CHAPTER 3
MATERIALS AND METHODS

3.1 GENERAL

The experimental studies of the thesis work were grouped under two broad headings. (1) Microbial analysis of the dairy activated sludge and (2) Zone settling experiments on dairy activated sludge. With the result of these studies a model was developed for the design of secondary clarifier optimizing the efficiency of the clarifier in the activated sludge process. Process control parameters were also established to facilitate the designers and plant operators to adopt immediate control measures in case of the sudden variations in operating parameters and bring back the normal operational condition of the activated sludge system.

3.2 DAIRY WASTE SAMPLES AND ACTIVATED SLUDGE

The raw dairy wastewater samples were collected from the milk processing industry, Coimbatore District Milk producer’s Union Limited, Coimbatore. The samples of 2 liters were collected at intervals of one hour for a sampling period of 5 hrs from the collection well of the treatment plant located in the Industry. All such samples were mixed at the end of the sampling period and collected in a single container. This composite sample was then stored in the refrigerator at 4°C, until the study was carried out. However for the microbial study, in order to identify the predominant bacteria, the samples were collected daily for a period of 10 days. The
activated sludge for seeding was collected from the recycle line, which carried the sludge from the secondary clarifier to the aeration tank in the treatment system of the same dairy Industry.

### 3.3 SOURCES AND QUANTITY OF WASTEWATER IN THE DAIRY INDUSTRY

The industry is collecting about 2,50,000 liters of milk daily, out of which 1,50,000 liters processed milk are sold to the market. The excess milk received is processed for milk powder, ghee, butter and bottled milk manufacturing. The dairy industry generates on an average of 6 liters of wastewater for every liter of milk that is handled. The waste in the Industry originate from reception tog, dump tank, chiller unit, and pasteurization unit, homogenizing Unit, butter plant, pre melting unit, and ghee converter unit. A large quantity of wash water, join as wastewater through cleaning of vessels, cans, bottles, equipments and plant’s floors.

![Wastewater flow diagram](image)

**Figure 3.1 Typical flow diagram of waste generation in dairy process**

At the reception tog, the milk is received and empty cans are rinsed, washed, sterilized and are returned to the farmers. At the dump tank, the raw
milk delivered by the receiving station is stored. The processing includes, chilling, pasteurization, homogenizing and bottling (Figure 3.1).

In the above two sections, the liquid waste originates out of rinsing and washing of bottles, cans and equipments and thus contains milk drippings, chemicals and detergents used for cleaning them.

In the creamery process, the whole milk is preheated to about 30°C to separate the cream from milk. In the butter plant, the cream is pasteurized and ripened with a selected acid and a bacterial culture. This is then churned at a temperature of about 7-10°C, to produce butter granules. On consolidation of the butter, the butter milk is drained out of the churn and the butter is washed and after standardization, it is packed for sale. While cleaning the churns, small quantity of butter and wash water with butter milk is let out. In addition to all the above, some quantity of cooling water comes as waste that is re-circulated. The nature and composition of wastewater also depend on the type of products produced and the size of the plants.

3.4 WASTEWATER CHARACTERIZATION

The characteristics of raw dairy wastewater, was analyzed as per Standard methods for the examination of water and wastewater (APHA 2005) in order to obtain the information regarding the general characteristics of the wastewater. The following tests were performed: pH, chlorides, sulphates, BOD, COD, total solids and suspended solids. The results are tabulated in Table 4.1 in chapter 4.

3.5 THE MICROBIAL ANALYSIS OF DAIRY WASTEWATER

The microbial analysis comprised of 1) Isolating and identifying the predominant bacteria present in raw dairy wastewater. 2) Assessing the
treatment efficiencies with native inoculum of bacterial isolates in the dairy wastewater. 3) Identifying the extracellular enzymes produced by the isolated predominant biodegrading bacteria. 4) Standardizing the optimum parameters for the maximum enzyme production. 5) Analyzing the causes of bulking in the dairy activated sludge. 6) Isolating and identifying the filamentous bacteria present in the dairy activated sludge.

3.5.1 Isolation of Bacteria from the Mixed Microbial Population

The sample collected from dairy industry was initially subjected to the serial dilution; down to $10^{-9}$ level (1 ml of waste water was diluted in the 9 ml of the sterile distilled water to get the dilution of $10^{-1}$). From this diluted sample 1 ml was plated on Nutrient agar media (Composition :- Peptone – 5.0 g, beef extract – 3.0 g, Sodium chloride – 5.0 g, Agar – 15.0 g, and distilled water – 100 ml) by spread plate method to obtain bacterial colonies (Figure 3.2).

