# MATERIAL AND METHODS

BARR. BALASAHEB KHARDEKAR LIBRARY

## Study Area :

Present work was carried out in the preview of Satara district. Satara district is one of the important districts within the western Maharashtra. It occupies position on the east side of western ghats.

Satara district lies between 16° 15' and 18° 10' North latitude and 73° 45' and 75° 0' East longitude. It occupies an area of 4792 square miles (See Map I). For administrative purpose Satara is divided into eleven subdivisions - seven in the east (Koregaon, Khatao, Vaduj, Patan, Phaltan, Karad and Dahiwadi) and four on the west (Mahabaleshwar, Medha, Wai and Khandala). District Satara is the head of the valley of the Krishna River. Krishna river flows first to the south and then to the east, passing across the whole district from its north west to its south east corner. From the central plain of the Krishna, eight valleys branch to the hills. In the west the district is rugged and well watered. In the east it is flatter, but parched and barren.

The climate of Satara district is one of the best in western Maharashtra. The hot season generally sets in about the beginning of March. In ordinary years the heat reaches its maximum in April. In the early part of May the temperature somewhat declines. During the first half of June a gradual change is felt as soft and refreshing of the monsoon. For about a fortnight in July the rain falls heavily. But during the rest of the monsoon there are two or three heavy falls of a week. The temperature is cool and equable.

The Sahyadri ranges in the extreme west, the Mahadev range passing at right angles from the Sahyadris east across the north of the district and the spurs of the Sahyadris chiefly stretching east and south-east and the south – running spurs of the Mahadev hills divide Satara into three belts, a western, a central, and an eastern.

The Krishna and Koyna valleys are in the centre of the district. Krishna valley is the finest valley in the district. These broad valleys are the richest part of the district. Well watered black soil yields a succession of rich crops which keeps area green till February.

The Satara district contains two main systems of hills; the Sahyadri range and its offshoots, and the Mahadev range and its offshoots. Five spurs pass east and south east from the Sahyadris water parting between Venna on north west and Urmodi on south west becomes feeders of the Krishna.

The top of Mahabaleshwar, the highest point in the district is about 1427.27 meters above the sea.

Within Satara limits there are fifty six notable hills and hill forts. The seven hills in the Satara sub-division are Ajinkyatara, Yevateshwar, Sajjangad, Petova, Ghatai, Pateshwar and Shulpani, varying from 909.09 meters to 1212.12 meters above sea level. The Satara hill about 1002.12 meters above the sea level and 363.63 meters above the plain.

The whole of Satara falls within the Deccan trap area. In other parts of the west Deccan the hills are layers of soft trap separated by flows of hard basalt and capped by laterite or iron clay.

The Satara forest land belong to three groups, the evergreen Sahyadri forest land, the slopes of the spurs that run east from the Sahyadri and the bare or bush sprinkled hills to the east of the Krishna.

The soils of district belong to three main classes, red in the hills, black and light coloured in the plains. The Black or *Kali* soil is generally found in belts lying along the banks of the leading streams, the breadth of the belt varying with the size of the stream. The Krishna valley is found the broadest belt of this rich soil. The leading light coloured soils are the *mal ran* or *murum mal* a hard rocky soil commonest at the bases of the more eastern hills. The same soil mixed with red at the foot of the Sahyadies form one variety of the soil called as *tambadi* or red. Near the heads of the streams which issue from the Sahyadries, the soil of the valleys is red or *tambadi* and yields most of the rice grown in the district. On the hill tops where water cannot be sufficiently confined for rice tillage the soil used for Kumri or wood-ash tillage. There is also the soil called *chunkhadi* which is strongly charged with lime. Lime is also found in black soils near river beds. The soil of the Sahyadris west of the Yerla is generally good, and the soil of the Krishna valley is especially rich. East of the Yerla land becomes poorer and the proportion of black soil becomes much smaller.

Present work was carried out for:

1. Isolation of blue green algae
2. Purification of blue green algae
3. Characterization of blue green algae

Collections were made for the soil samples from especially western parts of Satara district. Reason behind this is the western part of Satara district (See map) is a hilly area with offshoots of Sahyadri ranges running eastward. Paddy is the major crop of this area. The cultivation of paddy practiced is different than that of eastern part. The paddy saplings are prepared in a nursery bed within the corner of the field and then

after a month or so are transplanted. Usually transplantation is made in a waterlogged condition of the field. Due to the water logging the conditions become conducive for the natural growth of blue green algae. After few weeks of transplantation of the saplings good amount of growth of blue green algae along with other filamentous green algae is seen. Samples were collected from these fields at both times. At first the samples were collected during the water logged condition of standing crop and secondly after harvest the soil samples were collected in order to raise pure cultures.

