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Fabrication of the microbial biosensor by using *Pseudomonas aeruginosa* Forl the estimation of Biochemical Oxygen Deman (BOD) by Amperometeric Technique

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Abstract

Fabrication protocol for a microbial BOD sensor has been described in this paper. A real time analysis of its performance is also presented

Keywords: amberometry, Biochemical Oxygen Demand (BOD), biosensor, environmental biotechnology, *Pseudomonas aeruginosa*

INTRODUCTION

As the world's population and industrialization grow, environmental pollution becomes a progressively more serious problem. Advances in microbial genetics and genetic engineering have given impetus to the field of environmental biotechnology.

Waste water generation and its subsequent treatment is a major problem for every industry and for the society as well. Prior to treatment, the waste waters need to be monitored so as to permit their discharge into the local water resources. Pathogenic organism pollution has been recognized from the middle of nineteenth century when water transmitted disease had been demonstrated.

Nowadays genetic engineering methods and immobilization techniques are used for the treatment of various industrial effluents. The major contaminants found in the effluents include biodegradable organic compounds, recalcitrant compounds, toxic metal ions, suspended solids, microbial pathogens and parasites

The amount of various municipal and industrial effluents are inevitably increasing as the world economy expands and new industries develop, which results in the increase of organic pollution due to waste water. These toxic pollutants cause changes in the aquatic environment such as changes of PH, temperature, dissolved oxygen concentration, species present in the domain. Biological oxygen Demand (BOD) is used as the index and as an important parameter for measuring organic pollution.

Amongst all the parameters for which the waste Waters are monitored, biochemical oxygen demand, (BOD), is one of the most important and frequently used parameters for estimating the level of water pollution. The control of waste water treatment plants is very difficult or even impossible using the classical determination method for BOD because of its high time consumption (3-5 days) (Rastogi *et al.,* 2003).

A Biosensor is a sensor that is based on the use of biological material for its sensing function. The biocomponent specifically reacts or interacts with the analyte of interest resulting in a detectable chemical or physical change. And also Biosensors are devices that have several unique features such as compact size, simple to use, one step reagentless analysis, low cost and quick real time results.

The conventional BOD measurement requires 3-5 days, which a microbial BOD biosensor sense within minutes. A number of microbial BOD sensors have been developed nationally and internationally. The drawback of such developed sensors is that they cannot be used for all types of industrial and domestic waste waters.

We developed a BOD biosensor based on a tested, synergistic formulated microbial consortium. It is capable to sense the BOD load of a wide variety of synthetic as well as industrial waste waters having low moderate-high biodegradability within minutes. The sensor BOD values show a good linear relationship with the BOD valves obtained using the conventional method up to a GGA concentration 60 ppm of BOD, values of real waste water samples from different industries viz, distillery, dairy and tannery were analyzed using the developed sensor. The BOD sensor results were found to be comparable with those obtained using the conventional method.

BIOCHEMICAL OXYGEN DEMAND

The amount of oxygen required by aerobic microorganism to decompose the organic matter is a sample of water such as that polluted by sewage. It is used as a Measure of the degree of water pollution and also called as Biochemical oxygen Demand.

The principle of the conventional method involves measuring the difference of the oxygen concentration between the sample and after incubating it for five days at 20 degree Celsius. Thus the conventional test

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is non suitable for process control and monitoring where a rapid feed back is desirable.

The control of waste water monitored by BOD is one of the most important and frequently used parameter for estimating the level of water pollution. The classical determination of BOD method is of very high time consumption (3-5 days) (Rastogi *et al*., 2003). So it needs to be fast & cost effective and has stimulated the production of a variety of field analytical tools such as Biosensors.

SENSOR

A sensor is a device that measures a physical quantity and converts it into a signal which can be reed by an observer (or) by an Instrument.

Sensor can be defined as a device which receives a signal and converts it in to electrical form which can be further used for electronic devices. A Sensor differs from a transducer in the way that a transducer converts one form of energy in to other form whereas a sensor converts the received signal in to electrical form only.

BIOSENSOR

A Biosensor is a device for the detection of an analysis that combines a biological component with a physiochemical detector component. It contains 3 parts.

i. **Sensitive Biological Element** (Biological material – tissues microorganism, cells, enzymes, antibodies, nucliec acids etc.,) a biologically derived material or Biomimic. These sensitive elements can be created by Biological engineering.

ii. T**ransducer detector element** (Electrochemical Method) that transforms the signal resulting from the interaction of the analysis with the biological element in to another signal that can be more easily measured & quantified.

iii. **Associated electronics / Signal processors** that are primarily responsible for the display of the results in a user friendly way.

Type of Biosensors

i. Calorimetric Biosensor

ii. Poteriometric Biosensor

iii. Amperometric Biosensor

iv. Optical Biosensor

v. Imonunosensors.

Application of Biosensors

i. Glucose monitoring in diabetics patient

ii. Other medical health related targets.

iii. Enviromental application - Detection of BOD, in contamination river, ponds & pesticides detection

iv Remote sensing of airborne bacteria

v. Detection of pathogens and foreign substances before and after remediation.

vi. Drug discovery and Protein Engineering, etc are various types of applications of Biosensors of different fields.

MICROBIAL BOD SENSOR

A Microbial Biosensors is an analytical device that couples microorganism with a transducer to enable rapid, accurate and sensitive detection of target analytes in fields as diverse as medicine, enviromental monitoring, defense, food processing and safety (Yu Lei, *et.al.,* 2006). Microbial sensor has been developed for the measurement of biochemical oxygen demand (BOD) for water samples in most simple and easy way. It can measure BOD for any kind of water such as pond water, municipal water and industrial waste water such as beverages waste. Paper mill effluents, pharmaceutical waste and waste from food process industry (Wise 1991).

Recently many kinds of biosensors have been developed for the determination of organic compounds and to estimate the value of BOD.A biosensor as an analytical device consisting of an immobilized layer of biological materials such as enzyme, antibody, hormone, nucleic acid and the whole cell connected to a transducer. The signal give in by the transducer is proportional to the chemical being analyzed (Karube *et al*., 1981).

