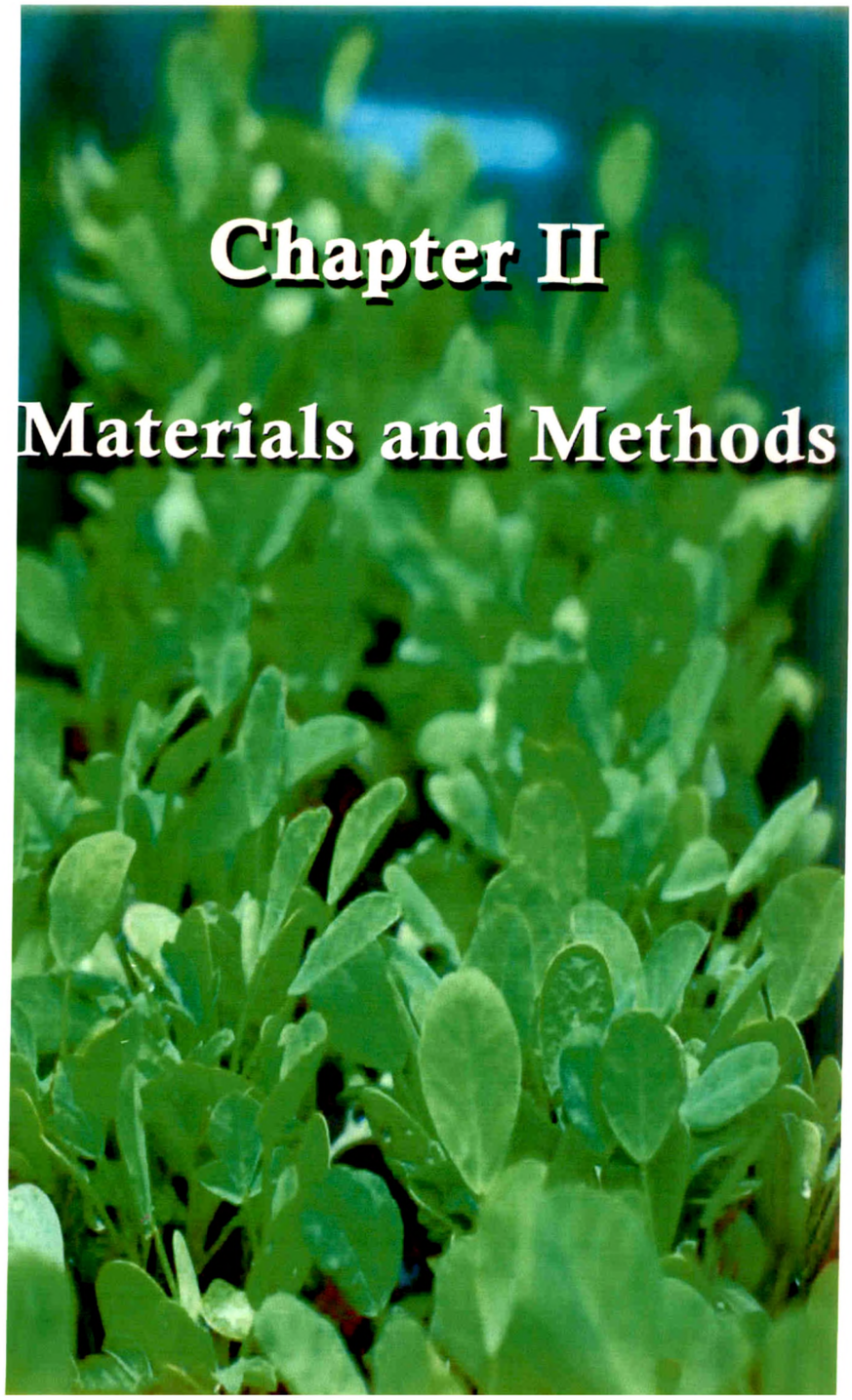


Chapter II

Materials and Methods

A. Study area

Coastal areas nearby Malvan and Kunkeshwar along the west coast of Maharashtra were considered for the survey and collection of seaweeds.

Malvan

Malvan is the southern most part of Maharashtra and located approximately 35km from Mumbai- Goa National Highway No. 17. It is a taluka place in the Sindhudurg district formed in 1981. Malvan town is bound by three small creeks viz., Karli, Kolamb and Kanlavali.

