

Microbial Fuel Cell-Type Biochemical Oxygen Demand Sensor

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1. INTRODUCTION

The biochemical oxygen demand (BOD) is an index for biodegradable organic compounds in water and wastewater, and widely used for the evaluation of water and wastewater quality [1]. However, the conventional method for determining the BOD is time-consuming (5 days of incubation) and usually requires experience and skill to achieve reproducible results [2]. Yet, for real-time control in the water and wastewater industry, the ability to determine the BOD on-line is needed, which has resulted in various extensive studies [3, 4].

1.1. Dissolved Oxygen Probe-Based BOD Sensors

A number of microbial BOD sensors have already been reported, mostly based on dissolved O₂ (DO) monitoring systems, and this topic has been well reviewed by Liu and Mattiasson [5]. These DO-based BOD sensors consist of a synthetic membrane with immobilized microorganisms as the biological recognition element, and an oxygen electrode as the physical transducer. The BOD is then determined by monitoring any decrease in the DO concentration due to the oxidation of biodegradable compounds by the microorganisms used. A variety of microorganisms have been tested for this purpose, including bacteria and yeast, where the selection was based on the ability of the microbial strain to assimilate a suitable spectrum of substances [6–13].

Although BOD sensors based on a single strain have a relatively good stability and longer lifetime, they have a limited capacity to metabolize a wide range of contaminants in samples, which can result in inaccurate BOD values. Thus, to alleviate this problem, mixed cultures [14] or activated sludge [3, 5, 15] have been used, and some of these BOD sensor systems have been commercialized [16, 17].

BOD sensors based on DO monitoring have a limited stability due to the nature of a DO probe. A typical Clark-type DO probe is a two-electrode system consisting of a silver anode and gold or platinum cathode [5]. However, the anode metal is oxidized during use, therefore, the electrolyte needs to be changed and the anode surface of DO probe cleaned regularly. Membrane fouling is also a serious problem in biosensors based on a DO electrode and frequent maintenance is required to maintain a high sensitivity. Furthermore, oxygen-based BOD sensors have a narrow response range, due to their limited oxygen solubility of less than 9 mg/L [18]. Fluctuating oxygen levels in a sample can also mean poor reproducibility and reliability, unless a sufficient oxygen supply is incorporated into the sensor [19].

1.2. Photometric BOD Sensors

Photometric techniques have been applied for a rapid estimation of the BOD based on the UV absorbance (at 254 nm) by dissolved organic matter in water and wastewater [20–22], and the fluorescent properties [23] of humic substances and organic matter in natural surface water [24–26].

Reynolds and Ahamd [27, 28] reported on the linear relationship between the BOD values of wastewater and the fluorescence intensities at 340 nm for organisms growing in wastewater. The reduced forms of pyridine nucleotides (NADH and NADPH) are fluorescent. Thus, in the presence of an electron donor, pyridine nucleotides are reduced and the ratio NAD(P)H/NAD(P)⁺ is related to the concentration of the electron donor.

The luminous bacterium *Photobacterium phosphoreum* can also be used for a similar purpose, where the intensity of the luminescence, which is proportional to the amount of

assimilable organic compounds in wastewater, is measured using a photodiode [29].

Yet, these techniques are not suitable for an accurate BOD determination due to the effect of interfering substances and the fact that some organic compounds absorb more UV (or are more fluorescent) than others.

1.3. Mediated Microbial Fuel Cell-Type BOD Sensors

A microbial fuel cell (MFC) is a microbial device that converts chemical energy into the form of electricity through the catalytic activity of microbes (see Fig. 3). In an MFC, microbes oxidize electron donors and the resulting electrons are transferred to the anode, which is separated from the cathode by a cation exchange membrane. The electrons from the anode are then transferred through the circuit to the cathode, which is aerated. Various microbes have been used to operate MFCs [30–40].

In most cases, intact microbial cells that contain active redox proteins are electrochemically inactive, as their cell walls and other surface structures are electrically non-conductive. Mediators can be used to facilitate the transfer of electrons from the microbial cells to the electrode [30–43]. When a mediator is present in the reaction medium, it acts as an electron acceptor and is preferentially reduced during the metabolic oxidation of organic substances. The reduced form of the mediator is then reoxidized at a working electrode (anode), which is maintained at a sufficiently high electric potential. Table 1 shows the bacterial strains and mediators that have been used in mediated MFCs, including thionine, methyl viologen, 2-hydroxy-1,4-naphthoquinone, neutral red, and other hydrophobic compounds [30–43].

An MFC system can be used for various purposes, including biosensors, bioelectrochemical synthetic processes, and electricity generation [44]. In particular, mediated MFCs have been studied as BOD sensors [18, 45, 46]. The sensor responses are linear from 2 to 200 mg/L and have a response time of 5 to 15 min. Yet, mediated MFCs are limited as regards long-term operations, as the mediators used in the device are toxic to the organisms used, which means the microorganisms need to be refreshed regularly. Typically a mediated MFC cannot be operated for more than 7 days.

As an alternative method for on-line BOD determination, Karube et al. [47, 48] investigated a hydrogen fuel cell coupled to anaerobic fermentation by *Clostridium butyricum* immobilized onto the electrode. A linear relationship was obtained between the steady state current and the BOD of a standard solution up to 300 mg/L. A steady state current was attained within 30–40 min, and the standard deviation was 2 mg/L. The BOD of industrial wastewater was estimated using this device, and the current output of the MFC was kept almost constant for 30 days. Yet, exact BOD values cannot be obtained using this system, as the bacterium does not metabolize non-fermentable contaminants.

