III MATERIALS AND METHODS
1. Plant material:

Parmelia simplicior (Male) and Leptogium azureum (SW.) Mont! are the common lichens found on tree trunk in open woods of Western Ghats. Healthy thalli of these lichens were collected for experimental purpose from the hill station called Panhala (954 m above M.S.L., receiving annual rainfall 180-200 cm), situated about 25 Km North West of Kolhapur. Unless otherwise stated the analyses were made of lichen thalli collected in three different seasons viz. Summer, Rainy and Winter to study seasonal variation in them.

Freshly harvested lichen thalli were packed in polythene bags and brought immediately to the laboratory. They were the washed with distilled water to remove debris and then blotted dry. Moisture content was determined by change in weight method for which the weighed quantity of lichen material was dried in an oven at 80°C and weighed. The process was repeated till it attained constant dry weight. For the purpose of experiments growing parts of the freshly harvested thallus of L. azureum and P. simplicior were chosen.

2. Experimental methods:

A) Inorganic constituents:

1) Estimation of minerals from lichen thalli:

Seasonal variation in mineral content was carried out from the acid digested solution of lichen material.
Acid digestion:

Cleaned and oven dried lichen material was digested by the standard method (Black 1965). About 3 g dry material was taken in a 75 ml clean corning beaker and to that 10 ml of acid mixture (mixed in the proportion of 75 ml HNO$_3$, 15 ml H$_2$SO$_4$ and 30 ml HClO$_4$) was added. It was kept for an hour in a fumina-hood and then digested gradually on a low flame first, then vigorously till clear white mixture remained. The cooled semisolid mass was dissolved in double glass distilled water (25 ml) and first filtered through acid washed Whatman filter paper No. 44 and then sintered glass funnel. The filtrate was made to volume (100 ml) and used for estimation of various inorganic elements such as potassium (K), Calcium (Ca), Magnesium (Mg), Sodium (Na), Manganese (Mn), Iron (Fe), Aluminium (Al), Copper (Cu), Cobalt (Co), Nickel (Ni), Cadmium (Cd), Lead (Pb), Zinc (Zn), Boron (B), Barium (Ba), Antimony (Sb), Lithium (Li), Germanium (Ge), Lanthum (La), Gold (Au), Strontium (Sr), Rubidium (Rb), Berellium (Be), Cesium (Cs), Molybdenum (Mo), Vanadium (V), Titanium (Ti), Zirconium (Zr), Arsenic (As) and Chromium (Cr). All these analyses were carried out with the help of Atomic Absorption Spectroscopy at Regional Sophisticated Instrumentation Centre, Indian Institute of Technology, Powai, Bombay.
ii) Estimation of mineral elements from the bark sustaining lichen:

All the above mentioned mineral elements were also analysed from the bark on which these lichens were growing. The external cortical layer having lichen thalli were removed from the tree trunk by taking 0.5 to 1 cm deep incision. The dried tree barks were then acid digested and used for mineral estimation as per the procedure mentioned above. The barks on which P. simplicior was growing were of Manoifera indica and Syzigium cummini whereas the bark over which L. azureum grew was of Bougainvila.

iii) Organic constituents:

a) Plant material:

For the estimation of organic constituents randomly sampled fresh and cleaned lichen material collected in the month of December was used.

b) Carbohydrates:

A plant material was crushed in a mortar with pestle and the sugars were repeatedly extracted in 80 % (v/v) ethanol and filtered through Buckner's funnel. The residue was saved for analysing starch content. The alcoholic extract was then condensed to aqueous medium: A part of an aqueous extract and residue were hydrolysed to estimate
total sugars and starch respectively. Thus they were determined spectrophotometrically by using arseno molybdate reagent (Moore 1974).

c) Proline:

Proline was estimated by the method of Bates et al (1973), where 0.5 g oven dried lichen material was homogenised in 10 ml of 3% aqueous sulfosalicylic acid. The homogenate was then filtered through Whatman No.1 filter paper. Two ml of the filtrate was mixed with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid in a test tube. The test tube was heated on water bath (100°C) for 1 h and then the reaction was terminated by dipping it in an ice bath. The reaction mixture was extracted with 4 ml toluene, mixed by stirring vigorously for 15-20 sec in a test tube. The ice cold chromophore containing toluene was aspirated from the aqueous phase and brought to room temperature. The proline in the filtrate was measured spectrophotometrically at 520 nm on Spectronic 21 (Baush and Lomb) against toluene as a blank. The proline concentration was determined from a standard curve and expressed on dry weight basis.

The acid ninhydrin reagent was prepared by dissolving 1.25 g ninhydrin in 30 ml acetic acid and 20 ml 6 M phosphoric acid. The contents were slightly warmed and stirred until dissolved. The reagent was stored for 24 h in the refrigerator.
d) **Nitrogen** :

Cleaned and oven dried 1 g lichen sample was used for determining nitrogen content colorimetrically employing the method of Hawk et al. (1948).

The lichen material was Kjeldhal digested in 10 ml H$_2$SO$_4$ (50%) with a pinch of microsalt. Glass beads were added to avoid bumping. The clear digested extract was made to volume filtered and used for nitrogen estimation. The nitrogen was determined spectrophotometrically using Nessler's reagent where ammonium sulphate was used as standard.

e) **Chlorophyll estimation** :

The chlorophyll contents were determined by the method of Arnon (1949). 1 g fresh lichen material was crushed in 80% acetone (v/v, containing 4 ml liquor ammonia/liter) with a pinch of MgCO$_3$ and then filtered through Buckner's funnel. The filtrate was centrifuged on refrigerated centrifuge to get clear supernatant. All these operations were carried out in dark at 0 to 4 °C. The chlorophyll in the centrifuged filtrate was measured spectrophotometrically. The O.D. was measured at 645 and 663 nm against 80% acetone as blank. The total chlorophyll content was calculated as follows:

$$\text{Chlorophyll (} \mu\text{g/ml)} = (20.2 \times \text{Absorption at 645}) + (8.02 \times \text{Absorption at 663 nm}).$$
After applying necessary correction factors for dilutions, the total chlorophyll content was calculated as mg chlorophyll 100 g⁻¹ fresh wt.

The levels of chlorophyll \(a\) and chlorophyll \(b\) were determined using the equation given below:

\[
\text{Chlorophyll } a \; (\mu g/ml) = [12.7 \times \text{Absorbance at } 663] - [2.69 \times \text{Absorbance at } 645].
\]

\[
\text{Chlorophyll } b \; (\mu g/ml) = [2.69 \times \text{Absorbance at } 645] - [4.68 \times \text{Absorbance at } 663 \, \text{nm}].
\]

The ratio of chlorophyll \(a\) to \(b\) was subsequently calculated.

\[\text{1. Photosynthesis:}\]

\[\text{a) } ^{14}\text{C} \text{- uptake by lichen thalli:}\]

Photosynthetically assimilated carbon from freshly harvested thalli of \(P.\) simplicior and \(L.\) azureum was studied (Patil and Hegde 1983) by allowing the lichen material to assimilate \(^{14}\text{CO}_2\) released from NaH \(^{14}\text{CO}_3\) of spec. act. 1.85 GBq mol⁻¹ (obtained from B.A.R.C. Bombay). The pieces of lichen thallus were set afloat in the mixture of 50 mM tris - HCl buffer pH 8.2 and the carrier Na\(^{14}\text{CO}_3\) taken in an Erlenmayer flask. The set up was allowed to equilibrate under artificial light generated through
HPL® (Phillins) lamp for 15 min. The quantum of light received at point of incidence was ca. 150 μE m⁻² s⁻¹.

