CHAPTER 3
MATERIALS AND METHODS

3.1. Materials

3.1.1. Collection and processing of lignocellulosic substrates

In the present investigation, following lignocellulosic agro wastes

- Wheat straw,
- Bagasse,
- Peels of fruits like Litchi (*Litchi chinensis*), Pineapple (*Ananas cosmosus*) and Sweet orange (*Citrus sinensis var mosambi*) were selected.

The substrates were collected from local farms (wheat straw) and from local fruit juice vendors (bagasse, peels of *L. chinensis*, *A. cosmosus* and *C. sinensis var mosambi*) of Gwalior (26°13′N 78°11′E / 26.22°N 78.18°E), Madhya Pradesh, India.

Wheat straw was dried in a forced-air oven at 55.8°C for 24 h and milled in a hammer mill to pass through a 1.27 mm screen. The milled wheat straw was stored at room temperature.

Bagasse was air-dried before it could be used for further process. It was then reduced to powdered form.

Peels of *C. sinensis var mosambi* were washed thoroughly to remove dirt and other associated particles. They were then air dried for 4-5 days.

Peels of *A. cosmosus* and *L. chinensis* have been air dried. Dried peels of all three substrates were separately ground into 100 mesh (0.15mm) fine particles by use of laboratory blender at 3000 rpm.

Wheat bran was purchased locally from Gwalior. Substrates and wheat bran were preserved in sealed plastic bags at 4°C to prevent any possible degradation or spoilage.
3.1.2. Culture Collection

The wild type strain used in the present investigation viz.

- *Pachysolen tannophilus* MTCC 1077,
- *Kluyveromyces marxianus* MTCC 4139,
- *Mucor indicus* MTCC 4349,
- *Saccharomyces cerevisiae* MTCC 3821 were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India and
- *Pichia stipitis* NCIM 3498 (which is now known as *Scheffersomyces stipitis*),
- *Trichoderma reesei* NCIM 1052 were procured from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India.

3.1.3. Chemicals

Synthetic microbial growth media components and quantitative analytical reagent were procured from Hi-Media Laboratories Pvt. Ltd., Mumbai, India and Sigma Aldrich Chemicals Pvt. Ltd., Bangalore respectively. Organic solvents were purchased from Sisco Research Laboratories (SRL) and Qualigens Fine Chemicals, India. Chromatographic material such as Silica G-60 was purchased from Selecto Scientific, USA. Crude calcium oxide (locally called chuna) was locally purchased.

3.2. Analysis of substrates before pretreatment

Composition of lignocellulose in terms of lignin, hemicellulose and cellulose was determined by the method of Goering and Van soest (1979). Determination of total carbohydrate was done by anthrone method (Hedge and Hofreiter 1962). The amount of reducing sugars was estimated by Dinitrosalicylic acid (DNS) method (Miller 1959). The Folin-Lowry method was used for protein assay (Lowry et al. 1951).
3.2.1. Estimation of the cellulose, hemicellulose and lignin

3.2.1.1. Neutral detergent fiber (NDF)

Neutral detergent solution: 18.61 g of disodium ethylenediamine tetraacetate and 6.81 g of sodium borate decahydrate were dissolved in 200 ml of distilled water and allowed to melt by heating. 30 g of sodium lauryl sulphate and 10 ml of 2-ethoxy ethanol were dissolved in 200 ml of distilled water. 4.5 g of disodium hydrogen phosphate was dissolved in 100 ml distilled water. All the contents were mixed and final volume was made up to 1 liter. pH was adjusted to 7.0.

