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BIOCHEMICAL ANALYSIS OF CYANOBACTERIA OF SAV HOTSPRING

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Hot Springs in India are usually formed due to volcanic or tectonic activities, symbolized by high temperatures. These thermal springs or hot springs are home to thermophilic and thermotolerant organisms. Cyanobacteria are among the few organisms that can tolerate and survive under high temperatures. Water samples for the Sav hot water spring in Mahad taluka of Raigad district in Maharashtra have been collected to conduct a biochemical analysis.

Keywords: Cyanobacteria, Hotspring, Maharashtra, Thermophiles, Tolerant species.

Introduction

Blue Green Algae are also termed as Cyanobacteria as they are more closely related to bacteria than algae. Cyanobacteria evolved about 2.8 billion years ago[1]. Cyanobacteria can be found in almost every terrestrial and aquatic habitat- oceans, freshwater, damp soil, temporarily moistened rocks in deserts, bare rocks and soil and even in extreme habitats. Among aquatic habitats, stream of hot water springs are one of the best habitats for blue-green algae[2,3]. Cyanobacteria can cope with extremes of pH, temperature, salinity, acidity and accumulation of heavy metals and hence are known as Extremophiles. Some can even thrive in combinations of more than one stress and hence are known as polyextremophiles. However, there is variation in the level of tolerance. Organisms that grow optimally at temperatures below 15°C are known as psychrophiles, while psychrotolerant organisms can survive at temperatures below 0°C but exhibit optimal growth at 20–25°C[4]. Thermophiles are organisms that live at relatively high temperatures of at least 60ºC. Those organisms with an optimal growth temperature between 60 - 80ºC are defined as thermophiles, whereas those growing optimally at temperatures of >80ºC are referred to as hyperthermophiles. *Thermus thermophilus* is a well-known thermophile. *Mastigocladus laminosus*is a cyanobacterium that occurs in thermal springs throughout the world[5,6].

A hot spring is a spring produced by the emergence of geo-thermally heated groundwater that rises from the earth's crust. While some of these springs contain water that is at a safe temperature for bathing, others are so hot that immersion can result in injury or death. Much of the heat is created by the decay of naturally radioactive elements. An estimated 45-90% of the heat escaping from the earth originates from the radioactive decay of elements mainly located in the mantle. The major heat-producing isotopes in earth are Potassium-40, Uranium-238, Uranium-235, and Thorium-232[7-9]

Material and Methods

During the field exploration in 2019, water samples were collected from the Sav hot water spring in Mahad taluka of Raigad district in Maharashtra for the study of extremophiles (Fig. 1). SAV site has two different Kunds (Water bodies). Samples collected from Kund A were labelled SAV A and those from Kund B were labelled as SAV B (Fig. 2). Soon after the collection of the sample TDS (total dissolved solids), pH, conductivity and temperature were recorded.

Fig. 1 - Google image of Sav hot springs

In the lab, the samples were kept in a water bath maintaining a temperature of 35-40ºc.

Further, BG11 (Blue-Green 11) nutrient media was used to culture the collected samples. Cultured samples were kept in a culture room for optimum growth (Fig. 3 & Fig. 4).

Biochemical analysis was carried out for the estimation of chlorophyll, water-soluble pigments, carbohydrates, and proteins.

For chlorophyll extraction and estimation: 1gm of algal sample was ground in 2-3ml of 80% acetone. This suspension was centrifuged at 3000rpm for 5 min, to remove the cell debris. The supernatant was collected, and absorbance was recorded at 663nm for chlorophyll a and 645nm for chlorophyll b.

The concentration of biomass was calculated by taking the absorbance of pigment extract at 663 nm for chlorophyll a and 645 nm for chlorophyll b against 80% acetone as the blank.

The following formulas were used in the calculation[10]

For Chlorophyll a (mg/l):

$$= (12.7 \times A_{663}) - (2.69 \times A_{645})$$

For Chlorophyll b (mg/l):

$$= (22.9 \times A_{645}) - (4.68 \times A_{663})$$

For total Chlorophyll (mg/l):

$$(a + b) = (8.02 \times A_{663}) + (20.2 \times A_{645})$$

For quantitative estimation of phycocyanin (PC), allophycocyanin (APC) and phycoerythrocyanin (PE) the algal sample was treated with 0.15M NaCl and the cells were disrupted by sonication for half an hour. Separation was achieved by centrifugation at 6000 rpm for 5 min. The supernatant was collected and absorbance was measured at 565 nm, 620nm and 650nm.

