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APPENDIX I

MEDIA COMPOSITION

1) Nutrient Agar

Peptone : 50g
Beef extract : 30g
Sodium chloride : 20g
Agar : 15g
Distilled water : 1000ml

2) Rose Bengal Agar

Mycological peptone : 5.0g
Glucose : 10.0g
Potassium dihydrogen phosphate : 1.0g
Magnesium sulfate : 0.5g
Rose Bengal : 0.05g
Chloramphenicol : 0.1g
Agar-agar : 15.5g
Distilled water : 1000ml

3) Kings B medium

Glycerol : 10ml
Proteose peptone : 20g
Dipotassium hydrogen orthophosphate : 1.5g
Magnesium chloride : 1.5g
Ampicillin : 100ppm
Chloramphenicol : 50ppm
Cycloheximide : 10ppm
Distilled water : 1000ml

4) Modified Burk’s medium
Sucrose : 3g
Dipotassium hydrogen orthophosphate : 1g
Magnesium sulphate : 1g
Calcium carbonate : 1g
Sodium chloride : 0.1g
Ferrous sulphate : 0.05g
Sodium molybdate : 5mg
Distilled water : 1000ml

5) Burk’s medium
Glucose : 20g
Potassium sulphate : 0.1g
Dipotassium hydrogen orthophosphate : 0.2g
Magnesium sulphate : 0.2g
Calcium carbonate : 5g
Sodium chloride : 0.2g
Sodium molybdate : 0.01g
Distilled water : 1000ml

6) Jarman’s medium
Sucrose : 40G
Dihydrogen potassium phosphate : 6.4mg
Dipotassium hydrogen orthophosphate : 0.26g
Magnesium sulphate : 1.6g
Sodium chloride : 1.6g
Sodium molybdate : 8mg
Calcium chloride : 0.34g
Ferric chloride : 17mg
Boric acid : 23mg
Cobalt sulphate : 0.8mg
Manganese chloride : 0.72mg
Zinc sulphate : 9.6mg
Distilled water : 1000ml

7) Basal medium

Sucrose : 10g
Dipotassium hydrogen orthophosphate : 1g
Magnesium sulphate : 0.5g
Calcium carbonate : 1g
Sodium chloride : 0.5g
Ferrous sulphate : 0.1g
Sodium nitrite : 0.1g
Sodium molybdate : 10mg
Distilled water : 1000ml
### APPENDIX II

**FT-IR Band Position (Standard)**

<table>
<thead>
<tr>
<th>Probable Assignment</th>
<th>Frequency (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free OH</td>
<td>3610-3645 (sharp)</td>
</tr>
<tr>
<td>Intramolecular H bonds</td>
<td>3450-3600 (sharp)</td>
</tr>
<tr>
<td>Intramolecular H bonds</td>
<td>3200-3500 (broad)</td>
</tr>
<tr>
<td>Chelate compounds</td>
<td>2500-3200 (very broad)</td>
</tr>
<tr>
<td>NH stretching vibrations</td>
<td></td>
</tr>
<tr>
<td>Free NH</td>
<td>3300-3500</td>
</tr>
<tr>
<td>H bonded NH</td>
<td>3070-3350</td>
</tr>
<tr>
<td>CH stretching vibrations</td>
<td></td>
</tr>
<tr>
<td>= -C-H</td>
<td>3280-3340</td>
</tr>
<tr>
<td>= -C-H</td>
<td>3000-3100</td>
</tr>
<tr>
<td>C-CH₃</td>
<td>2862-2882, 2652-2972</td>
</tr>
<tr>
<td>O-CH₃</td>
<td>2815-2832</td>
</tr>
<tr>
<td>N-CH₃ (aromatic)</td>
<td>2810-2820</td>
</tr>
<tr>
<td>N-CH₃ (aliphatic)</td>
<td>2780-2805</td>
</tr>
<tr>
<td>CH₂</td>
<td>2843-2863, 2916-2936</td>
</tr>
<tr>
<td>CH</td>
<td>2880-2900</td>
</tr>
<tr>
<td>SH stretching vibrations</td>
<td></td>
</tr>
<tr>
<td>Free SH</td>
<td>2550-2600</td>
</tr>
<tr>
<td>C= -C stretching vibrations</td>
<td></td>
</tr>
<tr>
<td>C= -CH (terminal)</td>
<td>2100-2140</td>
</tr>
<tr>
<td>C-C = C-C</td>
<td>2190-2260</td>
</tr>
<tr>
<td>C-C = C-C = -CH</td>
<td>2040-2200</td>
</tr>
<tr>
<td>C=O stretching vibrations</td>
<td></td>
</tr>
<tr>
<td>Nonconjugated</td>
<td>1700-1900</td>
</tr>
<tr>
<td>Conjugated</td>
<td>1590-1750</td>
</tr>
<tr>
<td>Amides</td>
<td>~1650</td>
</tr>
<tr>
<td>C = C stretching vibrations</td>
<td></td>
</tr>
<tr>
<td>Nonconjugated</td>
<td>1620-1680</td>
</tr>
<tr>
<td>Conjugated</td>
<td>1585-1625</td>
</tr>
<tr>
<td>CH bending vibrations</td>
<td></td>
</tr>
<tr>
<td>CH₂</td>
<td>1405-1465</td>
</tr>
<tr>
<td>CH₃</td>
<td>1355-1395, 1430-1470</td>
</tr>
<tr>
<td>C-O-C vibrations in esters</td>
<td></td>
</tr>
<tr>
<td>Formates</td>
<td>~1175</td>
</tr>
<tr>
<td>Acetates</td>
<td>~1240, 1010-1040</td>
</tr>
<tr>
<td>Benzoates</td>
<td>~1275</td>
</tr>
<tr>
<td>C- OH stretching vibrations</td>
<td></td>
</tr>
<tr>
<td>Secondary cyclic compounds</td>
<td>990-1060</td>
</tr>
<tr>
<td>CH out of plane bending vibrations in substituted ethylenic systems</td>
<td>905-915, 985-995, 650-750, 960-970, 885-895</td>
</tr>
</tbody>
</table>

