Research Paper

Evaluation of fungicidal activity of extracellular filtrates of cyanobacteria – possible role of hydrolytic enzymes

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A set of seventy axenised and unicyanobacterial isolates belonging to the genus *Anabaena* were evaluated for biocidal activity against a set of phytopathogenic fungi. Among them, 35 *Anabaena* strains showed zone of inhibition against one or more fungi. The extracellular filtrates from 4 and 8 weeks old cultures of these *Anabaena* strains were further evaluated in terms of hydrolytic enzymes, proteins and IAA employing standard methods. Significant differences were also observed among the strains in terms of their FPase, chitosanase and xylanase activity, while low and relatively similar values of CMCase, cellobiase and protease activity were recorded in the strains analyzed. IAA production was also observed in all the strains. Comparative evaluation of activity of hydrolytic enzymes and antifungal activity revealed that such enzymes may contribute to the fungicidal activity of the cyanobacterial strains, besides other bioactive compounds, including IAA, which are established promising traits for biocontrol agents. This study is a first time report on the production of hydrolytic enzymes by these oxygenic photosynthetic prokaryotes, which can be potential candidates for the development of biocontrol agent(s) against selected phytopathogenic fungi.

Keywords: Cyanobacteria / Fungicidal activity / Hydrolytic enzymes / Phytopathogenic fungi / IAA

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Introduction

Cyanobacteria represent a small taxonomic group of prokaryotes, which are equipped with the power to harm and help plants, animals and humankind, and possess a tremendous potential for producing a wide range of secondary metabolites [1]. It was in the 1930s that De [2] pointed out that the preponderance of cyanobacteria was responsible for the inherent and sustained fertility of rice fields which led to the cyanobacterial populations in soil being evaluated in terms of their diversity and utility as biofertilizers, not only for rice, but for other crops including wheat [3–5]. Cyanobacteria are also found commonly growing as blooms in eutrophic lakes, reservoirs and as floating assemblages in marine ecosystems. Such blooms have been notorious and associated with the production of toxins/allelopathic compounds, which provide a competitive advantage to these organisms [6–9]. This is one of the critical factors responsible for their abundance in diverse environments especially eutrophic water bodies. However, the chemical potential of these ubiquitous prokaryotes, widely distributed in diverse soil types, aquatic environments and ecologies, in terms of production of metabolites has not been much investigated.

Agriculture requires the extensive use of chemical pesticides to protect crops against pests and diseases. Several of these chemicals pollute our ground water and drinking water and therefore some governments have decided to reduce these chemical inputs substantially. This urges the need for alternative crop protectants. One of these alternatives is the use of biological control agents, among which are microorganisms that can protect the plants against diseases [10]. The coloni-

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zation and defensive retention of rhizosphere niche by microorganisms is enabled by the production of allelochemicals, antibiotics, biocidal volatiles and lytic/detoxification enzymes.

Terrestrial cyanobacteria such as *Anabaena laxa* are known to produce antifungal cyclic peptides such as laxaphycin A and B [11]. Casamatta and Wickstrom [12] reported that the exudates of *Microcystis aeruginosa* were inhibitory towards bacterial plankton communities. A number of nucleosides – tubercidin, toyocamycin and their corresponding derivatives isolated from Scytonemataceae members were observed to be toxic towards *Aspergillus oryzae, Candida albicans, Penicillium notatum* and *Saccharomyces cerevisiae* [13]. A diverse range of compounds are also known to exhibit a bioregulatory role as a result of their cytotoxic, immunosuppressive and enzyme-inhibiting activities which are of tremendous pharmaceutical significance [14, 15]. However, the role of hydrolytic enzymes, which are implicated in fungicidal activity of several biocontrol strains, has not been explored in these photosynthetic prokaryotes.

The present investigation describes the characterization of cyanobacterial metabolites from a set of *Anabaena* strains exhibiting biocidal activity, as a prelude to their utilization as biocontrol agent(s) in agriculture.

Materials and methods

Organisms

The available germplasm of seventy cyanobacterial strains, belonging to the genus *Anabaena* (isolates from rice fields, *Azolla* fronds and water bodies [5]) was screened for the biocidal activity against selected phytopathogenic fungi obtained from the Indian Fungal Type Culture Collection, Division of Plant Pathology, IARI, New Delhi.

Growth and maintenance

The cyanobacterial strains were axenised by standard procedures employing a set of antibiotics [16]. Such cultures were used for further work, after repeated sub culturing and grown under L: D (light: dark cycles – 16:18), white light (50–55 μmol photons $m^{-2} s^{-1}$) and 29 ± 1 °C in nitrogen free BG-11 medium [17] in Haffkine flasks. Potato dextrose medium was used for the growth and maintenance of the fungal cultures used. Incubation was done at 30 °C in a BOD incubator.