Pigment concentration was calculated by the following equations[11]

For C-Phycocyanin (PC):

$$= \frac{A_{650} - 0.7 \times A_{650}}{7.38}$$

For Allophycocyanin (APC):

$$= \frac{A_{650} - 0.19 \times A_{620}}{5.65}$$

For Phycoerythrocyanin (PE):

$$= \frac{A_{665} - 2.8 \times (PC) - 1.34 \times (APC)}{12.7}$$

Hedge and Hofreiter (1962) method was used for the carbohydrate estimation[12]. 1gm of the Sample was taken into a boiling tube and hydrolyzed by keeping it in a boiling water bath for three hours with 5ml of 2.5N-HCL. The sample was allowed to cool at room temperature and then it was neutralized with solid sodium carbonate until effervescence ceases. Further, the sample was centrifuged, and the supernatant was collected. 0.5 and 1.0ml aliquots were taken for analysis. However, the standards were prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of working standard. '0' served as blank, making the volume up to 1.0 ml in all the test tubes with distilled water then adding 4.0 ml of anthrone reagent heated for 8 min in a water bath and cooled rapidly and read the green to dark green color at 630 nm.

Bradford method (1976) was used for the protein content estimation[13]. For sample preparation, 50 ml of a sample was

(a) SAV – A (b) SAV – B

Fig. 2 - Sites of Sav hot springs

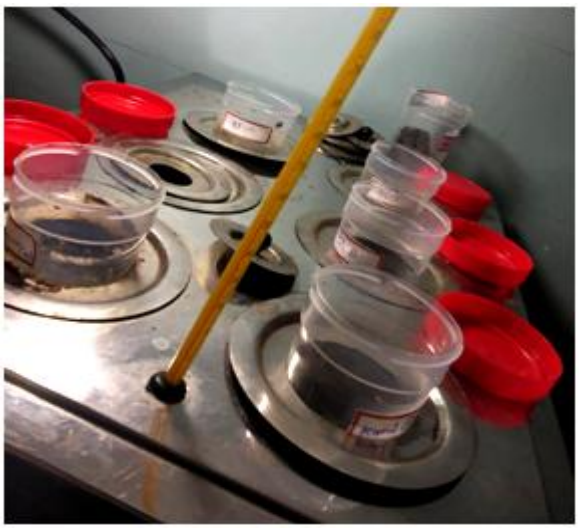

Fig. 3 - SAV-A samples kept in BG11 medium

Fig. 4 - Growth in the algal sample on cultured media after 3 weeks

taken in Phosphate buffer solution and ground in a cold mortar and pestle. The solution with extract was centrifuged at 8000 rpm for 10 min. Discard the chlorophyll containing supernatant and re-suspend the algal pellet in a small amount of buffer centrifuged at 8000 rpm for 5 minutes. The supernatant was stored for further procedures. The standards were prepared by taking 0.2-1.0 ml of working standard. 5 ml of Bradford reagent was added to each test tube and absorbance was taken at 595 nm and the readings were noted down.

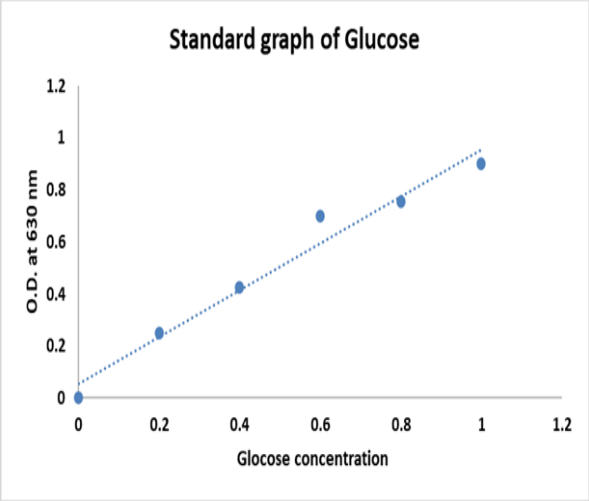

Fig. 5 - Standard graph of glucose using the readings obtained

A. Estimation Chlorophyll:

Chlorophyll a and b were estimated for both samples. The readings recorded are given in Table 2. Using the absorbance obtained, chlorophyll a, chlorophyll b and total chlorophyll were estimated and given in Table 3.