(Silverstein et al., 2005, Williams and Fleming, 2007)
AUTHOR’S PUBLICATIONS
RESEARCH ARTICLE

BACTERIAL ALGINATE CARRIER-BASED PREPARATIONS OF PLANT GROWTH-PROMOTING BACTERIAL INOCULANTS

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ABSTRACT

Bacterial alginate carrier-based preparations of two plant growth-promoting rhizobacteria (PGPR) viz. Bacillus subtilis and Pseudomonas fluorescens, developed in different formulations were evaluated for their growth promotion, rhizosphere colonization, and viability under storage. The effect of these formulations as fresh preparations, and after 6 months of storage at 4°C and room temperature was also determined. The bacterial inoculants in all the formulations were found to enhance the growth parameters of the test plant species; best results were obtained in case of bacterial alginate-based formulations. Maximum number of inoculated bacteria were recovered from the rhizosphere of bacterial alginate-based formulation-treated plants after 6 weeks of growth. Viability of bacterial inoculants was maximal in bacterial alginate beads, and bacterial alginate beads supplemented with skim milk formulations, after 180 days of storage at 4°C.

INTRODUCTION

Use of microorganisms for plant growth promotion and disease control is well recognized (Glick 1995; Whipp’s 2001). Importance of native strains and ecological specificity while selecting the microbial inoculants for a specific environment is also realized (Pandey et al. 1998). Isolation of microorganisms, screening for desirable characters, selection of efficient strains and production of inoculants are important steps for making use of this microbe-based technology. For field applications, the inoculum is required in an appropriate formulation. Viability of inoculum in an appropriate formulation for a certain length of time is important for commercialization of the technology (Bashan 1998).

With a view of developing microbial inoculants using bacterial alginate produced from mangrove isolate Azotobacter vinelandii for use in field regions of Chidambaram, a large number of bacteria were isolated from soil samples collected from mangrove sediments of Pichavaram. Two common plant growth promoting bacteria namely Bacillus subtilis and Pseudomonas fluorescens were selected based on their abundance in the soil. In the present investigation, the selected bacterial inoculants were prepared in four formulations and tested for their root colonization and growth promotion through bioassays using rice as a test species. The bioassays were conducted using fresh as well as stored preparations of the bacterial inoculants. The viability of the bacterial inoculants in all the formulations under storage at 4°C and at room temperature was evaluated over six months. The study has implications in field application and commercialization of the bacterial inoculants.

MATERIAL AND METHODS

Bacterial inoculants

Two selected species of bacteria, Bacillus subtilis and Pseudomonas fluorescens, isolated from the soil samples collected from Pichavaram mangrove and characterized using biochemical tests, were used as inoculants. The criteria for screening and selection of the bacterial species were their growth promotion, biocontrol and tolerance to low temperature. The cultures of Bacillus and Pseudomonas were maintained on slants of Tryptone yeast extract agar and Pseudomonas agar respectively, at 4°C.

Formulations

The bacterial inoculants were prepared in following five formulations: (1) bacterial alginate beads, (2) alginate beads supplemented with skim milk, (3) charcoal-based, and (4) broth-based preparations. Bacterial alginate was extracted and optimized by using the procedure mentioned by (LakshmiPriya and Sivakumar, 2013) and calcium chloride (0.1 M) were autoclaved separately. The bacterial strains were cultured in Tryptone yeast extract broth in a rotary shaker at 200 rev min⁻¹ at 28 ± 2°C for 24-48 h to obtain a final concentration of 10⁻¹ e.u. ml⁻¹. Fifty millilitres of bacterial suspension was aseptically mixed with bacterial alginate solution and stirred gently for 1 h in a rotary shaker at 100 rev min⁻¹. For preparing the beads this mixture was added dropwise with the aid of a micropipette into sterilized 0.1 M CaCl₂ at room temperature.
The resulting bacterial alginate beads entrapped the bacterial cells. The beads were maintained in the solution at room temperature for 1–3 h to obtain regular solid beads.

The CaCl₂ was then pumped out and the beads were washed twice with sterile tap water. After washing, the beads were incubated in fresh Trypont yeast extract broth for an additional 24 h in a rotary shaker at 28 ± 2°C to allow bacteria to multiply inside the beads. Then the beads were washed twice with sterile distilled water and collected. The beads were then allowed to dry overnight in a laminar flow hood. Alginate formulation containing skim milk as a food supplement was prepared in the similar manner supplementing 10% (w/v) sterile skim milk to the bacterial broth prior to alginate treatment.

