Materials and Methods
CHAPTER III

MATERIALS AND METHODS

3.1. GENERAL

In this chapter the materials and methods pertaining to the study on “Ethanol production from cheese whey supplemented with different substrates using different yeast species” are described in details:

3.1.1. Location

The experiments were conducted at the Faculty of Agriculture and Animal Husbandry, Gandhigram Rural Institute (Deemed University), Gandhigram, Dindigul District, Tamil Nadu.

3.1.2. Chemicals

The analytical reagent grade of chemicals and solvents of S.D. Fine, Reachem and Hi-media were used in the experiments. The rennet for the production of cheese was obtained from Dindigul Essence, Dindigul.

3.1.3. Safety Measures and Decontamination Methods Followed

As sulphuric acid is a toxic compound, protective clothing, hand gloves and safety goggles were used while handling the sulphuric acid. All the microbiological experiments were carried out in a fame hood and distilled 90 percent aqueous ethanol was used for disinfecting the hands.

3.1.4. Water

Distilled water for the routine experimental works was obtained from glass distillation unit.

3.1.5. Glassware

The glasswares were cleaned with commercially available soap solution (Clextran MA 02 Neutral) and then thoroughly washed with water. The glasswares thus cleaned were rinsed with double distilled water. All the glasswares were sterilized in hot air oven.
3.1.6. Authenticity of the Yeast Species Used

*Kluyveromyces marxianus* species MTCC 242, *Saccharomyces cerevisiae* MTCC 178 and *Achaetomiella fusispora* MTCC 1288 were procured from the Culture Collection Centre of the Institute of Microbial Technology (MTCC) at Chandigarh, India. *Kluyveromyces marxianus* species MTCC 242 and *Saccharomyces cerevisiae* MTCC 178 were maintained in Yeast Peptone Dextrose (YPD), while, *Achaetomiella fusispora* MTCC 1288 was maintained in Commeal medium and composition of these media are given below:

**Yeast Peptone Dextrose**

The Yeast Peptone Dextrose media was prepared as described by MTCC for *Kluyveromyces marxianus* species MTCC 242

Lactose 20 g
Bacto peptone 10 g
Malt extract 3 g
Yeast extract 5 g
Agar 20 g
Distilled water 1000 ml

**Yeast Peptone Dextrose**

The Yeast Peptone Dextrose media was prepared as described by MTCC for *Saccharomyces cerevisiae* species MTCC 178

Yeast extract 3 g
Peptone 10 g
Dextrose 20 g
Agar 15 g
Distilled water 1000 ml

The slant cultures of the above yeast species were incubated at 30°C for 48 h and preserved at 4°C for further uses.
**Corn Meal medium**

The Corn meal media was prepared as described by MTCC for *Achaetomiella fusispora* MTCC 1288

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commeal</td>
<td>30 g</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

The slants of the *Achatomiella fusispora* MTCC 1288 were incubated at 40°C for 72 h and preserved at 4°C for further uses. The colony morphology of *Kluyveromyces marxianus* species MTCC 242, *Saccharomyces cerevisiae* MTCC 178 and *Achatomiella fusispora* MTCC 1288 were shown in Plates 1, 2 and 3 respectively.

### 3.1.7 Sub-Culturing of Yeast culture

The *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* were sub-cultured from mother slants into 100 ml of YPD medium (Zafar and Owais, 2006) incubated at 30°C for 48H. Similarly, *Achaetomiella fusispora* yeast was sub-cultured in Commeal medium incubated at 40°C for 72 H. These cultures were used for further experiments and showed in plate 4.

### 3.1.8 Standardization of Yeast Volume

In our experiments 10 ml of yeast culture viz, *Kluyveromyces marxianus* species MTCC 242, and *Saccharomyces cerevisiae* MTCC 178 are containing the $3 \times 10^4$ cfu/ml, *Achaetomiella fusispora* MTCC 1288 containing the $2 \times 10^4$ cfu/ml (colony forming unit per millilitre). The dose of yeast culture was chosen at 10 ml based on the
Plate No. 1

A view of Klyveromyces marxianus
Plate No. 2

A view of *Saccharomyces cerevisiae*
Plate No. 3

A view of Achaetomiella fusispora
Plate No. 4

A view of sub cultures
Plate No. 5

A view of Cheese whey sample
Plate No. 6

A view of Molasses sample
Plate No. 7

A view of Sapota sample
Plate No. 8

A view of Sweet sorghum sample
previous report of Ozmichi and Kargi (2007) and various other researchers’ reports quoted by the same authors.