However, this chapter discusses the use of mediator-less MFCs as BOD sensors in terms of their fabrication and performance.

Table 1. Bacterial strains and mediators used in mediated microbial fuel cells.

Bacterial strains	Mediators used in anode compartment	Refs
<i>Actinobacillus succinogenes</i>	NR	[30]
<i>Alcaligenes eutrophus</i>	ABB, BV, BCB, DCPIP, GC, PES, RS, TH, DMSZ	[31, 32]
Alcalophilic organisms	BCB, Fe(III)EDTA, HNQ, MV	[33]
<i>Azobacter chroococcum</i>	BV, DCPIP, PES, ABB, DMST, MB, PTZ, TH, BCB, GC, RS	[32]
<i>Bacillus subtilis</i>	ABB, BV, BCB, DCPIP, GC, PES, RS, TH, DMSZ	[31]
<i>Clostridium butyricum</i>	MB	[34]
<i>Escherichia coli</i>	ABB, BV, BCB, DCPIP, GC, PES, RS, TH, DMSZ	[31]
<i>Escherichia coli</i>	HNQ	[35]
<i>Erwinia carotovora</i>	TH	[36]
<i>Erwinia dissolovens</i>	Fe(III)CyDTA, Fe(III)EDADPA, Fe(III)EDTA, Fe(III)TTHA	[37]
<i>Lactobacillus plantarum</i>	Fe(III)CyDTA, Fe(III)EDADPA, Fe(III)EDTA, Fe(III)TTHA	[37]
<i>Protues vulgaris</i>	ABB, BV, BCB, DCPIP, GC, PES, RS, TH, DMSZ	[31, 38]
<i>Protues vulgaris</i>	TH	[36, 39]
<i>Pseudomonas aeruginosa</i>	BV, DCPIP, PES, ABB, DMST, MB, PTZ, TH, BCB, GC, RS	[32]
<i>Pseudomonas putida</i>	BV, DCPIP, PES, ABB, DMST, MB, PTZ, TH, BCB, GC, RS	[32]
<i>Staphylococcus aureus</i>	MB	[34]
<i>Streptococcus lactis</i>	Fe(III)CyDTA, Fe(III)EDADPA, Fe(III)EDTA, Fe(III)TTHA	[37]
<i>Synechocystis</i> sp.	HNQ	[40]

ABB, alizarin brilliant blue; BV, benzyl viologen; BCB, brilliant cresyl blue; DCPIP, 2,6-dichlorophenolindophenol; DMST, *N,N*-dimethyl disulfonated thionine; Fe(III)CyDTA, ferric 1,2-cyclohexanediamine tetraacetic acid; Fe(III)EDADPA, ferric ethylenediamine diacetic acid dipropionic acid; Fe(III)EDTA, ferric ethylenediaminetetraacetic acid; Fe(III)TTHA, ferric triethylenetetraamine hexaacetic acid; GC, galloycyanine; HNQ, 2-hydroxy-1,4-naphthoquinone; MB, methylene blue; MV, methyl viologen; NR, neutral red; PES, phenazine ethosulphate; PTZ, phenothiazinone; RS, resorufin; TH, thionine

2. MEDIATOR-LESS MICROBIAL FUEL CELL (MFC)

2.1. Electrochemically Active Bacteria

A number of bacteria have been isolated based on their ability to use Fe(III) as a terminal electron acceptor [49, 50]. Although there is some evidence that soluble electron mediators are involved in the reduction of a water-insoluble electron acceptor [51, 52], direct contact between the bacterial cells and the electron acceptor is required for a dissimilatory Fe(III) reduction [53]. Among the Fe(III) reducers, *Shewanella oneidensis* (formerly *S. putrefaciens*) [54] and *Geobacter sulfurreducens* [55] are known to localize the majority of membrane-bound cytochromes on the outer membrane, plus they are electrochemically active [56–58] and can be used as the anode catalyst in an MFC. In addition, *S. putrefaciens* was found to grow on lactate in the absence of electron acceptors in an electrochemical fuel

cell, yet did not metabolize lactate when the anode was disconnected from the cathode [56, 57]. An MFC using *S. putrefaciens* can also be used as a lactate sensor [59], because lactate is one of the few electron donors used by this bacterium. Meanwhile, electrochemical activities have also been found in other Fe(III)-reducers, including *Clostridium butyricum* [60], *Aeromonas hydrophila* [61], *Rhodospirillum rubrum* [62], *Desulfobulbus propionicus* [63], and *Enterococcus gallinarum* [64].

MFCs using a single organism have an intrinsic disadvantage due to the limited range of fuel utilization. As such, electrochemically active microbial communities with different nutritional characteristics have been successfully enriched using fuel cell-type electrochemical cells (see below). Anode mediators were not used in these cases, and the coulomb yield was over 90% in some of the MFCs

Similarly, an electrode placed in marine sediment can collect electrons through microbial reactions when connected to another electrode placed at the aerobic surface [65–67]. Natural redox compounds, such as sulfur/sulfide, Fe(III)/Fe(II), and humic acid have also been suggested as possible mediators facilitating electron transfer from the microbial cells to the electrode [66], yet the addition of the humic acid analog anthraquinone-2,6-disulfonate was found to slightly increase the current production [65].

