Materials and Methods
Materials & Methods

I - GROWTH AND DEGRADATION

3.1.1 Cultures Used

Unicellular and filamentous non-heterocystous marine and fresh water cyanobacteria were obtained from the germplasm of National Facility for Marine Cyanobacteria (NFMC), Bharathidasan University, Tiruchirappalli, Tamilnadu, India.

3.1.1a Marine Forms

- *Gleocapsa* sp. BDU 110711
- *Croococcus turgidens* BDU 142111
- *Synechocystis pavalkii* BDU 130051
- *Spirulina subsalsa* BDU 141021
- *Oscillatoria salina* BDU 92071
- *Oscillatoria laetivirens* BDU 20801
- *Phormidium tenue* BDU 141753
- *Phormidium valderianum* BDU 20041
- *Phormidium corium* BDU 60121
- *Phormidium fragile* BDU 42911
- *Lyngbya* sp. BDU 90181
- *Lyngbya* sp. BDU 141961
3.1.1b Fresh Water Forms

- Phormidium sp.
- Oscillatoria annae

3.1.2 Media and Growth Conditions

Marine and fresh water cyanobacteria were grown and maintained in ASN III (Rippka et al., 1979) and BG11 medium (Rippka et al., 1979) respectively under white fluorescent light of 13.8 \mu E m^{-1} s^{-1} at 25 ±2°C with 14/10 D/L cycle.

3.1.2a Composition of ASN III Medium

<table>
<thead>
<tr>
<th>Name of the Chemicals</th>
<th>Amount (g L^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>25.0</td>
</tr>
<tr>
<td>MgSO_4.7H_2O</td>
<td>3.5</td>
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<tr>
<td>MgCl_2.6H_2O</td>
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<tr>
<td>KCl</td>
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</tr>
<tr>
<td>CaCl_2.2H_2O</td>
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</tr>
<tr>
<td>NaNO_3</td>
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</tr>
<tr>
<td>KH_2PO_4.3H_2O</td>
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<tr>
<td>EDTA disodium salt</td>
<td>0.0005</td>
</tr>
<tr>
<td>Citric acid</td>
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</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.003</td>
</tr>
<tr>
<td>*A5 micronutrients</td>
<td>1.0mL</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000mL</td>
</tr>
<tr>
<td>pH</td>
<td>7.2 - 7.8</td>
</tr>
</tbody>
</table>

*A5 Micronutrients

<table>
<thead>
<tr>
<th>Name of the Chemicals</th>
<th>Amount (g L^{-1})</th>
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</thead>
<tbody>
<tr>
<td>H_3BO_3</td>
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<tr>
<td>MgCl_2.4H_2O</td>
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<tr>
<td>ZnSO_4.7H_2O</td>
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<tr>
<td>NaMoO_4.2H_2O</td>
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</table>
CuSO₄·5H₂O 0.079
Co(NO₃)₂·6H₂O 0.494
Distilled water 1000mL

3.1.2b Composition of BG 11 Medium

<table>
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<th>Name of the Chemicals</th>
<th>Amount (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
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</tr>
<tr>
<td>K₂HPO₄·3H₂O</td>
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<tr>
<td>MgSO₄·7H₂O</td>
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<tr>
<td>CaCl₂·2H₂O</td>
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<tr>
<td>Citric acid</td>
<td>0.006</td>
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<tr>
<td>Ferric Ammonium Citrate</td>
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<tr>
<td>EDTA</td>
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<tr>
<td>*Trace metal mix</td>
<td>1mL</td>
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<tr>
<td>Distilled Water</td>
<td>1000mL</td>
</tr>
<tr>
<td>pH</td>
<td>7.1 ± 0.2</td>
</tr>
</tbody>
</table>

*Trace Metal Mix (g L⁻¹)

<table>
<thead>
<tr>
<th>Name of Trace Element</th>
<th>Amount (g L⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>H₃BO₃</td>
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</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>1.81</td>
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<tr>
<td>ZnSO₄·7H₂O</td>
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<tr>
<td>Na₂(MoO₄)·2H₂O</td>
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</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.079</td>
</tr>
<tr>
<td>Co(NO₃)₂·6H₂O</td>
<td>0.0494</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>1000mL</td>
</tr>
</tbody>
</table>
Materials & Methods

3.1.3 Lignocellulosic Materials

Coir pith (Plate 1b), a waste by-product of coir rope industry was collected from coir industries near Srirangam, Tiruchirappalli, Tamilnadu, India. Woody stems of Prosopis juliflora (Plate 1c) and Lantana camara (Plate 1d) were obtained from Bharathidasan University campus, dried and ground to pass a 0.5mm screen.

