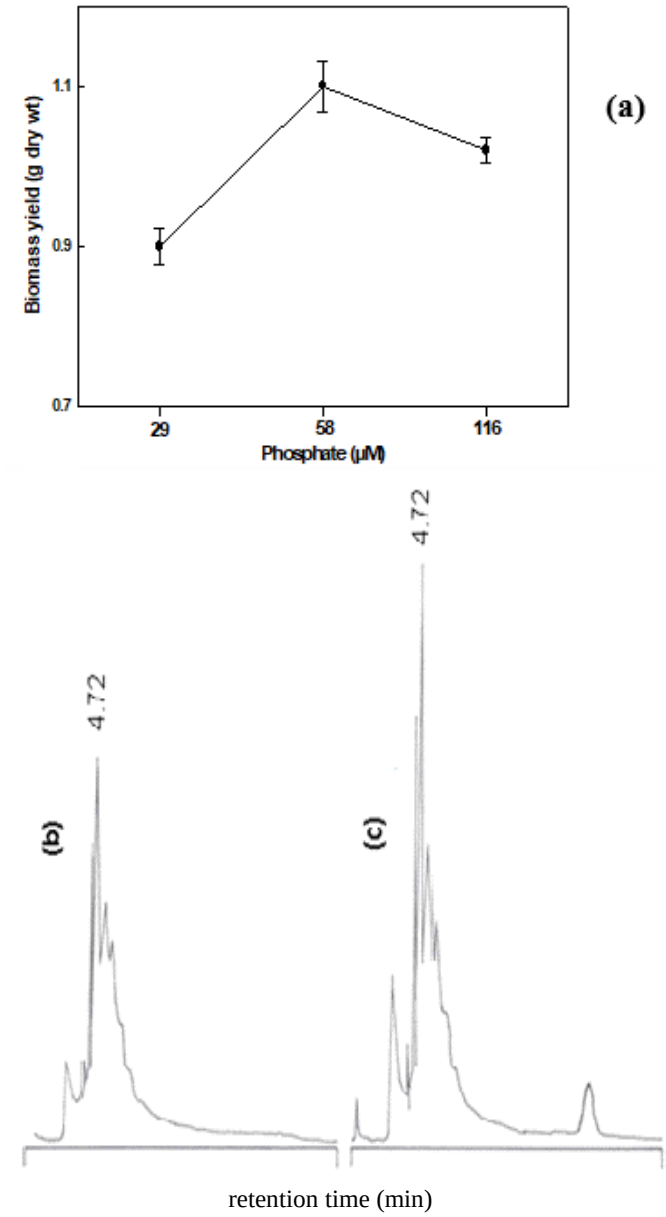


**Figure 5.** Biomass yield of *Nostoc* CCC537 in 29, 58 or 116 µM phosphate (a), HPLC profile of *Nostoc* CCC537 GLA(retention time 4.72 min) from cells grown in 29 µM (b) and 116 µM (c) phosphate Error bar denotes standard deviation.