In the case of SAV A sample, the Chlorophyll a estimated was 10.830mg/l and Chlorophyll b was 2.486mg/l. The total chlorophyll of the sample was estimated to be 13.276mg/l. In the case of SAV B sample, chlorophyll a was found to be 11.839mg/l and Chlorophyll b was estimated to be 0.934mg/l. The total chlorophyll of the sample was estimated to be 12.731mg/l.

Results and Discussion

The TDS, pH, conductivity, and temperature of the sample collected from SAV A and SAV B are given in Table 1. The TDS and temperature of the sample SAV A were 870 ppm and 40ºc, respectively. Similarly, the TDS and temperature of the sample SAV B were 766 ppm and 37ºc, respectively. The pH and conductivity of the collected water samples from SAV A and SAV B were 7.216, and 1982.33µs, respectively.

Biochemical assays were carried out for analysis and estimation of chlorophyll, soluble pigments, carbohydrates and protein.

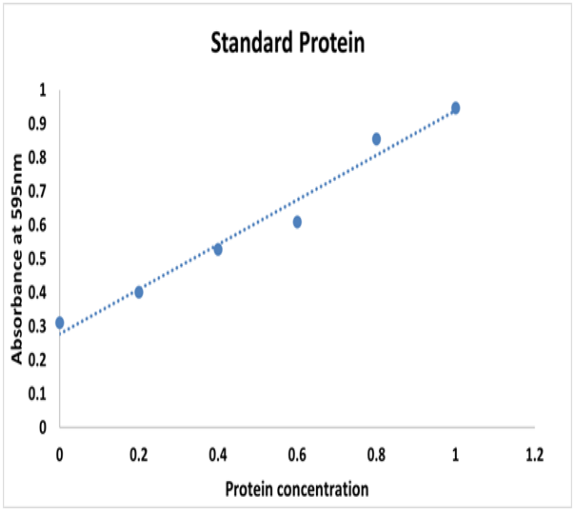

Fig. 6 - Standard graph of protein using the readings obtained

B. Estimation of water-soluble pigments (phycocyanin, allophycocyanin and phycoerythrin):

Cyanobacteria contain certain water-soluble pigments (phycocyanin, allophycocyanin and phycoerythrin) that cannot be found in any other algal species. Therefore, the occurrence of these pigments in algal extract indicates the presence of cyanobacteria. Hence it was analyzed and estimated and given in Table 4.

Using the absorbance, the concentration of Phycocyanin, Allophycocyanin and Phycoerythrin were estimated and given in Table 5:

Table 1: Water analysis (TDS, pH, conductivity, temperature).

Sr. No.	Analysis Parameters	SAV A	SAV B
1	TDS	870 ppm	766 ppm
2	pH	7.216	7.216
2	conductivity	1982.33µs	1982.33µs
2	temperature	40ºc	37ºc

Table 2: Absorbance of Pigment extract at 663 nm and 645 nm.

SAMPLE	Absorbance at 663 nm (Chlorophyll a)	Absorbance at 645 nm (Chlorophyll b)
SAV A	0.915	0.294
SAV B	0.983	0.240

Table 3: Chlorophyll concentration estimated for respective samples.

Sample	Chlorophyll a mg/l	Chlorophyll b mg/l	Total chlorophyll mg/l
SAV A	10.830	2.486	13.276
SAV B	11.839	0.934	12.731

Table 4: Absorbance recorded at 565nm, 620nm and 650nm.

