3. MATERIALS AND METHODS

3.1 Isolation and culturing of microorganisms from marine environment and preservation of stock cultures

3.1.1 Sampling

The choice of natural materials is often based on the assumption that samples from widely different locations are more likely to yield novel isolates which in turn might produce novel metabolites (Brown 1985).

Marine samples were collected from different parts of Tamilnadu (Cuddalore), Pondicherry and Karnataka (Mangalore) sea coast. The samples included sea water, wood pieces in the process of degradation, rock scrap, soil from vegetation in backwaters and sediment samples. The samples were collected in Sterile Polyethene zip bags and Borosil sampling flasks (500ml) and transported to the laboratory in thermocol boxes packed in ice and were preserved in refrigerator until further studies.

Sea Water for media preparation and dilution was collected from Pondicherry and Mangalore. The water sample was filtered using muslin cloth and stored in sterile cans under room temperature till further use. Sea water was autoclaved before use whereever required.

3.1.2 Isolation of Microorganisms

All the standard Media and Chemicals used for the present investigation were procured from HiMedia (Mumbai) and Sigma Chemicals.

Standard microbiological methods were followed for the purpose of isolation of both Bacteria and Fungi from the marine samples (Brown, 1985).
**Water Sample** – 1 ml was directly used for serial dilution

**Wood scrap, Rock scrapings**– were washed thoroughly in 100ml sea water and 1ml of this was used for serial dilution.

**Soil, Sediment** – 1g of the sediment was suspended in 100ml of sterile sea water and 1ml of this was used for serial dilution.

Serial dilution of the sample was carried out and one milliliter of the desired dilution \(10^{-3}, 10^{-5}, 10^{-7}\) for Bacteria and \(10^{-1}, 10^{-3}, 10^{-5}\) for Fungi) was transferred aseptically into different media for isolation of Bacteria (Nutrient Agar in marine water and Marine Agar in distilled water) and Fungi (Potato Dextrose Agar in marine water), by spread plate method.

The inoculated media plates were incubated at 28°C for 24-48 h for Bacteria and 5-7 days at room temperature for Fungi. Plates which showed colony counts between 30-300 were selected for identification and the colonies thus obtained on isolation were counted using a colony counter and the number and types of organisms were recorded for each dilution as no of CFUs x dilution factor.
Marine Agar

Peptic digest of animal tissue : 5 g
Yeast Extract : 1 g
Ferric Citrate : 0.1 g
NaCl : 19.45 g
Magnesium Chloride : 8.8 g
Sodium sulphate : 3.24 g
Calcium chloride : 1.8 g
Sodium bicarbonate : 0.16 g
Potassium bromide : 0.08 g
Strontium chloride : 0.034 g
Boric acid : 0.022 g
Sodium silicate : 0.004 g
Sodium fluorate : 0.0024 g
Ammonium nitrate : 0.0016 g
Disodium phosphate : 0.008 g
Agar : 15.00 g
pH : 7.6
Distilled water : 1000ml
**Nutrient Agar**

Peptone : 5g  
Beef extract : 3g  
Sodium chloride : 5g  
Agar : 15 g  
Sea water : 1000ml  
pH : 7.0

**Potato Dextrose Agar**

Potato infusion : 4g  
Dextrose : 20g  
Agar : 15g  
Sea water : 1000ml

**Bacteria:** After incubation, the media plates were checked for the growth of the colonies. Different types of colonies, no of colonies, colony morphology of each plate for different samples were documented. Individual colonies of each type were subcultured onto Marine agar plates and incubated for growth. Gram’s staining for each isolate was carried out and the Gram’s character and the cell morphology were documented to check for the purity of the culture. Individual isolate was then subcultured on to marine agar slants and maintained as pure culture.

**Fungi:** The Potato dextrose agar plates were checked for the growth of fungal colonies after an incubation period of 5-7 days. Different types of colonies, number of colonies and colony
morphology of each plate for different samples were documented. Individual colonies were further subcultured onto sterile PDA plates and incubated for growth of the fungi. Simple staining using Lacto phenol cotton blue was conducted on each isolate to check the characteristic feature of the hyphae, spores etc and the purity of the organism. Individual fungal isolate was then subcultured in PDA slants prepared in marine water and maintained as pure culture. Each of the isolated pure bacterial and fungal cultures was numbered and the colony characteristic feature documented.

