Variorum Multi-Disciplinary e-Research Journal
Vol.,-04, Issue-I, August 2013

Morphological and Biochemical Characterization of the Exocellular Investments of Polysaccharide-Producing Cyanobacteria

Shweta Sharma and **Om Prakash Chahar:** JJT University, Jhunjhunu

Abstract-

A total of 43 cyanobacterial strains, originally isolated from different habitats, were photoautotrophically grown in liquid cultures and tested for the presence of exocellular polysaccharidic investments surrounding the trichomes. However, 13 of them showed a significant presence of these structures, coupled with the release of polysaccharidic material (RPS) into the medium. A rather large number of different morphological forms was observed in the cultures during growth, but at the time of harvesting the predominant morphological form was, in most cases, the vegetative trichome. With regard to the exocellular mucilaginous investments, three main types of morphologies were observed: (i) capsules surrounded by an external pellicle, (ii) capsules with sharp outlines but without an external pellicle, (iii) slimy investments that either loosely surrounded the trichomes without following their shape or were organized in large globular lumps. Among the 13 strains that released polysaccharides into the culture medium, three showed mean daily productivities ranging from 0.20 to 0.28 mg (RPS). The morphological characteristics of the polysaccharidic investments produced by the cyanobacterial strains seem not to be related to their original habitats. Furthermore, the differences in RPS productivities observed among the strains seem not to be related to the shape of the mucilaginous exocellular investments. Chemical analysis of purified samples of the polysaccharides demonstrated that all the polymers possess an acidic nature, due to the presence of uronic acids, and that they are characterized by the presence of a peptidic moiety and of amino sugars.

Introduction-

Canobacteria are nitrogen-fixing, filamentous widely distributed in a large number of defferent habitats, where they play some very important roles (Dodds et al. 1995). In particular, they may contribute to the maintenance of soil fertility, by fixing atmospheric nitrogen in wetland soils (Rother & Whitton 1989; Sinha & HaÈ der 1996) or in symbiosis with higher plants (Bergman et al. 1992), or they may stabilize dried soils by producing abundant gelatinous capsules or slimes (Flaibani et al. 1989; Hill et al. 1994). Cyanobacteria characterized by a very complex life cycle, which includes a rather large number of morphological structures, namely vegetative trichomes, hormogonia, akinetes, heterocysts (Mollenhauer et al. 1994; Dodds et al. 1995), and three possible ways of multiplication have been described (Rippka et al.1979). Many cyanobacterial strains are characterized by the presence of external polysaccharidic investments (EPSs) such as capsules, sheaths or slimes (Bazzichelli et al. 1985, 1989; Mollenhauer et al. 1994; Huang et al. 1998), and by the release of water soluble polysaccharides (RPSs) that, like those produced by other microbial groups, may have interest for industrial applications (De Philippis & Vincenzini 1998). However, only fragmentary information is available about these outermost investments, since systematic studies aimed to characterize, on a large number of cyanobacterial strains, the morphology and the composition of the exocellular polysaccharidic envelopes surrounding the trichomes are lacking. In order to fill this gap, an investigation on forty three cyanobacterial strains, belonging to the culture collection at the Department of Microbiology, M.D.S. University, Ajmer. Collection and originally isolated from many different habitats, has been undertaken with the aim of investigating the life cycle of these strains

Variorum Multi-Disciplinary e-Research Journal
Vol.,-04, Issue-I, August 2013

growing in liquid cultures and to characterize their outermost polysaccharidic investments. The possible correlations between the morphology and the composition of the polysac- charidic envelopes and the characteristics of the original environments of the strains have also been investigated. Furthermore, the mean RPS productivity has been determined in order to pick out the most promising strains for the production of polysaccharides of possible biotechnological interest.

Materials and Methods

Forty three cyanobacterial strains, belonging to the culture collection at the Department of Microbiology, M.D.S. University, Ajmer, were photoautotrophically grown for 25-3 days, under axenic conditions, in 500 ml Erlenmeyer flasks filled with 200 ml of BG11 medium (Rippka et al. 1979), except strains incapable of N2 fixation which were grown on BG11 medium.Cultures were incubated with cool white fluorescent lamps with an irradiance of 1200-1500 lux in 12/12 hr L/D cycle at 30 ± 1^0C.

