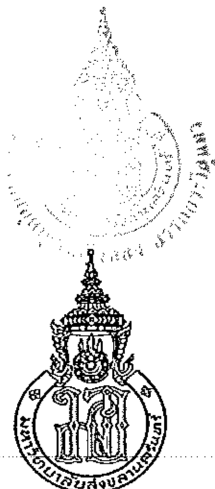

Cell-Based Biosensor for Organic Matter

Ampai Kumlanghan

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Prince of Songkla University
2008

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Major Program	Chemistry

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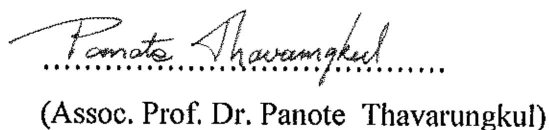

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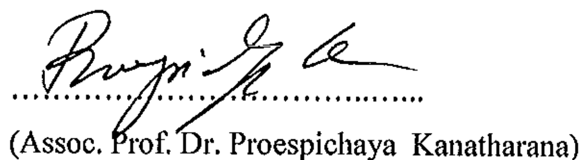

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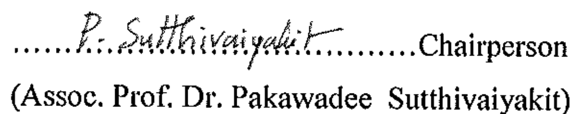
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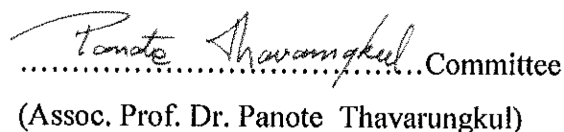
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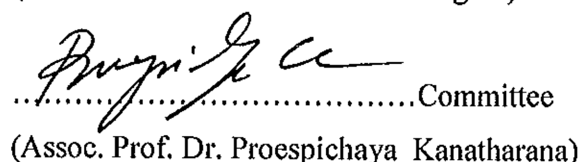
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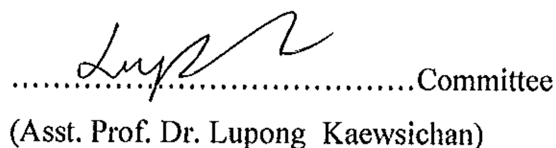
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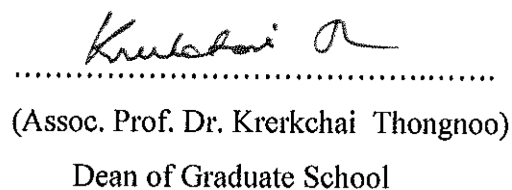
The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Chemistry



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ชื่อวิทยานิพนธ์ เซลล์เบสไบโอเซนเซอร์สำหรับสารอินทรีย์
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บทคัดย่อ

วิทยานิพนธ์นี้พัฒนาและทดสอบประสิทธิภาพของระบบเซลล์เบสไบโอเซนเซอร์สำหรับวิเคราะห์สารอินทรีย์ในรูปของค่าบีโอดี ระบบแรกพัฒนาขึ้นเพื่อใช้วัดค่าบีโอดีของน้ำเสียจากโรงงานน้ำยางข้นที่มีค่าบีโอดีสูง โดยใช้แอมเพอโรเมตริกไบโอเซนเซอร์ที่ใช้จุลินทรีย์หลายสายพันธุ์จากบ่อบำบัดน้ำเสียของโรงงานน้ำยางข้นเป็นวัสดุชีวภาพ และใช้ออกซิเจนอิเล็กโทรดเป็นทรานดิวเซอร์ในระบบไหลผ่าน ระบบเซนเซอร์ที่พัฒนาขึ้นนี้มีประสิทธิภาพดี โดยมีช่วงความเป็นเส้นตรงระหว่าง 2.0 – 25 และ 5.0 – 60 มิลลิกรัมต่อลิตร และมีขีดจำกัดการตรวจวัดอยู่ที่ 0.20 มิลลิกรัมต่อลิตรโดยใช้เวลาในการวิเคราะห์ประมาณ 10 – 15 นาที สามารถทำซ้ำได้โดยมีค่าเบี่ยงเบนมาตรฐานสัมพัทธ์อยู่ที่ 3.9% และสามารถใช้ได้เป็นเวลา 15 วัน (ค่าเบี่ยงเบนมาตรฐานสัมพัทธ์อยู่ที่ 0 - 6.7%) พบว่าเมื่อเทียบกับวิธีมาตรฐาน เทคนิคไบโอเซนเซอร์ที่พัฒนาขึ้นมีความแตกต่างเพียงร้อยละ 10

ทดสอบกระบวนการบำบัดน้ำเสียด้วยรีแอคเตอร์ไร้อากาศ โดยผ่านตัวอย่างน้ำเสียอย่างต่อเนื่องจากส่วนล่างถึงส่วนบนของรีแอคเตอร์ที่อัตราไหล 10, 20, 40 และ 80 มิลลิลิตรต่อวัน (30 วันต่อแต่ละอัตราการไหล รวมทั้งหมด 120 วัน) ที่อัตราไหล 10 มิลลิลิตรต่อวัน ให้ประสิทธิภาพสูงสุด สามารถลดปริมาณซีโอดีและบีโอดีของน้ำเสียได้ถึง 97% กระบวนการบำบัดน้ำเสียนี้มีประสิทธิภาพดีสำหรับน้ำเสียที่มีค่าบีโอดีสูง เทคนิคบีโอดีไบโอเซนเซอร์นี้สามารถใช้ได้ทั้งกับการตรวจวัดในกระบวนการบำบัดน้ำเสียด้วยรีแอคเตอร์ไร้อากาศแบบออนไลน์ และออฟไลน์

การศึกษาส่วนที่สองเป็นการพัฒนาเทคนิคไบโอเซนเซอร์โดยใช้หลักการของเซลล์เชื้อเพลิงจุลินทรีย์แบบแชมเบอร์เดียว (single-chamber) โดยบรรจุจุลินทรีย์ในส่วนแอโนดซึ่งแยกจากส่วนแคโทดด้วยเมมเบรนแลกเปลี่ยนโปรตอน (proton exchange membrane-PEM) เซนเซอร์นี้ใช้จุลินทรีย์ที่ไม่ต้องการอากาศในการหายใจหลายสายพันธุ์ในการวิเคราะห์การย่อยสลายสารอินทรีย์เมื่อจุลินทรีย์เร่งปฏิกิริยาออกซิเดชันทางชีวภาพของสารอินทรีย์จะเกิดการส่งผ่านอิเล็กตรอนไปยังขั้วแอโนดในการวิเคราะห์ แต่ละครั้งจะเปลี่ยนจุลินทรีย์ชุดใหม่ในปริมาณเท่าเดิม

ทุกครั้ง โดยการทดลองนี้ใช้สารละลายมาตรฐานกลูโคสเป็นตัวแทนของสารอินทรีย์ ศึกษาสภาวะที่เหมาะสมของระบบเซนเซอร์เซลล์เชื้อเพลิงจุลินทรีย์ประกอบด้วย การใช้ตัวต้านทาน 800 โอห์ม เป็นโหลดของวงจรภายนอกและใช้สารละลายฟอสเฟตบัฟเฟอร์เข้มข้น 25 มิลลิโมลาร์ และสารละลายโซเดียมคลอไรด์เข้มข้น 50 มิลลิโมลาร์ เป็นสารละลายในส่วนแคโทด อุณหภูมิที่เหมาะสมของส่วนแอโนดอยู่ที่ 37 องศาเซลเซียส ช่วงความเป็นเส้นตรงของสัญญาณการตอบสนองของสารละลายกลูโคสมาตรฐานให้ช่วงเส้นตรงจนถึง 25 กรัมต่อลิตร ขีดจำกัดการตรวจวัดอยู่ที่ 0.025 กรัมต่อลิตร มีความเป็นไปได้ที่จะนำเซลล์เชื้อเพลิงจุลินทรีย์ที่พัฒนาขึ้นนี้ไปใช้ในการตรวจวัดสารอินทรีย์แบบออนไลน์

การศึกษาส่วนที่สามเป็นการพัฒนาเทคนิคการวิเคราะห์ค่าบีโอดีในน้ำเสียโดยใช้เซลล์เชื้อเพลิงจุลินทรีย์แบบง่ายซึ่งใช้ยีสต์ *Saccharomyces cerevisiae* ในการย่อยสลายสารอินทรีย์ โดยใช้สะพานเกลือแบบง่ายแทน PEM ที่มีราคาแพง และกราไฟต์ไส้ดินสอเป็นอิเล็กโทรด ใช้สารละลายมาตรฐานกลูโคสเป็นสารอินทรีย์ในการทดสอบระบบ ได้ช่วงความเป็นเส้นตรงระหว่าง 8.0-100 มิลลิกรัมต่อลิตรและขีดจำกัดของการตรวจวัดที่ 0.50 มิลลิกรัมต่อลิตร ทำการทดสอบความสามารถในการทำซ้ำของเซลล์เชื้อเพลิงจุลินทรีย์ โดยใช้สารละลายกลูโคสมาตรฐานพบว่าหลังการวิเคราะห์ 5 ชั่วโมงและทำการวิเคราะห์ 30 ตัวอย่างมีค่าเบี่ยงเบนมาตรฐานสัมพัทธ์ที่ 14.7% เวลาที่ใช้ในการวิเคราะห์อยู่ที่ 3.0 – 10 นาที ระบบไบโอเซนเซอร์เซลล์เชื้อเพลิงจุลินทรีย์นี้สามารถใช้วิเคราะห์ค่าบีโอดีของน้ำเสียหลายประเภท สะพานเกลือที่ใช้มีขั้นตอนในการเตรียมง่ายและราคาถูกกว่า PEM มาก อย่างไรก็ตามควรเตรียมสะพานเกลือใหม่ทุกครั้งหรือทุกวัน

Thesis Title	Cell-Based Biosensor for Organic Matter
Author	Miss Ampai Kumlanghan
Major Program	Chemistry
Academic Year	2007

Abstract

This thesis focuses on the development and evaluation of the performance of cell-based biosensor systems for organic matter through the detection of biochemical oxygen demand (BOD). The first development was a sensor system for monitoring an anaerobic process for the treatment of high BOD levels in wastewater samples from a factory processing concentrated rubber latex. The amperometric cell-based biosensor based on flow injection analysis was investigated using mix cultures from activated sludge of a concentrated rubber latex factory as biological sensing element and oxygen electrode as a transducer. The sensor showed wide linear ranges, between 2.0–25 and 5.0–60 mg l^{-1}, and the limit of detection was found to be 0.20 mg l^{-1}. The sensor analysis time was around 10-15 min. The repeatability was observed with relative standard deviation of 3.9% and stability was over a period of 15 days (RSD of 0-6.7%). Good agreement between the results from biosensor method and standard method was obtained with a percentage difference of less than 10%. An anaerobic reactor treatment process was investigated by continuous feeding wastewater sample at flow rates 10, 20, 40 and 80 ml day^{-1} (30 days for each flow rate 120 days in total). At 10 ml day^{-1} the highest efficiency was obtained with percentage of COD and BOD reduction up to 97%. This treatment process showed good efficiency for high strength wastewater. The BOD biosensor was successfully applied to off-line and on-line monitoring of the anaerobic reactor treatment process.

The second study was to develop a biosensor based on a single-chamber microbial fuel cell in which anaerobes were retained in the anode compartment separated from the cathode compartment by a proton exchange membrane. In the sensor a replaceable anaerobic consortium was used for analyzing biodegradable organic matter. The anaerobes acted as biocatalysts in oxidizing

organic matter and transferring electrons to the anode. The biocatalysts were renewed for each sample analysis by replacing the old anaerobic consortium with an equal amount of fresh one. Glucose standard solution was used as the target substrate. To obtain the maximum sensor output, the MFC-based sensor system was optimized using an 800 Ohm resistor as the load to the external electric circuit and 25 mM phosphate buffer with 50 mM NaCl as catholyte in the aerobic compartment. The temperature of anaerobic compartment was maintained at optimal 37 °C. The cell potential across the electrodes increased with increasing loading of glucose. The sensor response was linear against concentration of glucose up to 25 g l^{-1}. The detection limit was found as 0.025 g l^{-1}. The microbial fuel cell with replaceable anaerobic consortium could be used as a biosensor for on-line monitoring of organic matter.

The third project was a simple microbial fuel cell for BOD analysis of wastewater using *Saccharomyces cerevisiae* to degrade the substrates. The MFC used a simple salt bridge to replace a more expensive proton exchange membrane (PEM) and pencil graphite carbon as the electrodes. Glucose standard solution was used as the substrate. The proposed system showed linear range between 8.0-100 mg l^{-1} and the detection limit was at 0.50 mg l^{-1}. The repeatability of the MFC was tested with the standard glucose solution producing a relative standard deviation of 14.7% for 30 samples tested over a period of 5 hours. The sensor response time was around 3.0 – 10 min. This MFC biosensor system can satisfactorily determine the BOD of several types of wastewater. The salt bridge was very simple to prepare and much cheaper than a PEM. However the salt bridge has to be freshly prepared.

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Ampai Kumlanghan

The Relevance of the Research Work to Thailand

The purpose of this Doctor of Philosophy Thesis in Chemistry (Analytical Chemistry) is to develop and evaluate the performances of BOD cell-based biosensors to analyze samples and to validate the results with the BOD_5 standard methods.

The developed BOD cell-based biosensor system can be applied for fast detection of BOD in wastewater samples. These systems can detect analyte with accuracy, good repeatability, using very short analysis time and no additional chemical is needed. Moreover, it is suitable for environmental on-line monitoring and process control. This will be useful for the evaluation of wastewater quality in Thailand.

The anaerobic treatment reactor can be applied to reduce BOD of wastewater from the rubber latex factory. This treatment process showed good efficiency for high strength wastewater. Thus it is possible that the anaerobic reactor can be applied to treat other wastewaters.

These techniques will be useful for the quantitative analysis of wastewater in environmental by several governmental and private organizations in Thailand which are the Ministry of Industry, the Ministry of Education and the Ministry of Science, Technology and Environmental.

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CHAPTER 1

Introduction

1.1 Background and Rationale

Wastewater generated by a variety of industries some of which contains high level of organic compounds. These include food processing (Nakhla *et al.*, 2006), latex rubber (Rakkoed *et al.*, 1999; Anotai *et al.*, 2007), pet food (Liu *et al.*, 2004; Kurian *et al.*, 2006) and bulk drug (Ganggagni Rao *et al.*, 2005). The discharge of such wastewater without prior treatment will have adverse effect on the environment. To prevent the release of harmful waste its treatment and monitoring before being discharged are necessary. The content of organic matter in wastewater is normally evaluated in terms of biochemical (biological) oxygen demand (BOD) which is one of the most widely used and important parameters for the estimation of water quality. The BOD standard method (BOD_5) to evaluate water quality requires the incubation of wastewater sample for 5 days at 20±1 °C in the dark. The determination of the oxygen consumption in the water is done at the end of the 5 days period by titrimetric method (APHA, 1992). Therefore, this standard method has the disadvantages of long analysis time and also requiring experience and skill to get reproducible results (Liu and Mattiasson, 2002). Due to its long analysis time, the wastewater needs to be retain, incurring much inconvenience, cost and space. Therefore, an alternative method which can rapidly determine BOD is necessary in order to overcome these disadvantages of the BOD_5 test. One promising method is biosensor.

A biosensor is an analytical device incorporating biological material, biologically derived material or biomimetic intimately associated with or integrated within a physicochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric or magnetic (Turner, 1999). Advantages of biosensors that are often cited are low cost, portable and simple-to-operate analytical tool (Mehrvar and Abdi, 2004). Biosensors for BOD determination have been developed using enzyme (Reiss *et al.*, 1998) and cells (Chee *et al.*, 1999;

Liu *et al.*, 2000). The limitation of enzyme-based BOD biosensor is its suitability for determining BOD of wastewater containing one or only a few types of macro-molecules (Reiss *et al.*, 1998). This limitation can be overcome by cell-based biosensor since cells can generally degrade several types of molecules (Wang *et al.*, 2005).

Cell-based biosensors developed for the measurement of BOD are generally based on respiratory activity of microorganisms. The response is usually detected as the change in concentration of dissolved oxygen (Liu and Mattiasson, 2002). This type of BOD biosensor has been applied to determine BOD in several types of wastewater such as dairy (Rastogi *et al.*, 2003; Tan and Wu, 1999), food processing (Liu *et al.*, 2000; Qian and Tan, 1998), pharmaceutical (Tan and Wu, 1999), distillery and tannery (Rastogi *et al.*, 2003). However, very few of these sensors were applied for online monitoring of BOD during the wastewater treatment process, for example the monitoring of the effluent from a municipal wastewater treatment plant (Vaiopoulou *et al.*, 2005). Since in Southern Thailand one of the most important industries is rubber and its wastewater contains high concentration of organics matter (Rakkoed *et al.*, 1999). Therefore, one of the aims of this thesis is to develop a cell-based BOD sensor and apply it for the monitoring of BOD during a treatment process of wastewater from concentrated rubber latex factory.

Fast determination of BOD has also recently been determined by microbial fuel cell (MFC). MFC is a device that converts chemical energy to electrical energy with the aid of the catalytic reaction of microorganisms (Jang *et al.*, 2004). The generated electric current output increases when the microorganisms oxidized substrate. The analyte concentration can then be measured from the generated current. The microbial fuel cell has an advantage over the oxygen electrode biosensor that it has long-term stability (Kim *et al.*, 2003). Therefore, another part of the thesis is devoted to the development of this MFC.

For the biological recognition part of the BOD biosensors a wide variety of microorganisms have been employed and the spectrum of substrates that can be measured depends on the types of microorgamisms (Liu and Mattiasson, 2002). Therefore, microorganism(s) that can degrade a wide range of substrates would be

preferred. These microorganisms can be single-culture or mix culture and this is also another subject that has been investigated in this work.

1.2 Objectives of the research

The objectives of this thesis are to develop and evaluate the performance of the cell-based biosensor technique for determination of organic matters in wastewater, use the BOD biosensor to monitor BOD during wastewater treatment and validate the results with standard method. To reach these objectives three subprojects using the cell-based biosensor were carried out as follows;

1. Microbial BOD sensor for concentrated rubber latex factory wastewater treatment monitoring where mix cultures from the concentrated rubber latex factory activated sludge was used as biological sensing element and oxygen electrode as a transducer. This system was applied to monitor the effluent of an anaerobic reactor used during the treatment process of wastewater from concentrated rubber latex factory.
2. Microbial fuel cell biosensor for fast analysis of biodegradable organic matter based on a single-chamber MFC separated by a proton exchange membrane where an anaerobic consortium was used to analyze biodegradable organic matters.
3. A simple microbial fuel cell for BOD analysis of wastewater using yeast to degrade the substrates and a simple salt bridge to replace a more expensive proton exchange membrane and pencil graphite carbon as the electrodes.

1.3 Benefits

It is expected that these cell-based biosensor techniques, with their several advantages i.e.: simple to construct, low cost, high stability, accuracy and precision and short analysis time, will become an alternative approach to detect organic matter in the environment.

CHAPTER 2

Cell-based Biosensor

2.1 Biosensor

A biosensor is an analytical device incorporating biological material, biologically derived material or biomimetic intimately associated with or integrated within a physicochemical transducer or transducing microsystem (Turner, 1999). The biological sensing element is responsible for the selective recognition of the analyte, generating the physicochemical signal monitored on the transducer and, ultimately, the sensitivity of the device. Many types of biological sensing element have been studied such as enzymes, organelles, whole cells or organisms and slices of animal or plant tissue. Another part of the sensor, a transducer serves to transfer the signal from the output domain of the recognition system to, mostly, the electrical domain. Detection principles commonly used in biosensor are electrochemical, optical, thermometric and piezoelectric (Collings and Caruso, 1997; Rogers, 2006; Turner, 1999; Thévenot *et al.*, 1999).

Figure 2.1 represents the principle of the operation of a biosensor. Analyte is first reacted with the biological sensing element such as enzyme, antibody, receptor and cell etc, which is connected to a suitable transducer such as electrochemical, optical, thermometric and piezoelectric etc. The transducer converts the biological signal into a measurable response such as current, potential or absorption of light through electrochemical or optical means, which can be further amplified, processed and stored for later analysis. The signal can result from a change in protons concentration, release or uptake of gases, light emission, absorption and so forth, brought about by the metabolism of the target compound by the biological sensing element (Lei *et al.*, 2006).

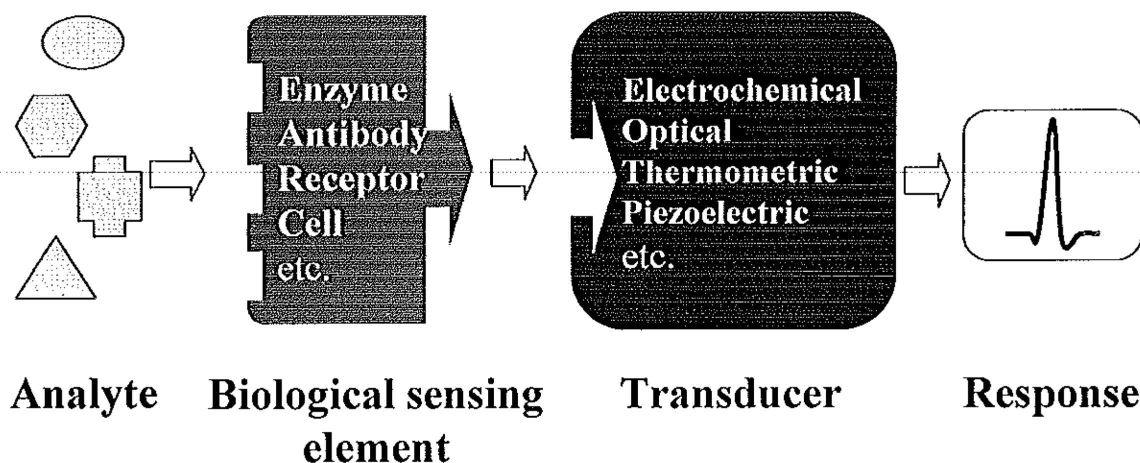


Figure 2.1 Schematic diagram of a biosensor. Analyte is reacted with the biological sensing element connected to a suitable transducer that converts the biological signal into a measurable response.

Biosensors have been applied in several fields, for example in process monitoring and control, medical, food and environmental monitoring (Yang and Bashir, 2008; Arya *et al.*, 2008; Song *et al.*, 2008; Rivas *et al.*, 2007; Wang *et al.*, 2005). Based on the reaction of the biological sensing element biosensors can be divided into two types, affinity and catalytic biosensors. Affinity biosensors are based on binding interactions between the immobilized sensing elements and the analytes of interest (Mattiasson, 1984; Taylor, 1991). Biological sensing elements in affinity biosensors include for example an antibody receptor and a single stranded DNA (Dong and Chen, 2002; Luppa *et al.*, 2001).

Affinity biosensor can be divided into two main categories; label-free (direct) and labeled (indirect). Label-free affinity biosensors measure the change in physical properties produce directly by the bind of affinity pair reaction. On the other hand, labeled affinity biosensors require a label on one of the biomolecules to produce a change in the property to be measured in a secondary reaction. Affinity biosensors have been used for detection of antigens, antibodies, proteins, DNA fragments and heavy metal ions (Iqbal *et al.*, 2000; Djellouli *et al.*, 2007; Corbisier *et al.*, 1999). Advantages of affinity biosensor are high sensitivity and selectivity but disadvantages are the troubles derived of regenerated electrode surface and cross-reactivity which is

often desirable in order to determine different congeners of the same family (Rodriguez-Mozaz *et al.*, 2004; D'Orazio, 2003; Berggren *et al.*, 2000)

The other type, catalytic biosensors are based on the detection of the change of solution property, consumption of substrate or the products of the convertion reaction of substrate by the biological sensing element. Changes cause by the catalytic reaction is detected by electrochemical, piezoelectric, optical and calorimetric transducers (D'Orazio, 2003; Thévenot *et al.*, 1999). The biological sensing elements in this category can be enzyme (mono or multi enzyme), whole cells (microrganisms, such as bacteria, fungi, eukaryotic cells and yeast), cell organelles and plant or animal tissue slice (Davis *et al.*, 1995; Dong and Chen, 2002; Gerard *et al.*, 2002; Luppa *et al.*, 2001; D'Orazio, 2003). Enzymes are suitable biological sensing elements because of their specificity and wide range of enzyme available, but the enzymes employed are generally expensive and unstable. To overcome this problem many cell-based biosensor has been developed.

Cell-based biosensor can be classified according to the different types of cells used for the biosensor construction. The most common are microbial biosensors that employ bacteria or yeast cells as biological sensing element (Baronian, 2004), mould (Baronian, 2004), tissue (Sidwell and Rechnitz, 1985; Lima *et al.*, 1997), fungi (Weitz *et al.*, 2002) and others types have also been studied.

2.2 Microbial biosensor

Microbes have a number of advantages as biological sensing materials in the fabrication of biosensors. They are present everywhere and are able to metabolise a wide range of chemical compounds. Microorganisms have a great capacity to adapt to adverse condition and to develop the ability to degrade new molecules with time. Microbes are also amenable for genetic modifications through mutation or through recombinant DNA technology and serve as an economical source of intracellular enzymes (D'Souza, 2001)

2.2.1 Natural microbes

The earlier microbial biosensors used the respiratory and metabolic functions of the microorganisms to detect a substance that is either a substrate or an

inhibitor of these processes (Lei *et al.,* 2006). To detect the substrate of microorganism's respiratory metabolic activity the reduction of dissolved oxygen is usually measured as in the case of BOD (Kim and Kwon, 1999; Liu *et al.,* 2000) and glucose (Choi *et al.,* 2003). On the other hand, the inhibition of bacterial respiration relies on the detection of the decrease of oxygen consumption rate. For examples, the use of *Trichosporon cutaneum* to monitor anionic surfactant in river water (Nomura *et al.,* 1998) and *Thiobacillus ferrooxidans* for the automated monitoring of KCN, Na_2S, and NaN_3 in water samples (Okochi *et al.,* 2004).

Cells have been used both in a viable or non-viable form. For viable cells pure culture has been investigated to determine several types of substrate such as biosensor for glucose (Niessen *et al.,* 2004; Choi *et al.,* 2003), lactate (Kim *et al.,* 2002), BOD (Kim and Kwon, 1999; Chee *et al.,* 1999; Konig *et al.,* 2000), *p*-nitrophenol (Lei *et al.,* 2002) and dichlorophenol (Tizzard *et al.,* 2004). Advantages of using pure culture are rapid, good stability, repeatability and selective. However, when a wide range of substrate need to be digested such as in the determination of BOD mix cultures and activated sludge are more useful.

Viable-mixed cultures bacteria have been used to determine BOD (Jia *et al.,* 2003) and the results showed that the biodegradable substrate spectrum could be expanded. Activated sludge collected from wastewater pond have also been applied for BOD determination (Liu and Mattiasson, 2002; Liu *et al.,* 2000; Rastogi *et al.,* 2003) and showed that it can be used to detect complex substrates in wastewater samples. It is rapid, simple, cost-effective with good repeatability and stability.

In addition to the use of viable cells, non-viable cells such as heat-killed, are sometimes employed. Heat-killed cells are generally investigated for their enzyme-cofactor systems which are sufficiently active to catalyse the substrates. For example, heat-killed bacteria were used to determine BOD of wastewater samples (Tan and Wu, 1999; Qian and Tan, 1998). Main advantages are the long stability, good repeatability and very convenient to store the sensor with out nutrients supply.

2.2.2 Genetic engineered cell

Recently, genetically engineered microorganisms have also been widely applied (Cai and Dobow 1997; Corbisier *et al.,* 1999; Sakaguchi *et al.,* 2003;

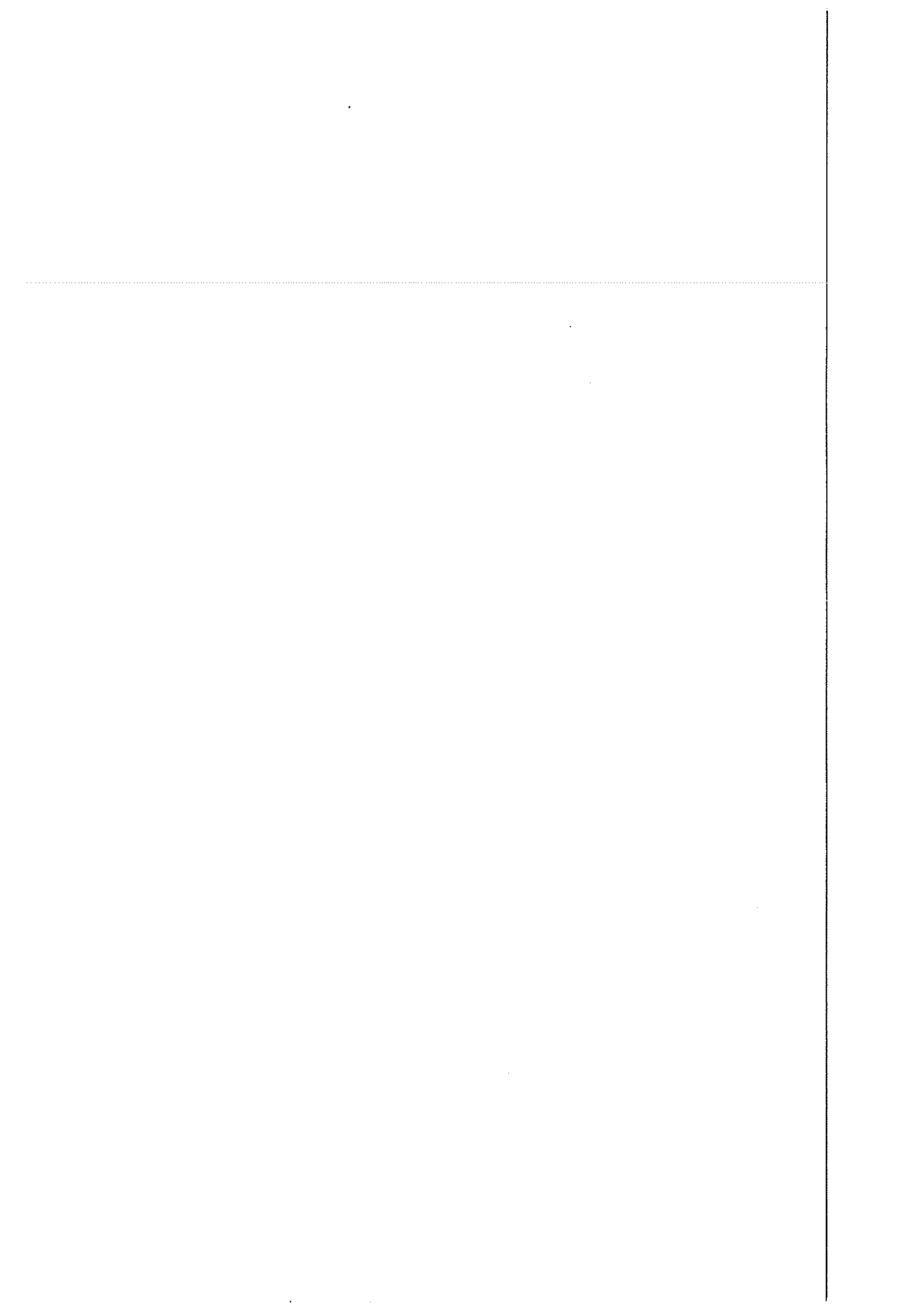
Islam *et al.,* 2005). Cells can be genetically altered to achieve fluorescence or bioluminescence when in contact with a targeted substance. Several genetically engineered bacteria have been developed to detect a wide range of analytes. For examples chromated copper arsenate (Cai and DuBow, 1997), heavy metal (Corbisier *et al.,* 1999) and BOD (Sakaguchi *et al.,* 2003). The advantages of using the genetically engineered bacteria are rapid and high sensitivity but it can be unstable.

2.3 Tissue biosensor

Tissue biosensors are based on the use of a slice of animal or plant tissue as biological sensing element. The tissue which contains high activity of the enzyme can be placed on the transducer surface. The enzyme will catalyze the conversion of substrate that is to be detected. Tissue-based biosensors have advantages such as low cost, simplicity of construction, and do not need cofactors for enzyme regeneration. It has been developed for application in food, agriculture, biotechnology, medicine, environmental studies and the military. Both plant and animal tissues have been investigated.

2.3.1 Plant tissues

The first tissue biosensor was made from banana pulp acting as a source of a polyphenol oxidase enzyme for sensing catecholamine (Figure 2.2) (Sidwell and Rechnitz, 1985). Several plant tissues are still widely studied because they are simple to prepare. These include the use of potato (*Ipomoea batatas* (L.) *Lam.*) as a source of peroxidase for determining hydroquinone in cosmetic creams (Vieira *et al.,* 2000). Avocado tissue (*Persea americana*) was developed as a source of polyphenol oxidase for the chronoamperometric determination of paracetamol in pharmaceutical formulations (Eatibello-Filho *et al.,* 2001). Polyphenol oxidase enzyme, present in the fibers of palm tree fruit (*Livistona chinensis*) tissue, was also proposed to determine epinephrine in pharmaceutical samples (S.Felix *et al.,* 2006). Mushroom (*Agaricus bisporus*) tissue containing alcohol oxidase activity was developed as a biosensor to determine ethyl alcohol (Akyilmaz and Dinckaya, 2000). This type of tissue was also used to detect phenolic compounds through the inhibition of polyphenol oxidase activity (Topcu *et al.,* 2004). Spinach (*Spinacia oleracea*)



tissue that contains an oxalate oxidase enzyme was used to determine oxalate in urine (Sezginturk and Dinckaya, 2003) and a glycolate oxidase enzyme in spinach leaf tissue was used to determine glycolic acid (Zhu *et al.,* 2004). Soybean tissue containing urease enzyme was developed to determine urea levels in urine sample (Qin *et al.,* 2000). Green bean (*Phaseolus vulgaris*) tissue, as a source of peroxidase, was used to determine caffeic acid in white wine samples (Fernandes *et al.,* 2007)

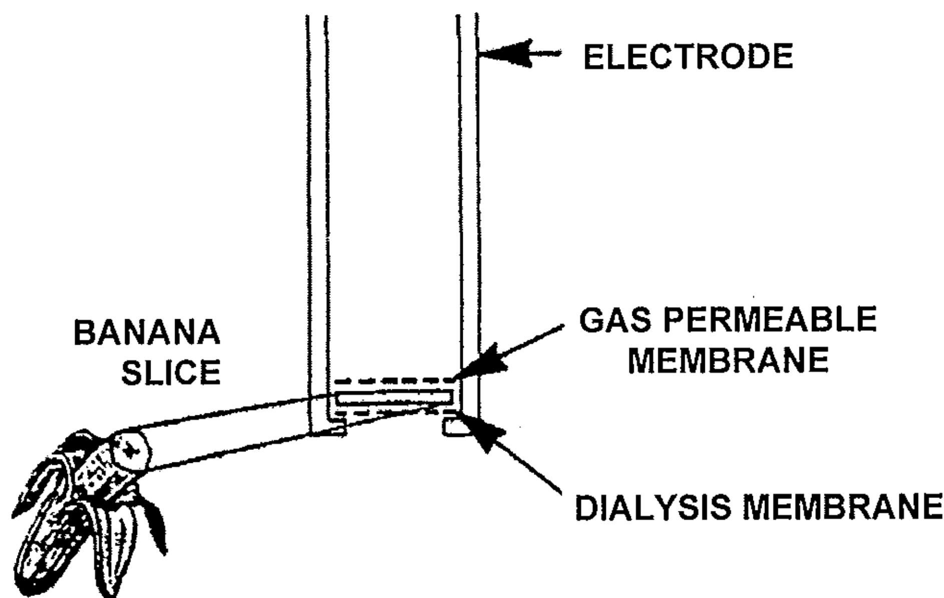


Figure 2.2 The physical construction of a banana tissue electrode (Sidwell and Rechnitz, 1985)

The advantages of plant tissue biosensor include short response times, low cost, simple and with no requirement for a co-factor for enzyme reactivation (Lima *et al.,* 1997; Sidwell and Rechnitz, 1985). Tissue-based biosensors have their own drawbacks. Their lifetime is comparatively short, and the reproducibility is not good (Sidwell and Rechnitz, 1985; Vieira *et al.,* 2000; S.Felix *et al.,* 2006; Eatibello-Filho *et al.,* 2001; Mei *et al.,* 2007).

2.3.2 Animal tissues

Animal tissues have also been applied in the detection of some analytes, although not as much as plant tissue. Recent applications are the liver tissue of pig. It contained urate oxidase and this was used to detect uric acid (Wu *et al.*, 2005). Porcine kidney tissue containing α-hydroxy acid oxidase has also been used to determine lactic acid (Wu *et al.*, 2005). The advantages of animal tissue biosensors are similar to plant tissue, i.e. low cost, and simplicity of construction but they often suffer from problems of long response times, low sensitivity (Wu *et al.*, 2005; Wu *et al.*, 2005).

2.4 Others

Several biosensors have employed some other biological sensing elements such as some fungi and moulds. For examples, the fungus *Aspergillus ustus* was used to determine tannic acid (Zhao *et al.*, 1998) and *Aspergillus niger* was used to detect hydrogen peroxide (Frebort *et al.*, 2000). The advantages of fungi are that often they will metabolise environmental compounds not metabolised by other biological sensors, they are suitable for use in the field, yield rapid results are simple to make but often loose their sensitivity quickly on storage (Zhao *et al.*, 1998; Frebort *et al.*, 2000).

2.5 Detection principle

Suitable transducing systems can be adapted for sensor assembly depending on the nature of the interaction with the biological detector and analyte. The physical transducers vary from electrochemical, spectroscopic, thermal, piezoelectric and surface acoustic wave technology (Gerard *et al.*, 2002). The most common transducers in cell-based biosensor are optical and electrochemical transducer and these will be reviewed here.

2.5.1 Optical transducer

The change of optical properties during interaction of the biocatalyst with the target analyte can be monitored by UV–vis absorption, bio- and chemi-luminescence, reflectance or fluorescence. The developments of whole cell optical

biosensors are mainly based on the detection of bioluminescence, fluorescence and the change of cell's color during the reaction.

2.5.2 Bioluminescence

Bioluminescence is associated with the emission of light by living microorganisms. The bacterial luminescence *lux* gene has been widely used as a reporter either in an inducible or constitutive manner (Lei *et al.*, 2006). The intensity of bioluminescence has to be linked to the concentration of the analyte. Bioluminescent microbial biosensors have been extensively investigated for the detection of heavy metals (Tibazarwa *et al.*, 2001; Tom-Petersen *et al.*, 2001), nutrients (Schreiter *et al.*, 2001), halogenated organic acids (Tauber *et al.*, 2001), and phenolic compounds (Philp *et al.*, 2003; Tiensing *et al.*, 2002). Advantages of bioluminescence include its rapid response with high sensitivity but methods to link the induction of the bioluminescence to the analyte can become complicated.

2.5.3 Fluorescence

Fluorescence spectroscopy has also been used as a reporter system in biosensors. Fluorescence biosensors have been prepared using the green fluorescence protein- and O_2-sensitive fluorescent material. In the former case the bioluminescent reporter *lux* gene, or the *gfp* gene coding for the green fluorescent protein (GFP), are fused to the host gene that allows for reporter activity to be examined in individual cells (Lei *et al.*, 2006). When a target compound passes through the cell membrane and binds to a regulatory protein this then activates transcription of the reporter gene, and subsequent translation of the reporter mRNA results in the production of a spectroscopically active reporter molecule as depicted in Figure 2.3. Detection of reporter molecules is most often achieved with standard colorimetry, fluorometry, or luminometry, so that the instrument response is related to the analyte e concentrations (Wells *et al.*, 2005).

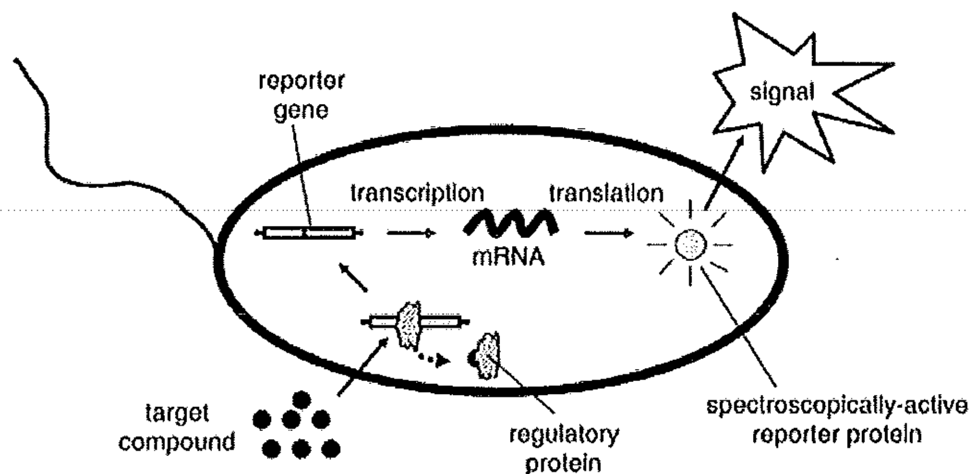


Figure 2.3 Generalized mode of bioreporter response to a target compound (Wells *et al.*, 2005).

The GFP-based microbial biosensor has been applied to investigate analytes such as arsenite (Wells *et al.*, 2005), toluene (Casavant *et al.*, 2003) and *N*-acyl homoserine lactones (Burmolle *et al.*, 2003). This type of biosensor can detect very low concentrations of analyte but modification of the cell that contains and produces the fluorescence is a complicated process.

In addition to the use of the green fluorescent protein, other fluorescent materials have been used in the construction of microbial biosensors. Recently, fiber-optical microbial sensors for determination of BOD have been reported (Dai *et al.*, 2004; Chee *et al.*, 2000).

This technique has many advantages as it is free of maintenance requirements, it shows a long-term stability, no calibration drift occurs, and it can detect very low concentrations of anlyte but it can give low reproducibility (Chee *et al.*, 2000).

2.5.4 Colorimetric biosensor

A biosensor based on a color change of cells has also been investigated. In the presence of toxins produced by microbial pathogens, the reporter cell undergoes a visible dose-dependant color change (Lei *et al.*, 2006). Some compounds

have been detected using colorimetric biosensor. For example, determination of organophosphates based on recombinant of *Escherichia coli* cells expressing organophosphorus hydrolase (OPH) on the cell surface that were immobilized in low melting temperature agarose on a nylon membrane and attached to a bifurcated fiber-optic bundle for light absorption detection of the products of enzyme-catalyzed organophosphate hydrolysis. The OPH-expressing *E. coli* cells catalyzed the hydrolysis of organophosphorus pesticides to form stoichiometric amounts of chromophoric products that absorb light at specific wavelengths (Mulchandani *et al.*, 1998). A colorimetric biosensor has advantages such as being very simple, low cost and high selectivity but they normally requires high concentrations of analyte and has low sensitivity (Mulchandani *et al.*, 1998; Lei *et al.*, 2006).

2.5.5 Electrochemical transducer

Four types of electrochemical transducers are used in microbial biosensors that is, amperometric, potentiometric, conductometric and fuel cell.

2.5.5.1 Amperometric transducer

Amperometry is the determination of the intensity of the current crossing an electrochemical cell under an imposed potential. This intensity is a function of the concentration of the electrochemically active species in the sample. Oxidation or reduction of a species is generally performed by a working electrode, and a second electrode acts as a reference. During electrolysis, the working electrode may act as an anode or a cathode (Canh, 1993). Amperometric cell-based biosensor operates at fixed potential with respect to a reference electrode.

Clark-type oxygen electrode is the most widely used amperometric transducer. It is used to measure dissolved oxygen change due to cell respiratory as shown in Figure 2.4.

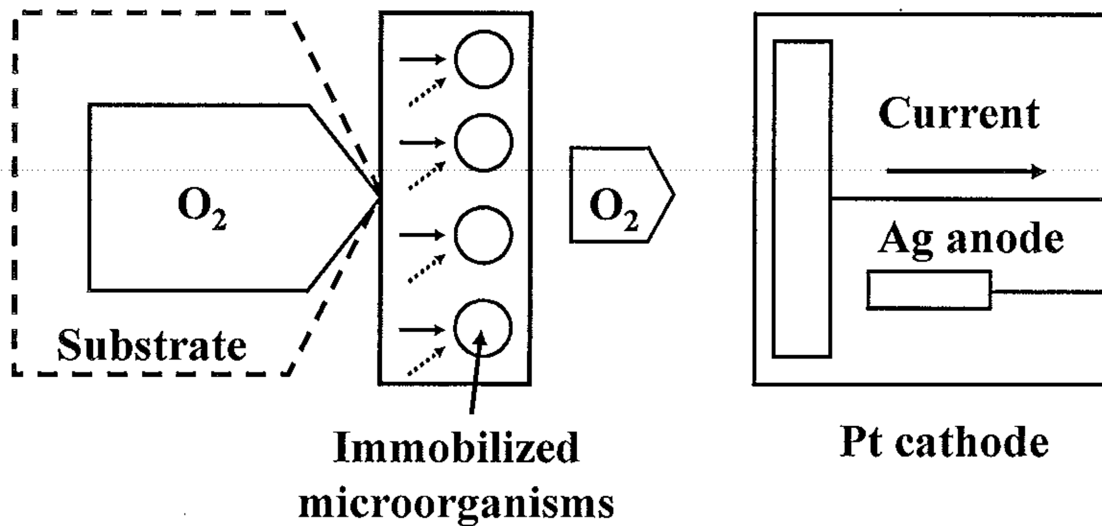
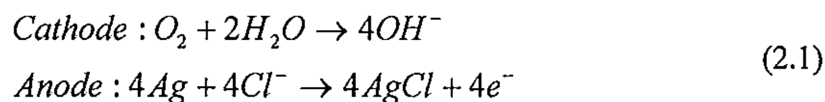


Figure 2.4 Respiration activity measurement using oxygen electrode (Karube and Nakanishi, 1994)

There are two types of the Clark-type oxygen electrodes polarographic and galvanic electrodes. A polarographic oxygen electrode consists of platinum (Pt) cathode and a silver (Ag) anode both immersed in the saturated potassium chloride solution, and for the membrane, a Teflon membrane is used (Figure 2.5(a)). A suitable polarization voltage between the anode and cathode selectivity reduces oxygen at the cathode, as shown in equation (2.1):

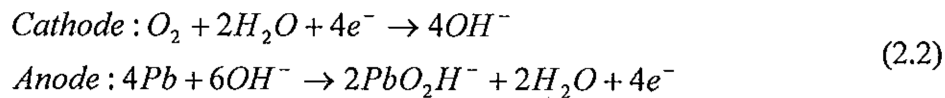


$$\begin{aligned} &Cathode: O_2 + 2H_2O \rightarrow 4OH^- \\ &Anode: 4Ag + 4Cl^- \rightarrow 4AgCl + 4e^- \end{aligned} \qquad (2.1)$$

These chemical reactions result in a current that is proportional to the dissolved oxygen concentration (Karube and Nakanishi, 1994).

Voltage is applied between the two electrodes so that the threshold diffusion current for oxygen is generated there. The oxygen which has passed through the membrane is reduced with the cathode. A reduction current in proportion to the dissolved oxygen is generated, and then the dissolved oxygen is measured.

A galvanic oxygen electrode is configured as shown in Figure 2.5(b). The cathode uses a noble metal (Ag), and the anode uses a base metal (Pb). For the electrolyte, a potassium hydroxide solution is used. For the membrane, a highly oxygen-permeable Teflon membrane is used. Therefore, it is a self driven electrode and dose not need an externally supplied voltage. The reactions are as follows in equation (2.2);



$$\begin{aligned} &Cathode: O_2 + 2H_2O + 4e^- \rightarrow 4OH^- \\ &Anode: 4Pb + 6OH^- \rightarrow 2PbO_2H^- + 2H_2O + 4e^- \end{aligned} \qquad (2.2)$$

Oxygen which has passed through the membrane is reduced with the working electrode. A reduction current in proportion to the concentration of the dissolved oxygen is generated, and then the dissolved oxygen is measured.

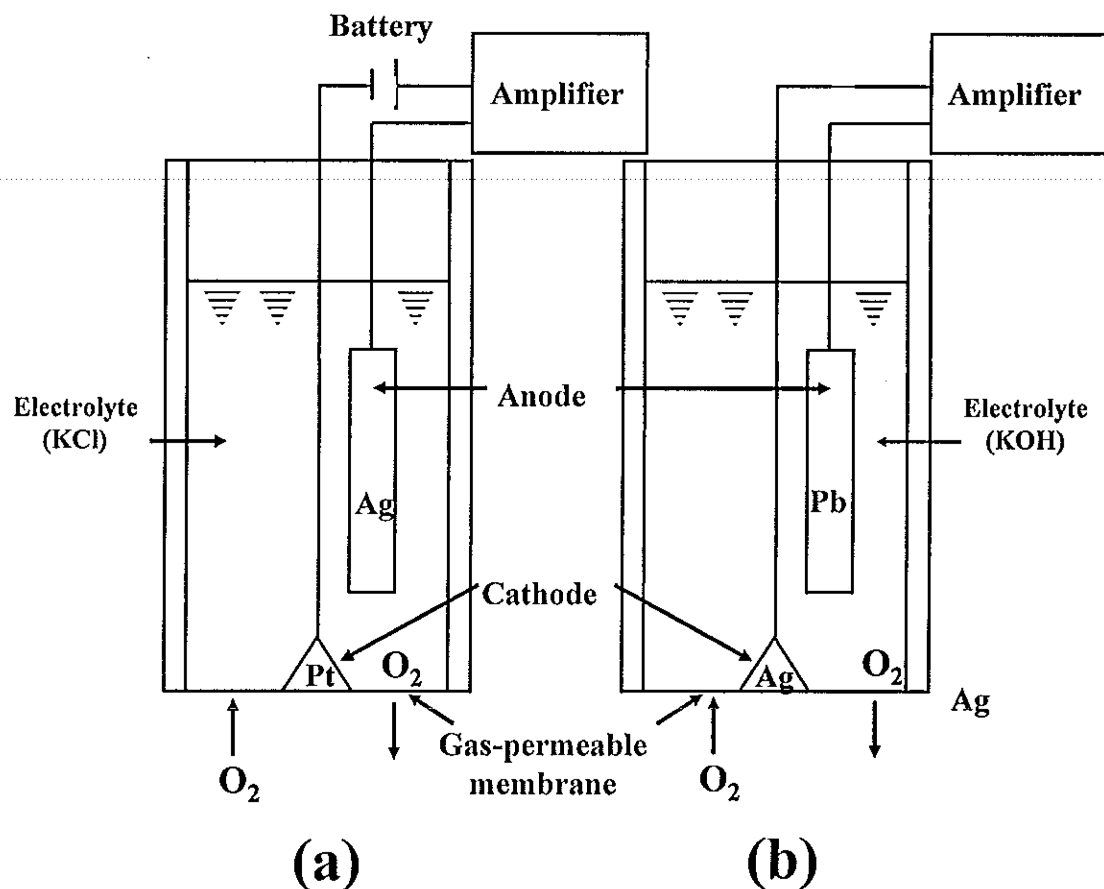


Figure 2.5 Principle of oxygen electrode. (a) Polarographic type (b) Galvanic type (Modified from Karube and Nakanishi, 1994)

The galvanic electrode is very simple and economical but it has disadvantages since it shows a slower response time and a shorter stability than a polarographic electrode (Karube and Nakanishi, 1994).

