Materials and Methods

Chemicals and reagents
General chemicals like potassium dihydrogen phosphate, ammonium sulfate, magnesium chloride, calcium chloride, sodium hydroxide, calcium carbonate, galacturonic acid, sugars like glucose, fructose, xylose, lactose and sucrose were procured either from Merck (India) or Qualigens (India). Acids like hydrochloric acid and sulphuric acid were purchased from Qualigens (India). Ethanol (99%) was procured from Changshu Yangyuan Chemicals, China. Sugar standards were procured from Sigma chemical company (St. Louis, USA). Enzymes like amylase, pectinase, cellulase and xylanase were purchased from Biocon Limited, India. Calcium ethylene diamine tetra acetic acid (Ca EDTA) was used for HPLC analysis. The commercial media components like agar powder, yeast extract were obtained from HiMedia (India).

Microorganisms explored in the study
Fruits, such as apple, sapota, grapes etc. were collected from the fruit market and were allowed to ripen for 8–10 days at 30 ºC and 37 ºC temperatures. After the incubation period, 2.5 g of ripened fruits were added to 45 ml of M21 medium prepared in 100 ml serum bottles sealed with rubber septum. The composition of the M21 medium is described in the Appendix. In addition to ripened fruits ethanol producing microbial strains were also enriched and isolated from raw honey, molasses, fermenting sugarcane juice. The inoculated bottles were incubated on a rotary shaker (150 rpm) at three different temperatures 23 ºC, 30 ºC and 37 ºC temperatures for 5 days. After three cycles of such enrichment, 1 ml of aliquot was diluted up to $10^8$ fold, and 100 μl of all the dilutions were plated on M21 agar plates. The bacterial colonies obtained were further purified on M21. Six different strains were enriched and isolated from above mentioned substrates. They were compared on the basis of their ethanol production ability.
A known ethanol producing bacterial strain *Zymomonas mobilis* MTCC 92 was procured from Microbial Type Culture Collection, Institute of Microbial
Technology (IMT), Chandigarh, India. The other bacterial strain *Z. mobilis* DSM 473 was procured from German Microbial Culture Collection centre.

**Preservation and maintenance of the strains**

Each of the purified bacterial isolates was given a code for cataloguing purpose. Frozen stock cultures of the bacterial strains were stored in 25% glycerol at -70 °C. Cultures were sub-cultured once, every 15 days and the purity of the bacterial strains was checked periodically. The glycerol stocks of all the bacterial strains were thawed and streaked over M21 medium. The plates were incubated at respective temperatures (30 °C and 37 °C) used for isolation and enrichment of bacterial strains in earlier experiments. Liquid cultures of bacterial strains required for ethanol production studies were also grown at the respective temperatures (30 °C and 37 °C), maintained during the isolation and enrichment procedures. For biochemical characterization and colony morphology studies, the bacterial strains were grown on M21 agar plates.

**Collection of Agro-industrial waste material**

**Fruit and vegetable waste**

In the present study, thirteen different fruits and vegetables that are found almost throughout the year were tested for initiating the ethanol fermentation. Before the fermentation experiments, characterization of all the thirteen fruit and vegetable waste was carried out. The waste material was shredded and smashed to extract juice, which was used as a substrate to study the ethanol production ability of strains, *Z. mobilis* MTCC 92, DSM 473 and an indigenous strain TERI SH 110. Generic process scheme for bioethanol production from agricultural waste followed in this work can be explained as Figure 3.1.
Materials and Methods

Seasonal production pattern analysis

Collection of fruit and vegetable

Analysis and characterization of individual components and mixture of fruit and vegetable residues

Extraction of liquid

Estimation of reducing sugars in liquid extract

Fermentation or storage of liquid extract at 4°C

Solid waste

Dilute acid hydrolysis

(2 step hydrolysis)