Culture samples were microscopically observed, before and after negative staining with India ink. (Reddy et al. 1996). All the morphological data reported in the paper are the result of microscopic observations. The cultures were harvested after 40-45 days of cultivation, under conditions of photolimitation and the exopolysaccharide released into the culture medium was recovered. Crude exopolysaccharide samples were obtained by addition of 2-propanol to the medium, after removing the cells by centrifugation. For the purification of crude polymers, RPS samples were solubilized again into distilled water, dialyzed for 24 h against distilled water and finally lyophilized. Total carbohydrate, uronic acid and hexosamine contents of purified RPS samples were colorimetrically determined by means of the Carbohydrate estimation (Roe, 1955) carbazole (Galambos 1967) and Ehrlich's reagent (Montreuil et al. 1986) methods, respectively. Elemental composition of the RPSs was determined by means of an automated Elemental Analyzer (Carlo Erba Strumentazione, Mod. 1106). Peptidic moiety of RPSs was calculated by multiplying the nitrogen content of the polymers, after subtracting the amount of N present in the hexosamines, by a factor of 6.25, that corresponds to the mean nitrogen content of the amino acids usually found in cyanobacterial proteins.

Table 1. Habitat of cyanobacterial strins

Sr.No.	Organism name	AZAR NO.	Mucilage /Sheath	Habitat
1.	*Gloeocapsa* sp.	1005	+	Fresh water lake
2.	*Lyngbya limnetica*	1104	-	Kolayatji lake
3.	*Lyngbya gardneri*	1106	-	Playa
4.	*Lyngbya gardneri*	1107	+	Sand stone
5.	*Lyngbya* sp.	1108	+	Lime soil water
6.	*Lyngbya infixa*	1109	+	Mud water
7.	*Lyngbya lagerheimii*	1110	-	Sand stone

Variorum Multi-Disciplinary e-Research Journal
Vol.,-04, Issue-I, August 2013

8.	*Lyngbya* sp.	1111	-	Mud water
9.	*Nostoc commune*	1203	++++	Crusted sand Suratgarh
10.	*Nostoc cacicola*	1205	++	LoosesoilSukha di river bed
11.	*Oscillatoria foreaui*	1303	-	Sand stone
12.	*Oscillatoria sp.*	1308	-	Lime soil water
13.	*Oscillatoria fremyii*	1309	-	Playa
14.	*Oscillatoria* sp.	1310	-	Lime soil water
15.	*Oscillatoria* sp.	1311	-	Mud water
16.	*Phormidium papyraceum*	1402	+	Barren soil
17.	*Phormidium molle*	1403	+	Rain water ditch
18.	*Phormidium foveolarum*	1404	-	Mud water
19.	*Phormidium bohneri*	1405	-	Sand stone
20.	*Phormidium* sp.	1406	+	Mud water
21.	*Plectonema* sp.	1502	-	Lime soil water
22.	*Synechococcus cedrorum*	1701	-	Soil crust near Prosopis
23.	*Anabaena anomala*	2200	-	Lime soil water
24.	*Anabaena anomala*	2201	-	Crust between dun
25.	*Anabaena* sp.	2202	-	Stone quarry
26.	*Anabaena oryzae*	2203	-	Anokhi lake
27.	*Anabaena* sp.	2204	-	Rain water Ditch
28.	*Anabaena variabilis*	2206	-	Sand stone
29.	*Anabaena variabilis*	2207	-	LoosesoilSukha di river bed
30.	*Anabaena variabilis var.ellipsospora*	2210	-	Rain water ditch
31.	*Anabaena anomala*	2215	-	Sand stone
32.	*Anabaena oryzae*	2221	+	Sand stone