![Figure 3.2 Serial dilutions for isolation of bacteria](image)

3.5.2 Isolation of Predominant Bacteria Present in the Wastewater

A loop full of the isolated bacterial colony was plated on the Macconkey agar media (Composition :- Peptone – 1.7 g, Protease Peptone –
0.3 g, Lactose – 1.0 g, bile salts – 0.15 g, Sodium chloride – 0.5 g, Neutral red – 0.0003 g, Crystal violet – trace, and Agar – 1.5 g.) prepared with prescribed ingredients dissolved in distilled water and sterilised in autoclave for 20 min. The lactose and non lactose fermenting organisms were clearly differentiated in the media. The morphological characteristics were studied with phase microscope and the results of series of bio chemical tests, compared with Bergy’s manual, confirmed the predominant bacteria as Bacillus sp. and Pseudomonas. 1 ml of suspended Bacillus sp solution was inoculated to 250 ml of sterile nutrient broth and shaked for 24 – 48 hrs at 30°C. For inoculum preparation, slant tubes were prepared with each strain and incubated at 37°C for 48 h. The Isolated bacteria were then checked for their proteolytic activity.

3.5.3 Proteolytic Activity Assays

For proteolytic activity assays, bacteria were grown on M₁ medium plates (Composition :- Tryptone 5.0 g, Yeast extract – 2.5 g, Glucose – 1.0 g, Skim milk – 20 ml, agar – 15 g, distilled water – 980 ml) and incubated at 30°C for 96 hrs. Colonies of microorganisms causing clearance from the plate medium were regarded as protease producers.

3.5.4 Biodegradation with Bacillus.sp

Batch tests were conducted in 500 ml Erlenmeyer flasks, containing 120 ml of the collected sample. Different concentrations of the prepared inoculum of the isolated Bacillus.sp varying from $1 \times 10^6$ CFU / ml to $5 \times 10^6$ CFU / ml were added in the test samples separately and incubated in the orbital shaker with a rotation of 120 rpm at 30°C. The growth of the bacteria in terms of optical density, the COD and the protein of the samples under incubation were measured periodically. The test was repeated with a commercial inoculum of Bacillus.sp (obtained from Bio Research Centre, Tamil Nadu Agricultural University, Coimbatore) in a separate flask for comparison.
3.5.5 Estimation of Protein Removal by Lowry’s method

The phenolic group of tyrosine residues present in the protein produced a blue – purple complex with a maximum absorption at 600 nm with folin – ciocalteu reagent which consisted of sodium tungstate, molybdate and phosphate.

The following reagents were used in this method.

- Solution A – 2% of sodium carbonate in 0.1N sodium hydroxide.
- Solution B – 0.5% of copper sulphate solution in 1% sodium potassium tartarate solution.
- Solution C - Fresh mixture of 50 ml of solution A and 1 ml of solution B.
- Folin – ciocalteau reagent was prepared freshly, before usage by mixing commercial reagent and distilled water in the ratio 1:1.
- Stock solution was prepared by dissolving 100 mg of Bovine serum albumin in a few ml of distilled water and made up to 100 ml with the same in a standard flask.
- Working standard was then prepared by diluting 10 ml of the stock solution to 100 ml in a standard flask with distilled water.

The following procedure was adopted to estimate the protein degradation capacity of the bacteria.

0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 ml of working standard protein solutions were taken in a series of test tubes. The volumes were corrected up
to 1 ml in each tube with distilled water. 1.0 ml of distilled water was taken as blank reagent solution. 0.4 ml of unknown protein solution was made up to 1 ml with distilled water. 5 ml of alkaline copper reagent was added in all the tubes and allowed to stand for 10 minutes. 0.5 ml of freshly prepared Folin – ciocalteu reagent was added to the above mixture and allowed to stand at room temperature for 30 min. The optical density was observed in a colourimeter at 600 nm. A standard graph was prepared by taking the concentration of protein in X- axis and optical density in Y- axis. From this standard graph, the amount of protein present in the samples were calculated, which directly represented the amount of protein degraded from the samples.