2.2. Enrichment of Electrochemically Active Bacterial Community

When the anode compartment of a fuel cell-type electrochemical device was inoculated with sludge collected from a wastewater treatment plant of a corn-processing factory and fed with wastewater from the same source with continuous aeration to the cathode compartment [68], an open circuit potential of around 0.5–0.7 volts was observed immediately after the inoculation, while a current of around 2.0 mA was generated stably within 3 weeks after the circuit, connecting the electrodes through a resistance of 10 Ω with the fuel consumption, was closed [Fig. 1(a)]. Accordingly, these results show that the inoculum contained electrochemically active microbes (EAM), and that the EAMs propagated in the fuel cell were used. Therefore, the anode was removed from the MFC, enriched, and run for about a year for microscopic observation. As shown in Fig. 1(b), a thick biofilm was observed on the surface of the electrode, indicating that the electrochemically active bacteria colonized on the electrode surface to use the electrode as an electron acceptor/sink in the absence of electron acceptors and the other bacteria associated with them (see below). The current generation was then the result of the electrons transferred to the electrode by the electrochemically active microbes after they metabolized electron donors in the wastewater. Plus, the current was generated stably for over 6 years with the consumption of the chemical oxygen demand. It is well documented that the microbial community in a biofilm is more stable than a free-living community, which would explain why the biofilm that developed on the electrode was better than artificially immobilized microbial cells for the performance of the microbial fuel cell.

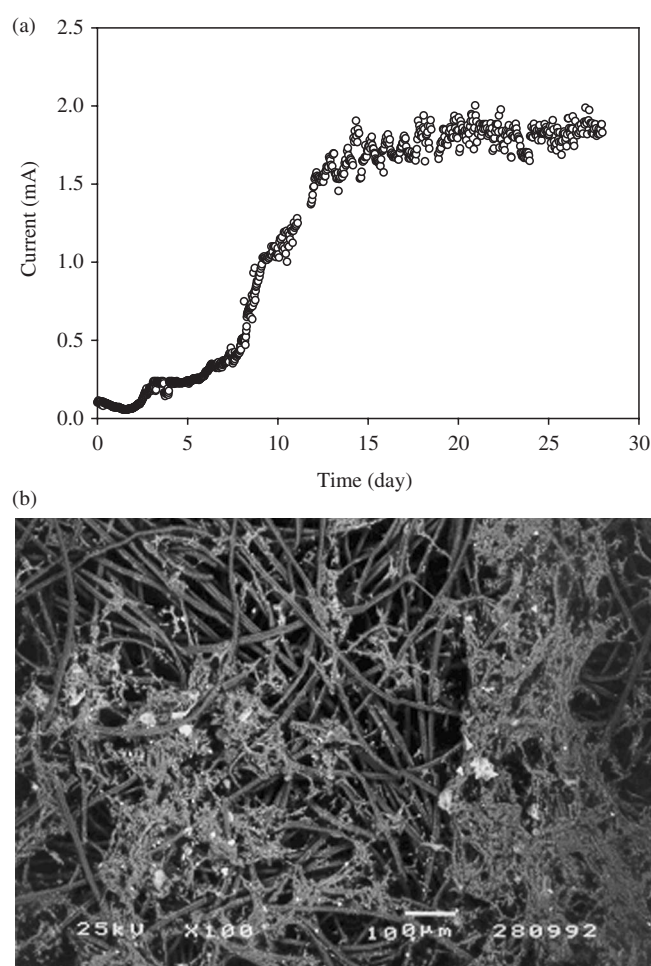


Figure 1. Current generation profile (a) and electron micrograph of enriched electrode (b).

Furthermore, enrichment cultures were made with various nutritional characteristics, including copiotrophic cultures enriched with artificial wastewater containing acetate [69] or artificial wastewater containing glucose and glutamate [70], and oligotrophic cultures with artificial wastewater or river water [71–73].

The coulomb generated from the MFC was directly proportional to the strength of the wastewater. Therefore, based on these observations, MFCs have been developed as BOD sensors [74, 75].

2.3. Microbiology of Mediator-Less MFC

In addition to the biofilm onto the electrode, surface electron microscopic observation revealed microbial clumps loosely attached to the electrode [68]. These clumps were not observed in MFCs enriched with a non-fermentable fuel, such as acetate [69]. Confocal laser scanning microscopic observation showed that the biofilm and bacterial clumps consisted of micro-colonies of Gram positive and negative bacteria [68]. Thus, it is hypothesized that the microbial clumps are inhabited by fermentative bacteria that ferment the fermentable substrate (complex electron donors) into fermentation products, then the microbes in the biofilm

oxidize the resulting fermentation products to transfer the resulting electrons to the electrode.

DNA was extracted from the anodes of the MFCs, enriched, and operated under various nutritional characteristics for molecular ecological analyses. Denaturing gradient gel electrophoresis (DGGE) revealed that the bacterial population in the MFC was different from that in the inoculum [68, 69, 73], and the microbial populations in the MFCs were dependent on the fuel used.

Analyses of the 16S rDNA sequences also showed that the dominant bacterial classes differed, thereby substantiating the DGGE results. In all cases, Gram negative bacteria were dominant. Table 2 compares the bacterial populations in the MFCs enriched with different fuels. The one enriched with glucose and glutamate was dominated by *Gammaproteobacteria* (36.5%), followed by *Firmicutes* (27.0%) [unpublished result], while around 70% of the bacterial population was *Deltaproteobacteria* with 17.3% *Gammaproteobacteria* in the MFC enriched with acetate [69]. It is interesting to note that a high population of *Firmicutes* (over 20%) was found in an acetate-enriched MFC in a marine environment [58], yet a low population found in a fresh water environment [69].