3.2 Substrates

The various substrates used in the present study includes Cheese whey, Pure lactose, Molasses, Sapota and Sweet sorghum and showed in Plates 5 to 8.

3.2.1. Procedure for Obtaining Cheese Whey from Milk

Cheese whey was obtained as a by product during the preparation of cheese as described by Sukumar Be (2005). For this purpose one litre of milk was heated to 80°C for a few seconds. The milk was cooled to 30° C. To this milk, 5 ml of butter milk (starter culture) was added and left for 30 minutes to increase the acidity. Subsequently, 5 ml of rennet was added and left for 30 minutes. The cheese whey was obtained by cutting the curd in length wise and then it was heated up to 44° C. The whey was drained off using muslin cloth and collected in a separate vessel. The various steps involved in the production of cheese whey at laboratory level are shown in Fig 1.

3.2.2. Collection of Sugar Supplements (Lactose, Molasses, Sapota and Sweet Sorghum)

Cheese whey produced in our laboratory contained 4.5% of lactose, 0.05 % fat, and 0.52 % protein and trace amount of ash. Pure lactose containing 100% of lactose was procured from the S.D. Fine chemicals, Mumbai. Molasses was collected from M/s. Rajashree Sugar Industries, Periyakulam, Theni District, Tamilnadu, India. The sample used in the present study, the molasses contained 46.0% of total sugar. This was stored in sterile brown colour bottle and kept at 4°C for further studies.
FIG. 1 FLOW CHART FOR THE PREPARATION OF CHEESE

Boiling milk at 80°C for few seconds

Cooling (32-35°C)

Adding butter milk and ripening for 30 min

Renneting

Cutting the curd

Cooking (40 - 44°C)

Draining of cheese whey
Damaged and waste sapota fruits were collected from the local market at Gandhigram. The fruits were cut open and the seeds and white crystal salt present inside the sapota fruits were removed physically. The remaining contents were crushed nicely using mixer grinder and this preparation was used for further experiment. Sapota fruit extract thus prepared contained 13.5% total sugars.

Sweet sorghum stalks were collected from the Tamilnadu Agricultural University, Coimbatore, Tamil Nadu. The stalk was stripped off and outer leaf covering was removed, then it was chopped in to very small pieces and crushed nicely using mixer grinder and this preparation was used for further experiment. The mixture thus prepared sweet sorghum contained 15% sucrose.

3.2.3 Standardization of Whey

The freshly prepared cheese whey was analysed to determine its lactose content. The lactose content was standardized to 4.5% either by addition of pure lactose or by the addition of distilled water.

3.3. Estimation of Lactose Level in Cheese Whey (Lane Eyon, 1981)

Transfer about twenty five ml of cheese whey to a 250 ml Erlenmeyer conical flask, heated near to boiling and cooled to room temperature. To this, 200 ml distilled water was added and then 3.75 ml of 10% acetic acid was added drop by drop to precipitate the non-carbohydrate compounds such as fat and protein and allowed to settle. The supernatant fluid containing lactose was decanted through WhatMan no 42 filter paper and collected. Fifty ml of this filtrate was transferred to a Burette.

Commercially available Fehling’s solution of 5ml of both A and B were taken in a 250 ml Erlenmeyer conical flask. To this 25 ml of
distilled water was added. This Fehling’s solutions were titrated with 1 ml of filtrate in the Burette and heated at 80°C for a few seconds. This process was repeated till the Fehling’s solution turned from blue to light green. Five drops of Methylene blue was added to the Fehling’s solution and titrated with 1 ml of filtrate in the Burette and heated at 80° C for a few seconds. This process was repeated till the Fehling’s solution turned from blue to red. The lactose level in the cheese whey was calculated following the formula shown below:

\[
\text{Lactose (\%)} = \frac{67.8}{\text{burette reading}} 
\]

3.4. Estimation of Total Sugar in Other Substrates (Somogy and Nelson, 1945)

Sample one gram was treated with hot aqueous ethanol (80%) twice (5ml each time) to extract the sugars. This suspension was centrifuged at 5,000 rpm for 10 min and the supernatant was evaporated by keeping it on a water bath at 80°C. Distilled water 10ml was added to the residue to dissolve the sugars. From this, 0.2 ml of solution was pipetted out to a clean test tube and the volume was made up to 2 ml with distilled water. To this, 1 ml of alkaline copper tartrate reagent was added and incubated in boiling water bath 10 minutes. After cooling the test tubes, 1 ml of arsénomolybodic acid reagent was added and the volume was made up to 10 ml with distilled water. The absorbance of this reaction mixture was red at 620 nm (Elicomake UV-VIS Spectrophotometer model no. SL159) against the blank solution after 10 minutes of adding the distilled water. The sugar concentration in the samples was calculated from the standard calibration curve.
Plate No. 9

A view of distillation process for ethanol production
3.5. Biomass Estimation (Zafar and Owasis, 2006)

Biomass was estimated in terms of dry weight. Samples of ten ml were centrifuged for 15 min at 5,000 rpm. The cell pellet was dried at 100°C for 24 h and then weighed.