3.5.6 Treatment with Bacillus sp and Pseudomonas

The bacterial isolates showing positive for the production of enzymes were selected and used in the treatment of dairy effluent. The bacterial isolates of Bacillus sp and pseudomonas were inoculated together into the dairy effluent with $2.4 \times 10^9$ CFU/ml and $2.7 \times 10^9$ CFU/ml respectively and incubated at 37 °C for 10 days at 120 – 130 rpm. Aliquots of the samples were removed after every 24 hrs and analyzed for the physicochemical characteristics of the effluent. The effluent samples were analyzed for their variation in the pH, total dissolved solids (TDS), hardness, chlorides and Chemical Oxygen Demand (COD).

3.5.7 Measurement of Growth Rate of Bacteria

Growth refers to both increase in population and enlargement of the sizes of microorganisms. Throughout the running of the reactor until attaining steady state, the optical density of the mixed liquor was found on a daily basis and the growth pattern of the microbial culture was plotted.
The mixed liquor sample was taken from the reactor. In a test tube the growth medium called the nutrient broth (5ml) was added. And then to the broth 1ml of sample was added. Three such tubes were prepared. All the tubes were cotton plugged and kept in a shaker for 24 hrs at a temperature of 37°C. After 24 hrs the test tubes were taken out and tested in a colorimeter at 520 nm and the optical density (OD) was noted. The OD readings were tabulated and a graph was plotted for the OD variations against time in days. Four recognizable phases were seen when the increase in cell number was determined in relation with time viz. Lag phase, log phase, Stationary phase and Death phase.

3.5.8  **Estimation of Enzyme Production**

Enzymes speed up the rate of hydrolysis of complex organic compounds and the rate of oxidation of simple compounds. The activities of enzymes are substantially affected by the parameters viz. pH, temperature and cell age. The optimal values of these parameters vary from enzyme to enzyme and hence these parameters have to be carefully worked out. The identified organisms were screened for their ability to produce extracellular enzymes mainly, cellulase, protease, lipase and amylase. Further, the optimum enzymatic activities were also standardized for the efficient microbial consortium, effective pH, temperature, incubation time, etc. for the treatment of dairy effluent

3.5.8.1  **Protease**

Isolates were streaked on gelatin – enriched nutrient agar medium. After 24 hrs of incubation at 37 °C, the plates were flooded with 10% mercuric chloride reagent. A zone of clearance around the colony indicated protease positive isolate. The isolates were inoculated into gelatin enriched nutrient broth, incubated at 37°C for 24 – 48 hrs. After incubation, the culture
broth was centrifuged at 3,000 rpm for 15 min. The crude enzyme supernatant was used for determining the activity of protease enzyme by the method described by Lowry et al (1951).

### 3.5.8.2 Lipase

The bacterial isolates were inoculated on sterile tween 20 – supplemented nutrient agar plates and incubated at 37 °C for 18 – 24 hrs. A clear zone of lipolysis around the colony indicated the production of lipase enzyme by the bacterial isolate. The isolates were inoculated into tween 20-supplemented nutrient broth, incubated at 37 °C for 24 – 48 hrs. After incubation, the culture broth was centrifuged at 3000 rpm for 15 min. The crude enzyme supernatant was assayed according to the method of Mustranta (1992) to determine the enzyme activity.

### 3.5.8.3 Amylase

Starch – enriched agar medium (1% starch) plates were prepared, inoculated with the isolates and incubated at 37 °C for 24 hrs. Lugol’s iodine solution (Lugol’s iodine: distilled water – 1:5) was flooded over the plates. A zone of clearance around the colony indicated production of amylase. The isolates were inoculated into starch enriched nutrient broth, incubated at 37 °C for 24 – 48 hrs. After incubation, the culture broth was centrifuged at 3,000 rpm for 15 min. The crude enzyme supernatant obtained after centrifugation was assayed for enzyme activity as described in DNS method.

### 3.5.8.4 Cellulase

Carboxy methyl cellulose enriched agar medium plates (composition: Yeast extract - 2.0 g/l, Carboxy Methyl cellulose - 5.0 g/l, Sodium Nitrate - 0.5/l, Dipotassium hydrogen phosphate - 10 g/l, Magnesium
sulphate - 0.5 g/l, Ferrous sulphate - 0.1 g/l, Potassium chloride - 0.5 g/l, Agar-15g/l) were prepared, sterilized, inoculated with the bacterial isolates and incubated at 37°C for 24 - 48 hrs. After incubation, the plates were flooded with 1% congo red solution for 15 min and washed with 1M NaCl for 15 – 30 min. A zone of clearance around the bacterial colonies indicated the production of cellulase enzyme. The isolates were inoculated into CMC broth, incubated at 37 °C for 24 – 48 hrs. After incubation, the culture broth was centrifuged at 3,000 rpm for 15 min. The crude enzyme supernatant was assayed according to the method of Sadasivam (1997).