Screening of the cultures for biocidal activity

All the seventy strains were tested for fungicidal activity utilizing double layer technique to obtain a uniform lawn of test organisms. Sterile filter paper discs of 5 mm diameter soaked in 100 μl filtrates (0.22 μm filter drawn) of cyanobacterial cultures (4/8 weeks old) were placed on the lawn of test organism. The phytopathogenic fungi, causing diseases of various crops, were used as test organisms. The fungus to be tested against was placed in the center as a disc; inhibition zone was measured around each well after incubation period of 7–8 d at 30 °C. The presence of inhibition zone was taken as positive indication, and the diameter of the zones measured. Antifungal sensitivity discs containing Nystatin (100 Units) obtained from Hi Media Laboratories, India were used as standard. The culture filtrates were also partitioned using two solvents – Ethyl Acetate and Dichloromethane and sterile discs soaked in the aqueous phase were placed in a similar manner for evaluating the water soluble nature of the toxic component of the filtrate.

Total proteins

The amount of proteins was determined spectrophotometrically according to [18] with Bovine serum albumin (BSA) as standard.

Chitosanase activity

The cell free culture filtrates were analysed for chitosanase activity (EC 3.2.1.4) by the spectrophotometric method using glycol chitosan as the assay substrate. One unit of chitosanase activity was defined as micromoles of N-Acetyl glucosamine released per min under the assay conditions [19].

Protease activity

Protease activity was assayed using casein as a substrate and measured by liberation of tyrosine from the incubation mixture [20]. The intensity of blue color was measured at 660 nm using spectrophotometer against the standard curve of Tyrosine, and expressed as International unit (IU), which was calculated as μmoles of tyrosine liberated min^{-1}.

Filter paperase/FPase (exo-*β*-1, 4-glucanase; EC 3.2.1.91) and CMCase activity (EC 3.2.1.4)

The enzyme activities of both enzymes were assayed spectrophotometrically using filter paper and carboxymethyl cellulose as substrates respectively [21]. Reducing sugars liberated were estimated at 575 nm against the standard curve of Glucose. One unit of enzyme represents I μmole of glucose liberated per ml of culture filtrate per min.

Xylanase activity

The xylanase activity was measured spectrophotometrically at 575 nm using xylan as substrate [22] against the

standard curve of xylose. One unit of xylanase activity represents I μmole of xylose liberated per ml of culture filtrate per min.

Cellobiase (*β*-D-glucosidase; EC 3.2.1.21) activity

The cellobiase activity was determined spectrophotometrically at 430 nm by the method outlined by [23] against the standard curve of p-nitrophenol.

IAA activity

IAA activity was measured by the method described by [24]. The intensity of pink color was measured at 530 nm and values were compared with those of the standard curve of IAA.

The activity of all the hydrolytic enzymes is expressed as standard International Unit (IU). The culture filtrates of the cultures of the cyanobacterial strains were also scanned in the UV-VIS range using Specord model spectrophotometer, and the peaks recorded for identification of novel compounds.

Statistical analyses

The data recorded in triplicate for the parameters in various strains was subjected to ANOVA (analysis of variance) in accordance with the experimental design (completely randomized block design) using MSTAT-C statistical package to quantify and evaluate the sources of variation. Duncan's Multiple Range Test (DMRT) was employed to compare the mean performances of different strains for the specific parameters under study and the rankings are denoted by superscripts in the relevant tables.

Results and discussion

Soil microorganisms are known to be an important determinant of allelopathic activity, besides leading to severe monetary losses as a result of soil borne pathogens of vegetables, fruits and field crops. Among them, fungal pathogens are a major problem in agriculture, as most of the fungicides employed exhibit lower potency under field conditions and have been a source of chemical pollution, poisoning the fruits and vegetables, which form an essential component of human diet.

Cyanobacteria are known to produce a large number of compounds with varying bioactivities, including the hepato- and neurotoxins, which are a cause for much concern in aquatic environments [6]. Extensive screening programs for bioactive molecules in these organisms have revealed the presence of protease inhibitors, cytotoxins to human and animal cells and compounds with antibacterial, antifungal and antiviral properties [25]. Despite the availability of a few patents [26] the industrial exploitation of cyanotoxins as fungicidal agents is limited and a need exists for identification of novel metabolites for developing commercial products. In this context, it would be valuable to analyse the bioactive compounds/enzymes from cyanobacteria for a better understanding of the mechanism of fungicidal activity.

Preliminary analyses of the culture filtrates of a set of 70 cyanobacterial strains against the selected phytopathogenic fungi (as measured by disc diffusion assay) revealed that only 35 strains produced a zone of inhibition of varying diameter, ranging from 10–20 mm, and the average size of the zone was measured as 10 mm (Fig. 1, Table 1). Comparative evaluation of filtrates from 4 and 8 weeks cultures (data not shown) had shown that the latter stage produced larger zones of inhibition. It is well known that biocidal compounds are secondary metabolites, produced at late log-stationary phase stage (more than 3 weeks of growth); hence, further evaluation was done with 8 weeks old culture filtrates. Among these, 23 strains of *Anabaena* inhibited the growth of *Fusarium moniliforme* while 17 strains inhibited *Alternaria solani* and *Aspergillus candida* was inhibited by 15 strains. Three strains each inhibited the growth of *Drechslera oryzae* and *Pythium aphanidermatum.* Three cyanobacterial strains – RP 8, 9 and 69 exhibited a broad spectrum of activity, inhibiting the growth of six phytopathogenic fungi. Strains RP 1