Charcoal-based preparations were made by growing the bacterial culture on Tryptone yeast extract agar at 28 ± 2°C for 24–48 h. The growth was harvested in sterile water and the cell numbers were adjusted to 10⁶ c.f.u. ml⁻¹. Hundred and fifty grams of sterile charcoal was mixed with 150 ml bacterial suspension and 10 g of gur (local sugar) was added. The slurry was mixed properly under aseptic conditions and air dried at 28 ± 2°C overnight in a laminar flow hood. Broth based formulation was prepared in Tryptone yeast extract broth. The broth was inoculated with a loop full of freshly grown bacterial culture and incubated at 28 ± 2°C for 24 h raising the final concentration of 10⁵ c.f.u. ml⁻¹. All the formulations were used as fresh, and after 6 months storage at 4 ± 2°C and at room temperature (Maximum 25.6 ± 2°C and minimum 5.2 ± 2°C) in hermetically-sealed flasks under laboratory conditions. All the media used were from Hi-media, Mumbai.

**Bioassay for evaluation of growth promotion and rhizosphere colonization by bacterial inoculants in various formulations**

The bioassay was conducted using rice as a test species. Rice seeds were grown in trays (32×32×10 cm) each containing sixteen cups. A total number of five treatments were taken; four inoculation treatments for each bacterial strain and one control (without any inoculation). For each treatment three trays were used. One plant was raised in each cup filled with local soil. Inoculations were carried out by adding 1 g of preparation in the case of bacterial alginate and charcoal-based formulations and 1 ml of bacterial broth in case of broth-based preparations, in the soil around each seed at the time of sowing. In the control treatment, seeds were sown without any inoculation. For evaluation of growth twenty five randomly selected plants from each treatment and control after 42 days of inoculation were uprooted and observations were recorded for increment in length and dry weight of root and shoot.

Dry weight was determined by placing the roots and shoots, separately into small, pre-weighed brown paper bags and drying them in an oven at 80°C for 48 h. For determining the rhizosphere colonization of the introduced bacteria, soil samples were collected from inoculated treatments. The enumerations for *Bacillus* and *P. fluorescens* were carried out from their respective treatments following serial dilution technique. The colonies of *Bacillus* were enumerated on Tryptone yeast extract agar supplemented with rifampicin (50 μg ml⁻¹) and colonies of *P. fluorescens* were enumerated on Pseudomonas agar supplemented with ampicillin (1000 μg ml⁻¹). Each experiment was conducted in triplicate.

**Enumeration of viable bacteria in various formulations under storage**

Viable bacteria in the formulations used in the study were counted by dissolving 1 g in case of solid formulation or 1 ml of liquid formulation in 9 ml potassium phosphate buffer (0.25 M, pH 6.8) in a test tube for 16–24 h at 28 ± 2°C. Further enumeration was carried out by dilution-plate technique using Tryptone yeast extract agar. The plates were incubated at 28 ± 2°C and c.f.u. were counted after five days. The viability tests were carried out with the fresh as well as stored preparations. The preparations were stored at two temperatures, 4 ± 2°C and at room temperature, and viability was checked at an interval of two months till six months. The plate counts were carried out in triplicate and the final values (log₁₀ c.f.u. g⁻¹ or ml⁻¹) of viable bacteria were the average of three readings.

**RESULTS AND DISCUSSION**

The effect of bacterial inoculations under different formulations on growth of rice showed varied results. The response of inoculations varied with different formulations as well as bacterial species used. The inoculations resulted in significant increase in most of the growth parameters with respect to control. Among the formulations, bacterial alginate-based formulations were more effective followed by charcoal and broth based formulations, respectively. There are reports of plant growth promotion ability of both the bacteria used in this study (Ryder et al., 1999; Pandey et al., 2000). Due to the limitations of direct inoculation and the use of various solid-phase bacterial inoculants, several polymer-based formulations, such as alginate beads, wet and dry alginate microbeads and gum-arabic preparations of bacterial species like *Azospirillum brasilense* Cd, *Pseudomonas fluorescens*, and *Rhizobium* sp. have been evaluated (Bashan, 1998; Forestier et al., 2001; Bashan et al., 2002).

Survival and establishment of the inoculated strains in the rhizosphere in competition with native microbial flora is important (Kim et al., 1997; Natuiyal, 1997). The use of generic markers such as intrinsic levels of resistance to various antibiotics is one of the simple and rapid method for strain identification, therefore it was used for evaluation of rhizosphere competitiveness of the bioinoculants in the present study. The ability of the bacterial inoculants to colonize the rhizosphere that was determined at weekly intervals varied with respect to different formulations (Fig.1).

**Fig.1. Colonization of rhizosphere of rice by bacterial inoculants in various formulations**
The bacterial populations that were recorded, initially lower in the case of bacterial alginate-based formulations, increased with time. In contrast to this, the maximum number of bacteria in the case of charcoal and broth-based formulations was recovered after 7 days of inoculation declined after 14 and 21 days in case of both bacteria. Maximum colonization of rhizosphere (5.319 and 5.231 log10 c.f.u. g-1 of soil) was recorded in bacterial alginate with skim milk by B. subtilis and in the bacterial alginate bead formulation by P. fluorescens respectively. Minimum colonization was recorded in case of the broth based formulation (3.125 and 3.410 log10 c.f.u. g-1 of soil) by B. subtilis and P. fluorescens respectively. Slow release of the entrapped bacteria and the protective environment created by encapsulation on alginate (Favel, 1985; Bashan, 1986), may be the reason for the initially low and subsequently higher populations of bacteria in the rhizosphere in case of alginate-based formulations. Greater ability of survival and colonization of rhizosphere by immobilized form of inoculants in comparison to free forms have been reported by (Hammad and El-Mohandes, 1999). For commercialization, viability of bioinoculant in a prescribed formulation for a certain period with preservation of strain characteristics is desirable (Fages, 1992; Smith, 1992). The effects of length of storage and temperature on the viability of B. subtilis and P. fluorescens in the various formulations are presented in Fig. 2(a) and 2(b).