3.2 Screening the organisms for production of cellulase, hemicellulase and ligninases under culture conditions

All of the isolated organisms were subjected to screening for the production of cellulases, hemicellulases and ligninases on chemically purified substrates CMC, Xylan and Lignin to check for degradation.

Three different assays were carried out to assess the degradation ability of the isolates for the respective substrates.

3.2.1 Cellulase

Screening for cellulose degradation by standard CMC agar method was carried out with both Bacteria and Fungi individually with all the isolates. Three methods were followed to analyze the degradation abilities of the isolates. The assay method followed was as per Pointing et al. 1999a.
Dye Staining of Carboxy Methyl Cellulose Agar (CMC Agar)

Actively growing culture was used to inoculate each assay medium, for bacteria 24-48 h culture was used and for fungi 5-7 days old culture was used.

CMB medium was prepared and supplemented with 2% w/v low viscosity CMC and 1.6% w/v agar and aseptically transferred to the petri dishes and these were inoculated with the test organisms, incubated at room temperature for 5 days. The plates were flooded with 2% w/v aqueous Congo red (C.I.22120) and left for 15 minutes. The stain was removed and the agar surface was washed with distilled water, the plates were flooded with 1M NaCl to de-stain for 15mins. CMC degradation around the colonies appeared as yellow-opaque area against a red color for un-degraded CMC. The organisms exhibiting maximum cellulolysis were chosen for further studies.

CBM (Cellulolysis basal medium)

L- Ammonium tartarate : 5g KH₂PO₄ : 1g
MgSO₄.7H₂O : 0.5g
Yeast extract : 0.1g
CaCl₂.2H₂O : 0.001g
Sea water : 1000ml
3.2.2 Lignin Assay

Lignin modifying enzyme assays

Actively growing culture was used to inoculate each assay medium, for bacteria 24-48 h culture was used and for fungi 5-7 days old culture was used to inoculate each assay medium individually.

Sterile LBM medium was prepared and 1 ml of separately sterilized 20% w/v aqueous glucose solution is added to each 100ml of LBM growth medium and was supplemented with 0.25% w/v lignin and 1.6% w/v agar. The media was then aseptically transferred to sterile petri dishes. The media was then inoculated with the test organism. The inoculated media was incubated at room temperature for 48 h for bacteria and 5-7 days for fungi. After the incubation period, the agar plates were stained with 1% ferric chloride and potassium ferri cyanide prepared freshly before use. Clear zones around colonies indicated oxidation of phenolic compounds against blue green undegraded lignin. Phenols in undegraded lignin will stain blue-green with clear zone around colonies indicating oxidation of phenolic compounds. (Buswell et al., 1996.)

LME basal medium (LBM) (g/1)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>1g</td>
</tr>
<tr>
<td>C₄H₁₂N₂O₆</td>
<td>0.5g</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>0.001g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5g</td>
</tr>
<tr>
<td>Fe₂(SO₄)₃</td>
<td>0.001g</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.001g</td>
</tr>
</tbody>
</table>
CaCl_2·2H_2O : 0.01g
Yeast Extract : 0.01g
Sea water : 1000ml

LBM medium, supplemented with 0.25% w/v lignin and 1.6% w/v of agar.

3.2.3 Hemicellulolytic (xylanolytic) enzyme assay

Relatively little attention has been given to qualitative assays for xylan utilization and only a few assay procedures have been described.

Actively growing culture was used to inoculate each assay medium, for bacteria 24-48hrs culture was used and for fungi 5-7 days old culture was used to inoculate each assay medium individually (Jorgensen et al., 2003.).

