Chapter 3

MATERIALS AND METHODS
Chapter – 3

MATERIALS AND METHODS

The details of materials and methods have been carried out under following headings:-

1) Selection of rice fields of Morigaon district.
2) Isolation of different species of BGA from different rice fields.
3) Characterization and identification of isolated BGA.
4) Distribution of isolated BGA.
5) Screening of efficient BGA.
6) Mass production of selected BGA.
7) Field application study.
8) Biofertilizer production.

3.1 SELECTION OF RICE FIELDS OF MORIGAON DISTRICT

Morigaon district has five different Developmental Blocks namely Bhurbandha, Kapili, Laharighat, Mayang and Moirabari. These blocks are divided into 12 A.E.O. circles as Bhurbandha, Gossorbori, Morigaon, Jaluguti, Mikirbheten, Bhuragaon, Gerua, Laharighat, Jagibhakatgaon, Jagiroad, Rajamayang and Moirabari. In each circle, there are a number of villages. The rice growing areas of these villages are located in different Pathar Parichalana Samities (P.P.S.). Soil samples from each area were collected.

3.2 ISOLATION OF DIFFERENT SPECIES OF BGA FROM RICE FIELDS

3.2.1 Soil sample collection

A bulk surface soil (0-15 cm.) was collected from 5-6 different paddy fields of each P.P.S. (Plate 1 A and B). Each collected sample were numbered and put in polythene bags. Thus,
altogether 198 numbers of randomized surface soil samples were collected. In view of the convenience of the investigation, samples from different P.P.S. of the same village were combined to make composite samples. Taking approximately 200 gm. from each sample made the composite samples. The composite samples thus obtained now served as representative samples from 12 A.E.O. circles. Altogether, 77 representative samples (Table 1) were obtained for isolation of BGA. Prior to isolation, soil samples were air-dried, ground to pass through 2 mm sieve (Plate 1 C) and packed in poly bags. A portion of the each representative sample was used for estimating physico-chemical properties of soil (Jackson 1973).

The nutrient status of soil samples was assessed prior to isolation of BGA (Table 2) using standard methods. Soil pH (2:1) was estimated by Potentiometric method using glass electrode, whereas available N, P and percent organic carbon was estimated following Micro Kjeldahl method (Jackson 1973), Bray and Kurtz's No1 method (Bray and Kurtz 1945) and Walkley and Black's method (Walkley and Black 1934) respectively. The remaining portion of the soil was used for isolation of BGA.

3.2.2 Isolation of BGA

Isolation of BGA was done following serial dilution method (Vincent 1970).

3.2.2.1 Culture media

Two types of culture media were used for isolation of BGA. These were Fogg's medium (1949) and BG 110 medium (Stanier et al. 1971). Nitrogen free Fogg's medium was used for the culture of possible heterocystous as well as non-heterocystous BGA, whereas BG 110 medium was specifically used for isolation of filamentous BGA forming heterocyst. For all aerobic nitrogen fixing BGA, both isolation and purification are best achieved with BG 110 medium i.e. medium BG 11, with the source of combined nitrogen (NaNO₃) omitted.
**Fogg's N₂ free medium (Fogg 1949)**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amount (g/l)</th>
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<tbody>
<tr>
<td>KH₂PO₄</td>
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</tr>
<tr>
<td>Mg SO₄ 7H₂O</td>
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</tr>
<tr>
<td>CaCl₂ 2H₂O</td>
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</tr>
<tr>
<td>A5 Micronutrient Solution</td>
<td>1.0</td>
</tr>
<tr>
<td>Fe-EDTA Stock Solution</td>
<td>1.0</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
<tr>
<td>Distilled Water</td>
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**A5 Micronutrient stock solution**

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<th>Amount (g/l)</th>
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<td>MoO₃</td>
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</tr>
<tr>
<td>ZnSO₄ 4H₂O</td>
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<tr>
<td>CuSO₄ 5H₂O</td>
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<tr>
<td>H₃BO₄</td>
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</table>

**Fe-EDTA Solution**

Dissolved 26.1 g of ethylene diamine tetra-acetic acid (EDTA) in 268 ml of 1N potassium hydroxide solution and added 24.9 g of ferrous sulphate. Made up the volume to 1 litre. Aerated the solution overnight to produce a stable complex marked by the change in colour to
dark brown. Made up the volume again to 1 litre. Added 1 ml of this stock solution to 1 litre medium to give 5 ppm of iron.

