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Screening of cyanobacterial strains for the selenium nanoparticles synthesis and their anti-oxidant activity

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ABSTRACT

Selenium (Se) is a crucial trace element required by all living organisms and its deficiency as well as excess is known to cause many diseases. Se substitutes when consumed in higher concentration cause toxicity. Therefore, research is now being focused to overcome the adverse effects of high Se doses. One possible way is to use them in form of Selenium nanoparticles (SeNPs). However, SeNPs synthesized through physical and chemical methods also show toxicity. Biologically synthesized SeNPs due to their lesser toxicity emerged as an alternative. Like plants, bacteria and other organisms, cyanobacterial extracts also have the ability to reduce the metal ions into nanoparticles due to the presence of many reducing biomolecules. The present study envisages screening of 20 cyanobacterial extracts for SeNPs synthesis based on time taken and particle size. The obtained synthesis time was in between two to five days and the range of SeNPs size was in between 11.8 to 60 nm. Synthesized SeNPs were characterized by UV–Vis spectroscopy, Scanning electron microscopy and Energy dispersive X-ray spectroscopy (EDX). Further, the anti-oxidant activities of best five cyanobacterial strains (*Anabaena variabilis* NCCU-441, *Arthrospira indica* SOSA-4, *Gloeocapsa gelatinosa* NCCU-430, *Oscillatoria* sp. NCCU-369, *Phormidium* sp. NCCU-104) were tested by the DPPH and SOR scavenging assays. The SeNPs synthesized by *Arthrospira indica* SOSA-4 showed the best anti-oxidant activity with $IC_{50} = 81.67 \pm 0.77$ μg/ml (SOR) and 73.94 ± 1.53 μg/ml (DPPH).

1. Introduction

Selenium (Se) is a crucial trace element for all living organisms and an important dietary component which incorporates into various proteins that assist the immune system to function properly, to prevent cell damage, and to regulate the function of thyroid gland (Zhang et al., 2005). It also acts as a potent anti-carcinogenic and anti-microbial agent (Wadhwani et al., 2016). Se occurs as a cofactor in various enzymes like thioredoxin reductases and glutathione peroxidases (Benko et al., 2012). Besides various advantages, high concentrations of Se intake can cause some side-effects. It is found that the reduction of selenate and selenite to selenium takes place by the reductase enzyme to form Selenium nanoparticles (SeNPs) (Wadhwani et al., 2016). To overcome the adverse effects of high Se doses, reduction in this dose through its consumption as SeNPs may be a good alternative (Ramamurthy et al., 2013).

SeNPs can be synthesized through various physical methods like UV radiations, laser ablation, hydrothermal techniques etc. (Iranifam et al., 2013; Quintana et al., 2002). They can also be synthesized by chemical methods like precipitation method, acid decomposition, reduction by using ascorbic acid, sodium dodecyl sulfate, glucose and sulfur dioxide etc. (Dwivedi et al., 2011; Zhang et al., 2010; Lin and Wang, 2005). But, all these methods require harsh chemicals, acidic pH and high temperature which make the nanoparticles unsafe for biomedical use (Wadhwani et al., 2016). SeNPs can also be bio-synthesized by using fungi (Zhang et al., 2019), bacteria (Wadhwani et al., 2017) and plants (Menon et al., 2019). Various bio-molecules like proteins, enzymes, phenols, sugars, flavonoids help in the reduction of ionic forms of selenium to SeNPs. Nowadays, biosynthesized SeNPs are gaining importance due to their various bio-medical applications viz. anti-oxidant (Forootanfar et al., 2014; Torres et al., 2012), anti-cancer (Ali et al., 2013), anti-fungal (Kazempour et al., 2013), anti-bacterial (Hariharan et al., 2012) and anti-inflammatory properties (Khurana et al., 2019). Biosynthesized SeNPs are inexpensive, eco-friendly and produce no

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toxic byproducts during their synthesis (Wadhwani et al., 2016). Different biomolecules act as reducing agent in the nanoparticle synthesis as well as they also act as stabilizers for the nanoparticles so that they do not aggregate with time. Proteins are found to be responsible for the synthesis and capping of SeNPs in different organisms like in bacteria- *Zooglea ramera* (Srivastava and Mukhopadhyay, 2013), *Bacillus* sp (Ikram and Faisal, 2010) (Tugarova and Kamnev, 2017), fungus-*Mariannaea sp. HJ* (Zhang et al., 2019) plants-*Diopyros Montana* (Kokila et al., 2017). Similarly, Polysachcharides have also been reported for the synthesis and stabilization of SeNPs in some organisms like cyanobacteria- *Spirulina platensis* (Yang et al., 2012), mushroom-*Pleurotus tuber--regium* (Wu et al., 2012). Secondary metabolites (phenols, alcohols, flavonoids, lignin, terpenes etc) are also found to be responsible for the formation of SeNPs in some plants like ginger (Menon et al., 2019), *vitis vinifera* (Sharma et al., 2014), *Psidium guajava* (Alam et al., 2019) etc.