After the equilibration, the reaction was seeded by adding 50 μl of NaH¹⁴CO₃ (Spec act. 1.85 GBq M⁻¹). Thus the final concentration of H¹⁴CO₃ in the reaction flask was 0.05 %. The temperature of the set up was maintained to 25 ± 2°C.

b) Time laps study:

For the time laps study the respective reaction was terminated with boiling ethanol (80 % v/v) after desired time intervals (5 sec, 10 sec, 1 min, 10 min, 30 min and 1 h).

c) Extraction:

The material was homogenized and the insoluble material was successively extracted first with 80, 60 and 40 % ethanol (all v/v), and then twice with boiling water. The extracts were pooled and concentrated to a small volume by evaporating in vacuo on water bath. The amount of ¹⁴CO₂ incorporated was determined by plating 100 μl aliquot of the combined extracts on aluminium planchet in a gas flow proportional counting system.

d) Chromatography and autoradiography:

The compounds formed during photosynthesis were separated by two dimensional paper chromatography and
activities in the compounds were counted on the chromatogram with the help of Liquid Scintillation Counter (by courtesy of BARC). The identity of the spots were confirmed by autoradiography. Method employed is of Benson et al (1950). To accomplish this two dimensional paper chromatography was employed. The known quantity of aliquot was loaded on Whatman No.1 filter paper. The chromatogram was run where the first solvent system used was phenol: water (80:20 v/v) and the second n-butanol:acetic acid:water (44:5:11 v/v). The compounds on the chromatogram was detected by spraying appropriate developer where amino acid was detected by ninhydrin, sucar was detected by anilinephthale, sugar phosphates by phosphomolybdic acid and organic acid with that of bromophenol blue. The activities in the compound were detected by counting the spots on the chromatogram with the help of automatic Hevlet Packard Liquid Scintilator with strip chart printer.

e) Pulse and chase experiment:

Freshly harvested healthy lichen thalli of Parmelia simplicior and Leptogium azureum were cut into small bits and exposed to NaH$^{14}\text{CO}_3$ for short durations. Lichen material was set afloat in Erlemayer flasks containing 50 mM tris- HCl buffer pH 8.2. The final concentration of CO$_2$ in the flask was 0.05%. The material after equilibration
and preillumination for 15 min was allowed to assimilate NaH$_{14}$CO$_3$ for 10 sec. To accomplish this the tissue were quickly transferred to reaction mixture where cold bicarbonate is replaced by NaH$_{14}$CO$_3$ (spec. act. 1.85 GBq mol$^{-1}$). The temperature was maintained to 25 ± 2°C. After 10 sec pulsation the activity at various time interval of 30 sec, 1 min, 30 min and 1 h was chased in 'cold' reaction mixture. The tissues harvested at different time interval were killed with the help of boiling 80% (v/v) ethanol. The method employed for further analysis is same as explained earlier.

f) Photosynthetic enzymes:
Ribulose 1, 5-bisphosphate carboxylase and Phosphoenolpyruvate carboxylase.

i) Extraction:
2 g sample of freshly harvested and cleaned lichen material was cut into 0.5 cm$^2$ pieces and ground vigorously in a chilled mortar with 4 volumes of extraction medium along with a pinch of acid free sand. The extraction medium consisted of 50 mM tris-HCl buffer, pH 7.8, 5mM DTT; 1 mM EDTA; 2 mM MnCl$_2$ and 10 mM 2 mercaptoethanol. The extract was filtered through four layers of muslin cloth and an aliquot was set aside for chlorophyll estimation according to the method of Arnon (1949). The filtrate was
then centrifuged at 10,000 g for 10 min in a refrigerated centrifuge. The clean supernatant was used for the enzyme assay. All the above operations were performed at 0 ± 4°C. The enzymes were assayed immediately.

ii) Assay of phosphoenolpyruvate (PEP) carboxylase (EC 4.1.1.31):