Enzyme sensors are highly specific for the substrates of interest, but the enzymes employed are generally expensive and unstable (Ibtisam 2001.) Thus the microbial biosensors act as an alternate for the enzyme sensors.

Some of the major types of biosensor include bioaffinity biosensor, thermistor biosensor, enzyme biosensor, microbial biosensor, conductimetric biosensor, optical microbial biosensor, bioluminescence biosensor, fluorescence biosensor etc.

SALIENT FEATURES OF MICROBIAL BIOSENSORS

The following are the salient features of microbial biosensors

- They are less sensitive to inhibition by solutes and are more tolerant of suboptimal pH and temperature values than enyzyme electrodes.

- They have longer life time than enzyme electrodes
- They are cheaper because an active enzyme does not have to be isolated.

The microbial biosensors developed so far involve the assimilation of organic compounds by microorganism, changes in respiration activity or the production of electrochemically active metabolities; the latter being monitored directly by an electrochemical device (Karube, *et.al.,* 1981).

THE WORKING PRINCIPLE

Microbial biosensors consist of a microorganism which is immobilized on a membrane with electrochemical devices. Microbial biosensors are of two types.

❖ Respiration activity measurement type.

❖ Electrochemistry active metabolites measurement type.

RESPIRATION ACTIVITY MESUREMENT TYPE

In this case, changes (normally increases) in respiration activity of microorganisms caused by assimilation are detected by an oxygen electrode from these changes, substrate concentrations are estimated. Aerobic microorganisms are used in these sensors. A microbial biosensor is dipped into a buffer solution saturated with oxygen, upon the addition of substrate; the respiration activity of the microorganism is increased, which causes a decrease in oxygen concentration near the membrane. Using an oxygen electrode, substrate concentration can be measured from the oxygen reduction (Karube, *et.al.,* 1981).

ACTIVE METABOLITES MEASURMENT TYPE

This type of microbial biosensor detects electrochemically active metabolites such as hydrogen, carbon dioxide, ammonia and organic acids which are secreted by the microorganism. This type of microbial biosensor is not limited to aerobes but can also employed anaerobic microorganism.

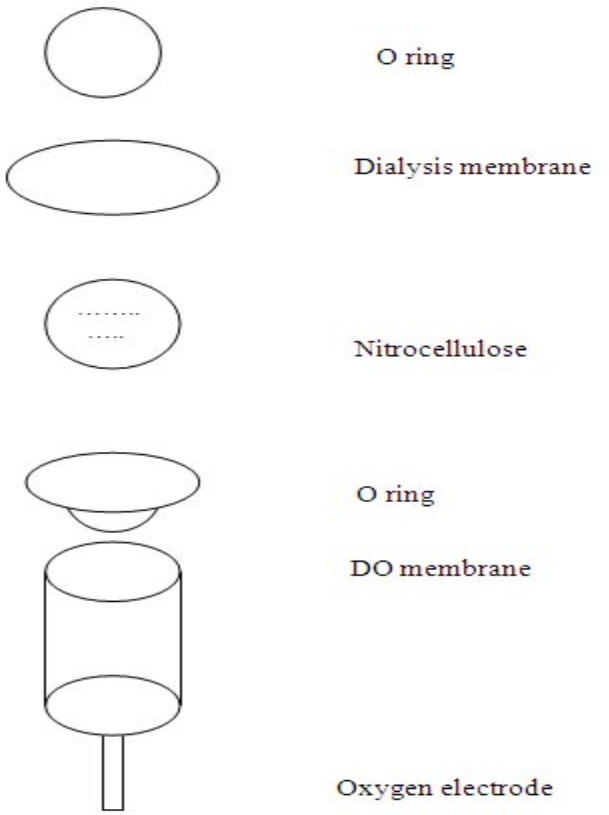
In any of the above case a very low microbial loading of the biosensor is pre request for a kinetically controlled respiration electrode and such sensor coupled with suitable immobilization of the microorganism as well as thin membranes have to be used. The sensitivity of this type of sensor is mainly determined by the cell activity. (Karube, *et.al.,* 1981).

AIM and OBJECTIVE

Aim of this experiment is to study the complete biochemical characterization of the pure culture like,

➢ Respiratory activity of Microorganism using BOD sensors.

Fig.1. **construction of the biosensor**



➢ Determination of Biochemical Oxygen Demand through assimilation studies of different substrates (sugars, alcohols, amino acids, aldehydes and organic acids).

➢ Biodegradable properties by doing various real sample analysis.

➢ Effect of pH and temperature on the sensor response.

REVIEW OF LITERATURE

Biosensor can be used for the estimation of hormones. Thyroxine hormone if less in children results in physical and mental retardation. Here hormone sensing molecules are used as biolayer.[Steve Prentis, *et.al.,* 1985].

Inner electrolyte solution is the medium in which the gas to be detected undergoes a reaction and produce an ion species that can be sensed by the indicator electrode. It should have no effect on the process of gas permeable membrance [Li, *et. al.,* 1994].

The first report of such microbial BOD sensor was published by Karube *et.al,* in 1976. Since 1986 a BOD sensor system based on microbial BOD sensor has been produced by Nisshin Electric co.Ltd (Karube, *et.al.,* 1981), Meanwhile commercially available BOD

sensor systms are being produced by AUCOTEAM GmbH Berlin, PGW GmbH Dresden and Dr.Bruno Lange GmbH Berlin.

Cupric and in particular silver ions significantly suppressed resulting in a lower measured BOD value of the substrate. The inference of silver and cupric ions on the BOD measurement were successfully eliminated by chelating these ions with EDTA or by phenotypic modification of the microbial system by pre-conditioning the sensor in BOD solution containing the interfering ions**.** [Li, *et.al.,* 1994].

A universal biosensor that can be used for analysis of all types of organic pollutants is the objective of current researches working in the area of biosensors. The BOD sensor employing *H.anamola* was fabricated and evolved in batch mode as well as under flow conditions [Sangeetha, *et. al.,* 1996].

A microbial biosensor based on thick film technology was developed. The microoorganisma**, *Arthrobacer nicotianae***, were immobilized in calcium alginate directly on the electrode surface. For the stability of the calcium alginate gel the addition of 0.5mM cacl12 the assay buffer was necessary. The sensor was used in a batch system and was applied to the determination of free fatty acids in milk.[Schmidt, *et.al.,* 1996].