Cyanobacteria are the photosynthetic prokaryotes and exist in a variety of forms like unicellular, filamentous and colonial. Previously, cyanobacteria and its byproducts have been extensively studied for agricultural and ecological applications due to the presence of wide spectrum of primary and secondary metabolites (Garlapati et al., 2019; Sami and Fatma, 2019). These metabolites also enable cyanobacteria to reduce the metal ions into metal nanoparticles. In past researches, most of the work in the field of biosynthesis of cyanobacteria mediated nanoparticles was only focused on the synthesis of silver and gold nanoparticles (Singh et al., 2013; Patel et al., 2015; Husain et al., 2015, 2019; Parial et al., 2016). Few researchers accidently noticed the formation of orange elemental SeNP granules in some species of cyanobacteria at high concentration of selenite exposure when they were studying the toxic effects of selenium on *Anacystis nidulans syn. Synechococcus leopoliensis* (Kumar and Prakash, 1971), *Spirulina platensis* (Pronina et al., 2002), *Phormidium luridum* var. *olivacea* (Sielicki and Burnham, 1973), but they have not studied the SeNPs in detail with respect to mechanism of synthesis, characterization and biological activities. For the first time, Yang et al. (2012) synthesized and characterized the *Spirulina* polysachcharides based SeNPs. Hnain et al. (2013) synthesized and characterized the intracellular SeNPs from *Synechococcus leopoliensis*. These preliminary studies prompted us to do a thorough screening of SeNPs synthesis by using crude extracts of 20 cyanobacteria with respect to nanoparticle size, reaction time and their bioactivity.

2. Materials and methods

2.1. Chemicals and reagents

For the media preparation, all analytical grade chemicals were purchased from Sigma while sodium selenite (Na_2SeO_3) was purchased from Hi-media.

2.2. Cyanobacterial culture collection and maintenance

20 Cyanobacterial strains were procured from CFTRI, Mysore; IARI, New Delhi; NFMC, Tiruchirapalli. Heterocystous and non-heterocystous strains were cultured in BG-11 (with and without Sod. Nitrate) (Stanier et al., 1971). *Spirulina* and *Arthrospira* strains were grown in Zarrouk's media (Zarrouk, 1966). Growth medium were illuminated with the fluorescent light with 2000 ± 200 lux intensity for 12 h light and 12 h dark cycle. For the large scale production of biomass, culturing was done at regular intervals. Biomass of all the strains was harvested by centrifugation except *Spirulina* and *Arthrospira* strains which were harvested by filtration. Biomass was thoroughly washed 3 times with double distilled water followed by freeze drying and crushing (in pestle and mortar).

2.3. Screening of cyanobacteria for the SeNPs synthesis

Freeze dried 1 g of powdered biomass was used to prepare 1 L of aqueous extract by keeping the mixture in water bath for 10 min at 60 °C. After that, it was centrifuged at 6000 rpm for 15 min at 4 °C and supernatant was pooled and filtered by whatman filter No.1. For the synthesis of SeNPs, reaction mixture (1 mM Sodium selenite and extract in 1:2 ratio) was placed at 32 °C. Sodium selenite solution (1 mM) without extract was also kept under identical conditions as control. Synthesized SeNPs were observed by change in solution colour. After the