Alkali hydrolysis

Enzymatic hydrolysis

Neutralization of the hydrolysate

Estimation of reducing sugars in hydrolysate

Fermentation or storage of hydrolysate at 4°C

**Figure 3.1** Flow Chart of general strategy of fruit and vegetable residues processing

Thippi

Starchy waste material, thippi is a waste by-product of the tapioca (*Manihot esculenta*) processing industry. It was obtained from Sri Laxmi industries, Tamilnadu, Southern India. Thippi was in dry pellet form and stored at room temperature. It was first ground to powder form before further processing and then was characterized. Before starting the experiment thippi was subjected to heat treatment at different temperatures. Initially, 10 g of thippi powder was taken in each of the flasks and incubated at three different temperatures of 30 °C, 55 °C and 121 °C. The heat treatment at 121 °C was given for 15 min and 30 min only while the incubation of flasks at 30 °C and 55 °C was extended up to 1 h, 3 h, and 6 h.

General scheme of process for thippi is presented in Figures 3.2 and 3.3.
Materials and Methods

Collection of thippi

Characterization of thippi

Pretreatment (steam 121 °C, 20 minutes)

Acid hydrolysis (0.75% H₂SO₄, 30 °C/ 55 °C/ 121 °C, 3 h)

Solid liquid separation

Reducing sugar analysis

Storage of hydrolysate at 4 °C or fermentation

Alkaline hydrolysis (0.75% H₂SO₄, 30 °C/ 55 °C/ 121 °C, 3 h)

Figure 3.2 Experimental layout of acid and alkaline hydrolysis and fermentation of thippi.
Characterization of thippi

Pretreatment (steam 121 °C, 20 minutes)

Collection of thippi

Solid liquid separation

Reducing sugar analysis in hydrolysate

Storage of hydrolysate at 4 °C or fermentation

Amylase enzyme (5 U g⁻¹ substrate, 55 °C, 3 h, pH3)

Pectinase enzyme (5 U g⁻¹ substrate, 55 °C, 3 h, pH3)

Cellulase enzyme (5 U g⁻¹ substrate, 55 °C, 3 h, pH3)

Figure 3.3 Experimental layout of enzymatic hydrolysis and fermentation of thippi.
Analysis of constituents of individual fruit and vegetable waste

Fruit and vegetable waste material was collected from local markets. A list of fruits and vegetables is presented in Table 3.1. Market survey on availability (whole year) of fruit and vegetable waste was done.

Table 3.1 List of fruits and vegetables selected for the study

<table>
<thead>
<tr>
<th>Fruits</th>
<th>Vegetables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple</td>
<td>Carrot</td>
</tr>
<tr>
<td>Mango</td>
<td>Tomato</td>
</tr>
<tr>
<td>Pineapple</td>
<td>Potato</td>
</tr>
<tr>
<td>Banana</td>
<td>Other seasonal vegetable</td>
</tr>
<tr>
<td>Orange</td>
<td></td>
</tr>
<tr>
<td>Grapes</td>
<td></td>
</tr>
</tbody>
</table>

Characterization of waste material

The selected substrates were characterized for total solids, moisture content, total sugar, total reducing sugar and pH. Total sugars were estimated using the Anthrone method (Dreywood, 1946). The total reducing sugars were estimated with the help of Dinitrosalicylic acid method (DNS Method, Miller, 1959). The amount of total reducing sugars was the key aspect for the selection of the substrates. Total solids, volatile solid, ash content and percentage moisture were estimated by the method described in Microbiological Aspects of Anaerobic Digestion, as given below,

Total solids and Moisture

A clean and empty silica crucible was heated at 103 °C to 105 °C for an hour in a hot air oven and allowed to cool down to room temperature in desiccators and weighed. Substrate (10 g) was weighed accurately in the silica crucible and dried at 103 °C to 105 °C to a constant weight. Moisture content and total solids were calculated. The dried samples were retained for further analysis. The dried samples then were incinerated at 600 °C for 6 h and the ash content was determined.
**Total sugars**

Numerous methods have been developed to quantitatively determine the carbohydrate content of various foods, feeds and other biomass related samples. In the present study total sugars were estimated using the Anthrone method as given below.