Dye staining of xylan agar (XBM)

XBM medium was prepared and incorporated with 4% w/v xylan and 1.6% w/v of agar and autoclaved. The sterile media was aseptically transferred to the sterile petri dishes and inoculated with the test organism individually. The plates were then incubated at room temperature for 48 h for bacteria and 5-7 days for fungi. The media after growth was flooded with iodine stain (0.25% w/v aqueous I_2 and KI) for 5 min, the stain was removed and the agar surface washed with distilled water. Xylan degradation around the colonies appeared as yellow-opaque area against a blue/reddish purple color for un-degraded. (Beilyet al., 1985 and Cai et al., 1994)
**Xylanolysis basal medium (XBM) (g/l)**

\[
\begin{align*}
\text{C}_4\text{H}_{12}\text{N}_2\text{O}_6 & : 5\text{g} \text{ KH} \\
\text{H}_2\text{PO}_4 & : 1\text{g} \\
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & : 0.5\text{g} \\
\text{CaCl}_2 \cdot 2\text{H}_2\text{O} & : 0.001\text{g} \\
\text{Yeast Extract} & : 0.1\text{g} \\
\text{Sea water} & : 1000\text{ml}
\end{align*}
\]

XBM medium, supplemented with 4% w/v xylan and 1.6% w/v of agar

### 3.3 Identification of the microorganisms with relevant literature

The isolates which exhibited maximal zone of degradation on all the three substrates were chosen as the test organisms for further studies. Four isolates, two bacteria and two fungi were identified to species level based on their morphological, biochemical and molecular characterization. These four organisms were used to check for the degradation of substrates chosen for the present study.

For bacteria the colony morphology of each organism was recorded, and identification was further done by **staining techniques** Gram staining. For fungi the colony morphology of each isolate was recorded and further identification was based on the morphology observed under the microscope stained with lactophenol cotton blue for spore morphology, arrangements and hyphal morphology.
Molecular characterization was done to identify the organism to the species level based on 16sRNA sequencing.

**DNA Extraction and 16S rDNA Sequencing**

The DNA extraction method presented in this paper is an improved method of the standard phenol/chloroform method (Neumann *et al.*, 1992) with following modification. Isolate was grown in LB broth at 37 °C for overnight. Culture was centrifuged at 10000 g for 10 min at room temperature in order to pellet the cells. After removing the supernatant, the cells were washed with 400 ul STE Buffer (100 mMNaCl, 10 mMTris/ HCl, 1 mM EDTA, pH 8.0) twice. Then 200 µl Tris-saturated phenol (pH 8.0) and 200 µl of chloroform was added and centrifuged at 10000g for 10 min. To the 160 µl supernatant 40 µl TE and 5 µl RNase (at 10 mg/ml) were added and incubated at 37 °C for 10 min to digest RNA. Then 100 µl chloroform was added to the tube, mixed well and centrifuged for 10 min at 10 000g at 4 °C. 150 µl upper aqueous phase was transferred to a clean 1.5 ml tube and DNA was precipitated by adding 100 µl iso-propanol and centrifuged for 10 min at 10,000g at 4 °C. The DNA pallet was dissolved in 50 µl TE buffer.

The isolated DNA was sent to **Bioserve, Hyderabad** for further identification based on molecular studies like 16sRNA sequencing and BLAST. The same were submitted to Gene bank for accession code.

Selected isolates which were identified based on the morphology, biochemical and molecular characterization were maintained in Marine agar (*Bacillus pumilus, Mesorhizobium sp.*) and PDA in sea water (*AspergillusnigerandTrichodermaviride)*.
3.4 Optimization of physical and chemical parameters for lignocellulose biodegradation by various enzymes

The various process parameters that influence the enzyme production during SSF were optimized over a wide range. The strategy adopted for standardization of fermentation parameters was to evaluate the effect of an individual parameter and to incorporate it at optimized level before standardizing the next parameter. Optimization of substrate, pH, Incubation Temperature, High Temperature, fermentation time, acid, alkali, carbon source, nitrogen source, and there concentration was carried out.