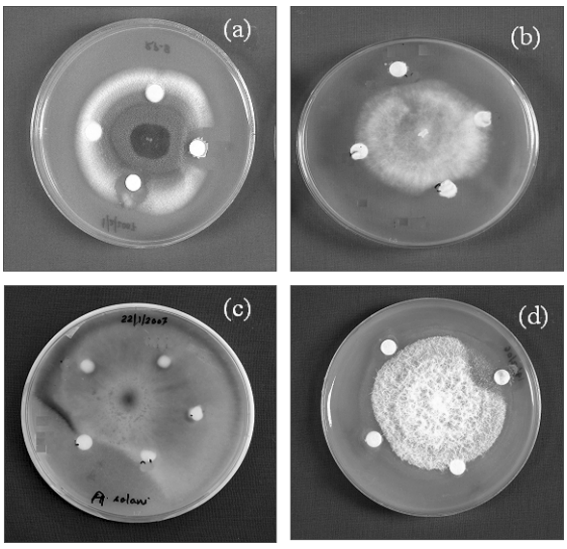

Figure 1. Zone of inhibition by filtrates of cyanobacterial cultures on the lawn of selected phytopathogenic fungi. (a) *Aspergillus candida* (b) *Pythium aphanidermatum* (c) *Alternaria solani* (d) *Fusarium solani*.

Table 1. Fungicidal activity of cyanobacterial culture filtrates in terms of zone of inhibition (values in parentheses indicate the diameter of inhibition zone (mm).

Anabaena strains	Phytopathogenic fungi tested						
	Macrophomina phaseolina	*Fusarium monoliforme*	*Alternaria solani*	*Pythium aphanidermatum*	Aspergillus *candida*	*Drechslera oryzae*	*Fusarium solani*
RP 1	–	+ (20)	+ (10)	–	–	–	+ (16)
RP 3	–	+ (11)	+ (11)	–	+ (10)	–	–
RP 4		+ (12)	+ (10)	–	+ (10)	–	+ (10)
RP 5	–	–	+ (10)	–	+ (10)	–	+ (13)
RP 6	+ (10)	+ (11)	+ (11)	–	+ (10)	–	+ (11)
RP 7	–	+ (11)	+ (10)	–	–	–	+ (10)
RP 8	–	+ (12)	+ (11)	–	+ (10)	–	+ (10)
RP 9	–	+ (12)	+ (11)	+ (12)	+ (10)	–	–
RP 11	–	–	+ (10)	–	–	–	–
RP 12	–	+ (11)	–	–	–	–	–
RP 14	–	+ (13)	–	–	–	–	+ (11)
RP 16	–	+ (10)	+ (11)	–	–	–	–
RP 17	–	–	–	–	–	–	–
RP 20	–	–	–	–	–	–	–
RP 21	–	–	–	–	–	–	+ (10)
RP 25	–	–	–	–	+ (10)	+ (10)	–
RP 26	–	–	+ (27)	–	+ (12)	+ (10)	–
RP 33	–	–	–	+ (12)	+ (14)	–	–
RP 34	–	+ (12)	–	+ (14)	–	–	–
RP 35	–	–	–	–	–	–	–
RP 45	–	+ (10)	–	–	–	–	+ (10)
RP 47	–	–	+ (10)	–	–	–	–
RP 49	–	+ (10)	+ (10)	–	+ (10)	–	–
RP 50	–	+ (16)	+ (10)	–	+ (12)	–	–
RP 52	–	+ (13)	+ (12)	–	–	–	–
RP 53	–	+ (12)	+ (28)	–	+ (10)	–	+ (14)
RP 56	–	+ (14)	–	–	–	–	–
RP 57	–	+ (10)	–	–	+ (10)	–	–
RP 58	–	–	–	–	–	–	–
RP 59	–	+ (10)	+ (40)	–	–	–	+ (10)
RP 63	–	+ (10)	–	–	–	–	+ (10)
RP 68	–	+ (11)	–	–	–	–	+ (11)
RP 69	–	+ (12)	–	–	+ (10)	+ (10)	+ (11)
RP 70	–	–	–	–		–	+ (11)
RP 71	–	+ (10)	–	–	+ (12)	–	+ (11)
Nystatin	+ (16)	+ (30)	+ (20)	+ (14)	+ (14)	+ (16)	+ (16)

and 59 were observed to be highly potent in terms of their fungicidal activity, as they produced a much larger zone of inhibition than those of standard antifungal agent – nystatin. The largest diameter of zone of inhibition of 40 mm was recorded by strain RP 59, followed by RP 53 (28 mm) against *Alternaria solani.* Other phytopathogenic fungi – *Pythium debaryanum, Rhizopus oryzae, Fusarium oxysporum pisi, Ustilago tritici, Fusarium graminearum, Rhizoctonia solani, Drechslera sorkiniana, Sclerotium oryzae* and *Alternaria triticiana* were also tested, however, no inhibition zone was observed.