3.6. Estimation of Ethanol in Culture Supernatant (William and Reese, 1950)

One millilitre of clarified supernatant sample was added to 24 ml of distilled water in a 250 ml capacity distillation flask and distilled at 78°C. The distillate was collected in a flask containing 25 ml of 3.4% (w/v) chromic acid. The final volume was made up to 50 ml with distilled water, mixed thoroughly and kept in a water bath at 80°C for 15 min. The absorbance was red at 600 nm (Elico make UV-VIS Spectrophotometer model no. SL159). The ethanol concentration was calculated from a standard graph of absorbance versus ethanol concentrations ranging from 1 to 5 %. The ethanol concentration was expressed as percentage. The process of ethanol in distillation unit shown plate 9.

3.6.1. Preparation of chromic acid reagent

The reagent was prepared by dissolving 34 g of K$_2$Cr$_2$O$_7$ in 350 ml of distilled water. This solution was kept in an ice bath and 325 ml of conc. H$_2$SO$_4$ was slowly added to it. After cooling, the volume was made up to 1 litre with distilled water and the reagent was stored in brown bottle.

3.7. Estimation of Reducing Sugar (Dubois et al., 1956)

Sample of 10 ml was taken after 72 h of incubation and the biomass was separated by centrifugation at 5,000 RPM for 10 min. 0.1 ml of the clarified supernatant solution was made up to 1 ml with distilled water. Phenol 10% (w/v) of 1 ml and 3 ml of concentrated sulphuric acid were carefully added along the sides of the tube, mixed and incubated at
room temperature for 15 min. The absorbance was read at 600 nm (Elico make UV-VIS Spectrophotometer model no. SL159) against the blank. The level of reducing sugar was expressed as %.

### 3.8 Formulae for Calculating the Ethanol Yield Co-Efficient, Fermentation Efficiency and Conversion Efficiency

**3.8.1 Ethanol yield coefficient**

\[
\text{Ethanol yield coefficient} = \frac{\text{Gram of ethanol produced}}{\text{Gram of substrate consumed}}
\]

**3.8.2 The fermentation efficiency (%)**

\[
\text{Fermentation efficiency} = \left( \frac{\text{Ethanol produced}}{\text{Theoretical maximum ethanol yield from sugar}} \right) \times 100
\]

(Theoretical maximum ethanol yield = 0.54 g ethanol per gram of lactose).

**3.8.3 Conversion efficiency (%)**

\[
\text{Conversion efficiency} = \left( \frac{\text{Initial sugar - residual sugar}}{\text{Initial sugar}} \right) \times 100
\]

**3.9. Procedure for Enrichment of Cheese Whey with Lactose, Molasses, Sapota and Sweet Sorghum**

Cheese whey of 100ml was transferred to a 250 ml of Erlenmeyer conical flask and to this different level of lactose, molasses (1, 2, 3 and
4g/100 ml) sapota and sweet sorghum extract (5, 10, 15 and 20g/100 ml) respectively were added. It was mixed thoroughly and sterilized by autoclaving.

3.9.1. Inoculation of Yeast Species

To 100 ml of sterilized cheese whey enriched with different levels of lactose, molasses, sapota and sweet sorghum with respective substrates, 10 ml of the respective yeast culture *Kluyveromyces marxianus* (3×10⁴cfu/ml), *Saccharomyces cerevisiae* (3×10⁴cfu /ml) and *Achaetomiella fusispora* (2×10⁴cfu/ml) of freshly prepared yeast species was inoculated and incubated at 30⁰C for 72 H. After incubating for 72 H, the ethanol production, reducing sugar, biomass, ethanol yield coefficient, fermentation efficiency and conversion efficiency were determined following the procedures/ formulae described in sections 3.5., 3.6, 3.7, 3.8.1,3.8.2 and 3.8.3 respectively.