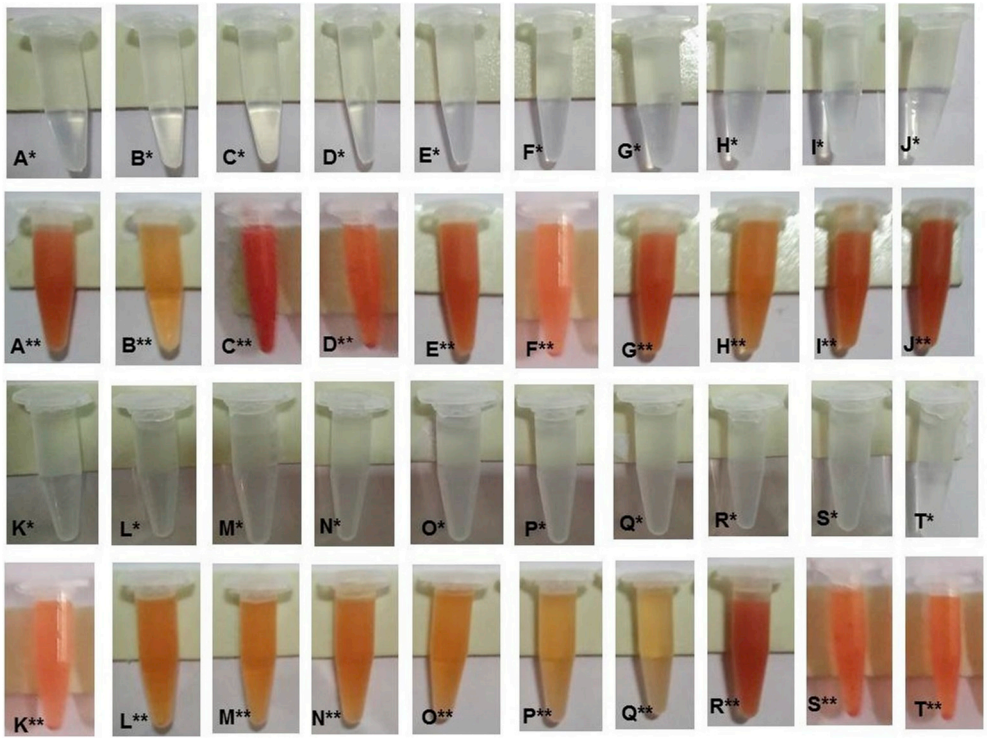

Fig. 1. Colour changes during SeNPs synthesis (* = Before synthesis, ** = After synthesis) A) *Oscillatoria* sp. NCCU-369, B) *Gloeocapsa gelatinosa* NCCU-430, C) *Westiellopsis prolifica* NCCU-331, D) *Anabaena variabilis* NCCU-441, E) *Arthrospira indica* SAE-84, F) *Spirulina* CPCC-695, G) *Arthrospira maxima* SAE-49-88, H) *Nostoc muscorum* NCCU-442, I) *Chroococcus* NCCU-207, J) *Arthrospira indica* SOSA-4. K) *Nostoc punctiforme,* L) *Lyngbya* NCCU-102, M) *Nostoc sphericum,* N) *Calothrix brevissema* NCCU-65, O) *Synechocystis* NCCU-370, P) *Plectonema* sp. NCCU-204, Q) *Spirulina platensis* NCCU-S5, R) *Microchaete* sp. NCCU-342, S) *Scytonema* sp. NCCU-1 26, T) *Phormidium* sp. NCCU-104. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Screening of the cyanobacterial strains for SeNPs synthesis.

S.No.	Cyanobacterial strains	Properties of Cyanobacterial SeNPs				
		Reaction time (in days)	Peak	Shape and color	Average Size	Weight (%)
1.	*Anabaena variabilis* NCCU-441	2	266	Spherical, Red	13.1	59.30
2.	*Arthrospira indica* SOSA-4	2	265	Spherical, Red	11.8	62.29
3.	*Arthrospira maxima* SAE-4988	3	270	Spherical, Red	16.2	6.79
4.	*Arthrospira indica* SAE-84	3	271	Spherical, Red	60	20.31
5.	*Calothrix brevissema* NCCU-65	2	262	Spherical, Red	25.6	16.59
6.	*Chroococcus* NCCU-207	3	265	Spherical, Red	18.2	36.66
7.	*Gloeocapsa gelatinosa* NCCU-430	2	270	Spherical, Red	13.2	50.68
8.	*Lyngbya* NCCU-102	2	264	Spherical, Red	18.9	10.12
9.	*Microchaete* sp. NCCU-342	2	265	Spherical, Red	15.2	26.21
10.	*Nostoc muscorum* NCCU-442	3	266	Spherical, Red	18.6	11.46
11.	*Nostoc punctiforme*	3	268	Spherical, Red	26	4.17
12.	*Nostoc sphericum*	2	268	Spherical, Red	22.4	40.56
13.	*Oscillatoria* sp. NCCU-369	2	270	Spherical, Red	13.6	54.35
14.	*Phormidium* sp. NCCU-104	2	259	Spherical, Red	14	51.84
15.	*Plectonema* sp. NCCU-204	3	270	Spherical, Red	18.3	12.36
16.	*Scytonema* sp. NCCU-126	3	274	Spherical, Red	24	7.60
17.	*Spirulina* CPCC-695	5	268	Spherical, Red	18.2	3.55
18.	*Spirulina platensis* NCCU-S5	3	268	Spherical, Red	21.4	4.48
19.	*Synechocystis* NCCU-370	4	265	Spherical, Red	18.9	21
20.	*Westiellopsis prolifica* NCCU-331	2	266	Spherical, Red	17.5	5.93

completion of reaction, the nanoparticles solution was centrifuged at 15000 rpm for 20 min at 4 °C. The obtained pellet was washed 3 times by centrifuging with double distilled water to remove unused substances. Then, the purified SeNPs was dried at 30 °C. Time taken for colour change during the synthesis of SeNPs was recorded.