The basis of antibiosis as a biocontrol mechanism has become increasingly better understood over the past two decades [27, 28]. A variety of antibiotics have been identified, including compounds such as amphisin, 2,4-di acetylphloroglucinol (DAPG), hydrogen cyanide, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, troplone, and cylic lipopeptides produced by pseudomonads, and oligomycin A, kanosamine, zwittermicin A, Xanthobaccin produced by *Bacillus* and *Stenotrophomonas* spp. [29]. However, a variety of microorganisms also exhibit hyperparasitic activity, attacking pathogens by excreting cell wall hydrolases, which has been a subject of intensive research in recent years. Lim and coworkers [30] isolated a strain of *Psuedomonas stutzeri* that produced extracellular chitosanase and laminarase, which could digest the mycelia of *Fusarium solani.* However, cyanobacteria are generally believed to

be obligate phototrophs, not requiring other C sources for their growth; therefore, no focused efforts have been undertaken on production of hydrolytic enzymes.

The filtrates from four and eight weeks old cultures were analyzed in terms of proteins, IAA and hydrolytic enzymes. A two-fold enhancement was observed in terms of proteins in the filtrates from 8 weeks old cultures, as compared to four weeks old cultures, indicative of release of proteinaceous molecules by the cells (Table 2). Extensive screening programs for bioactive compounds in cyanobacteria has revealed the presence of unique peptides with cyclic structures (cyanopeptolins, depsipeptides), besides linear peptides isolated from a number of strains belonging to genera – *Microcystis*, *Nostoc* and *Anabaena* [25]. The analyses of the culture filtrates are currently in progress as UV scans revealed the presence of novel compounds. Although toxins were the main subject of research on toxic cyanobacterial blooms, isolation of bioactive compounds other than biotoxins has now been recently stressed [1].

The production of phytohormones, earlier considered, as a trait of the plant kingdom is also widespread among soil and plant associated prokaryotes [31], especially those involved in plant microbe symbiotic or associative interactions or plant pathogenesis. Microorganisms inhabiting rhizosphere of various plants are likely to synthesize and release auxin as secondary metabolite because of rich supplies for substrates exuded from the roots compared with non-rhizosphere soils. IAA production was also observed in the cyanobacterial strains, which enhanced several folds with increasing age of the culture. Strains RP 53, 57 and 68 exhibited a 7–10 folds increase, indicative of the possible role in their interactions with the fungal pathogens. It has been suggested up to 80% of bacteria isolated from rhizosphere can produce IAA. The capacity for IAA biosynthesis was found in representative of free living and symbiotic cyanobacteria of the genera *Nostoc*, *Chlorogloeopsis*, *Calothrix*, *Plectonema*, *Gloeothece*, *Anabaena*, *Cylindrospermum* and *Anabaenopsis* [32, 33]. The tryptophan independent pathway, more common in plants, is also found in microorganisms – *Azospirilla* [31] and cyanobacteria [33]. Out of 34 free-living and symbiotically competent cyanobacterial strains, auxin like substances were detected in 38% of the free living and 83% of symbiotic isolates in the presence and absence of tryptophan. The endogenous accumulation of IAA was confirmed with immunological techniques (ELISA) and GC-MS techniques. Genes involved in IAA production via indole pyruvic acid pathway, such as *ipd*C, has also been sequenced in *Nostoc* PCC 73102 strain [33].

Table 2. Protein accumulation and IAA production in cyanobacterial strains at different stages of growth (4 and 8 weeks old cultures)*.

Strain no.	Protein (μg ml^{-1})		IAA (μg ml^{-1})	
	4 weeks	8 weeks	4 weeks	8 weeks
RP 1	80.00^{1}	130.00^{17}	0.361^{25}	1.920^{19}
RP 3	66.00^{3}	140.00^{16}	0.421^{24}	5.440^{8}
RP 4	31.00^{11}	190.00^{10}	0.532^{23}	4.200^{11}
RP 5	12.00^{19}	170.00^{13}	0.562^{23}	3.451^{14}
RP 6	14.00^{18}	143.00^{15}	1.690^{18}	1.056^{23}
RP 7	15.00^{18}	120.00^{19}	2.394^{14}	1.573^{21}
RP 8	20.00^{16}	220.00^{9}	2.441^{13}	1.769^{20}
RP 9	12.00^{19}	128.00^{18}	0.367^{25}	3.216^{15}
RP 11	8.00^{20}	410.00^{1}	1.173^{22}	3.440^{14}
RP 12	75.00^{2}	380.00^{3}	3.521^{6}	2.060^{18}
RP 14	4.00^{20}	280.00^{5}	3.286^{7}	2.580^{16}
RP 16	8.00^{20}	230.00^{6}	3.612^{4}	1.948^{19}
RP 17	42.00^{5}	171.00^{13}	2.582^{11}	0.915^{24}
RP 20	8.00^{20}	34.00^{25}	3.099^{8}	0.993^{23}
RP 21	26.00^{12}	400.00^{2}	3.560^{5}	1.364^{22}
RP 25	38.00^{8}	210.00^{8}	2.113^{15}	3.180^{13}
RP 26	62.00^{4}	220.00^{7}	0.113^{27}	1.900^{19}
RP 33	23.00^{15}	50.00^{24}	2.488^{12}	3.256^{15}
RP 34	25.00^{13}	192.00^{9}	1.314^{21}	6.550^{6}
RP 35	2.00^{22}	130.00^{17}	3.099^{8}	3.239^{15}
RP 45	7.00^{20}	60.00^{23}	3.897^{3}	0.191^{26}
RP 47	15.00^{18}	27.00^{27}	2.911^{10}	4.343^{10}
RP 49	25.00^{13}	67.00^{22}	3.568^{5}	4.250^{11}
RP 50	37.00^{9}	76.00^{21}	3.850^{3}	5.850^{7}
RP 52	7.00^{20}	34.00^{25}	3.005^{9}	7.230^{5}
RP 53	39.00^{7}	150.00^{14}	0.282^{26}	7.980^{3}
RP 56	19.00^{17}	100.00^{20}	3.239^{7}	2.448^{17}
RP 57	31.00^{11}	210.00^{8}	1.972^{16}	10.78^{2}
RP 58	35.00^{10}	310.00^{4}	4.319^{2}	5.378^{8}
RP 59	43.00^{5}	180.00^{12}	4.507^{1}	0.375^{25}
RP 63	40.00^{6}	182.00^{11}	4.534^{1}	2.410^{17}
RP 68	32.00^{11}	140.00^{16}	1.549^{19}	11.20^{1}
RP 69	26.00^{12}	75.00^{21}	1.455^{20}	3.992^{12}
RP 70	24.00^{14}	400.00^{2}	1.831^{17}	7.840^{4}
RP 71	20.00^{16}	30.00^{26}	2.394^{14}	5.188^{9}
SEM	0.820	0.816	0.0025	0.0365

