



## Isolation and identification of cyanobacterial species from selected regions of Trimbakeshwar, Maharashtra, India

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### Abstract

Cyanobacteria are considered as important members of ecosystem as they have immense potential for creating sustainable agriculture output. The prime existence in the rice fields is very well established due to many emerging benefits such as enhanced soil fertility, nitrogen fixation, P solubilisation and of course increased yields. Present study has screened many cyanobacterial species from various locations of Trimbakeshwar, Maharashtra. They have found majorly *Oscillatoria simplicissima* (5%) followed by *Anabaena constricta* (4.6%), *Nostoc spongiaeforme* (4.2%) species. They detected relative abundance of 7.4% in Pahine and Deogaon while minimum was found in Ghytipada, Behedpada, Harsul with RA of 3.1%. Furthermore Deogaon was found to be rich in cyanobacterial population with the highest Shannon index (4.0) followed by Alwand (2.7) and Pahine (2.65). Thus study put a light on the importance of cyanobacterial species in rice field which could be an important candidate for agricultural revolution in future.

**Keywords:** Cyanobacteria, nitrogen fixation, soil fertility, sustainability

### Introduction

Cyanobacteria (Blue green algae) are considered photosynthetic prokaryotes, which have the ability for fixing atmospheric nitrogen (Kondo and Yasuda 2003) [10]. They are ubiquitous and have the ability to grow even in adverse environmental conditions. However, their existence in tropical paddy fields is frequent, as they can carry out photosynthesis and nitrogen fixation even in submerged conditions (Thilak *et al.*, 2020) [19]. Besides these they have enormous potential for improving water holding capacity, soil texture, ability to increase availability of nutrients, scavenge sodium from soil, promotes binding of toxicants from the soil (Subedi, P., & Shrestha, 2015) [18]. Also, the algalization process dealt by BGA species is known to cause significant changes in soil structures due to the release of a number of compounds like polysaccharides, peptides, lipids etc. during their growth (Mishra & Pabbi 2004) [12]. The biomass of nitrogen-fixing cyanobacteria in rice fields can range from a few kg to about 500 kg dry weight per hectare land (Prasanna, R., & Nayak, S. (2007) [15]. These cyanobacteria can contribute about 20–30 kg N/ha/season with the additional advantage of increasing the organic matter content of the soil.

In India, rice is a predominant crop cultivated over an area of about 44 million hectares, which accounts to nearly 37 per cent of the total area under cereals (Krishna *et al.*, 2004) [11]. Although, rice cultivation has adopted since long time back in the country, however the present average rice productivity is about 124 million metric tons. Low productivity of rice is mainly due to adoption of old cultivation practices by small and marginal farmers who covers more than 85 per cent of the total area. The submerged conditions of a rice field provide congenial habitat for growth and development of blue green algae where they form the most efficient system in providing biologically fixed nitrogen to the crop (Singh and Bisoyi 1989) [17]. The importance of nitrogen fixing blue-green

algae responsible for the enhanced fertility of the rice fields (Anand, 1980, Kaushik, and R. Prasanna 2002, Dixit and Gupta, 2000) [1, 4, 8]. Nitrogen fixing blue green algae forms the most potential source of biologically fixed nitrogen under flooded conditions of rice.

Our work has focused for the study on the identification of cyanobacterial strains from various unidentified locations of the Maharashtra region. The study has further focused on analyzing their distribution, diversity, and abundance in selected ecologies.

### Material and method

#### 1. Study site description

The study was conducted in different districts of Maharashtra and the name and latitudes were presented in the table below. The soil samples from various rice fields in above mentioned locations were collected. The Meteorological data of such agriculture sites were collected from the district agriculture and meteorology office. The details about the locations were given in supplementary data.

#### 2. Collection and processing of soil samples

The soil samples from the rice fields of around fifty-seven given locations were collected. The sampling was randomly performed and each soil sample were carefully transferred to the polythene bags and dried in shed. Part of the soil was crushed, sieved and then it was used for the determination of physicochemical characteristics of the soil.