Sample	Absorbance at 565nm for phycocyanin	Absorbance at 620nm for allophycocyanin	Absorbance at 650nm for Phycoerythrocyanin
SAV A	0.745	0.118	0.889
SAV B	0.652	0.142	0.965

Table 5: The concentration of pigments in respective samples.

Sample	Absorbance at 565nm for phycocyanin	Absorbance at 620nm for allophycocyanin	Absorbance at 650nm for Phycoerythrocyanin
SAV A	0.036	0.153	0.030
SAV B	0.039	0.166	0.025

C. Estimation of Carbohydrates

By using Hedge and Hofreiter (1962) method carbohydrates were estimated. The readings were given in Table 6. Carbohydrate estimation was carried out to know the exact amount of carbohydrate present in the sample. Concentrations of glucose present in the samples were calculated from the graphical equation and the values were given in Table 7.

It indicated SAV B had a higher glucose level of 1.025 than that of SAV A which was found to be 0.884 (Fig. 5).

D. Estimation of Protein

Protein estimation was carried out to know the exact amount of protein that could be present in the sample. The readings recorded were given in Table 8. Concentrations of protein present in the samples were calculated from the graphical equation and the values were incorporated in Table 9.

In SAV A protein concentration was estimated to be 0.593 while that of SAV B was 0.479. Hence SAV A had a higher level of protein concentration (Fig. 6).

Conclusion

The algae collected from SAV hot water springs are not confined to a particular temperature range and they can survive a temperature range of 35-45ºc. The cyanobacteria were cultured in BG11 media at room temperature, and it showed positive results in three weeks. Hence, it can be concluded that the algae were not strictly thermal.

Table 6: Absorbance at different concentrations of glucose.

SL No.	Distilled Water (ml)	Anthrone (ml)	Glucose (ml)	Absorbance at 630 nm
1	1.0	4	0.0	0.000
2	0.8	4	0.2	0.252
3	0.6	4	0.4	0.424
4	0.4	4	0.6	0.698
5	0.2	4	0.8	0.756
6	0.0	4	1.0	0.899

Table 7: Concentration of Carbohydrate as calculated from the graph.

Sample	Aliquots of sample (ml)	Absorbance at 630 nm	Glucose concentration
sav A	0.5	0.620	0.628
sav A	1.0	0.849	0.884
sav B	0.5	0.281	0.250
sav B	1.0	0.976	1.025

Table 8: Absorbance at different concentrations of protein.

Sr. No.	Sample	Phosphate Buffered Saline (PBS)	Bradford reagent	Standard protein	Absorbance at 595 nm
1	Blank	1.0	5	0.0	0.311
2	BSA	0.8	5	0.2	0.401
3	BSA	0.6	5	0.4	0.528
4	BSA	0.4	5	0.6	0.609
5	BSA	0.2	5	0.8	0.856
6	BSA	0.0	5	1.0	0.948

Table 9: Concentration of proteins as calculated from the graph.

Sample	Aliquots of sample (ml)	Absorbance at 595 nm (y)	Protein concentration(x)
SAV A	0.5	0.653	0.567322239
SAV A	1.0	0.670	0.593040847
SAV B	0.5	0.546	0.405446293
SAV B	1.0	0.595	0.479576399

In the case of chlorophyll a, samples collected from SAV B site showed much higher concentration compared to SAV A samples. Thus, it may be concluded that SAV B site possess high phytoplankton biomass and faster eutrophication[14].

Whereas, in the case of chlorophyll b, samples collected from SAV A demonstrated higher amounts than that of SAV B sample. This might be because SAV A plants are shade-adapted since higher the amount of chlorophyll b, higher the range of wavelengths that can be absorbed[15]. Total chlorophyll for SAV A was slightly higher than SAV B.

Carbohydrate estimation indicated SAV B had a higher glucose level of 1.025 than that of SAV A which was found to be 0.884. The variation in carbohydrate content may be due to variations in temperature, llight intensity and nutrient conditions of the two sites[16].

As per our observations regarding protein concentrations, it can be concluded that SAV A had a higher level compared to SAV B. The differing protein content between microalgal species might be attributed to their different genetic traits. However, it might also be due to the variations in environmental conditions and the growth phase at which the microalgal biomass was harvested[17].

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