The loss in viability of B. subtilis and P. fluorescens was minimum in alginate bead formulations (with or without skim milk) over a period of 6 months. (Bashan, 1986) described that the immobilization procedure carried out in preparation of alginate based formulations results in the death of most bacteria, and therefore a second multiplication is required for increasing the number of cells in a formulation.

In the present investigation the decline in cell numbers in all the formulations of both the bacteria was greater at room temperature as compared with storage at 4°C. Alginate beads have been reported to preserve the beneficial properties of PGPRs under storage (Russo et al., 2001). Lewis & Papavizas (1985) suggested storage of alginate pellets of strains of Trichoderma viride and Gliocladium viride at 5°C. Lower temperatures (4–10°C) are known to retard division and metabolic activities of bacterial cells resulting in a reduced consumption of nutrients and reduced loss of moisture in the carriers favouring storage of inoculants (van Shreven, 1970).

In conclusion, the bacterial alginate bead-based formulation (with or without skim milk) of B. subtilis and P. fluorescens with their storage under refrigeration (4°C) was found to be the best carrier-based preparation for improving plant growth. The present investigation is a step towards field application and
commercialization of the bacterial inoculants selected for their use in the rice fields. The large scale production of the bacterial inoculants in bacterial alginate bead form is recommended for field application and commercialization.

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References


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STUDIES ON IMMOLIZATION AND STORAGE OF PLANT GROWTH PROMOTING INOCULANTS IN BACTERIAL ALGINATE BEADS

Lakshmipriya V. P., P. K. Sivakumaar And R. Parthasarathi
Department of Microbiology, Annamalai University, Annamalai Nagar, Tamil Nadu

Abstract: The growth and distribution of few plant growth promoting inoculants individually and as consortium, immobilized within bacterial alginate beads was assessed by viable cell counts on nutrient agar method and Scanning Electron Microscopy (SEM). Both techniques indicated that the inoculants survived and grown within alginate beads. The plant growth promotion was assessed by using tray culture. The recovery of the inoculants was observed after storage up to six months. It was found that the required inoculants cell load was maintained. The observations indicated a constant random distribution of cells within alginate beads for an extended period. This indicated that the spaces within the alginate bead were maximally supported for their survival in high cell densities.

Key words: Bacterial alginate, Immobilization, SEM, Plant Growth Promotion.

INTRODUCTION:
Importance of native strains and ecological specificity while selecting the microbial inoculants for a specific environment is realized (Pandey et al., 1998). Use of microorganisms for plant growth promotion and disease control is well recognized. Isolation of microorganisms, screening for desirable characters and selection of efficient strains and production of inoculums are important steps for making use of this microbe-based technology. For field applications, the inoculum is required in an appropriate formulation. Viability of inoculum in an appropriate formulation for a certain length of time is important for commercialization of the technology (Bashan, 1998).

With a view of developing microbial inoculants using bacterial alginate for use in plant growth promotion, alginate was produced by Azotobacter vinelandii and used for immobilization studies using various agricultural inoculants. The inoculants were prepared as individual as well as consortium for rice plant. The compatibility of the inoculants was analyzed using plate assay method.

MATERIAL AND METHODS
Bacterial alginate and inoculums used in the study
Three selected species of bacteria, Bacillus sp., Pseudomonas fluorescens and Azospirillum sp., isolated from the soil samples collected from Pichavaram mangrove and characterized using biochemical tests, were used as inoculants individually and as consortium. The criterion for screening and selection of the bacterial species was their growth promotion, biocontrol and survival under storage. The cultures of Bacillus, Pseudomonas and Azospirillum sp. were maintained on slants of Tryptone yeast extract agar, Pseudomonas agar and NB agar slants respectively at 4°C. The bacterial alginate was produced by Azotobacter vinelandii, isolated from Pichavaram mangroves by the method suggested by Lakshmipriya and Sivakumaar, (2013).

Immobilization technique
The microbial cells (Bacillus sp., Pseudomonas sp. and Azospirillum sp.) were immobilized as beads according to the procedure of Leung et al., (2000). Two percent bacterial alginate solution is prepared in sterile distilled water. Later 100 ml of the alginate is cooled to room temperature and 10% of the cell culture is added, the optimum condition was also studied as described above. The contents were mixed well by vigorous shaking to get a homogenised mixture. In a separate beaker 100 ml of 0.1 M calcium chloride solution was taken. The bacterial alginate containing cell culture suspension is extruded drop wise through a syringe and allowed to fall in the beaker containing calcium chloride solution. The beads formed are left in the beaker overnight for hardening. Then beads were washed and stored in distilled water at 28 ± 2°C.