2.4. Characterization of SeNPs for physical properties

After the completion of reaction, the SeNPs were characterized by UV–Visible spectroscopy, Scanning Electron Microscopy and Energy dispersive X-ray spectroscopy (EDX). UV–Vis spectra of SeNPs were

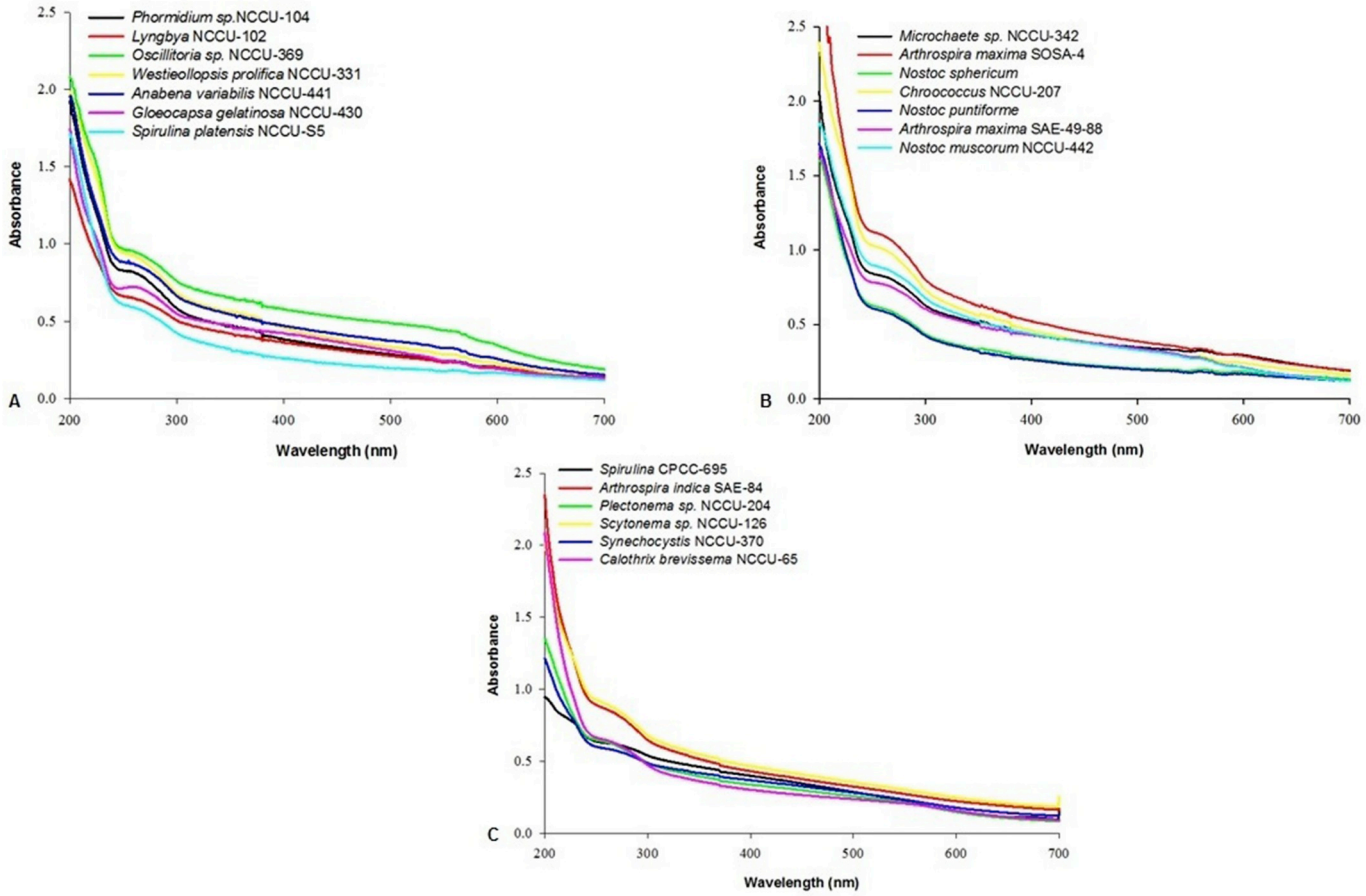

Fig. 2. UV–Vis absorption spectra of cyanobacterial strains A). *Phormidium* sp. NCCU-104, *Lyngbya* NCCU-102, *Oscillatoria* sp. NCCU-369, *Westiellopsis prolifica* NCCU-331, *Anabaena variabilis* NCCU-441, *Gloeocapsa gelatinosa* NCCU-430, *Spirulina platensis* NCCU-S5 B). *Microchaete* sp. NCCU-342, *Arthrospira indica* SOSA-4, *Nostoc sphericum, Chroococcus* NCCU-207, *Nostoc punctiforme, Arthrospira maxima* SAE-49-88, *Nostoc muscorum* NCCU-442 C). *Spirulina* CPCC-695, *Arthrospira indica* SAE-84, *Plectonema* sp. NCCU-204, *Scytonema* sp. NCCU-126, *Synechocystis* NCCU-370, *Calothrix brevissema* NCCU-65.

