II - MATERIALS AND METHODS

A. Collection of algal samples

The marine algal samples *viz.* Ulva, Chaetomorpha, Dictyota, Padina, Sphacelaria, Hypnea and Ceramium were collected in the month of September 1993 (post monsoon), December, 1993 (winter) and March, 1993 (Pre-monsoon).All collections were made during low tide at a rocky intertidal site near Sindhudurg, Malvan (North latitude 16.4' and East longitude 73.31') situated on the west coast of Maharashtra (India). Specimens were packed loosely in plastic bags along with sea water and brought immediately to the laboratory. Immediately after collection, a sample of each species was rinsed in sea water to remove epibiota and encrusting debris. The samples were then rinsed in distilled water in a laboratory to remove the surface salt and blotted dry. They were then dried on galvanized wire nets in sun-light and then in a hot air oven at 60°C. Once dry, samples were powdered and sieved to 80 mesh and were preserved in sealed bottles for analysis.

Sea water sample from which the algal samples were collected, was also collected at the same time and brought to the laboratory in polythene bottles, filtered through Whatman No.1 filter paper and used for analysis. The temperature, pH and salinity were recorded on the spot using mercury therma-meter,

pHep and salino-meter respectively. While conductivity was measured on conductivity meter (ELICO) and inorganic constituents were analysed on atomic absorption spectrophotometer.

B. Moisture content :

Moisture percentage was determined by drying 10 g cleanly washed fresh algal material to a constant weight in an oven at 60°C.

C. Ash content :

Ash content of the powdered material was obtained by burning triplicate subsamples (≥ 500 mg) for 4 h in a 500°C muffle furnace.

D. Inorganic constituents :

The powdered algal samples were acid digested by following the method of Toth et al. (1948). The residue obtained in this method was used for the estimation of crude silica and the filtrate was used for the estimation of various inorganic elements such as sodium (Na^{+}), Potassium (K^{+}), Calcium (Ca^{2+}), Manganese (Mn^{2+}), Magnesium (Mg^{2+}), Iron (Fe^{3+}), Zinc (Zn^{2+}), Copper (Cu^{+}), Cadmium (Cd^{2+}), Chromium (Cr^{3+}), Lead (Pb^{2+}) and Nickel (Ni^{2+}) on Atomic Absorption Spectrophotometer (Perkin-

Elmer Model 3030). Phosphorous (P^{5+}) was determined spectrophoto-metrically by using Molybdo-Vandate reagent (Sekine *et al.* 1965).

E. CHN analysis :

Total carbon, hydrogen and nitrogen contents of three aliquots of each of the powdered samples were determined with a Perkin-Elmer Model 240 B CHN analyzer operated by the National Facility for Blue Green Algal collection, Indian Agricultural Research Institute (IARI), at New Delhi by the courtesy of Dr. T.K.Biswas.

F. Lipid content :

Lipid content of three subsamples (≥ 200 mg) was extracted by heating at 60°C for 30 min with 20 ml 2:1 (V/V) chloroform : methanol (Folch *et al.*, 1957). Each solution was coarsely filtered through Whatman No.1 filter paper and 15 ml supernatant was collected. The supernatant was washed with 3 ml 0.7% NaCl and the resulting upper phase was discarded. After the solvent was evaporated, duplicate aliquots of the lower phase were assayed by charring in concentrated H_2SO_4 (Marsh and Weinstein, 1966) and comparing absorbances determined spectrophoto-metrically at 375 nm with those of palmitic acid standard curve.

G. Chlorophyll content :

Chlorophyll content was estimated from cleanly washed algal materials by the method of Arnon (1949). 0.5 g randomly sampled algal material was homogenised in dark in a chilled mortar with pestle using 80% acetone containing 4 ml liquor ammonia per litere. A pinch of magnesium carbonate ($MgCO_3$) was added while crushing. This extract was filtered through Buchner's funnel using Whatman No.1 filter paper, and the volume of filtrate was measured. In order to avoid chlorophyll destruction by light the flasks containing chlorophyll extract were covered with black paper and stored at low temperature in dark. The absorbance was measured at 645 and 663 nm on double beam spectrophotometer (Schimadzu). Chlorophylls (mg 100^{-1} g fresh wt) were calculated using the formulae given below :

Chlorophyll 'a' = 12.7 x A 663 - 2.69 x A 645 = X

Chlorophyll 'b' = 22.9 x A 645 - 4.68 x A 663 = Y

Chlorophyll a+b = 8.02 x A 663 + 20.2 x A 645 = Z

$$\text{Total chlorophylls (mg } 100^{-1} \text{ g fresh wt)} = \frac{X/Y/Z \times \text{volume of extract} \times 100}{\text{Wt. of plant material (g)} \times 1000}$$

H. Carotenoids :

Carotenoid content from cleanly washed fresh material was extracted and analysed by saponification of acetone extract by the method of Jenson (1978). A known quantity (1 g) of algal material was homogenised in 80% acetone in dark at 4°C and filtered through Buchnar's funnel using Whatman No.1 filter paper.