Oxygen electrode has been used with cell-based biosensor to detect several compounds (Table 2.1) such as polycyclic aromatic hydrocarbon (PAH) (Konig *et al.*, 2000), sulfuric acid (Dubey and Upadhyay, 2001), ethanol(Reshetilov *et al.*, 2001; Tkac *et al.*, 2002; Rotariu *et al.*, 2004; Akyilmaz and Dinckaya, 2005), Vitamin B1(Akyilmaz *et al.*, 2006), L-lysine amino acid (Akyilmaz *et al.*, 2007), surfactants (Taranova *et al.*, 2002), xylose (Reshetilov *et al.*, 1997), Choline (Stoytcheva *et al.*, 2006), benzene (Lanyon *et al.*, 2005), methanol, ethanol and

formaldehyde (Gonchar *et al.,* 1998), phenolic compounds (Timur *et al.,* 2004; Timur *et al.,* 2004), fructose (Heim *et al.,* 1999), *p*-nitrophenol (Mulchandani *et al.,* 2005), total sugar (Tkac *et al.,* 2000), biochemical oxygen demand (BOD) (Liu *et al.,* 2000; Chan *et al.,* 2000; Chen *et al.,* 2002; Jia *et al.,* 2003), amino acid, metal ions, polyphenols, glucose and ascorbic acid (Tai *et al.,* 1996).

A Clark-type oxygen electrode has also been used to determine hydrogen peroxide. In this case H_2O_2 is catalyzed by catalase from the bacteria to produce H_2O and O_2. The increase of O_2 detected by the oxygen electrode is proportional to the concentration of H_2O_2. This sensor has advantages such as long terms stability and high selectivity (Tai *et al.,* 1996).

In additional to the conventional oxygen electrode, a miniaturized oxygen electrode based on thick-film screen-printing has also been developed (Figure 2.6). The thick-film screen-printing technique was used to print the platinum-working electrode, Ag/AgCl reference electrode and platinum auxiliary electrode of the amperometric oxygen electrode on an inert substrate and potential was polarized at -900 mV (Heim *et al.,* 1999), carbon pastes electrodes have used to detect phenol (Mulchandani *et al.,* 2005), graphite electrodes also have used to measure total sugar (Tkac *et al.,* 2000).The detail of cell-based biosensor using oxygen electrode as the transducer is summarized in Table 2.1.

Table 2.1 Summary of recent cell-based biosensor using oxygen electrode

Microorganism	Analyte	Reference	Clark type		Other type
			Polarographic	Galvanic	
Sphingomonas yanoikuyae	PAH	Konig *et al.*, 2000			Chip fabrication electrode
Pseudomonas sp.	Sulfuric acid	Dubey and Upadhyay, 2001	√		
Pichia methanolica and *Gluconobacter oxydans*	Ethanol	Reshetilov *et al.*, 2001	√		
Gluconobacter oxydans	Ethanol	Tkac *et al.*, 2002			Glassy carbon electrode
Saccharomyces ellipsoideus	Ethanol	Rotariu *et al.*, 2004		√	
Candida tropicalis	Ethanol	Akyilmaz and Dinckaya, 2005		√	
Saccharomyces cerevisiae	Vitamin B1	Akyilmaz *et al.*, 2006		√	
Saccharomyces cerevisiae	L-lysine amino acid	Akyilmaz *et al.*, 2007	√		
Pseudomonas and *Achromobacter*	Surfactants	Taranova *et al.*, 2002	√		
Gluconobacter oxydans	Xylose	Reshetilov *et al.*, 1997	√		

Table 2.1 Summary of recent cell-based biosensor using oxygen electrode (continued)

Microorganism	Analyte	Reference	Clark type		Other type
			Polarographic	Galvanic	
Arthrobacter globiformis	Choline	Stoytcheva *et al.*, 2006		√	
Pseudomonas putida	Benzene	Lanyon *et al.*, 2005	√		
Hansenula polymorpha	Methanol, etnanol and formaldehyde	Gonchar *et al.*, 1998		√	
Pseudomonas putida	Phenolic compounds	Timur *et al.*, 2004			Screen printed graphite electrode
Pseudomonas putida	Phenolic compounds	Timur *et al.*, 2004			Thick film electrode
Rhodococcus erythropolis and *Issatchenkia orientalis*	Fructose	Heim *et al.*, 1999			screen-printed flow-through electrodes
Moraxella sp.	*p*-nitrophenol	Mulchandani *et al.*, 2005			Carbon paste electrode
Gluconobacter oxydans	Total sugar	Tkac *et al.*, 2000			graphite electrode

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Table 2.1 Summary of recent cell-based biosensor using oxygen electrode (continued)

Microorganism	Analyte	Reference	Clark type		Other type
			Polarographic	Galvanic	
Arxula Adeninivorans	BOD	Chan *et al.*, 1999		√	
Yeast	BOD	Chen *et al.*, 2002		√	
Trichosporon cutaneum and *Bacillus subtilis*	BOD	Jia *et al.*, 2003	√		
Activated sludge	BOD	Liu *et al.*, 2000	√		
Bacillus subtilis	amino acids, metal ions, polyphenols, glucose, and ascorbic acid	Tai *et al.*, 1996		√	

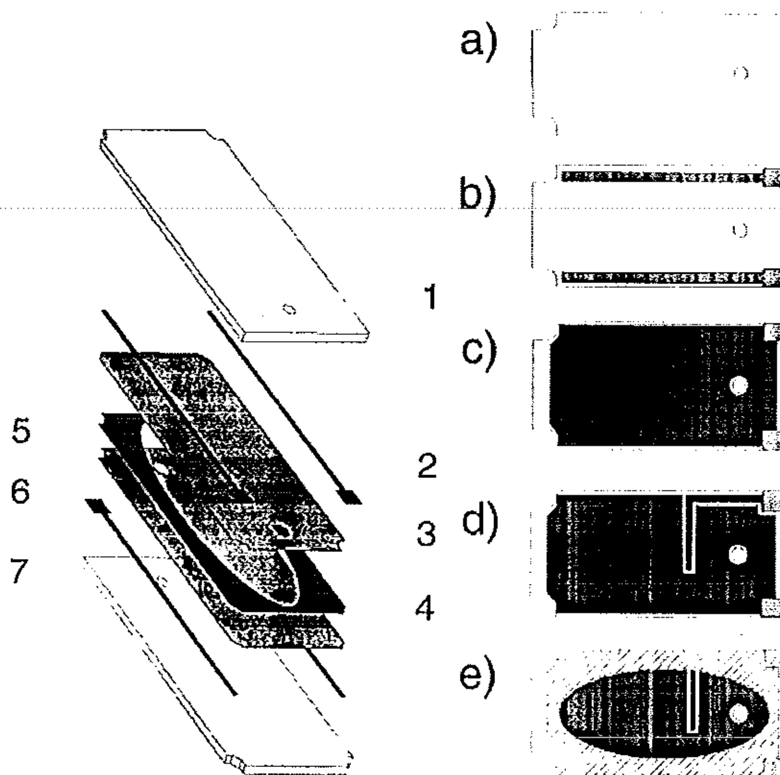


Figure 2.6 (a) Screen-printed flow-through sandwich electrode; 1= substrate (upper Electrode), 2= conducting wire and soldering wire, 3= surface of the electrode (e.g. Pt, Au or graphit), 4= flow channel, 5= surface of the lower electrode, 6= conducting wire and soldering wire, 7= substrate; (b)-(e) Same electrode design in another representation (Heim *et al.,* 1999).

2.5.5.2 Potentiometric transducer

In potentiometry the difference in potential between two electrodes immersed in a solution is measured. One of the electrodes probes the solution, while the other serves as a reference. The potentiometric measurements includes an ion-selective electrode (ISE), a reference electrode and a potential-measuring device (Figure 2.7)

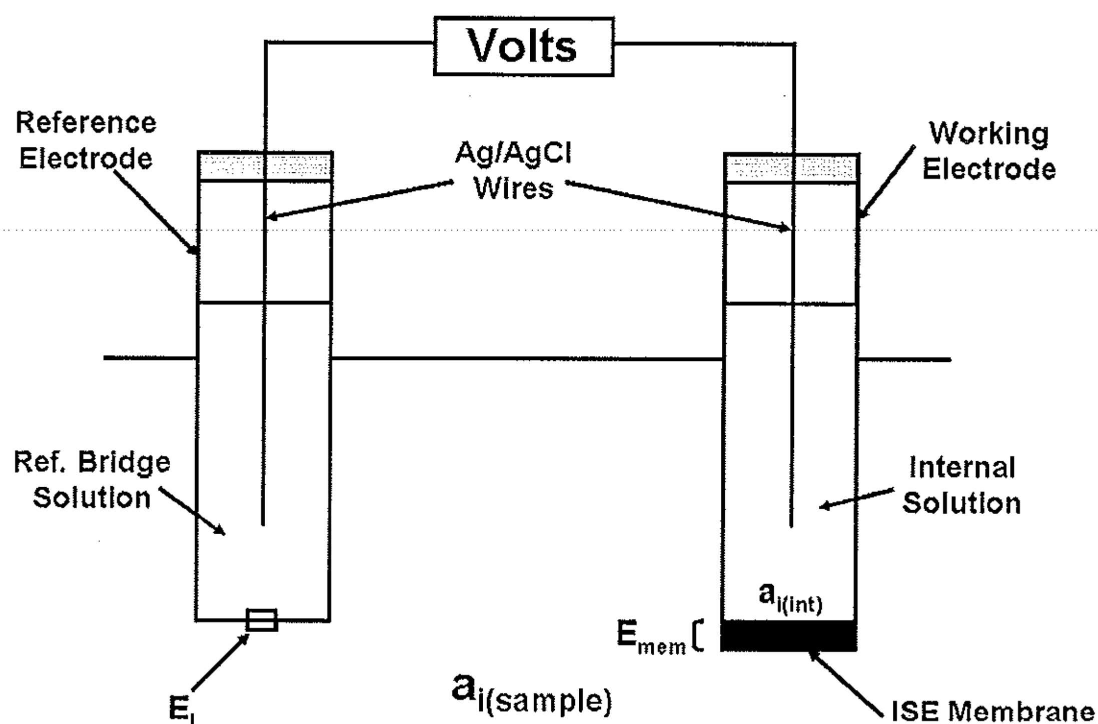


Figure 2.7 Schematic diagram of an electrochemical cell for potentiometric measurements (Wang, 2000).

Ion-selective electrodes (ISE)s are mainly membrane-based devices, consisting of a selectively permeable ion-conducting materials, that separates the sample from the inside of the electrode. The potential of ISE reflects the unequal distribution of the analyte ions across the boundary, is generally monitored relative to the potential of a reference electrode. Since the potential of the reference electrode is fixed, and the activity of the ion in the inner solution is constant, the measured cell potential reflects the potential of the ISE, and can thus be related to the activity of the target ion in the sample solution. The response of the ISE should be equation (2.3).

$$E = K + \left(2.303\frac{RT}{z_i F}\right)\log a_i \qquad (2.3)$$

Where E is the potential, and z_i and a_i are the ionic charge and activity, respectively. The constant K includes all samples –independent potential contributions, which depend upon various factors (Wang, 2000).

In a cell-based biosensor, a cell consuming analyte generates a change in potential resulting from ion accumulation or depletion and the signal is correlated to the concentration of the analyte. Several types of potentiometric cell-based biosensors have been studied. These include chloride and bromide electrodes with ion selective AgX-/Ag_2S membranes in combination with an Ag/AgC1 reference electrode to determine chlorinated and brominated hydrocarbons (Peter *et al.,* 1996), a pH sensitive field effect transistor (FET) contained either one or two FETs produced using a p-type silicon wafer, with Ta_20_5 as the pH-sensitive gate insulator to determine glucose (Reshetilov *et al.,* 1996). Another example is a chloride ion selective electrode based on a Ag_2S–AgCl membrane and a reference electrode for monitoring trichloroethylene (TCE) in batch and continuous modes in wastewaters (Han *et al.,* 2001; Han *et al.,* 2002). In this case the potentiometric oxygen electrode consists of a platinum electrode as redox indicator electrode and an Ag/AgCl electrode as reference electrode to detect sucrose (Rotariu *et al.,* 2002), or a potentiometric oxygen electrode to determine ethanol (Rotariu *et al.,* 2004).

For potentiometric cell-based biosensors the logarithmic relationship between the potential generated and analyte concentration, allows for the detection of a wide range of concentrations of analytes However, this method requires a very stable reference electrode, and this may be a limitation of these transducers (Lei *et al.,* 2006).

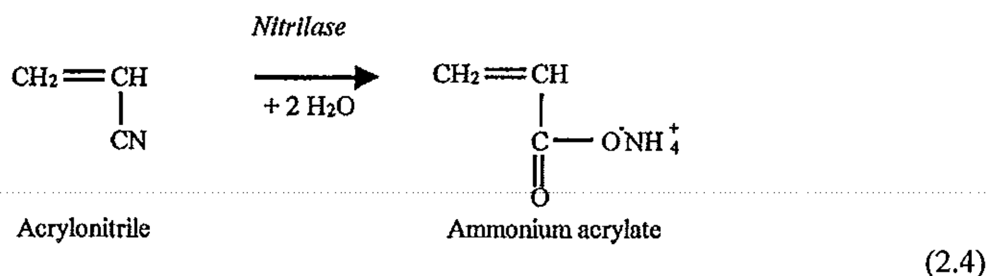
2.5.5.3 Conductimetric transducer

Conductivity measurements are made primarily to determine the concentration of a solution or to determine the relative amount of a salt in an aqueous solution. The principle employed is that of electrolytic conduction, in which the charge

carriers are provided by ionization. When a current flows through a volume of a solution, the soluble inorganic compounds in the solution will partially or completely separate into cation (positively charged ions) and anion (negatively charge ions). The ionizing current originates from two electrodes, a cathode and an anode. The cation will migrate to the cathode, at which they combine with the electrons of the current source to form hydrogen or metal atoms. The anions will migrate to the anode, at which the form neutral atom or molecules and liberate their electrons, which then flow to the current source (Norton, 1982).

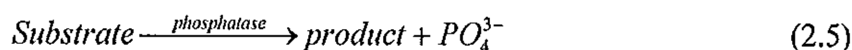
When the potential is applied across two electrodes, which are immersed in a solution, the current flowing through the circuit will be a function of the applied voltage and the resistance of the solution. This resistance, the reciprocal of conductance, in turn, is a function of the nature of solvent (water, and hence a constant factor, in the case of aqueous solution), of the number of ions present, and of the ion mobility (Norton, 1982; Mello and Kubota, 2002).

The conductivity measurement is given by the measurement of the conductance of the liquid solution between two electrodes. The most applications of conductimetric biosensors are based on the principle of changes in conductivity of the medium when microorganisms metabolize uncharged substrates, such as carbohydrates, to intermediates, such as lactic acid. The amount of the charged metabolite that accumulates is directly proportional to the growth rate of the organism and is easily quantifiable (Mello and Kubota, 2002). A conductimetric biosensor for the detection of acrylonitrile using *Rhodococcus ruber* was also developed. An ionic ammonium acrylate was produced from the non-ionic acrylonitrile results in a proportional increase in conductance (equation (2.4)), used to ascertain the acrylonitrile concentration of the solution (Roach *et al.*, 2003).



(2.4)

Chlorella vulgaris microalgae was used as a bioreceptor conductometric electrodes for alkaline phosphatase activity (APA) analysis. This sensor was used for the detection of toxic compounds, namely cadmium ions, in aquatic habitats. The detection of the local conductivity variations caused by algae enzymatic reactions could be achieved. The inhibition of *C. vulgaris* microalgae APA in presence of cadmium ions was measured. The principle of operation of such biosensor is based on the following reaction (equation (2.5)) (Chouteau *et al.*, 2004):



$$Substrate \xrightarrow{phosphatase} product + PO_4^{3-} \qquad (2.5)$$

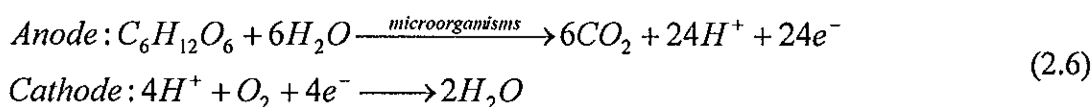
In addition, *Escherichia coli* sensors were used to investigate the effect chloride, nitrate, and sulphate species. The effect of changing salinity and nitrate concentration on the response of *E. coli* to 3,5-dichlorophenol and mercuric chloride was determined. The conductivity sensor proved robust enough to enable it to be used for repeat measurements (Bhatia *et al.,* 2003). The advantage of the conductrimetric transducer is its extremely sensitive conductance measurements and high stability but the detection of solution conductance is non-specific (Chouteau *et al.,* 2004; Roach *et al.,* 2003; Lei *et al.,* 2006).

2.5.5.4 Fuel cell

Fuel cells are galvanic cells, in which the free energy of a chemical reaction is converted into electrical energy (via an electrical current) and involves the use of an anode, a cathode, with a supporting electrolyte medium to connect the two electrode, and an external circuit to detect the electricity (Hobson *et al.,* 1996; Carrette *et al.,* 2001).

A microbial fuel cell (MFC) is a device that converts chemical energy to electrical energy with the aid of the catalytic reaction of microorganisms (Jang *et al.,* 2004). A microbial fuel cell is obtained through microorganisms in the form of metabolic intermediaries or as the final product of the microorganism's respiration.

A MFC system normally consists of an anode compartment with a negative electrode and a cathode compartment with a positive electrode (Figure 2.8). Two compartments are normally separated by a proton exchange membrane (PEM). In the anode compartment microorganisms oxidize substrate, e.g. oxidation of glucose results in generating electrons and protons (Grzebyk and Pozniak, 2004). The generated protons migrate from the anode compartment to the cathode compartment through the PEM. The produced electrons are transferred to the anode and then pass through an external electric circuit to the cathode, where they reduce oxygen to form H_2O. The reactions at the anode and cathode are:



$$\begin{aligned} &Anode: C_6H_{12}O_6 + 6H_2O \xrightarrow{microorganisms} 6CO_2 + 24H^+ + 24e^- \\ &Cathode: 4H^+ + O_2 + 4e^- \longrightarrow 2H_2O \end{aligned} \qquad (2.6)$$

The flow of electrons through the circuit forms a measurable current. The overall reaction requires the metabolism of a biodegradable organic matter to carbon dioxide and water with electricity as a product (Jang *et al.,* 2004; Bennetto, 1990; Kim and Park, 2004).

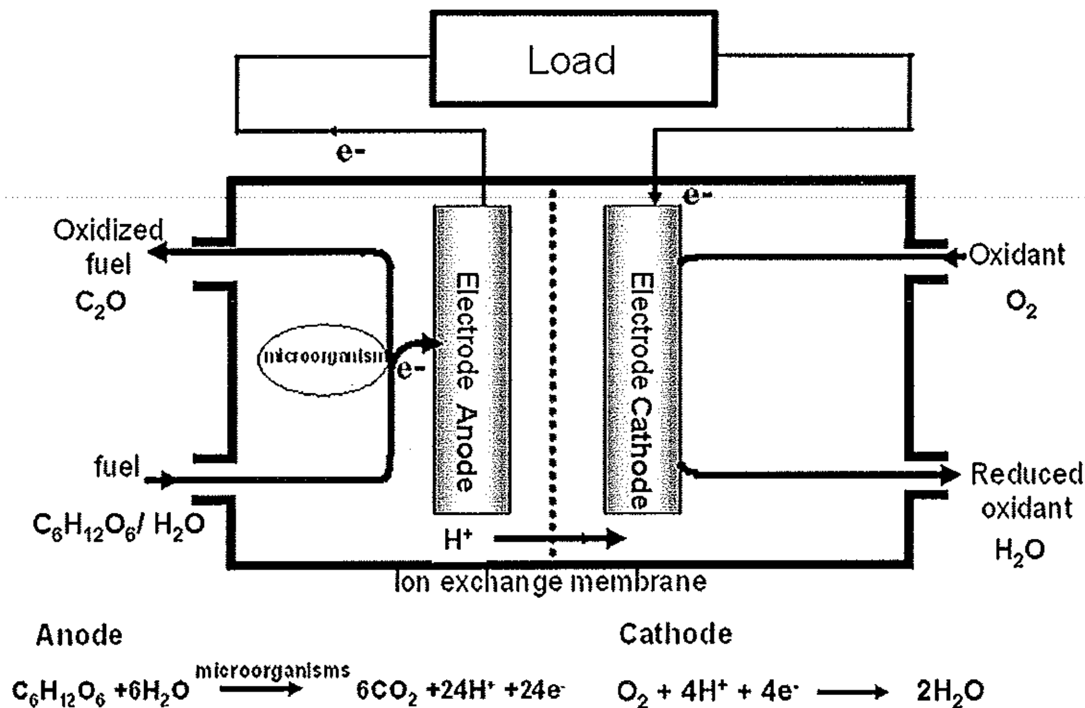


Figure 2.8 Schematic diagram of a microbial fuel cell. The microorganisms oxidize substrate in anode compartment to generate electrons and protons. The generated protons migrate to the cathode compartment through the PEM. The produced electrons are pass through the cathode, where they reduce oxygen to form H_2O.

Many types of feedstock have been used as the substrate for MFC, such as starch (Niessen *et al.*, 2004), glucose (Bennetto, 1990; Yogishita *et al.*, 2003; Niessen *et al.*, 2004), lactate (Kim *et al.*, 2002), cysteine (Logan *et al.*, 2005) and wastewater (Gil *et al.*, 2003).

Mediators have also been used to enhance the electron transfer (Figure 2.9). MFC-based sensors have the advantage that they have long-term stability (Kim *et al.*, 2000; Pasco *et al.*, 2004) and can be used continuously for on-line monitoring in particular for wastewater (Chang *et al.*, 2004; Kim *et al.*, 2000). More details are shown in Table 2.2

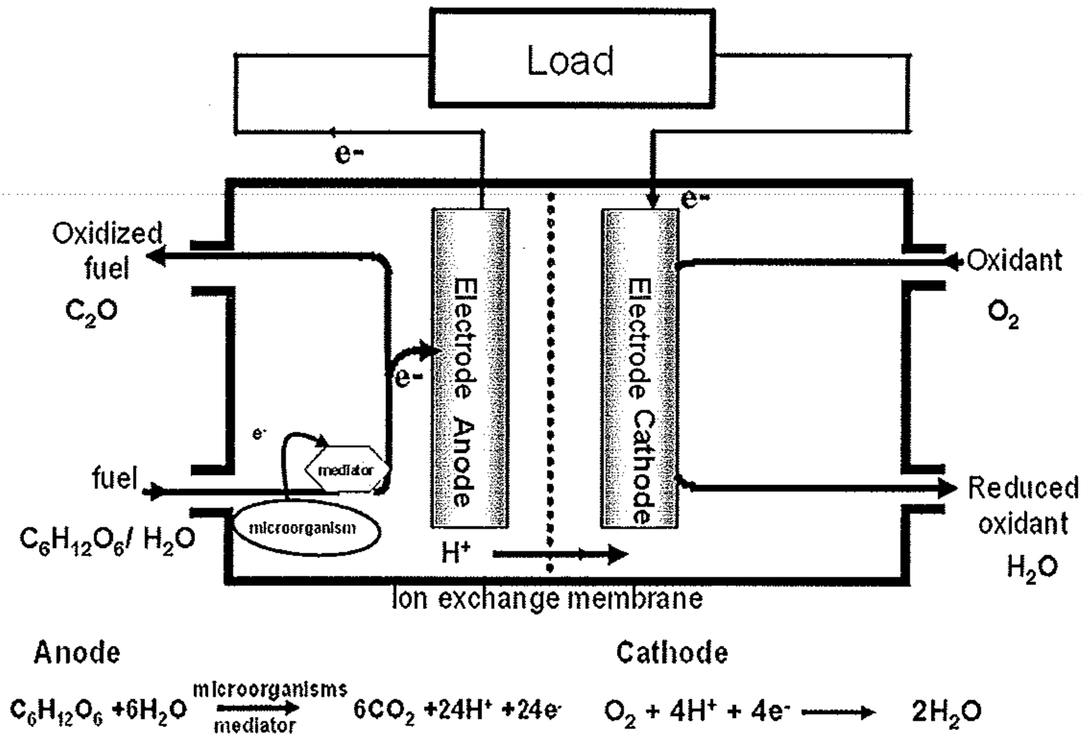


Figure 2.9 The principle of microbial fuel cell with the mediator. The microorganisms oxidize substrate with mediator in anode compartment to generate electrons and protons. The generated protons migrate to the cathode compartment through the PEM. The produced electrons are pass through the cathode, where they reduce oxygen to form H_2O.

Table 2.2 Summary of microbial fuel cell biosensor

Microorganism	Analyte	Reference	Mediator -less	Mediator
Clostridium spp.	Starch	Niessen *et al.*, 2004	√	
Escherichia coli	Glucose	Bennetto, 1999	√	
Escherichia coli	Glucose	Niessen *et al.*, 2004	√	
Synechocystis sp.	Glucose	Yogishita *et al.*, 2003	√	
Gluconobacter oxydans	Glucose	Lee *et al.*, 2002	√	
Shewanella spp.	Cysteine	Logan *et al.*, 2005	√	
Activated sludge	BOD	Chang *et al.*, 2004		√ (Azide and cyanide)
Proteus velgaris	BOD	Pasco *et al.*, 2004		√ (ferricyanide)
Proteus velgaris	Mono-and disaccharides	Kim *et al.*, 2000		√ (thionin)

2.6 Immobilization methods

Immobilization methods are an important step in the development of biosensors. It not only helps to achieve the required close proximity between the biological sensing element and transducer, but also to stabilize the biological sensing element for reuse (D'Souza, 2001). The aim of immobilization method is to produce a thin film of immobilized biologically active material on or near the transducer surface. The immobilization of cell-based biosensors can be divided into chemical or physical methods (Lei *et al.*, 2006).

2.6.1 Chemical methods

The chemical methods of cells immobilization compose of covalent binding and cross-linking.

2.6.1.1 Covalent binding

The immobilization of microorganisms by covalent binding can be achieved through covalent bonds between functional groups on the cell surface such as amines, carboxylic acids or sulphydryl groups and the transducer such as an amine, carboxylic, epoxy or tosyl derivative (Cohen, 2001; Lei *et al.,* 2006) (Figure 2.10). During the immobilization process cells are exposed to harmful chemicals and harsh reaction condition, and this may damage the cell membrane and decrease the biological activity or affect the cell's viability. Therefore, this method has not been used successfully for the immobilization of viable cells (D'Souza, 2001; Lei *et al.,* 2006).

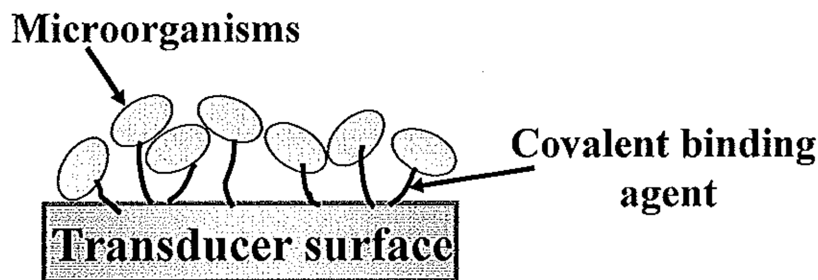


Figure 2.10 Immobilization of microorganisms on transducer surface by covalent binding.

2.6.1.2 Cross-linking

Cross-linking involves bridging between functional groups on the outer membrane of the cells by multifunctional reagents such as glutaraldehyde and cyanuric chloride, onto the transducer surface (Lei *et al.,* 2006) (Figure 2.11). Many types of chemicals have been used such as gelatine and glutaraldehyde (Akyilmaz *et al.,* 2007),

and egg white foam with glutaraldehyde (Marolia and D'Souza, 1999). The method is relatively easy to perform, however, cell viability and/or the cell membrane biomolecules can be affected by the cross-linking agents.

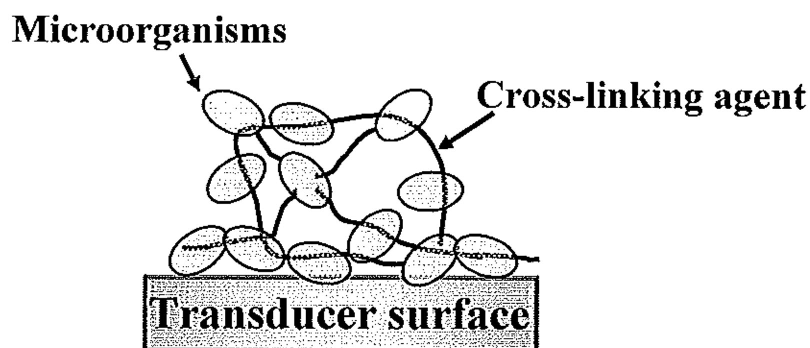


Figure 2.11 Immobilization of microorganisms on transducer surface by cross-linking.

2.6.2 Physical methods

Physical immobilization includes adsorption and entrapment

2.6.2.1 Adsorption

Physical adsorption involves van der Waals forces, ionic binding or hydrophobic forces. In early work, immobilization by adsorption was used successfully to couple proteins to various solid substrates, including derivatized glass, plastics and silicone rubber (Catt *et al.*, 1970).

The adsorption of cells is based on electrostatic interactions between the charged support or electrode and cells (Figure 2.12). Several types of supported matrices have been used such as a GF/A chromatographic paper (Lobanov *et al.*, 2001; Reshetilov *et al.*, 2001; Bhatia *et al.*, 2003; Guliy *et al.*, 2003; Emelyanova *et al.*, 2002), acetylcellulose membranes (Dubey and Upadhyay *et al.*, 2001) and polytetrafluoroethylene (PTFE) membranes (Han *et al.*, 2002).

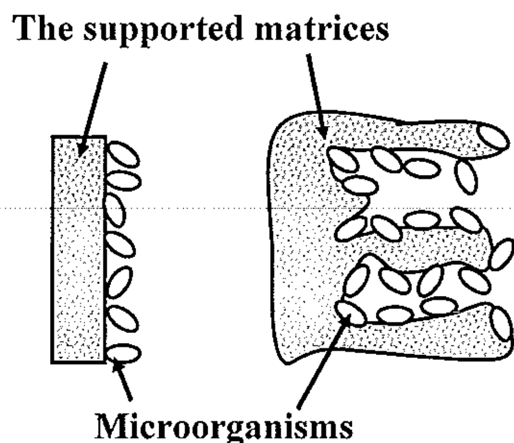


Figure 2.12 Immobilization of microorganisms on the supported matrices by adsorption.

The main advantage of the adsorption method is that it is a simple method that can be performed under mild conditions. However, biological sensing elements immobilized through adsorption exhibit a certain degree of reversibility, and with few exceptions, the forces involved in the binding are not very strong so it gives poor stability because of desorption of cells (Liu and Mattiasson, 2002; Lei *et al.*, 2006).

2.6.2.2 Entrapment

Immobilization of microorganisms by entrapment can be achieved by either retention of the cells in close proximity to the transducer surface using dialysis or filter membrane or in chemical/biological polymers/gels (Lei *et al.*, 2006). The entrapment of cells in polymer matrices is the most extensively studied method of cell immobilization (Kabube and Nakanishi, 1994; Liu and Mattiasson, 2002).

Gel entrapment methods mainly use gelation mechanisms to entrap cells into a porous polymeric structure, allowing transport of substrates and products but restraining the cells. Natural monomers are usually brought to gelation by either cooling and/or contact with a solution that contains different ions (Gerbsch and Buchholz, 1995). Several gel matrices have been used such as alginate (He *et al.*, 2004; Adinarayana *et al.*,

2004; Sohn *et al.,* 1995), K-carrageenan (Held *et al.,* 2002), agarose (Tammeveski *et al.,* 1998), and collagen (Medina., 2001). Among these entrapment of cells in alginate gel is popular because it is simple, low cost and very suitable for immobilization of viable cells.

Alginate, is a major structural polysaccharide of marine brown algae, and contains β-D-mannopyranosyl uronate and α-L-gulopyranosyl uronate in regular (1-4)-linked sequences. The alginate structures can be ionically linked by divalent ions such as calcium (Kierstan and Coughlan, 1985). This process is reversible and counteracted by chelating agents such as citric acid or EDTA. The chemical and macroscopical structures are presented in Figure 2.13. It usually forms in bead gel as shown in Figure 2.14. There have been several studies on the composition of alginate and their suitability for cell immobilization (Kierstan and Coughlan, 1985; Gerbsch and Buchhholz, 1995).

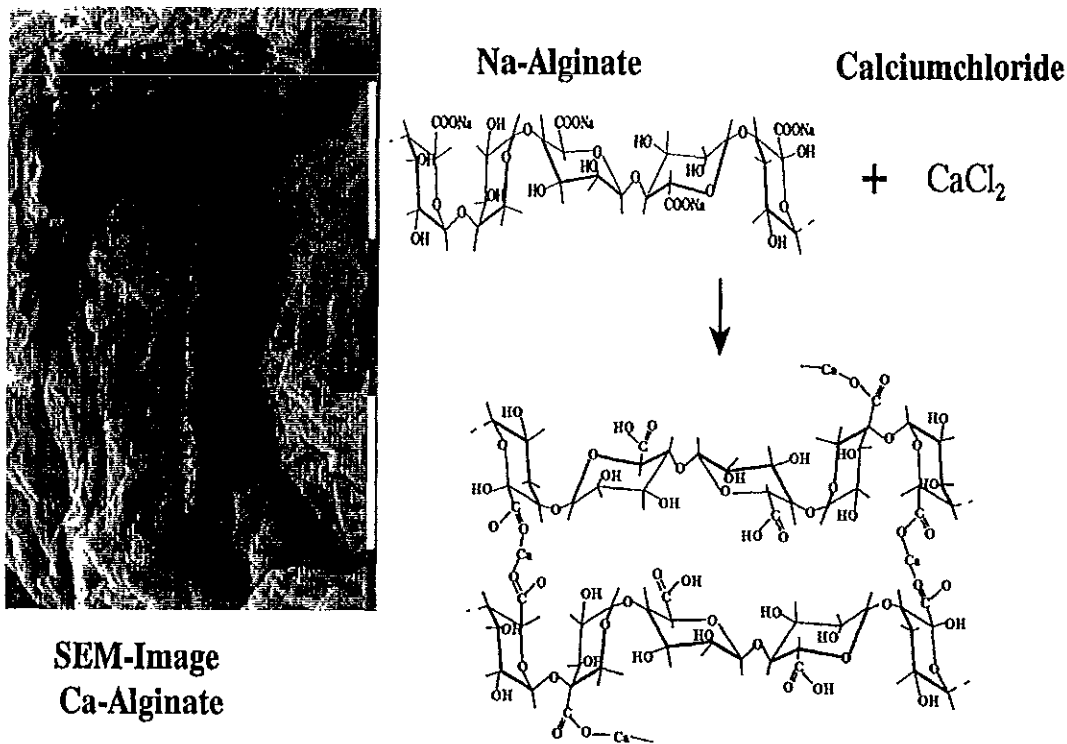


Figure 2.13 Calcium alginate: chemical and macroscopical structure (Gerbsch and Buchhholz, 1995).

Synthetic polymers can also be brought to gelation by a wide range of chemical or photochemical reactions. Polyacrylamide, cryopolyacrylamide (Guliy *et al.*, 2003) and poly(carbamoyl)sulfonate (Chan *et al.*, 2000) have been used for cell entrapment. This polymer is very simple to use (Chan *et al.*, 2000; Guliy *et al.*, 2003)

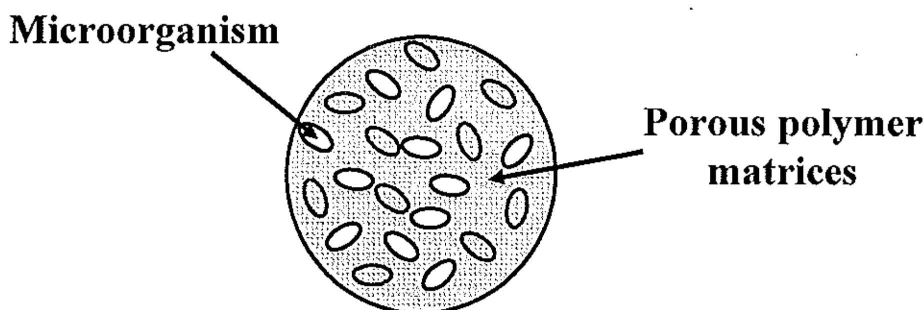


Figure 2.14 Immobilization of microorganisms in the porous polymer bead by entrapment.

For membrane entrapment used in biosensors the principle is the separation by membranes. Cells are enclosed on the transducer surface using dialysis or in chemical/biological polymers/gels such as a filter membrane (Yano *et al.*, 2001), or nucleopore membrane (Bhatia *et al.*, 2003). Figure 2.15 shows the preparation of an entrapped microorganism membrane. A Clark-type oxygen electrode consists of a Teflon membrane, a platinum cathode, an aluminium anode and saturated a potassium chloride electrolyte. The microorganism immobilized on the membrane is placed on the Teflon membrane and fixed in place using a bored cap or a dialysis membrane/ O-ring (Karube and Suzuki, 1990).

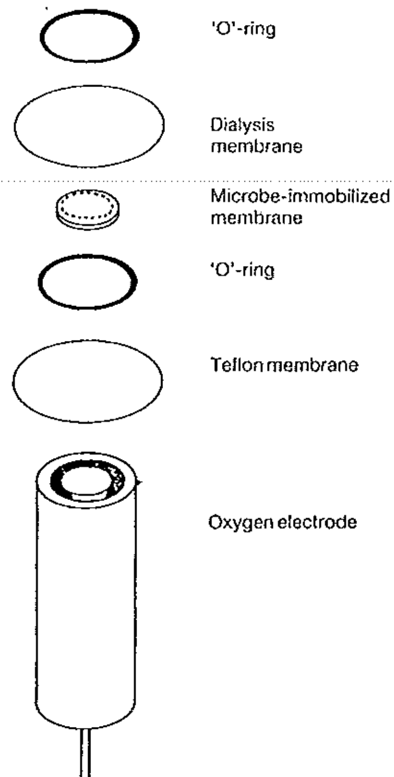


Figure 2.15 The preparation of a membrane with entrapped microorganism (Karube and Suzuki, 1990).

The advantages of entrapment immobilization are that the process is very simple and low cost but their disadvantage is the additional diffusion resistance offered by the entrapment material, which will result in lower sensitivity and detection limits (Lei *et al.*, 2006).

2.7 Applications of cell-based biosensor

Cell-based biosensors have a potential for continuous use and application in several fields such as environment monitoring, food analysis and clinical chemistry.

2.7.1 Environment

The major application of cell-based biosensors is presently applied in the environmental field. For instance, many substrates in wastewater samples have been determined including toxicants such as metal, alkanes, aromatic compounds, polycyclic aromatic hydrocarbons (PAHs), phenolic compounds (Nandakumar and Mattiasson, 1999), chlorophenol (Riedel *et al.*, 1995), cyanide (Ikebukuro *et al.*, 1996) and especially the organic matter that contributes to BOD (Karube and Nakanishi, 1994; Liu and Mattiasson, 2002; D'Souza, 2001). The advantages of these sensors are simplicity, low cost, good sensitivity, low detection limit and good correlation with the standard method. For the determination of toxic substances or to specifically monitor only one compound, it is necessary to adapt the selectivity of the biosensor before use.

2.7.2 Food analysis

Cell-based biosensors have been applied to the food industry for many objectives such as quality control, monitoring nutritional status and quality assurance. For example, a microbial biosensor based on immobilised yeast, *Saccharomyces cerevisiae,* using a potentiometric oxygen electrode has been developed to determine the amount of sucrose in soft drinks. Comparing results from the biosensor with those from a colorimetric spectrophotometric method produced a good correlation (Rotariu *et al.*, 2002). A similar cell-based biosensor was also developed by the same research group to analyze ethanol in alcoholic beverages. The advantage of these biosensors is their construction simplicity, low cost and low detection limit but there were few applications in food analysis since it may be the cause of contaminant (Lei *et al.*, 2006).

2.7.3 Clinical chemistry

Cell-based biosensors have also been used in clinical chemistry to determine and monitor substrates in blood or blood serum. For instance, a lactate biosensor has been developed using yeast *Hansenula anomala* to determine lactate in blood and blood plasma. Although this sensor gave a good reproducibility and good correlation to results from a spectrophotometric test but it required a long response time (Racek and Musil, 1987)

CHAPTER 3

Cell-based biosensors for BOD

Biochemical oxygen demand (BOD) is an important parameters in water quality control. It defined as the oxygen required by aerobic microbes to metabolise organic wastes over 5 days at 20±1 °C, and this is the standard method for detecting the amount of biodegradable organic material in water. The conventional BOD test has certain benefits such as being universally applicable to most wastewater samples, and, furthermore, no expensive equipment is needed. It has, however, the limitation of being time-consuming, and consequently it is totally unsuitable for online process monitoring. Thus, it would be advantageous to develop an alternative method that was still universally applicable but could circumvent the weakness of the conventional BOD test (Liu and Mattiasson, 2002). BOD biosensor is usually developed to reduce analysis time where microbial cells are the preferred sensing element. Rapid determination of BOD could be achieved by cell-based biosensor and this has been the subject of a number of review articles (D'Souza, 2001; Liu and Mattiasson, 2002; Lei *et al.*, 2006). Table 3.1 shows summary of recent cell-based biosensors which will be described in more detail in this chapter.

Table 3.1 Summary of recent cell-based biosensors

Microorganism	Transducer	Immobilization	response time	LOD	Linear range	Stability	Real sample	Reference
Trichoporon cutaneum	oxygen electrode	entrap with Ca-alginate bead	10 min		below 130 mg l^{-1}	60 day	wastewater from therefectories and the toilets of university building	Sohn *et al.*, 1995
Trichoporon cutaneum	light-addresable potentiometric	adsorption with acetylcellulose membrane	25 min		0-100 ppm	14 weeks	wastewater from a septic tank and a brewery	Murakami *et al.*, 1998
Trichoporon cutaneum	oxygen microelectrode		1 hour	200 mg l^{-1} (only one concentration)			secondary treated domestic wastewater	Catterall *et al.*, 2001
Trichoporon cutaneum	oxygen electrode	entrap with silica and the grafting copolymer of poly (vinyl alcohol) and 4-vinylpyridine (PVA-g-P(4-VP))	10 min	0.5 mg l^{-1}	1-60 mg l^{-1}	40 days	lake water and domestic wastewater	Jia *et al.*, 2003
Trichoporon cutaneum	oxygen microelectrode		15 min		up to 200 mg l^{-1}		biscuit factor, cake factory, cannery, brewery, sugar mill, carwash, pet food factory, butcher, bakery domestic, cafeteria and diary factory wastewater, wastwater treatment plant and pond water	Catterall *et al.*, 2003
Arxula adeninivorans	oxygen electrode	adsorption with Poly(carbomyl)sulfonate(PCS)	5 min		40-390 mg l^{-1}	5 days	monitor of industrial and domestic wastewater plant and seawater	Lehmann *et al.*, 1999
Arxula adeninivorans	thick-film oxygen electrode	adsorption with Poly(carbomyl)sulfonate(PCS)		2 mg l^{-1}	up to 550 mg l^{-1}	40 days	wastewater in coastal	Chan *et al.*, 1999
Arxula adeninivorans	thick-film oxygen electrode	adsorption with Poly(carbomyl)sulfonate(PCS)	100 s	1.24 mg l^{-1}		2 months	domestic wastewater	Chan *et al.*, 2000
Pseudomonas putida	oxygen electrode		5 min	0.2 mg l^{-1}			river water	Chee *et al.*, 1999
Pseudomonas putida	oxygen electrode	entrap on a PTFE membrane	2-15 min	0.5 mg l^{-1}	0.25-10 mg l^{-1}	10 days	river water	Chee *et al.*, 1999

Table 3.1 Summary of recent cell-based biosensors (continued)

Microorganism	Transducer	Immobilization	response time	LOD	Linear range	Stability	Real sample	Reference
Pseudomonas putida	fluorscence intensity	adsorption with membrane	15 min		1-10 mg l^{-1}		river water	Chee *et al.*, 2000
Pseudomonas putida	oxygen electrode	adsorption with bio-film	4 min	0.5 mg l^{-1}	1.5-6.0 mg l^{-1}		river water	Chee *et al.*, 2001
Pseudomonas putida	amperometric oxygen microelectrode		1 hour	200 mg l^{-1} (only one concentration)			secondary treated domestic wastewater	Catterall *et al.*, 2001
Pseudomonas putida	oxygen microelectrode			4.8 mg l^{-1}	up to 200 mg l^{-1}			Morris *et al.*, 2001
Pseudomonas putida	oxygen microelectrode		15 min		up to 200 mg l^{-1}		biscuit factor, cake factory, cannery, brewery, sugar mill, carwash, pet food factory, butcher, bakery domestic, cafeteria and diary factory wastewater, wastwater treatment plant and pond water	Catterall *et al.*, 2003
Pseudomonas putida	oxygen electrode	adsorption with bio-film	5-10 min	0.5 mg l^{-1}	up to 10 mg l^{-1}	40 measurement times	river water	Chee *et al.*, 2005
Pseudomonas putida	oxygen electrode	adsorption with bio-film	5 min	0.5 mg l^{-1}	0-10 mg l^{-1}		river water	Chee *et al.*, 2007
Proteus vulgaris	oxygen microelectrode		15 min		up to 200 mg l^{-1}		biscuit factor, cake factory, cannery, brewery, sugar mill, carwash, pet food factory, butcher, bakery domestic, cafeteria and diary factory wastewater, wastwater treatment plant and pond water	Catterall *et al.*, 2003

Table 3.1 Summary of recent cell-based biosensors (continued)

Microorganism	Transducer	Immobilization	response time	LOD	Linear range	Stability	Real sample	Reference
Pseudomonas fluorescens	oxygen electrode tip	entrap with poly (vinyl alcohol) –quaternized stilbazol (PVA–SbQ) photopolymer gel	15 min		15-200 mg l^{-1}	1 week	wastewater from a sewage treatment plant, a food plant and a factory cafeteria	Yoshida *et al.*, 2000
Pseudomonas fluorescens	amperometric fabrication of a disposable electrode	adsorption with bio-film	20 min		15-260 mg l^{-1}	35 days	food factory wastewater and wastewater from dinning room of a factory	Yoshida *et al.*, 2001
Sphingomonas yanoikuyae B1	oxygen electrode chip	entrap with poly vinyl alcohol	10 min	1 mg l^{-1}	up to 30 mg l^{-1}	40 days	wastewater from municipal sewage treatment plant	Konig et al., 2000
Candida parapsilosis	oxygen electrode chip	entrap with poly vinyl alcohol	10 min	1 mg l^{-1}	up to 30 mg l^{-1}	40 days	wastewater from municipal sewage treatment plant	Konig et al., 2000
Klebsiella sp.	oxygen electrode	adsorption withTeflon membrane		34 mg l^{-1}		50 hours	dairy product industrial wastewater	Kim and Park, 2001
Bacillus licheniformis	amperometric oxygen microelectrode		1 hour	200 mg l^{-1} (only one concentration)			secondary treated domestic wastewater	Catterall *et al.*, 2001
Bacillus licheniformis	fluorescence intensity	entrapment	30 min	0.9 mg l^{-1}		1 year	tide and refluent seawater	Lin *et al.*, 2006
Bacillus licheniformis	oxygen microelectrode		15 min		up to 200 mg l^{-1}		biscuit factor, cake factory, cannery, brewery, sugar mill, carwash, pet food factory, butcher, bakery domestic, cafeteria and diary factory wastewater, wastwater treatment plant and pond water	Catterall *et al.*, 2003

Table 3.1 Summary of recent cell-based biosensors (continued)