#### 3. Determination of the Physico-chemical properties of the soil

The pH, conductivity, and organic carbon (%) of the collected soil samples were determined by using digital pH meter and conductivity meter and colorimetric method respectively.

#### 4. Isolation and identification of cyanobacterial species

For the isolation of cyanobacterial species temporary slides were prepared and examination was performed using microscopic analysis. For this, approximately 1 g of the soil was aseptically transferred to freshly prepared BG11±N medium and it was incubated at 28±2°C with 16/8 light-dark cycle under 5 K lux intensity of light. After, incubation, the growth of cyanobacterial species in enriched cultures was used for further identification through standard literature and microscopic appearance. Part of their cultures was preserved in 4% formaldehyde solution for subsequent identification.

#### 5. Statistical analysis

The distribution pattern, relative abundance, species richness, and similarity coefficient were calculated for selected sites using standard equations. The sites were selected based on the frequency of the species collected. We have screened out the top 15 locations which have shown the maximum number of species.

#### Relative abundance

The relative abundance of a particular cyanobacteria type was calculated by employing the following formula:

$$\text{Relative abundance (RA)} = \frac{Y}{X} \times 100$$

Where

X = total number of samples collected

Y = number of samples from which a particular cyanobacteria type was isolated.

Diversity Index: The Diversity Index (ShannonWiener) was been studied following the formula:

$$\Sigma = - \sum_{i=1}^S (P_i \ln P_i)$$

$$H_s = - \sum_{i=1}^S (P_i \ln P_i)$$

Where

Hs - diversity in a sample of S species or kinds

S - the number of species in the sample

Pi - relative abundance of i<sup>th</sup> species or kinds measures, = ni / N

N - total number of individuals of all kinds

ni - number of individuals of i<sup>th</sup> species

ln - log to base 2

Table 1: Supplementary

Sr. No.	Sampling sites	No. of sample Collected	Latitude and Longitude
1	Khambale	03	19° 57'27"N & 73°37'36"E
2	Anjanneri	15	19° 56'00"N & 73° 34'53"E
3	Beze	04	19° 59'45"N & 73° 34'34" E
4	Divyachapada	06	20° 01'15"N & 73° 32'20" E
5	Malegaon	02	20° 02'01"N & 73° 32'15" E
6	Kone	05	20° 04'19"N & 73° 31'37" E
7	Jategaon Bk	07	20° 05'12"N & 73° 30'15" E
8	Sapatpali	02	20° 06'04"N & 73° 28'30" E
9	Dalpatpur	04	20° 07'30"N & 73° 26'15" E
10	Harsul	08	20° 06'55"N & 73° 26'55" E
11	Hattipada	06	20° 08'19"N & 73° 25'48" E
12	Torangan	11	20° 09'32"N & 73° 26'08" E
13	Khairapali	03	20° 08'32"N & 73° 24'31" E
14	Behedpada	08	20° 09'03"N & 73° 23'01" E
15	Deodongara	05	20° 11'01"N & 73° 19'53" E
16	Kas	12	20° 12'25"N & 73° 20'52" E
17	Goldari	09	20° 09'00"N & 73° 19'44" E
18	Khadakohol	06	20° 09'41"N & 73° 20'45" E
19	Boripada	03	20° 10'18"N & 73° 21'56" E
20	Thanapada	08	20° 07'16"N & 73° 24'00" E
21	Kalamuste	04	20° 05'55"N & 73° 22'48" E
22	Shirasgaon	07	20° 05'14"N & 73° 22'23" E
23	Koulpondha	08	20° 07'00"N & 73° 17'11" E
24	Khadakohol	09	20° 08'52"N & 73° 18'19" E
25	Ozarkhed	13	20° 07'14"N & 73° 16'12" E
26	Ghytipada	09	20° 06'28"N & 73° 16'41" E
27	Berwal	11	20° 06'52"N & 73° 19'05" E
28	Chourapada	14	20° 03'59"N & 73° 18'05" E
29	Sawarpada	06	20° 09'30"N & 73° 24'28" E
30	Mulvad	09	20° 05'30"N & 73° 17'43" E
31	Rayate	11	20° 04'44"N & 73° 19'40" E
32	Bhagohol	08	20° 04'20"N & 73° 20'39" E
33	Bhutmkhada	06	20° 04'58"N & 73° 20'54" E
34	Kotambi Harsul	09	20° 06'36"N & 73° 21'42" E
35	Kalamuste	14	20° 05'57"N & 73° 22'46" E