1. Saponification :

The acetone extract was saponified by adding equal volume of ether and 10% methanolic KOH in separating funnel. These funnels were stored in dark for 2 hours to get two distinct layers *viz.* upper carotenoid phase and lower chlorophyll phase. The lower phase was discarded and upper phase was treated with un-hydrous sodium sulphate at the rate of 10 g 100^{-1} ml ether phase and filtered through sintered glass funnel. The absorbance of filtrate was measured at 450 nm. The amount of carotenoids in mg was calculated by using the formula

$$\text{Carotenoids (mg)} = \frac{D \times V \times f \times 10}{2500}$$

where

D = Absorbance at 450 nm

V = Volume of extract

f = Dilution factor

$$\text{Dilution factor} = \frac{\text{Total volume of extract}}{\text{Moisture content in algal material used.}}$$

2. λ max of carotenoid :

The λ max of carotenoids of different algae were determined by measuring the absorption of saponified acetone extract at different wavelength ranging between 400 and 665 nm.

I. Polyphenols :

Polyphenols from dried algal powder were estimated following the method of Folin and Denis (1915). 1 g dried powder was homogenised in a mortar with pestle using 80% acetone. The extract was filtered through Buchnar's funnel using Whatman No.1 filter paper. The residue left on filter paper was washed several times with 80% acetone and the final volume of filtrate was adjusted to 50 ml using 80% acetone. 2 ml extract was taken in a Nessler's tube along with the series of standards (std. tannic acid having concentration 0.1 mg ml^{-1}) to which 10 ml 20% Na_2CO_3 was added. The volume was adjusted to 35 ml with distilled water. Then 2 ml Folin-Denis reagent was added to each test tube and the final volume was adjusted to 50 ml with distilled water. After

about 20-30 min absorbance was measured at 660 nm using reaction blank. Polyphenols were calculated from standard curve of tannic acid and values are expressed as g 100^{-1} g dry tissue.

1. Preparation of Folin-Denis Reagent :

100 g sodium tuntstate and 20 g phosphomolybdic acid were dissolved in 800 ml distilled water. To that 50 ml 80% phosphoric acid was added. The entire mixture was refluxed for 2 h on water bath using water condensor. After cooling to room temperature, the final volume of the mixture was made to 1 litre and stored in an ambar coloured bottle at low temperature.

Standard tannic acid was prepared by dissolving 250 mg tannic acid in 250 ml distilled water so as to get the final concentration in the range of 0.1 mg ml^{-1}.

J. Proline :

Proline content was estimated from dried powder by using the method of Bates et al. (1973). 0.5 g dried powder was homogenised in 10 ml 3% aqueous sulfosalicylic acid and the homogenate was filtered through Whatman No.1 filter paper. 2 ml filtrate was reacted with 2 ml acid ninhydrin and 2 ml glacial acetic acid in a test tube for 1 h at 100°C in water bath, and the tubes were transferred to ice bath immediately. After 15 min the reaction mixture was extracted weith 4 ml toluene by shaking

vigorously with a test tube stirrer for 20 sec. The red coloured toluene phase was separated from aqueous phase with the help of small separation funnel and warmed to a room temperature and the absorbance was measured at 520 nm using toluene as a blank on Shimadzu spectrophotometer. The proline concentration was determined from a standard curve on a dry weight basis.

1. Preparation of acid ninhydrin :

This reagent was prepared by warming 1.25 g ninhydrin (Unichem) in 30 ml glacial acetic acid (Merck) and 20 ml 6 M phosphoric acid (Glaxo) with agitation until dissolved. The reagent remain stable for 24 h at 4°C.

K. Crude Protein :

Crude protein was estimated using the formula N x 6.25 where N - the value of total nitrogen analysed with the help of CHN analyser.

L. Carbohydrates :

The sugars were estimated following the method of Nelson (1944). Carbohydrates were extracted from known quantity of dried algal powder with 80% ethanol. The extract was filtered through Buchnar's funnel using Whatman No.1 filter paper. The filtrate was then condensed on water bath to about 2-3 ml. To

this lead acetate and potassium oxalate (1:1) were added with constant stirring. To that about 30 ml distilled water was added and was once again filtered through Whatman No.1 filter paper and the volume of the extract was adjusted to 50 ml with distilled water. From this extract reducing sugars were determined.

The residue left over on filtere paper and aliquot of reducing sugar extract was hydrolyzed at 15 lbs pressure for 30 min. cooled to room temperature, neutralized with unhydrous Na_2CO_3 and filtered. The sugars from all the three filtrates were estimated by using arsenomolybdate and alkaline copper tartarate reagent (Nelson, 1944).