**BG 110 medium (Stanier et al. 1971)**

<table>
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<th>Amount (g/l)</th>
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<tr>
<td>Citric acid</td>
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</tr>
<tr>
<td>Iron ammonium citrate</td>
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<tr>
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<tr>
<td>H₃BO₃</td>
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<tr>
<td>Deionized water</td>
<td>1000ml</td>
</tr>
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<td>pH</td>
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</table>

**Preparation of liquid media**

Stock solution of all the ingredients were prepared and kept in screw cap bottles. The volume of each ingredient was taken accordingly for 1 litre of the medium. The pH of the medium was adjusted with 0.1N of KOH or NaOH. The liquid medium was poured in 250 ml conical flasks. The flasks were plugged tightly with non-absorbent cotton and sterilized at 121° C (15 lbs) for 15-20 minutes.
Preparation of solid media

In order to prepare solid media, bacto-agar @15-20g was dissolved in one litre of mineral medium to give a final agar concentration of $\frac{1}{10}$ (W/V) and autoclaved. The prepared medium was stored at low temperature ($4^\circ$ to $10^\circ$C). Any precipitation left was carefully discarded. The pH of the medium was adjusted before sterilization.

3.2.2.2 Sterilization of the medium and the glasswares

The media in conical flasks were sterilized at 15 lb/sq. inch for 20 minutes in an autoclave. All types of glassware like petri-plates, culture tubes, pipettes were sterilized in hot air oven at $160^\circ \pm 1^\circ$C for 2 consecutive hours.

3.2.2.3 Isolation of BGA (enrichment culture)

BGA enrichment cultures (Plate 1 E and F) were isolated from soil samples following serial dilution technique. In Fogg’s medium, both heterocystous and non-heterocystous forms could be cultured. However, in BG 11o only filamentous and heterocystous BGA were isolated.

A 10g soil sample was added to 90 ml BG 11o liquid medium taken in 250 ml to give $10^4$ dilution. The procedure was repeated by transferring 10 ml of suspension to next 90 ml BG 11o liquid medium ($10^3$), and so on up to $10^4$ dilution. The inoculated flasks were kept in the growth chamber for 15 days at <500 lux light intensity (Plate 1 D). During these days the temperature of the chamber and relative humidity were maintained at $25^0-28^0$C (diurnal) and 60-80% respectively.

3.2.2.4 Purification vis-à-vis isolation of unialgal culture

For isolation of unialgal culture, inoculum from each dilution (preferably from $10^3$ and $10^4$) was streaked in solid BG 11o medium, contained in sterile petri-plates. The plates were incubated as described (sect. 3.2.2.3), for 10–15 days until individual colonies appeared on the surface (Plate 2 A, B and C). Each colony represented a unialgal culture. The individual
colonies were picked up and transferred to fresh BG 11o liquid medium (100ml) and incubated in the growth chamber for multiplication and further studies (Plate 2 D, E and F).

3.3 CHARACTERIZATION AND IDENTIFICATION OF ISOLATED BGA

The isolated BGA grown in each liquid media in conical flasks were picked up carefully and observed under research microscope using photographic unit. The isolated pure cultures were characterized on the basis of thallus morphology and structure, vegetative cells, their shapes and sizes, presence or absence of sheaths, heterocysts, their shapes and sizes and position, constriction of trichomes, presence or absence of spores, numbers, shapes and sizes etc. as per procedure outlined by Deshikachary (1959); Ripka *et al.* (1979) and Santra (1993). On the basis of characterization, each species of BGA were taxonomically identified. Further, the identified isolates were micro-photographed (Plates 3, 4, 5, 6 and 7) and preserved for subsequent studies. Out of 77 nos. of soil samples, 38 nos. of BGA cultures were isolated (Table 3). Further, the soil samples were grouped as per the dominance of particular species (Table 4). Also, the total numbers of BGA isolated and identified in each agricultural circle were recorded (Table 5).