Climatology

Average atmospheric temperature at Malvan ranges from 16.5^{0}C to 33.1^{0}C with minimum and maximum values in January and May, respectively. Average relative humidity varies from 69.4% in April to 98% in July. The climate at Malvan is typical of monsoon regions, Cool and dry season is with low intensity of north-eastern winds from the land (November to February) hot–dry season from March to May followed by rainy season (June to September). The annual average rainfall ranges from 6.5 mm in April to 983 mm in July.

Currents

The coastal currents are clockwise or shoreward during February to September, while anti-clockwise during November to January and transitional in October.

Geomorphology

From Malvan Bay, a chain of submerged and exposed rocky islands, extends towards south up to 16^{o} 00' 00' N 16^{0} 05' 00' N Lat and 73^{0} 25' 00' E-73^{0} 30' 00' E Long. In this chain there are several islands including Sindhudurg Fort of Malvan at the northern tip. Other small islets around Sindhudurg fort are Mandal rock, Malvan rock, etc. There are numerous exposed outcrops in this area.

The coastline near Malvan is interrupted by sandy beaches and rocky cliffs. The most striking feature of the beach is littoral concrete and beach rock, which continues over long stretches. This littoral concrete occurs as a rocky beach either directly attached to the main land or separated by sandy or marshy areas. The rocky beach gives protection to the coast against strong waves. In some regions, the rocky beach occurs as a rim on banks enclosing marshy islands. Malvan is the best locality along the Maharashtra coast for the algal flora.

Ecological importance

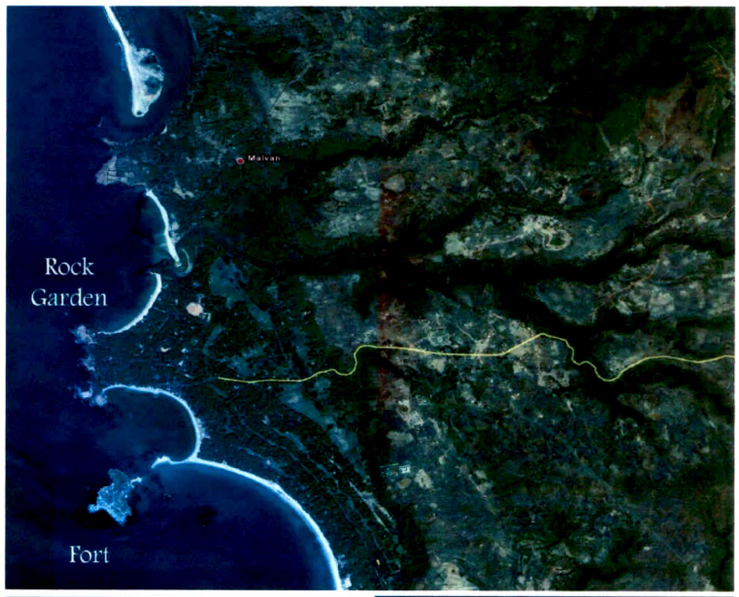
Malvan is an open coastal ecosystem, dominated by rocky outcrops with intermittent sandy beaches. Sindhudurg, a fort constructed by king Shivaji in 16^{th} century is on an island situated about a kilometer from the mainland. The sea in between and around the fort has many submerged and exposed rocks that provide an ideal substratum for marine biota and shelter to many organisms.