In a refluxing flask 0.5 g of substrate was taken to which 100 ml of cold neutral detergent solution, 2 ml of decahydronaphthalene (Decalin) and 0.5 g of sodium sulphite was added. This mixture was heated to boiling after which the heat was reduced to avoid foaming and refluxed for one hour. After cooling, a previously weighed crucible of G-1 grade was used to filter the sample under suction using a vacuum pump. The residue remained in the crucible was washed with hot water repeatedly. Finally the residue was washed twice with acetone. The crucible containing residue was dried at 100°C for 8 h in a hot air oven. Then it was cooled in a desiccator and the dry weight was recorded (Prasad 2008)

\[
\% \text{NDF} = \frac{Y - X \times 100}{W}
\]

Where,

\(Y = \) weight of crucible + NDF

\(X\) is weight of empty crucible

\(W\) is weight of the sample

3.2.1.2. Acid detergent fiber (ADF)

Acid detergent solution (ADS): 20 g of cetyl trimethyl ammonium bromide (CTAB) was dissolved in 1 L of 1N sulphuric acid.

The sample of 0.5 g was transferred to a refluxing flask. To this 100 ml of ADS and 2 ml of decahydronaphthalene were added. This mixture was heated to boiling and the heat was reduced to avoid foaming and refluxed for one hour. The mixture was then cooled and filtered through a previously weighed crucible under suction using a vacuum pump. Hot water was used to wash the samples in
crucible to remove acid and this was followed by two washings with acetone. The crucibles were dried at 100°C for 8 h in a hot air oven. The crucibles were cooled in a desiccator after eight hours and dry weight was recorded.

\[
\% \text{ ADF} = \frac{Y - X \times 100}{W}
\]

Where,

- \( Y \) = weight of crucible + ADF
- \( X \) is weight of empty crucible
- \( W \) is weight of the sample

3.2.1.3. Acid detergent lignin

Contents of crucibles (containing ADF) were layered with cooled 72% \( \text{H}_2\text{SO}_4 \). Frequent stirring was followed. After three hours of intermittent stirring, the contents were filtered off under suction using hot water. The crucibles with residues were then dried at 100°C for eight hours. After this, the crucibles were cooled in a dessicator and weighed (L). After weighing, the contents in crucibles were kept inside a muffle furnace for ashing at 500°C for 2 h. After the furnace temperature came down, the crucibles were taken out, cooled partially in air, followed by desiccator and the weight (A) of the ash was recorded.

\[
Y = \text{Weight of ADF + crucible}
\]

\[
L = \text{Weight of crucible + lignin}
\]

\[
A = \text{Weight of crucible + ash}
\]

\[
W = \text{Weight of the sample}
\]

\[
\% \text{ Hemicellulose} = \% \text{ NDF} - \% \text{ ADF}
\]

\[
\% \text{ Cellulose} = \frac{Y - L \times 100}{W}
\]

\[
\% \text{ Lignin} = \frac{L - A \times 100}{W}
\]
3.2.2. Estimation of total carbohydrates

**Principle**: In presence of dilute hydrochloric acid, carbohydrates are first broken down into simple sugars. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural (HMF). This compound forms with anthrone a green coloured product with an absorption maximum at 630 nm.

**Method**

Fresh anthrone reagent was prepared by dissolving 200 mg anthrone in 100 ml of ice-cold 95% H₂SO₄.

Standard glucose: Stock- 1mg/ml or 100 mg/100ml.

Working standard- 10 mg/100 ml.

- Different dilution of working standard were prepared.
- To this 4 ml of anthrone reagent was added.
- Tubes were heated for eight minutes in a boiling water bath.
- Tubes were cooled rapidly and absorbance was read at 630 nm.

100 mg of the sample was taken in boiling tube. It was hydrolysed by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N HCl and cool to room temperature. It was neutralized with solid sodium carbonate until the effervescence ceases. The volume was made up to 100 ml and centrifuged at 500 rpm for 10 min. 0.5 ml aliquot of supernatant was taken and procedure of anthrone was followed as mentioned above.

3.2.3. Estimation of reducing sugars

**Preparation of reagent**

Reagent was freshly prepared by dissolving of one gram of 3, 5-dinitrosalicylic acid (DNSA), 200 mg of crystalline phenol and 50 mg of sodium sulphite in 100 ml of 1% NaOH and was stored at 4°C.

Rochelle salt solution (40%): 40 g of potassium sodium tartrate was dissolved in 100 ml distilled water.