It is surprising to note the differences in the bacterial population between MFCs enriched with AWW containing glucose and glutamate and MFCs enriched with corn-processing wastewater [68]. The majority of the bacterial clones amplified from the latter were *Betaproteobacteria* (40.9%) and *Alphaproteobacteria* (27.2%), and devoid of *Deltaproteobacteria* and *Gammaproteobacteria*. Plus, the organic contaminants of the wastewater were mainly carbohydrates derived from corn.

Different microbial populations have been reported between oligotrophic MFCs enriched and run with AWW containing fuel at 10 mg/L as the BOD, and MFCs enriched and run with river water with a BOD of around 5 mg/L [73]. *Alphaproteobacteria* were identified as the major bacterial clones in the MFCs enriched with a low concentration of AWW, followed by *Betaproteobacteria*. The high population of *Alphaproteobacteria* was apparently due to the enrichment of nitrilotriacetate (NTA) oxidizing *Aminobacter aminovorans* [unpublished result], where NTA was used as the chelating agent to dissolve metal ions. Meanwhile, *Betaproteobacteria* were the major population in the river water-enriched MFCs. Thus, it would appear that *Betaproteobacteria* are the major electrochemically active bacteria under oligotrophic conditions.

It has been shown that *Deltaproteobacteria* are the major bacterial population in acetate-enriched MFCs [58, 69]. *Deltaproteobacteria* are believed to be able to transfer electrons to the electrode in an MFC. However, they were not found in the MFCs enriched with corn processing wastewater [68] and AWW with a low fuel concentration [73]. Instead, these MFCs were found to contain a high population of *Betaproteobacteria*, which was also the dominant bacterial population in the MFCs enriched with river water. *Rhodoferrax ferrireducens*, a member of *Betaproteobacteria*, can reduce an electrode [62]. High populations of *Gammaproteobacteria* were also found in most of the MFCs analyzed. Members of *Gammaproteobacteria*, known to be electrochemically active, include *Shewanella putrefaciens* [56] and *Aeromonas hydrophila* [61]. Thus, electrochemical activity may be a widely distributed characteristic among bacteria, including *Firmicutes* [60]. Consequently, novel methods should be developed to characterize their role in the natural ecosystem and confirm the hypothesis that electrode reduction is a form of anaerobic respiration.

Various metabolic inhibitors have been used to establish the electron transport chain of the electrochemically active bacterial population in an MFC. As such, the current increased slightly with the addition of terminal oxidase inhibitors, such as cyanide and azide, while antimycin A had no effect on the current generation. Meanwhile, the current generation was inhibited in the presence of rotenone, 2-heptyl-4-hydroquinolone-N-oxide (HQNO), p-chloromercuriphenylsulphonate (p-CMPS), dicyclohexylcarbodiimide (DCCD), and 2,4-dinitrophenol (DNP). Therefore, these results show that the electron transport in an MFC shares an early part of the electron transport chain (NADH dehydrogenase and coenzyme Q) with aerobic bacteria, although cytochrome b and terminal oxidases are not involved in MFCs [68, 69]. Figure 2 shows the proposed electron transport chain of an electrochemically active bacterial population in an MFC.

Studies have been conducted to determine the effects of alternative electron acceptors on the performance of an MFC. When MFCs were fed with fuel containing electron acceptors with a high redox potential, such as oxygen and nitrate, the current and coulomb generation was reduced. However, sulfate showed less deteriorating effects [68]. Accordingly, these results show that the microbes in the anode compartment use oxygen and nitrate preferentially over the electrode reduction. Yet, since nitrate is present

Table 2. Comparison of bacterial communities in MFCs enriched with different fuels.

Fuel (value as COD ^a)	Class						Refs.
	<i>Alpha-Proteobacteria</i>	<i>Beta-Proteobacteria</i>	<i>Gamma-Proteobacteria</i>	<i>Delta-Proteobacteria</i>	<i>Firmicutes</i>	Others	
Acetate (300 ^a)	7.0	1.7	17.3	68.8	1.0	3.8	[68]
Starch processing wastewater (400)	27.2	40.9	0	0	4.5	27.1	[64]
Copiotrophic AWW (200)	1.4	6.8	36.5	14.9	27.0	13.4	Unpublished work
Oligotrophic AWW (10)	64.4	21.1	3.3	0	0	11.1	[72]
River water (≈5)	10.8	46.2	12.9	12.9	0	17.2	[72]