Anthrone solution was prepared by dissolving 0.1 g of Anthrone (9, 10 – dihydro – 9-Ozanthracene) in 76% sulphuric acid to a volume of 100 ml. Glucose stock standard solution was prepared in double distilled water by dissolving 10 mg of glucose in 1 ml and making a working stock of 1 mg ml$^{-1}$. The standard stock solution was taken in aliquots of 0.5, 1.0, 1.5, 2, 2.5, corresponding to 50, 100, 150, 200 and 250 µg of glucose respectively in a set of five large test tubes. To these 4.5, 4, 3.5, 3, and 2.5 ml of distilled water was added to make up the volume to 5 ml. A reagent blank was also run along with the standards. All the tubes were transferred to an ice bath and 10 ml ice-cold anthrone reagent was added to each of the tubes gradually to avoid spurting. Afterwards the tubes were kept in boiling water bath for 16 min. During the heating, test tube mouths were closed either by keeping the test tubes caps or large marbles. The tubes were then transferred to an ice bath and allowed to stand for 10 minutes at room temperature. The blue green colour developed was read for absorbance at 620 nm of wavelength using a Spectrophotometer (U-2000, Hitachi, Japan). The samples were also treated in the same way and the total sugars were estimated using the K factor derived out of the standard curve.

**pH**

The pH of waste extract was checked using Orion pH meter after calibrating the instrument with standard pH buffers.

**Starch content (Thippi)**

Starch content of thippi was determined by acid hydrolysis method described elsewhere (Microbiological Aspects of Anaerobic Fermentation, 1986). Slurry of thippi was made in flasks by dissolving grinded thippi into 10% HCl solution. The solution was incubated at 100 °C for 3 h with shaking. After incubation, solid liquid separation was done by filtration and total reducing sugars were estimated in the hydrolysate.
Characterization of potential strain

The strains were studied for morphological characteristics by staining. Different morphological changes were studied in case of the selected indigenous strain. The culture was grown in different physical conditions like pH, temperature etc. Then the cultures were grown on M21 agar plates using thippi hydrolysate to observe their colony characteristics. Glucose of the medium was replaced by the thippi hydrolysate and salt components were added at the time of pouring the plates. After streaking plates were incubated at different temperatures and observed for colony morphology. Plates were prepared with different pH medium. For biochemical characterization, cultures were grown on different sugar media, glucose, xylose, fructose and sucrose. Sugar concentration and incubation time was optimized using these sugars.

Growth curve

The M21 medium was prepared with thippi hydrolysate medium. Medium was inoculated with the selected strain and samples were withdrawn at 4 h interval. Growth of the culture was monitored by measuring OD at 600 nm and protein concentration was estimated by Biuret method (Layne, 1957). Morphological changes were also monitored in every 4 H sample.

Strain Selection- Ethanol production potential of ethanologenic strains

The bacterial strains of Zymomonas mobilis were procured from German Microbial Culture Collection Center (DSM) and Microbial Technology Culture Collection (MTCC), Chandigarh. The two procured strains and isolated (in laboratory) strains were compared on the basis of their ethanol production potential. In M21 medium, the glucose was replaced by autoclaved and pH adjusted fruit waste extract/vegetable waste extract/thippi slurry. The medium was prepared in anaerobic bottles sealed with rubber septum. The concentration of reducing sugars in the media was adjusted to come to a final concentration of 50 g l⁻¹ using appropriate amount of fruit/vegetable waste extract or thippi slurry. The other components of the media i.e., yeast extract, glucose, magnesium chloride, ammonium sulphate and potassium dihydrogen phosphate were autoclaved separately and added at the time of inoculation. Anaerobic bottles containing media were inoculated with above mentioned strains and
incubated for a period of 96 h. The samples were withdrawn at every 24 h interval and analysed for reducing sugars and ethanol production.