Preparation of stock solution of glucose- 100 mg/100 ml
Preparation of working standard
About 10 ml of the stock was diluted to 100 ml with distilled water in a 100 ml volumetric flask to obtain the glucose concentration of 100 μg glucose/ml.

About 0.5 ml of sample was aliquoted from every treatment/sample into test tubes. The volume was made upto 3 ml using distilled water. 3 ml of DNS was added to each sample, vortexed. The reagent blank containing 3 ml of distilled water and 3 ml of DNS reagent was also prepared. Similarly, standards were also included whose glucose concentration ranged from 10 μg to 100 μg. All tubes viz., samples, standards and blank were kept on boiling water bath for 5 minutes. After this 1 ml of 40 % Rochelle salt solution was added when the reaction mixture was still warm. Then the tubes were cooled. The absorbance in terms of optical density of the standards and sample were read at 510 nm using UV Spectrophotometer (Systronics -117).

3.2.4. Protein estimation
Principle: With Folin- Ciocalteau reagent which consists of sodium tungstate molybdate and phosphate, the phenolic group of tyrosine and tryptophan residues (amino acid) in a protein produces a blue purple color complex, with maximum absorption in the region of 660 nm wavelength. The intensity of color depends on the amount of these aromatic amino acids present and will thus differ for various proteins or samples.

Materials
1. Stock solution of Bovine Serum Albumin (BSA) (1mg/ml)
2. Analytical reagents:
   (a) 50 ml of 2% sodium carbonate mixed with 50 ml of 0.1 N NaOH solution (0.4 gm in 100 ml distilled water.)
   (b) 10 ml of 1.56% copper sulphate solution mixed with 10 ml of 2.37% sodium potassium tartarate solution. Analytical reagents were prepared by mixing 2 ml of (b) with 100 ml of (a)
3. Folin - Ciocalteau reagent solution (1N) - was prepared fresh by diluting 2 N commercial reagent with an equal volume of water.

4. Standard protein was prepared by dissolving 1mg BSA in 1 ml distilled water.

**Procedure**

Different dilutions of BSA solutions are prepared by mixing stock BSA solution (1 mg/ml) and water in the test tube. The final volume in each of the test tubes is 5 ml. The BSA range is 0.05 to 1 mg/ml. From these different dilutions, 0.2 ml protein solution was pipetted to different test tubes and 2 ml of alkaline copper sulphate reagent (analytical reagent) was added followed by thorough mixing. This solution was incubated at room temperature for 10 mins. 0.2 ml of reagent Folin Ciocalteau solution (reagent solutions) was then added to each tube and incubated for 30 min. Absorbance was measured at 660 nm (Lowry et al. 1951).

**3.3. Pretreatment of substrates**

Three major physico-chemical pretreatments viz. solar pretreatment, microwave pretreatment and steam explosion pretreatment were given to all the above mentioned substrates. The use of microwave oven for pretreatment of lignocellulosic has been reported to make the substrate more susceptible to the subsequent enzymatic hydrolysis (Adrados et al. 2004, Adrados et al. 2005).

Various pretreatment techniques such as steam explosion in an autoclave at 15 psi (121°C) for 60 minutes; dilute sulphuric acid (1% v/v), concentrated sulphuric acid (10% v/v), sodium hydroxide (1% v/v), nitric acid (1% v/v) and calcium hydroxide (1% v/v) with steam explosion at 15 psi (121°C) for about 20 minutes, were adopted separately for the pretreatment of all substrate (Szczodrak and Fiedurek 1996). Lime pretreatment was selected as a model for study as this has gained industrial interest because of its perceived plus points over other pretreatment methods, including the use of a low-cost chemical (lime), lower reactor investment costs, as well as limited potential for the formation of degradation products as reported in chapter 2. This makes lime pretreatment environmentally attractive and economically interesting. The Liquid Hot Water method uses compressed hot liquid water (at pressure above saturation point) to
hydrolyse the hemicellulose. Only H₂SO₄ was tested for its effect on pretreatment at its higher concentration (10% v/v), just to make a comparative account of yield of sugars. Use of low concentration of bases are reported to be the best over their high concentrations by various researchers (Mirahmadi et al. 2010), hence we have chosen only 1% NaOH and 1% Ca(OH)₂, thereby avoiding huge and bulky experimentations. Economic and ecological concerns were behind the concept of selecting the concentration of these chemicals.