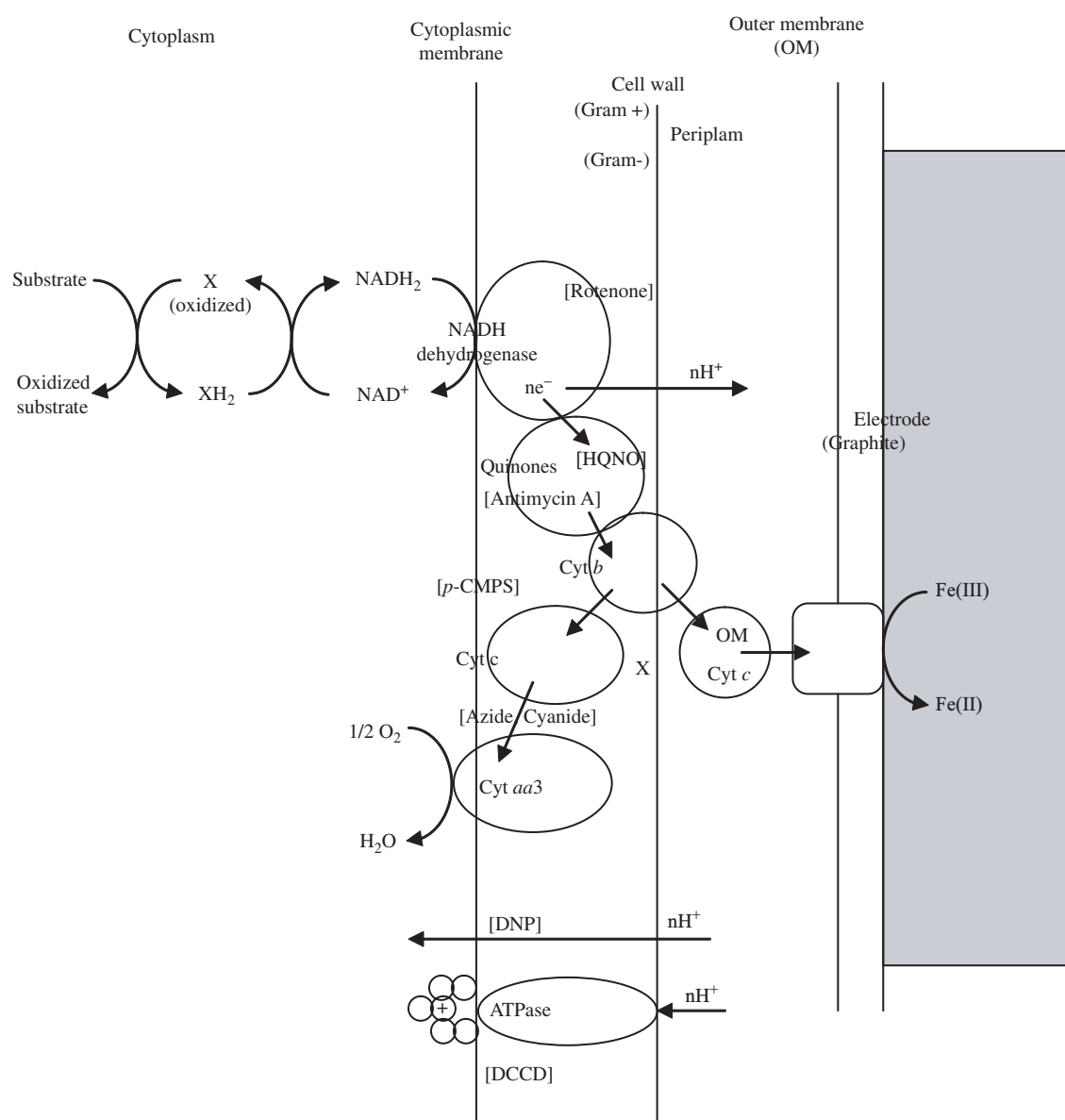


Figure 2. Proposed electron transport chain for electrochemically active bacteria in mediatorless microbial fuel cell.

in the effluent of wastewater treatment processes and oxygen is ubiquitous, their effects should be eliminated for an accurate BOD measurement [75].

2.4. Optimization of MFC Performance

It has been shown that the current generation from an MFC is determined by several physical and biochemical factors (Fig. (3)), including (1) the microbial activity to oxidize fuel, (2) electron transfer rate to the electrode from the microbes, (3) circuit resistance, (4) proton transfer from the anode compartment to the cathode compartment, (5) oxygen supply and reduction at the cathode, and (6) oxygen diffusion into the anode compartment through the membrane. Among these, the most serious problem in an MFC as a BOD sensor is the oxygen diffusion into the anode compartment, which consumes electrons in the anode compartment,

thereby reducing the coulomb yield. When the microbial population is properly enriched, the microbial activities to oxidize the fuel and transfer the electrons to the anode were not limited under experimental conditions where the physical factors were limiting.

Mediator-less MFCs have already been optimized in terms of their operating conditions [75–77]. The current generation is dependent on several factors, such as the pH, temperature, resistance, electrolyte used, and dissolved oxygen concentration in the cathode compartment. The highest current is generated at pH 7.0 and 35 °C [76].

As expected, the lower the resistance, the higher the current. Therefore, the MFCs were operated at 10 Ω. With a resistance lower than 500 Ω, the proton transfer and DO limited the cathode reaction. A high strength buffer reduced the proton limitation to some extent, and a larger membrane was found to increase the proton transfer rate, although

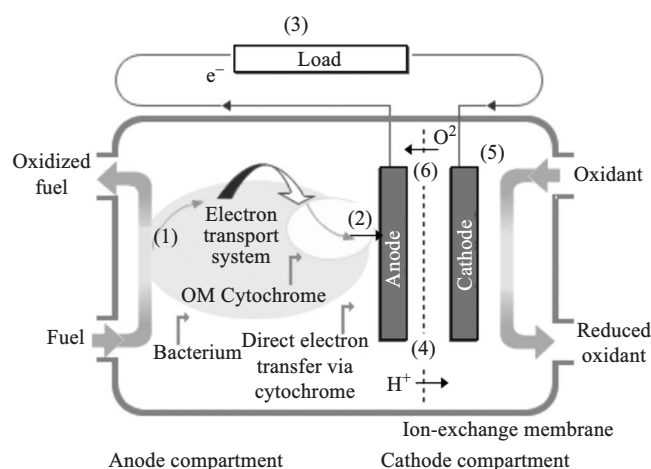


Figure 3. Possible rate-limiting steps in current generation by mediator-less microbial fuel cell. (1) Oxidation of fuel, (2) electron transfer from microbial cells to electrode, (3) electric load in circuit, (4) proton supply into cathode compartment, (5) cathode reactions, and (6) oxygen diffusion from cathode to anode.

the increased oxygen diffused in the opposite direction was a problem with the increased surface area. When graphite was used as the cathode, the critical oxygen concentration was around 6 mg/L. This high critical oxygen concentration was apparently due to the poor oxygen-reducing activity of the graphite electrode. The incorporation of a platinum-coated graphite cathode generated a maximum current that was 3–4 times higher than that with the graphite cathode, plus the critical oxygen concentration was reduced to 2.0 mg/L [78].