3.1.4 Screening of Cyanobacteria

The listed marine and fresh water cyanobacteria were grown with lignocellulosics (coir pith, P. juliflora and L. camara) separately at a dry weight ratio of 1:10 (cyanobacteria: lignocellulosics) in their respective media under above mentioned conditions (Ref. 3.1.2) for 15 days.

3.1.5 Optimization of Ratio

The ability of the selected cyanobacterium O. annae (Plate 1a) to grow in the presence of chosen lignocellulosics (coir pith, P. juliflora and L. camara) at varying dry weight ratios were tested in BG11 media under above mentioned conditions (Ref. 3.1.2) for 15 days. The supernatants were centrifuged and subjected to spectral and biochemical analysis using Jasco UV-vis spectrophotometer.

3.1.6 Selected ratio

Based on the spectral and biochemical analysis the optimum ratio was adjudged to be 1:10 for coir pith and 1:30 for P. juliflora and L. camara. Hence for further experiments the above ratios were maintained for the respective lignocellulosics.

3.1.7 Optimization of growth conditions for lab and field scale studies

The selected cyanobacterium was incubated along with lignocellulosics (coir pith, P. juliflora and L. camara in the ratio mentioned in 3.1.6) at 13.8μE at 25± 2°C in 14/10 D/L cycle laboratory conditions and 414 μE at 37± 2°C in field. After 15 days, the samples were used for the following estimations. Respective controls of untreated lignocellulosics and cyanobacterium alone were also maintained.

3.1.8 Optimization of Media

In order to select the best media suited for biodegradation of the lignocellulosics the screened cyanobacterium was allowed to degrade the lignocellulosic material in
different media (BG11, NPK, Urea medium) at the optimum ratio of cyanobacterium: lignocellulosics (Ref. 3.1.6).

<table>
<thead>
<tr>
<th>Name of the Chemicals</th>
<th>Amount (g L⁻¹)</th>
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<tbody>
<tr>
<td>NPK Medium</td>
<td></td>
</tr>
<tr>
<td>NaNO₃</td>
<td>1.5</td>
</tr>
<tr>
<td>K₂HPO₄·3H₂O</td>
<td>0.04</td>
</tr>
<tr>
<td>Urea Medium</td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>0.1</td>
</tr>
</tbody>
</table>

3.1.9 Estimation of Growth Parameters

3.1.9a Chlorophyll (Mac Kinney, 1941)

Reagent

80% methanol

Procedure

- The cyanobacterium treated and untreated lignocellulosics (coir pith, *P. juliflora* and *L. camara*) in the ratio (ref. 3.1.6) in the above mentioned conditions (ref. 3.1.7) were centrifuged at 5000 xg for 10 min.
- Washed the pellets twice with distilled water.
- Pellet was suspended in 4mL of methanol and vortexed thoroughly.
- Covered the mouth of the tubes with aluminium foils to prevent solvent evaporation.
- Tubes were incubated in a water bath at 60°C for 1hr in dark, with occasional shaking.
- Cooled the tubes and centrifuged at 5000 xg for 10 min.
- Transferred the supernatant to another tube and the pellet reextracted again as above till all chlorophyll was completely extracted.
- Pooled the supernatants and made up to a known final volume with 80% methanol
- Read the absorbance at 663nm in JASCO UV-Vis spectrophotometer against methanol as blank.
- Calculated the amount of chlorophyll using the following equation.
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**Amount of Chl. a = Absorbance at 663 × 12.63 × volume of sample**

*where 12.63 is correction factor*

- Expressed the results as μg chl a
- Each value is a mean of triplicates

### 3.1.10 Analysis of biochemical parameters

The supernatant obtained from the cyanobacterium treated and untreated lignocellulosics (*ref. 3.1.7*) after 15 days of incubation was used for the following biochemical estimations.