The enzyme was assayed by the method of Raghavendra and Das (1975) where its ability to fix CO$_2$ has been considered as a measure of its activity. Since oxaloacetate, the reaction product, was unstable under ordinary conditions the reaction was stopped with 2, 4-dinitrophenylhydrazine in HCl. The reaction mixture (2 ml) contained 50 mM tris-HCl buffer, pH 8.0; 2 mM MgCl$_2$; 2 mM NaH$_2$CO$_3$ (1.6 mCi/mM); 1 mM PEP and the enzyme. The reaction was stopped after 3 min by the addition of an equal volume of 1 N HCl saturated with 2, 4-dinitrophenylhydrazine. An aliquot was examined for incorporated radioactivity, using a continuous gas flow proportional counter.

iii) Assay of Ribulose 1-5-phosphate (RuBP) carboxylase (EC 4.1.1.39):

The activity was assayed by observing the incorporation of radioactive bicarbonate into acid stable products (Raghavendra and Das, 1977). The components of the reaction mixture (3 ml) were 50 mM tris-HCl buffer, pH 7.8;
10 mM MgCl$_2$; 3 mM DTT; 20 mM NaH$_2$CO$_3$ (0.3 mCi/ml); 0.5 mM RuBP, and the enzyme. After preincubation for 5 min the reaction was started with the addition of RuBP. The reaction was stopped after 5 min by adding 1 ml of 4 N HCl. An aliquot was examined for incorporated radioactivity, using a continuous gas flow proportional counter.

iv) Photorespiratory light to dark ratio:

The photorespiratory light to dark ratio was determined in the freshly harvested and excised lichen material (0.2 g). The method was essentially of Kisaki and Tolbert (1970) modified slightly to suit our condition (Patil and Hegde 1982). The lichen material was illuminated under light with HPLR lamps. The light incident was 150 μE m$^{-2}$s$^{-1}$. The temperature was maintained at 25 ± 2°C. The sample was allowed to assimilate at the rate of 1 μCi of Na- glycolate - $^{14}$C (spec. act. 1.9 m Ci mM$^{-1}$) per ml of 0.1 M phosphate buffer (pH 7.0) for 10 min. The surface washed lichen bits were then transferred to a jar (6 x 10 cm) containing 0.1 M phosphate buffer pH 7.0 and a small vial (2.5 x 3 cm) fixed inside with lanolin containing 0.2 ml of 20% KOH. Jars were made air-tight with ground glass stopper. A pair of set up was maintained for each lichen, out of which one was kept in light and the other was in dark for one our. After the experiment was over the KOH vial with the trapped $^{14}$CO$_2$ was
taken out. The $^{14}$CO$_2$ was precipitated out to Ba$^{14}$CO$_3$ with 5 ml of 2 % BaCl$_2$. The activity in of Ba$^{14}$CO$_3$ was counted on proportional counting system. The $^{14}$CO$_2$ evolved in light was compared to $^{14}$CO$_2$ evolved in dark.

vi) Isolation of algae from lichens:

The algae from lichen thalli isolated by the method of Green and Smith (1974).

Healthy lichen thalli were cleaned and kept on moist filter paper under light at 25$^\circ$C for about 24 h. The thalli were washed three times in distilled water and ground for about 2 min. in a mortar with a little distilled water. The resultant homogenate was filtered through a plastic strainer (0.5 mm mesh) and isolated following way.

Time to time the separation stages were checked under light microscope and the general routine was modified as required.
Centrifuge at 300 x g

Supernatant discarded
Green algal layer at top of pillet resuspended
Non-algal lower part of pillet discarded

300 x g

Supernatant discarded
Thin white skin on top of algal layer of pillet resuspended and discarded

Algal layer of pillet resuspended
Remainder of pillet discarded

Repeat (a)

30 x g

Supernatant discarded
Pillet discarded

Pillet discarded

30 x g

Supernatant discarded
Pillet
Resuspended and washed three times in distilled water

Final Algal Suspension