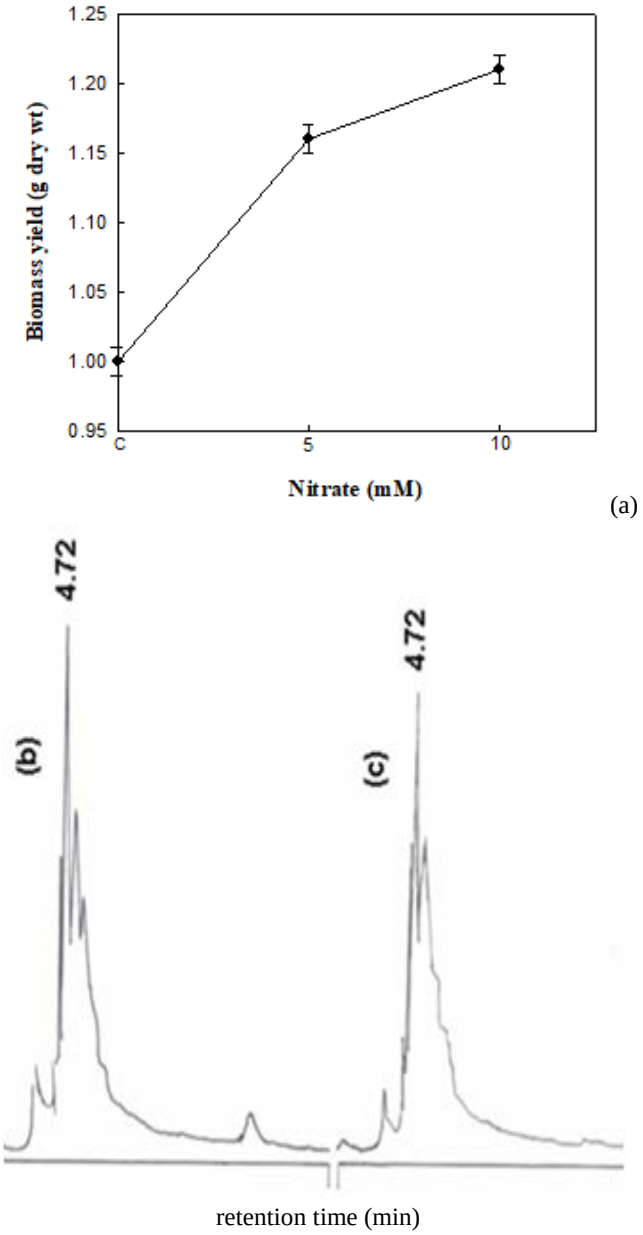


**Figure 6.** Biomass yield of *Nostoc* CCC537 in control (diazotrophic medium) and in, 5 or 10 mM nitrate (a) and HPLC profile of *Nostoc* GLA (retention time 4.72 min) from cells grown in 5 mM (b) and 10 mM (c) nitrate. Error bar denotes standard deviation.

pathogenic strains, isolated from noscomial infections. The target strain proved positive for GLA (Figure 1, 2), and the compound was active against both G (+) and G (-) bacterial strains in the sequence: *S. aureus* > *S. typhi* > *E. coli* > *P. aeruginosa* > *E. aerogenes* (Table 1). Therefore, *S. aureus*, a G (+) bacterium was the most sensitive against *Nostoc* GLA. In an analogous report, unsaturated fatty acids (including GLA) were antibacterial with MICs of 13.9-111.2 µg $ml^{-1}$ (0.05-0.4 mM) against G(+) bacteria, *S. aureus* and *S. pyrogens*, but ineffective against the G(-) *E. coli* and *P. aeruginosa*.[24] It is evident that GLA inhibition was concentration-dependent (Figure 3). Also, the time kill assay confirmed that bactericidal property of GLA depended on duration of contact (Figure 4). Giamarellos-Bourboulis *et al*.[5] deployed forty-two *E. coli* strains no other bacteria as used in the present work, to determine *in vitro* inhibitory activity of GLA and its influence on their susceptibilities to various antimicrobial agents. The GLA concentrations were relatively higher i.e., 100, 200 or 300 µg $mL^{-1}$. Lethality was restricted to 9.5 and 33.7% of strains at 50 and 300 µg $mL^{-1}$. Interestingly, *E. coli* strains recovered after GLA exposure also raised their MICs four-fold against many antimicrobials and most of these involved aminoglycosides. Moreover, the GLA was obtained from Sigma, while we have used cyanobacterium as the GLA source. Similar experiments extended to nineteen *P. aeruginosa* strains revealed lethality in 5-10% strains during 24h of GLA (300 µg $mL^{-1}$) exposure.[6] The survivors became resistant to aminoglycosides and ß-lactams. We also compared the potential of *Nostoc* GLA along-with the antibiotics rifampicin and streptomycin against the most resistant bacterium, *E. aerogenes.* Streptomycin, the aminoglycoside, is effective against staphylococcal and enterococcal infections, while rifampicin is the broad-spectrum antibiotics affecting RNA polymerase binding. As higher doses of both the antibiotics (45 µg $mL^{-1}$) produced smaller inhibition zones while the lower dose (30 µg $mL^{-1}$) was non-inhibitory to growth of the test bacterium (*E. aerogenes*), thus indicating superiority of *Nostoc* GLA in comparison. This potential was ascertained from a doubling of the MIC (16 µg $mL^{-1}$) against the same bacterium compared to just 8 µg $mL^{-1}$ for *Nostoc* GLA (Table 1).