Microorganism	Transducer	Immobilization	response time	LOD	Linear range	Stability	Real sample	Reference
Serratia marcescens LSY4	oxygen electrode	adsorption withTeflon membrane			up to 44 mg l^{-1}			Kim and Kwon, 1999
Escherichia coli	luminescence intensity		90 min		1-200 ppm		industrial, domestic and factory wastewater	Sakaguchi *et al.*, 2003
Escherichia coli	oxygen microelectrode			4.8 mg l^{-1}	up to 200 mg l^{-1}			Morris *et al.*, 2001
Bacillus subtilis	oxygen electrode	entrap in agarose gel	15-30 min			5 months		Tammeveski *et al.*, 1998
Bacillus subtilis	oxygen electrode	entrap with silica and the grafting copolymer of poly (vinyl alcohol) and 4-vinylpyridine (PVA-g-P(4-VP))	10 min	0.5 mg l-1	1-60 mg l^{-1}	40 days	lake water and domestic wastewater	Jia *et al.*, 2003
Bacillus subtilis	luminescence intensity	entrapment	20- 50 min		up to 25 mg l^{-1}	30 days	OECD, synthetic watsewater and domestic wastewater	Kwok *et al.*, 2005
Saccharomyces serevisiae	luminescence intensity			5.5 mg l^{-1}	11- 220 mg l^{-1}	8 days	river water	Nakamura *et al.*, 2007
Saccharomyces serevisiae	chronoamperometry		15 min	4.8 mg l^{-1}	6.6- 220 mg l^{-1}	14 days	seawater and river water	Nakamura *et al.*, 2007
Saccharomyces serevisiae	absorbance spectrometry		10 min	2.0 mg l^{-1}	up to 200 mg l^{-1}	21 days	river water	Nakamura *et al.*, 2007
Shewanella putrefaciens	microbial fuel cell		2 min					Kim *et al.*, 2002
Yeast	oxygen electrode	entrapment	15 min	1.0 mg l^{-1}	10-50 mg l^{-1}	30 days	wastewater from the food industry	Chen *et al.*, 2002
Exiguobacterium marius, Bacillus horikoshii and *Halomonas marina*	amperometric using a modified glassy carbon electrode	entrap in an ion-exchangable polysiloxane	30 min	0.8 mg l^{-1}	1.2-40 mg l^{-1}	0.5-4 hour	seawater	Chen *et al.*, 2008
Trichoporon cutaneum with *Bacillus subtilis*	oxygen electrode	entrap with silica and the grafting copolymer of poly (vinyl alcohol) and 4-vinylpyridine (PVA-g-P(4-VP))	10 min	0.5 mg l-1	1-60 mg l^{-1}	40 days	lake water and domestic wastewater	Jia *et al.*, 2003

Table 3.1 Summary of recent cell-based biosensors (continued)

Microorganism	Transducer	Immobilization	response time	LOD	Linear range	Stability	Real sample	Reference
Bacillus licheniformis, Dietzia maris with Marinobacter marinus	fluorcence intensity	entrap in a polyvinyl alcohol ORMOSILs	3.2 min	0.1 mg l^{-1}	0.3-40 mg l^{-1}	10 months	seawater	Jiang *et al.*, 2006
Bacillus licheniformis, Dietzia maris with Marinobacter marinus	fluorescence intensity	entrapment	3.2 min	0.2 mg l^{-1}	0.2-40 mg l^{-1}	1 year	tide and refluent seawater	Lin *et al.*, 2006
Bacillus licheniformis, Trichoporon cutaneum, Pseudomonas putida with *Proteus vulgaris*	oxygen microelectrode		15 min		up to 200 mg l^{-1}		biscuit factor, cake factory, cannery, brewery, sugar mill, carwash, pet food factory, butcher, bakery domestic, cafeteria and diary factory wastewater, wastwater treatment plant and pond water	Catterall *et al.*, 2003
Enterobacter cloaca, Citrobacter amalonaticus, Pseudomonas aeruginosa, Yersinia anterocolitica, Klebsiella oxytoca, Enterobacter sakazaki with *Serratia liquefaciens*	oxygen electrode	entrap in a porous nylon membrane	30 min	1.0 mg l^{-1}	up to 60 mg l^{-1}	180 days	food industrial, tannery and pulp and paper industrial wastewater	Rastogi *et al.*, 2003
Mixed culture of nitrifiers	oxygen microelectrode	adsorption with Poly(carbomyl)sulfonate(PCS)	6-12 min	0.07 mg l^{-1}	0.035-1.4 mg l^{-1}	4 days	wastewater of a municipal sewage plant	Konig *et al.*, 1999
Microbial consortium	oxygen electrode	enrap with teflon membrane	5-10 min	1.0 mg l^{-1}	30-90 mg l^{-1}	180 days	wastewater from mother dairy, distillery and tannery	Rastogi *et al.*, 2003
Activated sludge	oxygen electrode	entrap with dialysis membrane	4-8 min		3.5-40 mg l^{-1}	10 hours	food processing factory and municipal wastewater	Liu *et al.*, 2000
Activated sludge	oxygen electrode	entrap with dialysis membrane	10 min		5.0- 700 mg l^{-1}	18 days	effluent from the 2nd stage (anaerobic CSTR)	Liu *et al.*, 2003
Activated sludge	oxygen electrode	entrap in a porous nylon membrane		1 mg l^{-1}	up to 90 mg l^{-1}		mother dairy, tannery and distillery factory wastewater	Rastogi *et al.*, 2003
Activated sludge	microbial fuel cell		30 min to 10 hours	2.58 ppm	up to 206 ppm	over 5 years	wastewater	Kim *et al.*, 2003

Table 3.1 Summary of recent cell-based biosensors (continued)

Microorganism	Transducer	Immobilization	response time	LOD	Linear range	Stability	Real sample	Reference
Activated sludge	microbial fuel cell		1 hour	6 mg l^{-1}		over a year	surface water and artificial wastewater	Kang *et al.*, 2003
Activated sludge	microbial fuel cell	adsorption with bio-film			up to 50 mg l^{-1} (current), up to 400 mg l^{-1} (coulomb)	over 3 years	wastewater from starch processing plant	Gil *et al.*, 2003
Activated sludge	oxygen electrode	entrap with dialysis membrane	10 min	5.0 mg l^{-1}	5.0- 700 mg l^{-1}	1 week	effluent from microbial hydrolysis of sugar deet leaves	Liu *et al.*, 2004
Activated sludge	oxygen electrode	entrap with dialysis membrane	30 min			4.5 days	effluent from microbial hydrolysis of sugar deet leaves	Liu *et al.*, 2004
Activated sludge	oxygen electrode	entrap with dialysis membrane	10 min		5.0- 700 mg l^{-1}	2 weeks	effluent from an anaerobic cantact process (2nd stage)	Liu *et al.*, 2004
Activated sludge	microbial fuel cell		60 min	102.4 mg l^{-1}	up to 100 mg l^{-1}		strength artificial wastewaters	Kim *et al.*, 2004
Activated sludge	oxygen electrode	adsorption with bio-film	30 min		6-300 mg l^{-1}	15 hours	food industrial wastewater and municipal sewage	Sakai *et al.*, 2001
Activated sludge	microbial fuel cell		60 min		up to 100 mg l^{-1}		artificial wastewater	Chang *et al.*, 2004
Activated sludge	microbial fuel cell		2 hours	113.5 mg l^{-1} (only one concentration)			river water	Chang *et al.*, 2005
Activated sludge	luminescence intensity	entrapment	20- 50 min		up to 60 mg l^{-1}	30 days	OECD, synthetic wastewater and domestic wastewater	Kwok *et al.*, 2005
Activated sludge	CO_2 analyzer				up to 140 mg l^{-1}	3 months	effluent of a wastewater treatment plant	Vaiopoulou *et al.*, 2005
Activated sludge	microbial fuel cell		3-5 min	0.025 g l^{-1}	up to 25 g l^{-1}	11 days	glucose standard solution	Kumlanghan *et al.*, 2007
Activated sludge	oxygen electrode	entrap with Ca-alginate bead	10-15 min	0.2 mg l^{-1}	2.0-25 mg $l^{-1}$and 5.0-60 mg l^{-1}	15 days	wastewater from a rubber latex industry, hospital, a car transport, car spare parts, car wash company, instant food product,animal feed factory and monitor influent and effluent of anaerobic reactor	Kumlanghan *et al.*, 2008

Table 3.1 Summary of recent cell-based biosensors (continued)

Microorganism	Transducer	Immobilization	response time	LOD	Linear range	Stability	Real sample	Reference
Thermally killed *Bacillus subtilis*	oxygen electrode	adsorption with Poly(carbomyl) sulfonate(PCS)	15-30 min		up to 70 mg l^{-1}	134 days	sewage plant, food complex and food market wastewater	Qian and Tan, 1998
Thermally killed *Bacillus subtilis*	oxygen electrode	adsorption with Poly(carbomyl) sulfonate(PCS)		0.05 mg l^{-1}			glucose and glutamic acid standard solution	Qian and Tan, 1998
Thermally killed *Bacillus subtilis*	oxygen electrode	adsorption with Poly(carbomyl) sulfonate(PCS)		30 mg l^{-1} (only one concentration)				Qian and Tan, 1999
Thermally killed BODSEED	oxygen electrode	adsorption with Poly(carbomyl) sulfonate(PCS)	45-60 min		up to 45 mg l^{-1}	34 days	industrial wastewater, chemical waste and sewage plant	Tan and Wu, 1999
Thermally killed BODSEED	oxygen electrode	adsorption with Poly(carbomyl) sulfonate(PCS)	40-45 min		up to 40 mg l^{-1}	12 days	dairy and domestic wastewater	Tan and Lim, 2005

3.1 Biological sensing elements

Many types of microbial cells have been applied for the development of BOD biosensors. They can be pure or mix cultures as well as genetically engineered.

3.1.1 Pure culture

Pure microbial cultures often originate from a single strain isolated from the activated sludge of a wastewater treatment process that normally operates to treat the target sample (Kim and Kwon, 1999; Yoshida *et al.*, 2001; Kim and Park, 2001; Chee *et al.*, 2005). It can also be obtained from laboratory strains or from culture collections (Catterall *et al.*, 2001).

Several pure microbial cultures have been used for the BOD biosensor such as *Trichoporon cutaneum* (Sohn *et al.*, 1995; Murakami *et al.*, 1998; Catterall *et al.*, 2001), *Arxula adeninivorans* (Chan *et al.*, 1999; Lehmann *et al.*, 1999; Chan *et al.*, 2000), *Pseudomonas putida* (Chee *et al.*, 1999; Chee *et al.*, 1999; Chee *et al.*, 2000; Catterall *et al.*, 2001; Chee *et al.*, 2001; Morris *et al.*, 2001; Chee *et al.*, 2005; Chee *et al.*, 2007), *Pseudomonas fluorescens* (Yoshida *et al.*,2000; Yoshida *et al.*,2001), *Bacillus licheniformis* (Catterall *et al.*, 2001; Lin *et al.*, 2006), *Serratia marcescens* LSY4 (Kim and Kwon, 1999), *Candida parapsilosis* (Konig *et al.*, 2000), *Sphingomonas yanoikuyae* B1 (Konig *et al.*, 2000), *Escherichia coli* (Morris *et al.*, 2001), *Klebsiella* sp. (Kim and Park, 2001), *Bacillus subtilis* (Tammevesk *et al.*, 1998; Kwon *et al.*, 2005), *Shewanella putrefaciens* (Kim *et al.*, 2002), *Saccharomyces cerevisiae* (Nakamura *et al.*, 2007; Nakamura *et al.*, 2007; Nakamura *et al.*, 2007) and Yeast (Chen *et al.*, 2002). Advantages of pure culture BOD biosensors are good reproducibility for wastewater that has a fairly constant composition and ability to measure low BOD values (Sakaguchi *et al.*, 2003; Chee *et al.*, 1999; Chee *et al.*, 2000). Disadvantages are their short stability, low sensitivity, a need for purification steps for the culture and wastewater with a limited number of components because one organism will have only a limited metabolic potential (Kim and Kwon, 1999; Kim and Park, 2001; Chee *et al.*, 2005).

3.1.2 Mix cultures

Mixed cultures can be derived from activated sludge itself, other natural microbial consortia or those formed artificially. Examples of mixed cultures employed in BOD biosensors are *Exiguobacterium marius, Bacillus horikoshii* and *Halomonas marina* (Chen *et al.,* 2008), *Bacillus licheniformis, Dietzia maris* and *Marinobacter marinus* (Jiang *et al.,* 2006), *Enterobacter cloaca, Citrobacter amalonaticus, Pseudomonas aeruginosa, Yersinia enterocolitica, Klebsiella oxytoca, Enterobacter sakazaki* and *Serratia liquefaciens* (Rastogi *et al.,* 2003), microbial consortium (Rastogi *et al.,* 2003) and a mixed culture of nitrifiers (Konig *et al.,* 1999). In some applications the mix cultures from activated sludge were used without identifying the type of cells. Since the composition of an activated sludge consortium could be very difficult to determine. However, they will metabolise many substrates (Sakai *et al.,* 2001; Rastogi *et al.,* 2003; Liu *et al.,* 2000; Liu *et al.,* 2004; Liu *et al.,* 2004; Liu *et al.,* 2004; Chang *et al.,* 2004; Chang *et al.,* 2005; Kim *et al.,* 2003; Kim *et al.,* 2004; Gil *et al.,* 2003; Kang *et al.,* 2003; Kwok *et al.,* 2005; Kumlanghan *et al.,* 2007; Kumlanghan *et al.,* 2008). BOD sensors based on mixed cultures have a good detection capacity for a wide substrate spectrum, high sensitivity, fast response time, long stability (Liu and Mattiasson, 2002; Rastogi *et al.,* 2003) but can be difficult to maintain and reproduce

Some reports have compared the differences between pure and mixed culture by studied the responses of each pure cultures and the responses from the mixture of the pure cultures that have been tested such as *Trichoporon cutaneum* with *Bacillus subtilis* (Jia *et al.,* 2003), *Bacillus licheniformis, Dietzia maris* with *Marinobacter marinus* (Lin *et al.,* 2006), *Bacillus licheniformis, Trichoporon cutaneum, Pseudomonas putida* with *Proteus vulgaris* (Catterall *et al.,* 2003). A pure culture was generally reported to give better reproducibility compared to a mixed culture but the mixed culture gave results that were in better agreement with the BOD_5 value, were more stable and had a better detection limit and response time (Jia *et al.,* 2003; Lin *et al.,* 2006; Catterall *et al.,* 2003).

3.1.3 Thermally killed cells

BOD biosensors using thermally killed cells have also been reported such as with *Bacillus subtilis* (Qian and Tan, 1998; Qian and Tan, 1998; Qian and Tan, 1999) and BODseed (Cole-Parmer E-05466-00), a commercial microbial preparation from activated sludge. Their BOD_5 sensing characteristics were compared with those of a living cell sensor and the conventional APHA 5-day BOD measurement using the same BODseed microbial system (Tan and Wu, 1999; Tan and Lim, 2005). The thermally killed cells sensors had a good reproducibility and stability and it can be simply stored in buffer solution at room temperature without regular additions of nutrients and food as would be required for living cell biosensors but had a much shorter linear range (Qian and Tan, 1998; Qian and Tan, 1999; Tan and Lim, 2005). However, poor sensing of wastewater containing soluble starch could be due to high mass transfer resistance of the membranes, biofilm and the cell wall particularly for the thermally killed cells (Tan and Wu, 1999).

3.1.4 Genetically engineered cells

Some BOD sensors based on genetically engineered cells have been investigated. For example, *Escherichia coli* containing *lux A-E* genes from *Vibrio fischeri* (*E. coli* HB 101) (Sakaguchi *et al.*, 2003) has been applied to measure and detect organic pollution due to biodegradable substances in various wastewaters. However, the system based on the bioluminescence of recombinant *E. coli* HB 101 cells could not determine from BOD values over 250 ppm.

3.2 Measuring principal

Most BOD cell-based biosensors rely on measuring the respiratory activity of the cells using a suitable transducer (D'Souza, 2001; Liu and Mattiasson, 2002; Lei *et al.*, 2006). Sensors based on a respirometric principle include the use of an oxygen electrode (Suzuki, 2000; Liu *et al.*, 2004; Renneberg *et al.*, 2004), carbon dioxide analyzer (Vaiopoulou *et al.*, 2005), and an optical transducer (Chee *et al.*, 2000).

Microbial fuel cells have also been investigated as another detection method for BOD (Kwok *et al.*, 2005; Kim *et al.*, 2003).

3.2.1 Oxygen electrode

Many types of oxygen electrodes have been developed for BOD measurement. The Clark-type oxygen electrode was the first and is still the most widely used transducer to measure dissolved oxygen (Sohn *et al.*, 1995; Tammeveski *et al.*, 1998; Qian and Tan, 1998; Qian and Tan, 1998; Qian and Tan, 1999; Tan and Wu, 1999; Lehmann *et al.*, 1999; Kim and Kwon, 1999; Chan *et al.*, 1999; Liu *et al.*, 2000; Kim and Park, 2001; Catterall *et al.*, 2001; Chen *et al.*, 2002; Catterall *et al.*, 2003; Rastogi *et al.*, 2003; Rastogi *et al.*, 2003; Liu *et al.*, 2003; Rastogi *et al.*, 2003; Jia *et al.*, 2003; Liu *et al.*, 2004; Liu *et al.*, 2004; Liu *et al.*, 2004; Chee *et al.*, 1999; Chee *et al.*, 1999; Chee *et al.*, 2001; Tan and Lim, 2005; Chee *et al.*, 2005; Chee *et al.*, 2007; Kumlanghan *et al.*, 2008). A Clark-type probe that normally used for cell-based BOD sensor (Figure 3.1) consists of a platinum cathode as the working electrode, a silver anode as the reference electrode, and 0.1 M potassium chloride (KCl) electrolyte (Liu *et al.*, 2000).

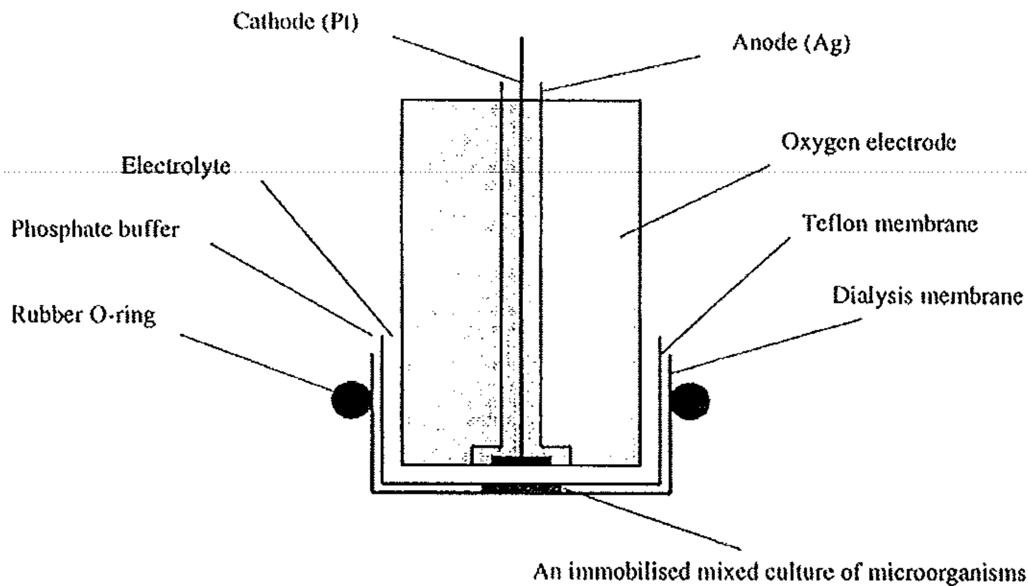


Figure 3.1 A Clark-type oxygen electrode (Liu *et al.,* 2000).

Other oxygen electrodes have also developed and used with BOD biosensors. These include a oxygen microelectrode (Konig *et al.,* 1999; Sakai *et al.,* 2001; Morris *et al.,* 2001; Catterall *et al.,* 2003), thick film oxygen electrode (Chan *et al.,* 1999; Chan *et al.,* 2000), oxygen electrode tip (Yoshida *et al.,* 2000) the BOD sensor chip fabrication as shown in Figure 3.2 (Konig *et al.,* 2000; Yoshida *et al.,* 2001) and the screen printed electrode as shown in Figure 3.3 (Karube *et al.,* 2001). These developed oxygen electrodes (Figure 3.2, 3.3) have short response times, are simple to make at low cost and therefore suitable as disposable sensors but they need a long recovery time (Konig *et al.,* 1999).

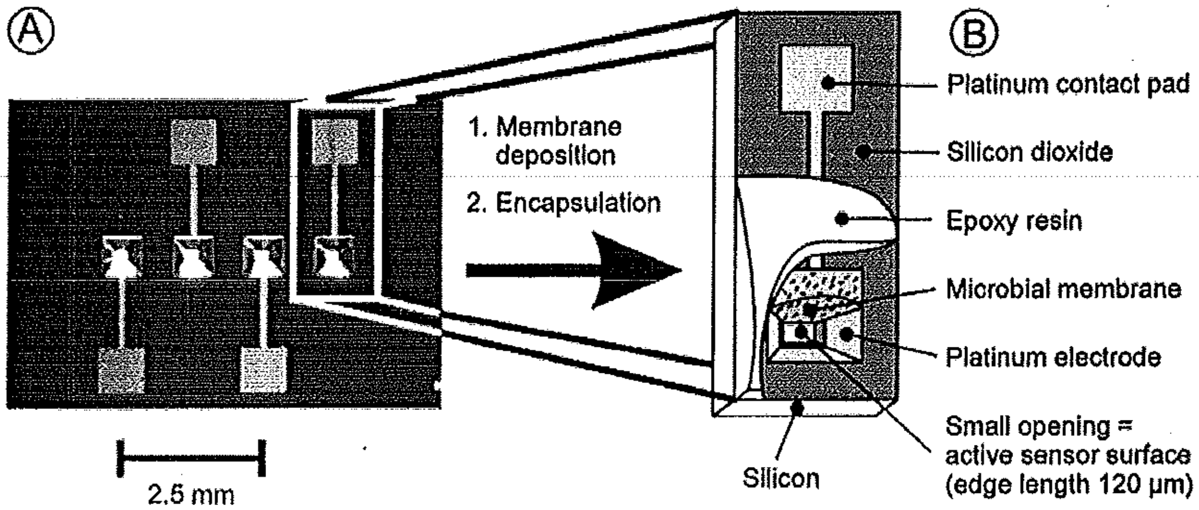


Figure 3.2 Photograph (A) and schematic drawing (B) of sensor chip and containment electrode (Konig *et al.*, 2000).

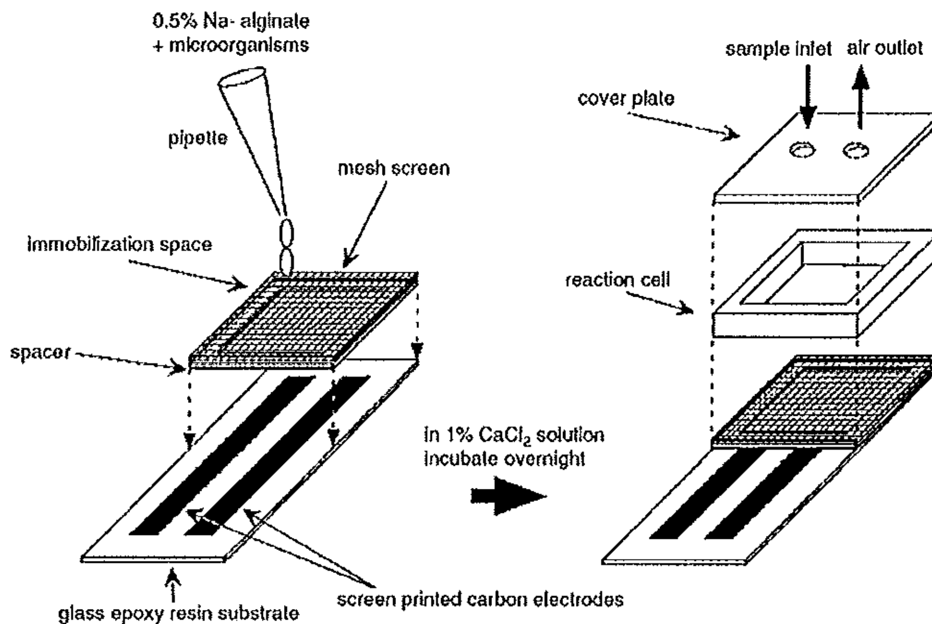


Figure 3.3 Schematic diagram of the disposable BOD sensor chip (Karube *et al.*, 2001).

3.2.2 Carbondioxide analyzer

The principle of the microbial BOD biosensor was based on the determination of CO_2 concentration in the off gas produced by microbial respiration activity during the degradation of organic compounds. The respiration activity of microbial population (CO_2 concentration in the off gas) was related to the extent of the current organic pollution (BOD). Consequently, continuous monitoring and control of wastewater treatment plant performance becomes feasible converting on-line data of CO_2 measurements to current BOD_5 values (Vaiopoulou *et al.*, 2005).

3.2.3 Optical detection

Optical cell-based biosensors have been developed by measuring fluorescence and luminescence intensities. Normally the intensity, related to oxygen concentrations, obeys the Stern-Volmer equation. (equation (3.1));

$$\frac{I_0}{I} = I = K_{SV}\left[O_2\right] \qquad (3.1)$$

where I_0 and I are the fluorescence intensities in the absence and presence of oxygen respectively, K_{sv} is the so-called Stern–Volmer constant and $[O_2]$ is the concentration of oxygen (Chee *et al.*, 2000). In some cases the intensity was represented by the output voltage of the photomultiplier tube (PMT). The Stern–Volmer equation becomes (equation (3.2)):

$$\frac{V_0 - V_f}{V - V_f} = 1 + K_{SV}\left[O_2\right] \qquad (3.2)$$

where V_0 and V are the output voltage of the PMT in the absence and presence of oxygen, respectively, V_f the PMT output voltage produced by 'false' light, K_{SV} is the Stern–Volmer constant and $[O_2]$ is the concentration of the oxygen (Kwok *et al.*, 2005).

Thus fluorescence and luminescence intensities can be used to determine the O_2 concentration during microbial respiration activity. Several the fluorescence (Chee *et al.*, 2000; Lin *et al.*, 2006; Jiang *et al.*, 2006) and luminescence (Kwok *et al.*, 2005; Nakamura *et al.*, 2007) cell-based BOD sensors have been developed with high sensitivity. Optical cell-based BOD sensors can have their sensitivity improved using a fiber optic transducer (Chee *et al.*, 2000; Kwok *et al.*, 2005; Lin *et al.*, 2006), a charge coupled device (CCD) camera and photomultiplication counter (Sakaguchi *et al.*, 2003). The repeatability of the optical fiber biosensor, however, was lower than the one of a previously described oxygen probe (Liu *et al.*, 2000; Liu *et al.*, 2004). This may be due to in optical fiber the shorter light wave has a large of scattered loss by Rayleigh Scattering (Chee *et al.*, 2000).

3.2.4 Microbial fuel cell

Microbial fuel cells (MFCs) can be used as transducers in biosensor systems. They convert chemical energy to electrical energy with the aid of the catalytic reaction of microorganisms (Jang *et al.*, 2004). The first microbial fuel cell BOD biosensor used the hydrogen produced by *Clostridium butyricum* immobilized on the electrode (Karube *et al.*, 1977). Following this publication, several types of MFC-based BOD biosensors have been developed such as MFCs detecting electron transfer (Pasco *et al.*, 2004; Chang *et al.*, 2005) and mediator-less MFCs (Chang *et al.*, 2004; Moon *et al.*, 2004; Kang *et al.*, 2003; Kim *et al.*, 2003; Gil *et al.*, 2003; Kumlanghan *et al.*, 2007). A proposed schematic diagram of the mediator-less microbial fuel cell is shown in Figure 3.4. In this case the electron transfer from the bacterial cells to the electrode also depends on physical contact between the cells and the electrode (Kim *et al.*, 1999). The MFCs-based sensors have the advantages that they have long-term stability (Kim *et al.*, 2003) and can be used continuously for on-line wastewater monitoring (Chang *et al.*, 2004). However, the measuring time of the MFCs-type BOD sensors are quite long, e.g. 60 min (Chang *et al.*, 2004), 1 hour (Kang *et al.*, 2003) and 30 min to 10 hour (Kim *et al.*, 2003),

when compared to other BOD biosensors 5-10 min (Chee *et al.*, 2005; Chee *et al.*, 2007), 15 min (Catterall *et al.*, 2003).

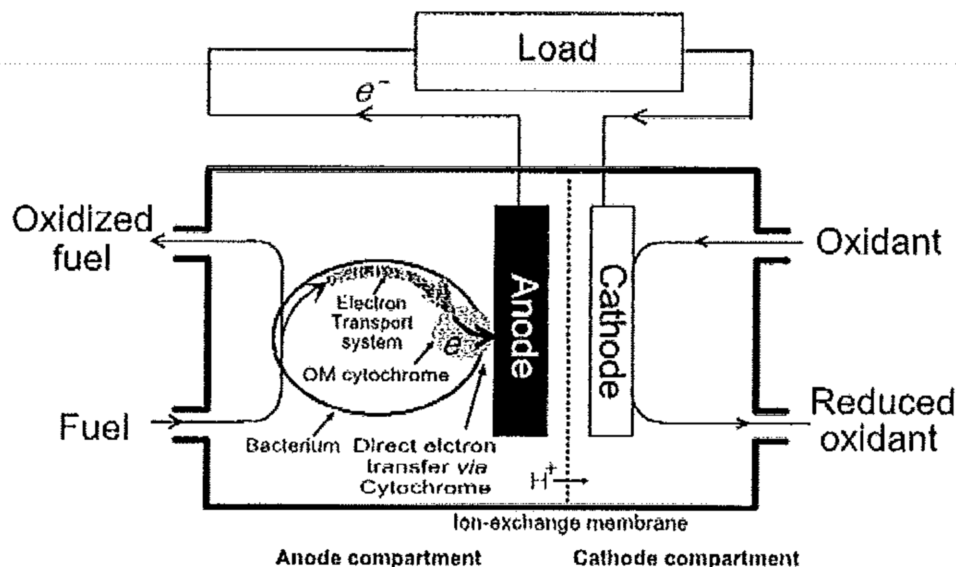


Figure 3.4 A proposed schematic diagram of the mediator-less microbial fuel cell. Bacterium can transfer electrons to the electrode without addition of a mediator. The electrochemical activity is believed to be due to the direct electron transfer from the electron carriers located on the cell surface, possibly outer membrane cytochromes, to the electrode (Kim *et al.*, 2002).

3.3 Immobilization

The use of immobilized cells has been a very important step in the development of cell-based biosensors. Microorganisms can be immobilized on either a transducer or a support matrix. Several methods have been used for immobilizing microbes such as adsorption and entrapment (Chan *et al.*, 2000; Sohn *et al.*, 1995).

3.3.1 Adsorption

Adsorption of cells is based on electrostatic interactions between the charged support or electrode and cells. Cells were immobilized by physical adsorption,

using for examples, Whatman GF/A chromatographic paper (Reshetilov *et al.,* 2001), porous cellulose nitrate membrane (Chee *et al.,* 1999), membrane (Chee *et al.,* 2000) and biofilm at the surface of electrode that make microbial clumps loosely associated with the electrode as shown in Figure 3.5 (Gil *et al.,* 2003).

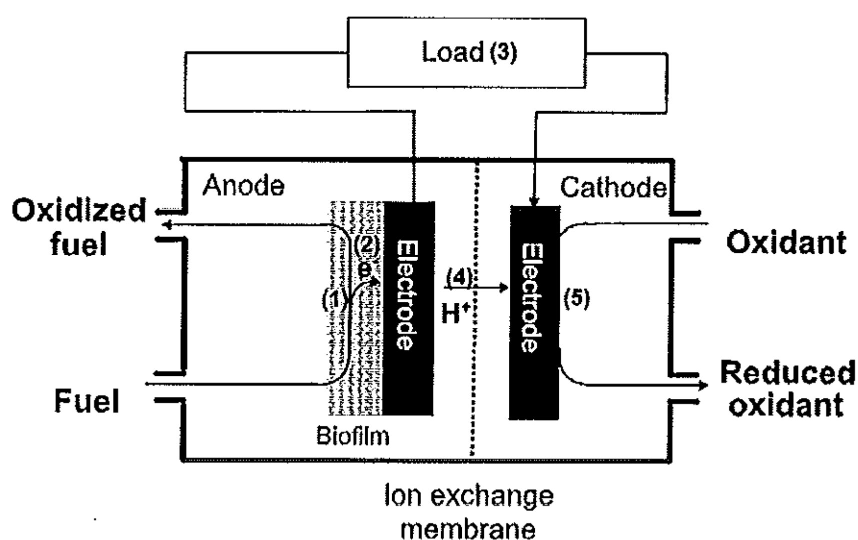


Figure 3.5 Schematic of a BOD microbial fuel cell. (1) Oxidation of fuel, (2) electron transfer from the microbial cells to the electrode, (3) electric load in the circuit, (4) Proton supply into the cathode compartment, and (5) Oxygen supply and reduction at the cathode.

The advantage of the adsorption method is that it is a simple method that can be performed under mild conditions. However, cells immobilized through adsorption exhibit a certain degree of reversibility, and with few exceptions, the forces involved in the binding are not very strong so it gives poor stability because of continued desorption of cells (Liu and Mattiasson, 2002; Lei *et al.,* 2006).

3.3.2 Entrapment

Entrapment method of cells for BOD sensor can be achieved by either retention of the cells in close proximity to the transducer surface using dialysis or filter membrane or in chemical/biological polymers/gels (Lei *et al.*, 2006). The immobilization

of cell in membrane retention is the most widely studied method. Cells are enclosed on the transducer surface using dialysis membrane (Liu *et al.*, 2000; Liu *et al.*, 2003; Liu *et al.*, 2004; Liu *et al.*, 2004; Liu *et al.*, 2004), teflon membrane (Kim and Kwon, 1999; Kim and Park, 2001; Rastogi *et al.*, 2003), PTFE membrane (Chee *et al.*, 1999)or an ion exchangeable polysiloxane (Chen *et al.*, 2008). Although there are several reports on BOD biosensors based on this electrode, this type of biosensor has disadvantages, such as the intrinsic limitation of oxygen solubility in aqueous solution and the short-term stability and calibration drift due to the lysis of bacteria (Liu and Mattiasson, 2002; Kang *et al.*, 2003).

Gel entrapment methods mainly use gelation mechanisms to entrap cells into a porous polymeric structure, allowing transport of substrates and products but restraining the cells. Several gel matrices have been used such as alginate (Sohn *et al.*, 1995; Karube *et al.*, 2001; Kumlanghan *et al.*, 2008), agarose (Tammeveski *et al.*, 1998), poly(carbamoyl)sulfonate pre polymer (Chan *et al.*, 2000). Among these entrapment methods the use of alginate gel is popular because it is simple, low cost and cells remain viable. In fact most immobilization methods for microbes originally did use gel entrapment methods because it has many advantages such as being simple, low cost and able to be produced with a high biomass concentration (Gerbsch *et al.*, 1995).

3.4 Performance of BOD cell-based biosensors

BOD cell-based biosensors normally produce a linear response with good response time and reproducibility and are stable. The linear range of BOD biosensors differs widely from 0.035-1.4 mg l^{-1} (Kong *et al.*, 1999) up to 700 mg l^{-1} (Liu *et al.*, 2004). The different ranges of the sensor signals are due to the sensor fabrication, type and density of cell preparation. BOD sensors with a high cell density biofilm are generally more sensitive in sample analysis but have a narrow linear range such as 0.035-1.4 mg l^{-1} (Konig *et al.*, 1999), 0.25-10 mg l^{-1} (Chee *et al.*, 1999) and 0-10 mg l^{-1} (Chee *et al.*, 2007). The different linear ranges are generally obtained for different composition of the organic content of the sample. For examples, natural water generally has low BOD

value and a sensor with the linear range in the lower concentrations, although narrow, can definitely be applied such as river water (1.5-6.0 mg l^{-1}) (Chee *et al.*, 2001), 1.0-10 mg l^{-1} (Chee *et al.*, 2000)) and seawater (0.30-40 mg l^{-1} (Jiang *et al.*, 2006), 1.2-40 mg l^{-1} (Chen *et al.*, 1999)). In the case of wastewaters with high BOD value a widely linear range such as up to 500 mg l^{-1} (Chan *et al.*, 1999), 40-390 mg l^{-1} (Lehmann *et al.*, 1999) is probably more useful.

For the response time of sensors for one measurement, it has been reported to be between 100 s up to 10 hours (Chan *et al.*, 2000; Kim *et al.*, 2003). The response depends on the type of transducer and the substrate concentration and composition in the sample (Liu and Mattiasson, 2002; D'Souza, 2001). In the case of transducer, the fluorescence intensity transducer was operated between 3.2-50 min to get the response (Jiang *et al.*, 2006; Lin *et al.*, 2006). Oxygen electrodes can obtain response between 5 min up to 1 hour (Lehmann *et al.*, 1999; Catterall *et al.*, 2001). Microbial fuel cells response times can range from 2 min up to 10 hour (Kim *et al.*, 2002; Kim *et al.*, 2003). Furthermore, the response time also depends on the substrate concentration and composition in the sample because more time will be spent for a sample with a higher concentration of substrates. High strength wastewaters need longer analysis time than domestic wastewater (Catterall et al., 2001). For examples, industrial and chemical wastewater required 45-60 min (Tan and Wu, 1999) whereas it takes only 2-15 min for the natural water analysis time (Chee *et al.*, 1999; Chee *et al.*, 2001).

In the case of repeatability, which is the standard deviation of a tested sensor, it was reported to be in the range of ±2.0 to ±15.0% depending on the type of cell and wastewater sample. The repeatability of previously reported pure culture is about 2.0 % (Kim *et al.*, 2001; Lin *et al.*, 2006) while the mix cultures varies between 2.0-8.4% (Konig *et al.*, 1999; Lin *et al.*, 2006). The BOD sensor using activated sludge had repeatability varying from 4.7-15.0% (Liu *et al.*, 2000; Liu *et al.*, 2004) depending on the applied wastewater samples (Liu *et al.*, 2000).

Stability is also a desired property of the sensor. The operational stability of BOD biosensors has been reported to be in the range of between 10 hours up to 5 years

(Liu *et al.,* 2000; Kim *et al.,* 2003). Normally cell-based biosensors give long term stability but it depends on several factors such as the immobilization method, type of cell and transducer (Karube and Nakanishi, 1994; Liu and Mattiasson, 2002; D'Souza, 2001). For example, in the case of immobilization method, the stability of the BOD sensor based on adsorption varies between 50 hours to 134 days (Qian and Tan, 1998; Kim and Park, 2001) and entrapment varies between 0.50 hours to 1 years (Lin et al., 2006; Chen et al., 2008).

3.5 Types of real samples

BOD measurements using cell -based biosensors have been used on several types of water samples including natural and wastewater. Many sources of natural water have been investigated such as seawater (Lehmann *et al.,* 1999; Jiang *et al.,* 2006; Lin *et al.,* 2006; Nakamura *et al.,* 2007; Chen *et al.,* 2008), lake water (Jia *et al.,* 2003), pond water (Catterall *et al.,* 2003), surface water (Kang *et al.,* 2003) and river water (Chee *et al.,* 1999; Chee *et al.,* 1999; Chee *et al.,* 2000; Chee *et al.,* 2001; Chee *et al.,* 2005; Chang *et al.,* 2005; Chee *et al.,* 2007; Nakamura *et al.,* 2007; Nakamura *et al.,* 2007). The sensor systems developed for the detection of BOD in this type of water need to be able to measure at low BOD values. Some of these biosensor have LOD $\leq$ 1.0 mg l^{-1} (Chee *et al.,* 1999; Chee *et al.,* 2000; Chee *et al.,* 2001; Chee *et al.,* 2004; Chee *et al.,* 2005).

Several types of wastewater sample have also been tested such as domestic wastewater (Chan *et al.,* 2000; Catterall *et al.,*2001; Sakagichi *et al.,* 2003; Jia *et al.,* 2003; Catterall *et al.,*2003; Kwok *et al.,* 2005; Tan and Lim, 2005), municipal wastewater (Konig *et al.,* 1999; Tag *et al.,* 2000; Liu *et al.,* 2000; Sakai *et al.,* 2001), food industrial wastewater (Murakami *et al.,* 1998; Qian and Tan, 1998; Liu *et al.,* 2000; Sakai *et al.,* 2001; Yoshida *et al.,* 2000; Yoshida *et al.,* 2001; Kim and Park, 2001; Chen *et al.,* 2002; Gil *et al.,* 2003; Rastogi *et al.,* 2003; Rastogi *et al.,* 2003; Catterall *et al.,*2003; Tan and Lim, 2005), starch industrial wastewater (Reiss *et al.,* 1998), tannery industrial wastewater (Rastogi *et al.,* 2003; Rastogi *et al.,* 2003), pulp and paper industrial

wastewater (Rastogi *et al.*, 2003), distillery industrial wastewater (Rastogi *et al.*, 2003; Rastogi *et al.*, 2003), industrial wastewater (Lehmann *et al.*, 1999; Riedel *et al.*, 1990; Sakagichi *et al.*, 2003; Kumlanghan *et al.*, 2008), wastewater from the refectories and the toilets of university building (Sohn *et al.*, 1995), wastewater in coastal (Chan *et al.*, 1999), effluent from microbial hydrolysis of sugar leaves (Liu *et al.*, 2004; Liu *et al.*, 2004), glucose or glutamic acid standard solution (Qian and Tan, 1998; Kumlanghan *et al.*, 2007) and artificial or synthetic wastewater (Kang *et al.*, 2003; Chang *et al.*, 2004; Kwok *et al.*, 2005). The wastewaters have wide range of BOD values that depend on origin of wastewater.

Cell-based biosensors have also been developed to monitor wastewater treatment processes (Lehmenn *et al.*, 1999; Liu *et al.*, 2003; Liu *et al.*, 2004; Vaiopoulou *et al.*, 2005). For example, *Arxula adeninivorans* sensor was used to monitor the BOD of a sewage treatment plant, as shown in Figure 3.6 (Lehmenn *et al.*, 1999).

The BOD cell-based biosensor systems that have been developed to monitor the performance of the treatment process showed that it could be applied for the on-line system (Liu *et al.*, 2004; Liu *et al.*, 2003; Liu *et al.*, 2004; Vaiopoulou *et al.*, 2005; Kumlanghan *et al.*, 2008) with short analysis time.

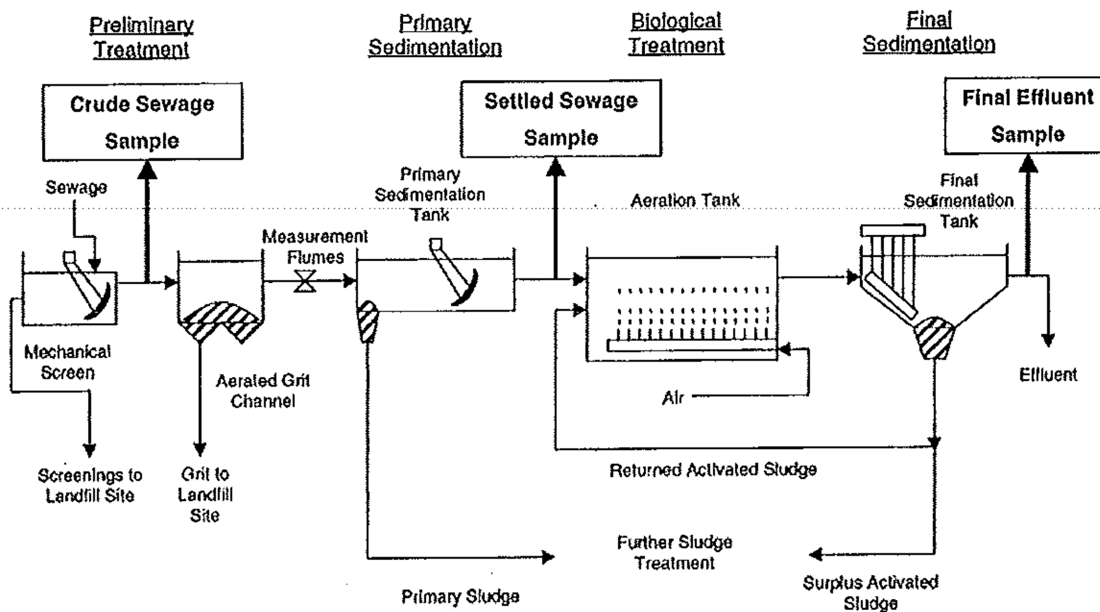


Figure 3.6 Flow diagram of the Sewage Treatment Works. The figure shows the sample collection spots for BOD monitoring (Lehmenn *et al.*, 1999).

Another example to monitor the BOD of wastewater both off-line in a laboratory and on-line in a wastewater treatment plant is shown in Figure 3.7. The sensors can be used to monitor different types of wastewater rapidly without pretreatment, and it can also be used for active process control. The sensor of Vaiopoulou *et al.* (2005) produced good agreement with the BOD_5 method and the rapid cell based biosensor.

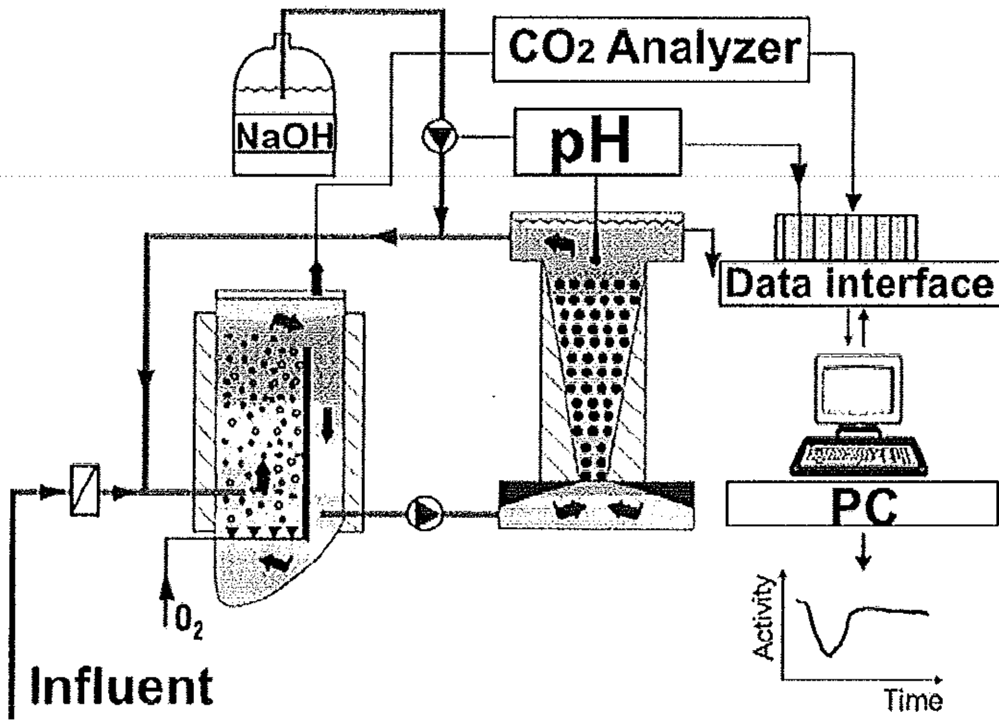


Figure 3.7 Schematic of the biosensor on-line in a wastewater treatment plant (Vaiopoulou *et al.*, 2005).

CHAPTER 4

Performance Criteria

For every analytical method, it is important to characterize its response. The factors to be evaluated depend on the purpose of the method (Taverniers *et al.*, 2004). In this case the performance criteria of the biosensor need to be evaluated. Some of the important performance criteria are listed as follows.

4.1 Linear range

The linearity of the response is the ability of the method to produce consistent test results that are directly proportional to the analyte concentration within the concentration range between the upper and lower levels of the analyte concentration that have been demonstrated to produce a linear response (Swartz and Krull, 1997). Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. To obtain linearity between assays and sample concentrations, the test data may have to be subjected to a mathematical transformation prior to the regression analysis (FDA, 1996). The linearity of a biosensor can be determined by plotting the response change *vs.* the analyte concentration or the response change *vs.* the logarithm of the analyte concentration (Thévenot *et al.*, 1999; 2001). The correlation coefficient, y-intercept and slope of the regression line should be determined (FDA, 1996).

4.2 Sensitivity

Sensitivity is a significant characteristic in all scientific disciplines that have to do with measurements. Sensitivity is defined, from the viewpoint of a measuring instrument, as the change in the response of a measuring instrument divided by the corresponding change in the stimulus (Danzer, 2007). In analytical chemistry, the

sensitivity S_{AA} of the analytical procedure (of the determination of an analyte A) is defined as the change in the measured value divided by the corresponding analytical value (analyte amount or concentration, respectively) as shown in equation (4.1):

$$S_{AA} = \frac{dy_A}{dx_A} \quad (4.1)$$

In case of a linear calibration function, the sensitivity becomes $S_{AA} = \frac{\Delta y_A}{\Delta x_A}$ and corresponds to the slope b of the calibration straight line (Figure 4.1(a)). If the calibration function is a curved line, then the sensitivity will vary according to the analyte amount or concentration as shown in figure 4.1(b) (Danzer, 2007).

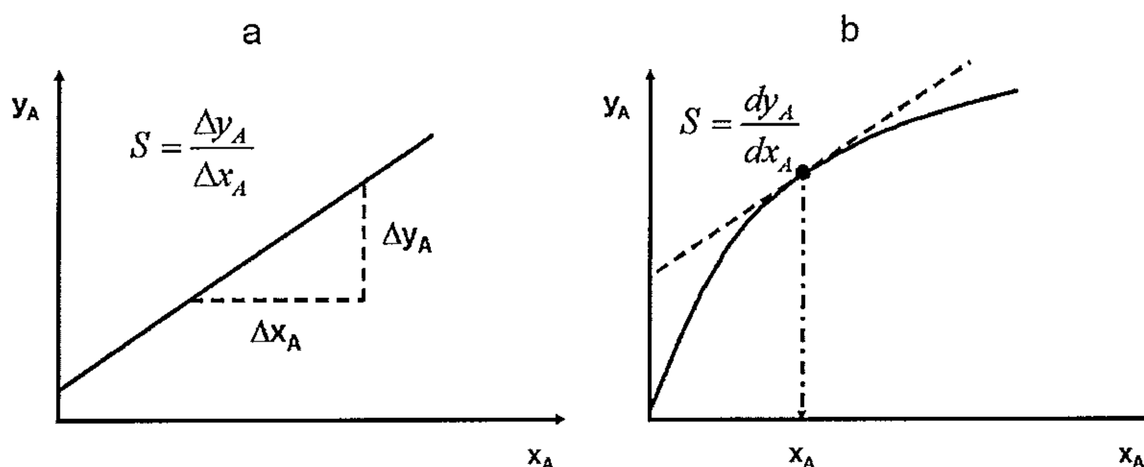


Figure 4.1 Sensitivity in the case of a calibration straight line (a) and a curved calibration function (b) (Danzer, 2007).

