A predefined microbial consortium was used for the development of the BOD biosensor. Software was developed which enabled automatic calculations for BOD values. Waste waters from different industries were procured at different periods of time over a period of one year for each industries and BOD values were determined with the developed BOD biosensor as well as with the conventional method. The reproducibility and repeatability of the developed biosensor was determined.

A microbial consortium for the determination of ultimate BOD was developed. The said microbial consortium was tested for the determination of BOD load of dairy waste water.

3.1. Chemicals, Equipments and Glassware

3.1.1. Chemicals

All chemicals used for the present study were of analytical grade. Nutrient broth, agar powder, Tween-80 and glycerol were obtained from Hi-Media, India. The routine chemicals were procured from S.D. fine, Qualigens and Merck India Limited. D-Glucose, D-glutamic acid and charged nylon membrane was procured from Sigma, Germany. Distilled water was used throughout the study.

3.1.2. Equipments

The equipment used during the present study includes laminar flow (Kartos International), electronic balance (Sartorius), pH meter (Lab India), autoclave (Yorco),
incubator shaker (New Brunswick – Innova 4300), BOD incubator (Grover enterprises),
centrifuge (Sorvall – RC 5B Plus), spectrophotometer (Pharmaspec UV - 1700,
Shimadzu), digital burette (Eppendorf) and COD digester (Spectralab), lyophilizer
(Vertis), multimeter (Model 2700, Keithley instruments), vacuum pump, vortex mixer
and magnetic stirrer were also used for the present course of study.

3.1.3. Glassware/Plastic Ware

Storage bottles, aspirator bottles, filtration assemblies and petri-dishes etc., were of
Tarsons make. Measuring cylinders, beakers, erlenmeyer flasks, BOD bottles and bulb
pipettes etc., were procured from M/s. Borosil.

3.2. Preparation of Media and Reagents

3.2.1. Media

(a) Nutrient Broth (NB) (pH- 7.4)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptic digest of animal tissue</td>
<td>5.0 g/l</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g/l</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.50 g/l</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.50 g/l</td>
</tr>
</tbody>
</table>

All of the above contents were dissolved in 1000 ml of distilled water and autoclaved at
121°C, (15 lbs pressure) for 15 min.

(b) Nutrient Agar (NA)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptic digest of animal tissue</td>
<td>5.0 g/l</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g/l</td>
</tr>
</tbody>
</table>
Yeast extract  1.50 g/l
Beef extract  1.50 g/l
Agar  20 g/l

All of the above contents were dissolved in 1000 ml of distilled water and autoclaved at 121°C (15 lbs pressure) for 15 min. After autoclaving, nutrient agar was poured in petri plates at 37°C and allowed to solidify for further use.

3.2.2. Reagents

3.2.2.1. Preparation of Reagents for Chemical Oxygen Demand (COD)

(a) **Potassium Dichromate Solution** (K₂Cr₂O₇) (0.25 N) - K₂Cr₂O₇ solution dried at 103°C for 2 hrs allowed to cool down in desiccators. Accurately weighed 6.1295 g of K₂Cr₂O₇ was added in 500 ml d/w followed by the addition of 0.06 g of sulphamic acid.

(b) **Ferrous Ammonium Sulphate** (FAS) (0.1 N) – accurately weighed 39 g of FAS was dissolved in 500 ml d/w followed by the addition of 20 ml of H₂SO₄. Finally, the volume was made up to 1000 ml with d/w.

(c) **Sulphuric Acid Reagent** (H₂SO₄) - accurately weighed 10 g of silver sulphate (Ag₂SO₄) was dissolved in 1 L of H₂SO₄ and left overnight for dissolution.

(d) **Ferroin Indicator** - an amount of 1.485 g of phenanthroline monohydrate and 0.6959 g of silver sulphate hepta hydrate (FeSO₄·7H₂O) was dissolved in 100 ml d/w.