3.4.1 Substrate Optimization

The substrates used for the present investigation were collected from different parts of rural Bangalore and were selected based on the ready availability, economic value and abundance. 18 substrates were used in the present study to check out for the degradation of cellulose and lignin using the isolated test organisms. The substrates were: Rice straw (Oryza sativa L.), Saw dust (DustaphobekL.), Paper, Ragi straw (EleusinecoracanaL.), Maize cobs (Zea mays L.), Maize leaves (Zea mays L.), Eucalyptus (Eucalyptus camaldulensisL.), Sugarcane waste (Saccharum officinarumL.), Teak leaf (Tectona grandisL.), Castor oil leaf (RicinuscommunisL.), Nerium (Nerium oleander L.), Champak (Magnolia champacaL.), Jack fruit waste (ArtocarpusheterophyllusL.), Ficus leaves (Opuntiaficus-indicaL.), Jamun leaves (JambulinaL.), Crotalaria leaves (Crotalaria L.), Honge leaves (PongamiapinnataL.) and Mango Leaves (MangiferaindicaL.).
Each of the substrate was individually inoculated with the four test organisms which were identified as *Bacillus pumilus, Mesorhizobium sp., Aspergillus niger* and *Trichoderma viride*. The results were documented individually for each organism and all the substrates.

The samples were air dried at room temperature for 3-5 days depending on the moisture content and then powdered. 5g of the substrate was mixed with 100ml of distilled water containing 0.8% Ammonium nitrate in 250 ml Erlenmeyer flasks and sterilized (Chundakkadu Krishna, 1999, Keikhosro Karimi, *et al.*, 2006). The substrates were inoculated with 3ml ($10^6$) of overnight incubated Bacterial cultures and two loopfull of 5-7 day old spores of fungal cultures. The inoculated substrates were incubated at room temperature for 8 weeks.

**Cellulase assay**

For the estimation of degradation of cellulose and release of simple sugars DNS assay (Reducing Sugar Assay) was carried out.

DNS assay was carried out at an interval of 7 days to check for cellulose degradation. Enzyme activity was calculated based on the amount of reducing sugar – glucose, released. (Acharya *et al.* 2008, Nutawan Yoswathana *et al.* 2009) Estimation of Sugar was carried out by the standard DNS method (Gail Lorenz Miller, 1959).

The inoculated and incubated substrate was filtered and 0.2ml of the supernatant was taken and 1.8ml of 1% CMC was added. The above mixture was incubated at 40°C for 30mins and 3ml of DNS reagent was added and kept at 90 °C for 15mins. 1ml of Rochelle salt solution was added. The absorbance was measured at 575nm. (Gail Lorenz Miller *et al.*, 1959).
**Lignin Assay**

The substrates were inoculated with 1ml (10^6 CFU/ml) of overnight incubated Bacterial cultures, and 2 loopfull of 5 day old fungal cultures. The inoculated substrates were incubated at room temperature. Lignin degradation assay, was carried out at an interval of 7 days to check for lignin degradation. Enzyme activity was calculated based on the oxidation of veratryl alcohol to veratryl aldehyde. (Acharya et al., 2008, NutawanYoswathana et al., 2009).

Lignin peroxidase (LiP) activity was measured using the standard method, the increase in absorbance at 310 nm due to oxidation of the veratryl alcohol to veratryl aldehyde was measured.

A reaction mixture containing 2.2 ml of sodium tartarate buffer (50 mM, pH 4 at 25°C), 40 µl of veratryl alcohol (2 mM) and 240 µl of the culture supernatant was prepared. The reaction was initiated by the addition of 20 µl of H_2O_2 (0.2 mM) to the reaction mixture. The absorbance was then measured at 310nm immediately. (Kirk and Brunow, 1998).

Based on the results obtained for the degradation of cellulose and lignin for all the substrates tested, four substrates **Eucalyptus, Paper, Rice straw and Ragi straw** as final substrates in the present study were further subjected to optimization of Physical and chemical parameters for maximal production of simple sugars for the production on bioethanol.

Optimization was carried out with individual substrate for both Physical and chemical parameters.