4.3 Limit of detection

The limit of detection (LOD) is defined as the lowest concentration of analyte in a sample that can be detected, though not necessarily quantitated (Swartz and

Krull, 1997). There are several methods to evaluate LOD (Long and Winefordner, 1982; Miller and Miller, 1993; Taverniers *et al.*, 2004; FDA, 1996). The LOD described in this thesis is based on the signal-to-noise ratio and the standard deviation of the blank (Swartz and Krull, 1997; FDA, 1996). Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte together with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected by exceeding the standard deviation of the blank signal. The LOD corresponds to that signal where the 'signal to-noise ratio' is 2:1 or 3:1 (Taverniers *et al.*, 2004)

The limit of detection based on the standard deviation of the blank was also studied to measure the magnitude of the analytical background response which is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses (Swartz and Krull, 1997; FDA, 1996). Blank injection was performed 20 times then mean values of the blank response, $\overline{X}_B$, and standard deviation (S_B) were calculated by (4.8) and (4.9).

$$\overline{X}_B = \frac{\sum_{j=1}^{n_B} X_{Bj}}{n_B} \tag{4.8}$$

$$S_B = \sqrt{\frac{\sum_{j=1}^{n_B} \left(X_{Bj} - \overline{X}_B\right)^2}{n_B - 1}} \tag{4.9}$$

In defining the smallest detectable signal (X_L), IUPAC state that

$$X_L = \overline{X}_B + kS_B \tag{4.10}$$

where k is a numerical factor chosen in accordance with the confidence level desired and the accepted value is 3 at a confidence level of 99.86%, S_B is the standard deviation for 20 times of injections. C_L was then obtained as a function of X_L as follows

$$C_L = \frac{(X_L - \overline{X}_B)}{m} \tag{4.11}$$

where m is the analytical sensitivity, and C_L is the smallest concentration that can be detected with reasonable certainty for a given analytical procedure. Because the mean blank reading, $\overline{X}_B$ is not always 0, the signal must be corrected for background. Equation (4.12) was obtained after substitution of equation (4.10) into (4.11).

$$C_L = \frac{kS_B}{m} \tag{4.12}$$

4.4 Repeatability

The definition of repeatability is the same for electrochemical biosensors as for any other analytical devices. Repeatability is a measure of the scatter or the drift in a series of observations or results performed over a period of time. It is generally determined for the analyte concentration within the usable range (Thévenot *et al.*, 1999; 2001). In this thesis, repeatability of the cell-based biosensor system was the standard deviation of the sensor tested under the same conditions. For BOD measurement, the accepted repeatability between replicate determinations should be at least ± 15 % (the relative standard deviation, % RSD) (Liu *et al.*, 2000).

4.5 Stability

The operational stability of a biosensor response may vary considerably depending on the sensor geometry, method of preparation, as well as on the applied receptor and transducer. Furthermore, it is strongly dependent upon the response rate-limiting factor, e.g. an external substrate or requirement for inner diffusion or rate of a biological recognition reaction. Finally, it may vary considerably depending on the operational conditions (Thévenot *et al.*, 1999; 2001).

For operational stability determination, it is recommended that consideration be given to the analyte concentration, the continuous or sequential contact

of the biosensor with the analyte solution, temperature, pH, buffer composition, presence of organic solvents, and sample matrix composition. The operational stability of the system presented in this thesis was primarily related to the stability of the cells which were both pure cultures and mixed cultures.

CHAPTER 5

Microbial BOD sensor for monitoring treatment of wastewater from a rubber latex industry

5.1 Introduction

Rubber industry is one of the most important industries in Southern Thailand and it usually generates large quantities of wastewater containing high concentration of organic matter, suspended solids and nitrogen (Rakkoed *et al.,* 1999). Thus, treatment of this wastewater under controlled conditions is a necessary first step in preventing the release of harmful wastes to the environment. Any treatment process requires monitoring and one of the most widely used and important parameter for the estimation of water quality is biochemical oxygen demand (BOD). The standard BOD method (BOD_5) is a measure of the amount of dissolved oxygen which is required for the biochemical oxidation of the organic compounds in five days from the time when microorganisms are inoculated into the water sample (APHA, 1992). Many local authorities demand that the BOD of wastewater released into any natural body of water must not exceed their designated BOD value. The major drawback of the standard method is the 5-day measurement time. In reality, the wastewater with a higher BOD than the limit could have been discharged to the environment long before the test result was known. Therefore, BOD_5 is not a suitable parameter for any form of active intervention for environmental monitoring and process control.

To overcome the problem, fast determination of BOD can be achieved by applying a biosensor-based method. Most BOD biosensors rely on the measurement of the respiratory activity of cells using a suitable transducer and this has been a subject of several recent reviews (D'Souza *et al.,* 2001; Liu and Mattiasson, 2002; Lei at al., 2006). Recent reports include the use of an oxygen electrode (Suzuki, 2000; Liu *et al.,* 2004b; Renneberg *et al.,* 2004), carbon dioxide analyzer (Vaiopoulou *et al.,* 2005), and an

optical transducer (Kwon *et al.*, 2005). The microbial fuel cell has also been investigated as another detection method for BOD (Kim *et al.*, 2003; Kumlanghan *et al.*, 2007). For cell-based systems many microbial strains have been used as biological sensing elements including both pure and mixed cultures (Liu and Mattiasson, 2002). Mixed cultures involve microbial consortia e.g. those from activated sludge treatments (Liu and Mattiasson, 2002; Vaiopoulou *et al.*, 2005; Rastogi *et al.*, 2003; Liu *et al.*, 2000). The advantage of BOD sensors based on mixed cultures is their good detection capacity for a wide spectrum of substrates (Liu and Mattiasson, 2002). Since the oxygen electrode is simple to use and economical, a BOD sensor based on an activated sludge consortium in combination with an oxygen electrode was considered in this study.

Wastewater obtained from industry processing concentrated rubber latex with a high organic content and generating malodor when discharged into the receiving water was selected for the study. Wastewater in this industry in Southern Thailand is generally treated in waste stabilization ponds (WSP) (Rakkoed *et al.*, 1999), by aerated lagoons and by an activated sludge processes (Boonreongkaow *et al.*, 2002). These treatments have good removal efficiency but still generate malodor. To our knowledge this wastewater has not yet been treated with an anaerobic reactor, a close system that may help solve the malodor problem. In addition methane generates from the reactor can also become a useful by product (Parawira *et al.*, 2004; Bjornsson *et al.*, 2004a). Some of the developed BOD biosensors have been applied successfully to monitor anaerobic processes such as the monitoring of the intermediate products of a two-stage anaerobic reactor (Liu *et al.*, 2004) and the BOD of effluent from a wastewater treatment plant (Vaiopoulou *et al.*, 2005). Therefore it would be an advantage if we could develop and apply such a BOD sensor to monitor an anaerobic treatment process for the wastewater from the concentrated latex process.

The objective of this work was to evaluate the possibility of monitoring an anaerobic treatment process for wastewater from a concentrated latex processing plant using a BOD-sensor with a sensor constructed with a mixed culture obtained from the treatment ponds at the factory. Finally the constructed microbial sensor was applied for

off-line and on-line tests of real wastewater samples from that latex processing plant and from various other industrial activities in the region. The BOD values obtained from the microbial sensor were compared with those obtained from the conventional BOD_5 analyses.

5.2 Materials and methods

5.2.1 Biosensor system

5.2.1.1 Cell-based biosensor

Figure 5.1(a) shows the BOD cell-based biosensor flow injection system. Aerated carrier buffer, 100 mM Tris-HCl pH 7.00, was continuously pumped (peristaltic pump, Gilson, France) through a small reactor column (30 mm length x 3.0 mm inner diameter), containing a consortium of immobilized cells, and then through a flow cell before being sent to waste. Dissolved oxygen in the carrier solution was measured by a Clark-type oxygen electrode (YSI Incoporated, Yellow Springs Instrument Co. Inc., USA) to obtain the baseline. The oxygen electrode was placed inside a chamber in the middle of a water insulation jacket used to control the temperature of the oxygen electrode (30 °C). When a sample of wastewater (500 μl) was injected by means of an injection valve (VICI Valco Europe, Switzerland) and then flowed through the reactor the concentration of dissolved oxygen decreased because oxygen was consumed by the immobilized cells during the degradation of organic matter. The response was recorded on a chart recorder (Ross Recorders, USA). The change in concentration of dissolved oxygen is related to the metabolic degradation of organic matter in the water by the immobilized mixed culture.

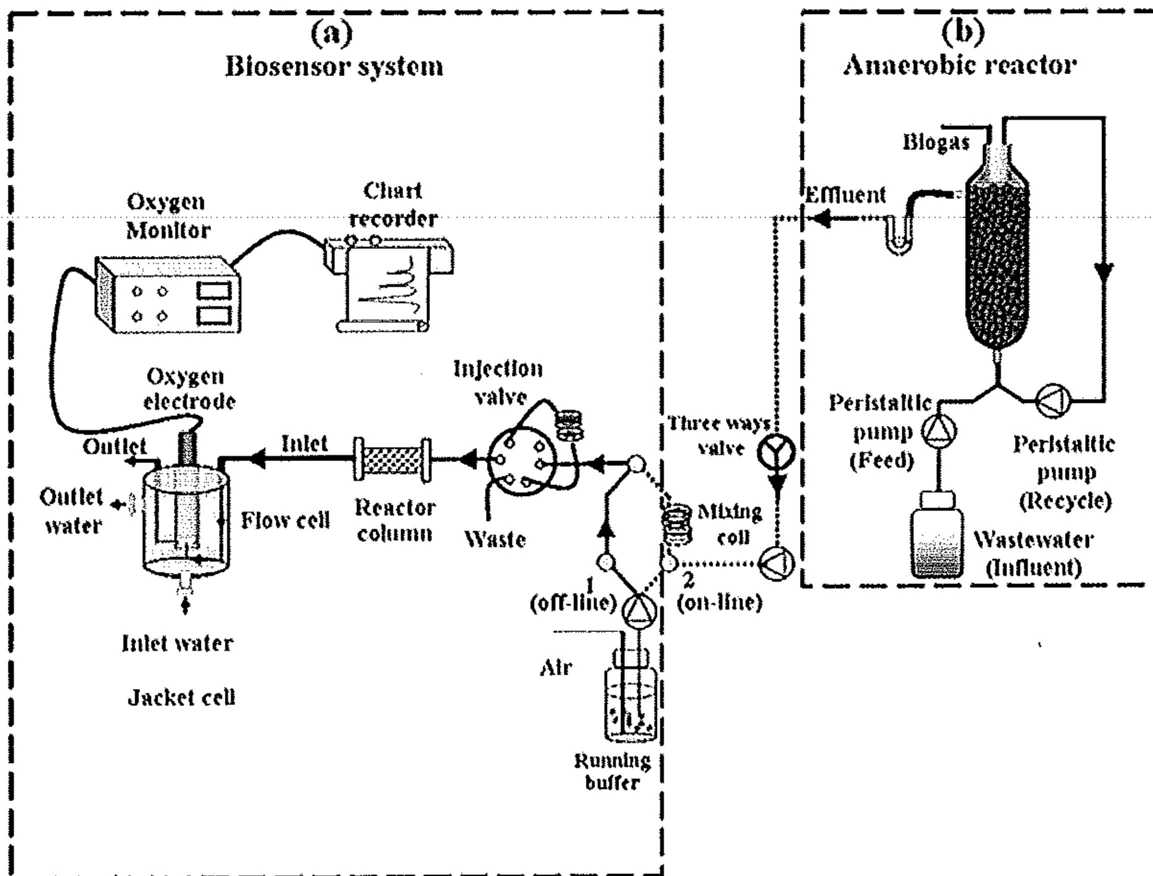


Figure 5.1 Schematic diagram of the system used for on-line monitoring of an anaerobic reactor consist of (a) BOD biosensor system (b) an anaerobic reactor.

5.2.1.2 Microorganisms

Activated sludge was collected from a wastewater treatment pond of a rubber latex factory and was stored at 4.0 °C until it was used. Fifteen millilitres of activated sludge were mixed with 150 ml of wastewater from the rubber latex factory in a 500 ml flask and the mixture was cultivated at room temperature on a rotary shaker (HL instruments, Thailand) at 150 rpm for 24 hours. The cells were harvested by centrifugation (15 min, 860 × g) (Dupont Instruments, USA), washed twice by resuspending in 10 ml of saline solution (0.90% w/v NaCl (Carlo Erba, France)) and centrifuged under the same conditions. Approximately 10 g of wet weight cells were

obtained from each cultivation. This constituted the mixed culture later used for constructing the biosensor.

5.2.1.3 Immobilization

Cells were entrapped in a Ca-alginate gel according to the method described by Thavarungkul *et al.* (1991). Sodium alginate (Fluka AG, Switzerland), 0.30 g, in 10 ml saline solution was stirred for 15 min to obtain a thick uniform solution, mixed with the appropriate amount of cells (see later) and stirred for another 10 min to obtain a uniform mixture. A peristaltic pump was used to pump the mixture at a flow rate of 4.0 ml min^{-1} through an auto pipette tip (0.50-10 μl AxyGen,Inc., USA) into an Erlenmeyer flask containing 0.10 M $CaCl_2$ (Merck, Germany) stirred at 400 rpm while a gentle suction was applied at the neck of the flask using a water pump. The formed gel beads with entrapped mixed culture of microbes were then sieved to obtain the selected size fraction (see later). They were allowed to stabilize overnight in 0.10 M $CaCl_2$ at 4.0 °C. After washing with 0.10 M Tris-HCl buffer pH 7.0 containing 10 mM $CaCl_2$ 0.70 g of the beads were packed into a reactor (30 mm length x 3.0 mm inner diameter). When not in use the reactor was kept in 0.10 M Tris-HCl buffer pH 7.0 with 10 mM $CaCl_2$ at 4.0 °C.

5.2.1.4 Calibration solution

The stock solution used to calibrate the biosensor system was a synthetic wastewater according to the recipe of OECD (Organisation for Economic Cooperation and Development) (OECD, 1984). This was prepared with distilled water and contained 7.5 mg l^{-1} peptone(Merck, Germany), 5.5 mg l^{-1} beef extract(Merck, Germany), 1.5 mg l^{-1} urea (Riedel-de Haen, France), 0.35 mg l^{-1} NaCl (Carlo Erba, France), 0.20 mg l^{-1} $CaCl_2.2H_2O$ (Merck, Germany), 1.4 mg l^{-1} K_2HPO_4 (AnalaR, England), 0.10 mg l^{-1} $MgSO_4.7H_2O$ (J.T. Baker Inc., USA), 75 mg l^{-1} D-glucose (Riedel-de Haen, France) and 75 mg l^{-1} glutamic acid (Merck, Germany) (Liu *et al.*, 2000). The BOD_5 of this synthetic wastewater was 160 ± 5.0 mg l^{-1} (n = 30). Calibration solutions were obtained by diluting

this stock solution with Tris-HCl buffer. BOD value of each calibration solution was tested by the standard BOD_5 method (n = 3).

5.2.1.5 Optimization

Parameters affecting the performances of the system were optimized and these are summarized in Table 5.1. OECD synthetic wastewater with BOD values of between 0.50-15 mg l^{-1} were used in the optimization process. Initial conditions were a flow rate of 0.50 ml min^{-1}, gel bead size 1.00-1.75 mm with the amount of cells 3% w/v, 100 mM Tris-HCl buffer pH 7.00 containing 10 mM $CaCl_2$ was the carrier buffer. Optimization was done by changing one parameter and keeping others constant. When an optimum value was obtained it was used to optimize the next parameter. The optimum conditions were considered to be those that produced a good balance between sensitivity (slope of the calibration curve) and analysis time.

Table 5.1 Optimized parameters and values for the BOD biosensors

Parameter	Value
Sample volume (ml)	0.50, 1.0, 1.5, 2.0
Flow rate (ml min^{-1})	0.10, 0.20, 0.30, 0.40, 0.50
Gel bead size (diameter-mm)	0.50-1.00, 1.00-1.75, 1.75-2.00, 2.00-2.75, 2.75-3.00, 3.00-3.50, 3.50-3.75
Tris-HCl Buffer	
pH	5.0, 5.5, 6.0, 6.5, 7.0
Concentration (mM)	25, 50, 100, 150
Calcium chloride (mM)	5.0, 10, 15, 20, 25, 30, 35, 40
Amount of microorganisms in alginate solution (%w/v)	1.0, 5.0, 7.0, 10, 15, 20

5.2.1.6 Conventional BOD method

BOD_5 of the OECD synthetic wastewater and all wastewater samples were determined by the Standard BOD_5 Method (APHA, 1992) describes in the appendix of this chapter.

5.2.1.7 Real samples

The BOD of real samples was determined by the biosensor under optimum conditions. Calibration of the system was done prior to the measurement using the OECD synthetic wastewaters. The calibration curve was prepared by plotting the dissolved oxygen change versus corresponding BOD values (mg l^{-1}). Samples were then injected into the system. The change in dissolved oxygen of each sample was used to calculate the BOD value from the calibration equation.

A total of 31 wastewater samples were collected from wastewater ponds of several types of industries. These include eleven samples from a rubber latex factory, one each from two hospitals, three from a transportation company, three from a car spare parts manufacturer, four from a car wash company, three from a colour manufacturing firm, three from a fish processing factory and two from an animal feed factory.

All samples were stored at 4 °C until they were tested by the BOD biosensor and compared with the BOD_5 standard method. The analysis of each sample was carried out in triplicate. Solid particles were removed from all samples by filtration before analysis (Whatman diameter 185 mm, Particle Retention 11 µm, Whatman International Ltd Maidstone England).

5.2.2 Anaerobic reactor

A lab-scale glass reactor with an effective volume of 0.50 L (4.0 cm internal diameter) was used for the treatment of wastewater (Figure 5.1(b)). The reactor contained 400 ml of a 0.50-1.0 cm diameter Poraver® glass carrier (Dennert Poraver Gmbh, Germany) used as a support. Sludge (300 ml) from a treatment pond at a rubber latex factory was passed through a sieve with a pore size of 0.50 mm and used as the

inoculum for the anaerobic reactor. The sludge was adapted for a period of two months by feeding with 1.0 ml of anaerobic medium once a day. The concentrations of nutrients in the medium were: D-glucose 10 g l^{-1}, NH_4Cl 100 g l^{-1}, NaCl 10 g l^{-1}, $MgCl_2.6H_2O$ 10 g l^{-1}, $CaCl_2.2H_2O$ 5.0 g l^{-1}, K_2HPO_4 152.6 g l^{-1}, 50 ml $NaHCO_3$ 52 g l^{-1}, $FeCl_2.4H_2O$ 2,441.7 mg l^{-1}, H_3BO_3 50 mg l^{-1}, $CuCl_2.2H_2O$ 38 mg l^{-1}, $AlCl_3.6H_2O$ 90.4 mg l^{-1}, $NiCl_2.6H_2O$ 91.6 mg l^{-1}, $Na_2SeO_3.5H_2O$ 100 mg l^{-1}, $ZnCl_2$ 50 mg l^{-1}, $MnCl_2.2H_2O$ 41 mg l^{-1}, $CoCl_2.6H_2O$ 50 mg l^{-1}, EDTA 500 mg l^{-1}. The reactor was operated at ambient temperature (30-37 °C), sealed at the top with a rubber stopper to maintain anaerobic condition. The first two months were only devoted to the microorganisms growth to attach to the support and start multiplying. Then, wastewater sample was continuously fed from the bottom to the top of the reactor using a peristaltic pump (Micro tube pump MP-3, EYEL 4, TOKYO PIKAKIKAJ CO, LTD, Japan) where flow rates at 10, 20, 40 and 80 ml day^{-1} were investigated, 30 days for each flow rate 120 days in total. These flow rate correspond to hydraulic retention times (HRT, the average time a liquid medium that continuously flows through a reactor stays within the reactor (IUPAC, 1997)) of approximately 50, 25, 12 and 6 days, respectively. Continuously during this operation the liquid in the reactor was also recirculated at a constant flow rate of 20 ml day^{-1} from the top to the bottom of the reactor (Figure 5.1(b)).

5.2.3 The monitoring system

The performance of the anaerobic reactor with respect to reduction of BOD of concentrated wastewater from an industry processing concentrated latex was investigated.

5.2.3.1 Characteristics of wastewater

The latex industry wastewater used in the experiments was collected and stored at 4 °C until used. The characteristics of the wastewater, i.e., BOD, COD, pH,

suspended solids and total dissolved solids, before and after treatment by the anaerobic reactor were analysed.

5.2.3.2 Off-line BOD monitoring analysis

The BOD content in wastewater was monitored prior to and after treatment. The Influent was wastewater from the rubber latex factory and the effluent is treated wastewater that came out from the anaerobic reactor after treatment (Figure 5.1(b)). These two samples were collected once a day, every day during the 120 days of running the anaerobic reactor. They were stored at -20 °C until analysis. Before storage the influent was filtered through a filter paper to remove suspended solids.

Influent and effluent samples of the anaerobic reactor operated at Hydraulic Retention Times (HRTs) of 50 to 6 days were analyzed for their BOD using standard method (BOD_5) (APHA, 1992) and biosensor (off-line Figure 5.1 at position 1).

5.2.3.3 On-line BOD monitoring analysis

The proposed on-line system for measuring BOD was also applied to test the effluent from the reactor outlet during the first 60 days. Running buffer and effluent were pumped through a three way valve (Figure 5.1 at position 2) into a mixing coil. A dilution factor between 17 and 54 times can be adjusted by varying the flow rates of effluent and running buffer lines. These dilution factors can be used to automatically dilute sample that have BOD values between 180-300 mg l^{-1} to fall within the linear range of the biosensor system (see 5.3.2.1). The diluted sample passed though the sampling loop of the injection valve where 1.0 ml was collected and injected into the biosensor system. The response was used to calculate the BOD using a calibration curve of standard solutions. BOD values obtained from this on-line biosensor system were then compared with off-line values obtained from both the off-line biosensor system and the BOD_5 standard method. This online analysis was tested during the operation of the anaerobic reactor at flow rates of 10 and 20 ml day^{-1} (HRT of approximately 50 and 25 days), once a day (n=3).

5.3 Results and discussion

5.3.1 The biosensor system

To optimize each operating condition (Table 5.1) four concentrations of synthetic wastewater with BOD values of between 0.50 and 15 mg l^{-1} were tested and the sensitivities (slopes) of the calibration curves of different tested values were compared. The one that gave the highest sensitivity was chosen as the optimum value.

5.3.1.1 Sample volume

The effect of sample volumes was investigated between 0.50 and 2.0 ml to obtain the highest sensitivity with short analysis time. Each sample volume was tested with five concentrations of synthetic wastewater with BOD between 0.50 and 4.0mgl^{-1}. The sample volume of 1.0 ml gave the highest sensitivity (Figure 5.2) with the analysis time between 25 and 30 min. At 1.5 and 2.0 ml the analysis time were longer with lower sensitivity. Therefore a sample volume of 1.0 ml was chosen for further experiment.

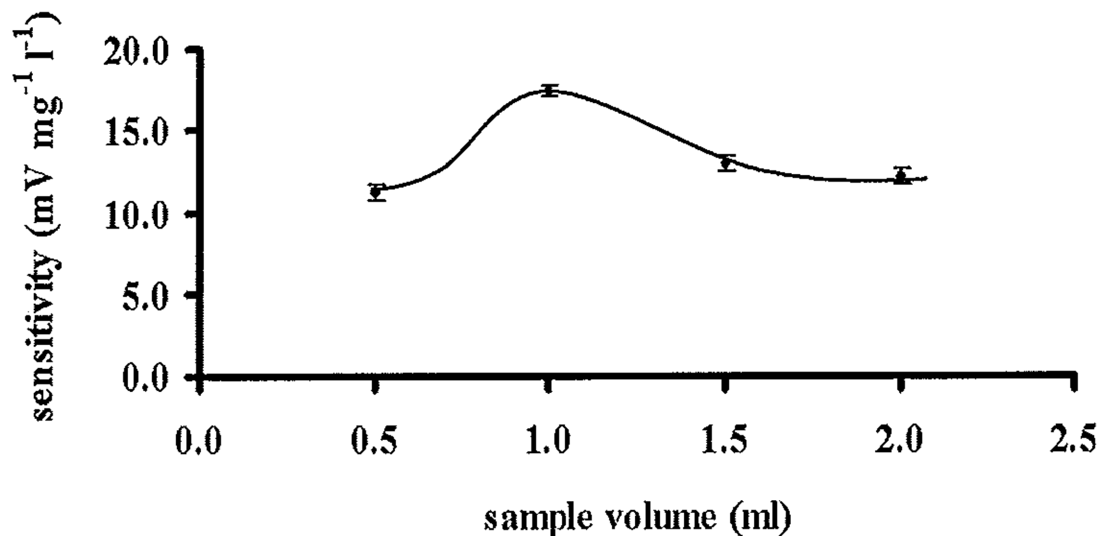


Figure 5.2 The effect of sample volume on the sensitivities of BOD calibration curve.

5.3.1.2 Flow rate

In a flow injection analysis system, flow rate of the sample is one of the parameters that can affect the response. For each flow rate four concentrations of synthetic wastewater with BOD between 1.0 and 4.0 mg l^{-1} were tested and the flow rate of 0.20 ml min^{-1} gave the best sensitivity (Figure 5.3). At higher flow rate lower responses and sensitivities were obtained. This was probably because the microbe had less contact time with the wastewater sample and substrates might not incorporate into the immobilized cells, hence, less reaction (Ikebukuro *et al.,* 1996). On the other hand, the lower flow rate of 0.10 ml min^{-1} gave broad peaks and the analysis time was very long, 30 and 35 min. This is because at very slow flow rate these is more dispersion of the injected sample into the carrier buffer, and this will dilute the concentration of the analytes, provide a broad peak and longer analysis time.

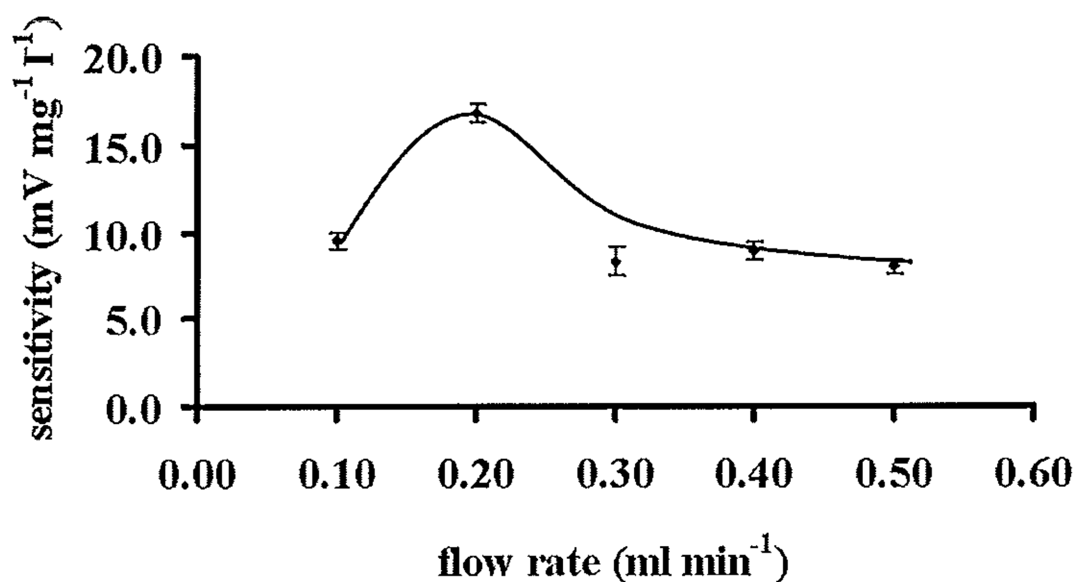


Figure 5.3 The effect of flow rate on the sensitivities of BOD calibration curve.

5.3.1.3 Gel bead size

Different gel bead sizes were obtained by vary the flow rate of the alginate-cells mixture passing though the pipette tip during the immobilization process. Bead size was found to be dependent upon the flow rate of the fed alginate solution. The effect of various bead sizes was studied (Table 5.1). For each gel bead size four concentrations of synthetic wastewater with BOD between 3.0 and 15 mg l^{-1} were tested. The results showed that bead size 1.0-1.75 mm gave the highest sensitivity (Figure 5.4). In the case of bigger bead sizes (1.75-2.00, 2.00-2.75, 2.75-3.00, 3.00-3.5 and 3.50-3.75 mm), low responses and sensitivity are probably due to less surface area. On the other hand for smaller size (0.50-1.0 mm.), gel beads were tightly packed so it is difficult for the sample to pass through.

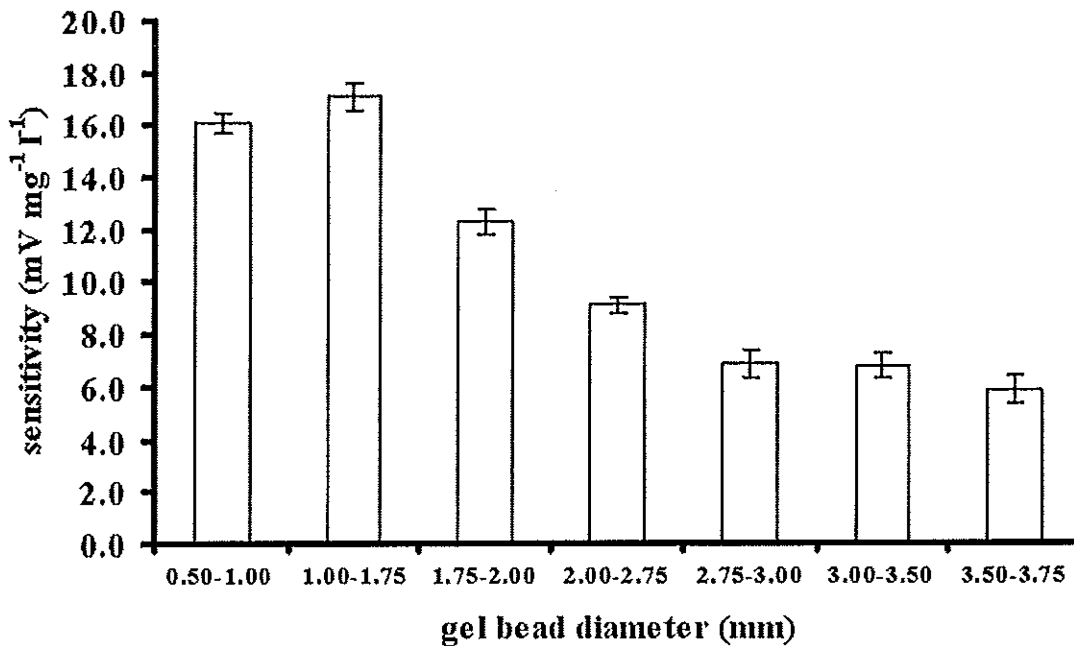


Figure 5.4 The effect of gel bead size on the sensitivities of BOD calibration curve.

5.3.1.4 pH of buffer solution

pH can affect the activity of cells, i.e. they can become inactive at too high or too low pH (Han *et al.,* 2002; Jia *et al.,* 2003). The effect of the pH of 100 mM Tris-HCl buffer with 10 mM $CaCl_2$ was investigated between pH 5 and 7. For each pH five concentrations of synthetic wastewater with BOD between 1.0 and 5.0 mg l^{-1} were tested. The sensitivity increased with the pH and gave a maximum at pH 6.5 and then decreased (Figure 5.5). This may be because pH 6.5 is closed to the concentrated rubber latex factory wastewater of 6.3±0.40 where these cells live.

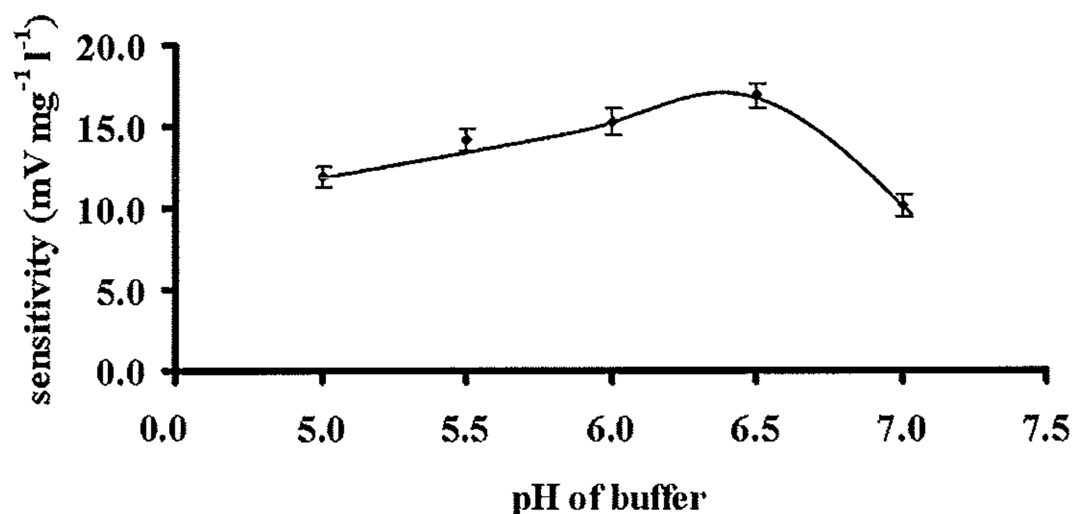


Figure 5.5 The effect of pH of buffer solution on the sensitivities of BOD calibration curve.

5.3.1.5 Buffer concentration

The effect of ionic strength on the response can be investigated by study the effect of difference concentrations of buffer solution (Wu *et al.,* 2004; Wu *et al.,* 2005). Four concentration of Tris-HCl buffer were investigated. For each concentration four concentrations of synthetic wastewater with BOD between 1.0 and 15.0 mg l^{-1} were tested. At 50 mM, the highest sensitivity was obtained (Figure 5.6). This may be due to

ionic strength or ionic concentration in the buffer solution effect to cell's activity (Tan and Qian, 1997). The observation that the mechanism for bacterial attachment differs when the electrolyte concentration is changed which has been reported previously in studies of the effects of different ionic strengths on bacteria adhesion (Zita and Hermansson, 1994). Moreover, Moghadam and coworkers (2005) investigated the effect of ionic strength on settling of activated sludge. Showed that reversible increased with adhesion of bacteria to a surface increased with increasing electrolyte concentration and finally reached 100 mM. Our results agree well with this finding result, indicating that there are similar mechanisms involved in the maintenance of an activated sludge.

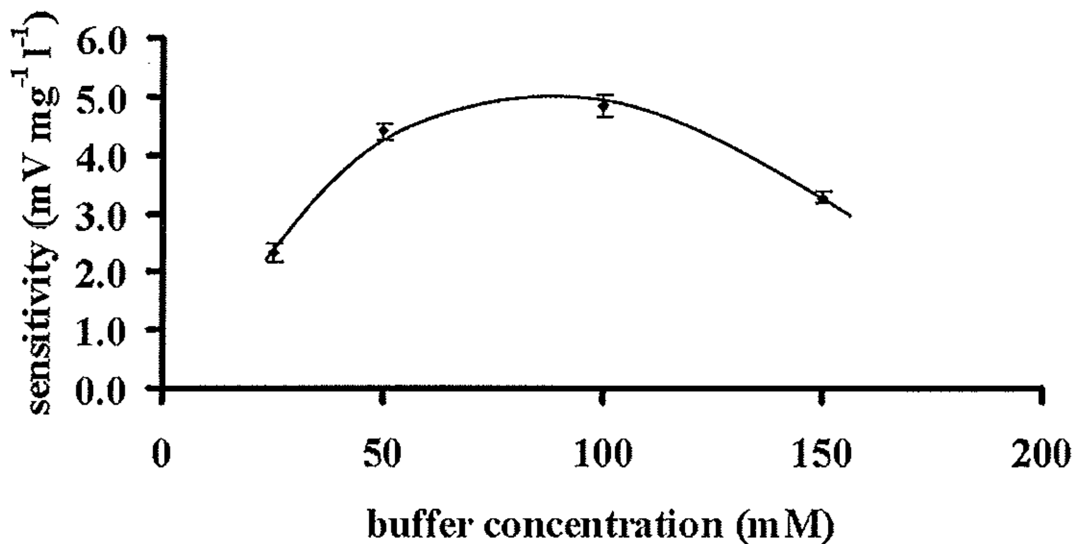


Figure 5.6 The effect of buffer concentration on the sensitivities of BOD calibration curve.

5.3.1.6 Calcium chloride in buffer solution

Calcium chloride has a significant effect on the gelling behaviour of alginate (Adinarayana *et al.*, 2004). However, it is an inorganic salt which can also affect the activity of immobilized microorganisms (Jia *et al.*, 2003). Therefore, the effect of the calcium chloride concentrations in buffer solution was investigated. Four concentrations of synthetic wastewater with BOD between 1.0 and 4.0 mg l^{-1} were tested. The sensitivity

of the BOD biosensor first increase with $CaCl_2$ concentration reaches a maximum at 20 mM and then decreased (Figure 5.7).

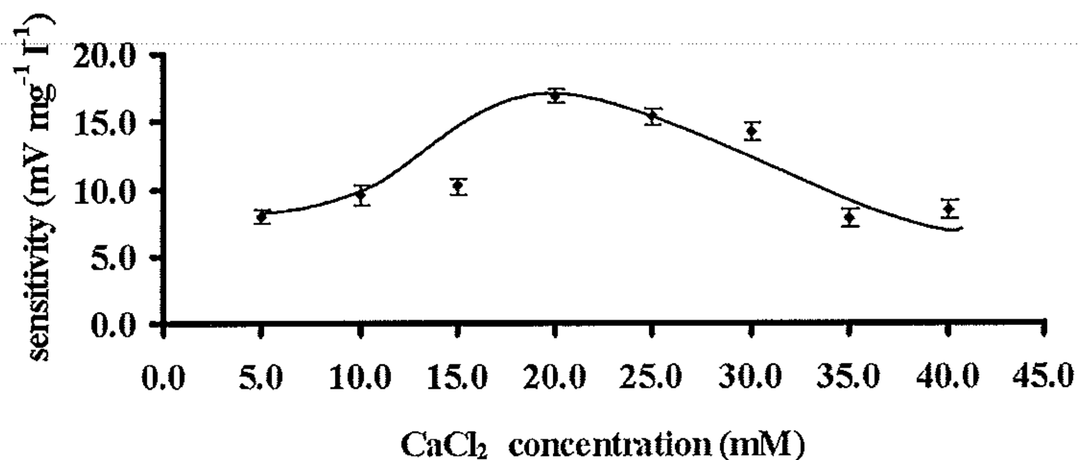


Figure 5.7 The effect of $CaCl_2$ concentration on the sensitivities of BOD calibration curve.

5.3.1.7 The amount of microorganisms

As the magnitude of the sensor signal is related to the amount of biological sensing element (Reshetilov *et al.*, 2001), the dependence of signal amplitude on the amount of immobilized microorganisms need to be studied. The effect of the amount of microorganisms was investigated at 1.0, 5.0, 7.0, 10, 15 and 20% w/v wet weigh of microorganisms per volume of alginate solution. Six concentration of synthetic wastewater with BOD between 0.5 and 15.0 mg l^{-1} were tested. The results showed that the sensitivity of the biosensor increased with the amount of microorganisms and 15% w/v gave the highest sensitivity (Figure 5.8). At 20% w/v, the permeability of dissolved oxygen may have been limited, because the density of the cells in the beads was too high (Sohn *et al.*, 1995).

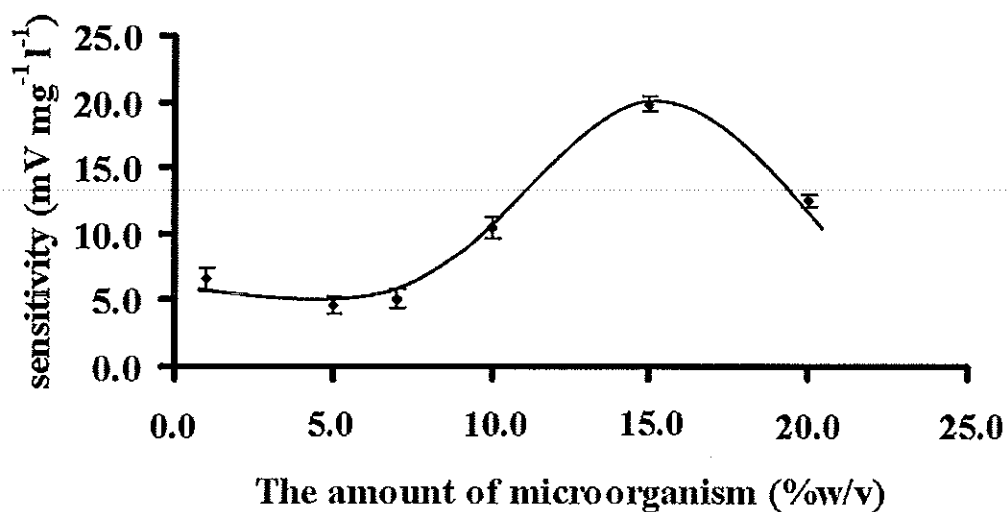


Figure 5.8 The effect of the amount of microorganisms flow rate on the sensitivities of BOD calibration curve.

Optimum conditions of all parameters are summarized in Table 5.2

Table 5.2 Optimum values of biosensor operating conditions.

Parameter	Optimum
Sample volume (ml)	1.0
Flow rate (ml min^{-1})	0.20
Gel bead size (diameter-mm)	1.00-1.75
Tris-HCl Buffer	
pH	6.5
Concentration (mM)	100
Calcium chloride (mM)	20
Amount of microorganisms in alginate solution (%w/v)	15

5.3.2 Performance characteristics of the BOD biosensor system

Using the obtained optimum conditions (Table 5.2) performance characteristics of the BOD biosensor were studied.

5.3.2.1 Linear range and limit of detection

The collected sludge was cultivated at different time interval over a 3 months period. Each cultivated mixed culture was used to prepare gel beads for one column. In total 17 different columns were prepared and tested in the biosensor system. The responses of the biosensor system were investigated using OECD standard wastewater with BOD values in the range 1.0 to 100 mg l^{-1}. The linear ranges were between 2.0-25 and 5.0-60 mg l^{-1} (Table 5.3). Since preparation conditions were the same the difference in the sensitivity and linear range of each column can only be attributed to the different activity of the mixed culture in each preparation. Hence system calibrations, using standard solutions, were generally performed before sample analysis. Average peak height from 20 blank injections was used to calculate the limit of detection (LOD) using the IUPAC method (S/N $\geq$3) (Long *et al.*, 1983) and was found to be 0.20 mg l^{-1}. The analysis time was around 10-15 min.

Table 5.3 Summary of the linear range and limits of detection (LOD) for 17 different columns in the biosensor system.

Column	Linear equation y (mV), x (mg l^{-1})	Linear range	R^2	LOD (mg l^{-1})
1	y= (2.50±0.37)x-(2.26±0.76)	2.0-35	0.9889	0.18
2	y= (2.53±0.11)x-(2.49±0.85)	2.0-35	0.9989	0.18
3	y= (2.59±0.17)x-(2.24±0.12)	2.0-35	0.9961	0.19
4	y= (3.24±0.81)x-(12.63±5.30)	5.0-60	0.9959	0.19
5	y= (3.28±0.82)x-(13.88±5.31)	5.0-60	0.9947	0.19
6	y= (3.31±0.78)x-(14.89±5.06)	5.0-60	0.9936	0.19
7	y= (3.26±0.93)x-(12.28±6.03)	5.0-60	0.9917	0.19
8	y= (3.09±1.06)x-(1.99±0.54)	3.0-30	0.9805	0.20
9	y= (2.88±0.75)x-(6.41±3.69)	3.0-30	0.9848	0.19
10	y= (1.63±0.21)x-(1.30±0.76)	3.0-30	0.9907	0.19
11	y= (0.98±0.07)x-(1.40±1.67)	2.0-50	0.9946	0.22
12	y= (1.11±0.27)x-(9.17±1.25)	2.0-25	0.9820	0.20
13	y= (1.85±0.24)x-(2.56±1.10)	2.0-25	0.9884	0.21
14	y= (1.85±0.51)x-(2.56±0.78)	2.0-25	0.9984	0.19
15	y= (0.98±0.07)x-(1.40±0.67)	2.0-50	0.9946	0.19
16	y= (0.91±0.12)x-(1.28±0.56)	2.0-50	0.9946	0.19
17	y=(111±0.27)x-(9.17±1.25)	5.0-30	0.9820	0.19

5.3.2.2 Repeatability

The repeatability of the sensor was studied by injecting 1.0 ml of 5.0 mg l^{-1} BOD of OECD synthetic wastewater standard solution 20 times over 15 hours. In this time the sensor gave a fairly constant response, (43.5 ± 1.7 mV), during the testing period with a relative standard deviation of ± 3.9 % (Figure 5.9). The repeatability of some previously reported BOD sensors varies from ±2.4% to ±10% for the single-strain sensors (Liu and Mattiasson, 2002) and ±1.3% to ±12.4% for multi-strain based sensors

The operational stability of previously reported biosensors varied widely depending on the sensor configuration, the microbes used, the immobilization method and the operational conditions (Liu and Mattiasson, 2002). The stability can be about a week (Liu *et al.*, 2004b), a month (Renneberg *et al.*, 2004; Kwon *et al.*, 2005) or up to a few months (Vaiopoulou *et al.*, 2005). For our system the sensor can be applied for at least 15 days. If a longer operation time is required changing the column containing the immobilized cells is quite straightforward.

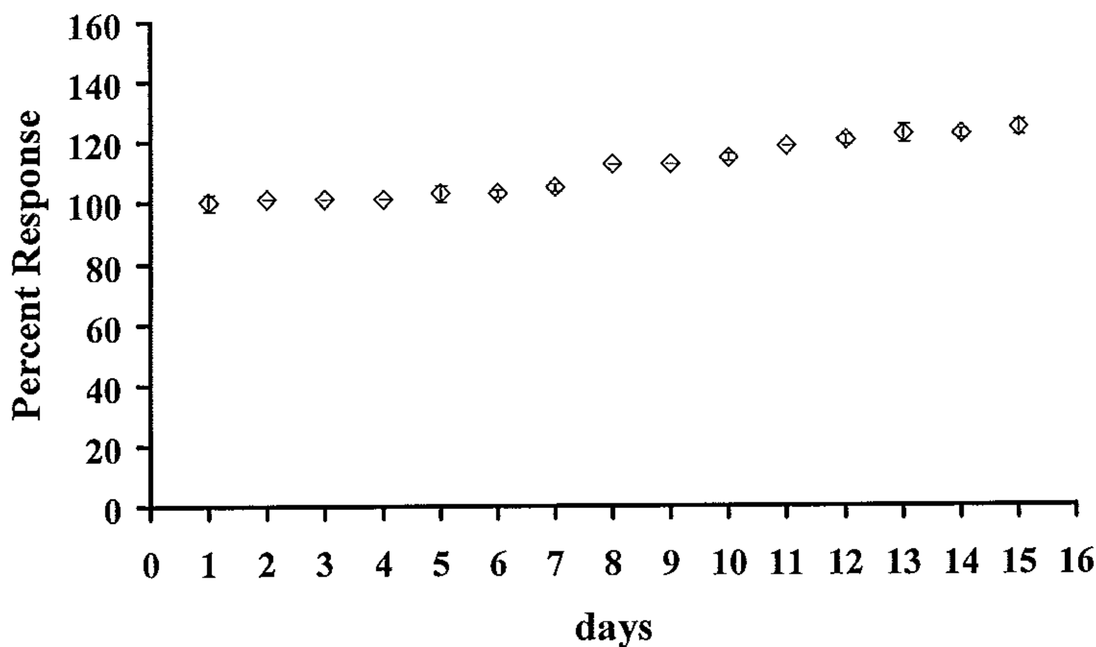


Figure 5.10 The stability of the sensor (n=3) showing the responses to 1.0 ml of 5.0 mg l^{-1} BOD of OECD synthetic wastewater.

5.3.3 Estimation of BOD in samples of different origin

Wastewater samples from different origin were analyzed by the BOD biosensor and BOD_5 standard method. The results are shown in Table 5.4. While there was good agreement between values from the biosensor and BOD_5-methods for samples from the rubber latex factory (number 1-11), it was clear that analytical results on samples from other sources showed pronounced differences between the two methods. These differences are possible to explain when considering the fact that the biosensor is constructed from a mixed culture that has been growing in one of the treatment ponds at the factory. The mixed culture thus has adapted to that specific wastewater. However, other wastewaters may contain chemicals that the cells are not adapted to deal with and therefore, the short analysis time used for the biosensor is not sufficient for degradation to take place. Therefore, larger differences are observed between the biosensor response and that from the longer incubation, the BOD_5. It is also possible that some macromolecules might be in the samples and the cells need longer time for digestion. To test this hypothesis some chemicals used at these other factories from where wastewater was collected were added into the wastewater from concentrated rubber latex production and tested with both methods. The results in Figure 5.11 confirm that in the presence of molecules normally not present in the concentrated latex wastewater BOD values obtained from biosensor were lower than those from the BOD_5-analysis since in the latter the microbes have longer time for metabolizing these compounds.

Table 5.4 Comparison of BOD values estimated by biosensor and the BOD_5 standard method (APHA, 1992) for wastewater samples (n=3) of different origin. Percentage difference is calculated from (biosensor-BOD_5)*100/BOD_5.