Scanning electron microscopic studies
Scanning electron microscopy (SEM) evaluation of the immobilized alginate beads with and without inoculum was carried out to examine surface morphology. Beads were mounted on metal stubs with carbon tape and the sputtered with a 150 A thick layer of gold in a Bio-Rad apparatus. A SEM (Jeu- Model 6390 Device in 30 kV) was used to evaluate surface characteristics.
Bioassay for evaluation of growth promotion

The bioassay was conducted using rice as a test species by the method of Trivedi et al., 2005. Rice seeds were grown in trays (32×32×10 cm) each containing sixteen cups. A total number of five treatments were taken; three inoculation treatments with each bacterial strain (Bacillus sp., Pseudomonas sp., and Azospirillum sp.), one consortium (Bacillus sp. + Pseudomonas sp. + Azospirillum sp) and one control (without any inoculation). For each treatment three trays were used. One plant was raised in each cup filled with local soil. Inoculations were carried out by adding 1 g of preparation of bacterial alginate. In the control treatment, seeds were sown without any inoculation. For evaluation of growth twenty five randomly selected plants from each treatment and control after 42 days of inoculation were uprooted and observations were recorded for increment in length and dry weight of root and shoot. Dry weight was determined by placing the roots and shoots, separately into small, pre-weighed brown paper bags and oven drying at 80°C for 48 h.

Recovery of plant growth promoting bacteria from bacterial alginate beads

The method of Pankaj Trivedi and Anita Pandey (2007) was followed for the recovery process. The viability of bacteria in alginate beads was observed by dissolving 1.0 g of alginate beads in 1.0 ml of respective broth containing 9.0 ml of potassium phosphate buffer (0.25 M, pH 6.8) in a test tube for 16-24 h at 28 ± 2°C. Further the growth of the PGPR was observed by pour plate technique. The viability test was carried out following serial dilution technique with freshly prepared alginate beads and as well as alginate beads stored at 4°C at an interval of 1 month up to a period of 6 months of study.

RESULTS AND DISCUSSION

The surface morphology of the prepared beads was studied by scanning electron microscopy (SEM). SEM photographs of the blank beads (Figure 1) compared with inoculums loaded beads show a difference in surface morphology. Smoothness increased when inoculum was loaded in the beads. The SEM photographs of the inoculum loaded beads (Figure 2) show that the culture is dispersed in the polymeric matrix, which further confirms that this system is a polymeric matrix system for beads prepared.

In terms of growth, both viable cell counts and visual observations indicated that the culture cells did survive and grow within alginate beads. Growth is referred to as change in total population, rather than an increase in the size or mass of an individual microorganism (Pelczar et al., 1986). Unlike Wada et al. (1980) and Shinmyo et al. (1982), who reported growth to be limited to the outer layer of the bead, our observations indicated that growth occurred in the cavities all over the bead showing no remarkable preferences between the outer layer and the central part. Cells were observed in the central part of the alginate beads even two weeks after incubation.
The surviving populations were 78.66× 10^8 cfu g^-1 for Bacillus sp., 77.88× 10^8 cfu g^-1 for Pseudomonas sp., 56.66× 10^8 cfu g^-1 for Azospirillum and consortium (Bs+Ps+Azp) in Alginate beads.

The observations in this study indicated a constant distribution and outer layer preference depending on the bead size and the aeration conditions.

Slow release of the entrapped bacteria and the protective environment created by encapsulation on alginate (Fravel, 1985; Bashan, 1986), may be the reason for the initially low and subsequently higher populations of bacteria in the rhizosphere in case of alginate-based formulations. Greater ability of survival and colonization of rhizosphere by immobilized form of inoculants in comparison to free forms have been reported by (Hammad and El-Mohandes, 1999).

For commercialization, viability of bioinoculant in a prescribed formulation for a certain period with preservation of strain characteristics is desirable (Fages, 1992; Smith, 1992).

### Table 1: Survival of individual Bacillus sp., Pseudomonas sp., Azospirillum and consortium (Bs+Ps+Azp) in Alginate beads

<table>
<thead>
<tr>
<th>Storage period (months)</th>
<th>Bacillus (Bs)</th>
<th>Pseudomonas (Ps)</th>
<th>Azospirillum (Azp)</th>
<th>Consortium (Bs+Ps+Azp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>73.00× 10^8</td>
<td>74.00× 10^8</td>
<td>75.00× 10^8</td>
<td>76.00× 10^8</td>
</tr>
<tr>
<td>1</td>
<td>76.00× 10^8</td>
<td>77.00× 10^8</td>
<td>78.00× 10^8</td>
<td>79.00× 10^8</td>
</tr>
<tr>
<td>2</td>
<td>70.00× 10^8</td>
<td>71.00× 10^8</td>
<td>72.00× 10^8</td>
<td>73.00× 10^8</td>
</tr>
<tr>
<td>3</td>
<td>65.00× 10^8</td>
<td>66.00× 10^8</td>
<td>67.00× 10^8</td>
<td>68.00× 10^8</td>
</tr>
<tr>
<td>4</td>
<td>60.00× 10^8</td>
<td>61.00× 10^8</td>
<td>62.00× 10^8</td>
<td>63.00× 10^8</td>
</tr>
<tr>
<td>5</td>
<td>55.00× 10^8</td>
<td>56.00× 10^8</td>
<td>57.00× 10^8</td>
<td>58.00× 10^8</td>
</tr>
<tr>
<td>6</td>
<td>50.00× 10^8</td>
<td>51.00× 10^8</td>
<td>52.00× 10^8</td>
<td>53.00× 10^8</td>
</tr>
</tbody>
</table>

Values in parenthesis are log10 transformed values.