Physical parameters included pH, high temperature, incubation time, temperature and chemical parameters included acid treatment, alkali treatment, carbon source and nitrogen source (Schulein, 1998).
Optimization of Physical parameters

3.4.2 pH

The substrates were individually used for the study. 5g of the substrate was ground into small pieces and transferred to flasks containing 100 ml of water. Different pH 4, 5, 6, 7,8 and 9 were set using 1N NaOH and 1N HCl (Pezsa and Ailer, 2011). The flasks were autoclaved at 121°C for 15 minutes at 15psi. Once the flasks had cooled, they were inoculated with Bacillus pumilus, Mesorhizobium sp., Aspergillusniger and Trichoderma viride. Reading was taken for reducing sugar estimation using DNS (Miller, 1959) and lignin degradation assay using veratryl alcohol (Frederick, 1992). The readings were further taken weekly for 8 consecutive weeks.

3.4.3 Incubation temperature

5 g of substrate was ground into small pieces and added into flasks each containing 100ml of water. Autoclaved at 121°C for 15 minutes at 15 psi. Once the flasks had cooled, they were inoculated with Bacillus pumilus, Mesorhizobium sp., Aspergillusniger and Trichoderma viride. After inoculation, the flasks were incubated at 4 different temperatures to study the impact of the incubation temperature on lingocellulose degradation. The temperatures at which the incubation was done were 25, 30, 37 and 40°C (Charitha Devi et al., 2012). Reading was taken for reducing sugar estimation using DNS (Miller 1959) and lignin degradation assay using veratryl alcohol (Frederick, 1992). The readings were further taken weekly for 8 consecutive weeks.

3.4.4 High temperatures

5 g of substrate was ground/cut into small pieces and was added in flasks containing 100ml of water and kept at different temperatures i.e., 100°C, 150°C, 200°C and 250°C for 1hr (Brownell
and Saddler, 1986). This was done in hot air oven. The flasks were kept without cap in hot air oven. Once the flasks had cooled, they were inoculated with *Bacillus pumilus*, *Mesorhizobium sp.*, *Aspergillus niger* and *Trichoderma viride*. Reading was taken for reducing sugar estimation using DNS (Miller, 1959) and lignin degradation assay using veratryl alcohol (Frederick, 1992). The readings were further taken weekly for 8 consecutive weeks.

**Optimization of Chemical parameters**

### 3.4.5 Acid Treatment

5 g of substrate ground into small pieces and was added into flasks containing 100ml of different concentrations of acid 0.1%, 0.3%, 0.5%, 0.7%, 0.9% and 1% (Leenakul and Tippayawong, 2010, Nutawan*et al.*, 2010, AshishVyas*et al.* 2005). The flasks were left at room temperature for 24 hours. The substrates were then neutralized (Umbrin*et al.*, 2011) and autoclaved at 121°C for 15 minutes at 15 psi. Once the flasks had cooled, they were inoculated with *Bacillus pumilus*, *Mesorhizobium sp.*, *Aspergillus niger* and *Trichoderma viride*. Reading was taken for reducing sugar estimation using DNS (Miller, 1959) and lignin degradation assay using veratryl alcohol (Frederick, 1992). The readings were further taken weekly for 8 consecutive weeks.

### 3.4.6 Alkali Treatment

5 g of substrate was ground into small pieces and was added into flasks containing 100ml of different concentrations of alkali - 0.1%, 0.3%, 0.5%, 0.7%, 0.9% and 1% (AshishVyas*et al.* 2005). The flasks were left at room temperature for 24 h. The substrates were then neutralized (Umbrin*et al.*, 2011) and autoclaved at 121°C for 15 minutes at 15 psi. Once the flasks had cooled, they were inoculated with *Bacillus pumilus*, *Mesorhizobium sp.*, *Aspergillus niger* and *Trichoderma viride*. Reading was taken for reducing sugar estimation using DNS (Miller 1959).
and lignin degradation assay using veratryl alcohol (Frederick, 1992). The readings were further taken weekly for 8 consecutive weeks.