#### 3.1.10a Estimation of Sugar (Miller, 1959)

**Reagent**

A. 1% Dinitrosalicylic acid (DNSA)
   - Dissolved DNSA in 20mL of 2N NaOH and made up to 100mL with distilled water

B. Dissolved 30g of Rochella (Sodium potassium tartrate) in 1% DNSA solution

**Procedure**

- Equal volume of supernatant and reagent B was added.
- The tubes were kept in boiling water bath for 10 min and cooled.
- The contents were made up to 10mL with distilled water.
- Read the absorbance at 540nm in JASCO UV-Vis spectrophotometer
- The amount of sugar released was calculated from standard graph of glucose
- Results are expressed as μg mL⁻¹
- Values are the mean of triplicates

#### 3.1.10b Estimation of Phenol (Bray and Thorpe, 1954)

**Reagent**

A. 12% Sodium carbonate solution

B. Folin Ciocalteu’s reagent: Diluted the reagent with equal volume of distilled water

**Procedure**

- The reaction mixture consisted of 1mL of culture filtrate, 2.5mL of reagent A and 0.75mL of reagent B
Materials & Methods

- Vortexed the samples thoroughly and incubated for 1 hr at room temperature
- Measured the absorbance at 725 nm in JASCO UV-Vis spectrophotometer
- The amount of phenol released was determined using a standard curve made with phenol.
- Expressed the results as μg phenol mL⁻¹ of culture filtrate.
- Results are average of triplicates

3.1.11 Pretreatment of lignocellulosics (Tappi, 1992)

Reagents

Ethanol: benzene mixture

Procedure

- To known amount (2 g) of the untreated and *O. annae* treated lignocellulosics (ref. 3.1.7) were taken in a porous thimble and placed in a soxhlet apparatus.
- Added 250mL of ethanol:benzene mixture and extraction was carried out for 6 hrs.
- Cooled and the alcohol:benzene treated contents were scrapped out of the porous thimble.
- Dried the pretreated contents in a thermostat controlled oven at 100°C until consecutive weights were obtained.
- Equal quantities of the alcohol benzene pretreated lignocellulosics were further used for lignin and holocellulose estimation.

3.1.11a Estimation of Klason Lignin (Tappi, 1992)

Reagent

Concentrated H₂SO₄

Procedure

- Added 2mL of concentrated H₂SO₄ to a known amount of extract free lignocellulosics and incubated for 1 hr with occasional stirring
- Added 56mL of distilled water after incubation
- Closed the tubes tightly and autoclaved and allowed to cool
- Filtered the contents through Whatmann No. 1 filter paper
- Dried the filter paper containing the contents at 70-80°C for 48-72 hr.
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- The resulting dried contents was weighed and the lignin content was expressed as %
- The results are average of triplicates

3.1.11b Estimation of Holocellulose (Tappi, 1992)

Reagent
- Sodium chlorite – 1.5 g
- Glacial acetic acid – 0.5 mL

Procedure
- Treated a known amount extractive free lignocellulosics with sodium chlorite and glacial acetic acid in an Erlenmeyer flask containing distilled water (160mL).
- Repeated the process at least 5 times till the lignocellulosics became white.
- Filtered the lignocellulosics thus obtained through G2 crucible.
- Washed with distilled water followed by acetone washing.
- The crucible was dried to constant weight in an oven at 100°C.
- The resulting dried contents were weighed and the lignin content was expressed in %.
- Values are average of triplicates.

3.1.12 Enzyme assays

3.1.12a Enzyme extraction

Reagents
- Phosphate buffer: 50 mM, pH 7.5

Procedure
- About 500mg of 15 day old control O. annae and O. annae exposed to respective lignocellulosics fresh culture were taken for enzyme extraction.
- Homogenized the samples at 4°C in the cold room with ice-cold phosphate buffer by using pre-chilled mortar and pestle.
- Centrifuged the homogenate at 15,000 xg for 30 min at 0-4°C
- The supernatant cell-free extract was used as the enzyme enzyme source.
- The protein content of the enzyme extract was determined according to the method of Lowry et al., (1951) with BSA as a standard.
3.1.12b Protein content (Lowry et al., 1951)

The enzyme assays were carried out with uniform amount of the protein content for the enzyme extracts following Lowry et al., 1951.