A novel electrochemical biosensor for nitric oxide based on electro polymerized film of o-amino benzaldehyde-ethylene-diamine nickel was developed byLangun Mao *et.al.,* (1999).

A novel biosensor for the determination of Biochemical Oxygen Demand [BOD] was developed using potassium hexa cyano ferrate a meditor. The sensor element consists of a three electrodes system, with both working and counter electrodes integerating as a disposable using etching and electroplating processes. *Pseudomonas flourescense biovar v* [isolated from a waste treatment plant] was immobilized on the surface of the working electrode using polyvinyl alcohol.[Yoshida *et.al.,* 2000].

The possibility of direct amperometric detection of Ag-Ab interaction on the surface of molecular films chemisorbed on the gold support and modified by gamma chain specific antihuman IgG was checked. The reproducibility, sensitivity and selectivity of Ag-Ab detection depended on the quality of coverage of gold support. The addition of IgG resulted in decrease of conductivity of molecular layers composed dodecanathiol, [Snejdarkova *et.al.,* 2000].

Synthetic waste water described by the organization for economic co-operation and development [OECD] was used as a standard solution instead of Glucose-Glutamic acid + 600mv operating potential. The sensor response was linear from 15 mg to 200mg. To demonstrate the wide metabolic range of activity of the sensor, the sensor response to 14 substances in 4 categories of organic compounds was investigated**.** [Yano *et.al.,* 2000].

Large numbers of biochemical activities and electron behavior have a very close relationship with life from and living cells which makes the study of physiological and biochemical characteristics, an important area analytical biochemistry**.** [Sreenath Subrahmaniyan *et.al.,* 2001].

Monitoring using a meditor type BOD sensor consisting of *Pseudomonas fluorescens biovar v* using sludge extract solution as standard solution for calibrations was carried out by Takashi Morita *et.al.,* [2001].

The field of biosensors for measuring BOD is reviewed particularly. BOD sensors constructed on the biofilm configuration are discussed regarding performance characteristics like linearity, response time, precision, agreement between BOD values obtained from the biosensors and the conventional 5 days test as well as toxic resistance to various compounds and operational stability. The techniques for improving the agreement between the sensor BOD and BODS are described. Information provided also includes BOD biosensors based on respirometers and other measuring principles, the commercial BOD instruments as well as the current limitations of BOD biosensor development. [Chinnayelka and Shane (2002)].

The property of amperometric glucose biosensor utilizing glucose oxidase immobilized in Naffion over glassy carbon electrode modified by Prussian blue and electro polymerized poly aniline are described. The biosensor was not sensitive to acetaminophen and ascorbic acid at an operating potential 0.0v. The decrease in current responses to glucose in the reaction of ascorbic acid with enzymatically formed H_20_2.[Garjonybe and Malinavskas., 2003].

Sampling and laboratory analysis are not well adapted to waste water quality monitoring in a process control or hazards prevention context, for which on line / on-site measurements is preferable. Before considering the implementation and constraints of on –line systems, the reasons for and ways of monitoring are discussed. The main existing and comingup solutions are then presented, showing that with respect to the number of parameters and substances to be monitored, for regulations purpose. For (eg) only a few of them are measurable with on line devices. [Oliver Thomas and Marie Florence Povet, 2005].

Recent advances in the development band applications of biosensors the environment analysis and monitoring are reviewed in this article, several

examples of biosensors developed for relevant environmental pollutants and parameters are briefly overviewed. Special attention is paid to the application of biosensors to real environmental samples, taking in to consideration aspects such as sample pretreatment,matrix effects and validation of biosensor measurements. Current trends in biosensor development are also considered and commented on this work. In this context, nanotechnology, miniaturization assay development especially biotechnology arise as fast growing areas that will have a marked influence on the development of new biosensing strategies in the near future**.** [Sara Rodriguez-Mozaz, *et.al.,* 2006].

Water quality is an important aspect of water management concerning pollution control. The removal of biodegradable organic substances is a very important aspect of evaluation of the treatment efficiency in a wastewater treatment plant (WWTP).

Two amperometric biosensors based on glassy carbon electrodes (GC) modified with Mg/Al layered double hydroxides (LDHs) containing ferrocene-carboxylate (Fc-CO_2H) or ferrocena-sulfonate (Fc-SO_3H), as interlayer anions, and glucose oxidase (Gox) are presented. Amperometric detection of glucose involves the electrochemical oxidation of hydrogenperoxide mediated by the ferrocene derivative.

Heavy metal ions such as cadmium, mercuric, lead, chromic, stanuous, ferrous, ferric, and aluminium at a concentration of 4mM have negligible or no effect on the BOD sensing of a mixed *Bacillus subtilis* and *Bacillus licheniformis* 7B microbial BODsensor.[Li, *et.al.,* 1994,].

An amperometric alcohol biosensor by using electroless nickel as working electrode was developred. The sensing results may be affected by the electrolyte composition, deposition time and temperature of the electroless nickel[Yun-Ying and Liao, 2000].

A new glucose biosensor was developed based on the sandwich configuration of organically modified sol-gel glasses. The new sol-gel glasses were used to develop glucose biosensors that differ in absence and the presence of graphite powder [Pandey, *et.al.,* 1999].

A universal biochemical oxygen demand (BOD) sensor that can be used for the analysis of all types of organic pollutants is the objective of current researches working in the area of biosensors.Development of such type of sensors involves the proper choice of a micro organism that can assimilte a wide spectrum of organic compounds. The BOD sensor employing *Torulopsis candida* was fabricated and evaluated in the batch mode as well as under flow conditions .[Sangeetha, *et.al.,* 1996].

A disposable bacterial CO_2 microbial sensor employing a miniature Clark Type oxygen electrode and autotrophic bacteria, to make the sensitive area less vulnerable to stress, to facilitate CO_2 diffusion through the sensitive area and to make the application to biosensors easier, the sensor structure was simplified by immobilizing the bacteria in an oxygen electrode cell along with a 0.1M KCl electrolyte solution [Hiroaki Suzuki, *et,al.,* 1991].