A considerable amount of oxygen diffuses from the cathode compartment to the anode compartment, thereby reducing the coulomb yield. However, a coulomb yield of over 90% was achieved in an MFC fed with a fuel concentration of over 100 mg/L, whereas an oligotrophic MFC fed with a fuel concentration of 10 mg/L showed a coulomb yield of less than 30%. Thus, to reduce the oxygen diffusion through the membrane, an oligotrophic MFC was designed with a reduced membrane size [71].

3. DESIGNS AND PERFORMANCE

Mediator-less MFCs can be used to determine the BOD by reading the current under continuous conditions or by measuring the coulomb in a batch mode. Since the form and concentration of the fuel determine the microbial population in an MFC, MFCs should be enriched using samples for an analysis of the fuel. For this reason, the measurement of high and low BOD values are discussed separately.

3.1. MFC to Measure BOD Values Higher than 10 mg/L

3.1.1. MFC Design

Polyacrylic plastic is used to construct the frame of a C-type MFC [79], consisting of anode and cathode compartments separated by a cation exchange membrane sealed with silicon rubber gaskets. Graphite felt is used as the electrode after being cleaned with 0.1 N HCl and thoroughly rinsed

with deionized water. Platinum wire is then used to connect the electrodes and the electronic part, and the MFC is installed in a temperature-controlled chamber. A cross-section of each compartment reveals a rectangular shape with a narrow width through which fuel or air-saturated tap water flows as the catholyte flows upward. As such, this design minimizes the fuel channeling and flow short-circuiting problem in a continuous operation. Each compartment is installed with two ports at the top and bottom, respectively. The electrodes are packed to occupy the entire anode and cathode compartments so as to have direct contact between the cation-specific membrane and the electrodes. The anode and cathode are connected through a voltmeter with a resistance of 10 Ω to monitor the current under close circuit conditions. The current is calculated as $I = V/R_{load}$, where V is the potential drop across R_{load} . Figure 4 shows a schematic diagram of a typical BOD monitoring system, including an MFC as the sensor.

3.1.2. Enrichment and Operation

The anode compartment of the MFC is loaded with a freshly collected sludge suspension, while the cathode compartment is fed with air-saturated tap water. The anode compartment should be kept as anoxic as possible. The sample is purged with oxygen-free nitrogen gas in a pretreatment tank before being fed to the anode compartment at a hydraulic retention time of about 14 min. Normally, within 3 weeks of the fuel feeding a stable current is generated, suggesting that electrochemically active bacteria have been successfully enriched. The MFC can be then used to measure the BOD. The peak current or coulomb is monitored for BOD estimation in a batch operation, while the current is monitored in a continuous operation. Meanwhile, a precise sample amount needs to be supplied for a batch operation, whereas a constant feeding speed is required for a continuous measurement.

3.1.3. Performance

The performance of an MFC as a BOD sensor is described here according to the criteria suggested by Liu and

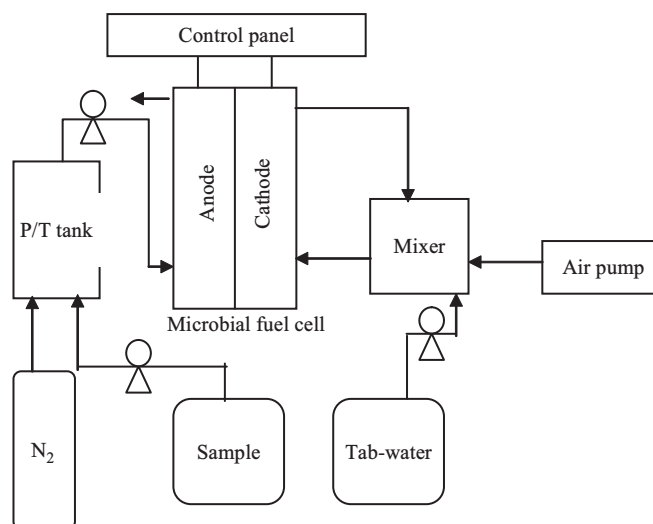


Figure 4. Schematic diagram of microbial fuel cell-type BOD monitoring system.

Mattiasson [3]. As the current from a copiotrophic MFC has a linear relationship with a fuel concentration of 20 to 400 mg/L, a BOD sample over 400 mg/L needs to be diluted to this range.

A short response time is important for on-line BOD monitoring and varies from 5 min to 10 hr depending on the microbial fuel cell type, operational conditions, and BOD concentration. A non-ideal plug flow in the anode compartment results in an increased response time, therefore, the feeding rate needs to be determined to reduce a non-ideal flow [79]. The smaller the MFC, the shorter the response time. The measuring time is shorter with a continuous operation than a batch operation. Thus, at optimal operating conditions using an MFC with an anode volume of 5 mL, the response time can be reduced to 5 min.