## Isolation of blue green algae from the soil samples:

For the isolation of blue green algae from soil samples, samples were collected in the month of December to January, after the harvest of the paddy and sufficient drying of the soil. During collection soil sample was collected removing the upper 1-2cm crust of soil and taking the lower soil between 5cm depth. The samples collected were brought in the laboratory, after homogenizing the larger clumps of soil, the samples were made into uniform coarse silt of coarse size. The soil samples were then dried under shade for a week or so. 10gm such dried soil sample was inoculated in respective culture medium and kept for incubation. Growth in the flasks was observed frequently.

## Preparation of glass ware:

Glass ware required was petridishes, 1ml pipette, 2ml pipette, screw capped glass bottles, 250 ml conical flasks etc.

All glass wares were cleaned using chromic acid. Then they were washed with detergent and distilled water. Before use they were again washed with distilled water and sterilized. Pipettes were plugged with cotton at their sucking ends and wrapped in craft paper. Petri dishes were also wrapped in a craft paper and then sterilized in a hot air oven at $180^0$C for 1hr or in an autoclave at 15 lb/in$^2$ pressure for 20 min.

## Culture Media:

Variety of culture media for growing algae are available for studying the entire algal component, media containing combined nitrogen is used. However, for nitrogen fixing diazotrophic cyanobacteria, media without nitrogen is used. However, it is advisable to raise the enrichment cultures separately in media and without nitrogen.

Generally, Fogg's medium without N is used for nitrogen fixing and with N (0.5g $KNO_3$/liter) for non-nitrogen fixing blue green algae.

## Composition of Culture Media:

For enumeration of algae in soil, the following procedure is adopted-

- **Salt concentration:** The total salt concentration, mostly dependent on the ecological origin of the organism.
- **Major ionic components:** The composition and concentration of major ionic components such as potassium, magnesium, sodium, calcium, sulphate and phosphate.
- **Nitrogen source:** Nitrate, ammonia and urea are widely used as the Nitrogen sources which may depend upon species performance and the pH optimum. For nitrogen fixing organisms, no nitrogen supply is required in the culture medium.
- **Carbon source:** Inorganic carbon is usually supplied as $CO_2$ gas in 1-5% mixture with air. Another means of supplying carbon is as bicarbonates.
- **pH:** Optimum pH required is 7.0 to 7.5.Slightly acidic pH may be used to prevent precipitation of calcium and magnesium and some of the trace elements.
- **Trace elements:** usually supplied in a mixture at concentrations essential for growth and maintenance for the stability of trace elements, chelating agents such as citrate and EDTA are used.
- **Vitamins:** Few may require vitamins such as thiamine and cobalamine for growth.

## Composition of Media:

**A. Fogg's Medium** (Fogg, 1949)

| | |
|---|---|
| $KH_2PO_4$ | 0.2 gm/lit |
| $MgSO_4.7H_2O$ | 0.2 gm/lit |
| $CaCl_2.2H_2O$ | 0.1 gm/lit |
| $A_5$ Micronutrient solution | 1.0 ml |
| Fe- EDTA stock solution | 1.0 ml |
| pH | 7.0 |

**B. BG – 11** (Staneir et al, 1971)

| | |
|---|---|
| $NaNO_3$ | 1.5gm/lit |
| $KH_2PO_4$ | 0.04 gm/lit |
| $MgSO_4.7H_2O$ | 0.075 gm/lit |
| $CaCl_2.2H_2O$ | 0.036 gm/lit |
| $Na_2CO_3$ | 0.02 gm/lit |
| Ferric Ammonium citrate | 0.006 gm/lit |
| $C_6H_8O_7.H_2O$ | 0.006 gm/lit |
| $A_5$ Micronutrient solution | 1.0 ml |
| Fe- EDTA stock solution | 1.0 ml |
| pH | 7.5 |

**C. $A_5$ Micronutrient solution**

| | |
|---|---|
| $H_3BO_3$ | 2.86 gm/lit |
| $Mn_2Cl_2.6H_2O$ | 1.81 gm/lit |
| $ZnSO_4.7H_2O$ | 0.222 gm/lit |
| $Na_2MoO_4.2H_2O$ | 0.0177 gm/lit |
| $CuSO_4.5H_2O$ | 0.079 gm/lit |

The pH is adjusted before autoclaving the medium. For solid media agar is added at 1.5% (w/v).