A microbial sensor consist of a transducer in conjuction with immobilized viable or non viable microbial cells. Bioluminescence-based biosensor have been developed using genetically engineered microorganisms constructed by fusing lux gene with an inducible gene promoter for toxicity and bioavalability testing [Dsouza, 2001].

Recently genetically engineered microorganisms based on fusing of the Lux,or lacZ gene reporter to invisible gene promoter widely apply to assay toxicity and bioavalability [Yu Lei,*et,al.,* 2006].

Developed with the help of *Aspergillus nige* by coupling this fungus onto an oxygen electrode are the highly selective ethanol sensors. Air as a carrier gas had no influence on alcohol detection, but with the nitrogen replaced, as expected, there was an increase in the sensitivity of the sensor [Subrahmaniya,*et,al.,* 2001].

A microbial fuel cell type of biosensor was used to determine the Biochemical Oxygen Demand (BOD) of wastewater. The biosensor gave a good correlation between the BOD value and the coulomb produced. The BOD sensor has been operated for over 5 years in a stable manner without any servicing. This is much longer that that of previously reported BOD biosensors [Kim,*et,al.,* 2003].

MATERIALS AND METHOS

CULTURE

The microorganisms used for this work were obtained from 3 different textile industries**.** From that *Pseudomonas aeroginosa* were used.

CULTURE CHARACTRISTICS

- Growth condition:Aerobic
- Temperature : 25 c
- Incubation Time: 24hrs-48hrs
- Sub culture : 30 days
- Microbial media ; Nutrient medium
- Hi medium ; *Pseudomonas asparagines* medium

Most of the cultures in which the BOD sensor maintained were carried out using nutrient broth medium. About 100ml of nutrient both and *Pseudomonas asparagine*s medium were prepared and autoclaved for the inoculation of the culture.

GGA SOLUTION

Synthetic OD nutrient solution was prepared for this work using 1:1 ratio of Glucose: Glutamic acid to standardize the solution. This Synthetic BOD Solution was prepared by adding 250mg of Glucose and 250 mg of glutamic acid was make up to 100ml of Milliq water.

PHOSPHATE BUFFER

Phosphate Buffer was preared [pH-7.0] with potassium dihydrogen orthophosphate and 1.2gm sodium hydroxide. It was made up to 1 liter with triple distilled water.

SUBSTRATE PREPARATION

To study the assimilation of substrates by the cultures, about 0.5gm of the substrate was dissolved in 50ml of the triple distilled water.

All the reagents used in this work were laboratory reagent [LR] grade and were used as received.

EQUIPMENTS

DO PROBE

A Dissolved Oxygen [DO] proe was developed by CECRI using a gold cathode, a platinum counter electrode and an Ag/Agcl reference electrode and inner electrolyte solution.

GAS PERMEABLE MEMBRANE

The gas permeable membrane used in the probe was purchased from Century Instruments and Co., Chandigarh.

NITROCELLULOSE MEMBRANE

Nitrocellulose [NC] membrane [pore size <0.25 micron] procured from Millipore was used as the matrix for immobilization.

BOD METER

The BOD meter instrument has been designed to have operational sensitivity to concentration level ranging from 0 to 60 ppm. The BOD meter is used to carry out BOD measurements.

OTHER MATERIALS USED

Micropipette, stop watch, forceps, scissors, culture tube, conical flasks, measuring cylinders, beakers, glass vials, eppendors, reagents bottles, Petrplates**.**

CHEMICALS USED

Potassium dihydrogen orthophosphate, Sodium hydroxide, Potassium chloride, Disodium hydrogen phosphate, Borax, Sodium bicarbonate were purchased from Rancem and were of analytical grade for preparation of buffer solution.

METHODOLOGY

CULTURE INOCULATION

- The microorganisms were grown in a nutrient broth and agar containing yeast extract, glucose, malt extract and peptone in 100 ml.
- The nutrient broth was then sterilized in an autoclave. Then it was cooled to ambient temperature under sterile condition in laminar air flow chamber.
- Then the required microorganism was inoculated and it was allowed to grow under aerobic condition at 37 °C for 48hrs in an incubator.
- At the tensure of the incubation time the broth became turbid and the microorganism was ready for harvest.
- The culture broth was centrifuged at 10,000 rpm at 10 c in a cooling centrifuge.

SUBCULTURE

- The Nutrient Agar Medium was prepared for the sub culturing of grown microorganism from the Nutrient Broth. It should be incubated for 24-48 hr at 37 °C.
- After the culture was grown in the nutrient medium, again it should be allowed for sub culturing. Then only we can get a pure culture.

PURE CULTURE

- Prepare the 100ml of *Pseudomonas asparagine* medium & broth was sterilized in an autoclave.
- Take a single colony from the sub cultured plate and it should be inoculated in the *Pseudomonas asparagine* medium.
- And it should be incubated for 24 – 48 hours at 37°C.
- After incubation the plates were observed for the growt of the pseudomonas culture.
- For the confirmation the biochemical test and gram staining were performed.
- Again the plates were observed under the UV iliminator for the observation of fluorescent colour production.
- The wet weight of the isolated microorganism was taken and dispersed in the phosphate buffer solution [H-7] and stored in a refrigerator at 4 °C.

- A solution of 1.0gm of microorganism in 100ml of phosphate buffer was then used for an important process of immobilization.

STRAIN IMMOBILIZATION

- As discussed earlier microbial sensors employ microorganism as the biological sensing material coupled to a suitable transducer.
- The performance and longevity of the microbial biosensor depends on number of parameter and the important being the method of immobilization.
- The immobilization of the microorganism for analytical purpose should result in the following effects. Increased working stability of the organisms of the biosensor.
- Reusability of the organisms because of their increased storage stability.
- The immobilization of the microorganisms was carried out by means of a physisorption technique.

Physisorption is the simplest method for microbe immobilization. Typically a microbial suspension was incepted and the electrode or an immobilization nature such as alumina and glass bead, followed by rinsing the buffer to remove unabsorbed cells.