3.5.9 Standardization of Enzyme Production System

After screening for the extracellular enzymes producing strains, the enzyme production system was standardized for incubation time (24, 48, 72 and 96 hrs), pH (5.0, 6.0, 7.0, 8.0 and 10.0) and temperature (25, 30, 37, 45 °C). The enzymes cellulase, protease, lipase and amylase production were standardized for the optimum incubation time, pH and temperature for the efficient treatment of dairy effluent.

3.5.10 Analysis of Causes of Bulking in Activated Sludge

Activated sludge bulking is defined as the phenomenon, which occurs in activated sludge plants when the sludge occupies excess volume and does not readily settle so that in extreme cases the effluent contains excessive suspended solids. According to the filamentous backbone theory, bulking is due to the excessive growth of filamentous bacteria, that interfere with the compaction and settling of the activated sludge either by producing a very diffuse floc structure or by growing in profusion beyond the confines of the floc into the bulk medium and bridging between flocs (Jenkins et al 1993).
Bulking is caused by an overabundance of filamentous microorganisms known filamentous bulking. Low DO Concentration, low F/M, septicity, nutrient deficiency and low pH are the specific environments for bulking in the activated sludge in the settling tank (Michael Richard 1993).

During the running of the reactor, the DO of the mixed liquor was maintained in the range of 2 to 2.5 mg/L. The F/M ratio was maintained at 0.2. Since the reactor was aerated continuously, there was no chance for any septicity. The pH was also checked daily and maintained. The remaining cause for sludge bulking was nutrient deficiency. To check this, the amount of BOD, Nitrogen and Phosphorus in the activated sludge sample was analyzed.

3.5.11 Identification of Filamentous Bacteria in Activated Sludge

Activated sludge reactors contain complex microbial ecosystems in which, bacteria play a vital role. There are two types of bacteria, one floc forming and the other filamentous. As the problem of bulking is generally encountered in the settling tank due to the presence of excessive filamentous bacteria, it is essential to identify them. Hence, with the objective of finding the type of bacteria and its population in the activated sludge, the sludge was subjected to series of microbiological tests.

3.5.11.1 Gram Staining Technique

Gram staining is an indispensable aid for identifying bacteria. This staining first colours the bacteria blue using carbol gentian violet. The cells are then washed with an alcohol solution. The cells of some bacterial strains re-release the absorbed blue dye during this process. These bacteria are known as gram-negative. In the case of gram-positive bacteria, the absorbed carbol gentian violet cannot be removed by washing with alcohol. The colourless gram-negative bacteria are subsequently re-stained with safranine,
which gives them a red colour. This is the result of differences between gram-positive and gram-negative bacteria in the composition of the cell wall.

The following solutions were used

A  Carbol gentian violet solution  Prepared by diluting 10 ml of the stock solution with 90 ml of a 5% phenol solution. Stock solution (Carbol gentian violet 10 g, alcohol (96%) 90 ml.)

B  Lugol’s iodine solution  Prepared by dissolving 3 g KI in a few ml of distilled water, then mixed in 1g I₂ and diluted to 300 ml with distilled water.

C  Alcohol solution  Prepared by diluting 7 ml of the stock solution with 1000 ml (96%) alcohol. Stock solution (I₂ 100 g, KI 40 g, alcohol (96%) 1250 ml distilled water 100ml).

D  Safranine solution  Prepared by dissolving 0.25 g safranine in 10 ml (96%) alcohol and dilute with 100 ml distilled water.

Identification of filamentous bacteria was made by the following procedure.

A fixed smear was prepared. Solution A was applied for a contact period of 60 sec, subsequently the excess dye was allowed to run off the slide. Solution B was applied for a contact period of 60 sec. subsequently the excess dye was allowed to run off the slide. The slide was dipped in solution C for 30 sec. The slide was moved gently to and fro in this solution. The slide was rinsed and cleaned with tap water by allowing the water to flow gently over the back slide. Solution D was applied for a contact period of 120 sec. subsequently, the slide was rinsed again with tap water. The slide was allowed to dry and viewed with a 100X bright field objective. A blue filter strengthened the contrast. Drying was speeded up by first removing most of the water with filter paper.
Gram-negative and gram-positive bacteria were stained red and blue respectively. The blue colour varied from light blue to almost black. The presence of gram-positive species contributed to a more robust floc. Fungi and protozoa/metazoa did not stain evenly, or not at all, with this staining method.