Number	Sample	Biosensor (mg l^{-1})	BOD_5 (mg l^{-1})	% Difference
1	rubber trap pond1	530 ± 6	560 ± 6	-5.4
2	rubber trap pond2	640 ± 10	700 ± 6	-8.6
3	rubber trap pond3	810 ± 6	880 ± 10	-8.0
4	rubber trap pond4	940 ± 6	1,000 ± 0	-6.0
5	aerobic pond1	1,120 ± 6	1,130 ± 6	-0.88
6	aerobic pond2	2,390 ± 6	2,500 ± 6	-4.4
7	aerobic pond3	3,970 ± 6	4,200 ± 10	-5.5
8	stabilization pond1	4,260 ± 6	4,300 ± 10	-0.93
9	stabilization pond1	4,030 ± 6	4,200 ± 100	-4.0
10	stabilization pond1	4,120 ± 6	4,230 ± 10	-2.6
11	stabilization pond1	4,140 ± 6	4,200 ± 6	-1.4
12	Hospital1	6.48 ± 0.03	6.60 ± 0.08	-1.8
13	Hospital2	1.68 ± 0.02	2.0 ± 0.1	-16
14	car transport1	20.00 ± 0.08	25 ± 1	-20
15	car transport2	19.4 ± 0.2	20.5 ± 0.5	-5.3
16	car transport3	19.5 ± 0.4	25 ± 1	-22
17	a car spare parts1	19.7 ± 0.2	25 ± 1	-21
18	a car spare parts2	21.0 ± 0.2	25 ± 1	-16
19	a car spare parts3	19.5 ± 0.3	24 ± 2	-17
20	car wash company1	19.5 ± 0.3	23.5 ± 0.5	-17
21	car wash company2	20.0 ± 0.5	24.8 ± 0.7	-19
22	car wash company3	19.5 ± 0.3	24.9 ± 0.5	-22
23	car wash company4	22.8 ± 0.2	25 ± 1	-8.8
24	color manufacturing1	4.6 ± 0.2	5.0 ± 0.5	-8.8
25	color manufacturing2	4.6 ± 0.3	5 ± 0	-7.6
26	color manufacturing3	22.9 ± 0.1	46.0 ± 0.1	-50
27	instant food product1	10.6 ± 0.5	12 ± 2	-12
28	instant food product2	14.0 ± 0.1	21 ± 1	-33
29	instant food product3	53 ± 1	63 ± 2	-15
30	animal feed factory1	20.7 ± 0.3	26 ± 3	-20
31	animal feed factory2	13 ± 1	17 ± 2	-19

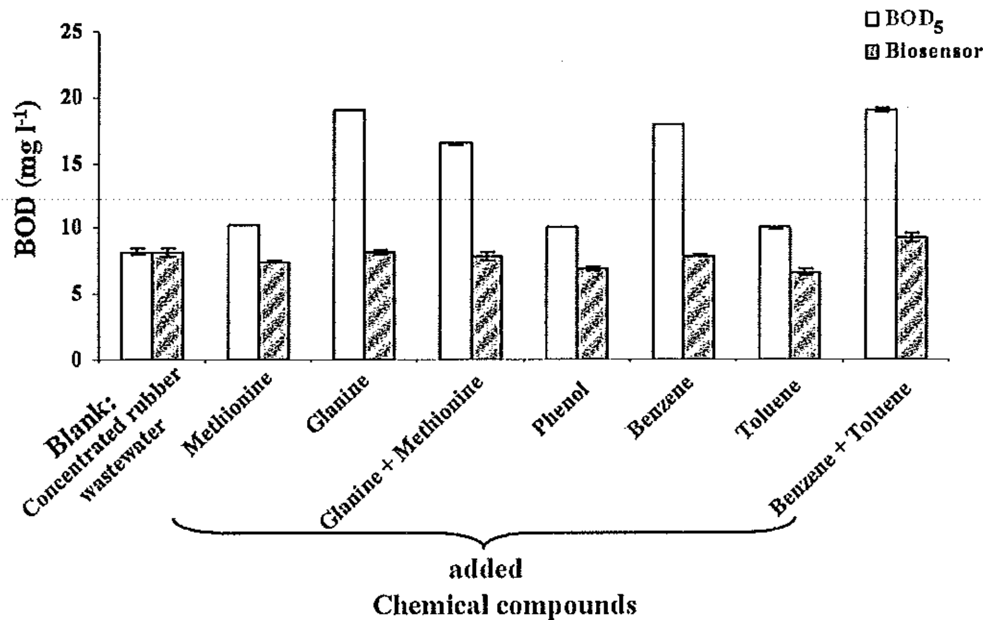


Figure 5.11 Comparison of BOD values (n=3) from BOD_5 and Biosensor from 5 mg l^{-1} BOD of OECD synthetic wastewater with and without (blank) added chemicals.

5.3.4 The anaerobic reactor

Wastewater was collected from a factory where the main production is concentrated latex. In the process field latex is centrifuged to separate two fractions of the latex from each other, i.e. the upper cream phase and the lower skim phase. The cream phase, concentrated latex, contains about 60% rubber content. Although the skim phase is mainly aqueous (serum) it still contains as much as 10% rubber material (Rippel *et al.*, 2003). The small rubber content in the skim phase is coagulated by acid then goes through several steps to remove the water inside. Then it is either pressed to produced the skim block or creep through a pair of driven rolls to create the skim crepe (Wongniramaikul, 2006). The combined wastewater from the two processes generally contains high level of suspended solid, from the concentrated latex process, as well as high level of BOD_5 and COD, mainly from the skim crepe process due to the high content of organic constituents in the serum (Wongniramaikul, 2006). The wastewater is

also contaminated with different kind of impurities including rubber fragments and chemicals. The efficiency of the anaerobic reactor was investigated and during the 120 days of operations BOD, COD and pH were tested daily while suspended solids and total dissolved solids were tested every 5-6 days. The characteristics of the performance of the bioreactor are summarized in Table 5.5.

Table 5.5 Performance of the anaerobic reactor.

Flow rate (l d^{-1})	Hydraulic Retention Time(HRT) (day)	Organic Loading Rate(OLR) yield (gCOD $l^{-1}d^{-1}$)	COD removal efficiency (%)	BOD removal efficiency (%)	
				Biosensor (%)	BOD_5 (%)
0.01	50	0.2	95 ± 2	96.1 ± 0.2	96.2 ± 0.2
0.02	25	0.4	87 ± 4	95.4 ± 0.3	95.3 ± 0.3
0.04	12	0.8	80 ± 7	91.5 ± 0.5	91.4 ± 0.5
0.08	6	1.6	41 ± 17	47 ± 3	47 ± 3

5.3.4.1 BOD monitoring system

5.3.4.1.1 Off-line monitoring

A total of 240 samples, 120 influent samples and 120 effluent samples (30 samples per each flow rate) were tested. Influent samples were analyzed after diluting the samples 50-200 times. A hundred and twenty effluent samples from the reactor when operated at flow rates of 10 to 80 ml day^{-1} were also analyzed after proper dilution. BOD values of the influent samples from both the BOD_5 standard method and biosensor were shown in Figure 5.12(a). BOD_5 and biosensor gave BOD values in the range of 6,070-6,430 mg l^{-1} and 5,820-6,090 mg l^{-1}, respectively. Between day 1 and 120, effluent samples were also analyzed off-line. The results showed that analyses using BOD_5 and biosensor gave BOD in the range of 220-3,480 mg l^{-1} and 200-3,300 mg l^{-1}, respectively (Figure 5.12(b)). The anaerobic bioreactor showed efficient BOD-reduction at high HRT

(low flow rate) (Figure 5.12(c)). Percentage differences between results from the off-line biosensor and the BOD_5 standard method for influent and effluent samples were -5.2 ±1.6 and -4.4 ±2.2%, whereas % difference of previously reported biosensors varied in the ranges of 0.10-27.30% (Tan and Lim, 2005), 2.2-9.0% (Jia *et al.,* 2003) and 0.10–43.00% (Rastogi*etal.,*2003).

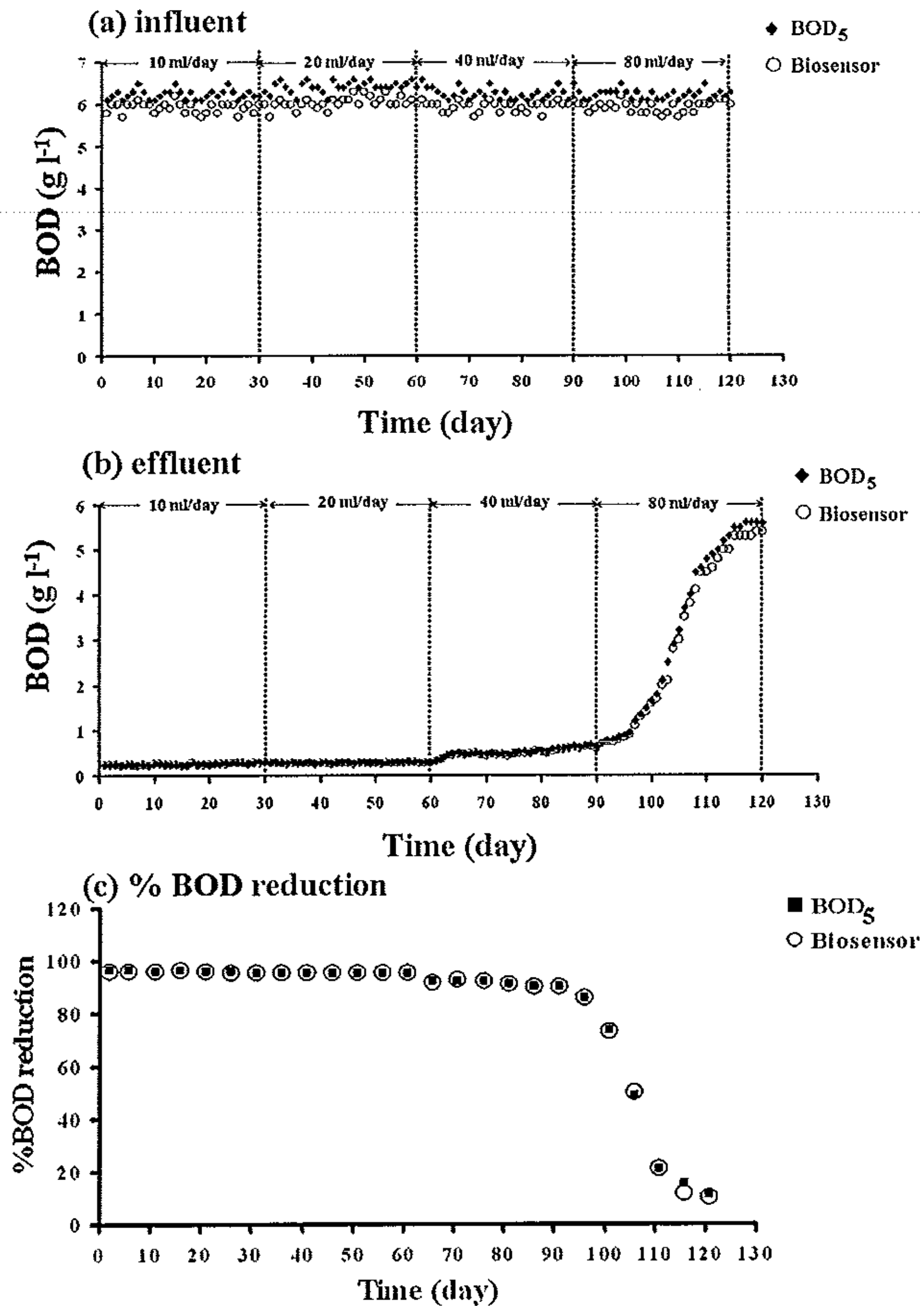


Figure 5.12 BOD values from BOD_5 standard method and biosensor methods. Biosensor results in (a) and (b) were analysed off-line. Percentages BOD reduction are shown in (c) where the data were plotted at 5 day intervals.

5.3.4.1.2 On-line monitoring

For the off-line system, samples have to be manually collected, diluted and injected to the biosensor system. When the biosensor system is applied for on-line monitoring of the anaerobic treatment process, the samples are automatically loaded. This was done by connecting the biosensor system to the effluent of the treatment process using a three-way valve (Figure 5.1). The effluent samples were automatically diluted with buffer solution in a mixing coil (Figure 5.1(a) at position 2). Since the dilution factor of the system was limited by the tube size and the speed of the pump it could only be between 17 and 54 times. Therefore, it can only be used with wastewater with not so high BOD and this system might be suitable for the determination of BOD which is less than 300 mg l^{-1}. The on-line system was used to analyze 30 effluent samples per each hydraulic retention time (HRT) when the reactor was operated at HRT approximately 25 and 50 days. The BOD_5 and biosensor gave BOD values in the range of 220-300 mg l^{-1} and 200-290 mg l^{-1}, respectively.

A regression line was used to compare between biosensor (off-line) versus BOD_5 and biosensor (off-line) versus biosensor (on-line). The comparison between biosensor (off-line) and BOD_5 (Figure 5.13(a)) shows that the values obtained from the biosensor are about 5.0% less than the BOD_5. This is because the short analysis time of the biosensor (10-15 min) cannot assimilate some hard to degrade compounds while the BOD_5 standard method has a much longer incubation time. For the comparison between BOD from biosensor operated off-line and on-line, the results show practically the same values (Figure 5.13(b)).

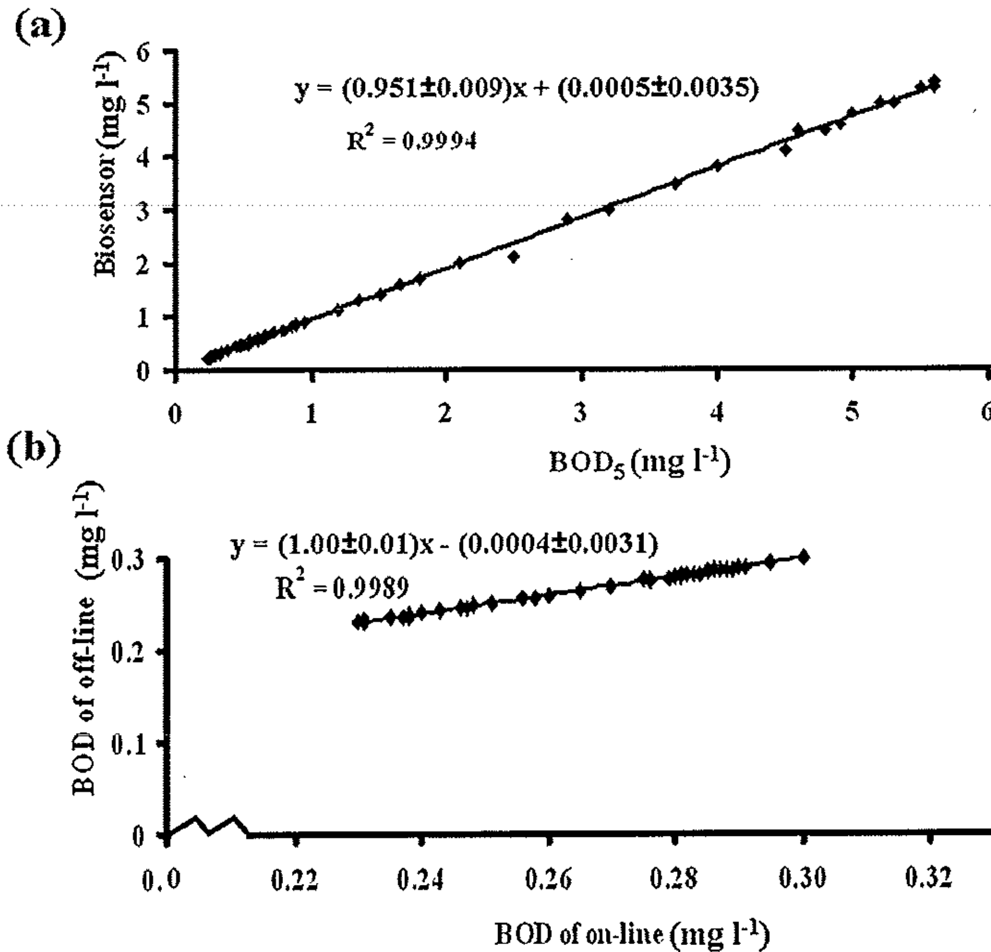


Figure 5.13 Regression line to compare between (a) biosensor (off-line) and BOD_5, (b) between biosensor (off-line) and biosensor (on-line).

5.3.4.2 Determination of COD

The influent and effluent samples were collected once a day, analyzed for COD and the results were calculated in terms of %COD reduction as shown in Figure 5.14. At a low flow rate of 10 ml day^{-1} (HRT approximately 50 days) the percentage COD reduction was constant at 97% for the first 20 days and decreased slightly to about 90% after that. When the flow rate was increased to 20 ml day^{-1} (HRT approximately 25

days) the percentage of COD reduction decreased to 85% during the first few days and increased to a stable value around 87%. At 40 ml day^{-1} (HRT approximately 12 days) initially there was no change in percentage of COD reduction. It remained around 87% for 10 days and then decreased slightly to be around 75%. At 80 ml day^{-1} (HRT approximately 6 days), percentage of COD decreased rapidly from 75% to 21%. From the results it shows that the studied lab scale reactor can operate sufficiently at HRTs down to 25 or maybe 12, but not at HRT of 6 days where reactor performance is influenced both by the ability of the organisms to deal with the substrate fed to them, and also with potential risk of wash-out of the reactor.

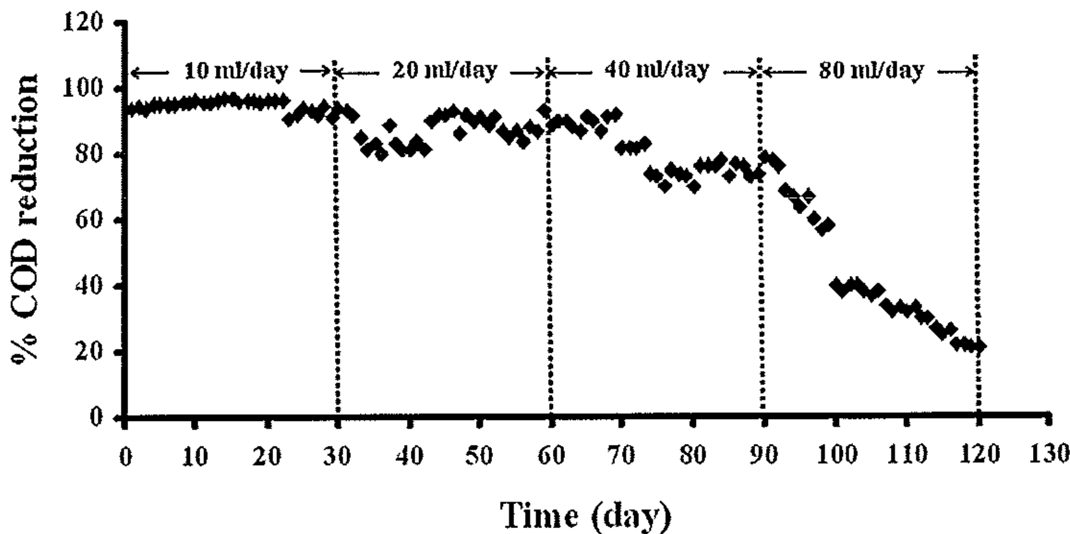


Figure 5.14 Percentage COD reduction of the anaerobic reactor at different flow rates of influent.

5.3.4.3 Determination of pH

pH of the wastewater was around 6.3 before treatment and it raised to almost 8.0 after passage through the anaerobic reactor (Figure 5.15). When the influent passes through the reactor the various acids are degraded by the microbes resulting in the decrease of these acids (Wongniramaikul, 2006) and hence the increase in pH. This is in

agreement with other previous investigations where the treated wastewater gave an increase in pH (Rakkoed *et al.,* 1999; Kaewyod, 1997; Anotai *et al.,* 2007).

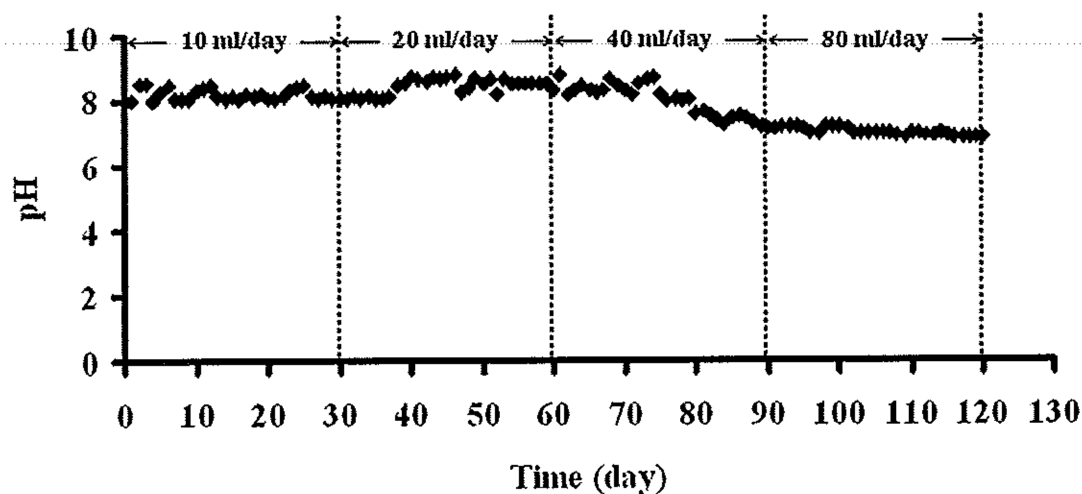


Figure 5.15 pH of effluents of the anaerobic reactor at different flow rates of influent.

5.3.5 Other characteristics of wastewater

Suspended solids (SS) and total dissolved solids (TSD) of influent and effluent samples were also analyzed. As shown in Table 5.5 SS of raw wastewater is 5,500 ± 120 mg l^{-1}. Most of the suspended solids came from the latex process. A possible minor source could be the release during the skim crepe process, a by-product from the production of the concentrated latex when acid was added to coagulate the rubber particle (Wongniramaikul, 2006). After treatment the reduction of SS in the wastewater depended on the hydraulic retention times used (Table 5.5). That is, with the anaerobic reactor the rubber particles could be separated from the liquid and this contributed to reduce the content of suspended solids. Total dissolved solids (TDS) were also reduced by the anaerobic treatment.

Treatment of wastewater at flow rate 10 ml day^{-1} gave the best results (Table 5.5). The pH value of 8.7±0.1 is quite acceptable, however, the other parameters still did not meet the required value (Ministry of Science, Technology and Environmental,

Thailand, 1996) (Table 5.5). The use of another reactor where the feed is the effluent from this first reactor would help to increase the treatment efficiency.

Table 5.6 Characteristics of the raw wastewater and treated wastewater.

Parameters	Raw	Treated waste water				The set value*
		10 (ml d^{-1})	20 (ml d^{-1})	40 (ml d^{-1})	80 (ml d^{-1})	
BOD_5 (mg l^{-1})	6,280 ± 150	239 ± 19	295 ± 9	530 ± 80	3,290 ± 190	20
BOD(Biosensor) (mg l^{-1})	5,960 ± 130	229 ± 20	281 ± 8	510 ± 80	3,120 ± 182	20
COD(mg l^{-1})	8,800 ± 470	440 ± 8	1,144 ± 311	1,760 ± 408	5,280 ± 870	120
pH	6.3 ± 0.4	8.7 ± 0.1	8.5 ± 0.5	8.5 ±0.2	8.9 ± 0.2	5.5-9.0
Suspended Solid (SS,mg l^{-1})	5,500 ± 120 (n=10)	1,100 ± 150 (n=3)	1,750 ± 120 (n=5)	1,800 ± 150 (n=5)	2,050 ± 150 (n=10)	50
Total Dissolve Solid (TDS,mg l^{-1})	5,220 ± 150 (n=10)	4,300 ± 250 (n=3)	4,340 ± 230 (n=5)	4,750 ± 150 (n=5)	5,080 ± 140 (n=10)	3,000

* Ministry of Science, Technology and Environmental, Thailand, 1996

5.4 Conclusions

A microbial BOD sensor was developed by using an oxygen electrode as the transducer and mixed culture of activated sludge from a concentrated rubber latex production as the biological sensing element. The biosensor was applied to monitor the BOD of wastewater. The sensor showed good performance, wide linear range, good repeatability and long term stability.

An anaerobic reactor treatment process was applied to treat the wastewater from the concentrated latex process. The performance of the anaerobic reactor was studied by feeding wastewater at different flow rates. At 10 ml day^{-1} the highest efficiency was obtained with percentage of COD and BOD reduction up to 97%. This is similar to those reported using stabilization pond system with aerobic treatments where %COD and %BOD reduction in the range 97-99% (Rakkoed *et al.,* 1999; Anotai *et al.,* 2007). This treatment process showed good efficiency for high strength wastewater but most parameters still exceeded the required values of discharged wastewater. The treatment efficiency might be increased by feeding the effluent back to the same reactor or to another reactor at the lowest flow rate.

The biosensor system that was employed to monitor the performance of the anaerobic reactor show that off-line and on-line assays could be carried out with short analysis time. The microbial BOD biosensor system has proven to be a suitable monitoring sensor for concentrated wastewater samples from the rubber latex process.

Biochemical oxygen demand (BOD_5) standard method, (APHA, 1998)

1. General Discussion

a. Principle: The method consists of filling a bottle of the specified size with sample to overflowing making it airtight and incubating it at the specified temperature for 5 days. Dissolved oxygen is measured initially and after incubation, and the BOD is computed from the difference between initial and final DO. Because the initial DO is determined shortly after the dilution is made, all oxygen uptake occurring after this measurement is included in the BOD measurement.

b. Sampling and storage: Samples for BOD analysis may degrade significantly during storage between collection and analysis, resulting in low BOD values. Minimize reduction of BOD by analyzing samples promptly or by cooling it to near-freezing temperature during storage. However, even at low temperature, keep the holding time to a minimum. Warm chilled samples to 20 ± 3°C before analysis.

1) Grab samples – If analysis is begun within 2 h of collection, cold storage is unnecessary. If analysis is not started within 2 h of sample collection, keep sample at or below 4°C from the time of collection. Begin analysis within 6 h of collection; when this is not possible because the sampling site is distant from the laboratory, store at or below 4°C and report length and temperature of storage with the results. In no case start analysis more than 24 h after collection of a grab sample. When samples are to be used for regulatory purposes make every effort to deliver samples for analysis within 6 h of collection.

2) Composite samples – Keep samples at or below 4°C during compositing. Limit compositing period to 24 h. Use the same criteria as for the storage of grab samples, starting the measurement of the holding time from the end of the compositing period. State storage time and conditions as part of the results.

2. Apparatus

a. Incubation bottles: Use 300-ml glass bottles bottles (Figure 5.15) having a ground-glass stopper and a flared mouth are preferred. Clean bottles with a detergent, rinse thoroughly, and drain before use. As a precaution against drawing air into the dilution bottle during incubation, use a water seal. Obtain satisfactory water seals by inverting bottles in a water bath or by adding water to the flared mouth of the special BOD bottles. Place a paper or plastic cup or foil cap over flared mouth of bottle to reduce evaporation of the water seal during incubation.

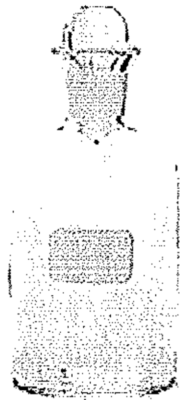


Figure 5.16 The 300 ml BOD bottle

b. Air incubator, thermostatically controlled at 20 ± 1°C. Exclude all light to prevent possibility of photosynthetic production of DO.

3. Reagents

Prepare reagents in advance but discard if there is any sign of precipitation or biological growth in the stock bottles. Commercial equivalents of these reagents are acceptable and different stock concentrations may be used if doses are adjusted proportionally.

a. Phosphate buffer solution: Dissolve 8.5 g KH_2PO_4 (AnalaR, Englang), 21.75 g K_2HPO_4 (AnalaR, Englang), 33.4 g $Na_2HPO_4 \cdot 7H_2O$ (Merck, Germany), and 1.7 g

NH_4Cl (Merck, Germany) in about 500 ml distilled water and dilute to 1 L. The pH should be 7.2 without further adjustment. Alternatively, dissolve 42.5 g KH_2PO_4 (AnalaR, Englang) in about 700 ml distilled water. Adjust pH to 7.2 with 30% NaOH (Merck, Germany) and dilute to 1 L.

b. Magnesium sulfate solution: Dissolve 22.5 g $MgSO_4 \cdot 7H_2O$ (Merck, Germany) in distilled water and dilute to 1 L.

c. Calcium chloride solution: Dissolve 27.5 g $CaCl_2$ (Merck, Germany) in distilled water and dilute to 1 L.

d. Ferric chloride solution: Dissolve 0.25 g $FeCl_3 \cdot 6H_2O$ (J.T. Baker Inc., USA) in distilled water and dilute to 1 L.

e. Acid and alkali solutions, 1*N,* for neutralization of caustic or acid waste samples.

1) Acid – Slowly while stirring, add 28 ml conc sulfuric acid (J.T. Baker Inc., USA) to distilled water. Dilute to 1 L.

2) Alkali – Dissolve 40 g NaOH (Merck, Germany) in distilled water. Dilute to 1 L.

f. Sodium sulfite solution: Dissolve 1.575 g Na_2SO_3 (Merck, Germany) in 1000 ml distilled water. This solution is not stable; prepare daily.

g. Glucose-glutamic acid solution: Dry reagent-grade glucose Riedel-de Haen, France) and reagent-grade glutamic acid (Merck, Germany) at 103°C for 1 h. Add 150 mg dried glucose and 150 mg dried glutamic acid to distilled water and dilute to 1 L. Prepare fresh immediately before use.

h. Ammonium chloride solution: Dissolve 1.15 g NH_4Cl (Merck, Germany) in about 500 ml distilled water, adjust pH to 7.2 with NaOH solution, and dilute to 1 L. Solution contains 0.3 mg N/ml.

i. Dilution water: Use demineralized, distilled or natural water for making sample dilutions.

4. Procedure

a. Preparation of dilution water: Place desired volume of water in a suitable bottle and add 1 ml each of phosphate buffer, $MgSO_4$ (J.T. Baker Inc., USA), $CaCl_2$, and $FeCl_3$ solutions/L of water. Test dilution water as described in dilution water blank so that water of assured quality always is on hand.

Before use bring dilution water temperature to 20 $\pm$ 3°C. Saturate with DO by shaking in a partially filled bottle or by aerating with organic-free filtered air. Alternatively, store in cotton-plugged bottles long enough for water to become saturated with DO. Protect water quality by using clean glassware, tubing, and bottles.

b. Dilution water storage: Source water may be stored before use as long as the prepared dilution water meets quality control criteria in the dilution water blank. Such storage may improve the quality of some source waters but may allow biological growth to cause deterioration in others. Preferably do not store prepared dilution water for more than 24 h after adding nutrients, minerals, and buffer unless dilution water blanks consistently meet quality control limits. Discard stored source water if dilution water blank shows more than 0.2 mg/L DO depletion in 5 days.

c. Glucose-glutamic acid check: Because the BOD test is a bioassay its results can be influenced greatly by the presence of toxicants or by use of a poor seeding material. Distilled waters frequently are contaminated with copper; some sewage seeds are relatively inactive. Low results always are obtained with such seeds and waters. Periodically check dilution water quality, seed effectiveness, and analytical technique by making BOD measurements on a mixture of 150 mg glucose/L and 150 mg glutamic acid/L as a "standard" check solution. Glucose has an exceptionally high and variable oxidation rate but when it is used with glutamic acid, the oxidation rate is stabilized and is similar to that obtained with many municipal wastes. Alternatively, if a particular wastewater contains an identifiable major constituent that contributes to the BOD, use this compound in place of the glucose-glutamic acid.

d. Sample pretreatment: Check pH of all samples before testing unless previous experience indicates that pH is within the acceptable range.

1) Samples containing caustic alkalinity (pH > 8.5) or acidity (pH < 6.0) - Neutralize samples to pH 6.5 to 7.5 with a solution of sulfuric acid (H_2SO_4) or sodium hydroxide (NaOH) of such strength that the quantity of reagent does not dilute the sample by more than 0.5%. The pH of dilution water should not be affected by the lowest sample dilution.

2) Sample temperature adjustment - Bring samples to 20 ± 1°C before making dilutions.

e. Dilution technique: Make several dilutions of sample that will result in a residual DO of at least 1 mg/L and a DO uptake of at least 2 mg/L after a 5-day incubation. Five dilutions are recommended unless experience with a particular sample shows that use of a smaller number of dilutions produces at least two bottles giving acceptable minimum DO depletion and residual limits. A more rapid analysis, such as COD, may be correlated approximately with BOD and serve as a guide in selecting dilutions. In the absence of prior knowledge, use the following dilutions: 0.0 to 1.0% for strong industrial wastes, 1 to 5% for raw and settled wastewater, 5 to 25% for biologically treated effluent, and 25 to100% for polluted river waters.

Prepare dilutions in graduated beaker glassware, and then transfer to BOD bottles. Either dilution method can be combined with any DO measurement technique. The number of bottles to be prepared for each dilution depends on the DO technique and the number of replicates desired.

1) Dilutions prepared directly in BOD bottles - Using a wide-tip volumetric pipet, add the desired sample volume to individual BOD bottles of known capacity. Add appropriate amounts of seed material either to the individual BOD bottles or to the dilution water. Fill bottles with enough dilution water, seeded if necessary, so that insertion of the stopper will displace all air, leaving no bubbles. For dilutions greater than 1:100 make a primary dilution in a graduated cylinder before making the final dilution in the bottle. When using titrimetric iodometric methods for DO measurements, prepare two bottles at each dilution. Determine the initial DO on one bottle. Stopper second bottle tightly, water-seal, and incubate for 5 day at 20°C. If the membrane

electrode method is used for DO measurement, prepare only one BOD bottle for each dilution. Determine initial DO on this bottle and replace any displaced contents with dilution water to fill the bottle. Stopper tightly, water-seal, and incubate for 5 day at 20°C.

Use of the azide modification of the iodometric method is explained below.

Reagents of the iodometric method

a. Manganous sulfate solution: Dissolve 480 g $MnSO_44H_2O$, in distilled water, filter, and dilute to 1 L. The $MnSO_4$ solution should not give a color with starch when added to an acidified potassium iodide (KI) solution.

b. Alkali-iodide-azide reagent:

1) For saturated or less-than-saturated samples—Dissolve 500 g NaOH and 135 g NaI (or 150 g KI) in distilled water and dilute to 1 L. Add 10 g NaN_3 dissolved in 40 ml distilled water. Potassium and sodium salts may be used interchangeably. This reagent should not give a color with starch solution when diluted and acidified.

2) For supersaturated samples—Dissolve 10 g NaN_3 in 500 ml distilled water. Add 480 g sodium hydroxide (NaOH) and 750 g sodium iodide (NaI), and stir until dissolved. There will be a white turbidity due to sodium carbonate (Na_2CO_3), but this will do no harm.

c. Sulfuric acid, H_2SO_4, conc: One milliliter is equivalent to about 3 ml alkali-iodide-azide reagent.

d. Starch: Use either an aqueous solution or soluble starch powder mixtures.

To prepare an aqueous solution, dissolve 2 g laboratory-grade soluble starch and 0.2 g salicylic acid, as a preservative, in 100 ml hot distilled water.

e. Standard sodium thiosulfate titrant: Dissolve 6.205 g $Na_2S_2O_35H_2O$ in distilled water. Add 1.5 ml 6*N* NaOH or 0.4 g solid NaOH and dilute to 1000 ml. Standardize with bi-iodate solution.

f. Standard potassium bi-iodate solution, 0.0021*M:* Dissolve 812.4 mg $KH(IO_3)_2$ in distilled water and dilute to 1000 ml.

3. Procedure of the iodometric method

a. To the sample collected in a 250- to 300-ml bottle, add 1 ml $MnSO_4$ solution, followed by 1 ml alkali-iodide-azide reagent. If pipettes are dipped into sample, rinse them before returning them to reagent bottles. Alternatively, hold pipette tips just above the liquid surface when adding reagents. Stopper carefully to exclude air bubbles and mix by inverting the bottle a few times. When precipitate has settled sufficiently (to approximately half the bottle volume) to leave a clear supernatant above the manganese hydroxide floc, add 1.0 ml conc H_2SO_4. Re-stopper and mix by inverting several times until dissolution is complete. Titrate a volume corresponding to 200 ml of the original sample after correction for sample loss by displacement with reagents. Thus, for a total of 2 ml (1 ml each) of MnSO4 and alkali-iodide-azide reagents in a 300-ml bottle, titrate $200 \times 300/(300 - 2) = 201$ ml.

b. Titrate with 0.025*M* $Na_2S_2O_3$ solution to a pale straw color. Add a few drops of starch solution and continue titration to first disappearance of blue color. If end point is overrun, back-titrate with 0.0021*M* bi-iodate solution added dropwise, or by adding a measured volume of treated sample. Correct for the amount of bi-iodate solution or sample. Disregard subsequent recolorations due to the catalytic effect of nitrite or to traces of ferric salts that have not been complexed with fluoride.

4. Calculation of the iodometric method

a. For titration of a 200 ml sample, 1 ml 0.025*M* $Na_2S_2O_3$ = 1 mg DO/L.

5. Precision and Bias of the iodometric method

DO can be determined with a precision, expressed as a standard deviation, of about 20 mg l^{-1} in distilled water and about 60 mg l^{-1} in wastewater and secondary effluents. In the presence of appreciable interference, even with proper modifications, the

standard deviation may be as high as 100 mg l^{-1}. Still greater errors may occur in testing waters having organic suspended solids or heavy pollution. Avoid errors due to carelessness in collecting samples, prolonging the completion of the test, or selecting an unsuitable modification.

f. Determination of initial DO: If the sample contains materials that react rapidly with DO, determine initial DO immediately after filling the BOD bottle with diluted sample. If rapid initial DO uptake is insignificant, the time period between preparing dilution and measuring initial DO is not critical but should not exceed 30 min.

g. Dilution water blank: Use a dilution water blank as a rough check on the quality of unseeded dilution water and cleanliness of incubation bottles. Together with each batch of samples incubate a bottle of unseeded dilution water. The DO uptake should not be more than 0.2 mg l^{-1} and preferably not more than 0.1 mg l^{-1}. Discard all dilution water having a DO uptake greater than 0.2 mg l^{-1} and either eliminate source of contamination or select an alternate water source for dilution.

h. Incubation: Incubate at 20°C ± 1°C BOD bottles containing the desired dilutions.

i. Determination of final DO: After 5 day incubation determine DO in sample dilutions and that blanks were checked.

6. Calculation

For each test bottle meeting the 2.0-mg l^{-1} minimum DO depletion and the 1.0- mg l^{-1} residual DO, calculate BOD_5 as shown in equation 5.1:

$$BOD_5, mgl^{-1} = \frac{D_1 - D_2}{P} \tag{5.1}$$

where:

D_1 = DO of diluted sample immediately after preparation, mg l^{-1}

D_2 = DO of diluted sample after 5 d incubation at 20°C, mg l^{-1}

P = decimal volumetric fraction of sample used,

If more than one sample dilution meets the criteria of a residual DO of at least 1 mg l^{-1} and a DO depletion of at least 2 mg l^{-1} and there is no evidence of toxicity at higher sample concentrations or the existence of an obvious anomaly, average the results in the acceptable range.

In these calculations, do not make corrections for DO uptake by the dilution water blank during incubation. This correction is unnecessary if the dilution water meets the blank criteria stipulated above. If the dilution water does not meet these criteria, proper corrections are difficult; do not record results or, as a minimum, mark them as not meeting quality control criteria.

CHAPTER 6

Microbial fuel cell-based biosensor for fast analysis of biodegradable organic matter

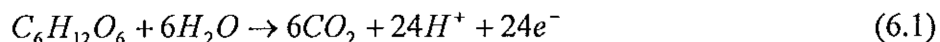
6.1 Introduction

The content of organic matter in wastewater is normally evaluated in terms of biochemical oxygen demand (BOD). The conventional methods for BOD analysis take 5 or 7 days incubation at 20±1 °C in the dark (APHA, 1992; SIS, 1979). BOD_5 and BOD_7 tests are good methods for evaluating water and wastewater quality. It is a universal method of measuring most wastewater samples. No expensive equipment is needed. It has, however, the limitation of being time consuming and also requiring experience and skill to get reproducible results (Liu and Mattiasson, 2002). Thus, the conventional BOD methods are not suitable for on-line monitoring and control of biological wastewater treatment processes where a rapid feedback is essential. It is therefore necessary to develop alternative methods that could give rapid measurements to illustrate the dynamic changes in the treatment process.

Fast determination of BOD could be achieved by biosensor-based methods and this has been a subject of several biosensors review articles (D'Souza, 2001; Liu and Mattiasson, 2002; Lei *et al.*, 2006). Most of previously reported BOD biosensors can be generally classified as bio-layer and respirometer type. Both types of sensor are based on a respirometric principle (Liu and Mattiasson, 2002; Spanjers *et al.*, 1998). The sensor response is expressed as the difference between two steady states or initial rate of change in the dissolved oxygen concentration before and after addition of the sample solution. Although there are several reports on developing oxygen electrode-based BOD biosensors, this type of biosensor has many disadvantages, such as intrinsic limitation of oxygen solubility in aqueous solution and short-term stability and calibration drift due to the lysis of immobilized bacteria (Liu and Mattiasson, 2002; Kang *et al.*, 2003). It is

therefore of great interest to develop alternative methods for compensating these shortages of existing BOD sensors for being operated under tough conditions and a microbial fuel cell is seem as an interesting approach.

A microbial fuel cell (MFC) is a device that converts chemical energy to electrical energy with the aid of the catalytic reaction of microorganisms (Jang *et al.*, 2004). The MFC system often consists of an anaerobic compartment with a negative electrode and an aerobic compartment with a positive electrode. Two compartments are normally separated by a proton exchange membrane (PEM). In the anaerobic compartment microorganisms oxidize substrate, e.g. oxidation of glucose results in generating electrons and protons, as shown in equation (6.1) (Grzebyk and Poznian, 2004).



$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^- \quad (6.1)$$

The generated protons migrate from the anaerobic compartment to the aerobic compartment through the PEM. The produced electrons are transferred to the anode and then pass through an external electric circuit to the cathode, where they reduce oxygen to form H_2O, as shown in equation (6.2).

$$4H^+ + O_2 + 4e^- \rightarrow 2H_2O \quad (6.2)$$

The flow of electrons through the circuit forms current and can be measured by a multi-meter. The overall reaction is the combustion of biodegradable organic matter to carbon dioxide and water with electricity as a product (Jang *et al.*, 2004; Bennetto, 1990; Kim *et al.*, 2004; Liu and Logan, 2004; Chaudhudi and Lovley, 2003). Pure cultures of microorganisms, such as *Shewanella putrefaciens* (Kim *et al.*, 2002), *Clostridium butyricum* (Park *et al.*, 2001), *Rhodoferax ferrireducens* (Chaudhuri and Lovley, 2003), *Geobacter sulfurreducens* (Bond and Lovley, 2003; Pham *et al.*, 2003; Ieropoulos *et al.*, 2005), *Geobacter metallireducens* (Min *et al.*, 2005), *Escherichia coli* (Ieropoulos *et al.*, 2005), *Proteus vulgaris* (Choi *et al.*, 2003), have been used for MFC construction. Mixed cultures, such as anaerobic sludge, have also been reported for MFC construction (Min *et al.*, 2005; Ieropoulos *et al.*, 2005). Many types of feedstock were used as the substrate for MFC, such as starch (Niessen *et al.*, 2004), glucose (Bennetto,

1990; Chaudhuri and Lovley, 2003; Niessen *et al.*, 2004; Choi *et al.*, 2003), lactate (Kim *et al.*, 2002), cysteine (Logan *et al.*, 2005) and wastewater (Gil *et al.*, 2003).

Beside the use of MFC to generate electricity the principle of MFCs has also been applied to construct biosensors for fast BOD estimation, in which a biological sensing element (bacteria in the anode compartment) and a transducer (electrodes and proton exchange membrane) are combined. Karube *et al.* (1977) developed a microbial fuel cell BOD biosensor using the hydrogen produced by *Clostridium butyricum* immobilized on the electrode. Following this work, several types of MFC-based BOD biosensors have been developed such as MFCs with mediated electron transfer (Pasco *et al.*, 2004; Chang *et al.*, 2005) and mediator-less MFCs (Chang *et al.*, 2004; Moon *et al.*, 2004; Kang *et al.*, 2003; Kim *et al.*, 2003; Gil *et al.*, 2003). The MFC-based sensors have the advantage that they have long-term stability (Kim *et al.*, 2003) and can be used continuously for on-line wastewater monitoring (Chang *et al.*, 2004). However, the measuring time (i.e. response time + sensor recovery time) of these MFC-type BOD sensors varies significantly from 1 hour up to several hours (Chang *et al.*, 2004; Kang *et al.*, 2003; Kim *et al.*, 2003).

In this study, a novel design of a MFC-based sensor system was developed for rapid estimation of content of organic matter where glucose was used as a preliminary substrate to evaluate performance of the MFC sensor. The sensor system was operated incorporating an anaerobic bioreactor for continuous supply of stable anaerobic consortium. Replacement of bacteria in the anode compartment was carried out for each sample analysis without using mediator. Therefore, a shorter measuring time is required since there is no need to wait for the metabolic recovery of anaerobic consortium. The sensor measurement was optimized and sensor characteristics were investigated.

6.2 Materials and methods

6.2.1 Experimental setup

As illustrated in Figure 6.1, the measuring system consists of a single chamber MFC acting as a biosensor (Figure 6.1(a)), an anaerobic reactor that was operated in continuous mode to provide a stable anaerobic consortium (Figure 6.1(b)), and a computer-based data acquisition system to record the signal. The MFC was made of glassware that had anaerobic and aerobic compartments separated by a piece of PEM (5.0 × 5.0 cm, Nafion™ 117, Dupont Co., Wilmington, USA). The membrane was pre-treated by immersing in 0.10 M HCl for 4 hours at room temperature. Graphite rods (Spektralkolstav, diameter 0.90 × 30.5 cm, ISOAB, Germany) were used as the anode, and a piece of graphite roll (Spektralkolstav, 1.0 cm × 11 cm × 12 cm, ISOAB, Germany) was used as the cathode. The apparent surface areas of anode and cathode were 134 cm^2 and 310 cm^2, respectively. The volumes of anaerobic and aerobic compartments were 100 and 1,000 ml, respectively. Mixing was provided inside the anaerobic compartment by using a magnetic stirrer to enhance the distribution of the substrate and mass transfer. The aerobic compartment was continuously aerated with air pump for oxygen supply. An external electrical circuit, through which the electrons were transported, connected the anode and cathode. A variable resistor served as the load to the MFC.

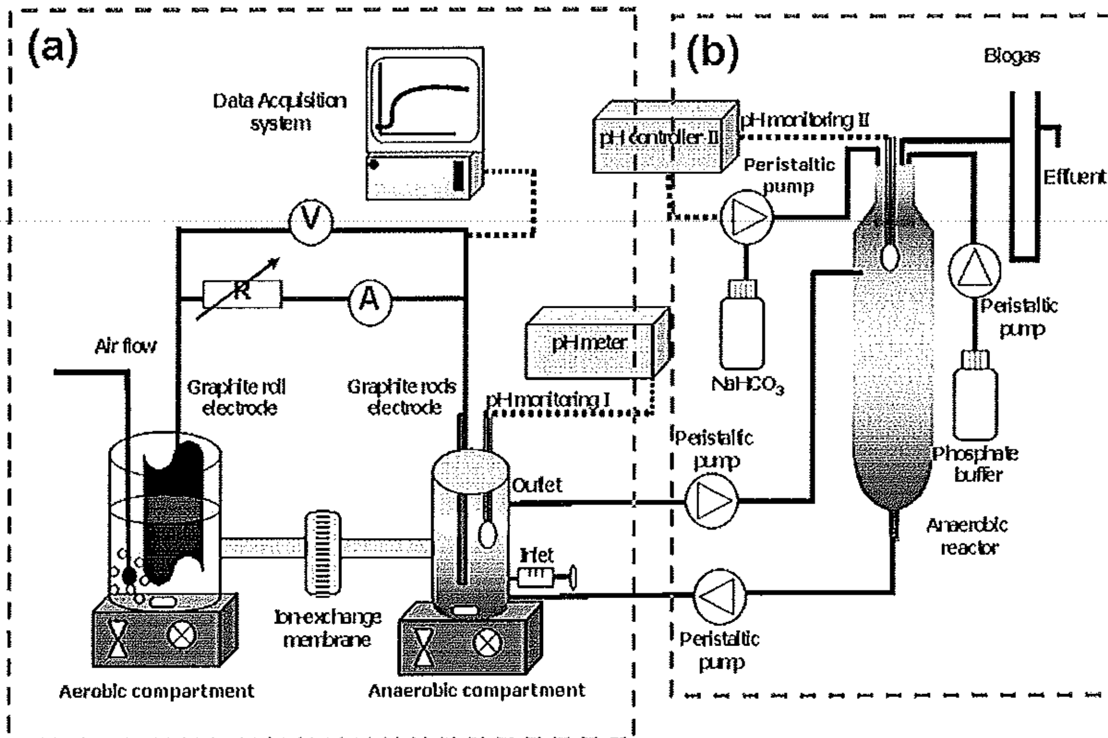


Figure 6.1 Schematic diagram of the MFC-type biosensor system (a) MFC biosensor system (b) anaerobic reactor.

An anaerobic reactor had been operated continuously in this study to provide bacteria consortium for MFC sensor system (Figure 6.1(b)). The reactor system consists of a cylindrical column made from jacketed glass with a total volume of 2.5 l, and a gas–liquid separation unit. The reactor temperature and the anode compartment were kept at 37 °C by pumping heated water through the reactor jacket. The anaerobic reactor was fed with 50 mM phosphate buffer (pH 7.5) at a flow rate of 0.70 ml min^{-1} through out the experiment to maintain a stable buffer capacity in the fermentation broth. It was considered a stable anaerobic consortium should be induced under the operational condition where type of feedstock, organic loading rate, temperature, alkalinity of the fermentation broth and pH were kept stable over a period of months. This consortium, originated from anaerobic sludge, was then used as biological recognition element in the MFC sensor. The anaerobic reactor was integrated into the MFC sensor system as shown

in Figure 6.1. For each sample analysis, 100 ml of fresh anaerobic sludge was pumped into the anaerobic compartment of the MFC sensor from the anaerobic reactor. After the sample was analyzed, the anaerobic sludge was then pumped back to the reactor. For an intensive sample analysis, a slight drop of the reactor pH might occur due to the formation of acidic compounds from the fermentation of organic substrates that were transferred together with anaerobic sludge from the MFC to the reactor. The anaerobic reactor was then equipped with an automatic pH control unit using 50 mM $NaHCO_3$ (pH = 8.0) to maintain a stable pH at 7.5 through out the experiment.

6.2.2 Anaerobic inoculumn and chemical

Anaerobic sludge which contained anaerobic consortium was collected from a pilot-scale biogas reactor at Biogas Research Station (Anneberg, Sweden) and was used as inoculum for a laboratory-scale anaerobic reactor. All chemicals used in this study were analytical-reagent grade and all solutions were prepared with deionized water.