- The microbes are immobilized due to adsorptive interactions such as ionic, polar or hydrogen bonding, hydrophobic interactions.
- The loading of microorganism on to nitrocellulose membrane was carried out under sterile condition in a laminar air flow and the process of technique is discussed below.
- The nitrocellulose membrane was placed in a suction filter and it was wetted thoroughly by means of spirit.
- Then 0.5ml of the microbial suspension is added on the top of the membrane and it was allowed to filter across the membrane slowly under suction.
- After the microbial suspension passed through the membrane it was removed carefully from the suction and gently rinsed with the phosphate buffer to remove any loosely bound microorganism from the membrane. The membrane was then stored in a refrigerator at 4 °C when not used.

MICROBIAL BOD SENSOR CONSTRUCTION

The microbes immobilized membrane were used for the fabrication of the respiratory electrode for evaluating the assimilation characteristics of microorganisms coupled with DO probe with the aid of a dialysis membrane as depicted in figure 1..

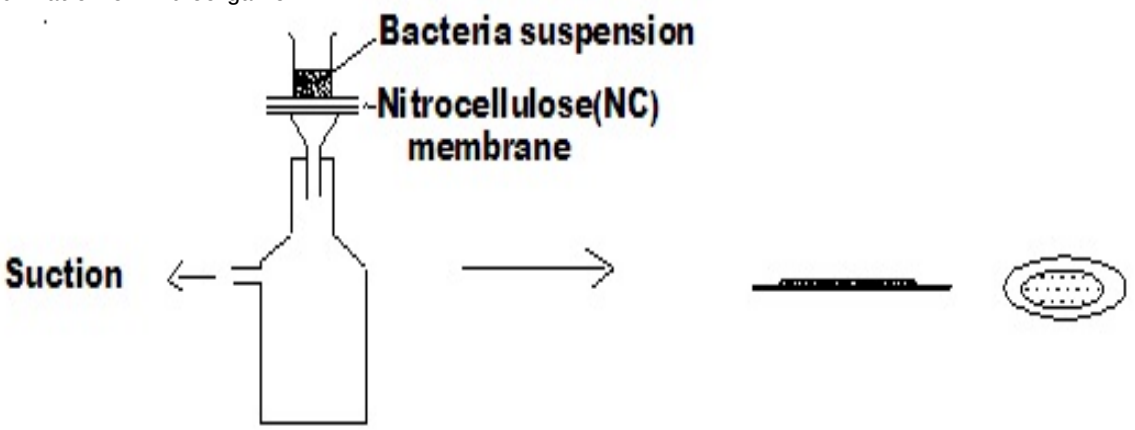
The selection of an appropriate gas permeable immobilized membrane is an essential step in fabricating a sensor. The body of the DOand the microbe immobilized membrane was placed on the DO probe. Then a dialysis membrane was kept on the microbe immobilized membrane and an O ring was used to keep intact the microbe immobilized membrane between the oxygen permeable membrane and dialysis. The DO body was then taken out of the assembly system and inserted into the DO probe body to constitute the respiratory electrode. The electrode was evaluated using synthetic BOD sample, viz. Glucose and glutamic acid mixture and also with a variety of organic compounds.

FABRICATION OF DO PROBE

The polarographic oxygen probe works on the principle of amperometric detection. The conventional and commercial DO probes are all two electrodes configuration, viz a noble metal cathode and silver counter electrode, the electrode system often encountered problems associated with drift in the potential and also at times shift in the zero calibration. Hence a DO probe has been developed as a part of the in house project on BOD sensor was made, which was used extensively in this study.

The DO probe consists of three electrodes. A gold electrode was used as a working electrode with an Ag/Ag CL reference electrode and a platinum

Fig.2. Immobilization of microorganism



electrode as the counter electrode. The gold cathode was fabricated by moldings method using a gold tripped brass rod with high temperature setting epoxy resin.

The DO probe works on the principle of amperometry. The DO probe comprises of two outer portions, the bottom made of poly carbonate which was affixed with the hydrophobic oxygen permeable membrane and the probe was filled with phosphate buffer with KCL as the electrolyte solution. The top portion comprises of three electrode configuration, the working cathode surrounded by platinum foil as the counter electrode and silver per silver chloride is used as the reference electrode.

The gold cathode for the DO probe was procured from fabricated electrode by casting the gold tipped brass rod using high temperature epoxy resin at 100 degree Celsius for a period of 1 hour. The gold surface was exposed from the resin casting by grinding and the electrode was subsequently polished with fire emery sheets and then with alumina powder.

EVALUATION OF THE DO PROBE

The DO probe so fabricated was evaluated under different experimental conditions. As indicated already DO probe works on the principle of amperometry. The concentration of the DO probe at the interface is a function of the amount of dissolved oxygen in the test solution in addition to the number of other parameters.

In our experiment 10ml of air saturated phosphate buffer was taken and the DO probe was dipped in the solution and the solution was stirred at constant speed using a magnetic stirrer. The DO probe was connected to the potentiostat and a potential of 0.6v us AG/ AGCL was impressed.

The Sodium sulphide was added to the test solution. Sodium sulphide is an oxygen scavenger and will consume all the oxygen from the solution. In the current time response curve the oxygen reduction current fell shortly to zero after the addition of sodium sulphide as the DO probe was depleted in the zero solution.

Experiments were carried out using DO probe by varying the experiment condition. The evaluation of DO probe was carried out as mentioned above and instead of sodium sulphide addition, the solution was deaerated using nitrogen and the oxygen reduction current dropped to zero gradually as the oxygen from the test solution was expelled due to nitrogen through the system.

The DO probes performance was evaluated over a period of time and found to be highly reproducible and maintenance free.

INSTRUCTMENTAL PROTOCOL

Amperometric detection technique is one of the most important analytical techniques commonly employed to measure the concentration of organic compound.

Amperometry in normally carried out in stirred or flowing solution at a rotated electrode current measured as the compound undergoes oxidation or reduction at working electrode (gold) held at a fixed operating potential .

Amperometry is a potentially controlled analytical technique which is enforces and maintains a predetermined potential of the working electrode of an electrochemical cell against a reference electrode and the cell current is monitored as a function of time.

Here the working electrode was maintained at a constant potential and the species of interest gets discharged at the electrode.