In all the three major pretreatments, 1 g of substrate was mixed with 10 ml of above mentioned chemicals/10 ml distilled water. Solar pretreatment was performed in open Petri dishes exposed to sunlight for about 6 hrs (from 10 am to 4 pm), at temperature of 34 ± 3°C. Microwave pretreatment was given to the substrates for 5.0 min in a microwave oven (LG Electronics Tianjin Appliances Co., Ltd., Tianjin, PR China). The oven’s irradiation power of 700 W was used in all pretreatment processes. Prior to the microwave treatment, soaking time of 90 min was given to all substrates with their respective chemicals (Zhu et al. 2006). Steam explosion of raw material was carried out at flask level. 1g of raw material along with its chemical/ distilled water was taken in the 100 ml flask, and was subjected to sudden steam depressurization in an autoclave (15 psi, 20 min, and 121°C) by fully opening the steam exhaust valve.

After pretreatment, the supernatant was filtered through wire gauze, collected in screw cap tubes and stored at 4°C for further biochemical tests. The residue left behind was thoroughly washed twice with distilled water to remove all associated chemicals and were dried in an oven at 70°C for 24 h, after which they were reweighed and some of them were further used for various studies.

3.4. Analysis of substrates after pretreatment

3.4.1. Determination of cellulose, hemicellulose and lignin, total carbohydrate, amount of reducing sugars was done as mentioned in section 3.2.1.
3.4.2. Bial’s test for pentose sugar

**Principle**: Qualitative assay of pentoses in the hydrolysates was done by Bial’s test. It is specific for pentoses. They get converted to furfural. In the presence of ferric ion, orcinol and furfural condense to yield a coloured product.

**Procedure**:
Bial’s reagent: 1.5 g orcinol was dissolved in 500 ml of concentrated HCl and 20 to 30 drops of 10% ferric chloride were added. To 5 ml of Bial’s reagent 2-3 ml of filtrate was added and warmed gently. Tubes were cooled under the tap when bubbles started rising to the surface. Appearance of green colour or precipitate confirms presence of pentose sugars (Sadasivam and Balasubramanian 1985).

3.4.3. Thin layer chromatography of hydrolysed products

**Principle**: One of the most important parameters in chromatography is the retention factor, \( k \). It is simply the additional time that the analyte takes to elute from the column relative to an unretained or excluded analyte that does not interact with the stationary phase and which by definition, has a \( k \) value of 0. Thus:

\[
Rf(k) = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}
\]

**Procedure**:
The filtrates obtained after pretreatment were centrifuged at 10,000 g for 10 min to get the supernatant. The hydrolysed products were assayed by spotting the supernatant on silica gel G plate using an ethyl acetate: isopropanol: water: pyridine (26:14:7:3, v/v/v/v) solvent system. The chromatogram was developed by spraying with aniline-diphenylamine (5 volume of 10 g/l aniline and 5 volumes of 10 g/l diphenylamine in acetone with 1 volume of 85% phosphoric acid) followed by heating at 105°C for 10 min. While drying coloured spots appear. The Rf values were compared with that of the standard sugars (Plummer 2007, Chandraju et al. 2011).

Corresponding standards i.e. lactose, maltose, sucrose, galactose, glucose, mannose, sorbose, fructose, arabinose and xylose were used as standard. 10 mg of
each sugar was dissolved in 1ml of deionised water, 1µl of which was applied to the chromatoplate (Silica gel G) and developed as above given protocol.