(e) **Glucose-Glutamic Acid Solution** – prior to use, glucose and glutamic acid was dried at 103°C for 1 hr and cooled in a dessicator. 0.15 g of glucose and 0.15 g of glutamic acid was weighed and dissolved in small volume of d/w. Finally volume was made up to 1 L.
3.2.2.2. Preparation of Reagents for Biochemical Oxygen Demand (BOD)/Ultimate BOD

(a) Preparation of Dilution Water

(i) Phosphate Buffer Solution (pH 7.0, 0.05 M), was prepared by using these chemicals:

- Potassium dihydrogen phospahte (KH$_2$PO$_4$) 8.5 g
- Dipotassium monohydrogen phosphate (K$_2$HPO$_4$) 21.7 g
- Disodium hydrogen phosphate hepta hydrate (Na$_2$HPO$_4$.7H$_2$O) 33.4 g

All the above constituents were dissolved in the 600 ml of distilled water and then the pH was adjusted to 7.0. Then, 1.7 g of NH$_4$Cl was added and finally the volume was made up to 1 L.

(ii) Magnesium Sulphate Solution (MgSO$_4$): accurately weighed 22.5 g of MgSO$_4$ was dissolved in 1 L of distilled water.

(iii) Calcium Chloride Solution (CaCl$_2$): an amount of 27.5 g of CaCl$_2$ was dissolved in 1 L distilled water.

(iv) Ferric Chloride Solution (FeCl$_3$.6H$_2$O): FeCl$_3$.6H$_2$O was weighed 0.25 g and dissolved in 1 L distilled water.

(b) Alkaline Iodide Azide Solution following chemicals was dissolved in 500 ml of distilled water.

- Sodium hydroxide (NaOH) 250 g
- Sodium Iodide (NaI) 67.5 g
- Sodium Azide (NaN$_3$) 5.0 g

(c) Manganese Sulphate (MnSO$_4$.7H$_2$O): Manganese sulphate 91.06 g was dissolved in 250 ml of DW.
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(d) Sodium Thio Sulphate Solution \((Na_2S_2O_3.5H_2O)\) (Hypo) 0.125 N: 
Na$_2$S$_2$O$_3$.5H$_2$O was weighed 3.102 g and dissolved in 60 ml of distilled water. Finally volume was made 100 ml with distilled water. The working solution (0.125 N) was made fresh prior to each analysis.

(e) Potassium Iodate (KIO$_3$) (0.0125 N): an amount of 0.466 g KIO$_3$ was dissolved per liter of d/w.

(f) Potassium Iodide (KI) (0.5 g) added directly while the standardization of sodium thio sulphate is being carried out.

(g) Starch Indicator Solution (2%): accurately weighed 2 g of starch was dissolved in 100 ml of hot distilled water.

(h) Glucose-Glutamic acid Solution: glucose and glutamic acid were dried at 103°C for 1 hr and cooled in a dessicator. 0.15 g of glucose and 0.15 g of glutamic acid were first dissolved in small volume of d/w. Finally volume was made up to 1 L. The solution was prepared fresh for each analysis.

(i) Allylthiourea (ATU) Solution: 2.0 g allylthiourea was dissolved in about 500 ml water and dilute to 1 L.

3.2.2.3. Preparation of Reagents for BOD Biosensor

(a) Sodium Phosphate Buffer (50 mM, pH- 6.8) was prepared by using these chemicals

- Sodium dihydrogen orthophosphate monohydrate 3.519 g
- Disodium hydrogen phosphate anhydrous 3.479 g

All the above constituents were dissolved in the 1 L of distilled water.

(b) Glucose-Glutamic Acid Solution: glucose and glutamic acid was dried at 103°C for 1 hr and cooled in a dessicator. 0.6 g of glucose and 0.6 g of glutamic acid was first dissolved in small volume of d/w. Finally volume was made up to 100 ml. The solution was prepared fresh for each analysis.
3.3. Analytical Tests

3.3.1. Chemical Oxygen Demand

Chemical oxygen demand was determined in accordance with the method described by APHA 1998.