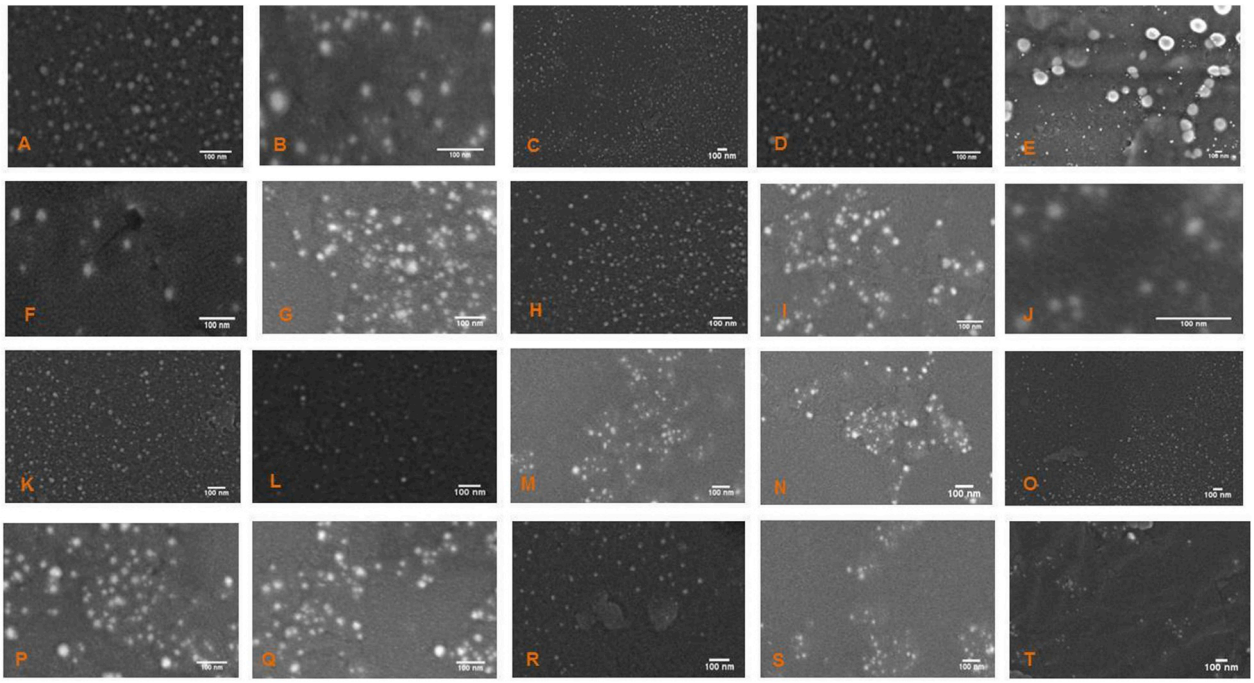

Fig. 3. SEM images of SeNPs synthesized from cyanobacterial strains: A). *Oscillatoria* sp. NCCU-369, B). *Gloeocapsa gelatinosa* NCCU-430, C). *Westiellopsis prolifica* NCCU-331, D). *Anabaena variabilis* NCCU-441, E). *Arthrospira indica* SAE-84, F). *Spirulina* CPCC-695, *G). Arthrospira maxima* SAE-49-88, H). *Nostoc muscorum* NCCU-442, I). *Chroococcus* NCCU-207, J). *Arthrospira indica* SOSA-4. K). *Nostoc punctiforme,* L). *Lyngbya* NCCU-102, M). *Nostoc sphericum,* N*). Calothrix brevissema* NCCU-65, O). *Synechocystis* NCCU-370, P). *Plectonema* sp. NCCU-204, Q). *Spirulina platensis* NCCU-S,5 R). *Microchaete* sp. NCCU-342, S). *Scytonema* sp. NCCU-126, T). *Phormidium* sp. NCCU-104.

scanned in the range of 200–700 nm by using spectrophotometer (Labtronics LT-2800) to observe the absorption maxima (λ_{max}). For SEM, SeNPs were dispersed in water and then sonicated in Ultra-sonicator (Thermotech PID- 41 S), thin films of the SeNPs were formed by just placing a drop of the sample on carbon coated copper grid followed by coating of gold, and then the micrographs were captured on SEM (Nova NanoSEM 450), at accelerating voltage of 5 keV. Average size of SeNPs was calculated by Image J software. EDX was also performed by Nova NanoSEM 450 (Bruker Espirit software) to determine the purity and elemental composition of synthesized SeNPs, which was operated at 20 keV.

2.5. Determination of anti-oxidant activities

2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay was performed by using the modified method of Muniyappan and Nagarajan (2014). For this, 0.1 mM of DPPH was prepared in 80% ethanol. 3 ml of DPPH was mixed in the 1 ml of SeNPs having different concentrations. Mixture was kept in the dark for 30 min and then absorbance was taken at 517 nm. 3 ml DPPH was mixed in 1 ml of water which was used as control and same procedure was further followed. Superoxide radical scavenging assay was performed by the modified method of Zhishen et al. (1999). Riboflavin (3×10^{-6} M), methionine (1×10^{-2} M) and nitroblue tetrazolium (NBT) (1×10^{-4} M) were mixed together in 0.05 M of potassium phosphate buffer to form a mixture of 50 µM (pH 7.8). 300 µl of SeNPs having different concentrations (25–1000 µg/ml) were added to 3 ml to the above mixture and kept in light for 30 min. A similar set was kept in dark for the same time as blank. Absorbance was taken at 560 nm. Percentage scavenging of both the free radicals was calculated by the following formula.

$$\%\ Scavenging\ activity\ of\ free\ radical = \frac{(Ac - At)}{Ac} \times 100$$

where, Ac = Absorbance of control, At = Absorbance of the test.