## Preparation of EDTA

Dissolve 26.1 g of (EDTA) ethylene diamine tetra-acetic acid (disodium salt) in 268 ml of 1N potassium hydroxide solution and add 24.9 g of ferrous sulphate. Make the volume to 1 liter. Aerate the solution overnight to produce a stable complex marked by the change in color to dark brown. Make up the volume again to 1 liter. Add 1 ml of this stock solution to 1 liter medium to give 5 ppm of iron.

All the stock solutions were prepared and kept in clean glass bottles. Using those stock solutions medium was prepared. For adjusting the pH, 0.1N HCl and 0.1N NaOH solutions were used.

Media was poured in clean screw capped bottles (100ml in each bottle). Medium was sterilized at 15 $lb/in^2$ pressure for 20 min. in an autoclave.

For preparation of solid medium agar was added at 1.5% (w/v). Medium was prepared and sterilized at 15 lb/in pressure for 20 min. in an autoclave. Sterilized solid medium was poured in previously sterilized petri dishes aseptically using Laminar flow and spirit lamps. Medium was allowed to cool and solidify. After solidification it was ready for use.

## Isolation and Purification:

For the isolation of blue green algae from soil samples various methods have been followed in different laboratories, number of protocols are also available, which may be browsed on internet or can be read in respective literature. (Andersen, R. A. 2005, Bagchi, S. N. et al, 2010, Hellebust J. A. and J. S. Craigie, 1973)

We have designed our protocol which is combination of different protocols available.

1. Collect surface soil samples within depth of 1 - 5 cm.
2. Air dry the sample in shade and homogenize into fine form (remove the debris carefully to avoid contamination)
3. Weigh 10 gm of soil samples in and transfer to 100ml sterilized medium.
4. Flasks are shaken vigorously and incubated undisturbed in a growth chamber under optimal condition.
5. Observe the culture successfully for 25 to 30 days regularly.

Culture flasks were observed regularly for the growth of different forms and when apparent, 5 - 6 wet mounts from each flask were prepared lifting the algal growth.

The wet mounts were lifted from glass surface, surface of the medium, surface of the soil as well as from the floating growth. The mounts were observed under the microscope. For isolation individual colonies were lifted using inoculation needle and suspended in 5ml sterile medium in a culture tube. Culture tubes are vigorously shaken to make a homogenize suspension. 0.5ml of this suspension was inoculated in fresh sterilized culture medium in another culture medium flask. In order to have accuracy in isolation 0.5ml suspension was also inoculated on agar plates. (containing culture media). The plates were rolled for even distribution of suspension and incubated under appropriate growth conditions. Initially the plates were placed in upright position for 1 day and then in an inverted position. The plates were also observed regularly and isolated colonies were picked up and examined under microscope. When found unialgal cultures, similar colonies were picked up and transferred to fresh culture medium for further growth.

The algal growth observed in the culture flasks was most of the time mixture of different forms. Our aim in culturing the blue green algae was to isolate some forms in pure and axenic cultures. All the forms grown in the medium were of course observed and identified to species level using standard literature (Frisch 1955, Smith G. M. 1950, Stanier 1979, Ripkka 1979, Santra 1993).

However those found promising cultural characteristics were isolated and practiced in axenic cultures. Numbers of methods have been employed for purification of various algae so that only desired forms can be isolated and propagated further.

During our experiment we followed the serial dilution method which was most convenient for its simplicity. Following protocol was followed for isolation and purification.

1. 1ml mixtures of algal growth suspend in 9ml sterile distilled water and shake vigorously.
2. Take out 1ml from the above and dilute it serially till dilution $10^4$.
3. Inoculate 1ml of above dilution in culture media and observe the unialgal growth.

Another simple method used during our experiment was the use of Pascheur pipette. For this method a glass capillary was drawn into a fine tip on a burner. This fine tip pipette is used to pick up either single filament or a colony to be inoculated in the medium.

Both the methods described above yielded good results for the isolation of pure forms to be grown into axenic cultures.

## Maintenance:

The cultures were maintained at 27±1$^0$C and 3000-4000 lux illumination under the photoperiod 16 : 8 hrs. The cultures were frequently observed whenever needed or medium is exhausted were sub-cultured to fresh medium. The process was continued to about one year till the completion of study.

Unialgal cultures are maintained on agar slants with appropriate medium contained in a screw capped culture tubes. The inoculated slants in 10 ml culture tubes with cotton pugs are incubated in a growth room for 7-10 days. The cotton plugs are then replaced by pre-sterilized, Bakelite screw caps provided with a rubber liner under aseptic conditions. Once sufficient algal growth appears on agar slants, culture tubes are then transferred to stock culture room for maintenance.