3.4.4. High-Performance Liquid Chromatography (HPLC)

The sugar concentrations in the samples were analyzed using high performance liquid chromatography (HPLC, Waters) with sugar pak column (Waters USA) and a system composed of a 510 pump, a refraction index differential detector (RI 2414 USA) and a data processor with register (Waters, USA). The samples were filtered through membrane filters 0.45 µm (Millipore) before injection. The temperature of the column was maintained at 70°C by column oven (Dyna, Mumbai) with injection valve of 20 µl. The RI detector was operated at 30°C and the solvent system used was water as mobile phase at flow rate of 0.2 ml/min. Calculations and analysis were performed using Empower 2 software Build 2154 (Waters). All the experiments were carried out in duplicate and all the reported results were the mean value. The average standard deviation of the achieved results was less than 4%.

**Calculation of sugar Percentage was done by the following formula**

\[
\text{Sample area} \times \frac{\text{Standard Weight}}{\text{Sample weight}} \times \frac{50}{10} \times \frac{\text{Potency}}{100} \times 100 = \% 
\]

Where

- Standard weight = 10mg
- Sample weight = 1gm (1000mg)
- 50ml = dilution
- 10ml = dilution of standard
- 100 = potency of standard
- 100 = percentage

3.4.5. Fourier- Transform Infrared (FTIR) Spectroscopy

The structural characteristics of polysaccharide sample were recorded on a Fourier- transform infrared spectrophotometer (IR Affinity- 1, Shimadzu, Japan).
The sample was ground with KBr powder (spectroscopic grade) and then pressed into 1mm pellet for FT-IR measurement in the frequency range 4000-400 cm$^{-1}$, with a spectral resolution of 0.5 cm$^{-1}$. The spectra would be obtained with an average of 64 scans. Analysis was performed on both the native and pretreated samples. The baselines of the spectra were adjusted and normalized with the IRsolution software, and the absorption bands at 1427 and 898 cm$^{-1}$ were used to calculate the crystallinity index (Mirahmadi et al. 2010).

3.5. Neutralization & detoxification

Fermentative inhibitors are common in acidic hydrolysis. Hydrolysates were detoxified effectively using calcium hydroxide overliming. The acid hydrolysates were detoxified following the standard methods for overliming, neutralization and treatment with activated charcoal as described by Carvalheiro et al. (2005). These detoxifying agents were used directly without any preparation. The calcium oxide was added to high alkalinity (pH 12-13). Hydrolysate was then neutralized with HCl. After neutralization the mixture was left for 30 min under moderate mixing followed by vacuum filtration to remove precipitates and other salts. 2.5% activated charcoal was then added and shaked for 30 min in orbital shaker. The reaction mixture was filtered twice to remove charcoal and the pH was maintained at 6.0-6.5 (Chandel et al. 2011, Prakasham et al. 2009).

3.6. Simultaneous Saccharification & Fermentation (SSF)

3.6.1. Production of crude cellulase

Production of crude cellulase was done from Trichoderma reesei NCIM 1052 (Abadulla et al. 2000). Culture of T. reesei NCIM 1052 was procured from NCIM, Pune and was maintained on Potato Dextrose Agar (PDA) medium as will be mentioned later in section 3.6.2.

The medium for crude cellulase enzyme extraction was prepared in medium with following composition (g/l) wheat bran, 45; yeast extract, 15; glucose, 10; NH$_4$Cl, 2.5; thiamine hydrochloride, 0.5; K$_2$HPO$_4$, 2.0; MgSO$_4$.7H$_2$O, 0.5; CaCl$_2$, 0.1; and KCl, 0.5 (Abadulla et al. 2000). This medium
was aseptically inoculated with actively growing *T. reesei* NCIM 1052. The flasks were incubated at 28°C for 10 days on a rotary shaker. After 10 days of incubation the culture broth was centrifuged at 10,000 rpm for 20 min to remove mycelia and spores. The supernatant was collected aseptically and stored at 4°C as the source of enzyme.

### 3.6.1.1. Filter paper assay for saccharifying cellulase

Reagents required: Sodium citrate (0.05 M), DNS reagent, 40% Rochelle salt and standard sugar (glucose).