The resulting cell current was a measure of the concentration of ions which in turn was the indication of biochemical oxygen demand (BOD) value.

Amperometry, the incorporated analytical technique enables BOD meter when coupled with microbial sensor to analyze the BOD values for various organic and inorganic compounds in nA current [nA-the unit of current] and BOD analysis for real test samples in ppm level.

THE EXPERIMENTAL PROTOCOL

The experimental set up was shown in Figure 3.The assimilation characteristics of the microorganisms were studied using the respiratory electrode with different immobilized microorganism.

All the studies were carried out with 5ml of phosphate buffer and the sensor was placed in the glass cell. The buffer was saturated with dissolved oxygen and was stirred magnetically during the measurement using a magnetic strirrer.

When the steady state current was attained, about 10 ppm of GGA solution was injected in to sensor cell and the reading was observed with the help of a BOD meter for every five minutes.

The steady current indicates that the consumption rate of oxygen by the microorganisms and the diffusion rate of oxygen from the solution to the membrane are equal.

The sensor is washedand the process is repeated till the microbial BOD sensor gets fully activated.

When the microbial sensor was removed from GGA solution and placed in buffer solution free of glucose, the current of microbial sensor gradually increases and attains the initial level.

RESULTS

This study was carried out in the estimation of the BOD by using microbial biosensor in the presence of organism, pH, temperature and a suitable synthetic and real samples. For this BOD estimation , textile effluent samples were collected for strain isolation .The Pseudomonas aeruginosa were cultured in a pseudomonas asparagines medium and isolated.

For the confirmation, identification tests were carried out.

Grams staining:

Gram negative rods

Biochemical test:

Catalase test - Positive

Oxidase test - Positve

UV-illuminator:

Under the uv illuminator it emits the bluish green fluorescence colour at 365 nm long.

Scanning electron microscope: (SEM IMAGE)

Long rod shapes structures appeared.

ASSIMILATION OF SUBSTRATES:

The microorganism enters the log phase and takes twenty days for the respiratory activity to be enhanced. At this stage there is full development of culture accompanied by degradation of organic substrates. Hence the decomposition of organic nutrient is monitored. Organic nutrients like carbohydrates, amino acids, alcohols, organic acids and aldehydes are added and the assimilation efficiency is manifested by the rate of change of oxygen consumption. The organic nutrient concentration was taken in mg.

These organic nutrients have been added and their assimilation capacity is monitored by manipulating the rate of oxygen consumption. The standard graph using GGA constructed is taken as the standard and then from the difference in nA current the rate of oxygen consumption is determined in ppm.

Determining the Respiratory activity of Microorganism:

From the figure and its corresponding standard graph figure entry of the microorganism in to the log phase could be referred .It takes twenty days for the respiratory activity to be enhanced .The steady state response in the beginning of measurements shows the endogenous respiration of immobilized microorganism .In a continuously mixed system dissolved oxygen diffuses from the aerated phosphate buffer to the bio membrane ,where part of the oxygen is consumed by the microorganism .The remainder diffuses through the oxygen sensors polymer membrane and is detected by the oxygen electrode.

Using oxygen electrode substrate concentration can be measured from oxygen decrease. When the substrate is added to the system it diffuses also into the bio membrane and is assimilated by microorganism, resulting in the increase of the amount of oxygen consumed .As a result, the concentration of oxygen on the membrane of sensor decreases leading to a decrease in the biosensor output signal (current).

REAL SAMPLE ANALYSIS

Real sample analysis was carried out to check the biodegradable properties of microorganism by measuring the oxygen level. The effluent raw samples analysed were first diluted approximately depending on their BOD load. The diluted samples are then incubated for 24 hours in order to estimate the BOD of the samples. These incubated samples are subjected to aeration by purging oxygen through an aerator. Real sample analysis was carried out for domestic and industrial effluents and industrial samples. The observations were tabulated.

REAL SAMPLE ANALYSIS

EFFECT OF PH

The performance of the respiratory electrode was evaluated under varying pH of the solution using GGA solution as the substrate. In a typical experiment, 5ml of the solution with the required pH was taken in the glass cell and the respiratory electrode was dipped in the solution. An impressed potential -0.6v VS the reference electrode was applied to the sensors and the initial steady state current was observed in the absence GGA mixture. Then 10 ppm GGA solution

Fig.3. The experimental setup.

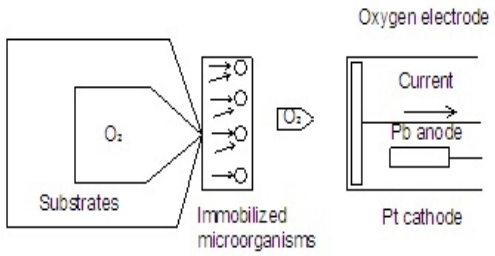


Table.1. Determination of the Respiratory activity

S.No	Concentration (ppm)	Current (nA)	Difference in current (nA)
1.	0	225	0
2.	10	242	13
3.	20	223	32
4.	30	208	47
5.	40	194	61
6.	50	180	75