(a) Procedure

- Pre-dried COD tubes were placed in the COD digestion chamber.
- Accurately measured 0.4 g of HgSO₄ was added to each tube followed by the addition of 20 ml of sample.
- Amount of 10 ml K₂Cr₂O₇ solution was added in each tube.
- Then, 30 ml of H₂SO₄ was added slowly to the tubes.
- The tubes were connected to the condenser and refluxed at 150°C for 2 hrs.
- After 2 hrs of incubation, the tubes were allowed to cool down to 35-40°C, and then, 80 ml of distilled water was added to rinse the condenser. The contents of the tubes were emptied in separate Erlenmeyer flasks.
- Approximately 4-5 drops of ferroin indicator was added in each of the digested samples prior to titration.
- The digested samples were titrated against 0.1 N FAS (ferrous ammonium sulphate), which was standardized before every titration.
- The end point of the titration was green to wine red.

Standardization of Ferrous Ammonium Sulphate (FAS): To 90 ml distilled water, 10 ml of K₂Cr₂O₇ was added followed by the addition of the 30 ml conc. H₂SO₄. This reaction mixture was allowed to cool down to room temperature and titrated against FAS using ferroin as an indicator. End point: green to wine red was observed.
(b) Calculations

\[
\text{COD (mg/l)} = (A-B) \times N_{\text{FAS}} \times 8000 / \text{ml of sample}
\]

Where,

- \( A \) = ml of the FAS used for the blank
- \( B \) = ml of the FAS used for the sample
- \( N_{\text{FAS}} \) = Normality of the FAS

\[= \text{Milli-equivalent weight of oxygen (8) } \times 1000 \text{mg/l}.\]

3.3.2. Biochemical Oxygen Demand/Ultimate BOD

Biochemical oxygen demand was determined in accordance with the method described by APHA 1998. Prior to analysis, the waste water samples were appropriately diluted because of the limited solubility of oxygen in water. Based on the following formula the dilutions were determined from COD values.

(a) Procedure

- Single distilled water was kept for aeration for 20-25 hrs.
- Following chemicals (0.1%) were added in the aerated d/w in the given order:
  - Phosphate buffer
  - Magnesium sulphate (MgSO₄)
  - Calcium chloride (CaCl₂)
  - Iron (III) chloride (FeCl₃)
  - Seeding material (Sewage)
- Samples were appropriately diluted depending on their COD values, using dilution water.
• BOD bottles were rinsed with appropriate samples and filled up to the brim.

• The bottles to be used for the estimation of Dissolved oxygen (DO) of zero days were kept aside, while the rest of the bottles were incubated at 27°C for 3 days in dark in a BOD incubator for carbonaceous demand whereas for the determine water nitrogenous demand bottles were incubated at 20°C for 90 days.

• **Determination of Initial DO**
  
  ▪ Fixation of the BOD bottles was done at 0 day and similarly on 3\textsuperscript{rd} day and for the nitrogenous demand the bottles were fixed for alternate days till 90 days of time by adding 2.0 ml of MnSO\textsubscript{4} followed by 2.0 ml of alkali azide to the BOD bottles.
  
  ▪ Bottles were shaken and precipitates were allowed to settle down for 20 to 30 min.
  
  ▪ Then, 2.0 ml of H\textsubscript{2}SO\textsubscript{4} was added.
  
  ▪ Bottles were shake properly for no particulate matter.
  
  ▪ 100 ml of this reaction mixture was transferred to Erlenmeyer flask and titrated against the freshly prepared 0.0125 N Hypo solution using starch as an indicator.

**Standardization of Sodium Thio Sulphate (Hypo):** To 90 ml of TDW, 10 ml of KIO\textsubscript{3} was added followed by H\textsubscript{2}SO\textsubscript{4}. This was immediately titrated against 0.0125 N hypo solution after the addition of 0.5 g KI using starch as an indicator. End point to be observed was blue to colorless.
(b) Calculation

\[ \text{BOD mg/l} = \frac{(D_1 - D_3) - (B_1 - B_3)}{\% \text{ of sample used}} \]

\[ D_1 = \text{DO of the dilute sample immediately after preparation (mg/l)} \]

\[ D_3 = \text{DO of diluted sample after the incubation for 3 days (mg/l)} \]

\[ B_1 = \text{DO of the blank immediately after preparation (mg/l)} \]

\[ B_3 = \text{DO of blank after incubation for 3 days.} \]