*Nostoc* CCC537, as the GLA resource can only be used in future provided the optimization of its production is secured. As the cyanobacterium grew optimally at 20° C (specific growth rate, $0.0128h^{-1}$), all the experiments were based on the biomass produced at 20° C. There are many studies on GLA overproduction in *Spirulina* sp. through manipulations in the nutrient regimes and also temperature.[25,26] GLA overproduction was also reported by mutating the *Spirulina* sp.[22,27] Recently, GLA content increased up to 2% (w/w) in *S. platensis* by using fatty acid supplement and light-dark illumination.[23] We observed decrease in the biomass production in *Nostoc* CCC537 in half the prescribed phosphate (58 µM) concentration in BG-11, but a doubling to 116 $\mu$M promoted GLA production 1.2-fold (Figure 5). This suggests the scope for manipulating the phosphate concentration if biomass production is not the actual goal. Increase in the nitrate concentration (5 or 10mM) stimulated biomass production (1.16 -1.21 fold) contrary to GLA production (Figure 6). *Nostoc* CCC537 grown at lower temperatures (10 or 15° C) than the routine one (20° C), had the biomass yield decreased by 47.8% or 17.4% at respective low temperatures (Table 4). We have also isolated GLA from the cyanobacterium growing at the above-mentioned temperatures and adopting the equal biomass for GLA extraction. The equal volume (10µL) from each temperature set was subjected to antibacterial assay. The inhibition zone size on lawn of test bacterium clearly indicated although indirectly, higher GLA content for cells grown at 10° C (Table 4). Thus, lowering in the growth temperature could have the important role in promoting GLA production irrespective of biomass production. Our observations are in tune with other workers *albeit* in *Spirulina* sp. that temperature was most crucial in optimizing the GLA rather than nitrate with the negative effect.[12, 28] By these workers it was also inferred that interaction of both the factors (nitrate concentration and temperature) were significant in production of GLA rather than in isolation. Colla *et al.* (2007) also reported that sodium nitrate concentration in the growth medium reduced to 1/4th (0.625g $L^{-1}$) favoured maximum GLA production in *Spirulina* sp.[28] Piorreck *et al.* (1984) also observed that the cyanobacterial total lipid content remained unchanged for the nitrate concentrations tested (0.001-1%).[29] According to Cohen et al. (1987), the varying N levels cannot be the reliable means of manipulation of lipid and fatty acid content in cyanobacteria.[21] The increase in GLA content in *Spirulina* sp. was also observed under the nitrogen-deficient growth medium.[30]

**Table 4.** Biomass yield and GLA production in *Nostoc* CCC537 at different temperatures and antibacterial assay against *E. coli* using 10 µl of eluate obtained from each sample.

| Temperature (°C) | Biomass yield (g $L^{-1}$) | GLA (mg $g^{-1}$ dry wt.) | Inhibition Zone dia. (mm) |
|---|---|---|---|
| 10 | 4.69 | 7.02 | 17 |
| 15 | 9.0 | 5.85 | 14 |
| 20 | 10.9 | 4.68 | 4 |

Low temperatures induce desaturation in fatty acids of glycolipids in the biological membranes.[8] Desnium et al. (2000) observed enhanced expression and mRNA stability of the desD gene (Delta6) in *S. platensis* strain C1, responsible for the introduction of a third double bond into fatty acids by a temperature shift from 35 to 22° C, but not the shift from 35 to 40° C.[31] The acclimatization to low temperatures induced both unsaturation of fatty acids in the membrane and also a number of metabolic modifications.[32] Therefore, the very survival of *Nostoc* CCC537 at low temperatures is attributed to the increased desaturation of fatty acids.[33]

In conclusion, we demonstrated that *Nostoc* CCC537 produced GLA more in high phosphate concentration (116 µM) and at low temperature (10° C). The increased nitrate concentrations (5 or 10mM) did not favour GLA production. The Antarctic *Nostoc* CCC537 can thus be taken as the ideal source of candidate gene (s)

for extremozyme(s) to be deployed in biotechnological applications. Although GLA content was *Nostoc* CCC537 7.02 mg $g^{-1}$ dry wt. for cells grown at 10° C (0.7%), the value is very close to *Spirulina* sp. (1-1.2%). However, there is a promise for GLA overproduction through manipulation of nutrient levels and the growth temperature regimes.

## EXPERIMENTAL SECTION

### Materials and Methods

### Organism and growth conditions

The Antarctic strain *Nostoc* CCC537 (Centre for Conservation and Utilization of Blue Green Algae, Indian Agriculture Research Institute, New Delhi- 110012) a kind gift from PA Broady, NewZealand) was grown in Chu-10 medium[34] lacking combined N-source with periodic shaking and continuous illumination (tungsten + fluorescent, 20.1µmol photons $m^{-2}$ $s^{-1}$) at 20±1 ºC, and specific growth rate calculated according to the Kratz and Myers (1955).[35] Purity of the culture was routinely checked by transferring aliquots to Luria Broth with incubation in dark at 37 ºC (24 h). As the organism was slow-growing, 60 d old batch cultures (10 g fresh wt $L^{-1}$) were processed. The cyanobacterium was grown in 2L capacity conical flasks (working volume, 1.5L). The pelleted biomass from culture (1L) was rinsed twice with deionized (sterile) water and lyophilized. In order to know the GLA level for different phosphate, nitrate or temperature regimes, the cyanobacterium was grown in the medium supplemented with half (29 µM), prescribed (58 µM) or double (116 µM) phosphate concentration. As the strain is diazotrophic, the nitrate lacking set was the control while nitrate ($KNO_3$) level was maintained at 5 mM or 10 mM. The cyanobacterium was also grown at 10º, 15º and 20 º C for monitoring the GLA production.