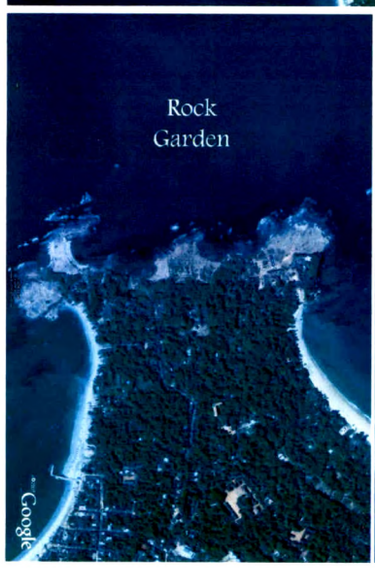
Plate I - Study area - Location Map

* www.googleearth.com

Plate II

Map showing collection sites at Malvan

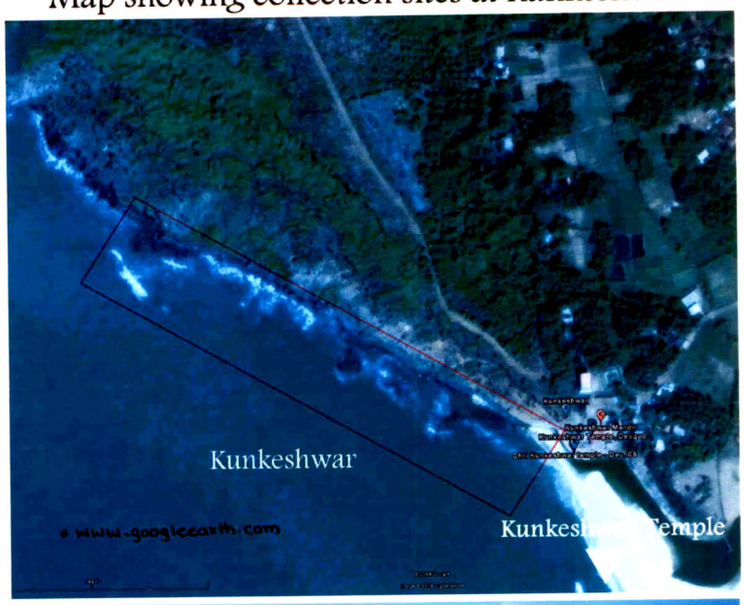




* www.googleearth.com

Plate III

Map showing collection sites at Kunkeshwar



Malvan coast has six types of habitats viz., rocky shore, sandy shore, rocky island, estuarine, muddy and mangrove habitats.

The rocky coastline of Malvan is primarily composed of sedimentary rocks. These rocks are soft and easily eroded by both wave and wind action, which attract animals that prefer a burrowing existence. Many crevices in the rocks serve as ideal place for sheltering, feeding and breeding grounds for many invertebrate and also an ideal substratum for harbouring marine algae (seaweed).

Kunkeshwar

Kunkeshwar is a village in Taluka Deogad southwards along the coast, beyond the creek at Mumbri. The place is famous for the Shiva temple built around 1000AD and situated along the sea coast. The substratum mostly consists of granite and laterite rocks. These rocky beaches are directly attached to the mainland. Intertidal expansion areas are more than those at Malvan

Climatology

Average atmospheric temperature ranges from 17.1°C to 32.3^{0}C with minimum and maximum values in January and May, respectively. The average relative humidity is around 70% and average rainfall is 3287 mm.

Ecological importance

Kunkeshwar is an open coastal ecosystem dominated by rocky outcrops with a sandy beach. The rocky coastline of Kunkeshwar is primarily composed of sedimentary rocks. These rocks are soft and easily eroded by both wave and wind action, and provide ideal substratum for development of marine algae.

The sea behind the temple has many submerged and exposed rocks that provide an ideal substratum for the growth and development of marine biota. The coast has two types of habitats viz., rocky and sandy

B. Materials

1. Seaweeds used

Seaweeds are macroscopic marine algae that grow in the intertidal and subtidal zones with a hold-fast attached to the substratum. Topography, nature of the substrata, salinity, current, nutrients, tidal action and other factors of the marine environment play an important role in the distribution, zonation and growth of seaweeds.

Coastal regions of Malvan and Kunkeshwar were surveyed and algal biodiversity was recorded. Out of the species observed along the west coast, following three species were considered for the present investigation. A brief description of these species follows.

1. *Ulva fasciata* Delile.

CLASSIFICATION

Division – Chlorophyta

Order – Ulvales

Family – Ulvaceae

Genus - *Ulva*

Species - *fasciata*

Distribution: Maharashtra, Karnataka, Goa, Gujarat, Kerala, Lakshadweep.

Ecological status : Open coast (intertidal), Estuaries and mangroves.

IUCN status : LR

Uses : Food, animal feed, medicine

Characteristics

- Plants 1-15 cm. tall.
- Cells of the midline region much taller than those of the margin.
- Thallus much thicker, 100 μ or more.
- Margins entire to irregularly ruffled and crenate with a somewhat paler central portion.
- Base of the blade cuneate, above expanding irregularly lobed, generally irregularly or sometimes pinnately divided into ligulate or linear lobes which may become several decimeters long.

2. *Sargassum ilicifolium* (Turner) Agardh

CLASSIFICATION

Division - Phaeophyta

Order - Fucales

Family - Sargassaceae

Genus - *Sargassum*

Species - *ilicifolium*

Distribution : Gujarat, Maharashtra, Goa, Karnataka, Lakshadweep,

Ecological status: Mangrove swamp, Intertidal in open coast.