* Superscripts denote DMRT ranking in descending order

In our investigation it was interesting to observe that the cyanobacterial strains exhibiting fungicidal activity produced one or more hydrolytic enzymes. The hydrolytic enzymes such as protease, CMCase, Cellobiase and Fpase also showed a wide range of values in the cyanobacterial strains tested (Table 3). The activity of CMCase and FPase enzymes were enhanced in 8 weeks old cultures. Protease activity in the culture filtrates of the 4 weeks old cultures ranged from 0.01–0.362 Uml^{-1}, which however dropped to 0.001–0.012 Uml^{-1} in 8 weeks old cultures. A similar pattern was observed for cellobiase activity, which was in general, very low. Chitosanase activity was remarkably high in all the strains, especially at 4 weeks stage, with values ranging from 0.040 to 0.482 Uml^{-1} (Fig. 2). Xylanase activity

showed an almost two folds enhancement in activity in eight weeks old culture filtrates (Fig. 3). Strains RP 69, 70 and 71 were observed to show much higher values for these parameters, especially in the 4 weeks old cultures, however, strains RP 9 and 45 were the highest ranked strains, when 8 weeks old cultures were analysed.

On analyzing the overall performance of the strains in terms of biocontrol traits, strain RP 1 was observed to be among the five top ranked strains in terms of diameter of zone of inhibition and chitosanase activity, while RP 9 was top ranked in terms of chitosanase activity, besides exhibiting high levels of activity of other hydrolytic enzymes and inhibited the growth of five out of seven fungi tested. The top ranked strains in terms of their hydrolytic enzyme activity were compared with respect to the antifungal activity (inhibition zone) shown by culture filtrates and aqueous phase after partitioning with Ethyl acetate and Dichloromethane as solvents, which revealed the significant role of the enzymes.

The excretion of hydrolytic enzymes is known to be a common trait of plant pathogens/symbionts, which promotes a closer association with plant roots/target organisms and improve the stability of such associations. Chitosanases are known to selectively degrade chitosan/chitin by hydrolysis of the β-1, 4-glycosidic bonds that link N-acetyl glucosamine residues of chitin and form the basis for antifungal activity. The chitosanase/chitinase produced by *S. plymuthica* C48 inhibited

Table 3. Screening the activity of selected hydrolytic enzymes in the 4 and 8 weeks old culture filtrates of cyanobacterial strains*.