### Extraction of liquid

Lipids from the lyophilized cyanobacterial biomass (1g) was extracted using chloroform: methanol (1:2) as prescribed by Bligh and Dyer.[36] The chloroform layers were pooled and subjected to a "Folch wash" to remove all non-lipid contaminants.[37] The mixture was washed with one-fourth of the volume of 0.88% (wt/v) potassium chloride, followed by methanol/saline solution (1:1, v/v). The purified chloroform extract was then evaporated under room temperature and total lipid content was then converted to Fatty Acid Methyl Esters (FAMEs). FAME of the extracted lipid and standard GLA were formed by heating (80 °C, 2 h) in MeOH:HCl (10:1) and extracted in hexane.[38] The FAME of both the extracted GLA and standard (100 µg $mL^{-1}$) was applied to the TLC plate (Merck Silica Gel-60) with solvent system hexane:diethylether:acetic acid (60:40:1, v:v), and visualized in iodine vapour. The spot-on TLC plate parallel to the GLA (standard) lane (same $R_f$) was eluted in methanol (1 mL) for further use.

### Identification and quantification of GLA

The identification and quantitative measurement of FAME was done by the modified method of Mansour (2005), in Waters Associate model 501, HPLC using a 25 cm x 4.6 mm Biophase ODS 5 µm particle reverse phase column ($C_{18}$). The mobile phase consisted of methanol and 1mM phosphate buffer (pH 7.4) (95:5, v/v) with flow rate of 1mL $min^{-1}$. The fraction containing GLA was collected at 4.72 min and the amount calculated using the peak area relative to GLA standard.

### Antibacterial assay

GLA extract (non-methylated) was bio-assayed against *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC25992, *Salmonella typhi* MTCC3216 (IMTECH, India), *Pseudomonas aeruginosa* ATCC27853 and *Enterobacter aerogenes* MTCC2822 using slightly modified disc diffusion method of Bauer et al. on 3.8% Mueller-Hinton (MH) agar.[39] The inoculum from Luria Bertani (LB) grown cells (37 °C, 18 h) was suspended in 0.85% NaCl and turbidity adjusted to $10^8$ cfu $mL^{-1}$ corresponded to 0.5 Mac Farland standard according to National Committee on Clinical Laboratory Standards (NCCLS) now Clinical and Laboratory Standards Institute (CLSI).[40] The cell suspension in each case, was inoculated onto MH agar with the help of sterile, non-toxic cotton swab and incubated (37 °C, 20 min). GLA (10 µl, 25 µg $mL^{-1}$) was spotted on plates keeping standard as positive and methanol as negative control, and incubated (37 °C, 18 h). Antibacterial potential assay is based on the inhibition zone size (dia. in mm). Potency equivalence test was also conducted by keeping the most resistant test strain (*E. aerogenes*) as the target and adopting 25, 30 and 45 µg $mL^{-1}$ of antibiotics (rifampicin and streptomycin) along with GLA (derived from the cyanobacterium).

### Minimum inhibitory concentrations (MIC) determination

MICs were determined according to the standard methods of NCCLS (1997). Mueller-Hinton agar plates with the GLA extract (0.002- 512 µg $mL^{-1}$) were spotted with the test bacterial inocula ($10^7$ cfu $mL^{-1}$) in normal saline (0.85%), and incubated (37 °C, 20 h). The lowest GLA concentration that did not permit bacterial growth, was taken as the MIC.

### Time- kill assay

The time-kill assay involved change in the viable cell count of bacterial targets exposed to selected GLA concentrations. *S. aureus* (most sensitive) was adopted to evaluate the bactericidal potential of GLA in reference (10, 20, and 30 µg $mL^{-1}$) during common contact duration (0-120 min) along with GLA-lacking control. A comparison was made for the viable cell counts during liquid contact duration (0-60 min) of *S. aureus E. coli, S. typhi, P. aeruginosa* or *E. aerogenes* with the native GLA at a common dose (25 µg $mL^{-1}$ ). Exponential phase bacterial cells ( $10^7$ cfu $mL^{-1}$) were diluted in fresh nutrient broth to monitor the cell number (expressed as percentage control). Control sets had 5µl of methanol as it was used to make the GLA solution. Aliquot (100 µl) was removed at various time intervals and diluted with 10 mL sterile broth. The cells from such broth were inoculated onto petri plates (3.8% Mueller-Hinton agar) and incubated (37 °C, 24 h) to account for the cfu.

### 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 has been used to test the significant difference among the mean level of bacterial count at different time interval, However,S-N-K test has been used in multiple comparison to find out the pair-wise significant

difference. All the experiments were carried out in triplicate with standard deviation (SD) represented as bar.

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