Reagents

i. *Alkaline sodium carbonate solution*: 2g of sodium carbonate was dissolved in 0.1N sodium hydroxide

ii. *5% Copper sulphate solution*: 5g of copper sulphate was dissolved in 100mL of distilled water.

iii. *Sodium potassium tartrate solution*: 10g of sodium potassium tartrate was dissolved in 100mL of distilled water.

iv. *Copper sulphate - Sodium potassium tartrate solution*: One part of copper sulphate solution was mixed with one part of sodium potassium tartrate solution and eight parts of distilled water was added.

v. *Alkaline reagent*: Prepared freshly by mixing 50mL of alkaline sodium carbonate solution and 1mL of copper sulphate - sodium potassium tartrate solution.

vi. *Folin – Ciocalteu reagent*: The reagent was diluted with equal amount of distilled water.

vii. *Standard Protein*: Bovine serum albumin (BSA) - 100 µg/mL

Procedure

1. An aliquot of 0.1mL from each enzyme extract (ref 3.1.10) was made upto 1mL with distilled water.
2. Added 4.5mL of alkaline reagent and incubated for 3min.
3. Added 0.5mL of Folin – Ciocalteu’s phenol reagent and allowed to stand for 30min.
4. Read absorbance at 750nm in JASCO UV-Vis spectrophotometer
5. Calculated the amount of protein in each sample using standard curve prepared using bovine serum albumin and expressed as µg protein
6. Values are average of triplicate
3.1.13 Assay of lignin degrading enzymes

Lignolytic enzymes of *O. annae* were studied colorimetrically by estimating the activity of manganese independent peroxidase, polyphenol oxidase and laccase. The respective enzyme activity was studied at varying pH with citrate phosphate buffer (pH 4.0–7.0) and Tris-HCl buffer (pH 8.0 and 9.0) and temperature regimes of 25 and 37 ± 2°C.

3.1.13a Manganese independent peroxidase (MnIP) (*Caramelo et al.*, 1999)

- 0.1mL of enzyme sample was added to 2mL sodium tartrate buffer containing 0.1mM 2,6-dimethoxy phenol.
- 4mM H$_2$O$_2$ was added and the OD was taken at 469nm and was expressed as Unit activity.

3.1.13b Polyphenol oxidase (*Caramelo et al.*, 1999)

- 0.1mL of enzyme sample was added to 2mL sodium tartrate buffer containing 0.15M O-catechol.
- OD was taken at 420 nm for 2 min. and the reaction was stopped by 0.5mL of 5% H$_2$SO$_4$ and was expressed as Unit activity.

3.1.13c Laccase (*Caramelo et al.*, 1999)

- 0.1mL of enzyme sample was added to 0.9mL of sodium acetate buffer containing 10mM guaiacol.
- OD was taken at 470nm and was expressed as Unit activity.


**Reagent**

- A. Phenol
- B. 4-aminoantipyrine
- C. Potassium phosphate buffer pH 6.9 (0.1M)
- D. Horseradish peroxidase
- E. Hydrogen peroxide
Procedure

➢ The reagent solution (100 mL) was prepared using 0.234g reagent A, 0.10g reagent B and 1 mL of reagent C and contains 2x10^-8 M reagent D.
➢ The reaction mixture (4 mL) was mixed with the peroxide sample (control O. annae and O. annae exposed to coir pith, P. juliflora and L. camara) and made upto 10 mL with double distilled water.
➢ The change in absorbance at 505 nm was measured until a constant reading was obtained (approximately 5 min. at ambient temperature).
➢ A 4 mL aliquot of the reagent solution made upto 10 mL with double distilled water served as reference.
➢ The amount of hydrogen peroxide released by O. annae was calculated from a standard curve prepared with varying amounts of standard hydrogen peroxide.
➢ The results are expressed as μmol hydrogen peroxide per g dry weight and are average of triplicates.

3.1.15 Mass cultivation of O. annae with lignocellulosics waste

Large scale cultivation of O. annae was carried out initially in boxes, tanks and further extended to outdoor pits on ground and covered with polythene sheets. Cyanobacteria with coir pith, P. juliflora and L. camara was inoculated in separate pits in urea media. After 15 days of incubation, the degraded wood materials were collected and separated from cyanobacterial mat and air dried for further use as source for bioethanol. The medium used for mass cultivation was air dried and tested for its plant growth promoting ability as foliar spray.
II - BIOETHANOL PRODUCTION

Bioethanol production was performed following dilute acid saccharification followed by detoxification and fermentation using the yeast *Pichia stipitis*. Bioethanol production from untreated and *O. annae* treated lignocellulosics (coir pith, *P. juliflora* and *L. camara*) were compared statistically to find its significance.

3.2.1 Microorganism and Medium

*Pichia stipitis* NCIM 3498 was obtained from the collection of National Chemical Laboratory, Pune, India. The culture was maintained in a medium containing peptone 2.0 g L⁻¹, yeast extract 2.0 g L⁻¹, (NH)₂SO₄ 3.0 g L⁻¹, KH₂PO₄ 2.0 g L⁻¹, MgSO₄ 1.0 g L⁻¹ and xylose 10.0 g L⁻¹.