3.4 DISTRIBUTION OF ISOLATED BGA

3.4.1 Frequency distribution and abundance

The frequency distribution and abundance of genera in the study circles were calculated by the following formulae:

Frequency of a genus in study area (%) = \( \frac{\text{Total no. of circles in which genus occurred}}{\text{Total no. of circles studied}} \times 100 \)

Abundance of a genus in study area = \( \frac{\text{Total no. of individual genus in all circles}}{\text{Total no. of circles in which genus occurred}} \)
From the above, the frequency distribution and abundance of the species of some dominant genera were calculated by the following formulae

Frequency of a species within a genus in study area (%) = 
Total no. of circles in which species occurred ÷ Total no. of circles studied X 100

Abundance of a species within a genus in study area = 
Total no. of individual species in all circles ÷ Total no. of circles in which species occurred

3.5 SCREENING OF EFFICIENT BGA

In the present investigation, the criteria taken for screening efficient BGA are

1) biomass production
2) total N content and
3) estimation of chlorophyll.

3.5.1 Biomass production

33 nos. of unialgal isolates were selected to assess their biomass on the basis of visual growth and presence or absence of heterocysts. An experiment was set up with 3 sets of flasks for each isolates. Individual isolates were inoculated in 100 ml of pre sterilized BG 11o liquid medium in 250 ml flasks. The inoculated flasks with one loopful of unialgal culture were incubated under natural conditions (inside the net house) where maximum sunlight could penetrate. The cultures were allowed to grow for a period of 28 days. Altogether 3 sets (99 nos.) of flasks were maintained. One set of flasks was drawn at 15 days while other two sets were drawn at 21 and 28 days after inoculation respectively to assess total biomass production. Biomass was assessed by filtering each culture using Whatman no. 41 filter paper. In the present investigation, the final biomass was recorded at 21 days of incubation period since the period showed maximum growth. Cultures in filter paper was dried to constant
weight at 60° C. Total biomass was calculated on the basis of weight loss. A total number of 24 isolates were selected on the basis of maximum biomass production for further evaluation.

3.5.2 Total N content

A 0.1 gm dry algal flake previously collected after biomass determination was digested and total N content was determined by Micro-Kjeldahl method (Jackson 1973). It was observed that the biomass production and total N content of 24 nos. of isolates varied considerably among the BGA isolates. However, in the present investigation, the isolates that showed maximum biomass production and N content were taken as one of the criteria for selection of potential isolates. Thus, out of 24 BGA isolates, 19 numbers showed positive correlation in terms of biomass production to that of N content. Therefore, these isolates were selected for estimation of chlorophyll ‘a’.

3.5.3 Measurement of chlorophyll ‘a’

Chlorophyll ‘a’ was estimated as an index of algal growth. The pigment was extracted and estimated by using Cold-extraction method (Arnon 1949) and expressed in mg/ml of fresh culture.

3.6 MASS PRODUCTION OF SELECTED BGA UNDER NATURAL CONDITION

The isolates that showed maximum biomass production as well as N-content, also the isolates having high chlorophyll ‘a’ content were selected for mass multiplication. Thus, altogether 10 numbers of isolates were selected for mass production.

Pit method was used for multiplication of selected algae. This method is easy and less expensive and recommended where farmers have to produce the biofertilizer for their own use. The field level mass culture was conducted in Telahi Chariali P.P.S. of Morigaon district.
1) Shallow pits measuring 1.5m X 1.5m X 0.20m were dug in the ground. Each pit was
 leveled uniformly and layered with a thick polythene sheet (0.5mm thickness) to hold water
 (Plate 8 A).

2) Finely powdered loamy soil (0.5 kg.) was spread in the pit and 200 gm. single super
phosphate (SSP) was added. The pH of soil was adjusted around neutral with lime. A 10 litres
BG 11o liquid medium was poured in each pit (Plate 8 B).