3.10 Experimental Protocols

3.10.1 Optimization of pH

Cheese whey of 100ml as control and 100 ml of cheese whey supplemented with lactose, molasses, sapota and sweet sorghum at the rate of 1 gram of respective substrates were adjusted to pH 4, 5 and 6 by using sodium thio-glycolate (98%). The pH adjusted medium was sterilized by autoclaving at 15 psi for 20 min. The autoclaved media were inoculated with 10 ml of the respective culture *Kluyveromyces marxianus* (3×10⁴/ml), *Saccharomyces cerevisiae* (3×10⁴/ml) and *Achaetomiella fusispora* (2×10⁴/ml) and incubated at 30⁰C for 72 H. After incubation for 72 H, ten ml of culture was centrifuged for 15 min. at 5,000 rpm and the supernatant was used for estimating ethanol and reducing sugar. The ethanol production, biomass and reducing sugar were determined
following the procedures described in sections 3.5, 3.6 And 3.7 respectively.

Treatments details

$T_1$ - pH 4
$T_2$ - pH 5
$T_3$ - pH 6

Design: RBD; Replications: 7/ treatments.

3.10.2. Optimization of Temperature

Cheese whey of 100ml as control and 100 ml of cheese whey enriched with lactose, molasses, sapota and sweet sorghum at the rate of 1 g of respective substrates were adjusted to pH 5 by using sodium thiglycolate (98%). This medium was sterilized by autoclaving at 15 psi for 20 min. The autoclaved media were inoculated with 10 ml of the respective culture *Kluyveromyces marxianus* ($3 \times 10^4$/ml), *Saccharomyces cerevisiae* ($3 \times 10^4$/ml) and *Achaetomiella fusiispora* ($2 \times 10^4$/ml) and incubated at different temperature regimes $25^0$C, $30^0$C and $35^0$C for 72 h. Ten ml of culture was centrifuged for 15 min at 5,000 rpm and the supernatant was used for estimating ethanol and reducing sugar. The biomass, reducing sugar and ethanol were determined following the procedures described in sections 3.5, 3.6 And 3.7 respectively.

Treatments details

$T_4$ - $25^0$C
$T_5$ - $30^0$C
$T_6$ - $35^0$C

Design: RBD

Replications: 7/ treatments

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3.10.3. Optimization of Incubation Time

Cheese whey of 100ml as control and 100 ml of cheese whey enriched with lactose, molasses, sapota and sweet sorghum at the rate of 1 g of respective substrates were adjusted to pH 5 by using sodium thiglycolate (98%). This medium was sterilized by autoclaving at 15 psi for 20 min. The autoclaved media were inoculated with 10 ml of the respective culture Kluyveromyces marxianus (3×10^4 cfu/ml), Saccharomyces cerevisiae (3×10^4 cfu /ml) and Achaetomiella fusiopora (2×10^4 cfu /ml) and incubated for 24, 48, 72 and 96 h at 30°C. Culture of 10ml was centrifuged for 15 min at 5,000 rpm and the supernatant was used for estimating ethanol and reducing sugar. The biomass, reducing sugar and ethanol were determined following the procedures described in sections 3.5, 3.6 and 3.7 respectively.

Treatments details

T_7 - 24 h
T_8 - 48 h
T_9 - 72 h
T_10 - 96 h

Design: RBD
Replications: 6/ treatments

3.10.4 Effect of Cheese Whey

Cheese whey of 100ml was adjusted to pH 5 by using sodium thiglycolate (98%). This medium was sterilized by autoclaving at 15 psi for 20 min. The autoclaved media were inoculated with 10 ml of the respective culture Kluyveromyces marxianus (3×10^4 cfu/ml), Saccharomyces cerevisiae (3×10^4 cfu /ml ) and Achaetomiella fusiopora (2×10^4 cfu /ml) and incubated at 30°C for 72 h. Sample of 10 was centrifuged for 15 min at 5,000 rpm and the supernatant was used for
estimating ethanol production, reducing sugar, the biomass, ethanol yield co-efficient, fermentation efficiency and conversion efficiency were determined following the procedures/formulae described in sections 3.5, 3.6, 3.7 3.8.1,3.8.2 and 3.8.3 respectively

**Treatments details**

T<sub>1</sub> . 100 ml of cheese whey + *Kluyveromyces marxianus*

T<sub>12</sub> . 100 ml of cheese whey + *Saccharomyces cerevisiae*

T<sub>13</sub> . 100 ml of cheese whey + *Achaetomiella fusiopora*

Design : RBD

Replications : 7/ treatments

**3.10.5. Effect of Different Levels of Lactose, Molasses, Sapota and Sweet Sorghum on Ethanol Production by *Kluyveromyces marxianus, Saccharomyces cerevisiae* And *Achaetomiella fusiopora.***