One ml of 0.05 M sodium citrate (pH 4.8) was added to a test tube. To this 0.5 ml of enzyme was added. One strip of 50 mg Whatman No.1 filter paper was put into test tube. Care was taken to push down filter paper strip whenever it winds up the test tube. The tube along with blank was kept in a water bath at 50°C for one hour. After one hour, the tubes were taken out and DNS method was followed further to determine the amount of sugars released by the cellulase (Singhania 2006). 3 ml of DNS reagent was added to the tubes. The tubes were kept on a boiling water bath for 5 min. One ml of 40% Rochelle salt solution was added when the tubes were still warm. After cooling, 1.5 ml of distilled water was added to bring the reaction mixture to a volume of 7 ml. The contents were vortexed. Then the filter paper pulp was allowed to settle down. After the filter paper pulp had settled down, the absorbance was recorded at 510 nm using UV-spectrophotometer-117 (Systronics, India) by transferring the supernatants in a cuvett. The amount of sugars in terms of glucose was known by referring to standard graph of glucose.

### 3.6.1.2. Treatment of substrates with crude cellulase

Five grams of the delignified samples (all five substrates of 500 μ size) were taken in Erlenmeyer flask of 100 ml capacity separately. The crude cellulase extracts of the fungi were used to treat substrates at substrate to enzyme ratio 1:5 (5 g substrate: 25 ml crude enzyme). The flasks were incubated at 50°C in a water bath for 48 h (Singh et al. 1990). Then the reaction of hydrolysis was ceased by
holding flasks at 80°C for 5 min. The amount of reducing sugars was estimated by DNS method as described by Miller (1959).

3.6.2. Maintenance of culture for Simultaneous Saccharification & Fermentation

Yeast cultures (Pachysolen tannophilus MTCC 1077, Kluyveromyces marxianus MTCC 4139, Saccharomyces cerevisiae MTCC 3821, Pichia stipitis NCIM 3498) were maintained separately on Malt Yeast Agar medium with following composition (g/l)- Malt extract, 3; Yeast extract, 3; Peptone, 5; Glucose, 10; Agar, 20, pH: 7.0 ± 0.2. The strain of Trichoderma reesei NCIM 1052 and Mucor indicus MTCC 4349 were maintained separately on PDA slants consisted of (g/l): potato, 200; dextrose, 20; agar, 25, pH: 4.8 ± 0.2. Stock cultures were stored at 4°C. The liquid medium for the growth of inoculum for yeast was YEPD medium consisted of (g/l): yeast extract, 10; peptone, 20; dextrose, 20, pH: 5.0 ± 0.2 for 48 h at 28 ± 0.5°C (Pasha et al. 2007). Inocula were grown aerobically in 250 ml Erlenmeyer flasks containing the above mentioned medium at 28°C in an Environmental Shaker (Remi Scientific) at 200 rpm for 48 h. Active cells were centrifuged at 1200 rpm for 10 min, washed with sterile water, and were used as inoculum.

3.6.3. Immobilization of yeast

Immobilization of yeast was done by using sodium alginate (Smidsrod and Skjakbraek 1990). 2% sodium alginate slurry was prepared and sterilized. 2 ml, 4 ml and 6 ml (i.e. 2%, 4% or 6 % v/v) of yeast suspension is prepared under aseptic conditions. 0.2 M calcium chloride solution is prepared and sterilized. Sodium alginate slurry is mixed with yeast suspension under aseptic conditions. With the help of sterilized syringe the above mixture is added drop wise into 0.2 M calcium chloride solution. Beads thus formed are left in calcium chloride solution for 1 hour for curing. Cured beads were then washed with sterilized water and will be inoculated during simultaneous saccharification and fermentation.
Free cells of *M. indicus* MTCC 4349 were used as an inoculum of various concentrations viz. 2%, 4% and 6% w/v

3.6.4. Study of effect of operational parameters on Simultaneous Saccharification & Fermentation (SSF).

Parameters, like temperature, inoculum level and nutrients were chosen as the most significant ones, like:

**3.6.4.1. Inoculum levels** (% v/v): Three inoculum levels were tested viz. 2%, 4% and 6%.

**3.6.4.2. Nutrients**: In media composition, different nutrients like nitrogen, phosphate & growth factors of different compounds were tested. Three nutrient factors were designed to study the effect of different sources of nitrogen, phosphorus and growth factors on the ethanol production capacity of test organisms. These factors are given below in table 1:

**Table 1- Nutritional components used in various Nutrient Factors (NF)**

<table>
<thead>
<tr>
<th>Components</th>
<th>NF 1</th>
<th>NF 2</th>
<th>NF 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen source</td>
<td>Ammonium sulphate (0.3%)</td>
<td>Sodium nitrate (0.3%)</td>
<td>Urea (0.3%)</td>
</tr>
<tr>
<td>Phosphorus source</td>
<td>Potassium dihydrogen phosphate (0.15%)</td>
<td>Di potassium hydrogen phosphate (0.15%)</td>
<td>Sodium dihydrogen phosphate (0.15%)</td>
</tr>
<tr>
<td>Growth factors</td>
<td>Yeast extract (0.5%)</td>
<td>Malt extract (0.5%)</td>
<td>Meat extract (0.5%)</td>
</tr>
<tr>
<td></td>
<td>Peptone (0.5%)</td>
<td>Soya Peptone (0.5%)</td>
<td>Tryptone (0.5%)</td>
</tr>
</tbody>
</table>

**3.6.4.3. Temperatures**: Three different temperatures were tested viz. 30°C, 32°C and 34°C.

Batch experiments were conducted in a 250 ml capacity of Erlenmeyer flask. The process was conducted at the initial substrate concentration of 10 g/l
pretreated substrate (i.e. bagasse/wheat straw/ peel of L. chinensis/A. cosmosus/C. sinensis var mosambi) and 100 ml citrate buffer (pH 5.0 ± 0.2, 50 mM) followed by sterilization for 15 min, at 15 psi (121°C). The substrate soaked in citrate buffer was supplemented with cellulase, at substrate to enzyme ratio of 1:5 (10 g pretreated substrate: 50 ml crude cellulase) or 5 FPU of cellulase was used for hydrolyzing per gram of substrate. The saccharification was done for 24 hrs at 50°C after which simultaneous fermentation was conducted in same vessel by addition of 50 ml of sterilized detoxified hydrolysate (obtained after pretreatment) and different nutrients (nutrient factors 1/2/3, as given in table 1) at lower temperatures (30°C / 32°C / 34°C). Immobilized yeast cells were used separately as inoculum at different concentrations viz. 2%, 4% or 6%. MgSO₄, 0.5 g/l; KCl, 0.5 g/l and FeSO₄ 0.01 g/l were used as common nutrients in all fermentation experiments other than different nutrient parameters mentioned above. Fermentation was carried out for 72 h under anaerobic condition, after which samples were withdrawn and centrifuged in a laboratory centrifuge at 1200 rpm, and the supernatants were analyzed for ethanol concentration (Chandel et al. 2009).

3.6.5. Quantitative estimation of ethanol
The ethanol was estimated as described by Caputi et al. (1968).

**Preparation of reagents**
0.23 N Potassium dichromate was prepared by adding 34 g of K₂Cr₂O₇ in 500 ml of distilled water. To this 325 ml of concentrated H₂SO₄ was added and the volume was made upto 1000 ml with distilled water.

**Preparation of stock solution**: standard ethanol is 10 mg/1 ml.

**Procedure**: 3 ml of representative sample from each SSF experiments was transferred to 100 ml round bottom flask connected to the condenser and was diluted with 30 ml distilled water. The sample was distilled at 74-75°C. The distillate was collected in 25 ml of 0.23 N K₂Cr₂O₇ reagent, which was kept at the receiving end. The distillate containing alcohol was collected till total volume of 45 ml was obtained. Similarly, standard (5-100 mg ethanol) were mixed with 25
ml of $K_2Cr_2O_7$ separately. The distillate containing alcohol was collected till total volume of 45 ml was obtained. These samples and standards were kept in water bath at 60°C for 20 min and were cooled. The volume was made upto 50 ml with distilled water and optical density was measured at 660 nm using spectrophotometer-117 (Systronics, India). The standard curve was plotted considering the concentration against absorbance.