3.4.7 Nitrogen source

5 different Nitrogen sources were selected for the study namely, ammonium nitrate, ammonium sulfate, urea, sodium nitrate and potassium nitrate. Different concentrations (0.5%, 1.0%, 1.5% and 2.0%) of each source were taken into flasks. 5 g of substrate was ground/cut into small pieces and was added into flasks containing 50ml water with different concentrations of nitrogen sources - 0.5%, 1.0%, 1.5% and 2.0%. The flasks were then autoclaved at 121°C for 15 minutes at 15 psi. Once the flasks had cooled, they were inoculated with Bacillus pumilus, Mesorhizobium sp., Aspergillusniger and Trichoderma viride. Reading was taken for reducing sugar estimation using DNS (Miller 1959) and lignin degradation assay using veratryl alcohol (Frederick, 1992). The readings were further taken weekly for 8 consecutive weeks.

3.4.8 Carbon source

5 different carbon sources were selected for the study namely, glucose, maltose, starch, dextrose and fructose. Different concentrations i.e., 0.5%, 1.0%, 1.5% and 2.0% (Mehdi Dashtbanet al., 2011) of each source were taken into flasks. 5 g of substrate was ground/cut into small pieces and was added into flasks containing 100ml water with different concentrations of carbon sources - 0.5%, 1.0%, 1.5% and 2.0%. The flasks were then autoclaved at 121°C for 15 minutes at 15 psi. Once the flasks had cooled, they were inoculated with Bacillus pumilus, Mesorhizobium sp., Aspergillusniger and Trichoderma viride. Reading was taken for reducing sugar estimation using DNS (Miller, 1959) and lignin degradation assay using veratryl alcohol (Frederick, 1992). The readings were further taken weekly for 8 consecutive weeks.
The readings for all the individual optimization conditions were documented and used further for optimization in the production of bioethanol.

The statistical analysis of the optimization and process parameters results was done using Microsoft Excel and Standard deviation.

3.5 Purification of the Enzyme

The growth medium after the specified incubation time was centrifuged at 10,000 RPM and the supernatant which contained the enzyme was further purified by ultra filtration.

Purification of the enzyme was done initially with the salt precipitation methodology and the precipitate so obtained was re-suspended in phosphate buffer and final purification for the assay was done by Ion exchange chromatography using DEAE Sephadex columns and eluted with buffer. Elutent was subjected to thin layer chromatography to check the zones for purity.

3.6 SDS-PAGE

SDS PAGE was carried out to determine the homogeneity and molecular weight of Cellulase enzyme as it was found to be predominant in the conversion of complex polysaccharides into simple sugars. SDS-PAGE was carried out with the crude enzymes produced from all the four isolates. Polyacrylamide gel electrophoresis in the presence of SDS separates the polypeptide chains according to their molecular weight. Thus the molecular weight of the polypeptide chains of a given protein can be determined by comparing their electrophoretic mobility on SDS gels to the mobility of marker proteins with polypeptide chains of known molecular weights.
**Procedure**

The plates for casting the gel were assembled together with spacers tightly by clipping them and ensuring the assembly leak proof. Silicon grease is usually applied to the spacers to make a good watertight seal. 5 ml of separating gel mix is prepared and poured between the plates till the level was below 3-4 cm from the bottom to top of notched plate. The surface was adjucted even with the help of water.

2 ml of stacking gel mix was prepared and poured directly on to the polymerized separating gel. An appropriate comb made up of Teflon was placed into the gel solution carefully without trapping any air bubbles out. The gel was allowed to solidify.

After the stacking gel was set, the comb was removed carefully and the wells were washed immediately with distilled water to remove the non polymerized acryl amide. The bottom spacer was then removed carefully and fixed to the PAGE apparatus with tris-glycine buffer. Any bubble caught between the plates at the bottom of the gel was removed through a syringe with a bent needle. The buffer was also added to the top reservoir. The sample was heated at 100°C for 5’ with 1X SDS gel loading buffer to denature the proteins and loaded on to the wells with a micro pipette. The bottom reservoir was connected to +ve electrode and the upper reservoir is connected to –ve electrode. Electrophoresis apparatus was attached to an electric supply and the voltage is applied 200 to 600volt. The gel was run until the dye front came to the bottom of the gel. The gel was later stained by removing the gel from the plates and stained by using staining dye with shaking at room temperature for 30-60 minutes. The sample was destained by using de-staining dye with continuous shaking until the background was cleared. The gel was then finally viewed against a bright background.
3.7 Estimation of bioethanol produced from sugars that are products of biodegradation of lignocellulose

The individual substrates (Eucalyptus, Paper, Ragi straw, Rice straw) were subjected to various physical and chemical parameters using the isolates (*Bacillus pumilus*, *Mesorhizobium sp.*, *Aspergillus niger* and *Trichoderma viride*) for the maximum production of sugars. The conditions which showed maximum yield of sugars were further used for the production of bioethanol individually for each substrate and test organism.