Variorum Multi-Disciplinary e-Research Journal
Vol.,-04, Issue-I, August 2013

33.	*Anabaena* sp.	2222	-	Loose barren sand
34.	*Anabaena* sp.	2223	-	Sand dune
35.	*Anabaena sp.*	2224	++	Sand dune
36.	*Aulosira prolifica*	2300	+++	Sand dune
37.	*Chroococcus minutus*	2400	+	Loose barren sand
38.	*Chroococcus cohaerens*	2403	-	Loose barren sand
39.	*Chroococcus cohaerens*	2405	-	Lime soil water
40.	*Chlorella*	5106	-	Sand dune
41.	*Dunaliella* sp.	5201	-	Sand dune
42.	*Chlamydomonas* sp.	5700	-	Kyar-50 Marwad Balliya
43.	*Chlamydomonas* sp.	5701	-	Salt crust

Table 2. General composition of the polysaccharides released by the cyanbacterial stains

Organism Name	**Chl.*a*** **µg ml^{-1}**	**Carbohydrate µg ml^{-1}**		**Protein µg ml^{-1}**		**η Cp**	**Uronic acid**	**Hexosamines**	**Peptidic moiety**
(AZAR NO.)		**Total**	**Extra-cellular**	**Intra-cellular**	**Extra-cellular**				
***Aulosira prolifica* (2300)**	22.48	276.36	71.44	273.82	38.63	2.23	82.9	1.8	3.12
***Anabaena* sp. (2224)**	15.64	176.86	25.10	194.89	65.11	1.60	51.0	0.9	2.60
Nostoc commune (1203)	12.50	196.90	46.69	138.74	21.41	2.26	55.1	2.1	1.60
***Chroococcus minutus* (2400)**	9.69	550.94	36.92	205.22	13.73	0.90	88.0	1.6	2.18
***Nostoc cacicola* (1205)**	11.56	200.77	76.22	204.43	40.48	2.32	40.1	1.58	2.44
Anabaena oryzae (2221)	2.58	214.83	62.30	181.38	50.81	2.51	32.2	2.19	2.32
***Phormidium papyraceum* (1402)**	29.18	163.15	13.29	623.54	50.54	0.90	29.3	1.37	6.83
***Lyngbya* sp. (1108)**	22.02	227.84	9.98	501.71	126.03	0.89	27.3	1.98	6.27

***Phormidium molle* (1403)**	17.40	70.68	11.78	253.42	74.91	0.92	21.2	2.20.	3.28
***Gloeocapsa* sp. (1005)**	6.77	405.39	86.32	180.59	29.36	0.98	72.9	1.89	2.09
***Lyngbya infixa* (1109)**	6.73	360.03	145.98	68.29	29.62	0.90	76.0	2.18	0.97
***Lyngbya gardneri* (1107)**	13.41	338.59	20.75	154.63	72.00	0.91	81.2	2.89	2.26
***Phormidium* sp. (1406)**	17.17	610.01	117.85	202.04	56.11	0.93	79.3	1.67	2.58