**Optimization of hydrolysis process**

*Acid hydrolysis of the substrate*

**Fruit and vegetable waste**

Fruit and vegetable were shredded and were soaked in to the different concentrations of sulphuric acid (0.25%, 0.5%, 0.75%, 1.0%, 2.0%, 3.0% and 4.0% v/v) solution. The mixtures were then incubated at 37 °C and 55 °C for 3 h with shaking. Release of reducing sugars in the hydrolysate was estimated.

For further optimization, National Renewable Energy Laboratory (NREL) protocol was followed for standardizing acid hydrolysis of fruit and vegetable waste to maximize reducing sugar content. Standardization was carried out using a two-step hydrolysis process.

**1st step hydrolysis**

Fruit and vegetable waste mixture was soaked into 0.75% sulphuric acid (optimized concentration) and kept at 55 °C for 3 h. Afterwards it was heated at 150 °C for 10 min to hydrolyse the hemicellulose. This slurry was pressed to obtain liquid portion and neutralization was done by adding lime, which forms gypsum precipitate. Gypsum was then removed by solid-liquid separation.

**2nd step hydrolysis**

Remaining solid was impregnated into 0.75% H₂SO₄. This acid soaked portion was kept at 55 °C for 3 h again, then heated at 180 °C for 5-7 min, neutralized by adding lime and stream was subjected to fermentation process.

**Thippi**

Thippi pellets were grinded into small granules before processing. Standardized quantity of water was then added to the granules and the mixture was treated with steam at 121 °C and 15 lb pressure for 20 min before hydrolysis process.

Thippi pellets were ground and treated with steam before hydrolysis process. Steam pre-treated thippi were subjected to dilute acid treatment with different
Materials and Methods

strength (0.25%, 0.5%, 0.75%, 1.0%, 2.0%, 3.0% and 4.0% v/v) of sulphuric acid in water. Different concentrations of H$_2$SO$_4$ solutions were added to respective bottles of the substrate and incubated at 37 °C and 55 °C with shaking. The release of reducing sugars was estimated. NREL protocol was followed for further optimization of acid hydrolysis process. Two-step hydrolysis was done with 0.75% (v/v) H$_2$SO$_4$. Waste material was soaked into 0.75% (v/v) H$_2$SO$_4$ and incubated at 55 °C for 3 h, then, heated at 150 °C for 10 min. This slurry was pressed to separate liquid and solid. Then remaining solid was again impregnated into 0.75% (v/v) H$_2$SO$_4$ followed by incubation at 55 °C for 3 h and heating at 180 °C for 5-7 min. Lime was added to increase the pH of the liquid to 6.0. There was gypsum formation at the bottom of the hydrolysate, which was removed by solid liquid separation and liquid stream was subjected to fermentation process after reducing sugar analysis.

Alkaline hydrolysis

Fruit and vegetable wastes were soaked into the different concentrations of alkali solution as explained for acid hydrolysis procedure. Mixture was incubated at 37 °C and 55 °C with shaking. The release of reducing sugars was analysed.

Thippi pellets were steam pre-treated before hydrolysis process. Afterwards thippi slurry was subjected to hydrolysis with different alkaline concentrations (0 to 4% of NaOH) and release of reducing sugars was estimated.

Enzymatic hydrolysis

Fruit and vegetable waste

Fruit and vegetable waste were sorted and individual components of the waste were subjected to enzymatic hydrolysis. The enzymatic hydrolysis was standardised for enzyme concentration, pH, incubation temperature and time. The hydrolysis of apple, carrot, mango, sapota and pineapple was done by adding three different enzymes i.e., amylase, pectinase and xylanase.

Standardization Incubation time

A fifty gram pre-weighed substrate was incubated with different enzymes (1 ml each of the three different enzymes in different flasks). The reactions were
Materials and Methods

sampled after specified intervals of time (0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 h). The total reducing sugar released after a specific incubation time was estimated.