Fig. 4 Standard Graph for determination of resipiratory activity

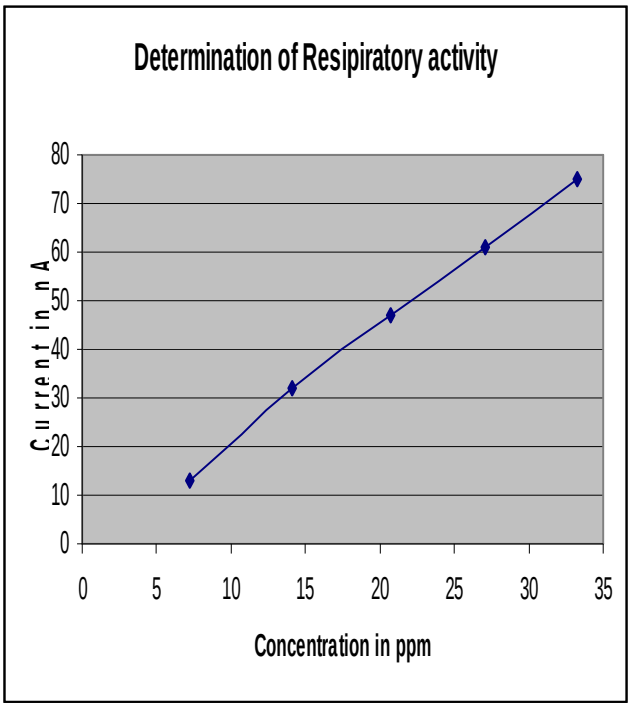


Table. 2. Assimilation of Carbohydrates

S.No	Carbohydrates	Concentration of Substrates (mg/lt)	Current I (nA)	Differences in current Δi (nA)
1.	Fructose	0	338	
		10	312	26
2.	Sucrose	0	311	
		10	224	87
3.	Glucose	0	356	
		10	332	24
4.	Galactose	0	352	
		10	329	23
5.	Maltose	0	359	
		10	314	45
6.	D-Arabinose	0	407	
		10	393	14
7.	Lactose	0	393	
		10	380	13
8.	Raffinose	0	406	11
		10	395	
9.	Mannose	0	347	
		10	335	12

Table.3. Assimilation of the Amino acids

S.No	Aminoacids	Concentration of Substrates (mg/lt)	Current I (nA)	Differences in current Δi (nA)
1.	Asparagine	0	355	
		10	266	89
2.	Leucine	0	351	
		10	290	61
3.	Alanine	0	382	
		10	328	54
4.	Threonine	0	357	
		10	303	54
5.	Valine	0	348	
		10	311	37
6.	Proline	0	338	
		10	301	37
7.	Tyrosine	0	368	
		10	333	35
8.	Methionine	0	343	
		10	311	32
9.	Glycine	0	324	
		10	300	24
10.	Phenyl alanine	0	340	
		10	333	7

Table.4. Assimilation of Organic acids

S.No	Organic acids	Concentration of Substrates (mg/lt)	Current I (nA)	Differences in current Δi (nA)
1.	Gluconic acid	0	306	
		10	250	56
2.	Succinic acid	0	366	
		10	312	55
3.	Nicotinic acid	0	386	
		10	381	5
4.	Oxalic acid	0	396	
		10	392	4
5.	Citric acid	0	383	
		10	375	8
6.	Salycilic acid	0	387	
		10	382	4
7.	Ascorbic acid	0	329	
		10	306	23
8.	Tartaric acid	0	385	
		10	374	11
9.	Malic acid	0	385	
		10	382	3

Table.5. Assimilation of Alcohol

S.No	Alcohols	Concentration of Substrates (mg/lt)	Current I (nA)	Differences in current Δi (nA)
1.	Benzyl Alcohol	0	393	
		10	347	46
2.	Iso Butanol	0	303	
		10	243	60
3.	Methanol	0	375	
		10	350	25
4.	Dulcitol	0	348	
		10	319	39
5.	Ethanol	0	338	
		10	293	45
6.	Amyl Alcohol	0	384	
		10	331	53

Table.6. Assimilation of Aldehydes

S.No	Aldehydes	Concentration of Substrates (mg/lt)	Current I (nA)	Differences in current Δi (nA)
1.	Benzaldehyde	0	383	
		10	328	55
2.	Chinnamaldehyde	0	370	
		10	328	42
3.	Nitrobenzaldehyde	0	348	
		10	316	32
4.	Gluteraldehyde	0	375	
		10	359	16
5.	Formaldehyde	0	387	
		10	342	45

Table no.7

S.No	Concentration (ppm)	Current (nA)	Difference in current (nA)
1.	0	430	0
2.	10	405	25
3.	20	388	42
4.	30	378	52
5.	40	370	60
6.	50	360	70

Fig. 5. Standard Graph for real sample analysis

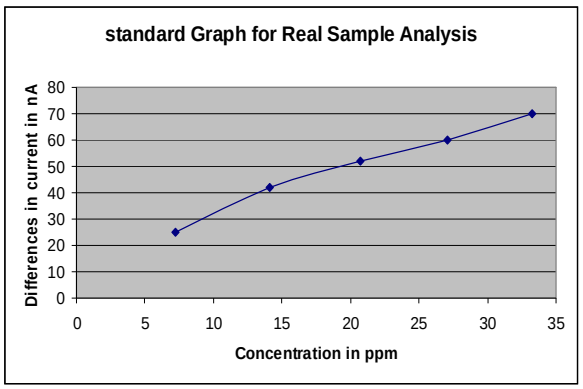


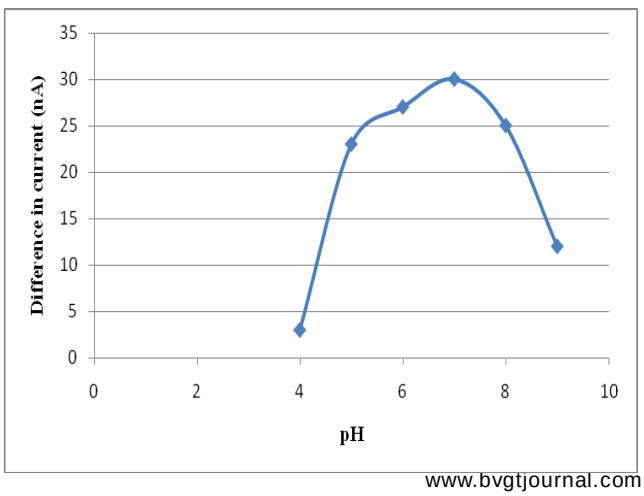
Table.8.