The immobilized biomass offers many advantages including better reusability, high biomass loading and minimal clogging in continuous flow systems (Holm and Volesky, 1998). Also, immobilized beads are hard enough to withstand the application, pressures, water retention capacity, porous, transparent to metal ion sorbent species and have high and fast sorption uptake even after repeated regeneration cycles. In addition because of immobilization, the biosorbents will have better shelf life and offer easy and convenient usage compared to free biomass, which is easily biodegradable (Volesky and May-Phillips, 2000).

In conclusion, the bacterial alginate bead-based formulation of various inoculants individual as well as consortium in accordance with their storage was found to be the best carrier-based preparation for improving plant growth. The present investigation is a step towards field application and commercialization of the bacterial inoculants selected for their use in the rice fields. The large scale production of the bacterial inoculants in bacterial alginate bead form is recommended for field application and commercialization.

### ACKNOWLEDGEMENTS

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### REFERENCES


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ORIGINAL RESEARCH ARTICLE

Isolation and Characterization of Total Heterotrophic Bacteria and Exopolysaccharide Produced From Mangrove Ecosystem

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ABSTRACT

Heterotrophic bacteria and their processes in the mangrove environment into focus, an understanding on their abundance, distribution, production and, their involvement in nutrient cycling and how they are at the base of microbial food web is essential. Total Heterotrophic Bacteria (THB) was screened from Pitchavaram mangrove sediment. Eight isolates were selected based on the colony morphology and of colonies were identified by phenotypic and biochemical character such as Bacillus subtilis, Strepococcus sp.. Staphylococcus sp., Pseudomonas sp. Photobacterium sp., Enterobacteriaceae sp., Escherichia coli and Azotobacter sp. All the isolates were screened for producing Exopolysaccharide (EPS), of which Azotobacter sp. and Pseudomonas sp. produced high appreciable amount of EPS.

Key words: Mangrove, Biochemical characterization, Heterotrophic bacteria and Exopolysaccharide

1. INTRODUCTION

Mangrove forests occupy several million hectares of coastal areas worldwide and distributed in over 112 countries and territories comprising a total area of about 1,81,000 km² in over one fourth of the world’s coastline[1,2]. According to Forest Survey of India (FSI) (State of Forest Report, 1999), out of 4,87,100 ha of mangrove wetlands in India, nearly 56.7% (2,75,800 ha) is present along the east coast, and 33.5% (1,14,700 ha) along the west coast and the remaining 19.8% (96,600 ha) is found in the Andaman and Nicobar islands. The largest single area of mangroves in the world lies in the Bangladesh part of the Sunderbans, covering an area of almost 6,00,000 ha including waterways. There are about 6.9 million ha in the Indo-Pacific region, 3.5 million ha in Africa, 4.1 million ha in Americas including the Caribbean.

Mangroves also survive in some temperate zones but there is a rapid decrease in the number of species including latitude[3,4,5]. Mangrove ecosystems are rich in bacterial flora. Fertility of the mangrove waters results from the microbial decomposition of organic matter and recycling of nutrients. Among the microbes, the bacterial population in mangroves is many-fold greater than the fungi. In tropical mangroves, bacteria and fungi constitute 91% of the total microbial biomass, whereas algae and protozoa represent only 7% and 2% respectively [6].

Most of the sea bacteria belong to Gram-negative [7]. Gram-positive bacteria are less than 10% of the total bacterial population and higher percentage in sediments. Arthrobacter and endospore producing forms Bacillus and Clostridium (Family: Bacillaceae) have also been isolated. Especially Bacillus species readily grow in medium containing nutrients. Majority of bacteria belong to the families Pseudomonadaceae and Vibrionaceae. Most marine bacteria are aerobic or facultatively anaerobic because large parts of the ocean are well oxygenated. Nitrogen-fixing bacteria such as members of the genera Azospirillum, Azotobacter, Rhizobium, Clostridium and Klebsiella were isolated from the sediments, rhizosphere and root surfaces of various mangrove species. Several strains of diazotrophic bacteria such as Vibrio campbelli, Listerella anguillarum, V. aestuarianus and Phyllobacterium sp. were isolated from the rhizosphere of the mangroves in Mexico [8].

Mangroves provide a unique ecological environment for diverse bacterial communities. The bacteria fill a number of niches and are fundamental to the functioning of these habitats. For example, sulfate-reducing bacteria (e.g., Desulfovibrio, Desulfotomaccula, Desulfosarcina, and Desulfococccus [9,10]) are the primary

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decomposers in anoxic mangrove sediments. These bacteria largely control iron, phosphorus, and sulfur dynamics and contribute to soil and vegetation patterns \(^{[11]}\). Methanogenetic bacteria are seasonally abundant in sediments where *Avicennia* species dominate \(^{[12,13]}\). Subsurface bacterial communities (along with epibenthic microalgae) may sequester nutrients and hold them within nutrient-limited mangrove muds \(^{[14]}\).

Alginates are exopolysaccharides synthesized by marine algae \(^{[15,16]}\), as well as by bacteria such as *Pseudomonas aeruginosa* and *Azotobacter vinelandii* \(^{[17]}\). *A. vinelandii* synthesizes a polymer that is similar to that of the algae \(^{[17]}\). \(^{[18]}\). The present study was aimed to isolate the total heterotrophic bacteria present in the mangrove sediments and rhizospheric soil and screening of exopolysaccharide producing bacteria.