## Biochemical characterization:

## Measurement of Chlorophyll as an Index of Algal Growth

Blue green algae have chlorophyll a as the major light harvesting pigment along with carotenoids and phycobillins, as accessory pigments. These pigments are extracted by using specific organic solvents and quantified in terms of absorbance at particular wave lengths. Absorbance was measured on UV-Visible spectrophotometer.

The measurement of the chlorophyll at different stages of growth is often used as a growth parameter.

**Chemicals:**

Acetone, methanol.

**Cold extraction method**

Procedure:

1. Homogenize the algal culture and make up the volume. Take 10ml from this and centrifuge for 5min at 3000rpm.
2. Suspend the algal pellet in 10ml of 90% acetone.
3. Shake the contents thoroughly and keep in a refrigerator for 20-24 hrs for complete extraction.
4. Remove the samples from the refrigerator, shake well and centrifuge at 3000 rpm for 5 min. Discard the pellet.
5. Make up the volume if necessary with 90% acetone.
6. Read the optical density (O.D.) at 665, 645 and 630 nm using 90% acetone as blank.

Calculations:

$$\text{Chl. a} = 11.6(\text{O.D.}_{665}) - 1.31(\text{O.D.}_{645}) - 0.14(\text{O.D.}_{630})$$

## Estimation of Carotenoides

**Chemicals:**

Acetone, petroleum ether, methanol, potassium hydroxide, sodium sulphate.

**Methodology:**

1. Take 10 ml of homogenized algal suspension and centrifuge at 3000rpm for 10 min.
2. Discard the supernatant and wash the pellet 2-3 times with distilled water to remove traces of adhering salts.
3. To the pellet, add 2-3 ml of acetone (85%) and either subject to repeated freezing and thawing or grind the pellet in a pestle mortar or a homogenizer.
4. Centrifuge and collect the supernatant containing pigment. Store at $4^0$C.
5. Repeat the extraction from pellet with acetone till the supernatant acetone becomes colourless.
6. Pool all the fractions of supernatants and make a final known volume.
7. Take the O. D. at 450nm using 85% acetone as blank. Calculate the total amount of carotenoides in mg/ml as follows

$$C = \frac{D \times V \times f}{2500 \times 100}$$

Where, D = O. D. at 450nm, V = Volume of extract and f = Dilution factor.
(The average extinction coefficient of pigment is assumed to be 2500)

The final extract must have negligible amount of chlorophyll in it. To check this, the O. D. of the extract is measured at 450 and 670 nm using 85% acetone as blank. If the former absorption is 10 times the later, the extract is considered to have negligible chlorophyll.

## Estimation of Carbohydrate

Blue green algae contain a great variety of carbohydrates ranging from mono to polysaccharides, containing both aldehyde and keto groups. They are important structural and nutritional components of the cell.

Obviously, no single reagent will determine all these substances and that so-called "Total Carbohydrate" estimation in fact; determine only the simple sugars and their polymers. All colorimetric methods of total carbohydrate determination are based on the well-known Molisch test, which involves heating the material with strong (20N on more) $H_2SO_4$ and a color developer, which is either an aromatic amine or phenol. The whole process involves:

i) Hydrolysis of polysaccharides to monosaccharides.
ii) Dehydration and rearrangement of monosaccharides to form furfural (in case of pentoses) or hydroxy-methyl furfural (in case of hexoses).
iii) Reaction of furfural with colour developer to form a coloured compound.

**Phenol-sulphuric acid method (Dubois et al., 1956)**

Furfurals produce an orange yellow color with phenol. This reaction can be used to quantify carbohydrate.

**Requirements:**

a. Phenol: Liquify phenol at $50^0$C. Weigh 5g in a clean 100ml beaker. Dilute and make up the volume to 100ml.
b. Concentrated sulphuric acid (AR, Specific gravity 1.84)
c. Standard glucose solution: Dissolve 10mg glucose in 100ml distilled water in a volumetric flask to prepare a working standard of 100 $\mu g. ml^{-1}$.

**Methodology:**

1. Mark different tubes. Take 1ml of distilled water or 1ml of sample in tubes.
2. Take 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0ml of standard glucose solution in different tubes. Make up the volume to 1ml in each tube with distilled water.
3. Add 1 ml of phenol reagent to all tubes. Mix well.
4. Add 5 ml of concentrated $H_2SO_4$ to all. Mix well.
5. Allow to stand at room temperature for 10-30 min.
6. Using distilled water blank, measure the absorbance of all tubes at 488 nm.
7. Prepare a standard curve and quantify carbohydrates (as hexose) in the sample.

**Sensitivity:** 10-100 µg.