IC_{50} values (concentration at which 50% of free radicals are scavenged) were calculated which were also compared with those of standard Ascorbic Acid solution.

3. Results and discussion

Synthesis of SeNPs was monitored by change in colour from sky blue to different shades of orange. All tested cyanobacterial extracts were capable of synthesizing SeNPs (Fig. 1). Orange to red coloured SeNPs had been also reported from fungi- *Gliocladium roseum, Mariannaea* sp. *HJ* (Srivastava and Mukhopadhyay, 2015; Zhang et al., 2019), bacteria-*Enterococcus faecalis* (Shoeibi and Mashreghi, 2017) and angiosperm-*Allium sativum* (Anu et al., 2017). Wadhwani et al. (2016) suggested that this change in colour is due to the reduction reaction (SeO_3^{2-} to Se^0). Time taken for such change was recorded as the reaction time and it was observed that 50% cyanobacterial strains took 2 days (Table 1). In previous studies, *Enterococcus faecalis* and *Zooglea ramigera* also took 2 days to synthesize SeNPs (Shoeibi and Mashreghi, 2017; Srivastava and Mukhopadhyay, 2013). According to Sharma et al. (2014), secondary metabolites of *vitis vinifera* are responsible for the reduction during SeNPs synthesis while Kessi et al. (1999) suggested the role of reductase enzymes in *Rhodospirillum rubrum*. Fast reduction reactions in some organisms may be due to the presence of higher quantity of enzymatic or non-enzymatic reducing substances present in them.

During scanning of 20 cyanobacterial extracts mediated SeNPs in wavelength range 200–700 nm by UV–Visible spectrophotometer, distinct SeNPs peaks were observed confirming the visible coloured observations. The λ_{max} for these peaks ranged from 259 to 274 nm (Fig. 2). Other workers have also reported almost similar λ_{max} for SeNPs e.g. at 245 nm in *Aspergillus terrus* (Zare et al., 2013), at 260 nm in garlic (Anu et al., 2017), at 261 nm in *Bacillus megaterium* (Mishra et al., 2011), at *Diospyros Montana* in 261 nm (Kokila et al., 2017) and at 271 nm in *Emblica officinalis* (Lokanadhan et al., 2019). Relatively higher λ_{max} is reported in many 330 nm in *Zooglea ramera* (Srivastava and Mukhopadhyay, 2013), 330 nm in *Gliocladium roseum* (Srivastava and