6.2.3 Standard solution

A glucose standard solution, used as a preliminary substrate to evaluate the performance of MFC sensor, was prepared by mixing D-glucose 10 g l^{-1} with a basic medium solution. The basic medium solution was prepared by mixing 10 ml l^{-1} of the solution 1 (NH_4Cl 100 g l^{-1}, NaCl 10 g l^{-1}, $MgCl_2.6H_2O$ 10 g l^{-1}, $CaCl_2.2H_2O$ 5 .0 g l^{-1}) and 2 ml l^{-1} of the solution 2 (K_2HPO_4 152.6 g l^{-1}), 50 ml of 52 g l^{-1} $NaHCO_3$ and 1 ml the solution 3 ($FeCl_2.4H_2O$ 2.4 g l^{-1}, H_3BO_3 0.05 g l^{-1}, $CuCl_2.2H_2O$ 0.040 g l^{-1}, $AlCl_3.6H_2O$ 0.090 g l^{-1}, $NiCl_2.6H_2O$ 0.090 g l^{-1}, $Na_2SeO_3.5H_2O$ 0.10 g l^{-1}, $ZnCl_2$ 0.050 g l^{-1}, $MnCl_2.2H_2O$ 0.040 g l^{-1}, $CoCl_2.6H_2O$ 0.050 g l^{-1}, EDTA 0.50 g l^{-1}). The glucose standard solution was used as the feedstock (10 ml day^{-1}) to the anaerobic reactor to induce a stable anaerobic consortium.

6.2.4 Analyses and signal recording

The closed-circuit potential (U) between the MFC anode and cathode across the selected resistance was measured and recorded in selected sampling frequency using an in-house developed computer-based data acquisition system. The resistance of the electric circuit could be regulated from 0 up to 2,000 Ω. After each sample injection, the potential signal increased until it reached a new steady state in 3-5 min. The response was calculated as the difference between two steady state potential before and after the sample injection. Current (I) could be calculated from the given resistance and the measured cell potential (U), i.e. $I = U / R$. After each run fresh anaerobic sludge was loaded. All sample analyses were carried out in triplicate and the mean values were calculated.

6.3 Results and discussion

Figure 6.2 shows an example of the potential signal when injected 10 ml of 25 g l^{-1} glucose standard solution. In order to maximize the sensor signal and evaluate the sensor performance, system optimization has been carried out, followed by a study on the sensor characteristics.

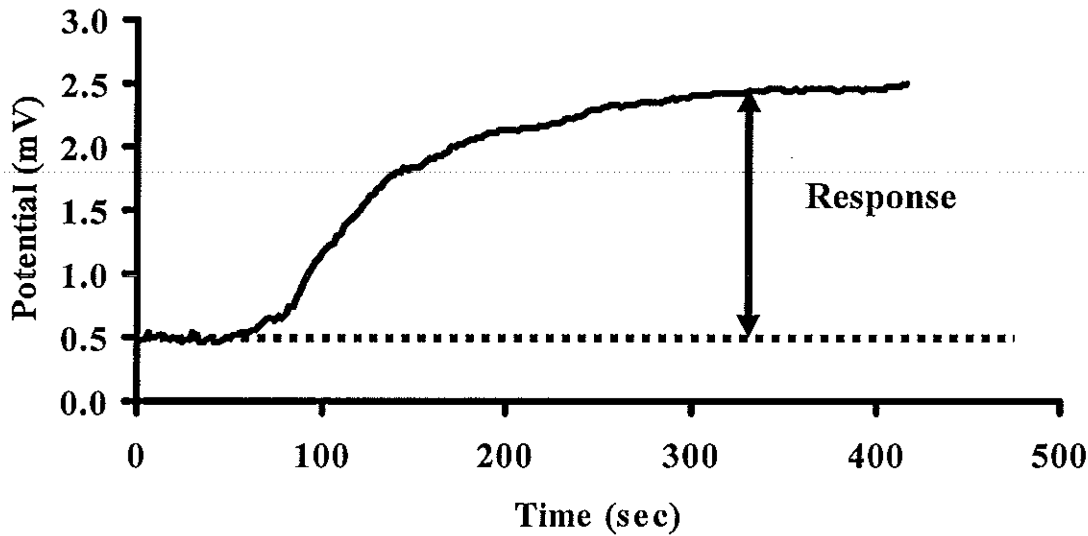


Figure 6.2 Response of MFC biosensor by injecting 10 ml of 25 g l^{-1} glucose standard solution. The microbial fuel cells were operated at 25 °C using 50 mM phosphate buffer solution as the electrolyte electrolyte.

6.3.1 Optimization of MFC sensor measurement

6.3.1.1 External circuit load

The potential output from the MFC sensor was evaluated with gradually increased circuit loads, from 50 to 1,200 Ω. Ten millilitres of 10 g l^{-1} glucose standard solution was injected for each analysis. The cell potential increased with resistance until 800 Ω, as shown in Figure 6.3. At higher resistance the sensor response started to decrease. It is possible that high resistance limits electron disposal through the electric circuit (Gil *et al.*, 2003). Therefore a resistor at 800 Ω was selected as the external circuit load.

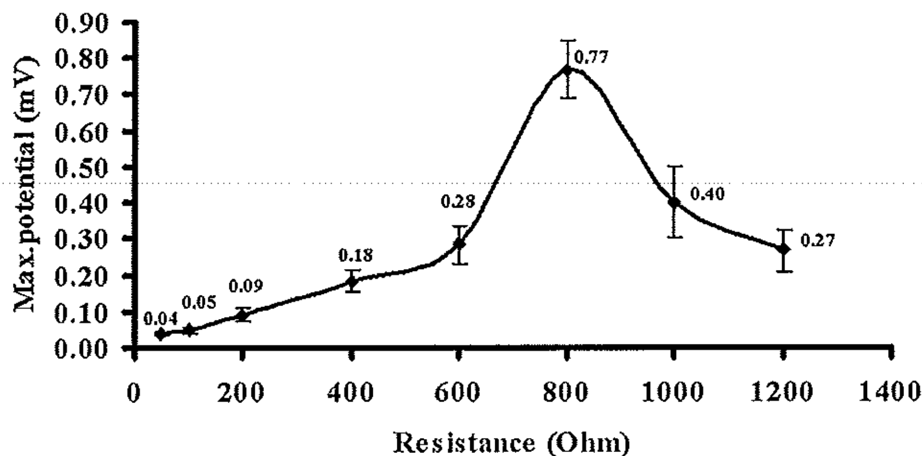


Figure 6.3 Potential generations from microbial fuel cells with different values of resistance with injecting 10 ml of 10 g l^{-1} glucose standard solution. The microbial fuel cells were operated at 25 °C using 50 mM phosphate buffer solution as the electrolyte electrolyte. 10 ml of glucose standard solution was injected for each analysis (n=3).

6.3.1.2 Electrolyte

Electrolyte is a chemical compound that conducts ions between anode and cathode compartment but it is impermeable to electrons (Fitzgerald R., 2001). The microbial fuel cells were tested with different solution in the cathodic compartment, i.e., distilled water, NaCl solution (100 mM), phosphate buffer (50 mM), phosphate buffer (50 mM) with NaCl (100 mM), phosphate buffer (25 mM) with NaCl (50 mM) and HCl solution (10 mM). The highest sensor signal expressed as potential output at 800 Ω was observed for the catholyte containing 25 mM phosphate buffer and 50 mM NaCl (Figure 6.4). Therefore, this type of electrolyte was selected in the subsequent experiment. The lowest potential was generated for the catholyte containing 10 mM HCl due to the inhibition effect of protons transportation from the anodic compartment. In such a case,

the resistance of protons migration through PEM could be hampered by the high proton concentration in the cathodic compartment.

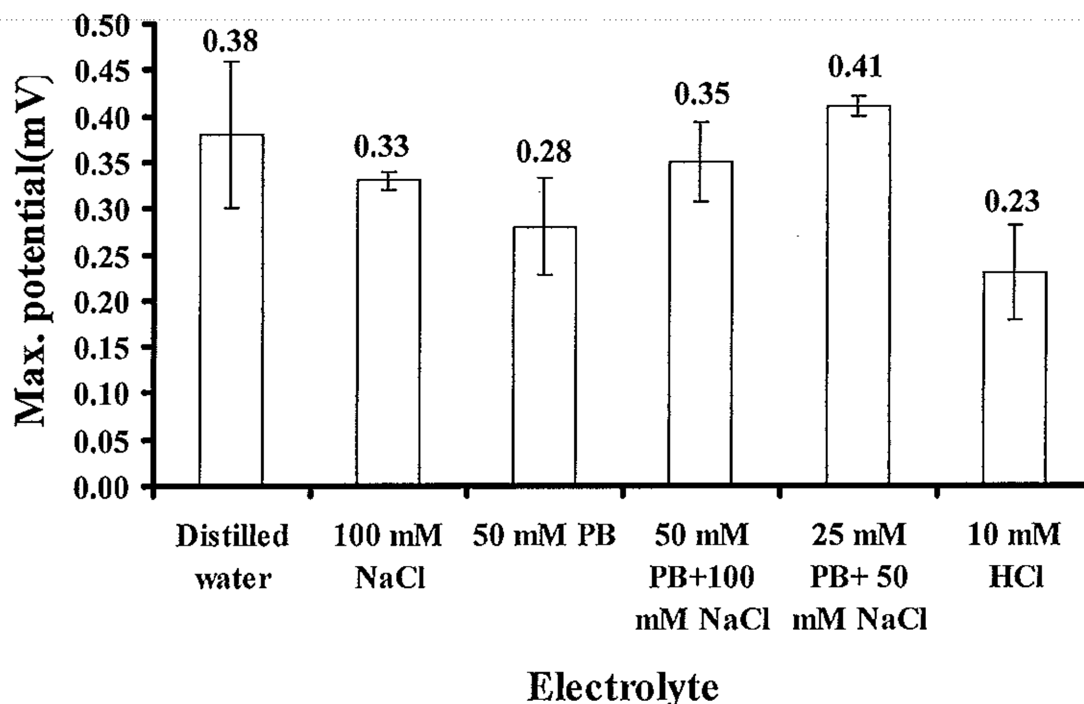


Figure 6.4 Cell potential with different types of catholyte (operational temperature: 25 °C, resistance of external circuit: 800 Ω). 10 ml of glucose standard solution was injected for each analysis (n=3).

6.3.1.3 Temperature

In the current study, the anaerobic compartment was equipped with the temperature control unit. Operational temperature from 20 to 40 °C was tested in order to find the best temperature for operating the MFC sensor. As shown in Figure 6.5, the highest cell potential was observed at 37 °C, whereas signal decreased when the temperature was set below or above this temperature. Thirty-seven degree Celsius was therefore selected as the optimal temperature for operating the MFC sensor system.

Optimum conditions of the MFC are summarized in Table 6.1.

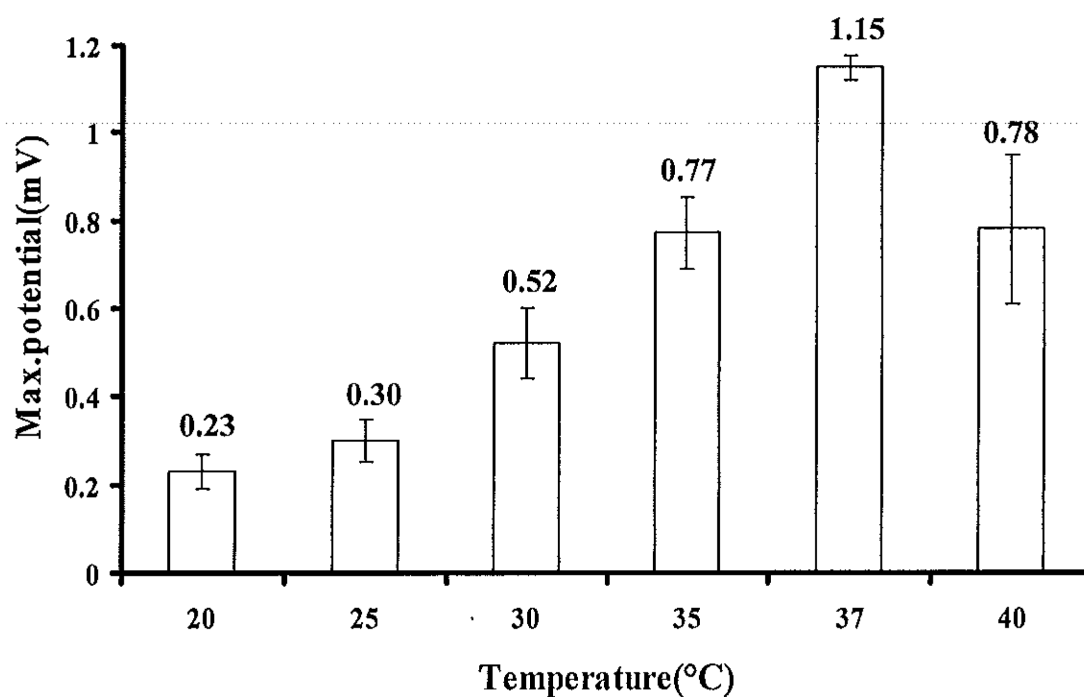


Figure 6.5 Effect of operational temperature on the cell potential (resistance of external circuit: 800 Ω, catholyte: 50 mM phosphate buffer and 25 mM NaCl). 10 ml of glucose standard solution was injected for each analysis (n=3).

Table 6.1 Optimized values of biosensor operating condition of MFC system

Parameters	Value	Optimum
Resistance (Ohm)	50, 100, 200, 400, 600, 800, 1,000, 1,200	800
Electrolyte	Distilled water, 100 mM NaCl solution, 50 mM phosphate buffer, 50 mM phosphate buffer with 100 mM NaCl , 25 mM phosphate buffer with 50 mM NaCl and 10 mM HCl solution	25 mM phosphate buffer with 50 mM NaCl
Temperature (°C)	20, 25, 30, 35, 37, 40	37

6.3.2 Performance characteristics of the MFC sensor

After the optimum conditions were obtained, performance characteristics of the MFC sensor were studied using these conditions (Table 6.1).

6.3.2.1 Linearity and the detection limit

System calibration was performed to evaluate the linearity of sensor response and sensor detection limit. A broad linear range when plotting response versus concentration of analyte is desirable for reliable and accurate measurement over the concentration range. Furthermore, the population of induced anaerobic consortium may still be varied over a longer period of time. This can give a negative effect on repeatability of the measurement. However, the problem can be simply solved by carrying out system calibration in a regular time base. As demonstrated in Figure 6.6, the linearity of MFC sensor response to the glucose standard solutions can be observed between 1.0 and 25 g l^{-1} ($y = (0.0514 \pm 0.0019)x + (0.267 \pm 0.052)$, $R^2 = 0.9914$). The detection limit is found to be 25 mg l^{-1} determined by comparing measured signals from analyte samples in low concentration with those of blank samples. The minimum concentration was established at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit (US DFA, 1996).

One of the bottlenecks for MFC is transport of ions in the electrolyte. For a good operation of the MFC, both proton and electrons need to migrate from the anode to the cathode at the highest possible rate (Lens *et al.*, 2005). Diffusion is often not sufficient to reach acceptable levels of current and cell potential. For the experimental setup in the current study, the PEM was placed in a specific modified plastic holder. Although the membrane area is considerably large, the connections to anodic and cathodic compartment were made through 40 mm long tubes with a diameter of 8 mm. Transport of proton is therefore relatively inefficient even through enhanced diffusion was achieved in the anodic and cathodic compartment through mixing and aeration, respectively. A preliminary test has shown a significant increase of the cell potential

when the connecting tubes of anodic and cathodic chambers became shorter as shown in Figure 6.7. A large cell potential and lower detection limit are, therefore, expected with improved cell configuration.

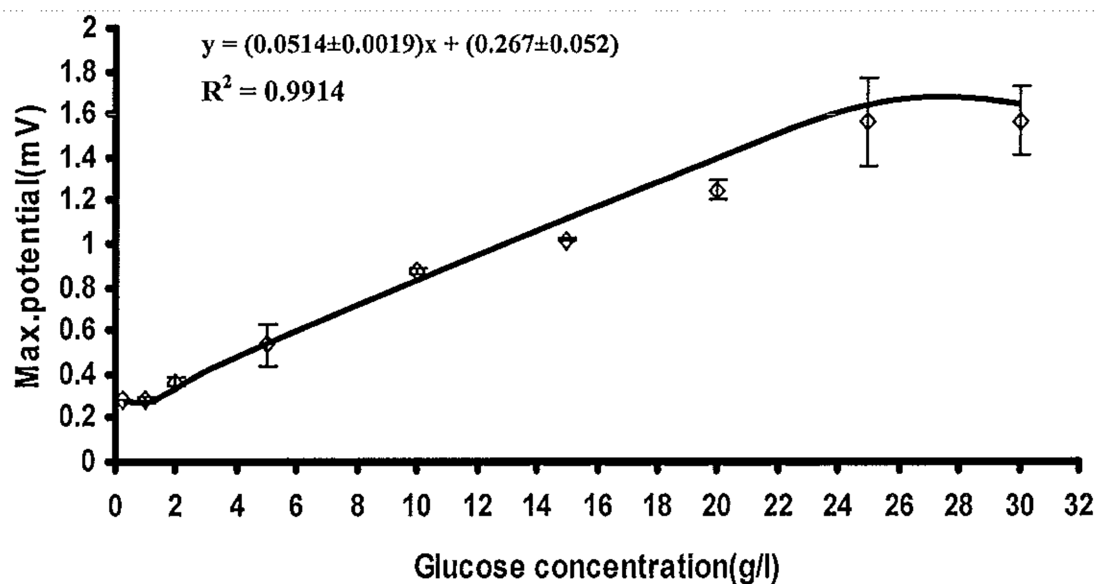


Figure 6.6 A calibration curve of the MFC sensor (operational temperature: 37 °C, resistance of external circuit: 800 Ω, catholyte: 50 mM phosphate buffer and 25 mM NaCl) (n=3).

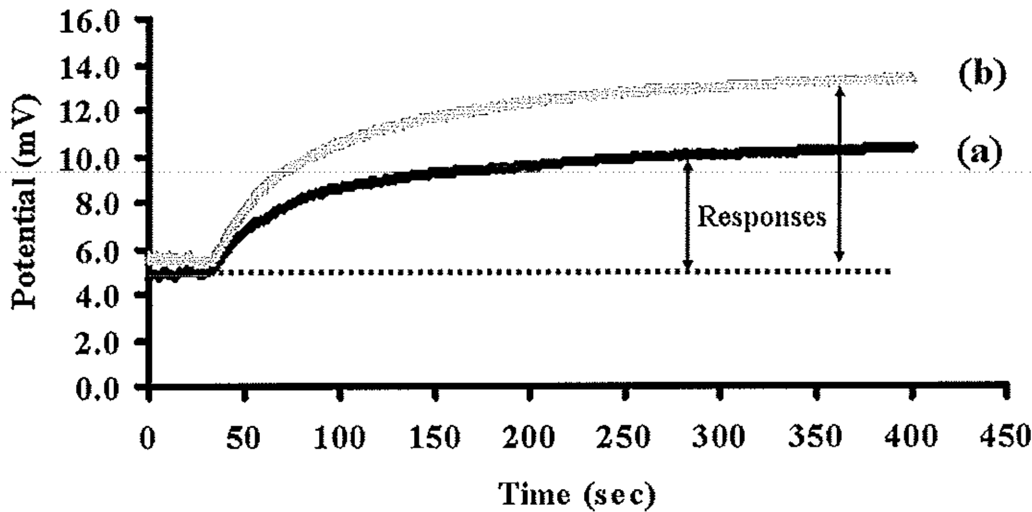


Figure 6.7 Preliminary test showing a significant increase of the cell potential when the connecting tube of anodic and cathodic chamber was reduced from (a) 10.0 cm and (b) 5.0 cm.

6.3.2.2 Repeatability

The repeatability of the MFC sensor has been studied by injecting 10 ml of the glucose standard solution 12 times over 15 hours. It was observed that the sensor gave fairly constant response (i.e. average cell potential was 1.11 ± 0.08 mV) during the testing period with relative standard deviation of ± 7.2 %, as shown in Figure 6.8. It should be noted that the biological recognition element was renewed before each sample analysis by replacing the old anaerobic consortium in the anodic compartment with the fresh anaerobic sludge from the anaerobic bioreactor. Still the system gives stable measurement with a good repeatability. The results could be compared to those obtained from conventional BOD 5-day tests, where ±15% is allowed (Liu *et al.*, 2000).

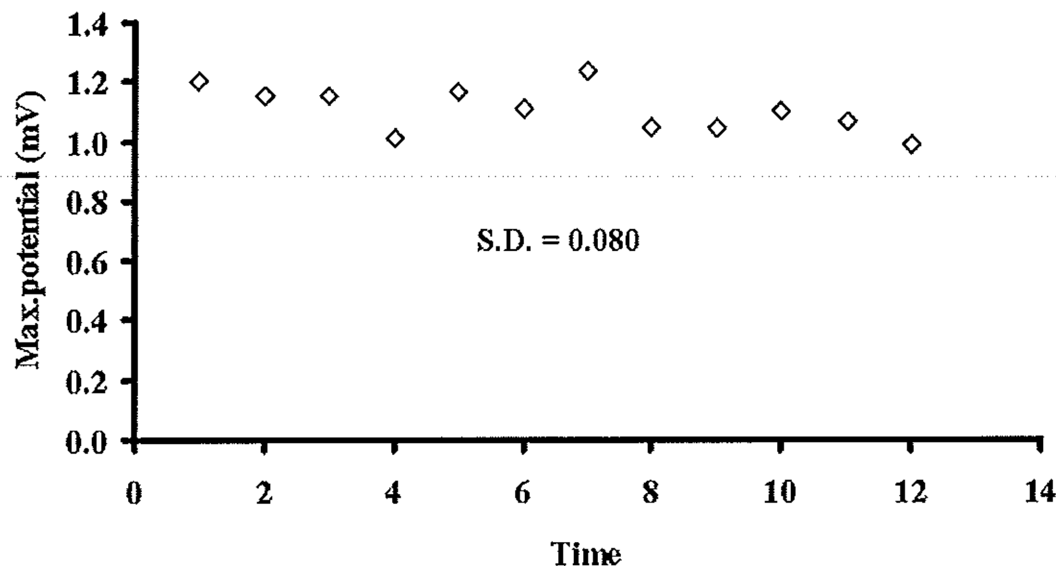


Figure 6.8 The repeatability of the MFC BOD biosensor system with injecting pulses of 10 ml of 10 g l^{-1} glucose standard solution. The microbial fuel cell was operated at 37 °C using a resistance of 800 Ω and 50 mM phosphate buffer with 25 mM NaCl solution as the electrolyte.

6.3.2.3 Stability

A stable sensor performance over a desired operational period is essential for a reliable biosensor system. The stability of the MFC sensor was evaluated by analyzing glucose standard solution over a period of 11 days. The analysis was carried out in triplicate by injecting 10 ml of glucose standard solution per analysis. The total analysing time was approximately 1 hour per each analysis which included pumping sludge from reactor to MFC chamber, injecting sample and pumping sludge back to reactor. As shown in Figure 6.9, the cell potential remained fairly stable over the whole testing period. The average cell potential varied daily between 1.00 and 1.10 mV with relative standard deviation from ±2.0 % to ±9.0 %. The average cell potential calculated from the 33 measurements of the whole testing period was 1.07 mV with relative standard deviation of ±5.9 %. It was observed that the stability of MFC sensor was

strongly affected by the surface condition of PEM. Nafion™ has been widely used as PEM for MFCs, and has the great advantage of being very selective for protons. However, this membrane contains sulfonic acid groups that are binding with ammonia present in the bacterial suspension. Hence, this membrane type scores high for selectivity but low for stability in a colloidal and nutrient-rich bacterial suspension (Lens *et al.*, 2005).

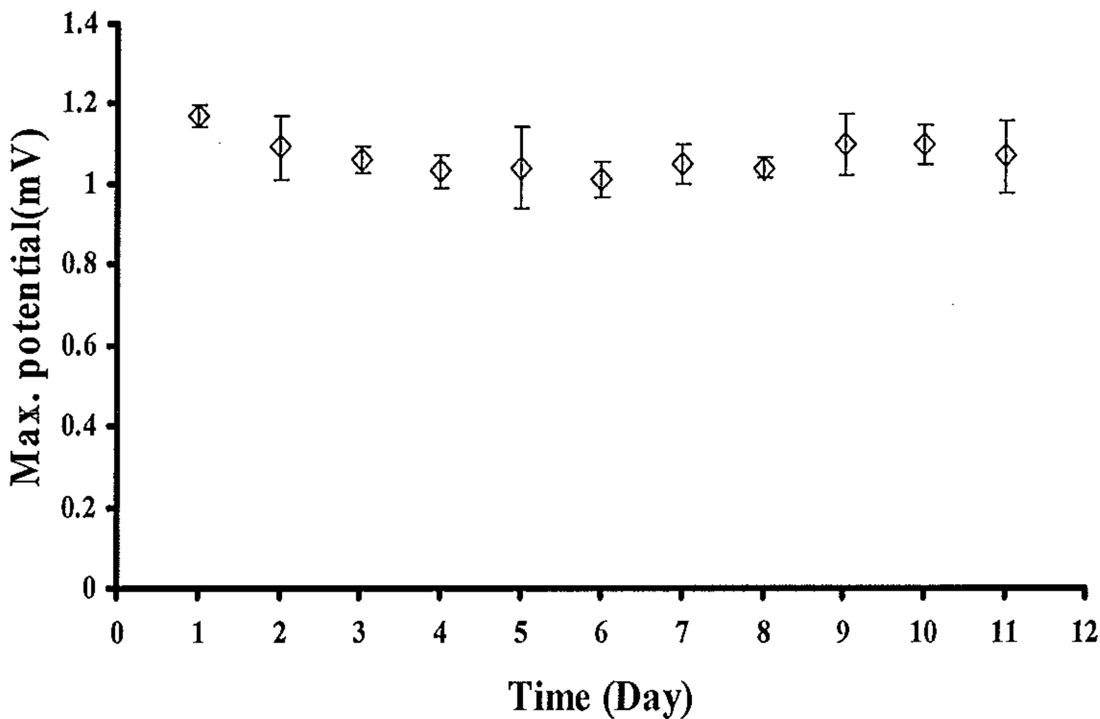


Figure 6.9 Stability of the cell potential (operational temperature: 37 °C, resistance of external circuit: 800 Ω, catholyte: 50 mM phosphate and 25 mM NaCl). 10 ml of glucose standard solution was injected for each analysis (n=3).

6.4 Conclusion

The current study shows the development of a MFC sensor system for fast estimation of easily biodegradable organic matter. The sensor system was operated by integrating with an anaerobic bioreactor for continuous supply of stable anaerobic consortium. Replacement of the biological recognition element was carried out for each

sample analysis. Still the system gives stable measurement with a good reproducibility. The sensor response time was estimated around 3–5 min and did not have to wait for the metabolic recovery of anaerobic consortium in the anodic compartment. This is considered as an advantage for this sensor system configuration. Although glucose was used as the only preliminary substrate in the current study to evaluate the sensor performance under the well-controlled condition, the MFC sensor system used mixed consortium originated from anaerobic sludge should also be able to degrade other kinds of organic matter even in more complex form. The MFC sensor needs, however, in its present configuration maintenance in terms of regular membrane cleaning. The sensitivity and the detection limit of the sensor system can certainly be improved even further by improving the efficiency of ions transport in the electrolyte.

CHAPTER 7

Simple to construct Microbial fuel cell using *Saccharomyces cerevisiae* for wastewater BOD analysis

7.1 Introduction

Microbial fuel cells (MFCs) are devices that use bacteria as the catalysts to oxidize organic and inorganic matter and generate current (Logan *et al.,* 2006). A typical MFC consist of two compartments, anodic and cathodic, separated by a proton exchange membrane (PEM) or salt bridge. When substrate are added and oxidized by the microbes in the anodic chamber electrons and positive ions are released. Electrons picked up by the anode, either through the diffusion of a secondary fuel, via a mediator molecule that repeatedly cycles or via a direct electron transfer between the two compartments, to the cathode (Oh et al., 2004; Min et al., 2005; Grzebyk and Pozniak, 2005; Ghangrekar and Shinde, 2007).

MFCs have been studied as an alternative source of electricity and this has been a subject of several recent reviews (Shukla *et al.,* 2004; Rabaey and Verstraete, 2005; Bullen *et al.,* 2006; Logan *et al.,* 2006; Lovley, 2006; Davis and Higson, 2007). They have also shown potential as sensors for fast determination of biochemical oxygen demand (BOD) using electrochemically active microorganisms that have been enriched and in some cases maintained in the MFC for a few years (Kang *et al.,* 2003; Kim *et al.,* 2003; Chang *et al.,* 2004; Moon *et al.,* 2004; Chang *et al.,* 2005). Recently an MFC "powered" by ordinary dry Baker's yeast, *Saccharomyces cerevisiae*, with glucose as substrate and methylene blue as an electron mediator was investigated (Walker and Walker, 2006). In this study aerobic respiration more efficiently utilizes glucose and produces more energy (i.e. electrons) than fermentation. At optimum conditions power peaks of 130 μW was obtained at about 45 ^{o}C. The advantage of using Baker's yeast is

that it is inexpensive and readily available, dose not require sterile conditions, is not substrate specific, and tolerates a wide range of environmental conditions (Walker and Walker, 2006). Therefore, it would be convenient to apply yeast MFC for BOD analysis.

Although electron mediator was used in the above study it has been shown in another work that transportation of electron from yeast metabolism to the electrode in a fuel cell can also be through reduced intermediate products (Holtmann *et al.,* 2006), hence no mediator is required. The explanation provided by Holtmann and coworkers was that when the substrate in the medium, S_{medium}, is transported into the cell the catabolism of substrate, S_{MO}, takes place (Figure 7.1) and reduced intermediates $P_{red,MO}$ are formed. Some of these intermediates, ($P_{red,medium}$) are released into the medium. When $P_{red,medium}$ is oxidized at the anode, the electrons are transferred to the anode. These electrons move through an external load to the cathode, causing a potential drop. The generated protons migrate from the anode compartment to the cathode compartment. At the cathode oxygen is reduced to water.

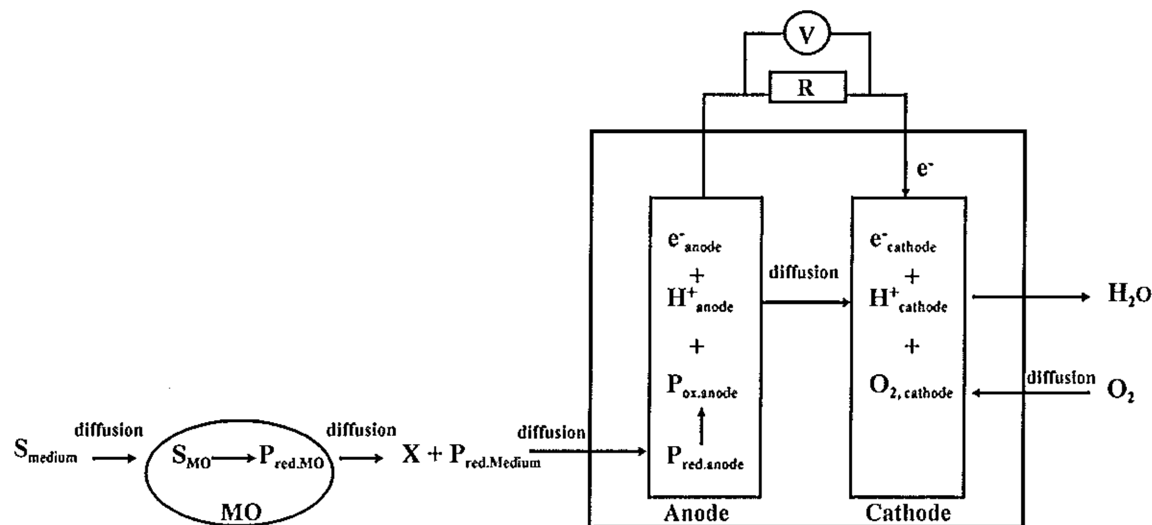


Figure 7.1 Principle of transportation of electron from yeast metabolism to the electrode in a fuel cell (S = substrate, P = product, MO = microorganisms, X = biomass, red = reduced, ox = oxidized) (Modified from Holtmann *et al.,* 2006).

In this study we focus on the development of a simple MFC using aerobic reaction of *Saccharomyces cerevisiae* without any added mediator to determine BOD in wastewater. Freeze dried Baker's yeast was activated by aerobic cultivation. A simple agar salt bridge was used to separate the anode and cathode compartments in place of the more expensive PEM. Common graphite pencil leads were used as electrodes. Parameters affecting the system were optimized and the performances were investigated. Wastewater samples from several sources were tested and the BOD values obtained from the MFC system were compared to the values obtained by standard BOD_5 method.

7.2 Materials and methods

All chemicals used in this study were analytical-reagent grade and all solutions were prepared with deionized water.

7.2.1 Preparation of microorganism

One gram of freeze dried Baker's yeast (*Saccharomyces cerevisiae*) (S.I.Lesaffre, France) were activated by aerobic cultivation for 24 h at room temperature on a rotary shaker (HL instruments, Thailand) operating at 150 rpm in a 500 ml flask containing 100 ml YPD medium consists of 10 g l^{-1} yeast extract (MERCH, Germany), 20 g l^{-1} peptone (MERCH, Germany) and 20 g l^{-1} dextrose (Himedia Laboratories Pvt. Ltd., India). Cells were centrifuged (15 min, 860 × g) (DUPONT instruments, USA) washed twice with saline solution (0.90% w/v NaCl (CARLO ERBA, France)) by resuspension and centrifuged again. Each cultivation gave around 5.0 g of wet weight cells. The cells were then cultivated again under the same condition by distributed 1.0 g of wet weight cells into 100 ml of liquid medium containing 1.0 g l^{-1} KH_2PO_4(AnalaR, England), 3.5 g l^{-1} $(NH_4)SO_4$ (Fluka, Switzerland), 0.30 g l^{-1} $MgSO_4$ (J.T. Baker Inc., USA), 0.10 g l^{-1} $CaCl_2$ (MERCH, Germany), and 20 g l^{-1} D-glucose (Riedel-de Haen, France). This cultivation gave around 10 g of wet weight cells and these were used for the MFC system.

7.2.2 MFC design and operation

Figure 7.2 shows schematic diagram of the MFC system with an anode and a cathode compartments in a Perspex chamber. The 2 compartments are separated by a salt bridge in the middle of the chamber. To prepare a salt bridge, two Perspex sheets was placed 1.0 cm apart. Agar, 2.0% w/v (Himedia Laboratories Pvt. Ltd., India), was boiled in saturated potassium chloride solution (Ajex Finechem, New Zealand) for 20 min (or until the solution was clear) and then poured between the Perspex sheets until it reached the height of 8.0 cm. When the agar was set the Perspex sheets were removed. One hundred milliliters of anode and cathode reagents were added to the compartments, now separated by a salt bridge 'membrane' with a 64 cm^2 surface area. Mixing was provided inside the anode compartment by using a magnetic stirrer (100 rpm) to enhance the circulation of the added substrate and mass transfer. "Electrode" in each chamber consisted of 12 graphite pencil leads (diameter 0.50 mm × length 60 mm) with a total surface area of 0.24 cm^2. The anode and cathode were connected by a 10 Ohm resistor, through which the electrons were transported. A multimeter and a chart recorder were used to record the current and voltage, respectively.

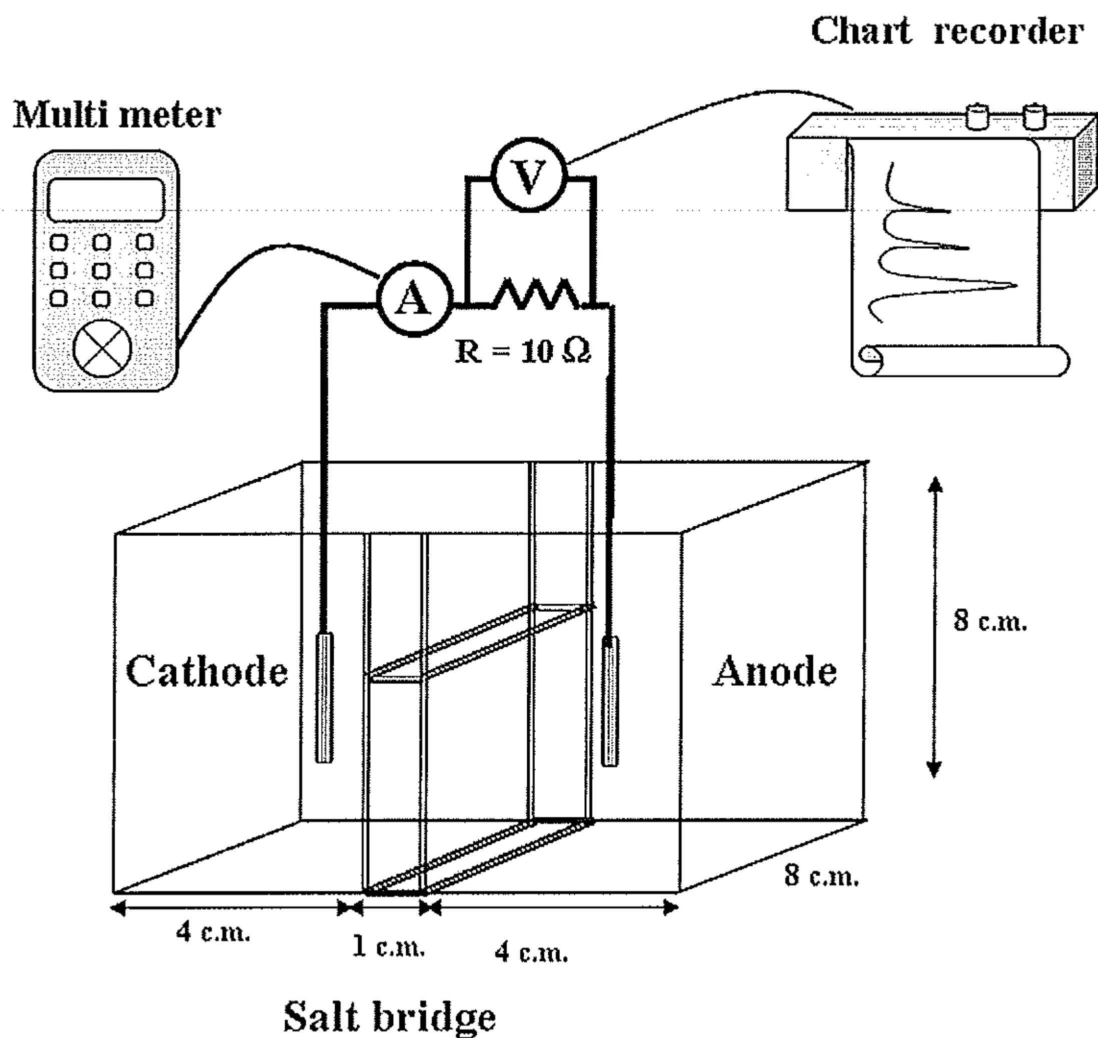


Figure 7.2 Schematic diagram of the MFC biosensor system.

7.2.3 Calibration solution

The system was calibrated by comparing three substrates; the OECD (Organisation for Economic Cooperation and Development) synthetic wastewater containing 7.5 mg l^{-1} peptone (MERCH, Germany), 5.5 mg l^{-1} beef extract (MERCH, Germany), 1.5 mg l^{-1} urea (Riedel-de Haen, France), 0.35 mg l^{-1} NaCl (CARLO ERBA, France), 0.20 mg l^{-1} $CaCl_2.2H_2O$ (MERCH, Germany), 1.4 mg l^{-1} K_2HPO_4 (AnalaR, England), 0.10 mg l^{-1} $MgSO_4.7H_2O$ (J.T. Baker Inc., USA), 75 mg l^{-1} D-glucose

(Riedel-de Haen, France) 75 mg l^{-1} glutamic acid (MERCH, Germany) (Liu *et al.*, 2000); YPD medium containing 10 g l^{-1} yeast extract (MERCH, Germany), 20 g l^{-1} peptone (MERCH, Germany), 20 g l^{-1} dextrose (Himedia Laboratories Pvt. Ltd., India); and glucose standard solution containing 75 mg l^{-1} D-glucose (Riedel-de Haen, France), 13.6 mg l^{-1} KH_2PO_4 (AnalaR, England) 17.4 mg l^{-1} K_2HPO_4 (AnalaR, England). For each solution a calibration curve was prepared by plotting the change in current against the corresponding BOD values (mg l^{-1}) tested using standard BOD_5 method (Kim *et al.*, 2003; Chang *et al.*, 2004). The sensitivities (slope) of the calibration curve of the different sample values were then compared.

7.2.4 Analyses and signal recording

Glucose standard solution, 500 µl, was added to the anode compartment using a micropipette. The potential and current signals increased, reaching peak values before slowly decreased to the base line. The generated current (I, ampere) was measured using a multimeter (FLUKE 87, JOHN FLUKE MKG Co., INC., Washinton, USA) and the closed-circuit potential (V, volt) across a load resistance was measured and recorded on a chart recorder (ROSS RECORDERS, USA). The peak height was used as the response signal that was dependent on the concentration of the sample solution. All sample analyses were carried out in triplicate and the mean values were calculated. These data were used to calculate the power (Power (P) = Potential (V) × Current (I)) of the microbial fuel cell.

7.2.5 Optimization of MFC sensor

In order to maximize the sensor signal and evaluate the sensor performance, optimization of the system was first carried out using 500 µl of 50 mM glucose standard solution. Optimization of the MFC sensor was done by changing one parameter while keeping the others constant. When an optimum value was obtained it was used to optimize the next parameter. The optimum conditions in this system were

considered by balancing between the generated current, potential and power and the analysis time. Optimized parameters and values are shown in Table 7.1.

Table 7.1 Optimized parameters and values for the MFC biosensor system

Parameters	Values
Resistance (Ohm)	10, 11, 15, 21, 19, 30, 38, 100, 117, 180, 200, 270, 330, 500, 1000, 1500
Cathode electrolyte	
Type	Ferric cyanide, Phosphate buffer pH=7.0, HCl, DI water, Phosphate buffer+ NaCl pH=7.0, NaCl
Concentration (mM)	25, 50, 75, 100
pH	6.5, 7.0, 7.5, 8.0
Cell loading in anode compartment(%w/v)	1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0

7.2.6 Real sample analysis

Fifteen wastewater samples from 7 locations were collected from wastewater ponds. Two samples were from 2 concentrated rubber factories, six from 4 hospitals, two from a car wash company, one from a color manufacturing plant, two from a rubber glove manufacturer and one from a gypsum production manufacturing company. All samples were stored at 4.0 °C until used and tested by the MFC sensor and the measured BOD values were compared to BOD_5 values obtained by the standard method. The analysis of each sample was carried out in triplicate. Any solid particles were first removed from each sample by filtration before analysis (Whatman diameter 185 mm, Whatman International Ltd Maidstone England, England).

The measurement of BOD in real samples was carried out under optimum conditions. Glucose standard solutions were first used to calibrate the system before analysis of real samples. The calibration curve plotted the generated current change

versus the corresponding BOD values (mg l^{-1}). Wastewater samples with high concentrations of organic matter were diluted using 100 mM phosphate buffer pH 7.0. The samples were then added into the anode compartment of the MFC. The change in generated current of each sample was used to calculate the BOD value of the sample from the calibration done prior to the test. The Wilcoxon signed rank test (Miller and Miller, 2000) was used to statistically test the results.

7.3 Results and discussion

Typical potential responses of the microbial fuel cell when injecting 500 µl of glucose are shown in Figure 7.3. In this case, peak height was used to interpret raw data since it was easily identified and directly related to the measurable signal. The response time was taken as the time after the response started to increase until it returned to baseline.

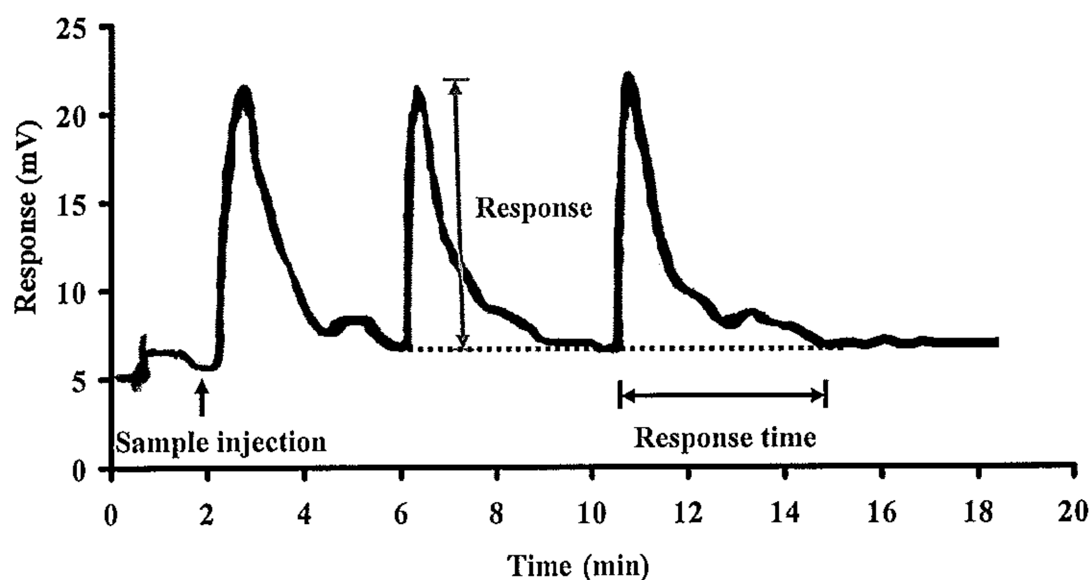


Figure 7.3 Typical potential responses of the microbial fuel cell when injecting 500 µl of 50 mM glucose (operational resistance of external circuit: 100 Ohm).

7.3.1 Optimization

7.3.1.1 Effect of external circuit load

The generated potential and current from the MFC sensor at different circuit load, (10 - 500 Ohm) were evaluated by injecting 500 μl of 50 mM glucose standard solution. The power was calculated from the measured potential and current (P=VI) as the external circuit loads were being increased (Figure 7.4). The maximum power of 8.11±0.05 μW was generated at an external circuit load of 200 Ohm. It might be because high resistance limits electron disposal through the electrical circuit (Gil *et al.*, 2003). Thus a resistor at 200 Ohm was selected as the external circuit load.

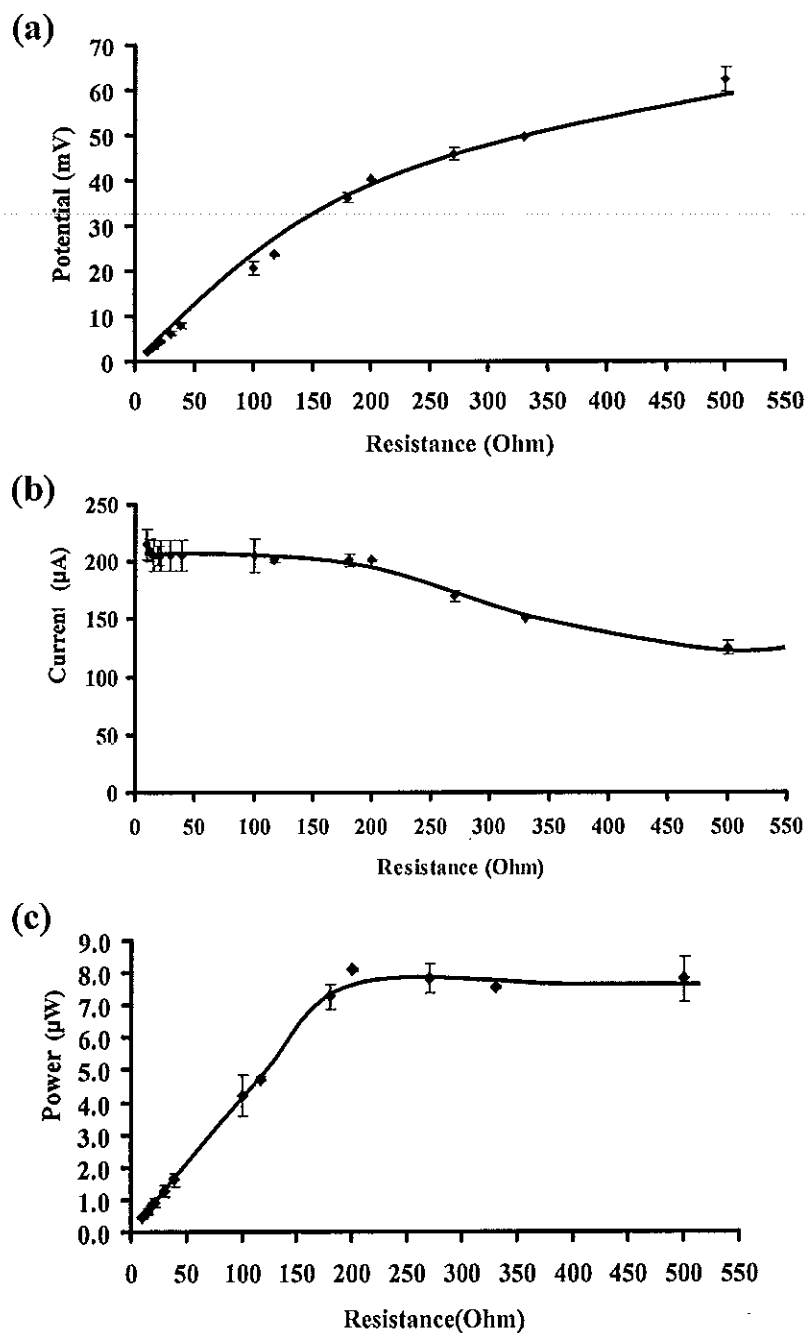


Figure 7.4 (a) potential, (b) current and calculated (c) power of MFC at different external loads, 500 µl of glucose standard solution was injected for each analysis ($n = 3$).