The fermentation medium of the yeast consisted of the hydrolysate as the sugar source, peptone 3.6 g L⁻¹, yeast extract 4.0 g L⁻¹, (NH)₂SO₄ 3.0 g L⁻¹, KH₂PO₄ 2.0 g L⁻¹ and MgSO₄ 1.0 g L⁻¹ at pH 5.8. Mineral salts were autoclaved separately at 121 °C for 20 min, and then added to the hydrolysate separately.

3.2.2 Inoculum and Fermentation Condition

*P. stipitis* inoculum (3.5 % (v/v)) was used for the fermentation studies. Ethanol fermentations by *P. stipitis* were evaluated at 30°C in 500 mL Erlenmeyer flasks containing 100 mL appropriate media in a shaker at 150 rpm. All experiments were carried out in triplicates.

3.2.3 Optimization of Acid Concentration

Dilute acid hydrolysis method by using H₂SO₄ was utilized for the saccharification process. Varying concentration of sulphuric acid (0.75, 1.5, 2.25, 3.0 and 3.75 % v/v) was tested for the maximum release of sugars from the wood materials.

3.2.4 Optimization of Temperature

Dilute acid hydrolysis was combined with heat for enhancing the release of sugars from the wood. Each concentration of dilute acid hydrolysis was combined with
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three different temperatures (100, 120 and 140°C) for 1 hour using a special Russian autoclave.

3.2.5 Detoxification

Several inhibitory compounds are formed during acid hydrolysis of lignocellulosic materials. Such compounds are divided into four groups: (a) sugar degradation products including furfural and hydroxymethylfurfural (b) lignin degradation products including several aromatic and polyaromatic compounds; (c) substances released from the hemicellulosic structure, including acetic acid, terpenes and tannins and (d) metals released from equipment like chromium, copper, iron and nickel (Olsson and Hahn-Hagerdal, 1996). In order to decrease the toxicity for *Pichia stipitis*, considerable efforts have been focussed on detoxification procedures prior to fermentation, including neutralization, overliming and overliming combined with charcoal adsorption.

3.2.5a Neutralization (Chandel et al., 2007)

- Added calcium hydroxide to the dilute acid hydrolysate to increase the pH up to 5.8.
- Stirred the mixture for 30 min.
- Filtered to remove any precipitate formed before using as substrate for fermentation.

3.2.5b Overliming (Martinez et al., 2001)

- Added Calcium hydroxide to the dilute acid hydrolysate to increase the pH up to 10.5.
- Stirred the mixture for 30 min.
- Filtered and the pH was adjusted to 5.8 using 2N H₂SO₄.

3.2.5c Overliming combined with Activated Charcoal (Gong et al., 1993)

- Added calcium hydroxide to the dilute acid hydrolysate to increase the pH to 10.5.
- Stirred the mixture for 30 min.
- Filtered and added 1% activated charcoal and stirred for 20 min.
- Filtration repeated.
3.2.6 Reducing Sugar Estimation (Miller, 1959)
As described in section 1.9.2

3.2.7 Estimation of Phenol (Bray and Thorpe, 1954)
As described in section 3.1.9b

3.2.8 Analytical Methods
Samples were withdrawn periodically during fermentation to determine yeast cell mass, reducing sugars and ethanol in the broth.

3.2.8a Estimation of Yeast Cell Mass

- Centrifuged the cell suspension (1mL) at 10,000rpm in preweighed eppendorf.
- Rinsed the cell pellets with distilled water, recentrifuged at 10,000rpm.
- Dried till constant weight at 60 °C.

3.2.8b Reducing Sugar Estimation (Miller, 1959)
As described in section 1.9.2

3.2.8c Bioethanol Estimation
From the supernatant obtained above the ethanol was estimated periodically.
Ethanol was estimated by gas chromatography (GC) (Perkin Elmer, Clarus 500) with an elite-wax (cross bond-Poly ethylene glycol) column (30m x 0.25 mm), at 120°C, flame ionization detector at 210°C and injector at 180°C using isopropanol as standard. The carrier gas was nitrogen (N₂). 0.2μL of sample was injected into the column for estimation.
III. BIOFERTILIZER

3.3.1 Plant chosen

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Plantae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Division</td>
<td>Magnoliophyta</td>
</tr>
<tr>
<td>Class</td>
<td>Magnoliopsida</td>
</tr>
<tr>
<td>Order</td>
<td>Asterales</td>
</tr>
<tr>
<td>Family</td>
<td>Asteraceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Tagetes L.</td>
</tr>
<tr>
<td>Species</td>
<td>erecta</td>
</tr>
</tbody>
</table>

3.3.2 Treatments

The culture filtrate was air dried and tested for its plant growth promoting ability as foliar spray. The air dried culture filtrate was dissolved in water to give three different concentrations (250, 500 and 1000ppm). Separate sprays were prepared for lignocellulosic controls (coir pith, P. juliflora and L. camara) and the respective O. annae treated lignocellulosics.