3.4. Immobilization of Predefined Microbial Consortium

3.4.1. Inoculum Preparation

The predefined microbial consortium was immobilized. The inoculum (mother culture) was prepared by inoculating one loopful of individual bacterial isolates, separately in 50 ml of sterilized nutrient broth having 0.01% Tween-80. The inoculated broths were incubated in an orbital shaker at 37°C for 16 hrs so as to obtain actively growing mother cultures.

3.4.2. Preparation of the Microbial Consortium

The above mentioned actively growing individual bacterial cultures were inoculated separately in 100 ml of sterilized nutrient broth and incubated at 37°C, 120 rpm for 16 hrs. Finally, these bacterial cultures were mixed in equal proportions on the basis of their optical density values at 600 nm. This microbial mixture was centrifuged at 6000 rpm for 10 min at 4°C. The cell pellet was washed twice with 50 mM sodium phosphate buffer and suspended in small volume of the same buffer. The resultant cell slurry was kept at 4°C till further use.
3.4.3. Immobilization on Nylon Membrane

For immobilization, appropriate aliquots of the cell suspension were filtered under vacuum on the commercially available membrane. The immobilized microbial membrane thus prepared was allowed to dry overnight at 25-30°C. Storage of the dried membranes was done at 4°C, in 50 mM phosphate buffer (pH 6.8), till further use.

Steps for Preparation of Immobilized Microbial Membrane

**Step 1**  Sub-culturing of individual cultures from slants to Nutrient agar plates

**Step 2**  Sub-culturing of individual mother cultures in 25 ml broth.

**Step 3**  Observing initial O.D. (0.2-0.3) at 600 nm of individual cultures

**Step 4**  Incubating at 30°C / 200rpm / 16-18 hrs

**Step 5**  Observing (a) final OD at 600 nm (OD$_{600}$ 1.0 – 1.7) of all cultures (b) Checking the purity of the final culture by streaking the cultures on nutrient agar plates.

**Step 6**  Inoculating final broth (100 ml) from mother cultures

**Step 7**  Observing initial O.D. at 600 nm of individual cultures (OD$_{600}$ 0.2-0.3)

**Step 8**  Incubating at 30°C / 200 rpm / 16-18 hrs

**Step 9**  Observing (a) final O.D. of cultures (OD$_{600}$ 1.0-1.7) (b) Checking the purity of the final culture by streaking the cultures on nutrient agar plates

**Step 10**  Mixing of all the cultures in 50 ml falcon.

**Step 11**  Centrifuging at 7000 rpm / 4°C / 20 min

**Step 12**  Collecting the pellet

**Step 13**  Washing the pellet by dissolving it in sodium phosphate buffer solution

**Step 14**  Centrifuging at 7000 rpm / 4°C / 20 min

**Step 15**  Re-suspending the washed pellet in phosphate buffer ≈ 2ml
Step 16  Placing the nylon membrane on membrane filter.

Step 17  Filtering the cells through the membrane

Step 18  Leaving the immobilized microbial membrane for drying for 18 – 20 hrs at room temperature

Step 19  Storing the immobilized microbial membrane in buffer at 4°C

3.4.3.1. Pre-conditioning of Immobilized Microbial Membrane

After the preparation of membrane, before use, pre-conditioning was required. Different parameters (Conditions and different GGA concentration) were considered for pre-conditioning of the membranes (Figure 3.1). For studying the first parameter conditions, stirring, non-stirring and shaking was considered. Simultaneously, different concentrations of GGA, low moderate and high concentration were taken into account. The microbial membranes were activated by immersing the membranes in sodium phosphate buffer (disodium hydrogen phosphate and sodium dihydrogen phosphate) containing different concentrations of glucose–glutamic acid (GGA 1920 – 12,000 mg/l) and studied by keeping the membrane for stirring, non-stirring and under shaking conditions.