2. MATERIALS AND METHODS

Sample collection

20 samples were collected from different sites in Pitchavaram. *Rhizophora apiculata* and *Rhizophora mucronata* plant rhizosphere and sediment (non-rhizosphere) samples were collected in the clean polyethylene bags and transported to the laboratory and processed within 3 hours and microbial analysis was carried within 4 hours. Mangroves sediment are acidic and clay in nature. Sediment becomes loose because of the presence of the decaying organic matter and sediment is black in colour.

Enumeration of microorganisms from rhizosphere and non-rhizosphere soil of mangrove ecosystem

The soil samples collected from various sources were serially diluted up to \(10^6\) dilution to determine the population of bacteria, fungi and Actinomycetes \(^{[19]}\). The \(10^6\) dilutions were plated on sterile Petri plates containing nutrient agar (NA) medium and incubated at \(28 \pm 2^\circ C\) for two days for enumerating the bacterial population, \(10^4\) dilutions were plated on sterile Petri plates containing Rose Bengal Agar medium (RBA) and incubated at \(28 \pm 2^\circ C\) for 3 days for enumerating fungal colonies and \(10^5\) dilutions were plated on sterile Petri plates containing KenKnight’s agar medium (KKK) and incubated at \(30 \pm 2^\circ C\) for 5 to 7 days for enumerating actinomycete colonies. After incubation the number of bacterial, fungal and actinomycetes colonies in the respective plates were counted and the population was expressed in terms of cfu g\(^{-1}\) soil on oven dry basis. The R: S ratio was calculated by using the formula proposed by Aneja\(^{[20]}\).

Screening and Identification of Total Heterotrophic Bacteria (THB)

The sediment sample were diluted and plated in Nutrient agar medium prepared with 50% aged seawater \(^{[21]}\). Triplicate plates from each dilution were incubated at \(28^\circ C\). After incubation the colonies were counted by colony forming unit (CFU) and subculture by colony morphology. The different morphological and biochemical characterization \(^{[22]}\) of the isolates were investigated according to the Bergey’s Manual of determinative bacteriology \(^{[23]}\).

Isolation of heterotrophic bacteria for EPS production

Culture conditions

The standard seed and production media contained per liter of distilled water: 6 g yeast extract; \(0.6 \text{ g (NH}_4\text{)}_2\text{SO}_4, 2 \text{ g Na}_2\text{HPO}_4; 0.3 \text{ g MgSO}_4.7\text{H}_2\text{O}; \text{pH was adjusted to 7. To assess the effect of cultural conditions on bacterial exopolysaccharide production, standard medium was supplemented with different amounts of glucose, (NH}_4\text{)}_2\text{SO}_4, \text{Na}_2\text{HPO}_4 and sodium acetate. Then, to limit the variation of pH within the range 7.2 - 6.7, this medium was buffered by adding 50 mM 3-(N-morpholino) propane-sulfonic acid (MOPS) and then adjusting the pH to 7.2 with NaOH \(^{[24]}\). All media used were sterilized at \(121^\circ C\) for 15 min.

The maintenance culture was transferred to 100ml Erlenmeyer flasks containing 25 ml of sterile medium each. The seed culture was incubated on a rotary shaker at 300 min\(^{-1}\) and 35°C for 24 hrs. A fraction (1.25 ml) of the vegetative seed culture was then used to inoculate a 100 ml Erlenmeyer flask containing 25 ml of the same medium which was incubated at different shaking speeds (25 and 450 min\(^{-1}\)) and temperatures (23 - 42°C) for 48 to 120 hrs.

3. RESULTS AND DISCUSSION

Enumeration of microbial biodiversity in rhizosphere and non-rhizosphere of mangrove soil

The enumeration of microorganisms in the rhizosphere and non-rhizosphere soil samples revealed that the rhizosphere soil sample contained higher microbial populations compared to non-rhizosphere soil (Table 1). The rhizosphere population was recorded as \(2.84 \times 10^9\) for bacteria, \(2.50 \times 10^7\) for fungi and \(1.12 \times 10^8\) for actinomycetes and in non-rhizosphere \(1.86 \times 10^9\), \(1.65 \times 10^7\) and \(1.00 \times 10^8\) for bacteria, fungus and actinomycetes respectively was observed.
Phenotypic Characterization

Bacteria inhabiting mangroves environment are dominant microorganisms fairly well adapted to the extreme condition of mangrove ecosystem. Recent bacteriological studies of mangrove environment concerned mainly their sanitary, pollution and bacterial number [25]. Eight different isolates were identified and sub cultured in the Nutrient agar (Table 2). The following isolates 2, 3 and 8 observed as round colonies, isolate 5 is transparent in nature. Rhizoid structure was observed in the isolate 4, isolate 1 and 6 was mucoid and isolate 7 was filamentous in nature. Colony size of the isolates also varied from the range of 0.1 - 1.5 mm. the colour was also varied as orange, yellow white and pale white.