### 3.5.11.2 Neisser Staining

Staining according to Neisser is a test for the presence of polyphosphates stored in the cells. This method is an indispensable aid to the identification of certain strains of filamentous bacteria. Furthermore, this staining method can make the Bio-P bacteria, responsible for biological phosphate removal, visible.

The following solutions were used:

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Methylene blue</td>
<td>0.1g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glacial acetic acid</td>
<td>5 ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol 96%</td>
<td>5 ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distilled water</td>
<td>100 ml</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Crystal violet, 10% in 96% ethanol</td>
<td>3.3 ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol 96%</td>
<td>6.7 ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distilled water</td>
<td>100 ml</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Chrysoidin Y, 1% aqueous solution</td>
<td>33.3 ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distilled water</td>
<td>100 ml</td>
<td></td>
</tr>
</tbody>
</table>

A fixed smear was prepared. A freshly made mixture of 2 parts solution A and 1 part solution B was placed onto the slide for a contact period of 10-15 sec. The excess dye was allowed to run off the slide. Solution C for a contact period of 45 sec was added. The slide was rinsed with tap water (with
the flow against the back of the slide). The slide was allowed to dry and then viewed with a 100X bright field objective. Drying was speeded up by removing most of the water carefully with filter paper.

3.6 SETTLING STUDIES ON DAIRY ACTIVATED SLUDGE

A bench scale reactor of 10 litres capacity was used for this study. The reactor was provided with air diffuser stones at the bottom and connected to air compressor to enable the supply of air to the reactor. Laboratory bench scale model of settling column was fabricated with internal diameter of 22.5 mm and a height of 650 mm to carry out the batch settling tests. The settling column made of glass facilitated the observation of solid liquid interface with respect to time.

The settling studies were carried out in different phases 1) to study the influence of chlorine in the settling characteristics of dairy activated sludge. 2) to find out the effect of chlorine in the design parameters of secondary settling tank 3) to determine the optimum dosages of chlorine for various MCRT values and use them for design of secondary clarifier by solid flux method and the process control of ASP.

3.6.1 Influence of Chlorine in the Settling Characteristics

Two commonly used measures, developed to quantify the settling characteristics of activated sludge are the sludge volume index (SVI) and the zone settling velocity (ZSV) which are essential for the design of secondary clarifier. On identifying the filamentous bacteria, the settling tests were conducted using dairy activated sludge generated from lab scale reactor of 10 liters capacity, operated for an MCRT of 5 days with addition of chlorine as a control measure for filamentous bulking and the settling characteristics with and without addition of chlorine for different MLSS concentrations were compared.
3.6.1.1 Bulking control with Chlorination

Chlorination is the most economical, non-specific method to control the excessive growth of filamentous microorganisms causing bulking in activated sludge plants. The goal of chlorination is to expose the activated sludge to sufficient chlorine to damage filaments extending from the floc surface while leaving organisms within the floc largely untouched. Chlorine effects on filaments include, in the order: a loss of intracellular sulfur granules; cell deformity and cytoplasm shrinkage; and finally filament breakup.

3.6.1.2 Chlorine Concentration in Stock Solution

The commercial bleaching powder normally contains low values of chlorine which vary from 25 – 30%. The chlorinating ability of bleaching powder varies from 65 -70% for high chlorine compounds. Before using the bleaching powder, its strength should first be checked in the laboratory. The stock solution was prepared by adding 5 gm of bleaching powder in 1000 ml distilled water. The concentration of chlorine in the solution was found from the laboratory tests.

3.6.1.3 Settling Characteristics with and without Chlorination

After attaining steady state condition sample settling studies were conducted using settling column (22.5 mm ID X 650 mm Ht) without chlorination and with chlorination of 3 ml stock solution for initial MLSS of 2 g/L, 3 g/L, 4 g/L, 5 g/L, 6 g/L, 7 g/L and 8 g/L for mean cell residence time of 5 days. The required MLSS concentrations were prepared by either concentrating or diluting the available mixed liquor. After filling the column with prepared activated sludge of the required initial MLSS concentration, the contents were agitated by stirring, to maintain uniform solids concentration throughout the depth of the column. The interface height was then recorded as
a function of time. A time interval of 1 min was kept initially to note the interface height with respect to time for the first 10 min as the fall of interface was rapid initially, then two-min interval was taken up for the next 10 min after which it was taken as 5 min up to 60 min.