![Figure 3.1. Different parameters for pre-conditioning of immobilized microbial membrane](image-url)
3.4.3.2. Stability Study of the Immobilized Microbial Membrane

The prepared immobilized microbial membranes were assessed for their stability during storage at different temperatures and pH. Stability of the prepared immobilized microbial membranes was assessed at intermittent time intervals, in terms of their response. The stability study was carried out while storing the prepared membranes at different temperatures viz., 4°C, 15°C, 25°C and 37°C and at different pH ranging from 6.0-8.0.

3.4.3.3. Viability Study of the Immobilized Microbial Membrane

The viability of the prepared membranes was assessed at regular time intervals during storage at different temperatures ranging from 4°C to 37°C and different pH varying between 6.0 - 8.0. For checking the viability of the bacterial strains immobilized on the membrane, the membrane was placed on an agar plate in an inverted position and incubated at 37°C for 16 - 20 hrs. The colonies were observed for growth on the agar plates.

3.4.3.4. Leaching Studies of the Immobilized Microbial Membrane

In order to check the leaching of the bacterial cells during storage, a loopful of buffer in which the membrane was stored, was streaked on nutrient agar plates. The plates were incubated at 37°C for 16-20 hrs and observed for growth. Simultaneously, the leaching rate of the bacterial strains from the membranes was assessed by observing the optical density of the storage buffer at regular time intervals.

3.5. Assembly of the Microbial Sensor and Measurement of Response

3.5.1. Assembly

The microorganisms were grown and immobilized on the nylon membrane. Immobilized microbial membrane along with the nylon cloth was attached with the DO electrode for the preparation of electrode assembly. The device was assembled by connecting the electrode assembly to multimeter which in turn was connected to PC
through an interface card. PC was installed with developed software “BioSens\textsuperscript{BOD}”, built on visual basic platform. This software takes “change in current” values in real time and manipulates them in desired graphical displays in mg/l BOD.

3.5.2. Measurement of Response

The response of the developed BOD sensor was measured by coupling the immobilized microbial membrane to cathode of the oxygen probe. Nylon net (400 mesh) was attached to the immobilized microbial membrane, and the two were fixed on the surface of the working and reference electrodes by means of a rubber O-ring. A Clark type probe for dissolved oxygen was used as a physical transducer, which consisted of a platinum cathode as the working electrode and a silver anode as the reference electrode. In order to stabilize the BOD sensor system, the sensor was immersed in 100 ml of the potassium phosphate buffer system under constant and moderate stirring. The working and reference electrodes of the BOD sensor were connected to a potentiostat and the output current was recorded through a multimeter, which amplified the response to nA. An applied potential of \(-0.650\) mV versus Ag/AgCl was delivered to the Pt-working electrode throughout all the measurements. The BOD sensor measurements were made using steady state method. The steady state indicates the consumption of oxygen by microorganisms which diffuse from a sample solution to the membrane at equilibrium. In steady state method, the difference of current between the two steady states reflects the respiration rate of substrate and was used for BOD estimation. The measuring time was 10-15 min followed by 15-16 min recovery time. In order to stabilize the BOD sensor system and measure the response of the immobilized microbial membrane, 100 ml of phosphate buffer (50 mM) pH 6.8 was placed in a thermo stated cell at \(35 \pm 2\)°C under constant and moderate stirring.
Aliquots of stock synthetic (GGA) were added after a stable current was attained for 30 min (initial steady state current). The current change (decrease) was observed after addition of the samples until a final steady state was reached. The response was calculated on the basis of current difference between the initial steady state current and the final steady state current.

3.6. Determination of BOD Values by Developed Biosensor

3.6.1. Sample Collection, Preservation and Storage of Samples from Different Industries

Samples of both inlet and outlet effluent were collected from industries situated in and around Delhi (beverage, dairy, sugar, distillery, tannery, pharmaceutical, paint) (Figure 3.2). The sampling was carried out from the same sites after regular time intervals for a period of at least one year. The effluent samples were collected in wide mouth bottles and before collection; the bottles were rinsed with the effluent. The collected effluents were stored at 4°C till further use.