Results

Cell cycle of the EPS-producing cyanobacterial strains Among the forty three cyanobacterial strains tested for the presence of exocellular investments, only thirteen (listed in Table 1) showed a significant presence of capsules or slimes, coupled with an appreciable release of polysaccharidic material into the liquid medium during photoautotrophic growth. The remainder, strains **AZARNO.**1104, 1106, 1110, 1111, 1303, 1308, 1309, 1310, 1311, 1404, 1405, 1502, 1701, 2200, 2201, 2202, 2203, 2204, 2205, 2206, 2207, 2210, 2215, 2222, 2223, 2403, 2405, 5201, 5700 & 5701 showed no significant RPS release. Microscopic observations of the cultures of the 13 EPS positive strains, done throughout the growth period, showed the presence of a rather large number of different morphological forms, quite similar to those previously described. Basically, these forms could be ascribed to two morphological shapes, depending on the presence or absence of a pellicle surrounding the capsule that may be present around the trichomes. Within each shape (pellicled or unpellicled) noticeable morphological differences, depending on the developmental stage, were also observed. From the numerous microscopic observations throughout the culture period, a general scheme of the cell cycle of the cyanobaterial strains growing in liquid medium was drawn. This scheme, reported in Figure-1 omitting those stages that were only rarely observed in the cultures (i.e. wound trichomes), proved to be useful to describe the morphological status of the cultures at the time of harvest. Indeed, when the cultures were harvested and the released polysaccharides were recovered from the culture medium, the type of predominant morphological form observed was different among the strains (Table 1). In most cases, the vegetative trichomes(stage 3 of Figure 1) predominated, even if a rather large number of mature trichomes (stage 4), characterized by a division plane not perpendicular to the longitudinal axis of the filament, was also observed.Out of 13 ruthenium red positive cyanobacteria five forms produced large amount of extracellular polymer (ECP). Maximum thickness of polysaccharidic material measured was 5.68 µm in *Nostoc commune (1203)* followed by *Aulosira prolifica (2300)* (4.25 µm), *Anabaena* sp. (2224), *Anabaena oryzae (2221)* (2.84 µm) and 1.42 µm in *Chroococcus* (2400). Other cultures showed very thin, tightly bound (<1 µm) layer of polysaccharidic material. Young trichomes (stage 2), sporadically surrounded by a gelatinous capsule, were quite unusual, being abundant only in strain AZAR NO1203 . Nine strains showed structures similar to those described above but surrounded by capsules possessing an external pellicle (Figure 2a). The pellicled stages 4P and 5P, that are the natural maturation of the vegetative trichomes 3P, were the predominant forms only in very few

strains, while pellicled wound trichomes (Figure 2b), probably originated from stage 4P, were only rarely observed.

Morphology of the Exocellular Investments and Polysaccharide Release

At the harvesting time, the EPS-producing cyanobacterial strains showed different types of exocellular investments (Table 1). Capsular envelopes were present in 13 strains but with different morphological characteristics: (i) capsules surrounded by an external pellicle which were observed in four strains; (ii) capsules devoid of pellicle which surrounded the trichomes of strain AZAR NO 1203 and (iii) capsules not homogeneously distributed along the filaments and without sharp outlines present in strains AZAR NO 2300. With regard to the remaining capsulated strains, in two of them (strains AZAR NO 1205 and 2224), the capsules surrounded only a limited number of trichomes and in the other nine a pellicle external to the capsule was only sporadically observed. When the cultures were stained with Alcian blue, a specific dye known to react with acidic polysaccharides, an intense pigmentation appeared in all strains, showing that their capsules are at least partially composed of polysaccharides containing acidic groups. In some cases, the Alcian blue staining also evidenced the presence of a pellicle inside the capsule, just surrounding the trichomes or of wide veils only partially linked to the trichomes most probably due to the staining of the aliquot of polysaccharide solubilized into the liquid medium.

More than 70% of the EPS-producing strains showed the formation of slimy investments. In most cases, these structures loosely surrounded a certain number of trichomes, which may be capsulated or devoid of capsules depending on the strain; in some cases, broad, irregular lumps of slime free of cells were also observed.

During photoautotrophic growth, all the strains released polysaccharidic material into the culture medium, with different RPS daily productivities (Table 1).

According to this parameter, the strains can be divided into three groups: a first one, composed of the most Table 1. morphological characteristics at the time of harvesting and mean RPS productivities of the AZAR productive strains (AZAR 1203, 2300), with productivities higher. (dried and dialysed RPS) l); a second group, including those strains that showed a mean RPS productivity in the range; and the last one composed of those strains that showed a very low mean productivity, below 1 µm mg (RPS) .