**Enzymatic hydrolysis of the individual waste substrates using pectinase, xylanase and amylase**

After optimization of incubation temperature, 50 g of each substrate was taken in 5 different flasks. Three such sets were made for the three enzymes. A positive control was also kept for each enzyme. The experiments were conducted in the similar manner for all the waste material: apple, pineapple, mango, carrot and sapota. Enzyme concentration of 0, 5, 10, 15 and 20 U g\(^{-1}\) was added to individual flask for each of the enzyme. The enzymes were added in citrate buffer. All the sets were incubated at 55 °C for 3 h and the release of reducing sugars was estimated.

**Scale up of the enzymatic hydrolysis of the fruit and vegetable waste**

A pre-weighed substrate of 500 g was taken in each of four different flasks and 5, 10, 15, 20 enzyme units of respective enzymes were added per gm of substrate to all the flasks. Optimum pH was adjusted and maintained throughout the process. A positive control (containing only substrate, without enzyme) was also kept and all the flasks were incubated at 55 °C for 3 h on a shaker.

**Thippi**

The thippi was then enzymatically hydrolysed using different enzymes. Optimization of enzyme concentration was done by using 5, 10, 15 and 20 U enzyme g\(^{-1}\) substrate. Thippi was hydrolyzed by adding optimized concentrations of different combinations of enzymes (5 U g\(^{-1}\) thippi). After optimization of enzyme concentration, 50 g of each substrate was taken in 5 different flasks. Two such sets were made for enzyme treatment and control. The flasks were incubated at 30 °C, 37 °C 50 °C, 55 °C and 60 °C for 3 h and the release of reducing sugars was estimated. Similar procedure was followed for all the enzymes. Then, steam pre-treated thippi was mixed with enzymes (5 U g\(^{-1}\)) at pH 5, 5.5 and 6. Reaction as incubated for 3 h and a control was kept for every pH.

After optimizing hydrolysis with individual enzymes, mixtures of enzymes were employed to treat thippi to obtain maximum available reducing sugars. Steam
pretreated thippi was weighted in different flasks and combinations of enzymes were added to those flasks. Combinations of thippi and enzymes were,

(i) Thippi + amylase,
(ii) Thippi + pectinase,
(iii) Thippi + cellulase,
(iv) Thippi + amylase + pectinase,
(v) Thippi + amylase + cellulase,
(vi) Thippi + pectinase+ cellulase,
(vii) Thippi + amylase + pectinase+ cellulase.

These combinations were incubated at optimized temperature, 55 °C for 3 h with shaking. After incubation, hydrolysate was obtained by solid-liquid separation and then reducing sugars were estimated in the hydrolysate.

Conversion efficiency of thippi into reducing sugars was standardized in different dilutions to obtain the maximum reducing sugars and hydrolysate volume. Thippi powder (100 g) was taken in five flasks and 200 ml, 300 ml, 400 ml 500 ml and 600 ml water was added to the respective flasks. Mixtures were steam pretreated and hydrolysed by using optimized amylase (5 U g⁻¹ thippi) enzyme at 55 °C for 3 h with shaking. Process was scaled up to hydrolyse 10 kg thippi. Reducing sugars released during hydrolysis were checked and conversion efficiency (thippi to reducing sugars) was calculated.

Evaluation of nutrient profile of the hydrolysate

After hydrolysis optimization studies, the hydrolysate was evaluated qualitatively as well as quantitatively. Quantitative analysis was done by reducing sugars analysis using DNS method. On the basis of reducing sugars yield, conversion efficiency was also calculated and substrates were compared. While qualitative analysis was done by High Performance Liquid Chromatograph (HPLC). Detail procedures are discussed later in the analytical methods section.