Figure 3.2. Map depicting the specific industrial location selected for the study.
3.6.2. Preparation of a Response Curve, Linearity, Calibration Curve for the Immobilized Microbial Membrane

3.6.2.1. Response Curve

Different concentrations 15 mg/l - 90 mg/l of GGA were checked for response over a period of 20 min. The depletion of total oxygen was observed.

3.6.2.2. Linearity Curve

Linearity in response time over a certain concentration range is a measure of the detection capacity to analyze waste water with varying concentrations. The BOD values of different concentrations of GGA as were determined by the developed sensor. The results were plotted and linearity observed.

3.6.2.3. Calibration Curve

Stock solution of GGA having a concentration of 12,000 mg/l was prepared. Different aliquots from the stock solution of GGA were added in the measuring cell of the BOD sensor system, to achieve the desired GGA concentrations of 15 - 300 mg/l (having a BOD of 11 - 220 mg/l). The response of the sensor with different GGA concentrations was observed and recorded. The readings were plotted and on the same graph, a second mantissa was drawn showing the conventional BOD (BOD₅) values against the same GGA concentrations as used with the developed BOD sensor.

3.6.3. Estimation of BOD Values of Real Waste Water Samples

A number of samples of beverage waste water collected at different timings were tried to determine the BOD load using BOD biosensor. COD of the samples was performed and COD value thus obtained was used for calculating the expected BOD value according to the following formula:

\[
\text{Expected BOD} = \text{COD} \times \% \text{ biodegradability of the sample}
\]
The percentage of the samples used for BOD analysis using sensor, were calculated from their expected BOD values, according to the following formula:

\[ \% \text{ of W.W} = \frac{\text{Linear range of sensor} \times \text{Vol. of buffer in the measuring}}{\text{BOD}_5 \text{ value of waste water}}. \]

The biosensor was used to estimate the BOD of a large number of samples and the dilutions need to be calculated manually for each sample so that BOD value falls within the range followed by the calculation of BOD. So it was necessary to develop software based on the extensive data collected so that the manual calculation could be eliminated and some aspects like generation of the dilution required for the estimation of BOD could be automated.

### 3.6.4. Software Development

Software, “BioSensBOD” was developed on visual basic platform meant for graphical displays, BOD calculation, online data acquisition and other calculations. The developed software “BioSensBOD”, which was developed on visual basic platform, has got two main parts:

In first part, users enter the parameters like sample description, membrane ID, % dilution, maximum time for taking readings, time interval between readings and output file name in the form “parameters” shown in Figure 3.3. Thereafter it shifts to the next form “Online Graph Plotting” (Figure 3.4). This form has got four command buttons i.e. Start, Stop, Print and Exit. By pressing Start button program starts acquiring data from the Keithley Multimeter (Model 2700) through RS232C interface and the date and time of starting of the acquisition of the data is displayed on the screen. The acquired data is also saved in the user defined output file. After the maximum time the plotted graph can optionally be printed on the printer.
The second part is offline part. This has got further three parts: (i) membrane data, (ii) BOD calculator and (iii) offline graph plotting.

(i) Membrane data: By selecting this, user can enter the new membrane reference data at different GGA concentrations along with its ID, modify the existing membrane data, delete any membrane reference data or can also undelete the deleted membrane reference data. The reference membrane data form is shown in (Figure 3.5).

(ii) BOD calculator: This calculator asks the user to enter the membrane ID, ΔI (i.e. I_{max} – I_{min}) at 60GGA, ΔI (i.e. I_{max} – I_{min}) of the under test sample and the percentage dilution of the sample used. Thereafter program compares the present ΔI
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Development of BOD Biosensor for Water Polluting Industries

60 GGA with the reference $\Delta I$ of the selected membrane and calculates the correction factor and applies it for the calculation of BOD level and the same is displayed in mg/l. If the equivalent $\Delta I$ for the sample reaches the saturation level, the program does the reverse calculation and gives the value of the dilution of the sample to be used for proper measurements. The BOD calculator data is shown in Figure 3.6 (a - c).