Chemical Characteristics of the Polysaccharides Released by the Cyanobacterial Strains

The RPS samples obtained from the cultures of the cyanobacterial strains were characterized by a rather wide range of concentration of phenol±sulphuric acid-reactive sugars, reported in Table 2 as percent of total carbohydrates on the dry weight of dialysed RPS.All the forms exhibited considerable amount of extracellular carbohydrate and an appreciable amount of extracellular protein also. Of the thirteen cultures tested *Phormidium* sp. (1406) produced maximum carbohydrate content of 610 µg ml^{-1} followed by *Chroococcus minutes* (2400) and *Gleocapsa* sp. (1005) which produced 550 and 405 µg ml^{-1} respectively. However the organisms did not show any profuse polysaccharidic contents on staining. The viscosity of the spent medium in these forms also ranged from 0.9 to 0.98 cps. Although *Anabaena oryzae (2221)* showed a total carbohydrate content of 215 µg ml^{-1} and thin layer of mucilage however the viscosity of the medium was a maximum of 2.512 cps followed by *Nostoc calcicola* (1205), *Nostoc commune (1203)*, *Aulosira prolifica (2300)* and *Anabaena* sp. (2224), that showed viscosity of >1 cps.

Variorum Multi-Disciplinary e-Research Journal
Vol.,-04, Issue-I, August 2013

All the released polysaccharides showed the presence of uronic acids (Table 2), thus confirming the acidic nature already evidenced by the Alcian blue staining. The concentration of uronic acids in the RPSs ranged from less than 1% to about 30% of the RPS dry weight.

Most RPSs also showed the presence of hexosamines, in some cases at rather high concentrations, exceeding 3.3% of RPS dry weight.The elemental analysis of the RPS samples evidenced that besides carbon, hydrogen and oxygen, nitrogen was always present in concentrations that were often much higher than those due to the presence of hexosamines.

Calculating the amount of the peptidic moiety from these data, it was evident that most RPSs contain a rather large amount of this constituent (Table 2); in particular, protein concentration was strikingly high in the polymer produced by strain AZAR 1108.

Discussion

The above results confirm the well-known morphological complexity of cyanobacterial and represent the first description of the cell cycle obtained with strains growing in liquid cultures instead of with colonies growing on solid media. It is interesting to stress that all the cyanobacterial strains tested, even those that did not show release of polysaccharidic material into the culture medium, maintained the capability to synthesize outermost poly-saccharidic investments even after several years of conservation in a culture collection, showing that this is a stable feature, as observed in many other EPS-producing cyanobacteria (De Philippis & Vincenzini 1998).

The thirteen RPS-producing cyanobacterial strains showed a wide heterogeneity in their exocellular mucilaginous investments; nevertheless, it is possible to identify three main types of morphological shapes: (i) capsules surrounded by an external pellicle, (ii) capsules with sharp outlines but without an external pellicle, (iii) slimy investments, that can give raise to large globular lumps or that can loosely surround the trichomes, without following their shape. These differences do not seem to be linked to the original environment of the strains;

A different picture arises from the RPS daily production:

the highest productivities were shown, by strains isolated from soils, suggesting that they need, in their natural habitats, to produce large amounts of mucilaginous material in order to create a more favourable microenvironment (e.g. in order to trap the quantity of water necessary to ensure the proper functionality of cells or to chelate cations essential to cell metabolism). On the other hand, the strains isolated from symbioses with plants showed lower productivities in comparison with most of the strains isolated from other environments. The results described above, dealing with a quite large number of strains originally isolated from different habitats and showing different types of exocellular envelopes, suggest that the general chemical characteristics of the RPSs are not correlable with the origin of the strains or with the morphology of the outermost investments. Furthermore, the composition of the RPSs released by the three symbiotic strains, characterized by low level of protein and a rather high level of uronic acid content, seems to exclude, at least for these strains, the previously suggested need to produce polysaccharides with a low content of uronic acids and a high content of protein in order to have a good adhesion of cells to plant roots (Gantar et al. 1995). As a final remark, it must be stressed that the RPS productivities are quite promising, being comparable with those shown by some of the most productive cyanobacterial strains so far described. However, the actual potential of these strains for the production of biotechnologically valuable polysaccharides may be envisaged only after the completion of further chemical and physico-chemical characterizations of the polymers.

Variorum Multi-Disciplinary e-Research Journal
Vol.,-04, Issue-I, August 2013

References

Bazzichelli, G., Abdelahad, N. & Ventola, F. 1985 Structural modifications in the extracellular investment of Nostoc commune VAUCH during the life cycle. I. Motile and nonmotile hormogo-nium, biseriate stage. Journal of Ultrastructure Research 91, 174-181.