3.7. Experimental design and Statistical Analysis

In the Central Composite Design (CCD), the total number of experimental combinations was $2^K + 2K + n_0$, where $K$ is the number of independent variables and $n_0$ is the number of repetitions of the experiments at the central point, which indicated that 20 experiments were required for this procedure. The dependent variable selected for this study was ethanol concentration, $Y$ (g/l). The independent variables chosen were incubation temperature (30°C, 32°C and 34°C) $X_1$, inoculum level (2%, 4% and 6%) $X_2$ and nutrients (1/2/3) $X_3$. A mathematical model, describing the relationships among the process dependent variable and the independent variables in a second-order equation, was developed (Giovanni 1983). Design-based experimental data were matched according to the following second-order polynomial equation (1).

$$Y = b_0 + \sum_{i=1}^{k} b_i x_i + \sum_{i=1}^{k} b_{ij} x_i^2 + \sum_{i<i}^{k} b_{ij} x_i x_j + e$$

Where, $i, j$ are linear, quadratic coefficients, respectively, while ‘b’ is regression coefficient, $k$ the number of factors studied and optimized in the experiment and ‘e’ is random error. The quality of fit of the second order equation was expressed by the coefficient of determination $R^2$, and its statistical significance was determined by $F$-test. The significance of each coefficient was determined using Student’s $t$-test. The student $t$-test was used to determine the significance of the parameters regression coefficients. The P values (Probability value) were used as a tool to check the significance of the interaction effects, which in turn may
indicate the patterns of the interactions among the variables. The response surface equation was optimized for maximum yield in the range of process variables using Design Expert software version 9.0.2. Analysis of variance (ANOVA) for the final predictive equation was done using the same software package. Isoresponse contour plots were obtained based on the effect of the levels of three parameters and their interactions on the yield of ethanol by keeping the other parameters at their optimal concentrations. From these contour plots, the interaction of one parameter with another parameter was studied. The optimum concentration of each parameter was identified based on elliptical structure of the contour plots (Sasikumar and Viruthagiri 2010).

3.8. Optimized Simultaneous Saccharification & Fermentation (SSF) in a modular fermenter

Batch experiment was conducted as per the central composite experimental design for ethanol production in a fermenter (BioFlo®/CelliGen® 115), with 2 L capacity, equipped with flat blade impeller, oxygen and pH electrodes, temperature and dO₂ (dissolved oxygen) probe. The equipment also monitored temperature, agitation speed, gas purging flow rate, pumping rates, antifoam addition and the vessel level. All processing parameters were online monitored, with the aid of BioXpert Lite 1.00 software. Other parameters, like temperature, inoculum concentration and nutrient factor, were chosen as the most significant ones, considering the experimental design. The process was conducted at the initial substrate concentration of 50 g/l (pretreated peel of L. chinensis) with the addition of citrate buffer (pH 5.0 ± 0.2, 50 mM) followed by sterilization for 15 min, at 15 psi (121°C). The substrate soaked in citrate buffer was supplemented with cellulase, at substrate to enzyme ratio of 1:5 (i.e. 50 g pretreated substrate: 250 ml crude cellulase) i.e. 5 FPU/g substrate. The saccharification was done for 24 hrs at 50°C after which simultaneous fermentation was conducted in same vessel by adding equal volume of sterilized detoxified L. chinensis peel hydrolysate (obtained after pretreatment) and components of nutrient factor 1. Immobilized Pachysolen tannophilus was used.
as inoculum at 6% concentration. Fermentation was carried out anaerobically for 72 h at lower temperature of 30°C after which samples were withdrawn and centrifuged in a laboratory centrifuge at 1200 rpm, and the supernatants were analyzed for ethanol concentration using Gas Liquid Chromatography with the following specifications-

**G.C System:** AGELIENT 7890

**Column** : DB1624 (30*0.5) 3Um

**Solubility** : Water

**Standard** : 10 μl of Ethanol was taken and dissolved in 10ml Water.

**Sample** : 2.7g of sample was dissolved in 5 ml of Water.

Fermented broth was distilled at 74-75°C to recover ethanol.