IUCN status : LRnt

Uses : Used as a source of alginate, fertilizer, medicine and animal feed

Characteristics

- Plants30-40 cm high with elliptical leaves in the upper part of the plant, 1-3 cm long and 8-15 mm broad.
- Margin is toothed, with minute and larger teeth mixed.
- Midrib is visible for 2/3 of the length of the leaf, vanishing near the tip.
- Mature receptacles are either flat or branched, 2-4 mm long or to 1cm long by a breadth of 1mm borne on a branched pedicel with spiny outgrowths.
- Vesicles are nearly globular, 3-5mm in diameter with a stalk of the same length.

3. *Gracillaria corticata* Agardh

CLASSIFICATION

Division - Rhodophyta

Order - Gracillariales

Family - Gracillariaceae

Genus - *Gracillaria*

Species - *corticata*

Distribution: Dwarka, Okha Bombay, Malvan, Ratnagiri, Goa, Karwar. Honawar, Bhatkal

Ecological status : Intertidal zone.

IUCN status :NE

Uses : Agar production, food, animal feed

Characteristics

- Plants 10-12cm long, reddish in colour.
- Thallus consists of bundles of flat and much divided blades with 2-3 mm broad segments.
- Branching is dichotomous in young blades.
- In older plants numerous marginal projections 0.5-2 cm long line the edges of the segments in a pinnate fashion.

2. Fenugreek : Agronomical aspects

Present study deals with the effect of seaweed fertilizer on the physiology of popularly consumed leafy vegetable fenugreek (*Trigonella foenum-graecum L.*). A short agronomical review of this vegetable crop follows hereafter.

Fenugreek commonly known as methi is a herbaceous plant belonging to family fabaceae (Nike, 1998) and native to southern Europe and Asia. It is cultivated in India and other parts of the world for leafy shoot and seeds. India produces about 31,200 (98.5 millions tones of vegetables on about 7 million hector) area per year. It is mainly cultivated in Rajasthan Madhya Pradesh, Punjab, Gujarat, Uttar Pradesh, Kashmir and Maharashtra.

Genus *Trigonella* comprises of 62 spp. of which two are economically important viz. *Trigonella foenum-graecum L.* (Common methi) and *T. corticulata* (Kasuri-methi) (Chaudhary, 1976). Kasuri methi is slow growing and its cultivation is confined to north India. The pods are sickle shaped and smaller than common methi.

Soil: - Fenugreek is grown successfully on all types of soil. The optimum soil pH should be between 6 to 7 for its better growth and development.

Climate: - Fenugreek is a cool season crop. It is fairly tolerant to frost and freezing weather. It can also be grown as hot weather crop. It is mainly cultivated in areas with a moderate or low rainfall.

Sowing: - Common Methi is sown from middle September to the middle of March and the duration of crop is 30 to 40 days. It can be successfully adapted in the multiple cropping systems (Singh and Joshi, 1960). The seed rate per hector for common Methi is 25kg. It requires 5-6 days to germinate but in cool climate complete germination needs a few days more.

A thorough soil preparation is necessary before sowing. Beds of convenient size should be prepared at the same time. The seeds are raked to cover them. Line sowing in rows, 20 to 25 cms apart facilitates weeding and intercultural operation during the initial stage of crop growth (Choudhary *et al.* 1976).

Manuring and fertilization: - Methi is commonly grown on the residual fertility of the previous crops. According to Singh and Joshi (1960) more succulent leafy growth could be obtained by ample supply of nitrogen, as a top dressing in three equal split doses, the first one is given to one month old plants and the rest two after every two cuttings.

Irrigation: - Frequent irrigation is necessary to obtain quick growth of the crop. First irrigation should be given just after cutting then according to necessity.

Interculture: - Weeding is necessary at early stage of crop as it grows slowly in the initial stage. The growth becomes vigorous after four to five weeks and crop does not allow weeds to compete with it.

Harvesting: - When common methi is used as a leafy vegetable, the young shoots are nipped off in about three weeks after sowing. A number of cuttings may be taken off from kasuri type. The first cutting is ready in about 20 to 30 days after sowing. The picking of leaves is done by nipping two cm above the ground level, leaving stub which produces new shoots, Subsequent cuttings may be taken after an interval of 10-15 days.