Bazzichelli, G., Abdelahad, N. & Ventola, F. 1989 Structural modifications in the extracellular investment of Nostoc commune VAUCH during the life cycle. III. Macrocolonies. Archiv fuÈr Hydrobiologie Suppl. S. 82, 365-370.

Bergman, B., Rai, A.N., Johansson, C. & SoÈ derbaÈ ck, E. 1992 Cyanobacterial-plant symbioses. Symbiosis 14, 61-81.

De Philippis, R. & Vincenzini, M. 1998 Exocellular polysaccharides from cyanobacteria and their possible applications. FEMS Micro-biology Reviews 22, 151-175.

Dodds, W.K., Gudder, D.A. & Mollenhauer, D. 1995 The ecology of Nostoc. Journal of Phycology 31, 2-18.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. & Smith, S. 1956 Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28, 350-356.

Flaibani, A., Olsen, Y. & Painter, T.J. 1989 Polysaccharides in desert reclamation: compositions of exocellular proteoglycan complexes produced by filamentous blue±green and unicellular green edaphic algae. Carbohydrate Research 190, 235-248.

Galambos, J.T. 1967 The reaction of carbazole with carbohydrates. I. Effect of borate and sulfamate on the carbazole color of sugars. Analytical Biochemistry 28, 350-356.

Gantar, M., Rowell, P., Kerby, N.W. & Sutherland, I.W. 1995 Role of extracellular polysaccharide in the colonization of wheat (Triticum vulgare L.) roots by N2-®xing cyanobacteria. Biology and Fertility of Soils 19, 41-48.

Huang, Z., Liu, Y., Paulsen, B.S. & Klaveness, D. 1998 Studies on polysaccharides from three edible species of Nostoc (Cyanobacte-ria) with di€erent colony morphologies: comparison of monosac-charide compositions and viscosities of polysaccharides from field colonies and suspension cultures. Journal of Phycology 34, 962-968.

Hill, D.R., Peat, A. & Potts, M. 1994 Biochemistry and structure of the glycan secreted by desiccation-tolerant Nostoc commune (Cyano-bacteria). Protoplasma 182, 126-148.

Martin, T.C. & Wyatt, J.T. 1974 Extracellular investments in blue green algae with particular emphasis on the genus Nostoc. Journal of Phycology 10, 204-210.

Mollenhauer, D., BuÈ del, B. & Mollenhauer, R. 1994 Approaches to species delimitations in the genus Nostoc VAUCHER 1803 ex BORNET et FLAHAULT 1888. Algological Studies 75, 189-209.

Montreuil, J., Bouquelet, S., Debray, H., Fournet, B., Spik G. & Strecker, G. 1986 Glycoproteins. In Carbohydrate analysis ± a practical approach, eds. Chaplin, M.F. & Kennedy, J.F., pp. 143± 204. Oxford: IRL Press. ISBN 0-947946-44-6.

Reddy, K.J., Soper, B.W., Tang., J. & Bradley, R.L. 1996 Phenotypic variation in exopolysaccharide production in the marine, aerobic nitrogen-fixing unicellular cyanobacterium Cyanothece sp. World Journal of Microbiology & Biotechnology 12, 311-318.

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. & Stanier, R.Y. 1979 Generic assignments, strain histories and properties of pure cultures of Cyanobacteria. Journal of General Microbiology 111, 1-61.

Rippka, R. & Herdman, M. 1992 Pasteur Culture Collection of Cyanobacteria. Catalogue and Taxonomic Handbook. Vol. 1:Catalogue of strains. Paris: Institute Pasteur.

Rother, J.A. & Whitton, B.A. 1989 Nitrogenase activity of blue green algae on seasonally ¯ooded soils in Bangladesh. Plant and Soil 113, 47-52.

Sinha, R.P.&HaÈ der, D.P. 1996 Photobiology and ecophysiology of rice field cyanobacteria. Photochemistry and Photobiology 64, 887-896.