36	Murambi	12	20° 04'05"N & 73° 22'36" E
37	Gavatha	09	20° 03'55"N & 73° 23'49" E
38	Deola	06	20° 02'23"N & 73° 23'17" E
39	Nandgaon	15	20° 01'54"N & 73° 26'08" E
40	Varasvihir	08	20° 03'27"N & 73° 26'55" E
41	Welunje	12	20° 01'18"N & 73° 29'08" E
42	Amboli	10	19° 59'03"N & 73° 28'35" E
43	Vaghera	12	20° 03'36"N & 73° 30'08" E
44	Gorthan	04	20° 02'17"N & 73° 29'50" E
45	Kachurli	09	19° 56'29"N & 73° 29'18" E
46	Talegaon trimbak	06	19° 55'26"N & 73° 30'25" E
47	Zarwad Kh	09	19° 53'57"N & 73° 29'44" E
48	Avhate	16	19° 51'42"N & 73° 28'25" E
49	Deogaon	19	19° 49'38"N & 73° 27'55" E
50	Alwand	17	19° 50'23"N & 73° 28'57" E
51	Vaviharsha	12	19° 49'11"N & 73° 29'04" E
52	Dapure	08	19° 51'03"N & 73° 30'47" E
53	Kharoli	09	19° 52'24"N & 73° 31'04" E
54	Samundi	11	19° 52'58"N & 73° 30'07" E
55	Kojoli	07	19° 53'09"N & 73° 32'16" E
56	Bhimal	13	19° 54'47"N & 73° 32'55" E
57	Pahine	19	19° 53'55"N & 73° 33'53" E

## Results and discussions

Rice fields are considered an important wetland ecosystem which has enormous biodiversity. They are the major habitats for cyanobacterial propagation, therefore the sustainable management of cyanobacterial populations the management and knowledge about the existing species are required. Cyanobacteria are considered as natural biofertilizers, therefore they play a major role in promoting the growth of rice crop even with less water requirements (Vaishampayan, *et al.*, (2001) [20]. In the present study, the diversity of cyanobacteria was recorded from different rice fields of the Trimbakeshwar area of Maharashtra, India. The location of all selected sites along with latitudes was provided in Supplementary. For the current study, we have screened top 20 locations for further study based of the frequency of isolated species of cyanobacteria. The rice fields provide a natural environment with sufficient light, water, temperature, humidity, and nutrients for the growth of cyanobacteria as reported earlier also. Besides these qualities, cyanobacteria are reported to have nitrogen-fixing abilities, phosphorous mobilization and adsorption of toxic compounds. Besides these actions they are very helpful in carrying significant changes in soil water interface attributing to the improvement in soil physicochemical properties (Chittora, *et al.*, 2020) [2]. In our investigation, we noticed that the soil parameters did not vary significantly in the selected locations. The physicochemical properties like pH, conductivity, organic carbon (%), from rice field soils of different locations of Nashik. Maharashtra was shown in Table 1. The pH of various locations was in the range of 5.7-7.1, with the highest value in Alwand in the neutral range. The value of EC was highest in Deogaon while lowest in the Rayate. The organic carbon was maximum in