Diseases and pests: - The crop is subjected to the attack of a number of diseases like powdery mildew (*Erysiphae polygoni*), root knot (*Uromyces anthyllisia*), downy mildew (*Perenospora trigonella*), leaf spot (*Cercospora traversiana*) damping of and root rot (*Rhizoctonia solani*).

Medicinal value: - Leaves are aromatic useful in external and internal swellings and hair fall. Seeds are hot antipyretic, appetizer, astringent to bowels. They are mucilaginous, aromatic, diuretic, nutritive and carminative, much used in colic flatulence, dysentery, diarrhea, dyspepsia with loss of appetite, dropsy. Powdered seeds are used in veterinary practices. Confection under the name of modak are used incase of dyspepsia and diarrhea of women in child birth and in rheumatism.

C. Methods

1. Collection of seaweeds

Seaweeds selected for the present study were *Ulva fasciata, Sargassum lilicifolium* and *Gracillaria corticata* belonging to the classes Chlorophyceae, Pheophyceae and Rhodophyceae, respectively. They were collected from the area of Malvan (73.30'E, 16.05'N) and Kunkeshwar (73.19^0 E to 16.40^0 N) along the west coast of Maharashtra, during Nov-Jan 2006 -07. The algal species were hand picked and washed thoroughly with seawater to remove all the unwanted impurities, adhering sand particles and epiphytes. Morphologically distinct thalli of algae were placed separately in new polythene bags and were kept in an ice box containing ice and transported to the laboratory. Samples were washed thoroughly using tap water to remove salt on the surface and then spread on a blotting paper to remove excess water. This fresh algal material was used to prepare liquid extract. A portion of this material was air dried and powdered. This powder was stored in dry air tight container and then used further to prepare extract.

2. Preparation of seaweed liquid fertilizer

Three types of liquid fertilizers designated as seaweed concentrate (SWC) were prepared as follows.

a) Fresh concentrate

For fresh extract (100g) were seaweeds cut into small pieces, blended in a mixer and then the extract was filtered. The volume of this filtrate was adjusted to 100 ml by adding distilled water. This filtrate was taken as 100% concentrate. From this, different grades (5%, 10%, 20%, 30%, 40%, 50% & 70%) were prepared by proper dilution with distilled water. As the liquid fertilizer contained organic matter, it was refrigerated between 0 to 4°c.

b) Boiled concentrate

After cleaning thoroughly, seaweeds were dried in sun light to constant weight and then crushed to make powder. This powder was stored in an airtight plastic container at room temperature (25°C). For preparation of fertilizer 50g of powder was boiled in 50ml of distilled water for an hour and filtered through four layers of muslin cloth. The volume of the filtrate was adjusted to 50ml and considered as 100% concentrate of the seaweed. From this; concentrate different dilutions (5 to 70%) were made using distilled water. This concentrate was also refrigerated between 0 to 4°C.

c) Soaked concentrate

Fifty grams dried powder of each of seaweed was soaked in 50ml distilled water for two days at room temperature. After two days it was filtered through four layered muslin cloth. The concentrate was refrigerated at 0-4^{0}C.

All the three types of extracts were prepared from *Sargassum*, *Ulva*, and *Gracillaria* and used for treatment of fenugreek.

3. Composition of Hoagland solution

The Hoagland solution (full strength) was made by following the method described by Epstein (1972). mixture of micronutrients i.e. (H_3PO_4, $MnCl_2.4H_2O$, $ZnCl_2$, $CuCl_2.2H_2O$ and $Na_2MO_4.2H_2O$) was prepared separately. Following ingredients were mixed with micronutrients (one ml) and final volume was made to1000ml with distilled water.

KNO_3 1M	6.0 ml
$Ca(NO_3)_2$ $4H_2O$ 1M	4.0 ml
$NH_4H_2PO_4$ 1M	2.0 ml
$MgSO_4$ $7H_2O$ 1M	1.0 ml

4. Procurement of seeds and seed treatment

Seeds of *Trigonella foenum-graecum* L. were obtained from Agriculture College, Kolhapur.

Healthy, mature seeds of fenugreek were surface sterilized in 0.1 % $HgCl_2$ and then soaked in different seaweed concentrate solutions for one hour. The concentrations used were 5, 10, 20, 30, 40, 50, 70 and 100%. After treatment, the seeds were washed thoroughly in distilled water and kept for germination in sterilized petri plates lined with Whatman No. 1 filter paper. Seeds soaked in distilled water for one hour simultaneously, served as the control. Hoagland nutrient medium at full strength was also employed for treatment of one set of seeds. The petri plates were kept in dark at room temperature for germination. The number of germinated seeds was counted and recorded every day.