3) Pit was filled with water up to 10cm. depth. This water table was maintained throughout
the growth period. To prevent breeding of mosquitoes and other insect larvae, furadon @ 3-5g
of 3% ai was added. The contents of pit was mixed thoroughly and allowed the soil to settle
down for one day.

4) 100 gm of pure culture of BGA (previously mass cultured in polypropylene tray; Plate 8 D)
was inoculated in each pit as starter culture for each alga. The selected 10 nos. of BGA
cultures were allowed to multiply further for two weeks until a thick mat was formed on the
surface (Plate 8 C, E and F).

5) From these, only 5 BGA cultures were selected to use as inoculum on the basis of their
ability to grow under natural environment and wide distribution.

Preservation of isolated BGA

The primary purpose of preservation is to slow down all the physiological processes and to
maintain isolated BGA in a viable state. Cultures of algae were concentrated by
centrifugation. Glycerol (5-10%w/v) was used as preservative agent as it decreases cell
mortality during freezing and thawing. The concentrated cultures were taken into screw cap
cubes with glycerol and placed into a deep freezer.
3.7 FIELD INOCULATION STUDY

3.7.1 Effect of algalization on rice production

A field experiment was conducted during January 2002 to assess the benefit of algalization on rice production. The experiment was conducted in Telahi Chariali P.P.S. of Morigaon district. Experiments were laid out in a simple Randomized Block Design (RBD).

3.7.1.1 Selection of rice variety

The rice variety 'Jaimati' was used as test crop. It is an early Ahu (Boro rice) grown under waterlogged condition, which is suitable for BGA multiplication. Boro rice is widely cultivated in Morigaon district. With the growing interest in production of Boro rice (early Ahu) under irrigated condition, it was believed that BGA biofertilizer may conveniently be used for improvement of productivity of the crop in this particular area as during this period the weather remains more or less stable with limited rainfall.

3.7.1.2 Plot preparation, fertilization and water management

Altogether 24 individual plots measuring 2m X 2m were prepared. Plots were 3-4 times ploughed followed by laddering. Plots were finely paddled and leveled properly to uniform water retention (Plate 9 A). Each plot received recommended dose of phosphorus as SSP @ 20 kg ha⁻¹ (50gm plot⁻¹) and potassium as MOP @ 20 kg ha⁻¹ (13.3 gm plot⁻¹). Nitrogen in the form of urea was added for standard treatment and other two combination treatments as @ 40 kg ha⁻¹ (34.8gm plot⁻¹), @20 kg ha⁻¹ (17.4gm plot⁻¹) and @ 10 kg ha⁻¹ (8.7gm plot⁻¹) respectively. The ingredients were thoroughly mixed with the soil and water was added to maintain 5 cm of standing water in each plot after 2-3 days of transplanting of rice seedling (Plate 9B). The water was maintained throughout the growing period.
3.7.1.3 Treatments

The treatment combinations included 4 N levels with or/and the composite culture of BGA. All the plots received recommended dose of P and K. Thus, altogether 8 treatments arising out of these were replicated thrice. The detailed treatment combinations were as follows -

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Composition</th>
</tr>
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<tbody>
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<td>T&lt;sub&gt;1&lt;/sub&gt;</td>
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</tr>
<tr>
<td>T&lt;sub&gt;2&lt;/sub&gt;</td>
<td>N&lt;sub&gt;0&lt;/sub&gt;P&lt;sub&gt;20&lt;/sub&gt;K&lt;sub&gt;20&lt;/sub&gt; + BGA</td>
</tr>
<tr>
<td>T&lt;sub&gt;3&lt;/sub&gt;</td>
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</tr>
<tr>
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</tr>
</tbody>
</table>

3.7.1.4 Seed Selection

Seeds of 'Jaimati' were put in plain water and the healthy seeds were selected for sowing.

3.7.1.5 Seed bed preparation

Two flat seed-beds (2m X 1.5m) were prepared. Pre-germinated seeds were sown on 01-01-2002.