Optimization of agitation, temperature and pH by Z. mobilis MTCC 92 and TERI SH 110
Materials and Methods

Optimum ethanol production conditions were determined by standardization process, which involved M21 medium, varying pH and incubation temperatures of 30 °C and 37 °C and with shaking at 100 rpm and 200 rpm. M21 medium (prepared with thippi hydrolysate, reducing sugar was 12%) was inoculated with 6% inoculum of overnight grown culture (OD 0.6). The culture was incubated at 30 °C and 37 °C with an agitation of 100 rpm and 200 rpm. The M21 medium was set at pH 5.4, 6.0 and 6.8.

**Fermentation of agro-industrial waste**

**Fruit and vegetable waste**

Total reducing sugars were estimated in the hydrolysate. Medium (M21) was then prepared with the optimum concentration of reducing sugars. Fermentation was done in 100 ml bottles (with 50 ml head space) sealed with rubber septum and aluminum cap. Other components of the medium, MgCl$_2$, (NH$_4$)$_2$SO$_4$ and KH$_2$PO$_4$ were autoclaved separately and added aseptically. Fermentation parameters were optimized and further fermentation studies were carried out with the optimized conditions. Overnight grown cultures *Zymomonas mobilis* MTCC 92 and *Candida tropicalis* TERI SH 110 were inoculated at 6% (v/v). The fermentation was carried out at 30 °C. The samples were collected at 12 h interval. Ethanol production and reducing sugars utilization were estimated. Reproducibility of the process was checked in six repeat runs with the above conditions. For optimization of scaling up process, fermentations were done either in 100 ml anaerobic bottles, a 1 L fermentor or a 5 L fermentor (Bioflo 3000, New Brunswick Scientific).

**Thippi**

Total reducing sugars were estimated in the hydrolysate and M21 medium was prepared with optimum concentration of reducing sugars. Fermentation was performed in bottles sealed with rubber septum and aluminum cap. Other components of the medium, KH$_2$PO$_4$, MgCl$_2$ and (NH$_4$)$_2$SO$_4$ were added aseptically. Fermentation studies were carried out with the optimized conditions. Overnight grown cultures of *Zymomonas mobilis* MTCC 92 (2.8 X 10$^8$ CFU ml$^{-1}$) and *Candida tropicalis* TERI SH 110 (1.2 X 10$^7$ CFU ml$^{-1}$) were used for seeding (6% inoculum) the fermentation broth. The fermentation was carried out at 30 °C. The samples were collected at 12 h intervals; the ethanol production and reducing sugars were estimated. Fermentation studies were
done with \textit{Z. mobilis} alone, \textit{C. tropicalis} alone and mixed culture of both. Different parameters like inoculum size, agitation, (NH$_4$)$_2$SO$_4$ concentration were changed among the batches. For optimization of scaling up process, fermentations were done in 100 ml, 250 ml anaerobic bottles, 1 L fermentor and 10 L fermentor (BIOFLO 3000, New Brunswick Scientific, Edison, N J, USA). Time course experiment was also performed at 1 L and 10 L level. In first run, reducing sugars in hydrolysate were 155.25 g l$^{-1}$, 6% inoculum was used and agitation was 150 rpm. Second run contained, reducing sugars 153.78 g l$^{-1}$, inoculated with 6% culture and fermentation was carried out at 150 rpm, (NH$_4$)$_2$SO$_4$ concentration was increased to 0.5% (w/v). Then, reducing sugars in the next run were 156.51 g l$^{-1}$ and medium was inoculated with 6% culture and (NH$_4$)$_2$SO$_4$ concentration was 0.5% (w/v), agitation was increased to 200 rpm. Reproducibility of the process was checked in repeat runs with the above conditions in 1 L and 10 L fermentor and all the conditions were kept same as above.

**Sugar tolerance studies**

The selected cultures were studies for their sugar tolerance capability and it was further increased by continuous adaptation of cultures to high sugar medium. Initially, M21 medium was prepared with thippi hydrolysate to obtain up to 150 gl$^{-1}$ reducing sugars in the media and ethanol production was observed. Then, cultures were gradually shifted to high sugar medium. After several such cycles, growth and ethanol production of the cultures was observed and compared. The adapted strains were inoculated into the 180 gl$^{-1}$ media and samples were withdrawn at every 24 h and reducing sugars, ethanol and growth were estimated.