From the settling test data, a plot between interface height and settling time was prepared. From the plot, the zone settling velocities were calculated as the slope of linear portion of batch settling curve. Settling velocities for various concentrations were found and tabulated.

Simultaneously, for each MLSS concentration, the prepared activated sludge was filled in a measuring jar of capacity 1litre and after allowing the contents to settle for a period of 30min, the interface height was noted as Sv30. The sludge volume index for MLSS concentrations of 2 g/L, 3 g/L, 4 g/L, 5 g/L, 6 g/L, 7 g/L and 8 g/L were calculated from the following relation.

\[
\text{Sludge volume index mL/g} = \frac{\text{Sv}30 \text{ mL/L}}{\text{MLSS g/L}} \quad (3.1)
\]

The settling velocities and SVI values of the experiments conducted with and without addition of chlorine were compared.

### 3.6.1.4 Influence of Chlorine Addition in the Design Parameters

Sample settling studies were conducted in the effluent from a batch reactor run for a \( \theta_c \) of 9 days with MLSS concentration of 5 g/L. The studies were carried out by varying the dosages of chlorine in order to determine the optimum dosage of chlorine. After determining the optimal chlorine dosage, its influence on the design of secondary settling tank was also analyzed.
The results of the settling tests with and without addition of chlorine were used for the design of secondary settling tank separately and the designed parameters were compared to determine the influence of addition of optimum dosage of chlorine in the settling tank. Talmadge and Fitch method was used for the design of secondary settling tank. Area needed for clarification and thickening were determined from the plot relating interface height with time as explained in section 2.9.1

3. 7 DESIGN OF SECONDARY CLARIFIER

It was ascertained with a sample test, that the addition of chlorine in the secondary clarifier, controlled the filamentous bulking, enhanced the settling velocity and eventually decreased the surface area required for settling in the secondary clarifier.

As it was established that addition of chlorine in the settling tank was beneficial in the design and enhanced the settling process, a series of zone settling tests were conducted with samples taken from the reactors which were run for different $\theta_c$ values (5, 7, 9, 11, 13 and 15 days). The tests were repeated for different MLSS concentrations and the optimum dosage of chlorine for each $\theta_c$ was obtained.

3. 7.1 Settling Tests with Optimum Dosages of Chlorine

Settling tests were repeated for the MLSS concentration of 5 g/L, without and with addition of varying chlorine doses to find the optimum chlorine dosages for $\theta_c$: 5, 7, 9, 11, 13 and 15 days respectively. After finding the optimum dosages for each $\theta_c$ value, different initial MLSS concentrations 2 to 20 g/L were prepared for each $\theta_c$ value and settling studies were conducted with the addition of respective optimum chlorine dosages.
The height of the solid-liquid interface with respect to time was noted for each case as done previously. Plots between interface height and time were prepared. Zone settling velocity was obtained as the slope of the initial settling straight line portion of the batch settling curve. Zone settling test data were subjected to the solid flux analysis and mathematical models were evolved for the design of secondary settling tank.

3.7.2 Analysis by Solids Flux Method

Solids flux is defined as the mass of solids per unit time passing through a unit area perpendicular to the direction of flow. In secondary clarifiers, it is the product of the solids concentration (mass/volume) and the velocity (length/time). The preferred unit is kilogram per square meter per hour (kg/m$^2$.h). Yoshioka et al. (1957) showed that slight modifications to the graphical approach (solids flux method) give greater flexibility for matching underflow concentrations to their associated limiting flux rates. To calculate the thickener area for the secondary clarifier, modified solid flux analysis (as explained in section 2.9.3) was used.