The APHA standard method for a 5-day BOD test allows a standard deviation of 30.5 mg/L using artificial wastewater containing glucose and glutamate with an average BOD₅ value of 198 mg/L as the repeatability, which is equivalent to a $\pm 15.4\%$ variation in repeatability. As shown in Table 3, the repeatability of MFCs ranges from ± 3 to $\pm 13\%$, which is within the acceptable variation set by APHA. The long-term stability of a mediator-less MFC-type BOD sensor is remarkable when compared with a DO electrode-based BOD sensor and mediator-aided MFC-based BOD sensor. Without any maintenance, a mediator-less MFC-type BOD sensor can be operated for over 6 years, and a mediator-less MFC enriched with the wastewater to be monitored used for a long period of time with good accuracy. An MFC-type BOD sensor system was tested under field conditions at a sewage plant [80], where the sensor was successfully operated in a batch mode with high accuracy and good stability for over 60 days. It took 45 min to measure a sample.

3.2. MFC to Measure BOD Values Lower than 10 mg/L

3.2.1. Background

The BOD sensors reported have almost all been developed to determine high BOD values in industrial wastewater. Yet, surface water and secondary effluents usually contain less biodegradable organic compounds, such as humic acid, lignin, tannic acid, gum Arabic, and surfactants at low concentrations [81, 82]. Some microorganisms are known to grow optimally in environments with low levels of nutrients. These microorganisms are known as “oligotrophs” in contrast to “copiotrophs,” which are common in environments with greater nutritional opportunities. The ability of oligotrophic microorganisms to grow in this way has a number

of important biotechnological, medical, and environmental implications, and the current authors have been involved in developing an MFC using oligotrophs obtained from surface water for the rapid determination of low BOD values.

The coulomb yield is very low, less than 10%, in oligotrophic MFCs enriched using a C-type fuel cell, as used in MFCs for measuring BOD values higher than 10 mg/L. This low yield is apparently due to fuel consumption through aerobic respiration in the anode compartment using O₂ diffused through the membrane [78]. Thus, a new oligotrophic MFC (O-type MFC) was designed as a low value BOD sensor to enhance the cathode reaction and lower the O₂ diffusion into the anode compartment by reducing the size of membrane [71, 83], and the performance of the new MFC was greatly improved in terms of the maximum current and coulomb yield.

3.2.2. Sensor Design and Performance

The O-type MFCs were inoculated with sediment collected from a local river and fed with river water collected from the same source to enrich electrochemically active oligotrophic bacteria. Alternatively, the MFCs were fed with artificial wastewater containing 10 mg/L glucose and glutamate as the BOD. The cathode was fed with air-saturated tap water continuously. Within 8 weeks of continuous fuel feeding, the MFCs generated a stable current of over 0.01 mA with a resistance of 10 Ω , showing that electrochemically active oligotrophs had been enriched under these conditions.

When the MFCs enriched with surface water were run in a batch mode, the maximum current generated was 0.02 mA with a coulomb yield of 20%, which was much higher than for an oligotrophic MFC enriched in a C-type MFC. In a continuous operation [83], the dynamic linear range of the calibration curve was 2.0 to 10.0 mg BOD/L. The response time to a change of 2 mg BOD/L was about 60 min. The current signal from the O-type MFCs increased with an increase in the salt concentration, however, this effect was eliminated using a 50 mM phosphate buffer.

3.3. BOD Determination of Samples Containing Oxygen and Nitrate

3.3.1. Oxygen and Nitrate Reduce Current Coulomb

As discussed above (Section 2.4), oxygen and nitrate are reduced preferentially over the electron transfer to the anode, thereby reducing the coulomb yield. Yet, the effluents

Table 3. Comparison of MFC-based BOD sensor performances.

Biological recognition element	Mediator usage	Operation mode	Measuring range (mg BOD/l)	Response time (min)	Repeatability ($\pm\%$)	Operational stability	Refs.
Copiotrophic microbial consortium	No	Batch	20–400	30–600	3–12	>5 years	[64, 73, 74]
Copiotrophic microbial consortium	No	Continuous	20–200	5–15	3–4	>1 year	[78]
Oligotrophic microbial consortium	No	Continuous	2–10	60	—	>1 year	[70, 82]
Single bacterium	No	Batch	<300	30–40	10	<30 days	[47]
Single bacterium	Yes	Batch	15–200	15	13 (reproducibility)	Disposable	[45]
Two yeast strains	Yes	Batch	2–100	5	10	<7 days	[18]

from sewage works and certain types of industrial wastewater contain high concentrations of nitrate [84], and oxygen can be easily dissolved into the samples. Thus, for an accurate BOD measurement, these electron acceptors with a high redox potential should be removed from the samples. Although oxygen can be purged from the samples using inert gases, such as nitrogen, before the samples are fed to the MFCs for BOD measurement [68, 70, 75], a considerable amount of oxygen still diffuses into the anode compartment through the ion-exchange membrane, which separates the anode compartment from the cathode compartment [78]. Plus, it is not easy to eliminate nitrate from the samples.

3.3.2. Use of Respiratory Inhibitors

Azide [85] and cyanide [86] are known to inhibit nitrate reductase, the key enzyme of dissimilatory nitrate reduction [87], and cytochrome oxidase, the terminal oxidase of aerobic respiration is inhibited by azide [88] and cyanide [89]. Thus, since azide and cyanide do not inhibit the current generation from MFCs [68], they have been used to improve the performance of an MFC as a BOD sensor. The signal from MFCs decreases in the presence of electron acceptors with a higher redox potential, such as nitrate and oxygen. Yet, the addition of azide or cyanide did not change the signal in the absence of the electron acceptors, thereby eliminating the inhibitory effects of the electron acceptors on the current generation from MFCs [77]. Similar results were also obtained using oligotrophic MFCs fed with an environmental sample that contained nitrate. Accordingly, the use of respiratory inhibitors is recommended for accurate BOD measurements of environmental samples containing nitrate and /or oxygen with an MFC-type BOD sensor. Nonetheless, aerobic respiration that is resistant to azide and cyanide is not uncommon in prokaryotes, therefore, studies are needed to see if inhibitor-resistant bacteria are enriched in MFCs during a long-term operation with the inhibitors. Alternating use of these bacteria may reduce their chances of enrichment.