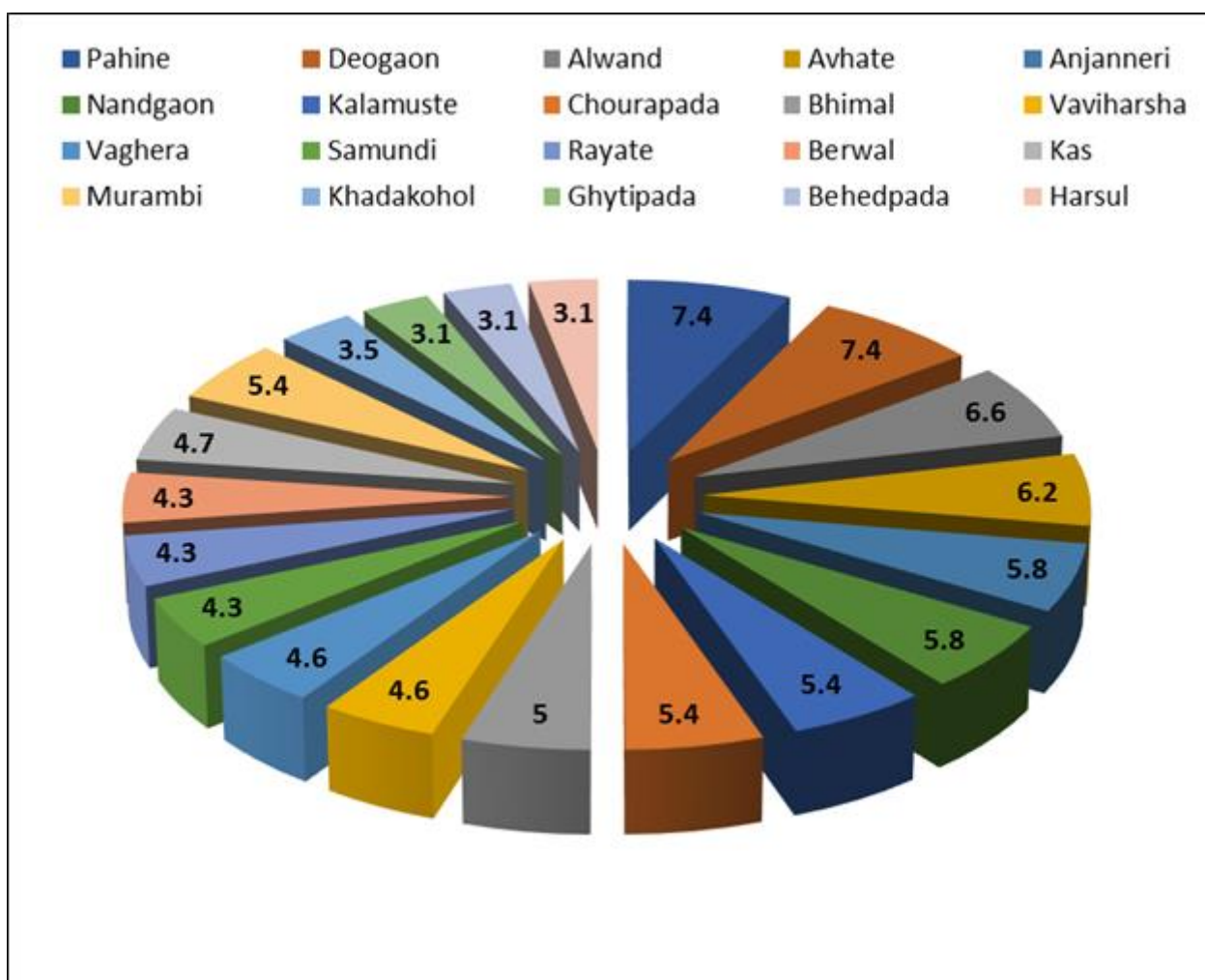
Pahine (0.63%) with the lowest reported value in Khadakohol (0.44%). The soil characteristics of the selected locations were neutral to alkaline, with sufficient OC and EC for the growth of cyanobacteria. In another study carried out by Jain *et al.*, (2022) [6], similar kinds of soil properties were reported in the Nasik district of Maharashtra region. Shirsath, (2021) [16] also showed alkaline soil with similar EC values of Satana (Baglan) Tahsil, District Nashik, Maharashtra. According o previous studies conducted by many authors the pH of the soil plays an important role in the proliferation of cyanobacterial populations. Dey *et al.*, (2010) [3] has shown a positive correlation between the soil pH and cyanobacterial population. The neutral-alkaline nature of soil of Ghagrahat, India recorded the highest MPN values in terms of N<sub>2</sub> fixers ( $67.8 \times 10^2$ ), while Hazaribagh recorded very high values of total cyanobacterial populations in neutral Ph of soil (Prassana and Nayak, 2007). Therefore it was concluded that pH was an important factor in the establishment of cyanobacterial diversity. Although it is difficult to conclude due to the involvement of various environmental factors responsible for the ubiquitous distribution of cyanobacterial growth. The distribution of various species was found at different selected locations where maximum no of species was detected in Pahine and Deogaon with the relative abundance of 7.4% while minimum was found in Ghytipada, Behedpada, Harsul with RA of 3.1%. The RA of various locations was shown in the Figure 1. Also, the effect of geographical locations was earlier reported for cyanobacterial populations but detailed studies about their diversity in such regions are still needed.