7.3.1.2 Effect of electrolyte type

An electrolyte is a chemical compound that conducts ions between the anode and cathode compartment but it is impervious to other electrons (Fitzgerald, 2001). The microbial fuel cell was operated at 200 Ohm with different electrolyte solution in the cathodic compartment, i.e., ferric cyanide, phosphate buffer (50 mM) pH=7.0, HCl solution (10 mM), distilled water, phosphate buffer (50 mM) pH=7.0 in 75 mM NaCl and NaCl solution (100 mM). The highest generated current was observed with the catholyte containing 50 mM phosphate buffer pH=7.0 in 75 mM NaCl (Figure 7.5). Therefore, this electrolyte was selected for the subsequent experiments. The lowest potential was generated by 10 mM HCl. This may due to its ability to inhibit proton transportation from the anodic compartment. In this case, the resistance to protons migrating through the salt bridge could be hampered by the high proton concentration in the cathodic compartment (Kumlanghan *et al.*, 2007).

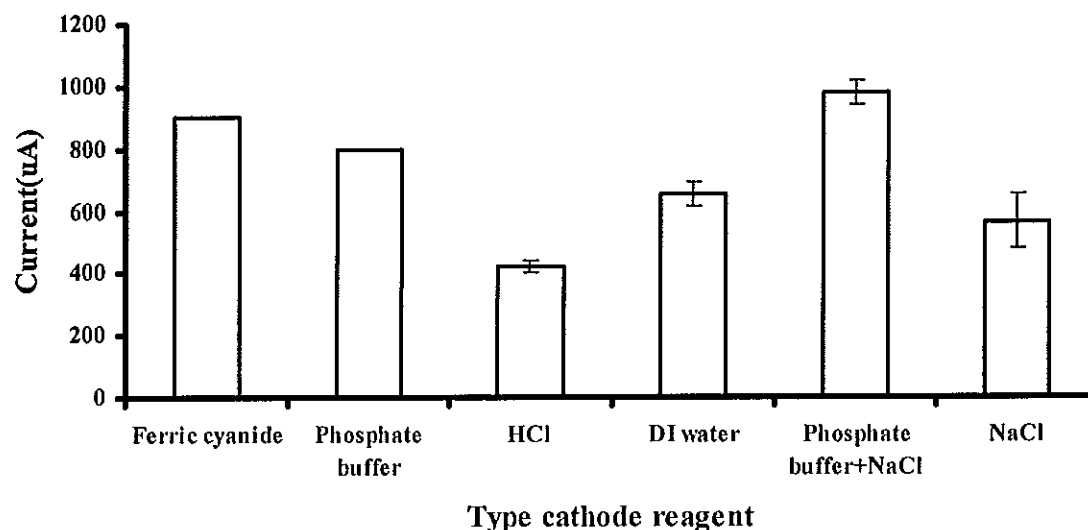


Figure 7.5 Effect of different cathode reagent (operational resistance of external circuit: 200 Ohm). 500 μl of glucose standard solution was injected for each analysis ($n = 3$).

7.3.1.3 Effect of electrolyte concentration

The effect of electrolyte concentrations was investigated with phosphate buffered solution pH=7.0 at 25, 50, 75 and 100 mM all in 75 mM NaCl. For each concentration 500 μl of 50 mM glucose standard solution was the test solution. A concentration of 50 mM phosphate buffer in 75mM NaCl gave the highest current, as shown in Figure 7.6.

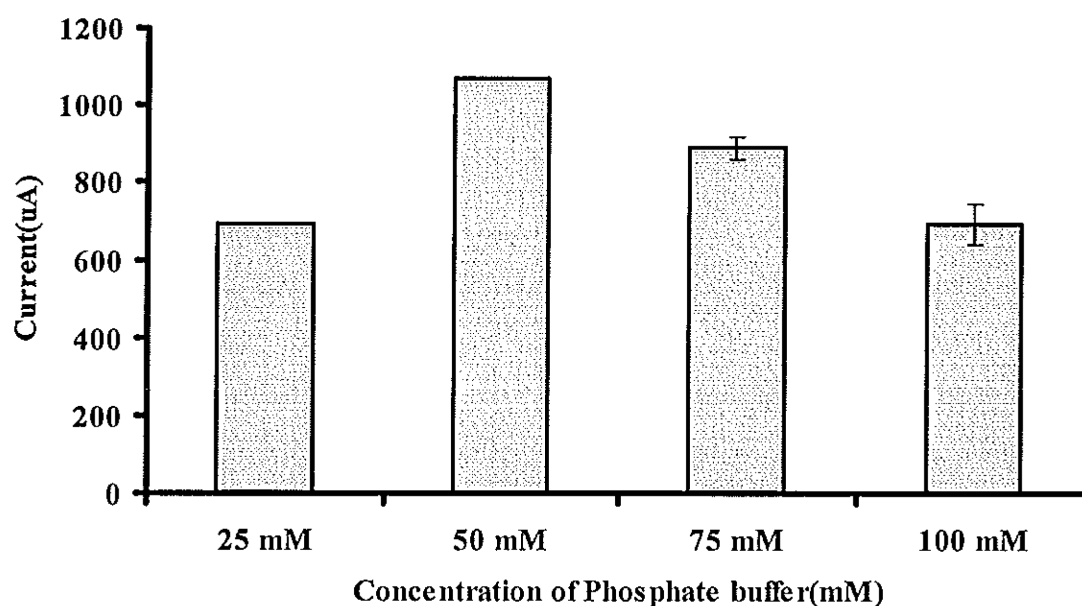


Figure 7.6 Effect of different electrolyte concentration (operational resistance of external circuit: 200 Ohm). 500 μl of glucose standard solution was injected for each analysis (n = 3).

7.3.1.4 Effect of electrolyte pH

The effect of different pH values (6.5, 7.0, 7.5 and 8.0) was investigated in the phosphate buffered solution. For each pH, 500 μl of 50 mM glucose standard solution was the test sample. The highest current was produced at pH 7.0 (Figure 7.7). This is probably due to the inactivation of the microorganism at lower and higher pH values (Jia

et al., 2003) where the observed pH reliance of the sensor response is dependent on the activity of the microbes (Han *et al.*, 2002).

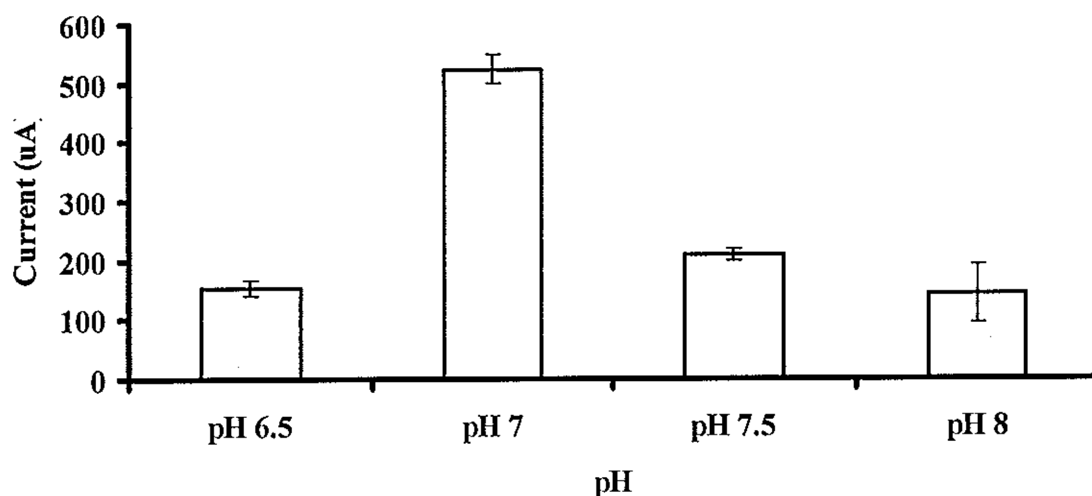


Figure 7.7 Effect of different electrolyte pH (operational resistance of external circuit: 200 Ohm). 500 μl of glucose standard solution was injected for each analysis ($n = 3$).

7.3.1.5 Effect of cell loading

The effect of yeast cells loading was investigated at 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0% w/v wet weigh of yeast cells per volume of 100 mM phosphate buffer. The results showed that the current increased until a cell loading of 6.0% w/v, after which the responses decreased (Figure 7.8). It is possible that at less than 6.0% w/v, the oxidation of the standard glucose solution is limited by the numbers of cells present. On the other hand at loadings of more than 6.0% w/v, the permeability of dissolved oxygen may have been limited, because of the density of the particles (in this case cells) being too high (Sohn *et al.*, 1995).

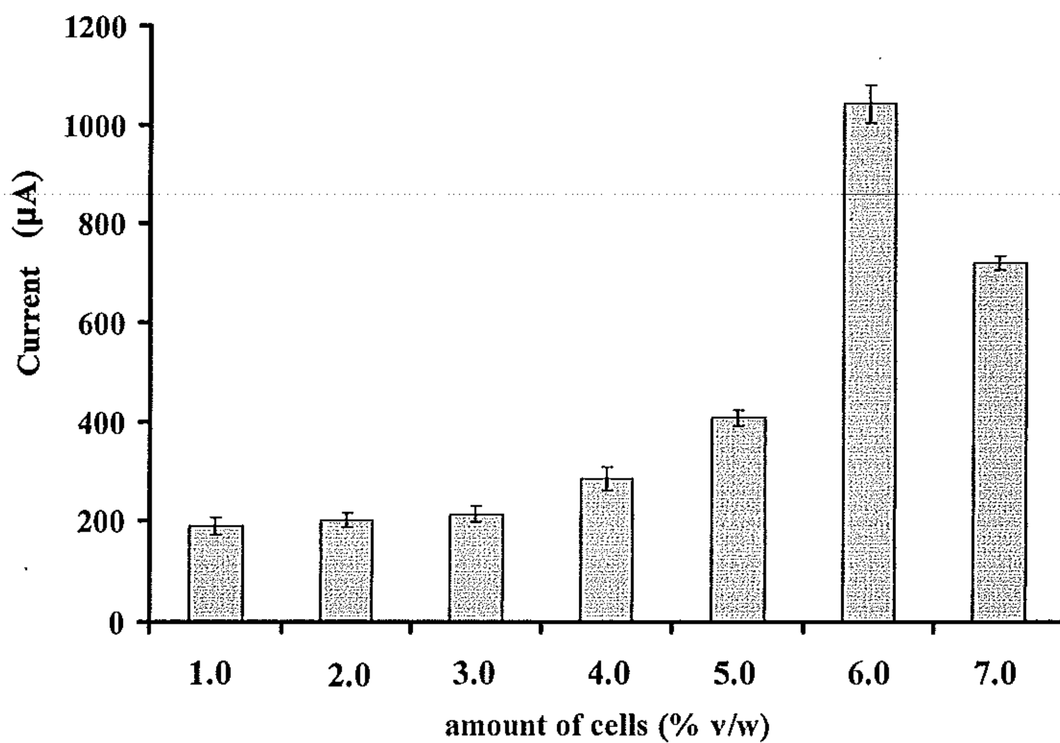


Figure 7.8 Effect of different amount of cells (operational resistance of external circuit: 200 Ohm). 500 μl of glucose standard solution was injected for each analysis ($n = 3$).

The optimum conditions of the MFC are summarized in Table 7.2

Table7.2 Optimized values of MFC biosensor operating conditions.

Parameters	**Optimum**
Resistance (Ohm)	200
Cathode electrolyte	
Type	Phosphate buffer+ NaCl
Concentration (mM)	50
pH	7.0
Cell loading in anode compartment (% w/v)	6.0

7.3.2 Characteristics of the MFC performance

The optimum conditions were used to study the performance characteristics of the MFC sensor.

7.3.2.1 Linearity and detection limit

The system was tested by comparing the responses to three standard substrates. The calibration curve for each test solution was prepared by plotting the current change versus BOD values (mg l^{-1}). Calibration of the system was performed to evaluate the linearity of the sensor response and the sensor detection limit. The linearity of the MFC sensor using the OECD synthetic wastewaters was observed between 15 and 750 mg l^{-1} BOD_5 ($y = 0.1862x + 7.865$, $r^2 = 0.9598$), for the YPD medium between 540 and 1300 mg l^{-1} BOD_5 ($y = 0.1489x + 71.348$, $r^2 = 0.8178$) and for the glucose standard solution between 40 and 350 mg l^{-1} BOD_5 ($y = 0.1862x + 7.865$, $r^2 = 0.9598$) (Figure 7.9).

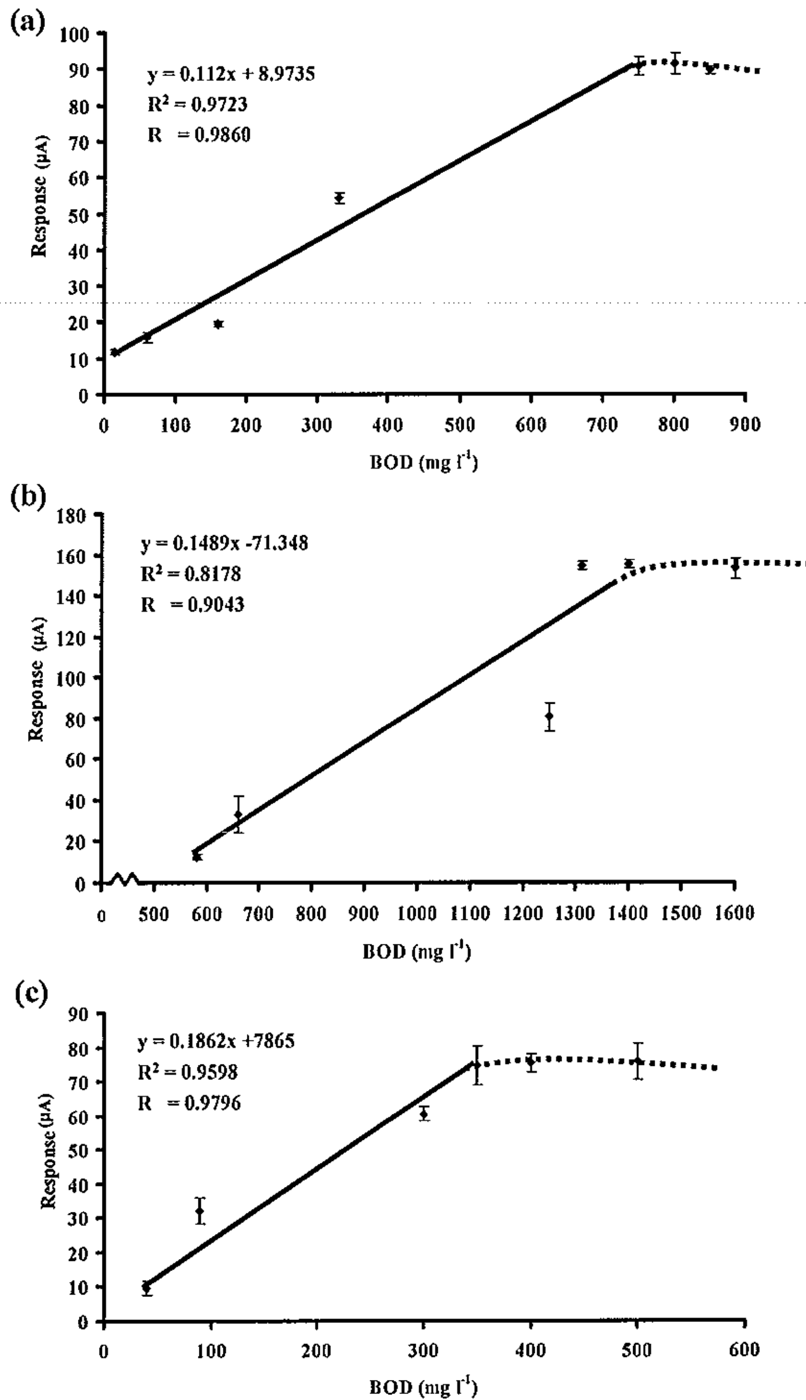


Figure 7.9 Calibration curves of different standard solutions (operational resistance of external circuit: 200 Ohm, electrolyte: 50mM phosphate and 75mM NaCl pH=7.0, amount of cell: 6.0% w/v) (a) OECD, (b) YPD medium, (c) glucose standard solution ($n = 3$).

When the calibration curves for the three test substrates were compared the glucose standard solution gave the highest sensitivity and the widest linear range. Therefore, glucose standard solution was chosen to calibrate the sensor before testing any real samples. The calibration curve of MFC using glucose standard solution was then retested in more detail and linearity was obtained between 8.0 and 100 mg l^{-1} BOD_5 ($y = 1.5131x + 15.987$, $r^2 = 0.9802$) (Figure 7.10). The detection limit was found to be 0.5 mg l^{-1} BOD determined by the measured signals from the analyte samples present at low concentration with those of a blank solution (S/N≥3) (FDA, 1996).

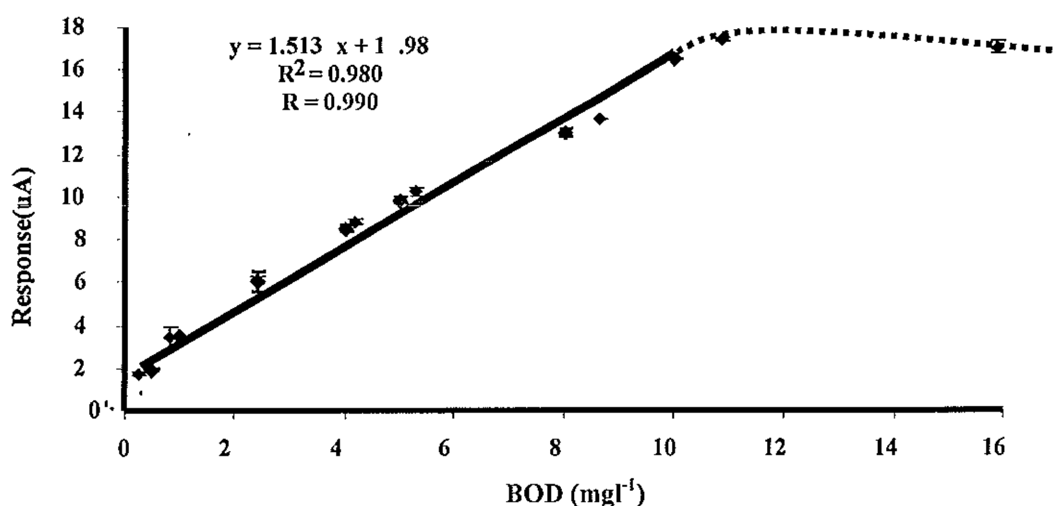


Figure 7.10 A calibration curve of the MFC sensor (operational resistance of external circuit: 200 Ohm, electrolyte: 50mMphosphate and 75mM NaCl pH=7.0, amount of cell: 6.0% w/v), 500 μl of glucose standard solution was injected for each analysis (*n* = 3).

7.3.2.2 Repeatability

The repeatability of the MFC sensor was studied by adding 500 μl of the 50 mM glucose standard solution 30 times over a 5 h period. The sensor gave a reasonably constant response (i.e. the average cell potential was 12.3 ± 1.8 mV and the current was 61 ±9 μA) with a relative standard deviation of ± 14.7 %, as shown in Figure 7.11. The system can therefore be considered to give stable measurements with a fairly good repeatability. For standard BOD_5 measurements 15% variation is allowable (Liu *et al.*, 2000).

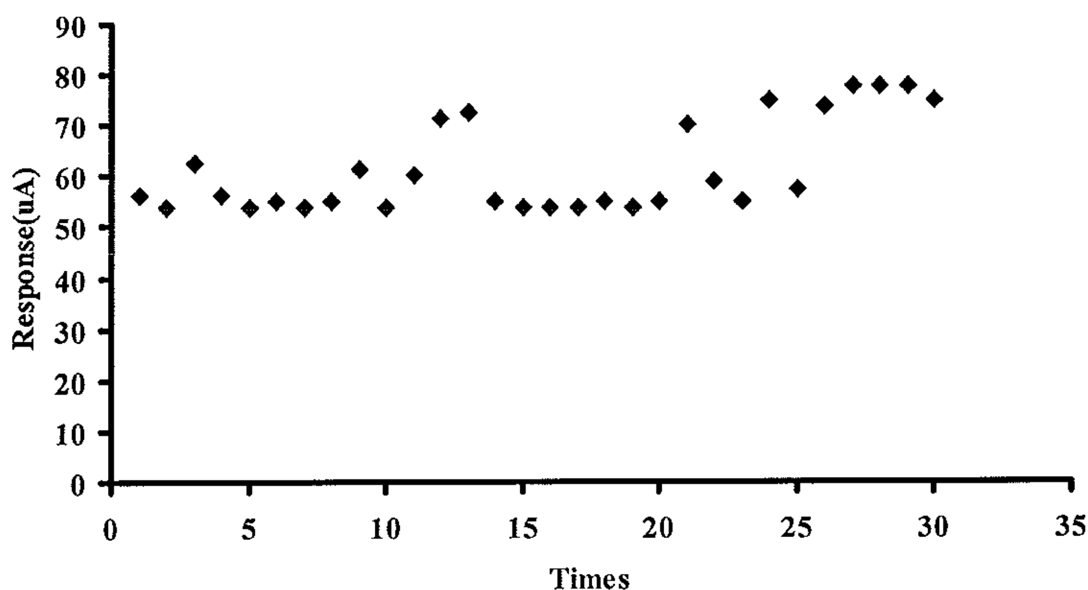


Figure 7.11 Repeatability of MFC sensor (operational resistance of external circuit: 200 Ohm, electrolyte: 50mMphosphate and 75mM NaCl pH=7.0, amount of cell: 6.0% w/v), 500 μl of glucose standard solution was injected for each analysis.

7.3.2.3 Stability of salt bridge

The stability of the salt bridge of the MFC sensor was evaluated by analyzing 500 μl of the 50 mM glucose standard solution for three times then, washing out the anode and cathode compartments with DI water and the system was re-established

with the same salt bridge. After the first wash the responses increased by 10%, but after the second wash the response decreased by 38% (Figure 7.12). The salt bridge was then wrapped in Para film and stored at 4.0 °C. The salt bridge was reused on the following day. This time after the first wash the response decreased by 59% compared to the last response on the first day. On the third day, compared to the first day, the response decreased by 74%, as shown in (Figure 7.13) and the salt bridge turned soft. Thus, the salt bridge can only be used only twice on the first day, i.e. it should be freshly prepared everyday.

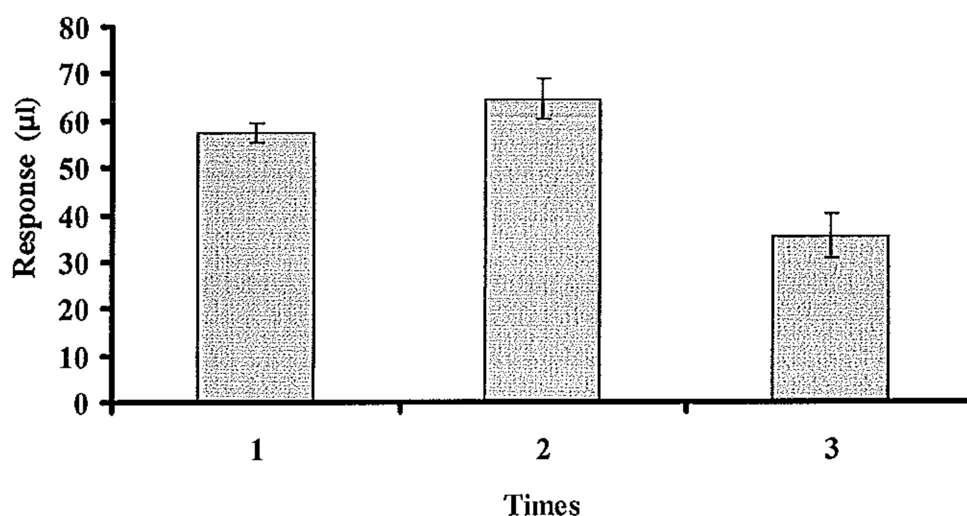


Figure 7.12 Stability of salt bridge of the MFC sensor within the same day (operational resistance of external circuit: 200 Ohm, electrolyte: 50mMphosphate and 75 mM NaCl pH=7.0, amount of cell: 6.0% w/v). 500 micro liters of glucose standard solution was injected for each analysis (n = 3).

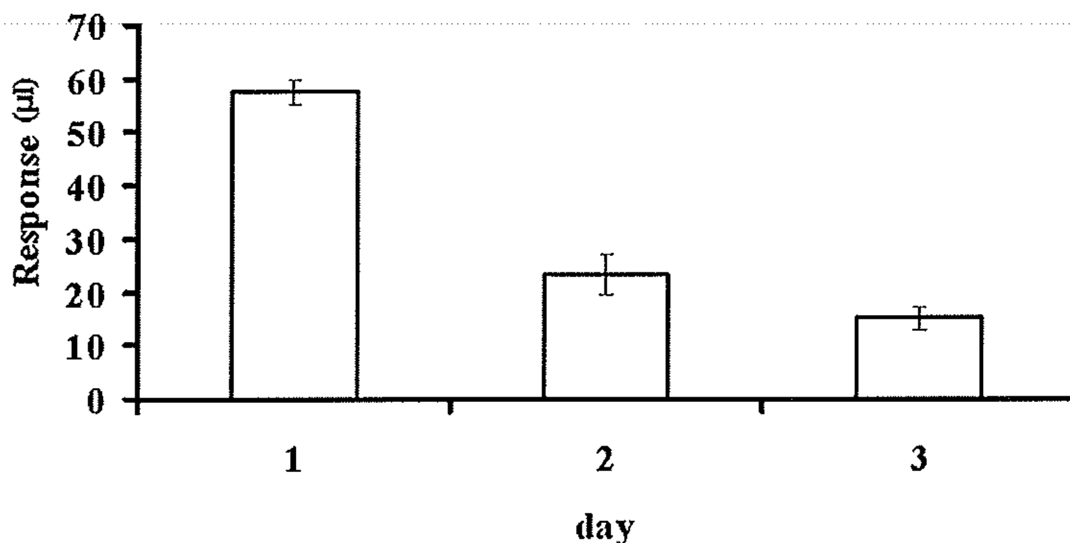


Figure 7.13 Stability of salt bridge of the MFC sensor on the first, second and third day of use (operational resistance of external circuit: 200 Ohm, electrolyte: 50 mM phosphate and 75 mM NaCl pH=7.0, amount of cell: 6.0% w/v). 500 µl of glucose standard solution was injected for each analysis (n = 3).

7.3.2.4 Real sample analysis

A total of 15 wastewater samples were collected from different origin. These include two samples from rubber latex factories, six from hospitals, two from a car wash company, one from a packaging company, two from a car spare parts manufacturer, one from gypsum industry and one from a municipal. The BOD values of wastewater samples obtained using the MFC BOD biosensor was compared with results from the BOD_5 standard method. The results are shown in Figure 7.14 and Table 7.3. The percentage differences are in the range of 2.5-20% whereas % difference of previously reported biosensors varies from 0.1 to 46% (Lehmann et al., 1999; Vaiopoulou et al., 2005). There was good agreement between values from the biosensor and BOD_5 method

for nine samples, two from rubber latex factories, four from hospitals, one from a packaging company, one from a car spare parts manufacturer and one from a municipal. While other wastewater samples showed difference between the two methods. It is possible that these wastewaters may contain some macromolecules and yeast need longer time for digestion. Thus, the short analysis time used for the MFC BOD biosensor is not enough for degradation to take place. The two samples from hospital 3 gave the percentage difference because the samples were collected from two different ponds. Sample 1 was collected from raw wastewater pond and sample 2 was collected from treated wastewater pond. In this case, some macromolecules in sample 2 might already be degraded so BOD value and the percentage difference were lower than sample 1.

Nine samples with percentage difference between 2.5-10% were statically tested using the Wilcoxon signed rank test (Table 7.4) the null hypothesis (there is no significant difference between the two methods) will be rejected if the test statistic is less than or equal to the critical value. At number of samples of 9 the critical value is 5 at a significance level of $P \leq 0.05$ (Miller and Miller, 2000). In this case the test statistic is 21 (the lower of the two rank sums). This is higher than the critical value and hence the null hypothesis is retained. That is, there is no significant difference between the two methods.

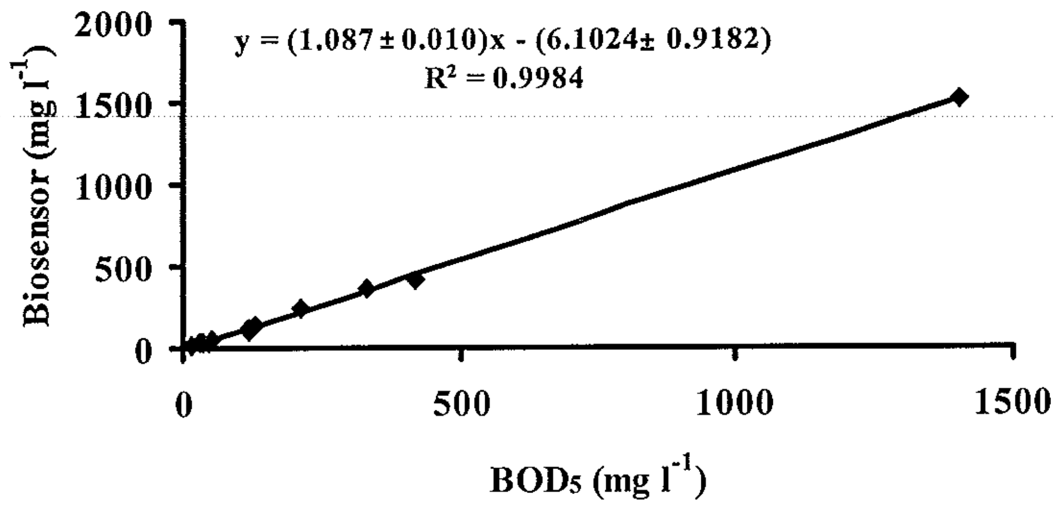


Figure 7.14 Regression line to compare between biosensor and BOD_5.

Table 7.3 Percentage difference is calculated from (biosensor - BOD_5)*100/ BOD_5

Number	Sample	Biosensor	BOD_5	% Different
1	Concentrated rubber factory	1524±14	1400±140	9.3
2	Rubber factory	362.9±1.7	330±14	10
3	Hospital 1	58.9±1.8	50±14	20
4	Hospital 2	31.85±0.45	31.0±1.4	2.8
5	Hospital 3-sample 1	36.89±0.45	46.0±2.8	-20
6	Hospital 3-sample 2	34.72±0.45	37.0±1.4	-6.1
7	Hospital 4-sample 1	36.24±0.22	38.0±2.8	-4.4
8	Hospital 4-sample 2	16.71±0.22	18.0±2.8	-6.4
9	Car wash company-sample 1	244±20	210±14	16
10	Car wash company-sample 2	39.31±0.89	33.0±1.4	19
11	Packaging company	139.0±3.9	130±14	7.2
12	Car spare parts manufacturer-sample 1	115.8±3.1	120.0±5.7	-3.3
13	Car spare parts manufacturer-sample 2	35.46±0.51	32.0±2.8	11
14	Gypsum industry	101.2±5.6	120.0±5.7	-15.6
15	Municipal	408.8±4.5	420±28	-2.5

Table 7.4 Application of the Wilcoxon signed rank test for the comparison of the BOD values in wastewater samples from the MFC biosensor and the BOD_5 standard method.

Number	Sample	Biosensor	BOD_5	Difference (biosensor-BOD_5)	Rank
1	Concentrated rubber factory	1524±14	1400±140	124	9
2	Rubber factory	362.9±1.7	330±14	33	8
3	Hospital 2	31.85±0.45	31.0±1.4	0.8	1
4	Hospital 3-sample 2	34.72±0.45	37.0±1.4	-2.3	-4
5	Hospital 4-sample 1	36.24±0.22	38.0±2.8	-1.8	-3
6	Hospital 4-sample 2	16.71±0.22	18.0±2.8	-1.3	-2
7	Packaging company	139.0±3.9	130±14	9	6
8	Car spare parts manufacturer-sample 1	115.8±3.1	120.0±5.7	-4.2	-5
9	Municipal	408.8±4.5	420±28	-11	-7
sum of negative					-21
sum of positive					24

The null hypothesis is rejected if the test statistic T is less than or equal to the critical value. In this test Critical value=5, $P \leq 0.05$.

Retain null hypothesis = there is no significant difference between the two methods

Reject null hypothesis = there is significant difference between the two methods

7.4 Conclusions

An MFC biosensor system operated by using a simple salt bridge to replace a proton exchange membrane (PEM), and *Saccharomyces cerevisiae* can satisfactorily determine the BOD of several types of wastewater. The salt bridge was very simple to prepare and much cheaper than using a PEM. However the salt bridge has to be freshly prepared before use. The repeatability of the MFC was tested 30 times over a period of 5 h with standard glucose solution producing an acceptable relative standard deviation of 14.7% (15% variation is allowed (Liu *et al.,* 2000)). The sensor response

time was around 3.0 – 10 min, similar to other cell-based BOD sensors (Liu *et al.,* 2000) but much shorter than the 5 days required in the standard method. Calibration of the MFC with the glucose standard solution gave a good agreement between sensor response and the standard BOD_5 method. Percentage differences between the BOD determined by the MFC sensor and the standard BOD_5 method are in the range of 2.8-20% (Table 7.3). When compared to other work that used *Saccharomyces cerevisiae* as a biological sensing element i.e. 1.4-90% (Nakamura *et al.,* 2007) our results are relatively better. High percentage differences are from wastewater of Siam gypsum industry (19%), Hat-Yai hospital (15%) and Phattalung hospital (25%).4.2-84% (Nakamura *et al.,* 2007) and 0-94% (Nakamura *et al.,* 2007). It is possible that some molecules might be in the samples and the cell need longer time for digest because in this case the cell was not adapted to any specific wastewater.

The results showed that the salt bridge "membrane" can be used as the proton conducting material to replace a proton exchange membrane (PEM) that is costly (Nafion^{TM} 117 costs about US $955/ m^2; Dupont Co., Wilmington, USA). The stability of salt bridge "membrane" will need to be improved. This may be done by incorporated into the agar some polymer, such as polypropylene (Scott *et al.,* 2007), poly(styrene-*b*-vinylbenzylphosphonic acid) (PS-*b*-PVBPA) (Cho *et al.,* 2008) and Poly(ether ether ketone) (PEEK) (Hasegawa et al., 2008) which may help to stabilize the membrane as well as facilitate the ion exchange. For example, Grzebyk and Pozniak (2005) synthesized the polyethylene/poly (styrene-co-divinylbenzene) [PE/poly(Stco-DVB)] membrane that has been applied to MFC system.

CHAPTER 8

Conclusions

In this thesis, the development and evaluation of the performance of BOD cell-based biosensor systems have been investigated. These BOD biosensors are based on amperometric and microbial fuel cell transducers for the determination of BOD in wastewater samples. All developed methods attempted to improve on the standard method (BOD_5). That is, these biosensors should be simple to construct, low cost, have high stability, accuracy and precision with short analysis time. This will become an alternative approach to detect organic matter in the environment.

The first development was the flow injection amperometric cell-based biosensor. The goal of this work is to develop the sensor and apply it for the monitoring of BOD during a treatment process of wastewater from concentrated rubber latex factory. Mix cultures from the factory's activated sludge were used as biological sensing element and oxygen electrode as the transducer. The sensor showed good performances, wide linear ranges, between 2.0–25 and 5.0–60 mg l^{-1}, and the limit of detection at 0.20 mg l^{-1}. The sensor analysis time was around 10-15 min. The repeatability was observed with relative standard deviation of 3.9% and good stability can be obtained over a period of 15 days (RSD of 0-6.7%). Good agreement between the results from biosensor method and standard method was obtained, with about 10 % difference.

An anaerobic reactor treatment process was investigated by continuously feeding wastewater sample through the reactor with flow rates 10, 20, 40 and 80 ml day^{-1} (30 days for each flow rate 120 days in total). At 10 ml day^{-1} the highest efficiency was obtained with percentage of COD and BOD reduction up to 97%. This treatment process showed good efficiency for high strength wastewater. The biosensor system that was employed to monitor the performance of the anaerobic reactor showed that off-line and online assays could be carried out with short analysis time. The microbial BOD biosensor

system has proven to be a suitable monitoring sensor for wastewater samples from the concentrated rubber latex process.

The second development was the microbial fuel cell (MFC) biosensor for fast analysis of biodegradable organic matter based on a single-chamber MFC separated by a proton exchange membrane (PEM) where an anaerobic consortium was used to analyze biodegradable organic matters. The sensor system was operated by integrating with an anaerobic bioreactor for continuous supply of stable anaerobic consortium. Replacement of the biological recognition element was carried out for each sample analysis. A glucose standard solution was used as the target substrate. To obtain the maximum sensor output, the MFC-based sensor system was optimized using an 800 Ohm resistor as the load to the external electric circuit and 25mM phosphate buffer with 50mM NaCl as catholyte in the aerobic compartment. The temperature of anaerobic compartment was maintained at optimal 37 °C. The cell potential across the electrodes increased with increasing loading of glucose. The sensor response was linear against concentration of glucose up to 25 g l^{-1}. The detection limit was found as 0.025 g l^{-1}. The system gives stable measurement with a good repeatability with a relative standard deviation 7.2% and long term stability (RSD less than 9.0%). The sensor response time was estimated around 3.0–5.0 min and did not have to wait for the metabolic recovery of anaerobic consortium in the anodic compartment. This is considered as an advantage for this sensor system configuration. The MFC sensor needs, however, in its present configuration maintenance in terms of regular membrane cleaning. The sensitivity and the detection limit of the sensor system can certainly be improved even further by improving the efficiency of ions transport in the electrolyte.

The third investigation was a simple MFC for BOD analysis of wastewater using *Saccharomyces cerevisiae* to degrade the substrates and a simple salt bridge to replace a more expensive PEM and pencil graphite carbon as the electrodes. The proposed system showed wide linear in the range of 8.0-100 mg l^{-1} and the detection limit was at 0.50 mg l^{-1}. The sensor response time was around 3.0 – 10 min. The repeatability of the MFC was tested with the standard glucose solution producing a relative standard

deviation of 14.7% for 30 samples tested over a period of 5 hour. The use of an MFC biosensor system can satisfactorily determine the BOD of several types of wastewater. The salt bridge was very simple to prepare and much cheaper than using a PEM. However the salt bridge has to be freshly prepared each time or each day.

These results show that the cell-based biosensor systems were successfully applied for rapid analysis (less than 15 min) of BOD that can overcome the major drawback of the standard method which requires 5 days. Therefore, the wastewater with higher BOD than the limit can be known before being discharged to the environment. In addition, it is suitable for environmental monitoring and process control.

Table 8.1 compared the performances of the oxygen electrode system developed in this work (CHAPTER 5) with other cell-based BOD biosensors. Although the response time is similar to other methods, the limit of detection of our system is much lower, i.e. in addition to wastewater with high BOD this system can also be applied to determine BOD of natural water which is normally quite low. Although the linear range is not as wide as other methods but high BOD samples can be analysed with on-line dilution. As for stability, this sensor can be applied for at least 15 days, much longer than the 15 h and 1 week reported by Sakai *et al.*, 2001 and Liu *et al.*, 2004, respectively. If a longer operation time is needed the cell's column can easily be changed.

The comparison of the analytical features of microbial fuel cell BOD biosensors (CHAPTER 6 and CHAPTER 7) with some similar works is also summarized in Table 8.2. The results showed that our systems required very short analysis time (3-10 min) and the detection limit was low enough to analyze both natural water and wastewater. A wide linear ranges were obtained similar to others works. The stability of MFC system using activated sludge (CHAPTER 6) was only tested for 11 days, however, since the activated sludge is maintained in an anaerobic reactor it is possible that the system can be operate with longer stability similar to other works (Kim *et al.*, 2003; Kang *et al.*, 2003; Kim *et al.*, 2004).

Table 8.1 Comparison of the analytical features of cell-based BOD biosensors based on oxygen electrode.

Microorganism	Immobilization	Response time (min)	LOD ($mg\ l^{-1}$)	Linear range ($mg\ l^{-1}$)	Stability	Analytical features
Activated sludge	entrap with Ca-alginate bead	10-15	0.2	2.0-25 and 5.0-60	15 days	CHAPTER 5
Trichoporon cutaneum	entrap with Ca-alginate bead	10	-	below 130	60 day	Sohn *et al.*, 1995
Activated sludge	adsorption with bio-film	30	-	6.0-300	15 hours	Sakai *et al.*, 2001
Activated sludge	entrap with dialysis membrane	10	5	5.0- 700	1 week	Liu *et al.*, 2004
Yeast	Entrapment	15	1	10-50	30 days	Chen *et al.*, 2002

Table 8.2 Comparison of the analytical features of cell-based BOD biosensors based on microbial fuel cell.

Microorganism	Response time	LOD	Linear range	Stability	Analytical feature
Activated sludge	3-5 min	0.025 g l^{-1}	up to 25 g l^{-1}	up to 11 days	CHAPTER 6
Saccharomyces serevisiae	3-10 min	0.5 mg l^{-1}	8.0-100 mg l^{-1}	1 day	CHAPTER 7
Activated sludge	30 min to 10 hours	2.58 ppm	up to 206 ppm	over 5 years	Kim *et al.*, 2003
Activated sludge	1 hour	6 mg l^{-1}	-	over a year	Kang *et al.*, 2003
Activated sludge	60 min	102.4 mg l^{-1}	up to 100 mg l^{-1}	-	Kim *et al.*, 2004

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Appendices

Appendix A

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BIOSENSORS BIOELECTRONICS

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Microbial fuel cell-based biosensor for fast analysis of biodegradable organic matter

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Abstract

The current study was made to develop a biosensor based on a single-chamber microbial fuel cell in which anaerobes were retained in the anode compartment separated from the cathode compartment by a proton exchange membrane. In the sensor a replaceable anaerobic consortium was used for analyzing biodegradable organic matter. The anaerobes acted as biocatalysts in oxidizing organic matter and transferring electrons to the anode. The biocatalysts were renewed for each sample analysis by replacing the old anaerobic consortium with an equal amount of fresh one. A glucose standard solution was used as the target substrate. To obtain the maximum sensor output, the MFC-based sensor system was optimized using an 800 Ω resistor as the load to the external electric circuit and 25 mM phosphate buffer with 50 mM NaCl as catholyte in the aerobic compartment. The temperature of anaerobic compartment was maintained at optimal 37 °C. The cell potential across the electrodes increased with increasing loading of glucose. The sensor response was linear against concentration of glucose up to $25\,g\,l^{-1}$. The detection limit was found as $0.025\,g\,l^{-1}$. The microbial fuel cell with replaceable anaerobic consortium could be used as a biosensor for on-line monitoring of organic matter.

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Keywords: Biosensor; Biochemical oxygen demand; Microbial fuel cell

1. Introduction

The content of organic matter in wastewater is normally evaluated in terms of biochemical oxygen demand (BOD). The conventional methods for BOD analysis take 5 or 7 days incubation at 20 ± 1 °C in the dark (APHA, 1992; SIS, 1979). BOD_5 and BOD_7 tests are good methods for evaluating water and wastewater quality. It is a universal method of measuring most wastewater samples. No expensive equipment is needed. It has, however, the limitation of being time consuming and also requiring experience and skill to get reproducible results (Liu and Mattiasson, 2002). Thus, the conventional BOD methods are not suitable for on-line monitoring and control of biological wastewater treatment processes where a rapid feedback is essential. It is therefore necessary to develop alternative methods that could give rapid measurements to illustrate the dynamic changes in the treatment process.

Fast determination of BOD could be achieved by biosensor-based methods and this has been a subject of several review articles (D'Souza, 2001; Liu and Mattiasson, 2002; Lei et al., 2006). Most of previously reported BOD biosensors can be generally classified as bio-layer and respirometer type. Both types of sensor are based on a respirometric principle (Liu and Mattiasson, 2002; Spanjers et al., 1998). The sensor response is expressed as the difference between two steady states or initial rate of change in the dissolved oxygen concentration before and after addition of the sample solution. Although there are several reports on developing oxygen electrode-based BOD biosensors, this type of biosensor has many disadvantages, such as intrinsic limitation of oxygen solubility in aqueous solution and short-term stability and calibration drift due to the lysis of immobilized bacteria (Liu and Mattiasson, 2002; Kang et al., 2003). It is therefore of great interest to develop alternative methods for compensating these

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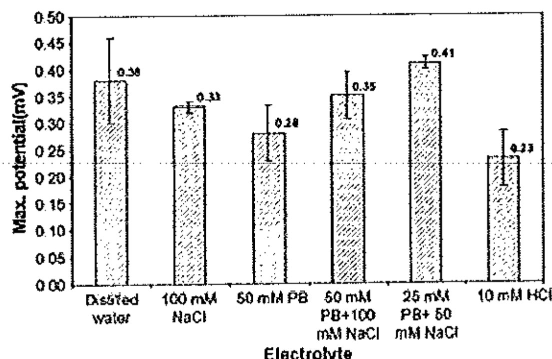

Fig. 2. Cell potential with different types of catholyte (operational temperature: 25 °C, resistance of external circuit: 800 Ω). Ten milliliters of glucose standard solution was injected for each analysis (n = 3).

decrease. It is possible that high resistance limits electron disposal through the electric circuit (Gil et al., 2003). Therefore a resistor at 800 Ω was selected as the external circuit load.

3.1.2. Electrolyte

Electrolyte is a chemical compound that conducts ions between anode and cathode compartment but it is impermeable to electrons (Fitzgerald, 2001). The microbial fuel cells were tested with different kinds of electrolyte solution in the cathodic compartment, i.e. distilled water, NaCl solution (100 mM), phosphate buffer (50 mM), phosphate buffer (50 mM) with NaCl (100 mM), phosphate buffer (25 mM) with NaCl (50 mM) and HCl solution (10 mM). The highest sensor signal expressed as potential output at 800 Ω was observed for the catholyte containing 25 mM phosphate buffer and 50 mM NaCl (Fig. 2). Therefore, this type of electrolyte was selected in the subsequent experiment. The lowest potential was generated for the catholyte containing 10 mM HCl due to the inhibition effect of protons transportation from the anodic compartment. In such a case, the resistance of protons migration through PEM was hampered by the high proton concentration in the cathodic compartment.

3.1.3. Temperature

In the current study, the anaerobic compartment was equipped with a temperature control unit. Operational temperature from 20 to 40 °C was tested in order to find the best temperature for operating the MFC sensor. As shown in Fig. 3, the highest cell potential was observed at 37 °C, whereas signal decreased when the temperature was set below or above this temperature. Thirty-seven degree Celsius was therefore selected as the optimal temperature for operating the MFC sensor system.

3.2. Performance characteristics of the MFC sensor

After the optimum conditions were obtained, performance characteristics of the MFC sensor were studied using these conditions.

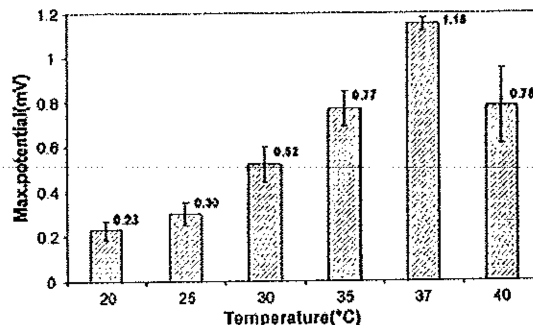

Fig. 3. Effect of operational temperature on the cell potential (resistance of external circuit: 800 Ω, catholyte: 50 mM phosphate buffer and 25 mM NaCl). Ten milliliters of glucose standard solution was injected for each analysis (n = 3).

3.2.1. Linearity and the detection limit

System calibration was performed to evaluate the linearity of sensor response and sensor detection limit. A broad linear range when plotting response versus concentration of analyte is desirable for reliable and accurate measurement over the concentration range. Furthermore, the population of induced anaerobic consortium may still be varied over a longer period of time. This can give a negative effect on repeatability of the measurement. However, the problem can be simply solved by carrying out system calibration in a regular time base. As demonstrated in Fig. 4, the linearity of MFC sensor response to the glucose standard solutions can be observed between 1 and 25 g l^{-1} ($y = 0.0514x + 0.2671$, $r^2 = 0.9914$). The detection limit is found to be 25 mg l^{-1} determined by comparing measured signals from analyte samples in low concentration with those of blank samples. The minimum concentration was established at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit (FDA, 1996). One of the bottlenecks for MFC is transport of ions in the electrolyte. For a good operation of the MFC, both protons and electrons need to migrate from the anode to the cathode at the highest possible rate (Lens et al., 2005). Diffusion is often not sufficient to reach acceptable levels of current and cell potential. For the experimental setup in the current

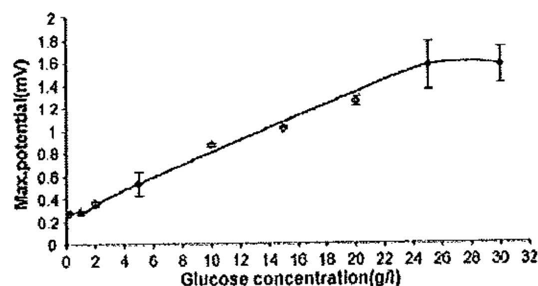

Fig. 4. A calibration curve of the MFC sensor (operational temperature: 37 °C, resistance of external circuit: 800 Ω, catholyte: 50 mM phosphate buffer and 25 mM NaCl) (n = 3).

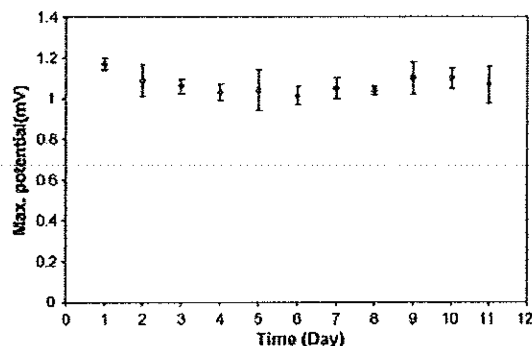

Fig. 5. The stability of the cell potential (operational temperature: 37 °C, resistance of external circuit: 800 Ω, catholyte: 50 mM phosphate and 25 mM NaCl). Ten milliliters of glucose standard solution was injected for each analysis ($n = 3$).

study, the PEM was placed in a specific modified plastic holder. Although the membrane area is considerably large, the connections to anodic and cathodic compartment were made through 40 mm long tubes with a diameter of 8 mm. Transport of proton is therefore relatively inefficient even though enhanced diffusion was achieved in the anodic and cathodic compartment through mixing and aeration, respectively. A preliminary test has shown a significant increase of the cell potential when the connecting tubes to the anodic and cathodic chambers became shorter (the data are not shown in the paper). A large cell potential and lower detection limit are, therefore, expected with improved cell configuration.