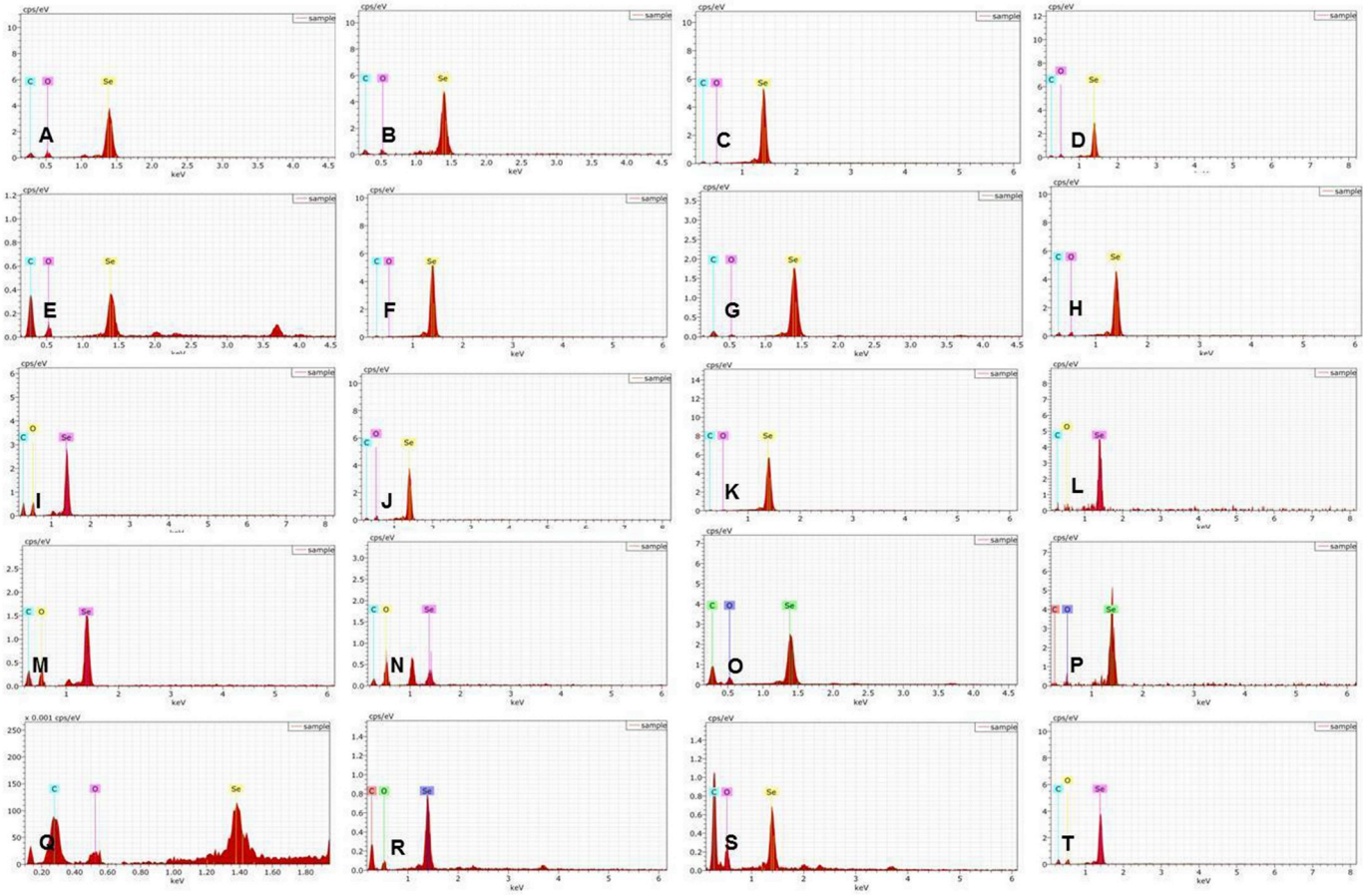

Fig. 4. EDX graph of SeNPs synthesized from different Cyanobacterial strains: A). *Oscillatoria* sp. NCCU-369, B). *Gloeocapsa gelatinosa* NCCU-430, C). *Westiellopsis prolifica* NCCU-331, D). *Anabaena variabilis* NCCU-441, E). *Arthrospira indica* SAE-84, F). *Spirulina* CPCC-695, *G). Arthrospira maxima* SAE-49-88, H). *Nostoc muscorum* NCCU-442, I). *Chroococcus* NCCU-207, J). *Arthrospira indica* SOSA-4. K). *Nostoc punctiforme,* L). *Lyngbya* NCCU-102, M). *Nostoc sphericum,* N*). Calothrix brevissema* NCCU-65, O). *Synechocystis* NCCU-370, P). *Plectonema* sp. NCCU-204, Q). *Spirulina platensis* NCCU-S5, R). *Microchaete* sp. NCCU-342, S). *Scytonema* sp. NCCU-126, T). *Phormidium* sp. NCCU-104.

Mukhopadhyay, 2015), 381 nm in *Psidium guajava* (Alam et al., 2019), 390 nm in *Terminalia arjuna* (Prasad and Selvaraj, 2014) and 395 nm in lemon (Prasad et al., 2013). Broad nature of SeNPs absorption peaks indicated the poly-dispersive nature of nanoparticles. Poly-dispersive SeNPs were also observed in *Emblica officinalis* and *Psidium guajava* (Lokanadhan et al., 2019; Alam et al., 2019).

As UV–Vis is not the confirmatory test due to its limitations, like it does not give any idea about the elemental nature, particles size and morphology. Analysis of biogenic SeNPs synthesized during present study was done by SEM and EDX. SEM results showed spherical and poly-dispersive nanoparticles (Fig. 3). Smallest size (11.8 nm) SeNPs were found in *Arthrospira indica* SOSA-4 and largest size (60 nm) in *Arthrospira indica* SAE-84 (Fig. 3). Angiospermic plants- *Emblica officinalis* and *Psidium guajava* are also reported to synthesize spherical SeNPs within the size range of 15–40 nm and 8–20 nm respectively (Lokanadhan et al., 2019; Alam et al., 2019). But Srivastava and Mukhopadhyay (2015) reported 20–80 nm sized SeNPs from fungus- *Gliocladium roseum.* According to Akhtar et al. (2013), the variation in size may be due to difference in chemical constituents present in organism employed for the synthesis.

EDX signals for SeNPs synthesized from all the cyanobacterial strains were obtained at 1.4 keV (Fig. 4). Similar EDX signal was observed in SeNPs synthesized by from *Gliocladium roseum* (Srivastava and Mukhopadhyay, 2015). Weight % of Se obtained during EDX analysis was within the range of 3.55–62.29%. However, the SeNPs synthesized from *Withania somnifera, Dyopyros Montana* and *Spirulina* polysachcharides gave the weight % as 50.79%, 94.44% and 86.3% (Alagesan and Venugopal, 2018; Kokila et al., 2017; Yang et al., 2012). On the basis of minimum synthesis time (2 days), minimum average size (11.8–14 nm) and percentage purity (>50%) of SeNPs, five cyanobacterial strains were selected viz., *Anabaena variabilis* NCCU-441, *Arthrospira indica* SOSA-4, *Gloeocapsa gelatinosa* NCCU-430, *Oscillatoria* sp. NCCU-369, *Phormidium* sp. NCCU-104 for determination of their oxidative potentials through DPPH and SOR assays. DPPH and SOR assay values showed free radical scavenging activity in the range 73.94 ± 1.53 to 161.07 ± 0.46 μg/ml and 81.67 ± 0.77 to 160.23 ± 0.21 μg/ml

Table 2
Antioxidant activity of best five Cyanobacterial strains.