**Analytical methods**

**Microbial growth-optical density**

Growth of cultures was monitored by measuring optical density. Broth cultures were withdrawn from the fermentation reaction and optical density of cultures was measured by spectrophotometer (U-2000 Hitachi, Japan) at 600 nm. Samples were then centrifuged, cell pellet was used for protein analysis by Biuret method and supernatant was used for ethanol and sugar analysis.
Protein estimation - Biuret method

Composition of Biuret reagent is mentioned in annexure. Initially a standard curve was prepared using protein Bovine Serum Albumin (BSA) as standard. A 10 mg per ml stock solution of BSA was prepared. Different volumes of this stock were taken into the test tubes. Volume of each test tube was made 2 ml by distilled water. To each of these test tubes 1 ml Biuret reagent was added and tubes were vortexed and incubated at room temperature for 10 minutes.

Sample analysis

Bacterial cell protein was estimated to check growth of isolates in different media. 1 ml culture was removed and centrifuged at 10,000 rpm for 10 min. Pellet was used to analyse total protein. It was dissolved in 0.2 ml distilled water. An aliquot (100 µl) of dissolved pellet was taken into a test tube. 1 ml of distilled water (1.9 ml) was added to this dissolved pellet. 1 ml of Biuret reagent was added to this mixture. Reaction mixture was vortexed and incubated in dark at room temperature for 15 min. Readings were taken at 540 nm using spectrophotometer (U-2000 Hitachi, Japan).

Sugar estimation

The total reducing sugars were estimated by DNS method, modified according to National Renewable Energy Laboratory, USA. Initially a standard curve was prepared using standard glucose solution (10 mg ml⁻¹). The standard curve was prepared as per following protocol (Table 3.2).
### Table 3.2 Protocol for reducing sugars estimation followed in present study

<table>
<thead>
<tr>
<th>Glucose concentration (mg)</th>
<th>Deionized Water (ml)</th>
<th>Incubation at 50°C (min)</th>
<th>Amount of DNS added</th>
<th>Incubation at 100°C (min)</th>
<th>Dilution of reaction sample</th>
<th>OD (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>1.5</td>
<td>60</td>
<td>3.0</td>
<td>5</td>
<td>1:10</td>
<td>540</td>
</tr>
<tr>
<td>0.5</td>
<td>1.45</td>
<td>60</td>
<td>3.0</td>
<td>5</td>
<td>1:10</td>
<td>540</td>
</tr>
<tr>
<td>1.0</td>
<td>1.40</td>
<td>60</td>
<td>3.0</td>
<td>5</td>
<td>1:10</td>
<td>540</td>
</tr>
<tr>
<td>1.5</td>
<td>1.35</td>
<td>60</td>
<td>3.0</td>
<td>5</td>
<td>1:10</td>
<td>540</td>
</tr>
<tr>
<td>2.0</td>
<td>1.30</td>
<td>60</td>
<td>3.0</td>
<td>5</td>
<td>1:10</td>
<td>540</td>
</tr>
<tr>
<td>2.5</td>
<td>1.25</td>
<td>60</td>
<td>3.0</td>
<td>5</td>
<td>1:10</td>
<td>540</td>
</tr>
</tbody>
</table>

The concentration of unknown samples were calculated by using the formula $K_{1x} = \text{Abs} + \ldots$
Qualitative sugar analysis by HPLC

Preparation of mobile phase

The mobile phase was prepared by dissolving the 50 mM calcium ethylene diamine tetra acetate acid (Ca EDTA) into HPLC grade water, Qualigens (India). This was used as mobile phase for doing HPLC of sugar standards and samples. The mobile phase was filtered through 0.22µ membrane filters (Millipore Corporation, BedFord, MA).