S.No	Samples	Concentration (mg/lt)	Current (nA)	Difference in current (nA)
1.	Sugar molasses	0	382	
		10	352	30
2.	Mud sample	0	401	
		10	372	29
3.	Biopharm sample	0	379	
		10	358	21
4.	Pond sample	0	380	
		10	355	25
5.	Dye sample A	0	403	
		10	358	45
6.	Dye sample B	0	388	
		10	380	8
7.	Cotton	0	424	
		10	420	4
8.	Paper	0	382	
		10	368	14
9.	Bagassee	0	426	
		10	390	36
10.	Lactate	0	372	
		10	352	20

Table. 9. effect of pH

S.No	pH	Current in nA
1.	4	3
2.	5	23
3.	6	27
4.	7	30
5.	8	25
6.	9	12

Fig. 6 Standard graph for pH



was added to the solution and the oxygen current decreased gradually and attained the steady state value. The change in oxygen reduction current were observed and tabulated above.(Table 1 -10)

EFFECT OF TEMPERATURE

The respiratory electrodes were evaluated to study the effect of temperature on the performance of the sensor. The experiments were carried out using phosphate buffer with 10ppm of GGA as the substrate. In a typical experiment the 5ml of phosphate buffer was taken in the glass and the sensor was inserted in it in the holder and then connected to the BOD meter. The system was thermo stated to the required temperature (10°C). The oxygen reduction was monitored after the system attained a temperature and the first steady state was value was obtained. Then GGA solution was added to the phosphate buffer. The oxygen reduction current started decreasing and second steady state value was obtained. Experiments were carried out for the sensor at different temperatures. The change in oxygen reduction current were observed and tabulated.

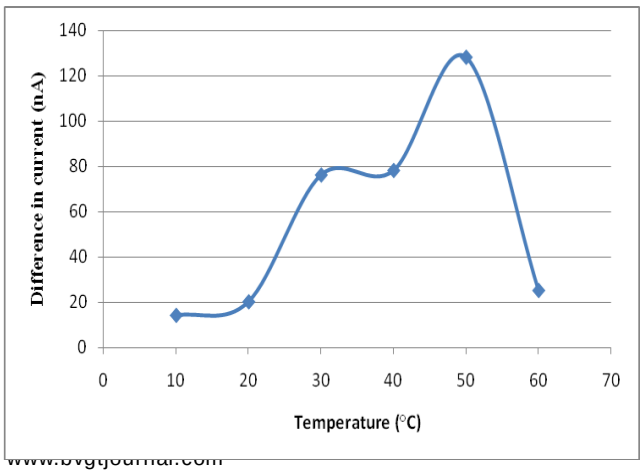
ASSIMILATION OF CARBOHYDRATES

The assimilating capacities of microorganism for carbohydrates were determined and a graphical representation was made for the determination of most assimilable carbohydrates. From the table it is inferred that assimilating capacity of the carbohydrates, the BOD of **Sucrose, Maltose and Fructose** when higher for this microorganism at its optimum pH and temperature.

Table. 10 Effect of pH

S.No	Temperature	Current
1.	10	14
2.	20	20
3.	30	76
4.	40	78
5.	50	128
6.	60	25

Fig. 7. Effect of temperature



ASSIMILATION OF AMINOACIDS

The assimilating capacities of microorganism for amino acids were determined and a graphical representation was made for the determination of most assimilable Amino acids. From the table it is inferred that assimilating capacity of the amino acids, the BOD of **Asparagines, Leucine and Alanine** were higher for this microorganism at its optimum pH and temperature.

ASSIMILATION OF ORGANIC ACIDS

The assimilating capacities of microorganism for organic acids were determined and a graphical representation was made for the determination of most assimilable organic acids From the table it is inferred that assimilating capacity of the Organic acids, the BOD of **Gluconic acid, Succinic acid and Ascorbic acid** were higher for this microorganism at its optimum pH and temperature.

ASSIMILATION OF ALCOHOLS

The assimilating capacities of microorganism for Alcohol were determined and a graphical representation was made for the determination of most assimilable alcohol. From the table it is inferred that assimilating capacity of the alcohol, the BOD of **Isobutanol, Benzyl alcohol and Amyl alcohol** were higher for this microorganism at its optimum pH and temperature.

ASSIMILATION OF ALDEHYDES

The assimilating capacities of microorganism for Aldehydes were determined and a graphical representation was made for the determination of most assimilable aldehydes. From the table it is inferred that assimilating capacity of the aldehydes , the BOD of **Benz aldehydes , Formaldehyde and Chinnamaldehydes** were higher for this microorganism at its optimum pH and temperature.

Real sample analysis

Real sample analysis is carried out to determine the biodegradable properties of microorganism .The oxygen consumed by the microorganism to degrade the substrate in an effluent is measured using the microbial sensor .The fresh effluent samples were first analyzed directly and then diluted approximately depending on their BOD load .When the samples were diluted there was a decrease in current difference compared to the results from the fresh samples .This so because change in composition would lead to

1. Increased in microorganisms respiration rate

2. High molecular weight substrates which were impermeable to the immobilized membrane become permeable.

3. On dilution the immobilized microorganism might identify and assimilate various organic substrates in distant metabolic pathways.

4. Resulting in different level of oxygen consumption.

Hence the dilution leads to the decrease in current difference,

The diluted samples were then incubated for 24 hours .Incubation leads to intense growth of microorganism and hence the overall oxygen content decrease and there is an increase in the Biological Oxygen Demand .The incubated samples were again aerated which again leads to an increase in overall oxygen concentration .A standard graph was drawn for estimation of real sample analysis and the oxygen concentration is monitored.

ASSIMILATION OF REAL SAMPLES

The assimilating capacities of microorganism for different types of real samples were determined.From the table the most assimilated samples are **Dye sample 'A' (deep pink colour effluent), Sugarcane Bagassee , Sugar molasses** in a higher amount at its optimum pH and temperature .These assimilable substrates contains **dye solution and fiber** material so the organism isolated from Textile effluent were assimilated it in a higher amount.

ASSIMILATION STUDIES FOR pH

It can be seen that the change in the oxygen reduction current varies as a function of the solution pH. A plot of solution pH vs. (current) is presented .It can be seen from the figure from that the change in the oxygen reduction current increases with the increase in the **pH 0.4** and then started decreasing after the pH value of **0.7**. It may be noted that the microorganism is highly active in **pH7** of the test solution. Prolonged stage at higher **pH>9** or lower **pH<4** deteriorated performance of the sensor.

ASSIMILATION STUDIES FOR TEMPERATURE

It shows that dissolved oxygen concentration increase with lowering temperature as the rate of reduction decreases in an exponential fashion with a temperature fall of **10**^{o}C.The optimum temperature for the microorganism is 30^{o}C. In this temperature microorganism shows maximum activity for assimilation and degradation of the substrates.

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