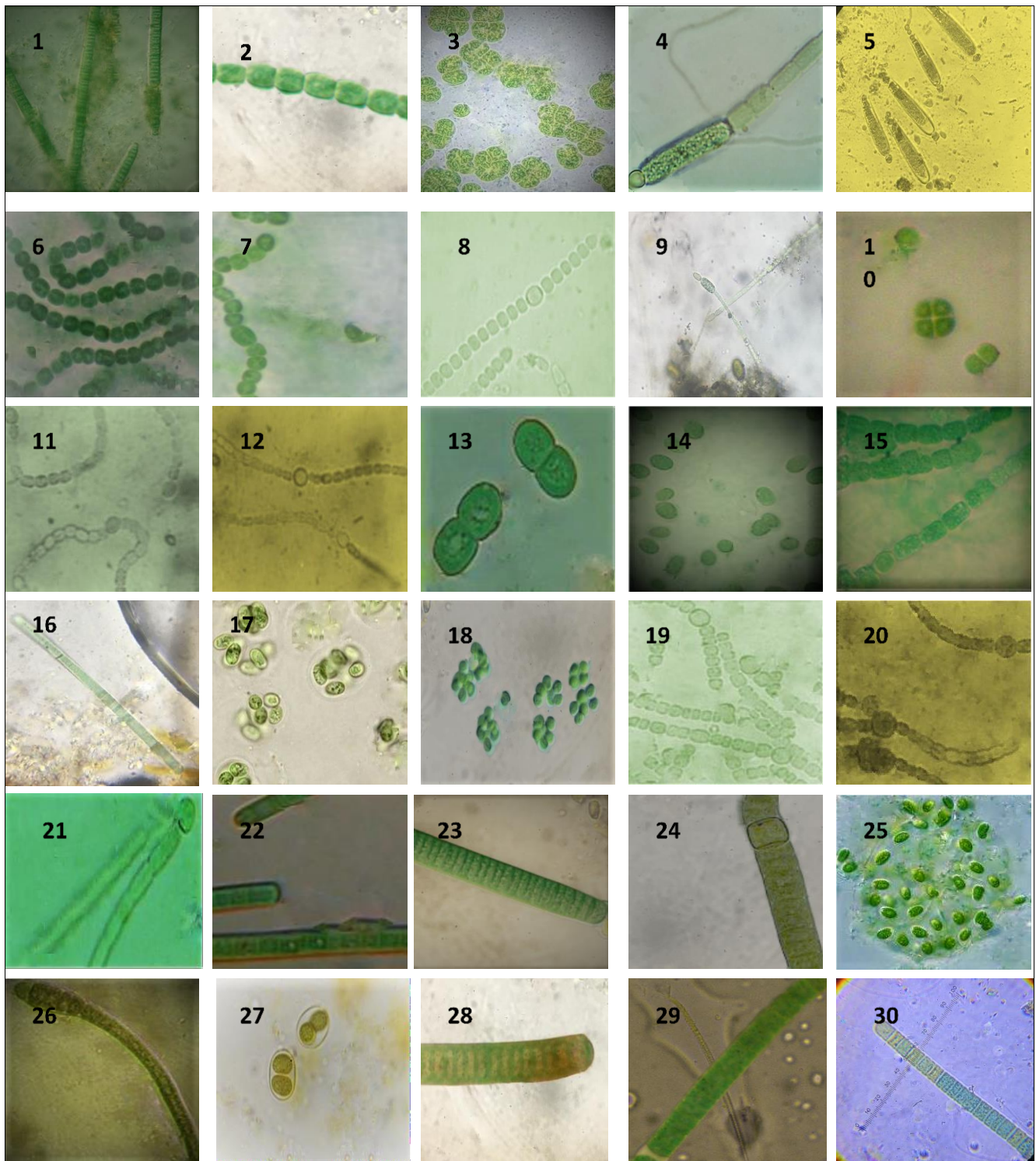
The details about the cyanobacterial species isolated from different locations were given in Table 1.