3.7.2.1 Generation of Flux Curves

The general procedure for determining the maximum allowable solids loading rate (the limiting solids flux) on an activated sludge clarifier begins with the development of a plot of the subsidence flux $G_s$ as a function of $C_o$. The data generated from settling experiments were utilized in developing the solid flux plots. Gravity flux curves were developed for $\theta_c$ 5, 7, 9, 11, 13 and 15 days for dairy wastewater.
3.7.2.2  Flux Curve Utilization for Clarifier Design

The values of zone settling velocity and gravity solid flux of dairy wastewater were calculated for MCRT 5, 7, 9, 11, 13 and 15 days. The limiting solids flux to achieve a desired underflow concentration was calculated using the gravity flux curve. A line was drawn tangent to the resulting flux curve with the intercept on X-axis at the desired underflow concentration $C_u$. The intercept on the Y-axis provided the maximum allowable clarifier solids loading rate, $G_L$ (for that particular $C_u$) based upon the clarifier feed MLSS concentration. This line is the underflow operating line and when it is tangential to the flux curve, the clarifier is said to be critically loaded. A line dropped from point of tangency to the X-axis, gives the maximum MLSS concentration $C_{\text{max}}$ to be maintained in the reactor. If $C_{\text{max}}$ is maintained in the reactor, the area of secondary clarifier will be sufficient to thicken the activated sludge to yield a desired underflow concentration.

This procedure was repeated for various underflow concentrations and the corresponding maximum solids loading rates, were determined each representing a limiting design and operating condition.

3.7.2.3  Evolution of Models with Regression Analysis

The solids flux curves for various MCRT 5, 7, 9, 11, 13 and 15 days were plotted and the limiting solids flux for the desired underflow concentrations were determined from the flux curves by drawing tangent to the curve from the $C_u$ under consideration. The data generated from the flux curves for all $\theta_c$ were given as input to a computer-aided multi regression analysis (SPSS) and for each $\theta_c$ an equation for the limiting solid flux as a function of the underflow concentration was evolved. Using the respective equations, for different $C_u$ values (with the increment of 0.10) corresponding
GL values were generated. The values of $C_o$, $A/Q$ were obtained using the equations 2.7 and 2.8. The recycling ratio $R$ was varied from 0.25 to 1.00 with the increment of 0.25. An attempt has been made to correlate the parameters $A/Q$, $C_o$, $C_u$, $R$ and $\theta_c$ by regression analysis.

### 3.7.2.4 Model Using ANN Analysis

Evolution of model for $A/Q$ was done based on the influencing input parameters like underflow concentration $C_u$, reactor MLSS concentration $C_o$, recycling ratio $R$ and mean cell residence time $\theta_c$. The networks of nodes were arranged in three layers – the input, hidden and output layers. $C_u$, $R$ and $\theta_c$ were taken as input parameters. The parameter $A/Q$ was taken as the output. Configuring hidden layers was done only by trials. The configuration, which showed maximum reduction in error after a fixed number of cycles, was accepted. The network was trained with the input and output data taken from the lab measurements for various conditions for appropriate error tolerance, learning parameters and number of cycles. Once the network was accepted with the acceptable tolerance, the output values of the network were used to form the regression model. The results of the ANN model, mathematical model and the actual experiments were compared.

### 3.8 PROCESS CONTROL

A good control strategy is vital to the successful operation of an activated sludge process. Such strategies, involve the application of both steady-state and dynamic-state control methods.

#### 3.8.1 Dynamic-state Control Method

The state point concept was used to develop a procedure for adjusting the recycle ratio $R$, when the activated sludge plant was subjected to
variation in flowrate, substrate concentration and combined variation in the flowrate and substrate concentration. Models were evolved for solid flux data generated from settling data using graphical procedure as explained in section 2.13.2

3.8.1.1 Generation of Data Based on Dynamic-State Analysis

A MATLAB program was developed for the generation of data, taking into account individual variations in hydraulic flowrate and substrate concentration in the influent and also both occurring simultaneously. From the solid flux curve developed for $\theta_c$ from 5 to 15 days (stationary phase), the underflow concentrations $C_u$, ranging from 14 to 21 Kg/m$^3$ with the increments of 1.0 were taken for analysis. The recycle ratio was varied from 0.25 to 1.00 with the increment of 0.25 for each case. The influent flowrate and substrate concentrations were assumed to vary from 50% to 150% of the original flowrate and substrate concentrations. The detailed results of the analysis are presented in chapter 7.

3.8.2 Steady-State Control Method

The steady-state control parameter chosen was $\theta_c$ and the control was exercised by varying the recycle rate, according to the change in influent flowrate and substrate concentration. Sludge recycle rates deserve careful consideration, since it affects the size of the secondary clarifiers without influencing the size of the aeration tank. The data generated based on steady-state control analysis were used for plotting the operational charts.