3.7.1.6 Transplanting

Seedlings with 5-leaf stage were transplanted on 20-02-2002 following agronomic practices (Plate 9 A). In each experimental plot 25 nos. of seedlings were planted.
3.7.1.7 Algal inoculation

It has been reported that composite cultures of BGA were more effective than single culture in improving the rice yield (Kannaiyan 1978; Nayak et al. 1996). Moreover, under natural condition no single BGA exist as monoalgal culture. Therefore, in the present investigation, a multi-inoculated trial was undertaken comprising 5 individual potential isolates of BGA (*Anabaena torulosa, Nostoc commune, Aulosira fertilissima, Calothrix marchica and Cylindrospermum majus*). These isolates were selected on the basis of biomass production, nitrogen content/chlorophyll ‘a’ content and their dominancy. The selected individual algal culture from the multiplication pit was collected (Plate 8 F) using a piece of porous cotton cloth and homogenized separately. Thereafter, the homogenized individual culture was taken in a bowl in equal amount and mixed (re-homogenized) thoroughly. This composite culture was inoculated @ 100 ml. per plot as per treatment combinations, 4 days after transplanting of seedlings. The crop was allowed to grow till maturity (Plate 9 C, D and E).

3.7.1.8 Plant protection measures

During the period of experiments, no serious incidents of disease and insect pests were observed. So, no special plant protection measures were adopted.

3.7.1.9 Harvesting and observations

The crop was harvested during the end of the month of May 2002 when the crop attained maturity and following observations were recorded.

i) Yield attributing parameters viz. plant height, no. of effective tiller and panicle number

ii) grain and straw yield

iii) population succession of BGA after harvest

iv) nutrient build up.
3.7.2 ANALYSIS OF DATA

3.7.2.1. Variance ratio

Mean data on plant height, number of tillers, number of panicle, grain yield and straw yield were worked out from three replications of each experiment. The data obtained from the experiments were subjected to statistical analysis to study the effect of applied inoculum and their interactions. The significance and non-significance of the variance was determined by calculating the variance ratio using Fischer’s method (Panse and Sukhatme 1989). The variance ratio significance at 1 per cent and 5 per cent level of probability was shown.

The analysis of variance table gives only a broad indication of performance of the inoculum with or without different level of nitrogen fertilizer as well as their interactions on selected parameters. But, in order to get a clearer appraisal of specific phenomenon of the different treatment combinations as well as the different parameters, the calculation of CD becomes essential. CD was calculated as follows:

\[ CD = \left( \sqrt{\frac{\text{Error MSS} \times 2}{\sqrt{n}}} \right) \times t \text{ value at 5% or 1% level for error degrees of freedom.} \]

where, \( n \) = total unit /individual units.

= the actual number used for calibrating the means.

The calculated value of CD was utilised in testing the difference between the two mean values as significant or not.

3.7.2.2 Standard Error

Standard errors of the means were calculated where the size or value of the sample was small.

Standard error was calculated by using the following formula:

\[ \text{SE for the mean} = \sqrt{\frac{\sum d^2}{N(N-1)}} \]

Where, \( d^2 = \text{Sum of individual deviation from the mean squared.} \)

\( N = \text{The number of observations.} \)
3.8 BIOFERTILIZER PRODUCTION

3.8.1. Preparation of immobilized inoculum

An effort was made for preparation of immobilized BGA cultures of finally selected 5 isolates, using paper strip method. In view of this, initially a homogenize mixture of the five isolates was prepared and finally cultured in a multiplication pit. The algal mat formed on the surface of multiplication pit was collected and spread over low-grade filter paper and sun-dried. The BGA coated filter papers were cut into small bits (1cm²), packed in polythene bags and preserved (Plate 10 A and B). The paper based immobilized BGA culture served as inoculum.

3.8.2. Multiplication of immobilized inoculum

Multiplication from paper bits prepared as immobilized inoculum was done after seven and fifteen months. The multiplication tray received 5 gm paper bits along with 0.1 gm S.S.P. and 2 liter of water. The water was maintained throughout the growth period. The trays were kept under bright sunlight (Plate 10 C, D, E and F).