From the germination study a few promising concentrations of fertilizer concentrate were selected for further study. Healthy fenugreek seeds were treated in these solutions and grown in sterilized coco peat and garden soil mixture (1:1) in plastic trays. Foliar sprays of selected concentration (10%, 25% and 50%) were applied after every five days interval. For spraying control plants distilled water was used. One set of plants was sprayed with Hoagland nutrient solution at full strength. Various growth parameter and a few biochemical components were analyzed after 25 days of growth of plants.

The methods used for biochemical analysis are given in brief in the following pages.

5. Biochemical analysis of fenugreek.

a) Photosynthetic Pigments:

i. Chlorophylls:

Chlorophylls were estimated following the method of Arnon (1949). Randomly sampled fresh leaves (0.5g) were homogenized and extracted in 80% chilled acetone containing 4ml ammonia per liter. A pinch of $MgCO_3$ was added to neutralize the acids released during extraction. The extract was filtered through Whatman No.1 filter paper using Buchner's funnel under suction. Final volume of the filtrate was made to 100 ml with 80% acetone. The filtrate was transferred into a conical flask wrapped with black paper to prevent photooxidation of the pigments. Absorbance was read at 663 nm and 645 nm on a double beam spectrophotometer (Shimadzu) using 80% acetone as a blank.

Chlorophylls ($mg100^{-1}g$ fresh weight) were calculated using the following formulae

For Chlorophyll 'a' (12.7 x A663) – (2.69 x A645) = X

For Chlorophyll 'b' (22.9 x A645) – (4.68 x A663) = Y

For Total chlorophylls (a+b), (8.02 x A663) + (20.20 x A645) = Z

$$\text{Chl.a/Chl.b/Total Chlorophylls } (mg100^{-1}g \text{ fresh weight}) = \frac{X/Y/Z \times \text{volume of extract} \times 100}{1000 \times \text{weight of plant material (g)}}$$

ii. Carotenoids:

Carotenoids were estimated from the same acetone extract of chlorophylls as per the method described by Kirk and Allen, (1965). The absorbance was recorded at 480 nm on a double beam spectrophotometer (Shimadzu). The amount was calculated using the following formula.

$$\text{Total Carotenoids } (g100^{-1}g \text{ fresh weight}) = \frac{A480 \times \text{volume of extract} \times 10 \times 100}{2500 \times \text{weight of plant material (g).}}$$

Where 2500 is average extinction at 480 nm

b). Organic constituents:

i. Total Carbohydrates:

Total carbohydrate content was estimated following anthrone method described by Sadasivam and Manickam, (1992) with slight modification. Five hundred mg oven dried plant material was taken in a 100 ml conical flask and five ml of 2.5 N HCl and 20 ml distilled water were added. The mixture was autoclaved at 15 lbs atmospheric pressure for 30 minutes. The contents were

cooled, neutralized with anhydrous sodium carbonate and filtered two times using Whatman No. 1 filter paper through Buckner's funnel. Final volume was made to 100 ml with distilled water. From this extract 1ml sample was taken for assay. It was mixed with four ml of cold anthrone reagent (200 mg anthrone dissolved in 100ml ice cold 95 % H_2SO_4. Prepared fresh before use) were added. The reaction mixture was heated for eight minutes in boiling water bath and rapidly cooled keeping in ice bath. Absorbance of green colour was measured at 630 nm on a double beam spectrophotometer (Shimadzu) using blank prepared as above but with distilled water in place of glucose solution. Amount of total carbohydrates was calculated with the help of a standard curve of sugar obtained by using different concentrations of standard glucose (0.1 mg/ml in d. w.). Values of carbohydrates were expressed in g 100^{-1}g dry weight.

ii. Total Proteins:

Total soluble proteins were estimated from the fresh thalli of seaweeds according to the method described by Lowry *et al.* (1951). Five hundred mg plant material was homogenized in 0.1 M phosphate buffer (pH 7), filtered through moist muslin cloth and centrifuged for 10 min at 5000 rpm. 0.1 ml supernatant was taken into a test tube followed by 5 ml alkaline copper tartarate solution [prepared by mixing 50 ml of reagent 'a' (20 % Na_2CO_3 in 0.1 N aqueous NaOH) with 1 ml of reagent 'b' (2% $CuSO_4$, $5H_2O$ in 1 % potassium sodium tartarate)]. After 15 minutes, 0.5 ml folin phenol reagent