S. No.	Cyanobacterial strains	SOR assay IC_{50} Value (μg/ml)	DPPH assay IC_{50} Value (μg/ml)
1	*Arthrospira indica* SOSA-4	81.67 ± 0.77	73.94 ± 1.53
2	*Anabaena variabilis* NCCU-441	85.581 ± 0.37	87.90 ± 1.34
3	*Gloeocapsa gelatinosa* NCCU-430	133 ± 0.96	128.34 ± 2.77
4	*Oscillatoria* sp. NCCU-369	147.58 ± 6.02	138.49 ± 0.81
5	*Phormidium* sp. NCCU-104	160.23 ± 0.21	161.07 ± 0.46
6	Ascorbic Acid (Standard)	75.63 ± 1.51	55.74 ± 1.40

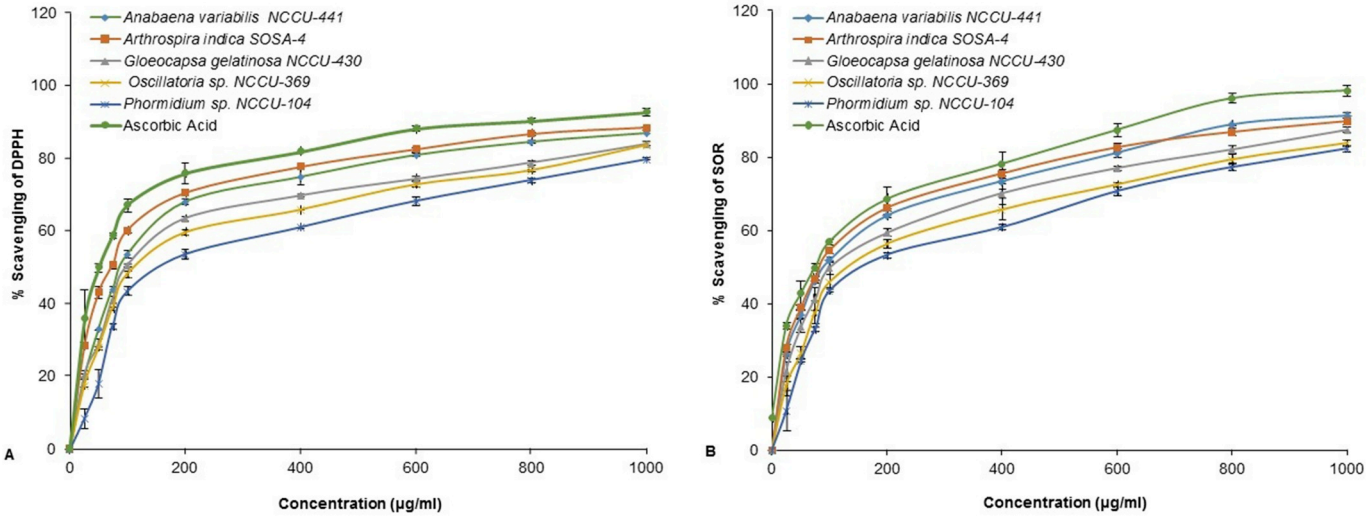

Fig. 5. Anti-oxidant activities of SeNPs from 5 best Cyanobacterial strains (*Arthrospira indica* SOSA-4, *Anabaena variabilis* NCCU-441, *Gloeocapsa gelatinosa* NCCU-430, *Oscillatoria* sp. NCCU-369, *Phormidium* sp. NCCU-104) A). DPPH scavenging assay B). SOR scavenging assay.

respectively (Table 2; Fig. 5A and B). Out of them, *Arthrospira indica* SOSA-4 mediated SeNPs showed best activity by both the assays and IC_{50} values were recorded as 73.94 ± 1.53 µg/ml (DPPH assay) and 81.67 ± 0.77 µg/ml (SOR assay). SeNPs synthesized from *Zingiber officinale* and *Emblica officinalis* showed the IC_{50} values 125 µg/ml and 15.67 respectively in anti-oxidant assays (Menon et al., 2019; Lokanadhan et al., 2019). It is also suggested that the small sized SeNPs possess higher anti-oxidant potential (Torres et al., 2012). Cyanobacterial SeNPs may serve as a promising anti-oxidant in drug and food supplement.

4. Conclusion

The present study suggests that cyanobacteria are capable of synthesizing small sized SeNPs. Out of 20 screened cyanobacterial strains, *Arthrospira indica* SOSA-4 was the best strain. It was capable of synthesizing SeNPs having small size (11.8 nm) SeNPs in two days of time with good anti-oxidant activity. Thus, this may be used as a future anti-oxidant drug or food supplement.

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