1. Preparation of alkaline copper tartate :

4 g $CuSO_4 \cdot 5H_2O$, 24 g anahydrous Na_2CO_3, 16 g sodium potassium tartarate and 180 g anhydrous Na_2SO_4 was dissolved in distilled water.

2. Preparation of arsenomolybdate reagent :

25 g ammonium molybdate was dissolved in 450 ml distilled water to which 21 ml concentrated H_2SO_4 was added. This was denoted as solution 'A'. 3 g of sodium arsenate was dissolved in 25 ml water. This was denoted as solution 'B'. Both A and B solutions were mixed well and kept in an incubator at 37°C for 48

h before use.

M. Chromatography of amino acids :

Amino acid composition of different algae was studied by using the technique of ascending unidirectional paper chromato graphy (Block et al. 1955).

1. Preparation of extract :

2 g dried powder of algae was repeatedly extracted in 80% ethanol and filtered through Buchanar's funnel using Whatman No.1 filter paper. The filtrate was condensed under reduced pressure up to 2 ml and transferred into a centrifuge tube and centrifuged at 5000 x g for 5 min and the supernatant was collected in a small glass vial and stored at 4°C until used.

2. Unidirectional paper chromatography :

The amino acids were separated by loading the aliquot of extract on chromatographic paper and by using the solvent system n-butanol : acetic acid : water (80:20:100 v/v). 0.5% ninhydrin prepared in 95% acetone was used as detecting reagent for amino acids. Identification of amino acids was made by using the chromatography of authentic standards and comparing their 'Rf' values. The colour intensity of spots were compared and according to their concentrations they were graded as least (+),

less (++), moderate (+++), high (++++) and very high (+++++).

N. Chromatography of Polyphenols :

1. Preparation of extract :

The polyphenols were extracted by the method of Glass and Bohm (1969). 2 g dried powder of algae was repeatedly extracted in 80% ethanol. The extract was refluxed for 2 h on water bath using water condensor and filtered hot through sintered glass funnel. The solvent was removed under reduced pressure. The residue was triturated in 50 ml hot water. The combined extract was reduced to 3 ml by condensation, centrifuged at 5000 g for 5 min and supernatant was used for the chromatography.

2. Unidirectional paper chromatography of polyphenols :

Unidirectional paper chromatography was performed using Whatman No.1 filter paper (size 28x14 cm) with slight modification in the method used by Shetty (1971). Chromatograms were spotted with known 20 µl extract using microsyringe with frequent drying. The sdolvent system used was n-butanol, acetic acid and water in the proportion of 80:20:44 (v/v). The position of individual phenolic compounds on chromatogram was determined by marking fluorescent area under UV light as well as under UV light in the presence of ammonia fumes. Phenolic compounds that could

not be located under UV and ;UV + NH_3' were detected by dipping the chromatogram in a mixture of 0.3% $FeCl_3$ and $K_3Fe(CN)_6$ in equal proportion.

The probable identification of the compounds was made by calculating the Rf values, observing the colour under UV and UV + NH_3 and by comparing with Rf values of authentic standards obtained from Dr. P. Neuman, University of Texas, Austin.

O. Estimation of Alginic acid :

Crude alginic acid was estimated by the method of Booth (1975). Known quantity of dried algal powder was extracted thrice with 3% sodium carbonate and filtered through 8 layered cheese cloth and the calcium salt is then precipitated by running alkaline extract into a concentrated solution of calcium chloride (25%), which yielded fibrous precipitate of calcium alginate. This precipitate was collected, dried and weighed.

P. Estimation of Agar-Agar :

Agar-agar was estimated in crude form using routine mthod. 10 g dried algal powder was mixed in 100 ml 4% NaOH solution and kept overnight. To that additional 30 ml 4% NaOH was added and boiled on hot plate, cooled to room temperature and filtered through coarse filter paper. The filtrate was denoted as 'A'. The residue was reextracted in 4% NaOH and filtered through

musline cloth and through coarse filter paper. This filtrate was denoted as 'B'.

Both the filtrates A and B were mixed together and the volume was made equal for all the algal samples with 4% NaOH. To this alkaline extract, thrice the volume of absolute alcohol was added to precipitate agar-agar. The precipitate was then collected, dried and weighed.

Q. Estimation of Mannitol :

The mannitol content was determined by the method of Barker (1955). 0.5 g fine powder of algal material was added in a conical flask containing 5 ml 0.1 N H_2SO_4 and 5 ml 0.1 M periodic acid. After exactly one minute 3 g potassium iodide and 20 ml 4 N H_2SO_4 was added in a conical flask, to liberate the iodine. This liberated iodine was titrated using 0.1 N sodium thiosulphate using starch as an indicator. A blank was carried out simultaneously. The mannitol content was calculated by considering the equation

1 g mol mannitol $\equiv$ 5 I_2 $\equiv$ l N sodium thiosulphate.