C - Control (untreated plants)

Untreated wood foliar spray – 250, 500, 1000 ppm
Cyanobacteria treated wood foliar spray – 250, 500, 1000 ppm

Analysis of Parameters

After a growth period of 60 days from the time of planting, the following parameters were examined for the Tagetes erecta.

3.3.3 Morphological Parameters

1. Length of shoot and root
2. Fresh and dry weight of leaves, shoot and root
3. Number of branches
4. Number of flowers and buds
5. Yield
3.3.4 Biochemical Parameters
3.3.4a Estimation of Chlorophyll (Arnon, 1949)

Reagents

80% acetone

Procedure

- Ground a known amount of fresh leaf in mortar and pestle with 80% cold acetone with a pinch of CaCO₃ to prevent pheophytin formation.
- Centrifuged the homogenate at 2500g for 10 min and the supernatant was made up to a known volume with 80% cold acetone.
- Measured the absorbance at at 645nm and 663nm against acetone as blank in JASCO UV-Vis spectrophotometer.
- Values are average of triplicate
- Chlorophyll a and b were calculated using Arnon’s formula.

Calculation

\[
\text{Chlorophyll } a \ (\text{mg/g}) = \frac{12.67A_{663} - 6.9A_{645}}{a \times 1000 \times W} \times V
\]

\[
\text{Chlorophyll } b \ (\text{mg/g}) = \frac{2.29A_{645} - 4.68A_{663}}{a \times 1000 \times W} \times V
\]

- a - Length of the path of light in the cell
- V - Volume of the extract
- W - Dry weight of the sample in g

The value was expressed as mg chlorophyll/g weight of the sample
3.3.4b Estimation of carotenoids (Siegelman and Kycia, 1978)

Reagents

80% acetone

Procedure

\[ \text{1 gm of leaf sample was ground in 3mL of acetone and the suspension was centrifuged at 5000 rpm for 10 minutes.} \]

\[ \text{The extraction was repeated till acetone and the suspension remained colorless. The supernatants were pooled and made to known volume with acetone. The absorbance was measured at 450nm against acetone blank.} \]

Calculation

\[
\text{Carotenoids} = \frac{D \times V \times F \times 10}{2500} \text{ mg/mL}
\]

D – absorbance at 450nm

V – Volume of sample

F – dilution factor

2500 – extinction coefficient

3.3.4c Estimation of Sugars (Yemm and Willis, 1954)

Reagents

Dissolve 0.2g of anthrone in 100mL of H\textsubscript{2}SO\textsubscript{4} made by adding 500mL of acid to 200mL of water. The reagent was allowed to stand for 30-40min with occasional shaking until it was perfectly clear. The reagent was freshly prepared each day and used within 12 hr.

Procedure

\[ \text{Known amount of the sample were exhaustively extracted with 70\% (v/v) ethanol and the extract was evaporated in vacuum.} \]

\[ \text{Evaporated residue was dissolved in distilled water to a final volume of 1mL which was kept in water bath at 30 °C.} \]
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- Added anthrone reagent (5mL) to the test (1mL) solution.
- Followed with 0.5mL of H₂SO₄ and cooled the solution for 5min.
- Tubes were loosely fitted with corks heated for 5min. and then cooled in water bath.
- The absorption spectra were determined in a spectrophotometer (JASCO UV-vis) at 600nm.
- Results are expressed in μg/mL using a standard graph
- Values are average of triplicates

3.3.4d Estimation of protein (Lowry et al., 1951)
Refer 3.1.11b

3.3.4e Estimation of Phenol (Bray and Thorpe, 1954)
Refer 3.1.9b

IV Data and Statistical Analysis

Only significant data were included in the tables and were presented in mean and standard error (±) of three replicates per treatment and repeated three times. Two factor analysis of variance (ANOVA) was used to assess the significance (p = 0.05) of the mean values of treatments and the differences were compared using Duncan’s Multiple Range Test.