3.2.2. Reproducibility

The reproducibility of the MFC sensor has been studied by injecting 10 ml of the glucose standard solution 12 times over 15 h. It was observed that the sensor gave fairly constant response (i.e. average cell potential was 1.11 ± 0.08 mV) during the testing period with relative standard deviation of ±7.2%. It should be noted that the biological recognition element was renewed before each sample analysis by replacing the old anaerobic consortium in the anodic compartment with the fresh anaerobic sludge from the anaerobic bioreactor. Still the system gives stable measurement with a good repeatability. The results could be compared to those obtained from conventional BOD 5-day tests, where ±15% is allowed (Liu et al., 2000).

3.2.3. Stability

A stable sensor performance over a desired operational period is essential for a reliable biosensor system. The stability of the MFC sensor was evaluated by analyzing glucose standard solution over a period of 11 days. The analysis was carried out in triplicate by injecting 10 ml of glucose standard solution per analysis. The total analysing time was approximately 1 h. As shown in Fig. 5, the cell potential remained fairly stable over the whole testing period. The average cell potential varied daily between 1.00 and 1.10 mV with relative standard deviation from ±2 to ±9%. The average cell potential calculated from the 33 measurements of the whole testing period was 1.07 mV with relative standard deviation of ±5.9%. It was observed that the stability of MFC sensor was strongly affected by the surface condition of PEM. Nafion™ has been widely used as PEM for MFCs, and has the great advantage of being very selective for protons. However, this membrane contains sulfonic acid groups that are binding with ammonia present in the bacterial suspension. Hence, this membrane type scores high for selectivity but low for stability in a colloidal and nutrient-rich bacterial suspension (Lens et al., 2005).

4. Conclusion

The current study showed the development of a MFC sensor system for fast estimation of easily biodegradable organic matter. The sensor system was operated by integrating with an anaerobic bioreactor for continuous supply of stable anaerobic consortium. Replacement of the biological recognition element was carried out for each sample analysis. Still the system gives stable measurement with a good reproducibility. The sensor response time was estimated around 3–5 min and did not have to wait for the metabolic recovery of anaerobic consortium in the anodic compartment. This is considered as an advantage for this sensor system configuration. Although glucose was used as the only preliminary substrate in the current study to evaluate the sensor performance under the well-controlled condition, the MFC sensor system used mixed consortium originated from anaerobic sludge should also be able to degrade other kinds of organic matter even in more complex form. The MFC sensor needs, however, in its present configuration maintenance in terms of regular membrane cleaning. The sensitivity and the detection limit of the sensor system can certainly be improved even further by improving the efficiency of ions transport in the electrolyte.

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Appendix B

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ENZYME AND MICROBIAL TECHNOLOGY

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Microbial BOD sensor for monitoring treatment of wastewater from a rubber latex industry

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Abstract

A cell-based biosensor system was designed for monitoring an anaerobic process for treatment of high biochemical oxygen demand (BOD) levels in wastewater samples from a factory processing concentrated rubber latex. The BOD biosensor used immobilized mixed culture of microorganisms as the biological sensing element and an oxygen electrode as the transducer. The assay principle is based on the determination of the oxygen consumption rate caused by microbial respiration. Synthetic wastewater according to the OECD specifications was used as standard solution for calibration of the BOD biosensor. Response time of the sensor was 10–15 min. The BOD of the influent and the effluent from an anaerobic reactor was measured using both the cell-based biosensor system and a standard method (BOD_5). Good agreement was achieved between the results from the two assay methods with a percentage difference of less than 10%. However, when exposing the mixed culture to wastewaters from other industrial plants the agreement between the results of the two assays was poor. The anaerobic treatment of the wastewater from the concentrated latex process resulted in a COD removal efficiency of 97% at a hydraulic retention time (HRT) of 50 days. The BOD biosensor was successfully applied to off-line and on-line monitoring of the anaerobic reactor treatment process.

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Keywords: Cell-based biosensor; Biochemical oxygen demand (BOD); Anaerobic reactor treatment; On-line monitoring

1. Introduction

Rubber industry is one of the most important industries in Southern Thailand and it usually generates large quantities of wastewater containing high concentration of organic matter, suspended solids and nitrogen [1]. Thus, treatment of this wastewater under controlled conditions is a necessary step in preventing the release of harmful wastes to the environment. One of the most widely used and important parameters for the estimation of water quality is biochemical oxygen demand (BOD). The standard BOD method (BOD_5) is a measure of the amount of dissolved oxygen which is required for the biochemical oxidation of the organic compounds in 5 days from the time when microorganisms are inoculated to the water sample [2]. The major drawback of the standard method is the 5-day measurement time. In reality, the wastewater with higher BOD than the limit could have been discharged to the environment long before the test result was known. Therefore, BOD_5 is not suitable for any form of active intervention for environmental monitoring and process control.

To overcome the problem, fast determination of BOD can be achieved by applying a biosensor-based method. Most BOD biosensors rely on the measurement of the respiratory activity of cells by a suitable transducer and this is a subject of several recent reviews [3–5]. Recent reports include the use of oxygen electrode [6–8], carbon dioxide analyzer [9], and optical transducer [10]. Microbial fuel cell has also been investigated as another detection method for BOD [11,12]. For cell-based systems many microbial strains have been used as biological sensing elements including both pure and mixed cultures [4]. Mixed cultures are

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different microbial consortia, e.g. activated sludge [4,9,13,14]. The advantage of BOD sensors based on mixed cultures is a good detection capacity for a wide substrate spectrum [4]. Since oxygen electrode is simple to use and economical, a BOD sensor based on activated sludge in combination with an oxygen electrode was considered in this study.

Wastewater from an industry processing concentrated rubber latex with a high organic content and generating malodor when discharged to the receiving water was selected for the study. Wastewater in this industry in Southern Thailand is generally treated by waste stabilization ponds (WSP) [1], aerated lagoon and activated sludge processes [15]. These treatments have good removal efficiency but still malodor is generated. To our knowledge this wastewater has not yet been treated with an anaerobic reactor, a closed system that may help solve the malodor problem and at the same time generates methane which is a useful by-product [16,17]. A few of the developed BOD biosensors have been applied successfully to monitor anaerobic processes such as the monitoring of intermediate products of a two-stage anaerobic reactor [18] and BOD of effluent from a wastewater treatment plant [9]. Therefore, it would be advantageous if we could develop and apply such a BOD sensor to monitor anaerobic treatment of wastewater from the concentrated latex process.

The objective of this work was to evaluate the possibilities to apply and monitor an anaerobic treatment process of wastewater from concentrated latex process and at the same time evaluate the possibilities to use a BOD sensor constructed based on a mixed culture from the treatment ponds at the factory. Finally the microbial sensor was applied for off-line and on-line tests of real wastewater samples from processing of concentrated latex and various other industrial activities in the region. The BOD values obtained from microbial sensor were compared with those obtained from conventional BOD_5 analyses.

2. Materials and methods

2.1. Biosensor system

2.1.1. Cell-based biosensor

Fig. 1(a) shows the BOD cell-based biosensor flow injection system. Aerated carrier buffer, 100 mM Tris–HCl pH 7.00, was continuously pumped (peristaltic pump, Gilson, France) through a small reactor column (30 mm length × 3 mm inner diameter), containing immobilized cells, and then through a flow cell before being sent to waste. A Clark-type oxygen electrode (Yellow Springs Instrument Co. Inc., USA) was used to measure dissolved oxygen in the carrier solution to obtain the baseline. The electrode was placed inside a chamber in the middle of a water insulation jacket used to control the temperature (30 °C). When a sample of wastewater (500 μl) was injected by means of an injection valve (VICI Valco Europe, Switzerland) and flowed through the reactor the concentration of dissolved oxygen decreased because oxygen was consumed by the immobilized cells during the degradation of organic matter. The response was recorded on a chart recorder (Ross Recorders, USA). The change in concentration of dissolved oxygen is related to the metabolic degradation of organic matter in the water by the immobilized mixed culture.

2.1.2. Microorganisms

Activated sludge was collected from a wastewater treatment pond of a rubber latex factory and was stored at 4 °C until it was used. Fifteen milliliters of activated sludge was mixed with 150 ml of wastewater from the rubber latex factory in a 500 ml flask and the mixture was cultivated at room temperature on a rotary shaker (HL Instruments, Thailand) at 150 rpm for 24 h. The cells were harvested by centrifugation (15 min, 860 × g) (Dupont Instruments, USA), washed twice by resuspending in 10 ml of saline solution (0.9%, w/v NaCl (Carlo Erba, France)) and centrifuged under the same conditions. Approximately 10 g of wet weight cells were obtained from each cultivation. This constituted the mixed culture used for constructing the biosensor.

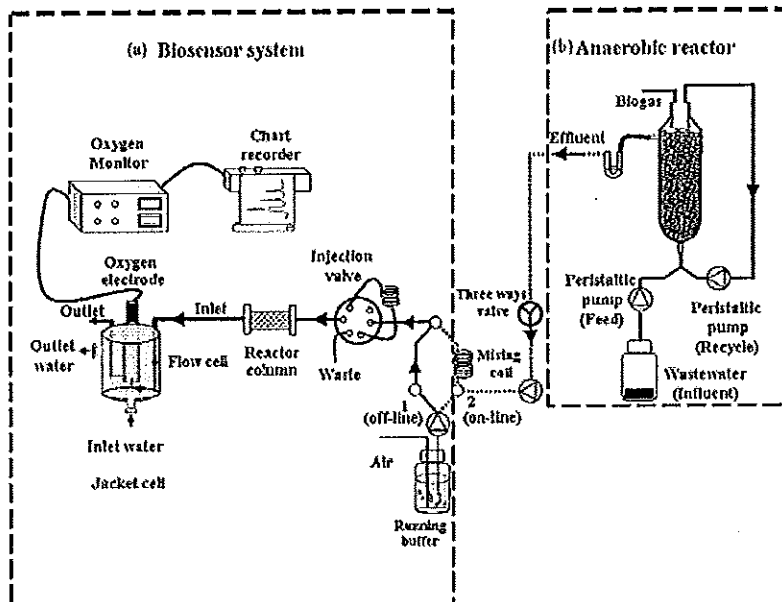


Fig. 1. Schematic diagram of BOD monitoring system for anaerobic wastewater treatment process consists of (a) BOD biosensor system (b) an aerobic reactor.

Cells were immobilized by entrapment in Ca-alginate gel according to the method described by Thavarungkul et al. [19]. Sodium alginate (Fluka AG, Switzerland), 0.3 g, in 10 ml saline solution was stirred for 15 min to obtain a thick uniform solution, mixed with appropriate amount of cells (see later) and stirred for another 10 min to obtain a uniform mixture. A peristaltic pump was used to pump the mixture at a flow rate of 4.0 ml min^{-1} through an auto pipette tip (0.5–10 μl) into an Erlenmeyer flask containing 0.1 M $CaCl_2$ (Merck, Germany) stirred at 400 rpm while a gentle suction was applied at the neck of the flask using a water pump. The formed gel beads with entrapped mixed culture of microbes were then sieved to obtain selected size fraction (see later). They were allowed to stabilize overnight in 0.1 M $CaCl_2$ at 4 °C. After washing with 0.1 M Tris–HCl buffer pH 7.0 containing 10 mM $CaCl_2$ 0.7 g of the beads were packed into a reactor (30 mm length × 3 mm inner diameter). When not in use the reactor was kept in 0.1 M Tris–HCl buffer pH 7.0 with 10 mM $CaCl_2$ at 4 °C.

2.1.3. Optimization

The stock solution used to calibrate the biosensor system was a synthetic wastewater according to the recipe of the Organisation for Economic Cooperation and Development (OECD) [20]. This was prepared with distilled water and contained 7.5 mg l^{-1} peptone (Merck, Germany), 5.5 mg l^{-1} beef extract (Merck, Germany), 1.5 mg l^{-1} urea (Riedel-de Haen, France), 0.35 mg l^{-1} NaCl (Carlo Erba, France), 0.2 mg l^{-1} $CaCl_2 \cdot 2H_2O$ (Merck, Germany), 1.4 mg l^{-1} K_2HPO_4 (AnalaR, England), 0.1 mg l^{-1} $MgSO_4 \cdot 7H_2O$ (J.T. Baker Inc., USA), 75 mg l^{-1} D-glucose (Riedel-de Haen, France) and 75 mg l^{-1} glutamic acid (Merck, Germany) [14]. The BOD_5 of this synthetic wastewater was 160 ± 5 mg l^{-1} ($n = 30$). Calibration solutions were obtained by diluting this stock solution with Tris–HCl buffer. BOD value of each calibration solution was tested by standard BOD_5 method ($n = 3$).

Parameters affecting the performances of the system were optimized and these are summarized in Table 1. OECD synthetic wastewaters with BOD values between 0.5 and 15 mg l^{-1} were used in the optimization process. Initial conditions were flow rate 0.5 ml min^{-1}, gel bead size 1.00–1.75 mm with the amount of cells 3% (w/v), 100 mM Tris–HCl buffer pH 7.00 with 10 mM $CaCl_2$ was the carrier buffer. Optimization was done by changing one parameter and keeping others constant. When an optimum value was obtained it was used to optimize the next parameter. The optimum conditions were considered by balancing between sensitivity (slope of the calibration curve) and analysis time.

2.1.4. Real samples

BOD of real samples was determined under optimum conditions. Calibration of the system was done prior to the measurement using the OECD synthetic wastewaters. BOD values of the OECD synthetic wastewater were determined by the standard BOD_5 method [2]. The calibration curve was prepared by plotting the dissolved oxygen change versus corresponding BOD values (mg l^{-1}). Samples were then injected into the system. The change in dissolved oxygen of each sample was used to calculate the BOD value from the calibration equation.

A total of 31 wastewater samples were collected from wastewater ponds of several types of industries. These include eleven samples from a rubber latex factory, one each from two hospitals, three from a transportation company, three from a car spare parts manufacturer, four from a car wash company, three from a color manufacturing firm, three from a fish processing factory and two from an animal feed factory.

All samples were stored at 4 °C until they were tested by the BOD biosensor and compared with BOD_5 standard method. The analysis of each sample was carried out in triplicate. Solid particles were removed from all samples by filtration before analysis (Whatman diameter 185 mm, Particle Retention 11 μm, Whatman International Ltd., Maidstone, England).

2.2. Anaerobic reactor

A lab-scale glass reactor with an effective volume of 0.50 l (4.0 cm internal diameter) was used for the treatment of wastewater (Fig. 1(b)). The reactor contained 400 ml of 0.5–1.0 cm diameter Poraver® glass carrier (Dennert Poraver® Gmbh, Germany) used as a support. Sludge (300 ml) from a treatment pond at a rubber latex factory was passed through a sieve with a pore size of 0.5 mm and used as the inoculum for the anaerobic reactor. The sludge was adapted for a period of 2 months by feeding with 1.0 ml of anaerobic medium once a day. The concentrations of nutrients in the medium were: D-glucose 10 g l^{-1}, NH_4Cl 100 g l^{-1}, NaCl 10 g l^{-1}, $MgCl_2 \cdot 6H_2O$ 10 g l^{-1}, $CaCl_2 \cdot 2H_2O$ 5.0 g l^{-1}, K_2HPO_4 152.6 g l^{-1}, 50 ml $NaHCO_3$ 52 g l^{-1}, $FeCl_2 \cdot 4H_2O$ 2441.7 mg l^{-1}, H_3BO_3 50 mg l^{-1}, $CuCl_2 \cdot 2H_2O$ 38 mg l^{-1}, $AlCl_3 \cdot 6H_2O$ 90.4 mg l^{-1}, $NiCl_2 \cdot 6H_2O$ 91.6 mg l^{-1}, $Na_2SeO_3 \cdot 5H_2O$ 100 mg l^{-1}, $ZnCl_2$ 50 mg l^{-1}, $MnCl_2 \cdot 2H_2O$ 41 mg l^{-1}, $CoCl_2 \cdot 6H_2O$ 50 mg l^{-1}, EDTA 500 mg l^{-1}. The reactor was operated at ambient temperature (30–37 °C), sealed at the top with a rubber stopper to maintain anaerobic condition. The first 2 months were only devoted to the microorganisms growth to attach to the support and start multiplying. Then, wastewater sample was continuously fed from the bottom to the top of the reactor using a peristaltic pump (Micro-tube pump MP-3, EYEL 4, Tokyo Rikakikai Co. Ltd., Japan) where flow rates at 10, 20, 40 and 80 ml day^{-1} were investigated, 30 days for each flow rate 120 days in total. Hydraulic retention times (HRT, the average time a liquid medium, that continuously flows through a reactor, stays within the reactor [21]) were controlled at approximately 50, 25, 12 and 6 days, respectively. Continuously during this operation the liquid in the reactor was also recirculated at a constant flow rate of 20 ml day^{-1} from the top to the bottom of the reactor (Fig. 1(b)).

2.3. The monitoring system

The performance of the anaerobic reactor with respect to reduction of BOD of concentrated wastewater from an industry processing concentrated latex was investigated.

2.3.1. Characteristics of wastewater

The latex industry wastewater used in the experiments was collected and stored at 4 °C until use. The characteristics of the wastewater, i.e., BOD, COD, pH, suspended solids and total dissolved solids (TDS), before and after treatment by the anaerobic reactor were analyzed.

2.3.2. Off-line BOD monitoring analysis

BOD content in wastewater was monitored prior to and after treatment. Influent is wastewater from the rubber latex factory and effluent is treated wastewater that came out from the anaerobic reactor after treatment (Fig. 1(b)). These two samples were collected once a day, every day during the 120 days of running

Table 1
Optimized values of biosensor operating conditions

Parameter	Value	Optimum
Sample volume (ml)	0.5, 1.0, 1.5, 2.0	1.0
Flow rate (ml min^{-1})	0.1, 0.2, 0.3, 0.4, 0.5	0.2
Gel bead size (diameter-mm)	0.50–1.00, 1.00–1.75, 1.75–2.00, 2.00–2.75, 2.75–3.00, 3.00–3.50, 3.50–3.75	1.00–1.75
Tris–HCl Buffer		
pH	5.0, 5.5, 6.0, 6.5, 7.0	6.5
Concentration (mM)	25, 50, 100, 150	100
Calcium chloride (mM)	5, 10, 15, 20, 25, 30, 35, 40	20
Amount of microorganisms in alginate solution (%w/v)	1, 5, 7, 10, 15, 20	15

Table 2
Summary of linear range and limit of detection (LOD) of 17 different columns in the biosensor system

Column	Linear equation	Linear range	R^2	LOD ($mg\,l^{-1}$)
1	$y=(2.50\pm0.37)x-(2.26\pm0.76)$	2–35	0.9889	0.18
2	$y=(2.53\pm0.11)x-(2.49\pm0.85)$	2–35	0.9989	0.18
3	$y=(2.59\pm0.17)x-(2.24\pm0.12)$	2–35	0.9961	0.19
4	$y=(3.24\pm0.81)x-(12.63\pm5.30)$	5–60	0.9959	0.19
5	$y=(3.28\pm0.82)x-(13.88\pm5.31)$	5–60	0.9947	0.19
6	$y=(3.31\pm0.78)x-(14.89\pm5.06)$	5–60	0.9936	0.19
7	$y=(3.26\pm0.93)x-(12.28\pm6.03)$	5–60	0.9917	0.19
8	$y=(3.09\pm1.06)x-(1.99\pm0.54)$	3–30	0.9805	0.20
9	$y=(2.88\pm0.75)x-(6.41\pm3.69)$	3–30	0.9848	0.19
10	$y=(1.63\pm0.21)x-(1.30\pm0.76)$	3–30	0.9907	0.19
11	$y=(0.98\pm0.07)x-(1.40\pm1.67)$	2–50	0.9946	0.22
12	$y=(1.11\pm0.27)x-(9.17\pm1.25)$	2–25	0.9820	0.20
13	$y=(1.85\pm0.24)x-(2.56\pm1.10)$	2–25	0.9884	0.21
14	$y=(1.85\pm0.51)x-(2.56\pm0.78)$	2–25	0.9984	0.19
15	$y=(0.98\pm0.07)x-(1.40\pm0.67)$	2–50	0.9946	0.19
16	$y=(0.91\pm0.12)x-(1.28\pm0.56)$	2–50	0.9946	0.19
17	$y=(1.11\pm0.27)x-(9.17\pm1.25)$	5–30	0.9820	0.19

the anaerobic reactor. They were stored at −20 °C until analysis. Before storage influent was filtered through a filter paper to remove suspended solid.

Influent and effluent samples of the anaerobic reactor operated at hydraulic retention times of 50–6 days were analyzed for their BOD using standard method (BOD_5) [2] and biosensor (off-line Fig. 1 at position 1).

2.3.3. On-line BOD monitoring analysis

A proposed on-line system for BOD was also applied to test the effluent from the reactor outlet during the first 60 days. Running buffer and effluent were pumped through a three-way valve (Fig. 1 at position 2) into a mixing coil. A dilution factor between 17 and 54 times can be adjusted by varying the flow rates of effluent and running buffer lines. These dilution factors can be used to automatically dilute sample which have BOD values between 180 and 300 $mg\,l^{-1}$ to fall within the linear range of the biosensor system (see Section 3.1.1). The diluted sample passed though the sampling loop of the injection valve where 1.0 ml was collected and injected into the biosensor system. The response was used to calculate the BOD using a calibration curve of standard solutions. BOD values obtained from this on-line biosensor system were then compared with off-line values obtained from both the off-line biosensor system and the BOD_5 standard method. This online analysis was tested during the operation of the anaerobic reactor at flow rates of 10 and 20 $ml\,day^{-1}$ (HRT approximately 50 and 25 days), once a day ($n=3$).

3. Results and discussion

3.1. The biosensor system

To optimize each operating condition (Table 1) four concentrations of synthetic wastewater with BOD between 0.5 and 15 $mg\,l^{-1}$ were tested and the sensitivities (slopes) of the calibration curves of different tested values were compared. The one that gave the highest sensitivity was chosen as the optimum value. Optimum conditions of the studied parameters are shown in Table 1. Using the obtained optimum conditions (Table 1) performance characteristics of the BOD biosensor were studied.

3.1.1. Linear range and limit of detection (LOD)

The collected sludge was cultivated at different time interval over a 3 months period. Each cultivated mixed culture was used to prepare gel beads for one column. In total 17 different columns were prepared and tested in the biosensor system. The responses of the biosensor system were investigated using OECD standard wastewater with BOD values in the range 1–100 $mg\,l^{-1}$. The linear ranges were between 2–25 and 5–60 $mg\,l^{-1}$ (Table 2). Since preparation conditions were the same the difference in the sensitivity and linear range of each column can only be attributed to the different activity of the mixed culture in each preparation. Hence system calibrations, using standard solutions, were generally performed before sample analysis. Average peak height from 20 blank injections was used to calculate the limit of detection using the IUPAC method ($S/N \geq 3$) [22] and was found to be 0.2 $mg\,l^{-1}$. The analysis time was around 10–15 min.

3.1.2. Repeatability

The repeatability of the sensor was studied by injecting 1.0 ml of 5.0 $mg\,l^{-1}$ BOD of OECD synthetic wastewater standard solution 20 times over 15 h. It was observed that the sensor gave fairly constant response during the testing period (43.5 ± 1.7 mV) with relative standard deviation of ±3.9%. The repeatability of some previously reported BOD sensors varies from ±2.4 to ±10% for the single-strain sensors [4] and ±1.3 to ±12.4% for multi-strains-based sensors [4,7,9,14]. That is this system gives stable measurement, compared well with other BOD biosensors with an improved repeatability when compared to the conventional BOD 5-day tests where ±15% is accepted [14].

3.1.3. Stability

The stability of the sensor was investigated by injecting, in triplicate per analysis one analysis per day, 1.0 ml of OECD synthetic wastewater standard solution (5.0 $mg\,l^{-1}$ BOD) over a period of 15 days. Between the assays, the column with immobilized cells was kept in 100 mM Tris–HCl buffer solution (containing 10 mM $CaCl_2$) pH 6.5 at 4 °C. The average response varied daily between 42.1 and 52.5 mV with relative standard deviation from 0 to ±6.7%. The average response

Table 3
Comparison of BOD values estimated by biosensor and the BOD_5 standard method (APHA) [2] for wastewater samples ($n = 3$) of different origin

Number	Sample	Biosensor ($mg\,l^{-1}$)	BOD_5 ($mg\,l^{-1}$)	% difference
1	Rubber trap pond1	530 ± 6	560 ± 6	−5.4
2	Rubber trap pond2	640 ± 10	700 ± 6	−8.6
3	Rubber trap pond3	810 ± 6	880 ± 10	−8.0
4	Rubber trap pond4	940 ± 6	1000 ± 0	−6.0
5	Aerobic pond1	1120 ± 6	1130 ± 6	−0.88
6	Aerobic pond2	2390 ± 6	2500 ± 6	−4.4
7	Aerobic pond3	3970 ± 6	4200 ± 10	−5.5
8	Stabilization pond1	4260 ± 6	4300 ± 10	−0.93
9	Stablization pond1	4030 ± 6	4200 ± 100	−4.0
10	Stabilization pond1	4120 ± 6	4230 ± 10	−2.6
11	Stabilization pond1	4140 ± 6	4200 ± 6	−1.4
12	Hospital1	6.48 ± 0.03	6.60 ± 0.08	−1.8
13	Hospital2	1.68 ± 0.02	2.0 ± 0.1	−16
14	Car transport1	20.00 ± 0.08	25 ± 1	−20
15	Car transport2	19.4 ± 0.2	20.5 ± 0.5	−5.3
16	Car transport3	19.5 ± 0.4	25 ± 1	−22
7	A car spare parts1	19.7 ± 0.2	25 ± 1	−21
18	A car spare parts2	21.0 ± 0.2	25 ± 1	−16
19	A car spare parts3	19.5 ± 0.3	24 ± 2	−17
20	Car wash company1	19.5 ± 0.3	23.5 ± 0.5	−17
21	Car wash company2	20.0 ± 0.5	24.8 ± 0.7	−19
22	Car wash company3	19.5 ± 0.3	24.9 ± 0.5	−22
23	Car wash company4	22.8 ± 0.2	25 ± 1	−8.8
24	Color manufacturing1	4.6 ± 0.2	5.0 ± 0.5	−8.8
25	Color manufacturing2	4.6 ± 0.3	5 ± 0	−7.6
26	Color manufacturing3	22.9 ± 0.1	46.0 ± 0.1	−50
27	Instant food product1	10.6 ± 0.5	12 ± 2	−12
28	Instant food product2	14.0 ± 0.1	21 ± 1	−33
29	Instant food product3	53 ± 1	63 ± 2	−15
30	Animal feed factory1	20.7 ± 0.3	26 ± 3	−20
31	Animal feed factory2	13 ± 1	17 ± 2	−19

Percentage difference is calculated from (biosensor − BOD_5) × 100/BOD_5.

calculated from the 45 measurements was 46.6 ± 3.9 mV with relative standard deviation of ±8.3%.

Operational stability of previously reported biosensors varied widely depending on the sensor configuration, the microbes used, the immobilization method and the operational conditions [4]. This can be about a week [7], a month [10] or up to a few months [9]. For our system the sensor can be applied for at least 15 days, if a longer operation time is required changing the column containing the immobilized cells is quite straightforward.

3.1.4. Estimation of BOD in samples of different origin

Wastewater samples from different origin were analyzed by the BOD biosensor and BOD_5 standard method. The results are shown in Table 3. While there was good agreement between values from the biosensor and BOD_5 method for samples from the rubber latex factory (number 1–11), it was clear that analytical results on samples from other sources showed pronounced differences between the two methods. These differences are possible to explain when considering the fact that the biosensor is constructed from a mixed culture that has been growing in one of the treatment ponds at the factory. The mixed culture thus has adapted to that specific wastewater. However, other wastewaters may contain chemicals that the cells are not adapted to deal with and therefore, the short analysis time used for the biosensor is not sufficient for degradation to take place. Therefore, larger differences are observed between the biosensor response and that from the longer incubation, the BOD_5. It is also possible that some macromolecules might be in the samples and the cells need longer time for digestion. To test this hypothesis some chemicals used at these other factories from where wastewater was collected were added into the wastewater from concentrated rubber latex production and tested with both methods. The results in Fig. 2 confirm that in the presence of molecules normally not present in the concentrated latex wastewater BOD values obtained from biosensor were lower than those from the BOD_5 analysis since in the latter the microbes have longer time for metabolizing these compounds.

3.2. The anaerobic reactor

Wastewater was collected from a factory where the main production is concentrated latex. In the process field latex is centrifuged to separate two fractions of the latex from each other, *i.e.*, the upper cream phase and the lower skim phase. The cream phase, concentrated latex, contains about 60% rubber content. Although the skim phase is mainly aqueous (serum) it still contains as much as 10% rubber material [23]. The small rubber content in the skim phase is coagulated by acid then goes through several steps to remove the water inside. Then it is either pressed to produced the skim block or creep through a pair of driven rolls

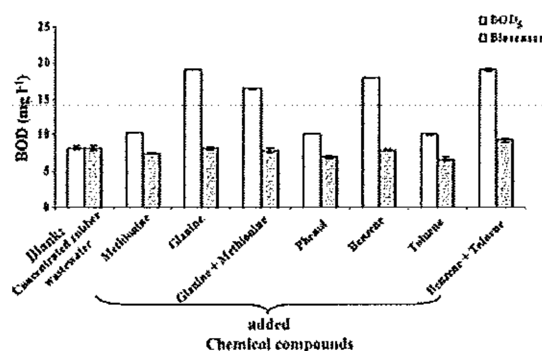


Fig. 2. Comparison of BOD values ($n = 3$) obtained from BOD_5 standard method and biosensor system of $5\ mg\ l^{-1}$ BOD of OECD synthetic wastewater with and without (blank) added chemicals.

to create the skim crepe [24]. The combined wastewater from the two processes generally contains high level of suspended solid, from the concentrated latex process, as well as high level of BOD_5 and COD, mainly from the skim crepe process due to the high content of organic constituents in the serum [24]. The wastewater is also contaminated with different kind of impurities including rubber fragments and chemicals. The efficiency of the anaerobic reactor was investigated and during the 120 days of operations BOD, COD and pH were tested daily while suspended solids and total dissolved solids were tested every 5–6 days. The characteristics of the performance of the bioreactor are summarized in Table 4.

3.2.1. BOD monitoring system

3.2.1.1. Off-line monitoring. A total of 240 samples, 120 influent samples and 120 effluent samples (30 samples per each flow rate) were tested. Influent samples were analyzed after diluting the samples 50–200 times. A hundred and twenty effluent samples from the reactor when operated at flow rates of $10–80\ ml\ day^{-1}$ were also analyzed after proper dilution. BOD values of the influent samples from both the BOD_5 standard method and biosensor were shown in Fig. 3(a). BOD_5 and biosensor gave BOD values in the range of 6070–6430 and $5820–6090\ mg\ l^{-1}$, respectively. Between days 1 and 120, effluent samples were also analyzed off-line. The results showed that analyses using BOD_5 and biosensor gave BOD in the range of 220–3480 and $200–3300\ mg\ l^{-1}$, respectively (Fig. 3(b)). The anaerobic bioreactor showed efficient BOD reduction at high HRT (see Fig. 3(c)). Percentage differences between results from the off-line biosensor and the BOD_5 standard method for influent and effluent samples were -5.2 ± 1.6 and $-4.4 \pm 2.2\%$, whereas percentage difference of previously reported biosensors varied in the ranges of 0.1–27.3% [25], 2.2–9.0% [26] and 0.1–43.0% [13].

3.2.1.2. On-line monitoring. For the off-line system, samples have to be manually collected, diluted and injected to the biosensor system. When the biosensor system is applied for on-line monitoring of the anaerobic treatment process, the samples are automatically loaded. This was done by connecting the biosensor system to the effluent of the treatment process using a three-way valve (Fig. 1). The effluent samples were automatically diluted with buffer solution in a mixing coil (Fig. 1(a) at position 2). Since the dilution factor of the system was limited by the tube size and the speed of the pump it could only be between 17 and 54 times. Therefore, it can only be used with wastewater with not so high BOD and this system might be suitable for the determination of BOD which is less than $300\ mg\ l^{-1}$. The on-line system was used to analyze 30 effluent samples per each hydraulic retention time when the reactor was operated at HRT approximately 25 and 50 days. The BOD_5 and biosensor gave BOD values in the range of 220–300 and $200–290\ mg\ l^{-1}$, respectively.

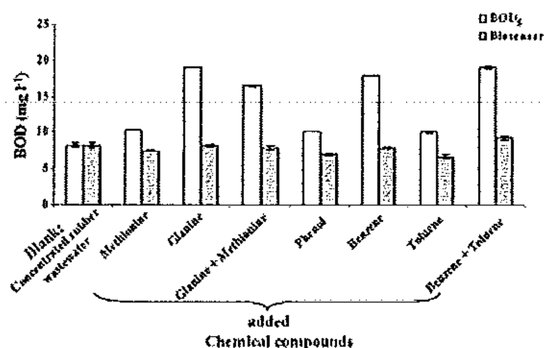
A regression line was used to compare between biosensor (off-line) versus BOD_5 and biosensor (off-line) versus biosensor (on-line). The comparison between biosensor (off-line) and BOD_5 (Fig. 4(a)) shows that the values obtained from the biosensor are about 5% less than the BOD_5. This is because the short analysis time of the biosensor (10–15 min) cannot assimilate some hard to degrade compounds while the BOD_5 standard method has a much longer incubation time. For the comparison between BOD from biosensor operated off-line and on-line, the results show practically the same values (Fig. 4(b)).

3.2.2. Determination of COD

The influent and effluent samples were collected once a day, analyzed for COD and the results were calculated in terms of %COD reduction as shown in Fig. 5. At a low flow rate of $10\ ml\ day^{-1}$ (HRT approximately 50 days) the percentage COD reduction was constant at 97% for the first 20 days and decreased slightly to about 90% after that. When the flow rate was increased to $20\ ml\ day^{-1}$ (HRT approximately 25 days) the percentage of COD reduction decreased to 85% during the first few days and increased to a stable value around 87%. At $40\ ml\ day^{-1}$ (HRT approximately 12 days) initially there was no change in percentage of COD reduction. It remained around 87% for 10 days and then decreased slightly to be around 75%. At $80\ ml\ day^{-1}$ (HRT

Table 4
Performance of the anaerobic reactor

Flow rate ($l\ day^{-1}$)	Hydraulic retention time, HRT (day)	Organic loading rate (OLR) yield ($gCOD\ l^{-1}\ day^{-1}$)	COD removal efficiency (%)	BOD removal	
				Biosensor (%)	BOD_5 (%)
0.01	50	0.2	95 ± 2	96.1 ± 0.2	96.2 ± 0.2
0.02	25	0.4	87 ± 4	95.4 ± 0.3	95.3 ± 0.3
0.04	12	0.8	80 ± 7	91.5 ± 0.5	91.4 ± 0.5
0.08	6	1.6	41 ± 17	47 ± 3	47 ± 3



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to create the skim crepe [24]. The combined wastewater from the two processes generally contains high level of suspended solid, from the concentrated latex process, as well as high level of BOD_5 and COD, mainly from the skim crepe process due to the high content of organic constituents in the serum [24]. The wastewater is also contaminated with different kind of impurities including rubber fragments and chemicals. The efficiency of the anaerobic reactor was investigated and during the 120 days of operations BOD, COD and pH were tested daily while suspended solids and total dissolved solids were tested every 5–6 days. The characteristics of the performance of the bioreactor are summarized in Table 4.

3.2.1. BOD monitoring system

3.2.1.1. Off-line monitoring. A total of 240 samples, 120 influent samples and 120 effluent samples (30 samples per each flow rate) were tested. Influent samples were analyzed after diluting the samples 50–200 times. A hundred and twenty effluent samples from the reactor when operated at flow rates of 10–80 ml day^{-1} were also analyzed after proper dilution. BOD values of the influent samples from both the BOD_5 standard method and biosensor were shown in Fig. 3(a). BOD_5 and biosensor gave BOD values in the range of 6070–6430 and 5820–6090 mg l^{-1}, respectively. Between days 1 and 120, effluent samples were also analyzed off-line. The results showed that analyses using BOD_5 and biosensor gave BOD in the range of 220–3480 and 200–3300 mg l^{-1}, respectively (Fig. 3(b)). The anaerobic bioreactor showed efficient BOD reduction at high HRT (see Fig. 3(c)). Percentage differences between results from the off-line biosensor and the BOD_5 standard method for influent and effluent samples were -5.2 ± 1.6 and -4.4 ± 2.2%, whereas percentage difference of previously reported biosensors varied in the ranges of 0.1–27.3% [25], 2.2–9.0% [26] and 0.1–43.0% [13].

3.2.1.2. On-line monitoring. For the off-line system, samples have to be manually collected, diluted and injected to the biosensor system. When the biosensor system is applied for on-line monitoring of the anaerobic treatment process, the samples are automatically loaded. This was done by connecting the biosensor system to the effluent of the treatment process using a three-way valve (Fig. 1). The effluent samples were automatically diluted with buffer solution in a mixing coil (Fig. 1(a) at position 2). Since the dilution factor of the system was limited by the tube size and the speed of the pump it could only be between 17 and 54 times. Therefore, it can only be used with wastewater with not so high BOD and this system might be suitable for the determination of BOD which is less than 300 mg l^{-1}. The on-line system was used to analyze 30 effluent samples per each hydraulic retention time when the reactor was operated at HRT approximately 25 and 50 days. The BOD_5 and biosensor gave BOD values in the range of 220–300 and 200–290 mg l^{-1}, respectively.

A regression line was used to compare between biosensor (off-line) versus BOD_5 and biosensor (off-line) versus biosensor (on-line). The comparison between biosensor (off-line) and BOD_5 (Fig. 4(a)) shows that the values obtained from the biosensor are about 5% less than the BOD_5. This is because the short analysis time of the biosensor (10–15 min) cannot assimilate some hard to degrade compounds while the BOD_5 standard method has a much longer incubation time. For the comparison between BOD from biosensor operated off-line and on-line, the results show practically the same values (Fig. 4(b)).

3.2.2. Determination of COD

The influent and effluent samples were collected once a day, analyzed for COD and the results were calculated in terms of %COD reduction as shown in Fig. 5. At a low flow rate of 10 ml day^{-1} (HRT approximately 50 days) the percentage COD reduction was constant at 97% for the first 20 days and decreased slightly to about 90% after that. When the flow rate was increased to 20 ml day^{-1} (HRT approximately 25 days) the percentage of COD reduction decreased to 85% during the first few days and increased to a stable value around 87%. At 40 ml day^{-1} (HRT approximately 12 days) initially there was no change in percentage of COD reduction. It remained around 87% for 10 days and then decreased slightly to be around 75%. At 80 ml day^{-1} (HRT

Table 4
Performance of the anaerobic reactor

Flow rate (l day^{-1})	Hydraulic retention time, HRT (day)	Organic loading rate (OLR) yield (gCOD l^{-1} day^{-1})	COD removal efficiency (%)	BOD removal	
				Biosensor (%)	BOD_5 (%)
0.01	50	0.2	95 ± 2	96.1 ± 0.2	96.2 ± 0.2
0.02	25	0.4	87 ± 4	95.4 ± 0.3	95.3 ± 0.3
0.04	12	0.8	80 ± 7	91.5 ± 0.5	91.4 ± 0.5
0.08	6	1.6	41 ± 17	47 ± 3	47 ± 3

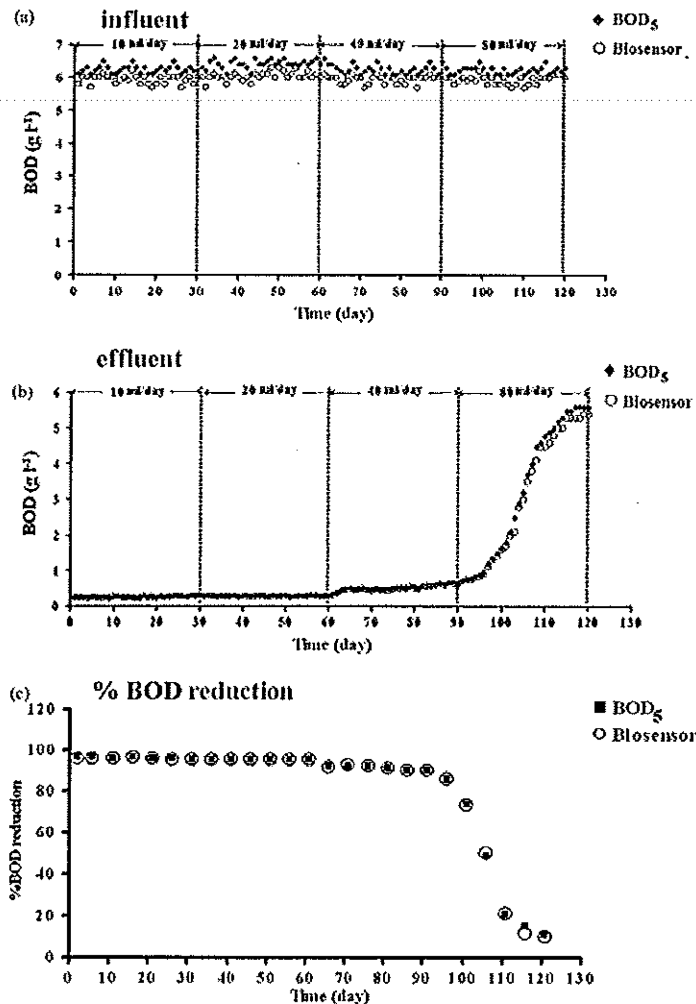

Fig. 3. BOD values from BOD_5 standard method and biosensor. Biosensor results in (a) and (b) were analyzed off-line. Percentages of BOD reduction are shown in (c) where the data were plotted at 5-day intervals.

approximately 6 days), percentage of COD decreased rapidly from 75 to 21%. From the results it shows that the studied lab scale reactor can operate sufficiently at HRTs down to 25 or maybe 12, but not at HRT of 6 days where reactor performance is influenced both by the ability of the organisms to deal with the substrate fed to them, and also with potential risk of washout of the reactor.

3.2.3. Determination of pH

pH of the wastewater was around 6.3 before treatment and it raised to almost 8.0 after passage through the anaerobic reactor (Fig. 6). When the influent passes through the reactor the various acids are degraded by the microbes resulting in the decrease of these acids [24] and hence the increase in pH. This is in agreement with other previous investigations where the treated wastewater gave an increase in pH [1,27,28].

3.2.4. Other characteristics of wastewater

Suspended solids (SS) and total dissolved solids of influent and effluent samples were also analyzed. As shown in Table 5 SS of raw wastewater is $5500 \pm 120\ \mathrm{mg\,l^{-1}}$. Most of the suspended solids came from the latex process. A possible minor source could be the release during the skim crepe process, a by-product from the production of the concentrated latex when acid was added to coagulate the rubber particle [24]. After treatment the reduction of SS in the wastewater depended on the hydraulic retention times used (Table 5). That is, with the anaerobic reactor the rubber particles could be separated from the liquid and this

Table 5
Characteristics of the raw wastewater and treated wastewater

Parameters	Raw	Treated waste water (ml day^{-1})				The set value[a]
		10	20	40	80	
BOD_5 (mg l^{-1})	6280 ± 150	239 ± 19	295 ± 9	530 ± 80	3,290 ± 190	20
BOD (biosensor) (mg l^{-1})	5960 ± 130	229 ± 20	281 ± 8	510 ± 80	3,120 ± 182	20
COD (mg l^{-1})	8800 ± 470	440 ± 8	1144 ± 311	1760 ± 408	5280 ± 870	120
pH	6.3 ± 0.4	8.7 ± 0.1	8.5 ± 0.5	8.5 ± 0.2	8.9 ± 0.2	5.5–9.0
Suspended solid (SS, mg l^{-1})	5500 ± 120 (n = 10)	1100 ± 150 (n = 3)	1750 ± 120 (n = 5)	1800 ± 150 (n = 5)	2050 ± 150 (n = 10)	50
Total Dissolve Solid (TDS, mg l^{-1})	5220 ± 150 (n = 10)	4300 ± 250 (n = 3)	4340 ± 230 (n = 5)	4750 ± 150 (n = 5)	5080 ± 140 (n = 10)	3000

[a] Ministry of Science, Technology and Environmental, Thailand, 1996.

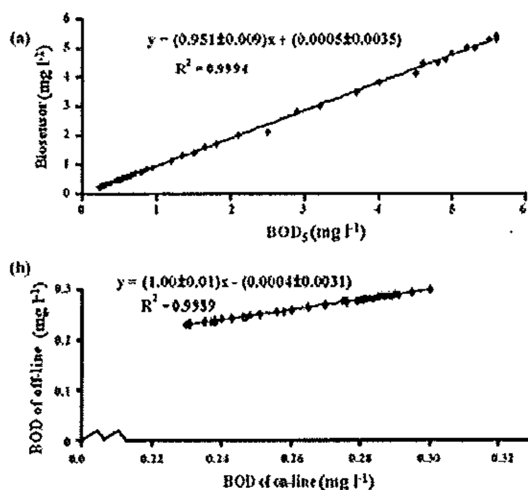

Fig. 4. Regression line comparing the BOD values obtained from two analysis systems (a) biosensor (off-line) and BOD_5 (b) biosensor (off-line) and biosensor (on-line).

helped to reduce the content of suspended solids. Total dissolved solids were also reduced by the anaerobic treatment.

Treatment of wastewater at flow rate 10 ml day^{-1} gave the best results (Table 5). The pH value of 8.7 ± 0.1 is quite acceptable, however, other parameters still did not meet the required value (Ministry of Science, Technology and Environmental, Thailand, 1996) (Table 5). The use of another reactor where the

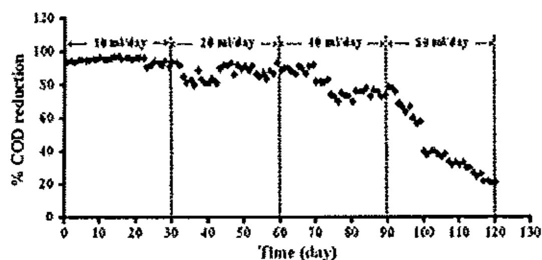

Fig. 5. Percentage COD reduction of the anaerobic reactor at different flow rates of influent.

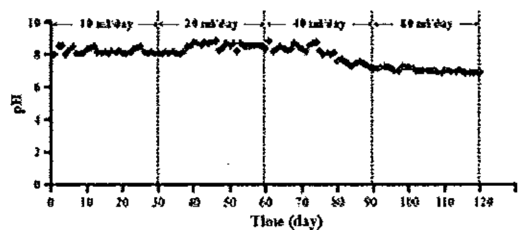

Fig. 6. pH of effluents of the anaerobic reactor at different flow rates of influent.

feed is the effluent from this first reactor would help to increase the treatment efficiency.

4. Conclusions

A microbial BOD sensor was developed by using an oxygen electrode as the transducer and mixed culture of activated sludge from a concentrated rubber latex production as the biological sensing element. The biosensor was applied to monitor the BOD of wastewater. The sensor showed good performance, wide linear range, good repeatability and long-term stability.

An anaerobic reactor treatment process was applied to treat the wastewater from the concentrated latex process. The performance of the anaerobic reactor was studied by feeding wastewater at different flow rates. At 10 ml day^{-1} the highest efficiency was obtained with percentage of COD and BOD reduction up to 97%. This is similar to those reported using stabilization pond system with aerobic treatments where %COD and %BOD reduction were in the range 97–99% [1]. This treatment process showed good efficiency for high strength wastewater but most parameters still exceeded the required values of discharged wastewater. The treatment efficiency might be increased by feeding the effluent back to the same reactor or to another reactor at the lowest flow rate.

The biosensor system that was employed to monitor the performance of the anaerobic reactor show that off-line and on-line assays could be carried out with short analysis time. The microbial BOD biosensor system has proven to be a suitable monitoring sensor for concentrated wastewater samples from the rubber latex process.

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Vitae

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2. VR (The Swedish Research Council) SIDA (The Swedish International Development Cooperation Agency) Research Links Programmes, Sweden

List of Publications and Presentations

Publications

1. Ampai Kumlanghan, Jing Liu, Panote Thavarungkul, Proespichaya Kanatharana and Bo Mattiasson. 2007. Microbial fuel cell-based biosensor for fast analysis of biodegradable organic matter. *Biosensors and Bioelectronics* **22**, 2939–2944.
2. Ampai Kumlanghan, Proespichaya Kanatharana, Punnee Asawatreratanakul, Bo Mattiasson and Panote Thavarungkul. 2008. Microbial BOD sensor for monitoring treatment of wastewater from a rubber latex industry. *Enzyme and Microbial Technology* **42**, 483–491.

Presentations

Poster Presentations

1. Ampai Kumlanghan, Jongjit Jantra, Proespichaya Kanatharana and Panote Thavarungkul. 2005."Environment Cell-based Biosensor for Organic Compounds". The International Conference on BioNanotechnology: A New Chapter of Life at BioThailand: Biotechnology Challenges in the 21st Century. The Queen Sirikit National Convention Center (QSNCC), Bangkok, Thailand. 2nd-5th November, 2005

2. Jongjit Jantra, Ampai Kumlanghan, Proespichaya Kanatharana and Panote Thavarungkul. 2006. "MICROBIAL BIOSENSORS FOR WASTEWATER MONITORING". 32nd Congress on Science and Technology of Thailand (STT.32) "Science and Technology for Sufficiency Economy". The Queen Sirikit National Convention Center, Bangkok, Thailand. 10th – 12th October 2006.

3. Ampai Kumlanghan, Jing Liu, Panote Thavarungkul, Proespichaya Kanatharana and Bo Mattiasson. 2007. "Novel Microbial Fuel Cell Biosensor for Organic Compounds". The 5th PERCH-CIC Annual Scientific Congress (PERCH-CIC Congress V). Pattaya, Thailand. 6th-9th May 2007.