Cheese whey of 100 ml enriched with lactose and molasses at the rate of 1, 2, 3 and 4 g however sapota and sweet sorghum at the rate of 5g, 10g, 15g and 20g of the respective substrates were adjusted to pH 5 by using sodium thio-glycolate (98%). This medium was sterilized by autoclaving at 15 psi for 20 min. The autoclaved media were inoculated with 10 ml of the respective culture *Kluyveromyces marxianus* (3×10<sup>6</sup>cfu/ml), *Saccharomyces cerevisiae* (3×10<sup>6</sup>cfu/ml) and *Achaetomiella fusiopora* (2×10<sup>6</sup> cfu/ml) and incubated at 30°C for 72 H. Sample of 10 ml was centrifuged for 15 min at 5,000 rpm and the supernatant was used for estimating ethanol production, reducing sugar, the biomass, ethanol yield co-efficient, fermentation efficiency and conversion efficiency were determined following the procedures/formulae described in sections 3.5, 3.6, 3.7, 3.8.1,3.8.2 and 3.8.3, respectively.
Treatments details

$T_{14} - 100$ ml of cheese whey + 1 g lactose
$T_{15} - 100$ ml of cheese whey + 2 g lactose
$T_{16} - 100$ ml of cheese whey + 3 g lactose
$T_{17} - 100$ ml of cheese whey + 4 g lactose

Design : RBD
Replications : 6/ treatments

Treatments details

$T_{18} - 100$ ml of cheese whey + 1 g molasses
$T_{19} - 100$ ml of cheese whey + 2 g molasses
$T_{20} - 100$ ml of cheese whey + 3 g molasses
$T_{21} - 100$ ml of cheese whey + 4 g molasses

Design : RBD
Replications : 6/ treatments

Treatments details

$T_{22} - 100$ ml of cheese whey + 5 g sapota
$T_{23} - 100$ ml of cheese whey + 10 g sapota
$T_{24} - 100$ ml of cheese whey + 15 g sapota
$T_{25} - 100$ ml of cheese whey + 20 g sapota

Design : RBD
Replications : 6/ treatments

Treatments details

$T_{26} - 100$ ml of cheese whey + 5 g sweet sorghum
$T_{27} - 100$ ml of cheese whey + 10 g sweet sorghum
$T_{28} - 100$ ml of cheese whey + 15 g sweet sorghum
$T_{29} - 100$ ml of cheese whey + 20 g sweet sorghum

Design : RBD
Replications : 6/ treatments
3.10.6 Combined Effect of *Kluyveromyces marxianus*, *Saccharomyces cerevisiae* and *Achaetomiella fusiopora* on Ethanol Production in Different Substrates.

Cheese whey of 100ml as control and 100ml of cheese whey enriched with lactose and molasses at the rate of 4 g and 100 ml of cheese whey enriched with sapota and sweet sorghum at the rate of 20g were adjusted to pH 5 by using sodium thio-glycolate (98%). This medium was sterilized by autoclaving at 15 psi for 20 min. The autoclaved media were inoculated with 10 ml of mixed culture and incubated at, 30°C for 72 hrs. Culture of 10 ml was centrifuged for 15 min at 5,000 rpm and the supernatant was used for estimating ethanol and reducing sugar. The biomass, reducing sugar, ethanol, ethanol yield co-efficient, fermentation efficiency and conversion efficiency were determined following the procedures/ formulae described in sections 3.5, 3.6, 3.7 3.8.1,3.8.2 and 3.8.3 respectively.

**Treatments details**

- **T₃₀** – 100 ml cheese whey
- **T₃₁** – 100 ml cheese whey + 4 g molasses
- **T₃₂** – 100 ml cheese whey + 4 g lactose
- **T₃₃** – 100 ml cheese whey + 20 g sapota
- **T₃₄** – 100 ml cheese whey + 20 g sweet sorghum

**Design** : RBD

**Replication** : 6/ treatments

3.11. COST ANALYSIS

The cost of production of ethanol from cheese whey and cheese whey supplemented with sugar supplements were worked out.
3.12. STATISTICAL ANALYSIS

The statistical analyses of the data were done based on the procedures and formulae given by Panse and Sukhatme (1985).