[A mixture consisting of 100 g of sodium tungstate (Na_2WO_4, $2H_2O$), 25 g of Sodium molybdate (Na_2MoO_4, 2H2O), 700ml of d. w., 50 ml phosphoric acid and 100 ml of conc. HCL in one liter volumetric flask was refluxed gently for 10 hrs. Then 50 g of lithium sulphate, 50 ml of d. w. and a few drops of bromine water were added. This mixture was boiled for 15 min. without condenser to remove excess of bromine. It was then cooled and adjusted to 1N acidity by titrating against 1 N NaOH to a phenolphthalein end point and filtered. Care was taken that the reagent did not have a greenish tinge] was mixed and it was kept for 30 minutes at room temperature. Absorbance of blue colour was read at 660 nm against blank prepared using distilled water. Amount of soluble proteins was calculated with the help of a standard curve obtained by using different concentrations of bovine serum albumin (0.1 mg/ml) by a similar procedure as employed for the plant extract.

iii. Total Nitrogen:

Total nitrogen was estimated colorimetrically following the method given by Hawk *et al.* (1948). Five hundred mg of oven dried plant material was digested in Kjeldahl's flask containing sulphuric acid (1:1) with a pinch of microsalt [Microsalt was prepared by grinding anhydrous $CuSO_4$ and potassium sulphate in proportion of 1: 40 i.e. 100 mg $CuSO_4$ + 4 g K_2SO_4] and a few glass beads (to avoid bumping) till a colorless liquid is obtained at the bottom of the flask. After cooling to room temperature, the contents were

diluted with distilled water to 100 ml. Then it was filtered through Whatman No. 1 filter paper and used for the estimation of nitrogen.

One ml of filtrate was taken in a Nessler's tube along with a drop of 8 % $KHSO_4$ and volume was adjusted to 35 ml with distilled water. Then 15 ml of freshly prepared Nessler's reagent [Reagent A-7 g KI + 1 g HgI_2 dissolved in 40 ml distilled water. Reagent B-10 g NaOH dissolved in 50 ml distilled water. Reagent A and B were mixed immediately prior to use] were added and mixed thoroughly. After 15 minutes, absorbance was measured at 520 nm on a double beam spectrophotometer (Shimadzu). The blank prepared contained all the ingredients except the nitrogen source. Standard curve was obtained by using different concentrations of ammonium sulphate {$(NH_4)_2$ SO_4 was kept in an oven at 60^0C for 10 h and 0.236 g of it was dissolved in distilled water. A few drops of concentrated H_2SO_4 were added to it and the volume was made to 1 liter. This solution contains 0.05 mg of nitrogen /ml} in place of filtrate. It was used to calculate the amount of total nitrogen and values were expressed as g 100^{-1}g dry weight.

iv. Free Amino acids:

A colorimetric method suggested by Sadasivam and Manickam, (1992) was used to determine the free amino acid content. 100 mg oven dried plant material was extracted in 5-10 ml of 80 % ethanol and filtered through Whatman No. 1 filter paper using Buchner's funnel. The filtrate was saved. The residue was again suspended in ethanol and again filtered. Procedure was

repeated once more. The total volume of filtrate was evaporated on a water bath to obtain 10 ml solution which was then used as the source of amino acids.

Estimation was carried out using ninhydrin as an oxidizing agent, which decarboxylates α-amino acids and gives bluish purple product. Extract (0.1 ml) was mixed with one ml of ninhydrin solution. [0.8 g SnCl2. $2H_2O$ in 500 ml of 0.2 M citrate. buffer (pH 5.0) + 20 g of ninhydrin in 500 ml of methyl cellosolve]. The volume was adjusted to two ml with distilled water and heated on a boiling water bath for 20 minutes. After cooling 5 ml diluent (equal volumes of water and n-propanol) solution was mixed in it. Absorbance of the purple colour was measured at 570 nm after 15 minutes. The blank was prepared by using 80% ethanol in place of the extract. Standard curve of amino acids was obtained using leucine (0.1mg/ml in d. w.) to calculate the amount. The values were expressed as g 100^{-1}g dry weight.

v. Total Polyphenols:

Polyphenols were estimated by the method of Folin and Dennis, (1915). Five hundred mg fresh plant material was extracted in 80% acetone. This extract was filtered through Buchner's funnel using Whatman No.1 filter paper. The residue on the filter paper was washed several times with 80% acetone and the final volume of extract was adjusted to 100 ml with 80% acetone.