Strain no.	Protease (IU uml^{-1})		CMCase (IU ml^{-1})		Cellobiase (IU ml^{-1})		FPase (IU ml^{-1})	
	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks
RP 1	0.263^{2}	0.005^{1}	0.007^{1}	0.035^{3}	0.008^{2}	0.003^{1}	0.032^{4}	0.102^{10}
RP 3	1.121^{1}	0.004^{1}	0.010^{1}	0.067^{2}	0.008^{2}	0.004^{1}	0.037^{4}	0.126^{8}
RP 4	0.298^{2}	0.005^{1}	0.026^{1}	0.117^{1}	0.009^{2}	0.003^{1}	0.034^{4}	0.084^{12}
RP 5	0.252^{2}	0.003^{1}	0.006^{1}	0.069^{2}	0.004^{2}	0.003^{1}	0.054^{3}	0.106^{9}
RP 6	0.220^{2}	0.012^{1}	0.008^{1}	0.043^{3}	0.007^{2}	0.003^{1}	0.040^{4}	0.100^{10}
RP 7	0.341^{2}	0.009^{1}	0.010^{1}	0.017^{3}	0.006^{2}	0.005^{1}	0.033^{4}	0.073^{15}
RP 8	0.290^{2}	0.003^{1}	0.029^{1}	0.031^{3}	0.007^{2}	0.002^{1}	0.033^{4}	0.137^{7}
RP 9	0.362^{2}	0.002^{1}	0.018^{1}	0.037^{3}	0.009^{2}	0.003^{1}	0.083^{3}	0.146^{5}
RP 11	0.362^{2}	0.001^{1}	0.013^{1}	0.031^{3}	0.009^{2}	0.006^{1}	0.061^{3}	0.168^{2}
RP 12	0.331^{2}	0.003^{1}	0.004^{1}	0.081^{2}	0.009^{2}	0.004^{1}	0.032^{4}	0.151^{4}
RP 14	0.341^{2}	0.005^{1}	0.015^{1}	0.025^{3}	0.008^{2}	0.003^{1}	0.027^{4}	0.112^{9}
RP 16	0.351^{2}	0.002^{1}	0.010^{1}	0.036^{3}	0.009^{2}	0.006^{1}	0.113^{2}	0.076^{13}
RP 17	0.331^{2}	0.005^{1}	0.008^{1}	0.022^{3}	0.077^{1}	0.004^{1}	0.027^{4}	0.095^{10}
RP 20	0.292^{2}	0.005^{1}	0.007^{1}	0.032^{3}	0.010^{2}	0.005^{1}	0.023^{4}	0.335^{1}
RP 21	0.292^{2}	0.005^{1}	0.007^{1}	0.039^{3}	0.013^{2}	0.003^{1}	0.039^{4}	0.090^{11}
RP 25	0.333^{2}	0.005^{1}	0.010^{1}	0.033^{3}	0.012^{2}	0.004^{1}	0.048^{4}	0.096^{10}
RP 26	0.311^{2}	0.008^{1}	0.004^{1}	0.041^{3}	0.008^{2}	0.003^{1}	0.038^{4}	0.093^{10}
RP 33	0.024^{2}	0.006^{1}	0.003^{1}	0.037^{3}	0.008^{2}	0.002^{1}	0.056^{3}	0.085^{12}
RP 34	0.020^{2}	0.006^{1}	0.004^{1}	0.031^{3}	0.013^{2}	0.006^{1}	0.075^{3}	0.074^{14}
RP 35	0.026^{2}	0.006^{1}	0.003^{1}	0.035^{3}	0.009^{2}	0.007^{1}	0.081^{3}	0.820^{13}
RP 45	0.028^{2}	0.005^{1}	0.006^{1}	0.039^{3}	0.008^{2}	0.008^{1}	0.059^{3}	0.073^{15}
RP 47	0.020^{2}	0.007^{1}	0.003^{1}	0.032^{3}	0.006^{2}	0.007^{1}	0.046^{4}	0.077^{13}
RP 49	0.022^{2}	0.006^{1}	0.006^{1}	0.023^{3}	0.008^{2}	0.003^{1}	0.070^{3}	0.074^{14}
RP 50	0.024^{2}	0.007^{1}	0.003^{1}	0.060^{2}	0.006^{2}	0.003^{1}	0.078^{3}	0.095^{10}
RP 52	0.030^{2}	0.008^{1}	0.004^{1}	0.043^{3}	0.008^{2}	0.003^{1}	0.056^{3}	0.062^{16}
RP 53	0.021^{2}	0.006^{1}	0.003^{1}	0.048^{3}	0.010^{2}	0.005^{1}	0.060^{3}	0.080^{13}
RP 56	0.012^{2}	0.007^{1}	0.016^{1}	0.031^{3}	0.008^{2}	0.003^{1}	0.040^{4}	0.081^{13}
RP 57	0.022^{2}	0.006^{1}	0.005^{1}	0.035^{3}	0.009^{2}	0.005^{1}	0.037^{4}	0.107^{9}
RP 58	0.026^{2}	0.007^{1}	0.008^{1}	0.035^{3}	0.009^{2}	0.002^{1}	0.049^{4}	0.097^{10}
RP 59	0.025^{2}	0.001^{1}	0.005^{1}	0.040^{3}	0.006^{2}	0.003^{1}	0.037^{4}	0.109^{9}
RP 63	0.018^{2}	0.007^{1}	0.017^{1}	0.028^{3}	0.008^{2}	0.003^{1}	0.055^{3}	0.130^{8}
RP 68	0.022^{2}	0.006^{1}	0.004^{1}	0.032^{3}	0.013^{2}	0.007^{1}	0.029^{4}	0.156^{3}
RP 69	0.022^{2}	0.006^{1}	0.004^{1}	0.036^{3}	0.009^{2}	0.003^{1}	0.050^{4}	0.151^{3}
RP 70	0.021^{2}	0.008^{1}	0.018^{1}	0.037^{3}	0.019^{2}	0.005^{1}	0.138^{1}	0.139^{6}
RP 71	0.026^{2}	0.005^{1}	0.002^{1}	0.036^{3}	0.009^{2}	0.004^{1}	0.063^{3}	0.155^{3}
SEM	0.2016	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025	0.0025

* Superscripts denote DMRT ranking in descending order.