3.8.2.1 Evolution of Constants for Zone Settling Velocity

Zone settling velocity of sludge solids was then calculated from fall in height with time. The relationship between $C_o$ and $V_o$ was most often characterized by the Vesilind (1974) equation as
\[ V = V_0 \cdot e^{-a \cdot C_0} \] (3.2)

where \( V_0 \) and \( a \) are the constants for a particular \( \theta_c \). The Vesilind parameters were determined from multiple batch test data for \( \theta_c \); 5, 7, 9, 11, 13 and 15 days. They were analyzed using a computer program for curve fitting.

### 3.8.2.2 Generation of Data for Steady-State Analysis

The operational chart consists of four quadrants relating the operational parameters of the activated sludge process. A MATLAB program was developed to obtain the data required for the development of chart

**First Quadrant**

The overflow rate \( Q/A \) (\( m^3/m^2\cdot h \)) was related with the total biomass per unit area \( VX/A \) (in \( kg/m^2 \)) for various values of initial soluble substrate concentration \( (S_o) \) raising from 1100 mg/L to 1900 mg/L for dairy waste and shown in the first quadrant of the operational chart. The values of \( S_o \) were so chosen, as the average \( \text{BOD}_5 \) of the raw dairy wastewater was taken as 1500 mg/L.

**Second Quadrant**

The total biomass per unit area \( (VX/A) \) was related with the biomass concentration per unit area \( (X/A) \) for the various volume ranges from 100 m\(^3\) to 900 m\(^3\) and shown in the second quadrant of the operational chart. The biomass concentration per unit area \( (X/A) \) varies from 0.01 to 0.1 Kg/m\(^3\).m\(^2\).

**Third Quadrant**

The biomass concentration per unit area of the secondary settling tank \( (X/A) \) was related with microbial concentration \( X \) Kg/m\(^3\) (MLVSS) for
the area of the secondary settling tank ranging from 10 to 90 m$^2$ and shown in the third quadrant.

**Fourth Quadrant**

The suspended solid (MLSS) concentration $C_o$ and overflow rate $(Q/A)$ for the various, values of recycling rate $(R)$ were related and shown in the fourth quadrant. In stationary phase, the recycling ratio varies from 0.2 to 1.2 and the MLSS concentration ranging from 2 to 12 kg/m$^3$ with an increment of 1 Kg/m$^3$. The steady state charts are presented in section 7.

### 3.8.2.3 Development of the Clarifier Operating Diagram

As a guide in operating an activated sludge plant, it is important to control the parameters that can be either controlled or measured directly. These include primary effluent $Q$, MLSS concentration $C_o$, SVI, recycle ratio $R$ or RAS concentration $C_u$.

From batch settling flux curve formed from the settling experiments, for all $\theta_c$ (5 – 15 days), the values of initial MLSS concentration $C_o$ were calculated using equation (3.3)

$$C_o = \frac{R}{1+R}C_u$$  \hspace{0.2cm} (3.3)

This equation was represented as a set of straight lines, with slopes being a function of recycle ratio. Based on the solid flux theory, a limiting value of underflow velocity, $V$ exists for each RAS concentration. Underflow velocity, $V$ is defined as the velocity of liquid resulting from sludge withdrawal at the bottom of clarifier as equal (Equations (3.4) and (3.5)).
\[ V = \frac{Q_R + Q_w}{A} \approx \frac{Q_R}{A} \]  \hspace{1cm} (3.4)

\[ V = R \cdot \frac{Q}{A} = Rq_H \]  \hspace{1cm} (3.5)

As RAS concentration increases, limiting underflow velocity decreases. For a specified clarifier loading \( q_H \), each value of RAS concentration, \( C_u \) can be associated with a particular value of \( R \). The profile thus obtained represents the limiting curve and this curve, along with the set of straight lines relates the operational parameters of ASP, at steady state flux limited condition.

The limiting curve divides the set of straight lines into two portions. The region to the right and above the curve represents the clarifier overloading conditions which results in sludge rising. The region to the left and below the curve represents conditions, when the clarifier operation is not limited by solids flux. So the operation can be moved closer to the limiting curve by increasing MLSS, RAS or \( R \) depending on the desired results.

The results derived out of the above experiments are presented in the subsequent chapters. The next chapter deals with the results of the microbial experiments carried out in this research.