Preparation of sugar standards

Sugar standards of glucose, sucrose fructose and xylose were prepared by using HPLC grade water, Qualigens (India). Sugars concentration used for the standards were 500, 750, 1000, 1250, 2500 and 5000 ppm. The standards were filtered through 0.22µ membrane filters (Millipore Corporation, BedFord, MA) using Swinnex mini filter assembly (Millipore Corporation, BedFord, MA).

Preparation of Samples for HPLC

Then, after acid/enzyme treatment, hydrolysate was obtained by solid-liquid separation and was centrifuged at 10,000 rpm for 10 min. pH of the acid hydrolysate was adjusted to neutral. The clear supernatant was double filtered by passing through 0.22µ membrane filters (Millipore Corporation, BedFord, USA) using Swinnex mini filter assembly (Millipore Corporation, BedFord, MA). The filtrate was dispensed in autosampler vials and mounted on the autosampler tray.

Samples analysis

The hydrolysate was analysed by HPLC (Agilent 1100 Series, Agilent Technologies) for the constituent reducing sugars, which were used for ethanol production. The sugars were analysed using HPLC fitted with Sugar-PAK I column 300 mm x 6.5 mm ID (Waters). The mobile phase constituted 50 mM Ca EDTA. The column temperature was set as 75 °C, and flow rate was kept at 0.5 ml min⁻¹. The sugars were detected by Refractive Index Detector (RID). The Hydrolysate sample (20 µl) was injected into column and samples were then analysed through settings on the software supplied by the manufacturer.
**Ethanol analysis**

The ethanol produced in the culture broth was analyzed with Gas chromatography (GC) by following methods;

1 ml of the culture broth was taken in 1.5 ml eppendorf tube from the actively growing culture medium at regular intervals of 24 h for a period of 120 h. The culture broth was centrifuged at 10,000 rpm for 5 min. The supernatant was again filtered through sterile 0.22 µ membrane filter. The filtrate was analysed by using Nucon GC. One micro litre of the filtrate was injected into the GC fitted with chromosorb-101 column at the chromatography conditions of 155 °C oven temperature, 175 °C injector temperature and 250°C detector temperature with nitrogen as carrier gas with a flow rate of 30 ml min⁻¹. An internal standard of isopropanol was used in the making of a standard curve along with 99% ethanol for estimating the ethanol concentration in the culture broth. Then, ethanol estimation was further standardised by following method;

Standardization of ethanol analysis was carried out with DB Wax ETR (60 mt x 0.32 mm) J & W 123-7364 USA column by Agilent Technologies 6890 N, USA gas chromatograph. 1 ml fermentation broth was collected and centrifuged at 10,000 rpm for 10 min, filtered with 0.22µ filter. Approximately 550 µl of the filtrate was added to each of the autosampler vial and the vials were mounted on to the autosampler. An injection split ratio of 1:5 was given. The oven temperature was set at 40°C for 5 minutes and a ramp of 10 °C per minute to 230 °C and 5 minute hold at 230 °C for oven. The injector temperature was 250°C and the detector temperature was 250 °C. The flow rate of helium mobile phase was kept at 5 ml per minute. Calibration curve was made as given above. Both qualitative and quantitative analysis was carried out using this method.
Statistical Analysis

The data generated during the study was processed using various statistical tests.

Data characteristics and Analysis of Variance

The data characteristics such as Mean, Standard deviation, range, etc. were determined. Analysis of variance (ANOVA) was used to test the hypothesis that several means are equal. This procedure is an extension of the two-sample ‘t’ test technique. In addition to determining the existence of differences among the means, the data was further processed to specifically check, which means differ, following the post hoc tests (as the post hoc tests are run after the experiment has been conducted).

Significance Level

The significance level was chosen to be 0.05 (or equivalently, 5%) by keeping in view the consequences of such an error. That is, we want to make the significance level as small as possible in order to protect the null hypothesis and to prevent, as far as possible, from inadvertently arriving at false conclusions.