**Table 1:** Physiochemical properties of soil of various locations

Locations	pH	EC	OC(%)
Pahine	6.9	0.42	0.63
Deogaon	7.0	0.54	0.6
Alwand	7.1	0.46	0.61
Avhate	7.0	0.36	0.59
Anjanneri	6.7	0.33	0.6
Nandgaon	6.3	0.32	0.54
Kalamuste	6.7	0.39	0.52
Chourapada	6.6	0.37	0.57
Bhimal	5.9	0.39	0.59
Vaviharsha	6.8	0.42	0.44
Vaghera	6.2	0.45	0.49
Samundi	6	0.3	0.51
Rayate	6.1	0.27	0.50
Berwal	6.2	0.38	0.58
Kas	6.3	0.32	0.59
Murambi	6.6	0.33	0.52
Khadakohol	6	0.36	0.44
Ghytipada	5.7	0.38	0.48
Behedpada	5.9	0.38	0.49
Harsul	5.8	0.31	0.50

A total of 258 species were purified and out of those 30 different species were identified (Fig 2).

**Fig 1:** Relative abundance among various locations



**Fig 2:** Identified species from different locations:

1. *Phormidium uncinatum*, 2. *Anabaena oryza*, 3. *Gloeocapsa calcarea*, 4. *Gloeotrichia natans*, 5. *Gloeotrichia intermedia*, 6. *Nostoc linckia*, 7. *Nostoc padulosum*, 8. *Nostoc spongiaeforme*, 9. *Cylindrospermum majus*, 10. *Chroococcus minor*, 11. *Nostoc calcicola*, 12. *Nostoc ellipsiformae*, 13. *Cyanothece aeruginosus*, 14. *Aphanothe ceconferta*, 15. *Anabaena constricta*, 16. *Oscillatoria simplicissima*, 17. *Gloeocapsa magma*, 18. *Microcystis flos-aquae*, 19. *Anabaena doliolum*, 20. *Anabaena varibilis*, 21. *Rivularia aquatic*, 22. *Oscillatoria boryana*, 23. *Oscillatoria princep*, 24. *Scytonema simplex*, 25. *Calothrix javanica*, 26. *Calothrix linearis*, 27. *Chroococcus indicus*, 28. *Oscillatoria irrigua*, 29. *Oscillatoria tenuis*, 30. *Oscillatoria subbrevis*

The species from heterogeneous and nonheterogeneous genera were *Phormidium uncinatum*, *Anabaena oryza*, *Gloeocapsa calcarea*, *Gloeotrichia natans*, *Gloeotrichia intermedia*, *Nostoc linckia*, *Nostoc padulosum*, *Nostoc spongiaeforme*, *Cylindrospermum majus*, *Chroococcus minor*, *Nostoc calcicola*, *Nostoc ellipsiformae*, *Cyanothece aeruginosus*, *Aphanothe ceconferta*, *Anabaena constricta*, *Oscillatoria simplicissima*, *Gloeocapsa magma*, *Microcystis flos-aquae*.

*Anabaena doliolum*, *Anabaena varibilis*, *Rivularia aquatic*, *Oscillatoria boryana*, *Oscillatoria princep*, *Scytonema simplex*, *Calothrix javanica*, *Calothrix linearis*, *Chroococcus indicus*, *Oscillatoria irrigua*, *Oscillatoria tenuis*, *Oscillatoria subbrevis*. The relative abundance data has shown the maximum abundance of *Oscillatoria simplicissima* (5%) followed by *Anabaena constricta* (4.6%), *Nostoc spongiaeforme* (4.2%) (Table 2).