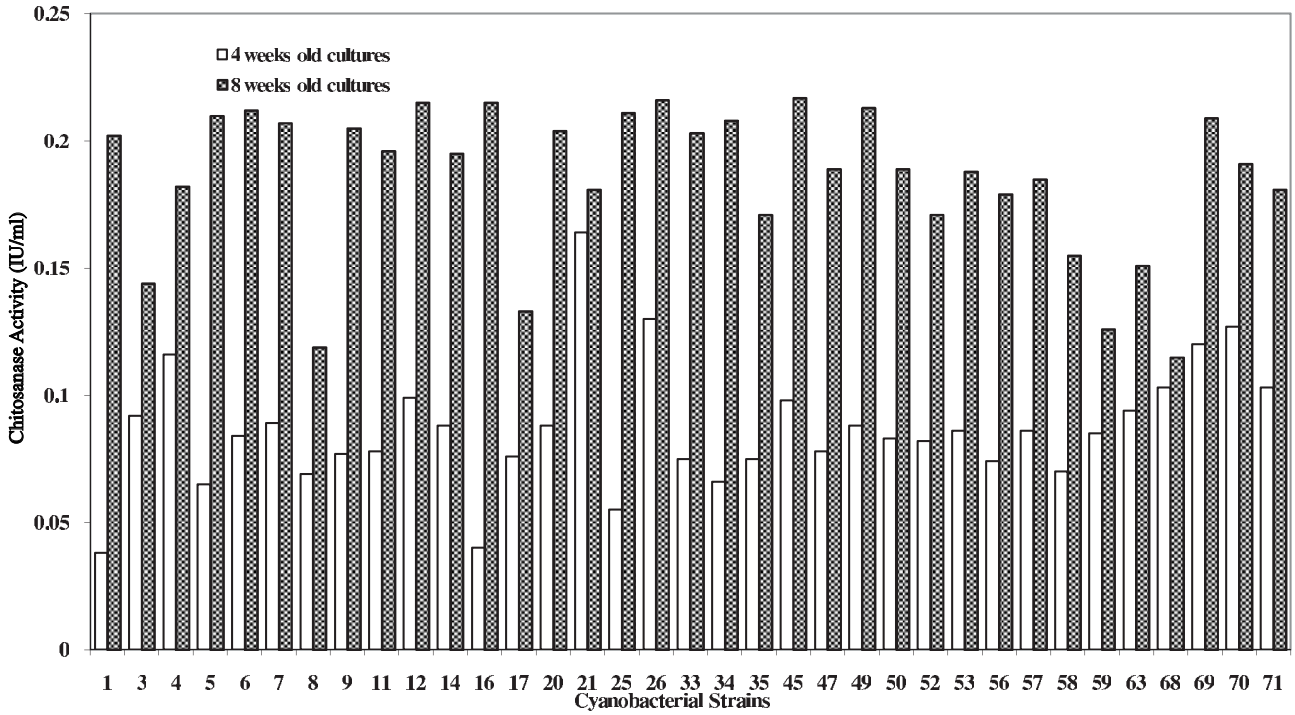

Figure 2. Chitosanase activities of 4 and 8 weeks old cultures of cyanobacterial strains.