**Paper**

Paper was cut into small pieces and 400 g of paper taken into four different Erlenmeyer flasks 100 g each for *Bacillus, Mesorhizobium, Aspergillus* and *Trichoderma*.

Pre-treatment was carried out with Acid treatment overnight with optimal 0.3%, 0.9%, 0.3% and 0.1% acid, high temperature treatment of 200, 200, 150, 100 for *Bacillus, Mesorhizobium, Aspergillus* and *Trichoderma* respectively. The treated substrate was then washed thrice with distilled water. The washed substrate was suspended in 1000 ml of distilled water for each organism and the flasks were autoclaved at 121°C for 15 minutes at 15 psi. The flasks were allowed to cool to room temperature. Once flasks were cooled, appropriate amount of carbon (maltose- 2% for *Bacillus, Mesorhizobium, Aspergillus* and *Trichoderma*) and Nitrogen (ammonium nitrate- 0.5% for *Bacillus, 0.5% for Mesorhizobium, 1.0% for Aspergillus* and 1.5% for *Trichoderma*) sources were added.
The pretreated flasks were inoculated separately with Bacillus, Mesorhizobium, Aspergillus and Trichoderma, the pH was set at 4, 4, 9, 9 and incubated at 30°C, 40°C, 25°C and 37°C respectively for 30 days.

The incubated substrates were taken and filtered with 0.45 micron Whatmann filter paper by using filtration unit (Fatma, 2010). The filtrate was collected separately for each organism in a sterile Erlenmeyer flask and inoculated with 3% Saccharomyces cerevisiae. The Saccharomyces cerevisiae inoculated flasks were incubated at 30°C for 40 days.

About 200ml of each sample were drawn on 10th, 20th, 30th and 40th day and distillation was carried out at 70°C. The amount of distillate collected was measured and alcohol estimation was carried out by potassium dichromate method.

**Eucalyptus**

Eucalyptus was cut into small pieces and ground and 400 g of the material was distributed into four different Erlenmeyer flasks 100g each for Bacillus, Mesorhizobium, Aspergillus and Trichoderma. Alkali treatment was carried out overnight with optimal 0.5%, 0.9%, 0.5% and 0.7% alkali, high temperature treatment of 200°C for Bacillus, Mesorhizobium, Aspergillus and Trichoderma.

The treated substrate was then washed thrice with distilled water. The washed substrate was suspended in 1000ml of distilled water for each organism and the flasks were autoclaved at 121°C for 15 minutes at 15 psi. The flasks were allowed to cool to Room Temperature.

Once flasks were cooled, appropriate amount of carbon (maltose- 2% for Bacillus, 1.5% for Mesorhizobium, 1.5% for Aspergillus and 2% for Trichoderma).and nitrogen (ammonium nitrate- 2.0% for Bacillus, 1.5% for Mesorhizobium, 2.0% for Aspergillus and 0.5% for Trichoderma) sources were added.
The pretreated rice straw was cut into small pieces and 400 g of rice straw taken into four different Erlenmeyer flask of 100g each for Bacillus, Mesorhizhobium, Aspergillus and Trichoderma. Acid treatment was carried out overnight with optimal 0.9%, 0.9%, 0.9%, 0.9% acid high temperature treatment of 200, 200, 150, 200 for Bacillus, Mesorhizhobium, Aspergillusniger and Trichoderma respectively. The treated substrate was then washed thrice with distilled water. The washed substrate was suspended in 1000ml of distilled water for each organism and the flasks were autoclaved at 121°C for 15 minutes at 15 psi. The flasks were allowed to cool to Room Temperature. Once flasks were cooled, appropriate amount of carbon (maltose- 2% for Bacillus, 2% for Mesorhizhobium, 2% for Aspergillusand 2% for Trichoderma) and nitrogen (ammonium nitrate- 0.5% for Bacillus, 0.5% for Mesorhizhobium, 0.5% for Aspergillus and 0.5% for Trichoderma) sources were added. These flasks were inoculated separately with Bacillus, Mesorhizhobium, Aspergillusniger and Trichoderma the pH was set at 4, 7, 7, 7 and incubated at 25°C, 25°C, 25°C and 25°C respectively for 30 days.
The incubated substrates were taken and filtered with 0.45 micron Whatmann filter paper by using filtration unit (Fatma, 2010). The filtrate was collected separately for each organism in a sterile Erlenmeyer flask and inoculated with 3% *Saccharomyces cerevisiae*. The *Saccharomyces cerevisiae* inoculated flasks were incubated at 30°C for 40 days.