**Table 2:** Species wise occurrence and relative abundance at different location

Species	Total Strains	Pahine	Deogaon	Alwand	Avhate	Anjanneri	Nandgaon	Kalamuste	Chourapada	Bhimal	Vaviharsha	Vaghera	Samundi	Rayate	Berwal	Kas	Muarmbi	Khadakohol	Ghytipada	Behedpada	Harsul
<i>Phormidium uncinatum</i>	5	0	0	1	0	1	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0
<i>Anabeana oryzae</i>	6	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0
<i>Gloeocapsa calcarea</i>	5	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Gloeotrichia natans</i>	8	1	0	1	1	0	0	1	0	1	0	1	0	0	0	0	0	1	0	0	1
<i>Gloeotrichia intermedia</i>	10	1	0	1	2	1	0	0	0	0	0	0	0	0	1	0	1	1	0	1	1
<i>Nostoc linckia</i>	8	0	1	1	0	0	1	0	0	1	0	0	0	1	0	0	0	1	2	0	0
<i>Nostoc padulosum</i>	8	2	1	0	0	1	0	1	0	1	0	0	0	2	0	0	0	0	0	0	0
<i>Nostoc spongiaeforme</i>	11	0	1	0	0	1	0	0	1	0	1	1	1	0	0	0	2	0	1	1	1
<i>Cylindrospermum majus</i>	5	1	1	0	2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Chroococcus mino</i>	9	2	0	0	1	0	0	1	1	0	1	0	1	0	0	1	0	0	0	0	1
<i>Nostoc calcicola</i>	5	0	1	1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
<i>Nostoc ellipsiformae</i>	10	1	2	0	0	0	0	0	1	0	1	2	1	0	0	0	0	0	1	0	0
<i>Cyanothece aeruginosu</i>	10	0	0	1	1	0	1	0	0	0	0	1	1	1	1	0	0	1	0	1	1
<i>Aphanothece ceconferata</i>	11	1	1	1	0	0	2	0	1	0	0	0	1	1	2	0	0	0	0	0	0
<i>Anabaena constricta</i>	12	1	0	0	0	0	0	1	0	1	1	0	1	0	1	1	2	0	1	1	1
<i>Gloeocapsa magma</i>	10	0	1	0	1	0	1	1	0	0	0	1	2	1	2	0	0	0	0	0	0
<i>Oscillatoria simplicissima</i>	13	0	2	1	1	1	0	0	1	0	1	1	0	1	2	1	1	0	0	0	0
<i>Microcystis flos-aquae</i>	8	0	0	0	0	0	1	0	0	1	0	0	2	0	0	1	1	0	1	1	0
<i>Rivularia aquatica</i>	8	1	0	1	0	1	0	0	1	0	1	0	0	1	0	1	0	1	0	0	0
<i>Anabaena varibilis</i>	11	0	0	2	0	1	0	1	0	1	0	0	0	0	0	2	1	1	1	1	0
<i>Anabaena doliolum</i>	8	2	1	0	1	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0
<i>Oscillatoria boryana</i>	9	0	0	0	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	0	0
<i>Calothrix javanica</i>	5	0	1	0	2	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
<i>Scytonema simplex</i>	10	2	1	1	1	2	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0
<i>Oscillatoria princeps</i>	10	0	0	0	0	0	1	1	0	2	0	2	1	0	0	0	0	0	1	1	1
<i>Calothrix linearis</i>	5	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
<i>Oscillatoria tenuis</i>	11	0	0	1	1	1	1	1	1	0	0	1	0	1	1	0	0	1	0	1	0
<i>Oscillatoria irrigua</i>	10	1	1	0	0	2	1	2	2	0	0	0	0	0	0	0	0	0	0	0	1
<i>Chroococcus indicus</i>	8	1	1	1	0	0	0	1	1	2	0	1	0	0	0	1	0	0	0	0	0
<i>Oscillatoria subbrevis</i>	9	0	0	1	0	0	2	0	0	2	2	0	0	1	0	0	1	1	0	0	0
TOTAL SPECIES NO	258	19	19	17	16	15	15	14	14	13	12	12	11	11	11	12	14	9	8	8	8