![BOD Calculator](image1)

![BOD Calculator](image2)

![BOD Calculator](image3)

**Figure 3.6. (a-c) BOD calculator**

(iii) Offline graph plotting: This form asks the user to enter the file name whose data is to be plotted on the screen and then plots the data after reading the file. The user can also view the data of any selected file on the screen. The offline graph plotting form is shown in Figure 3.7 (a,b).

![Offline Graph Plotting](image4)

![Offline Graph Plotting](image5)

**Figure 3.7. (a,b) Offline graph plotting**
3.6.5. BOD Estimation of Waste Water from Different Industries by Using Developed BOD Biosensor

The BOD biosensor was assembled by connecting the immobilized electrode to developed highly stable constant volt source and multimeter which in turn is connected to a mobile PC through RS232C interface. Mobile PC was installed with developed software “BioSensBOD” build on visual basic platform. This software acquires the real time current data from the multimeter and plots the same in the form of desired graphical display as well as save the same in the user defined file along with the time in seconds. Initially the, electrode was dipped in sodium phosphate buffer placed on the magnetic stirrer and external polarization voltage was applied through the highly stable developed voltage source. Current through the electrode was measured by the multimeter. Then stability of the immobilized microbial membrane was observed as displayed by developed software installed in a mobile PC which in turn is connected to a multimeter through RS232C interface. Change in current (i.e. ΔI = I max – I min) was calibrated for different concentrations of GGA. Sample solution was replaced now with fresh sodium phosphate buffer and assembly was stabilized. In order to calculate the BOD, the percentage of the waste water as determined by the software was added in sodium phosphate buffer. Finally, BOD value of waste water was calculated with the help of BOD calculator as mentioned above. This BOD value was compared to BOD_5 as determined by titration-based method.

3.6.6. Repeatability and Reproducibility

To check the reproducibility and repeatability of the immobilized membrane the glucose – glutamic acid (GGA) was used. Two membranes (A9 and A11) were used to study the response of 60 mg/l of GGA repeatedly. Similarly different membranes (A7, A9, A11 and A12) were used and the experiment was conducted with GGA as well as
waste water. The difference in current was measured and repeatability was observed. Likewise, single membrane was used by different operators utilizing GGA as a reference standard. The experiments were conducted with GGA as well as with waste water. Electrode attached with immobilized membrane and nylon cloth was dipped in sodium phosphate buffer and external polarization voltage was applied through the highly stable developed voltage source. Then reproducibility of the immobilized microbial membrane was observed by measuring change in current (i.e. $\Delta I = I_{\text{max}} - I_{\text{min}}$) for 60 mg/l GGA.

Number of membranes were prepared and their response curve, linearity and calibration curve was studied in order to check the repeatability and reproducibility.

3.7. Industry Specific BOD Biosensor: Restructuring BOD: COD Ratio

3.7.1. Isolation, Maintenance, Screening and Identification of Potential Bacteria for Dairy Industry

3.7.1.1. Isolation

(a) Sample Collection for Isolating Autochthonous Bacteria

For the isolation of microorganisms, samples were collected from dairy industry. The samples were collected in wide mouth sterile plastic bottles by immersing the bottles inside the sample. These samples were stored in an icebox (4°C) during transportation.

(b) Preparation of Enrichment Media

The samples were homogenized and suspended in nutrient broth, which consisted of 0.5% sodium chloride, 0.15% beef extract, 0.15% yeast extract, and 0.5% of peptic digest of animal tissue with 0.01% Tween-80. The inoculated medium was incubated at 37 ± 2°C for 48 hrs on a shaker.
(c) Isolation of Pure Cultures

For the isolation of pure cultures, serial dilutions of the incubated broth medium (up to $10^{-12}$) were prepared by adding 1ml of the incubated sample in 9 ml of sterilized normal saline (0.85% w/v sodium chloride in distilled water). 100 μl inoculum from the dilutions $10^{-8}$, $10^{-10}$, $10^{-11}$ and $10^{-12}$ was spread on nutrient agar plates (in duplicate). These plates were incubated at $37 \pm 2^\circ$C, for 16-24 hrs. Single isolated colonies, which appeared on nutrient agar plates were picked up with the help of sterile nichrome wire and streaked on fresh plates of the same medium. These plates were incubated at $37 \pm 2^\circ$C for 16-24 hrs in incubator.