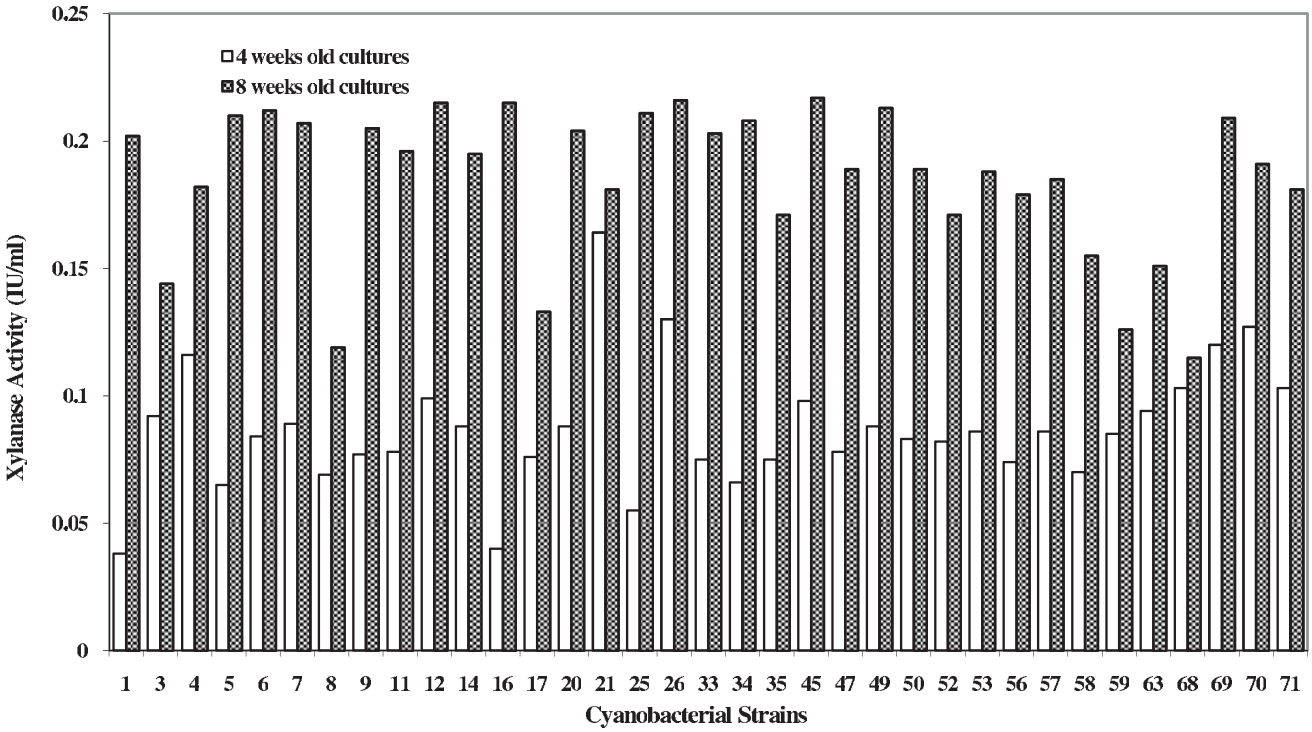

Figure 3. Xylanase activities of 4 and 8 weeks old cultures of cyanobacterial strains.

Table 4. Comparative evaluation of hydrolytic enzyme activity with inhibition zone produced by selected cyanobacterial strains on the lawn of phytopathogenic fungi.

	Hydrolytic enzymes evaluated						Phytopathogenic fungi tested		
Anabaena strains*	FPase	Cellobiase	CMCase	Protease	Chitosanase	Xylanase	*Fusarium moniliforme*	*Alternaria solani*	*Aspergillus candida*
RP 1	–	–	–	–	0.302	0.202	AP-DCM[b], AP-EA[c], CF[a]	CF[a]	AP-DCM[b], AP-EA[c]
RP 4	–	–	0.117	–	–	0.182	CF[a]	AP-DCM[b]	CF[a]
RP 6	–	–	–	0.012	–	0.212	CF[a]	CF[a]	CF[a]
RP 9	0.146	–	–	–	0.318	0.205	CF[a]	CF[a]	CF[a]
RP 11	0.168	–	–	–	0.286	0.196		AP-EA[c]	–
RP 12	0.151		0.081		0.293	0.215	AP-EA[c], CF[a]	–	AP-EA[c], CF
RP 16	–	–	–	–	0.306	0.215	CF[a]	CF[a]	AP-DCM[b], CF[a]
RP 20	0.335	–	–	–	–	0.204	AP-EA[c]	–	–
RP 26	–	–	–	0.008	–	0.216	–	CF[a]	CF[a]
RP 34	–	–	–	–	0.313	0.208	CF[a]	AP-DCM[b], AP-EA[c], CF	–
RP 45	–	0.008	–	–	–	0.217	CF[a]	CF[a]	AP-DCM[b], AP-EA[c]
RP 47	–	–	–	–	0.292	0.189	AP-DCM[b], CF[a]	CF[a]	AP-DCM[b], CF[a]
RP 50	–	–	0.060	–	–	0.189	CF[a]	AP-DCM[b], CF[a]	–
RP 53	–	–	–	–	0.295	0.188	CF[a]	CF[a]	–
RP 57	–	–	–	–	0.301	0.185	CF[a]	–	–
RP 68	0.156	0.007	–	–	0.278		AP-DCM[b], AP-EA[c], CF[a]	–	–
RP 69	0.151	–	–	–	0.282	0.209	CF[a]	CF[a]	–
RP 71	0.155	–	–	–	–	0.181	CF[a]	–	–

* Top ranked strains on the basis of DMRT analyses of activity of hydrolytic enzymes
[a] denotes Culture Filtrate
[b] denotes Aqueous Phase obtained using Dichloromethane as solvent
[c] denotes Aqueous Phase obtained using Ethyl acetate as solvent

spore germination and germ-tube elongation in *Botrytis cinerea*. The ability to produce extracellular chitosanases/chitinase is considered crucial for *Serratia marcescens* to act as antagonist against *Sclerotium rolfsii*, and for *Paenibacillus* sp. Strain 300 and *Streptomyces* sp. strain 385 to suppress *Fusarium oxysporum* f.sp. *cucumerinum* [34]. The regulation of siderophores and antibiotics, lytic enzyme production (proteases and chitosanases in particular) is known to involve the GacA/GacS regulatory systems and colony phase variation [35]. Similar studies on the genes involved need to be undertaken in cyanobacteria, for a better understanding of their relationship with biocidal activity.

Although the role of cyanotoxins/peptides/phenolic molecules cannot be ruled out in terms of biocidal activity, it becomes evident from this study that hydrolytic enzymes are definitely contributing to the inhibition of phytopathogenic fungi.

Conclusions

The present investigation, for the first time, illustrates the activity of hydrolytic enzymes in these ubiquitous photosynthetic prokaryotes and their possible role in biocidal activity against phytopathogenic fungi. The potential of these strains in developing biocontrol agents is immense – as these strains possess abilities for production of IAA and hydrolytic enzymes, besides the UV absorbing compounds (which are presently being characterized). Such multifaceted strains would possess a competitive edge over other rhizosphere microflora, against phytopathogenic fungi and need to be explored for the developing biocontrol agents.

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