Table 2: Showing the Morphological Characters of different bacterial isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Colony Colour</th>
<th>Colony Size (mm)</th>
<th>Morphology and Nature of the Colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>White</td>
<td>0.1 - 0.3</td>
<td>Round, mucoid</td>
</tr>
<tr>
<td>2</td>
<td>Orange</td>
<td>0.1 - 0.5</td>
<td>Round</td>
</tr>
<tr>
<td>3</td>
<td>Yellow</td>
<td>0.5 - 1.5</td>
<td>Round, Convex</td>
</tr>
<tr>
<td>4</td>
<td>White</td>
<td>0.5 - 1.0</td>
<td>Rhizoid, sticky</td>
</tr>
<tr>
<td>5</td>
<td>White</td>
<td>0.5 - 1.5</td>
<td>Transparent</td>
</tr>
<tr>
<td>6</td>
<td>Pale white</td>
<td>0.2 - 0.7</td>
<td>Mucoid, circular</td>
</tr>
<tr>
<td>7</td>
<td>White</td>
<td>0.7 - 1.5</td>
<td>Filamentous, irregular</td>
</tr>
<tr>
<td>8</td>
<td>White</td>
<td>0.1 - 0.6</td>
<td>Round, convex</td>
</tr>
</tbody>
</table>

Biochemical Characterization

There are many methods for identifying bacteria. Traditionally, an observational and biochemical approach has been used. Simply looking at (and even smelling) a bacterial colony growing on an agar plate can give an experienced researcher clues to a bacterium's identity. Bacteria are categorized as "Gram Positive" or "Gram Negative" according to whether or not they are stained by a chemical dye, a common biochemical technique [22]. The screened isolates were subjected in to the biochemical characterization, test results of Indole, Methyl red, Voges Proskauer, citrate utilization, carbohydrate fermentation (Glucose, Sucrose and Lactose), Catalase and Oxidase (Table 3).

The results obtained in this study portray the bacterial community associated to the mangrove rhizosphere as a dynamic one, experiencing important changes in abundance of both total and active bacteria. In contrast to the vertical exponential decline in the bacterial abundance often observed in muddy terrogenous sediments [26-27] the community analyzed here was, in terms of abundance, rather homogenous through the depth. The results from our study indicate a higher proportion of Gram negative bacteria than Gram positive bacteria among the species of heterotropic bacteria. The results were in agreement with the general rules that the proportion of Gram negative bacteria is much higher than the proportion of Gram positive bacteria in the ocean [28].

Table 3: Phenotypic and Biochemical characteristics of isolates

<table>
<thead>
<tr>
<th>Tests</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Indole</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Methyl red</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Voges proskauer</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Starch, hydrolysis</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

In our study, it indicated that *Pseudomonas* sp., *Bacillus* sp., *Enterobacteriaceae* sp., *Escherichia coli*, *Streptococcus* sp., *Azotobacter* sp., *Staphylococcus* sp. and *Photobacterium* sp. were abundant in the mangrove rhizosphere sediment samples. The result of *Streptococcus* and *Escherichia coli* were comparable to the earlier reports of Thompson [31][29][30] recorded 9 genera and Paramasivam (2002) recorded 10 genera of THB from Pitchavaram and Muthupettai mangrove environment respectively. The genus *Bacillus* comprises a phylogenetically and phenotypically heterogeneous group of species. Due to their ubiquity and capability to survive under adverse conditions, heterotrophic *Bacillus* strains are hardly considered to be species of certain habitats [32]. Several *Bacillus* strains from soils and mangrove sediments have already been reported as hydrocarbon degraders
and emulsifier producers. Macrae found bacilli as dominant rhizosphere organisms in mangroves and suggested that they should be targeted to provide microbial solutions which ameliorate polluted environments. All the isolates were further characterized based on EPS production. *Pseudomonas* sp. (Isolate 1) and *Azotobacter* sp. (Isolate 6) were found more efficient in producing EPS and were further characterized for alginate production.

### REFERENCES


### Table 4: Production of Exopolysaccharide (EPS) by different mangrove isolates

<table>
<thead>
<tr>
<th>S. No</th>
<th>Name of the Isolate</th>
<th>Production of EPS (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Isolate 1</td>
<td>6.8</td>
</tr>
<tr>
<td>2</td>
<td>Isolate 2</td>
<td>4.9</td>
</tr>
<tr>
<td>3</td>
<td>Isolate 3</td>
<td>4.8</td>
</tr>
<tr>
<td>4</td>
<td>Isolate 4</td>
<td>4.7</td>
</tr>
<tr>
<td>5</td>
<td>Isolate 5</td>
<td>5.1</td>
</tr>
<tr>
<td>6</td>
<td>Isolate 6</td>
<td>5.3</td>
</tr>
<tr>
<td>7</td>
<td>Isolate 7</td>
<td>3.0</td>
</tr>
<tr>
<td>8</td>
<td>Isolate 8</td>
<td>3.6</td>
</tr>
<tr>
<td>9</td>
<td><em>ATCC 14579</em></td>
<td>5.2</td>
</tr>
</tbody>
</table>

### 4. CONCLUSION

The present study concluded that the phenotypic and biochemical analysis is a suitable tool for characterize the Total Heterotrophic Bacterial (THB) community in Pitchavaram mangrove sediment and better understand the functioning of their related ecosystems. In addition to their distribution pattern and involved in nutrient cycling how they are act as a tool for biodegrading the nutrients in food web. *Pseudomonas* sp. (Isolate 1) and *Azotobacter* sp. (Isolate 6) were