3.7.1.2. Maintenance of Isolated Pure Cultures

In order to ensure the availability of pure microorganisms and their initial metabolic activities, the isolated cultures were sub-cultured periodically. Finally, the isolated pure cultures were stored. Generally, two storage methods viz., short term and long term storage methods have been used.

3.7.1.2.1. Short Term Storage Methods

These methods are applied when the microorganisms are in continuous use. They include preservation techniques such as, storage of microorganisms on agar plates and slants (Figure 3.8).

(a) Preparation of Nutrient Agar for Slants

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient Agar: Peptic digest</td>
<td>5.0g/l</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g/l</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.50 g/l</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.50 g/l</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g/l</td>
</tr>
</tbody>
</table>
Chapter 3  Materials and Methods

1. All of the above components were dissolved in 1000 ml of d/w and autoclaved at 121°C, 15 lbs pressure for 15 min.

2. To prepare slants, the autoclaved nutrient agar was poured in sterile glass vials when it was warm (~45°C) and the glass vials were tilted at 30-35°C until solidification.

3. The slants were incubated at 37°C (overnight) to check for contamination.

(a)                                                  (b)

Figure 3.8. Short term storage techniques for preservation of cultures a) plates b) slants.

3.7.1.2.2. Long Term Storage Methods

Methods such as the use of 50% glycerol, lyophilization of microorganisms and cryopreservation have been used for the long term storage of microorganisms (Figure 3.9).

(a) **Glycerol:** Double strength Nutrient broth media i.e. 13 g / 500 ml d/w was autoclaved at 15 psi for 15 min. The cultures were inoculated individually and the flasks were incubated at 37°C for 16 hrs at 200 rpm. The cultures were streaked for purity check and the plates were incubated (overnight) in incubator. Finally 500 µl of the cultures was inoculated in autoclaved* cryovials (1.5 ml capacity) containing 500 µl of glycerol.

* The cryovials containing glycerol were autoclaved at 10 psi for 10 min.
(b) **Lyophilization:** The bacterial cultures were grown in double strength medium and centrifuged at 6000 rpm for 10 min.Pellet thus obtained was re-suspended in phosphate buffer pH 6.8 and the slurry was layered on to round bottom flask using chilled acetone (-80°C). The layered suspension was freeze dried; bacterial powder was transferred to Duran bottle and stored at room temperature.

![Figure 3.9](image1.png)  
(a)  
(b)  

**Figure 3.9.** Long term storage techniques for preservation of bacterial cultures (a) glycerol stock (b) lyophilized powder.

### 3.7.2. Screening of Isolated Bacteria

Different bacterial isolates were screened for their ability to degrade dairy waste water. The inoculum (mother culture) was prepared by inoculating one loopful of all individual bacterial isolates in 25 ml of sterilized nutrient broth. The inoculated broths were incubated in an orbital shaker at 37°C for 16 hrs so as to obtain actively growing mother cultures. The above mentioned actively growing cultures were inoculated separately in 100 ml of sterilized nutrient broth and incubated at 35°C, 120 rpm for 16 hrs. All the cultures were centrifuged separately at 6000 rpm for 10 min at 4°C. After centrifugation, supernatant was discarded and the pellet was washed twice with 50 mM sodium phosphate buffer. Bacterial pellet was inoculated in 100 ml of waste water sample (sample and culture ratio was 1 : 1). Chemical oxygen demand and biochemical oxygen demand of the samples was estimated after 16 hrs incubation.
3.7.3. Identification of Selected Bacterial Isolates

Identification of all the individual bacterial strains comprising the selected formulated microbial consortium was carried out at the Institute of Microbial Technology (IMTECH), CSIR, Chandigarh.