Among all locations, Pahine and Deogaon were recorded for maximum cyanobacterial strains isolation i.e. 19. The prevalence of *Oscillatoria chalybea* (9.90%) was the most abundant cyanobacteria followed by *Oscillatoria subbrevis* (8.96%) was also reported in previous work (Dey *et al.*, 2010) [3]. Also in another study conducted in the Maharashtra region, India 93.65% of samples showed the occurrence of unicellular, heterocystous and non-heterocystous forms while 6.34% of soil samples showed only non-heterocystous forms where *Anabaena* and *Oscillatoria* were majorly found (Ghadage and Karande 2019) [5]. Generally, cyanobacteria form heterocyst during unfavorable conditions however the presence of nutrient and suitable environmental conditions for their growth refrain them from forming heterocyst. However, the report given by Indian Agricultural Research Institute for cyanobacterial diversity of rice fields the has found an abundance of genera–Nostoc, *Anabaena* and *Phormidium*, irrespective of chemical/biofertilizers treatments and stage of crop growth (Nayak *et al.* 2001, 2004) [13, 14]. They can exist as free living and symbiotic forms therefore capable of adapting to diverse ecological habitats.

The diversity index of cyanobacterial populations occurring in rice fields of various sites were calculated by Shannon-Wiener method (Table 3).

**Table 3:** Diversity indices, species richness and relative abundance of species at different locations

Locations	SR	RA	SI
Pahine	19	7.335907	2.65
Deogaon	19	7.335907	4
Alwand	17	6.563707	2.7
Avhate	16	6.177606	2.5
Anjanneri	15	5.791506	2.5
Nandgaon	15	5.791506	2.5
Kalamuste	14	5.405405	2.54
Chourapada	14	5.405405	2.54
Bhimal	13	5.019305	2.24
Vaviharsha	12	4.633205	2.36
Vaghera	12	4.633205	2.25
Samundi	11	4.247104	2.14
Rayate	11	4.247104	2.27
Berwal	11	4.247104	2.01
Kas	12	4.633205	2.36
MURAMBI	14	5.405405	2.44
Khadakohol	9	3.474903	2.19
Ghytipada	8	3.474903	1.9
Behedpada	8	3.088803	2.07
Harsul	8	3.088803	2.07

Among the locations, Deogaon was found to be rich in cyanobacterial population with the highest Shannon index (4.0) followed by Alwand (2.7) and Pahine (2.65). However, the species richness was maximum in Pahine and Deogaon as provided in the table. The high diversity indices are therefore representative of cyanobacterial isolates resilient to such interventions, with the potential to adapt to diverse chemical and ecological perturbations in the soil-water environment of the rice ecosystem. In India, on average, BGA accounts for about 33% whereas reports show that up to 50% of the total algae were BGA in some of the southern and eastern states. Most of the cyanobacterial population are reported to have certain adaption against inhospitable conditions also, therefore they stimulate the phosphorus solubilization, reduce sodium ion content, and increase

nutrient content in deficient soil systems also. Therefore the presence of an indigenous cyanobacterial population in such locations may help in ameliorating the land and making them suitable for obtaining higher yields as benefits, other than nitrogen fixation, including solubilization of phosphorus, improved soil structure and synthesis of growth-promoting substances (Khan *et al.* 1994, Saxena and Kaushik 1994) [7, 9].

### Conclusion

Enormous potential of cyanobacterial species such as improvement of soil physiochemical properties, act as biofertilizers, control of soil borne disease, improve grains quality and yield, add organic matter, remove toxics etc. has been reported earlier. Current study has successfully isolated and identified around 30 species from various new locations of Maharashtra regions. Maximum abundance of *Oscillatoria simplicissima* (5%) followed by *Anabaena constricta* (4.6%), *Nostoc spongiaeforme* (4.2%) was reported. However, the diversity indices were maximum in Deogaon followed by Alwand and Pahine. For wider understanding of role of each isolates detailed experimentation is required at fields scale.

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