About 200ml of each sample were drawn on 10th, 20th, 30th and 40th day and distillation was carried out at 70°C. The amount of distillate collected was measured and alcohol estimation was carried out by Potassium Dichromate method.

**Ragi straw**

Ragi straw was cut into small pieces and 400 g of ragi straw was distributed into four different Erlenmeyer flask 100g each for *Bacillus*, *Mesorhizobium*, *Aspergillus* and *Trichoderma*. Alkali treatment was carried out overnight with optimal 1.0%, 1.0%, 1.0%, 1.0% high temperature treatment of 200°C, 200°C, 150°C and 200°C for *Bacillus*, *Mesorhizobium*, *Aspergillus* and *Trichoderma*. The treated substrate was then washed thrice with distilled water. The washed substrate was suspended in 1000ml of distilled water for each organism and the flasks were autoclaved at 121°C for 15 minutes at 15 psi. The flasks were allowed to cool to room temperature.

Once flasks were cooled, appropriate amount of carbon (maltose- 2% for *Bacillus*, 2% for *Mesorhizobium*, 2% for *Aspergillus* and 1.5% for *Trichoderma*) and nitrogen (ammonium nitrate- 0.5% for *Bacillus*, 2.0% for *Mesorhizobium*, 0.5% for *Aspergillus* and 0.5% for *Trichoderma*) sources were added.

These flasks were inoculated separately with *Bacillus*, *Mesorhizobium*, *Aspergillus* and *Trichoderma* the pH was set at 7 and incubated at 25°C, 25°C, 25°C and 25°C respectively for 30 days.
The incubated substrates were taken and filtered with 0.45 micron Whatmann filter paper by using filtration unit (Fatma, 2010). The filtrate was collected separately for each organism in a sterile Erlenmeyer flask and inoculated with 3% *Saccharomyces cerevisiae*. The *Saccharomycescerevisiae* inoculated flasks were incubated at 30°C for 40 days. About 200ml of each sample were drawn on 10\textsuperscript{th}, 20\textsuperscript{th}, 30\textsuperscript{th} and 40\textsuperscript{th} day and distillation was carried out at 70°C. The amount of distillate collected was measured and alcohol estimation was carried out by potassium dichromate method.

The readings were individually documented for further analysis and interpretations.

### 3.7.1 Alcohol Estimation by Potassium dichromate method

Standard alcohol was prepared from 2% to 10% with blank. 1.5ml of sample distillates and standards (2%, 4% 6%, 8% and 10%) were taken in separate test tubes. 2.5ml of freshly prepared potassium dichromate solution (1g of Potassium Dichromate in 100ml of prechilled 6N H\textsubscript{2}SO\textsubscript{4}) was added to each test tube. Incubation was carried at 60°C for 30minutes (for color development) Tubes were allowed to cool to room temperature and absorbance was read at 600nm. (William, 1950).

### 3.7.2 Confirmative Qualitative and Quantitative Analysis

The confirmation of ethanol qualitatively and quantitatively was done by Gas Chromatography Analysis.

The statistical analysis of the optimization and process parameters results was done using Microsoft Excel, one way ANOVA and Standard deviation.