Two ml plant extract was treated with 10ml 20% Na_2CO_3 in Nessler's tube. The volume was adjusted to 35ml with distilled water. Then two ml Folin Dennis reagent [100 g sodium tungstate and 20 g phospomolybdic acid were

dissolved in 200 ml distilled water. 50ml 25% phosphoric acid were added and it was reflux for 2-3 hours using water condenser. The volume was adjusted to one litre with distilled water] were added to each test tube and the final volume was adjusted to 50 ml with distilled water. After about 20–30 min. absorbance of blue colour was read at 660 nm against a blank reaction mixture. Polyphenols were calculated from a standard curve of tannic acid (0.1mg/ml) in d.w. and were expressed in g 100^{-1} g dry weight.

vi. Total mineral content (ash content):

The mineral content was determined by charring a known amount of dry biomass in crucible in a furnace at 540° C for 5 hrs. The total mineral content (ash content) was then determined gravimetrically and expressed as percentage on dry weight basis.

6. Analysis of seaweeds

Seaweed used in the present investigation for treatment, were analysed for certain biochemical parameters. The parameters and methods used are described below.

a. pH

Hydrogen ion concentration of seaweeds was determined using the method of Singh and Purohit (2003). Extract was prepared by crushing 5 g of fresh algal material in d. w. which was then filtered through four layered muslin cloth. The volume was made to 50ml with distilled water. The glass

electrode of pH meter was standardized using buffers having pH 4 or 9. After standardization the electrode was washed with distilled water and they immersed in the seaweed extract. The reading was recorded.

b. Organic constituents:

i) Total lipid content

From air dried algal sample total lipid content was determined according to Bligh and Dyer (1959). The material (10 g) was extracted with chloroform: methanol: water (2:10:8 vol/ vol/ vol). The homogenized sample was transferred to a sepraratory funnel and the chloroform layer containing the lipid was collected. This chloroform layer was evaporated to obtain the total lipid sample. Total lipid content was determined gravimetrically and expressed as percentage.

Total carbohydrate, total proteins, total nitrogen and total minerals from seaweeds were determined by following the similar methods as used for fenugreek analysis and described earlier.

c. Inorganic constituents:

Acid digestion:

For the determination of mineral elements, acid digest was prepared following the method described by Toth *et al.* (1948). Five hundred milligram oven dried seaweed was treated with 20 ml concentrated HNO_3 in a beaker covered with a watch glass and kept till the primary reaction is subsided. It was then subjected to slow heating on a hot plate to dissolve the plant material.

After cooling to room temperature, 10ml of perchloric acid (70 %) was added into the beaker and it was heated again until reduced to 2–3 ml clear solution. This extract was cooled to room temperature, diluted to 100 ml with distilled water and kept overnight. Next day, it was filtered through Whatman No. 1 filter paper and stored at room temperature. This acid digest was used to estimate different inorganic elements.

i. Macro and micronutrients

Potassium, Calcium, Magnesium and Iron from acid digest was determined using an atomic absorption spectrophotometer (Perkin-Elmer 3030 model). The readings were recorded in ppm and then converted to g 100^{-1} g of dry weight.

Tract elements Manganese, Copper, Zink and Nickel were also measured using AAS from the same acid digest.

ii. Phosphorus

Phosphorus content was estimated according to the method of Sekine *et al.* (1965). When phosphorus reacts with molybdate vandate reagent a yellow coloured complex is formed. The intensity of this colour is measured colorimetrically and compared with that of the known standard of phosphorus.

Two ml of acid digest was taken in the test tubes and to this equal amount of 2 N HNO_3 and one ml of freshly prepared molybdate vandate reagent {(A) 25 g ammonium molybdate in 500 ml of distilled water. (B) 1.25g

ammonium vandate in 500 ml 1 N HNO_3, (A) and (B) were mixed at the time of using} were added. Then final volume of each test tube was adjusted to 10 ml with distilled water. The ingredients were mixed well and allowed to react for 20 minutes. After 20 minutes colour intensity was measured at 420 nm using a reaction blank without any phosphorus content.

Calibration curve of standard phosphorus was prepared from standard phosphorus solution (0.110 g KH_2PO_4 per litre = 0.025 mg p^{+5} ml^{-1}). With the help of standard curve, amount of phosphorus in the plant material was calculated in g 100^{-1} g dry weight.