4. CONSTRUCTION AND OPERATION

The aim of this section is to provide a protocol to construct an MFC-type BOD sensor and format for its start and operation. The representative MFC described here is a C-type MFC (Fig. 5).

4.1. Materials

4.1.1. MFC Construction

The materials necessary to construct a typical MFC are listed below:

- Two polyacrylic sheets ($50 \times 100 \times 30 \text{ mm}^3$) carved to form a chamber ($10 \times 50 \times 10 \text{ mm}^3$).
- Nafion membrane (Nafion® 450, Dupont Co., Wilmington, DE, USA) cut to fit between the polyacrylic sheets.
- Four graphite felt sheets, $10 \times 50 \times 5 \text{ mm}^3$ (GF series, Electrosynthesis, Lancaster, NY, USA)

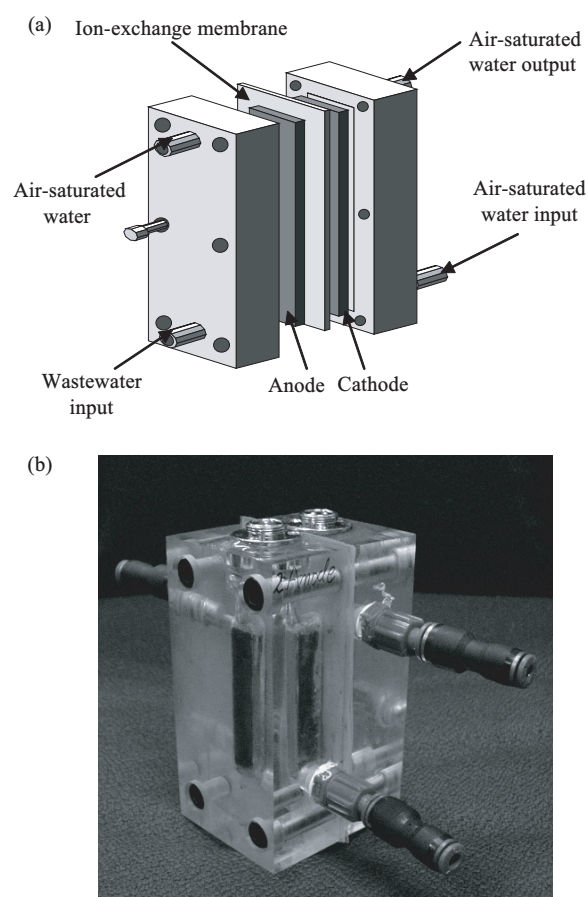


Figure 5. (a) Schematic diagram of microbial fuel cell. (b) Microbial fuel cell-type BOD sensor (C-type MFC).

- Platinum wire with a diameter of 0.5 mm for the electronic connection between the electrodes and the electronic port
- Electrical connector to connect the platinum wire to the electrical cable
- Silicone rubber gaskets with a thickness of 2 mm, cut to fit between the polyacrylic sheets.
- Nafion ionomer (5%, Dupont Co.) for platinum coating
- Platinum on carbon powder (40% platinum on Vulcan XC-72, E-TEK, Somerset, NJ, USA) for platinum coating
- Two 10 mL plastic syringes for inoculation
- 0.1 N HCl for electrode cleaning
- Isopropanol for platinum coating
- Spray gun for platinum coating

4.1.2. MFC-Type BOD Monitoring System

The materials and equipment required to make an MFC-type BOD monitoring system are as follows:

- Peristaltic pump for sample delivery
- Marprene II tubing
- Multimeter (Model 2700, Keithley, Cleveland, OH, USA) linked to a differential multiplexer (Model 7701, Keithley).
- Personal computer equipped with an IEEE-488 interface card (Model PCI-488, Keithley)

Table 4. Composition of artificial waste water.

Component	Composition
(NH ₄) ₂ SO ₄	0.56 g
MgSO ₄ 7H ₂ O	0.20 g
CaCl ₂	15 mg
FeCl ₃ 6H ₂ O	1 mg
MnSO ₄ H ₂ O	20 mg
NaHCO ₃	0.42 g
Trace mineral solution*	10 mL
Phosphate buffer (1 M, pH 7.0)	50 mL
Distilled water	940 mL
*Trace mineral solution [90]	Composition (g/L)
Nitrioltriacetic Acid (NTA)	1.5
FeSO ₄ 7H ₂ O	1.1
MnCl ₂ 4H ₂ O	0.1
CoCl ₂ 6H ₂ O	0.17
ZnCl ₂	0.1
CaCl ₂ 2H ₂ O	0.1
CuCl ₂ 2H ₂ O	0.02
H ₃ BO ₃	0.01
Na ₂ MoO ₃	0.01
Na ₂ SeO ₃	0.17
NiSO ₄ 6H ₂ O	0.26
NaCl	1.0

- Resistance, 10 Ω
- Electric wire
- Incubator
- Artificial wastewater as the BOD check solution with the composition presented in Table 4
- 1 M phosphate buffer (pH 7.0)
- Azide and cyanide for sample pretreatment

4.2. Electrode Preparation

The graphite felt sheets used for the electrodes are cleaned using 0.1 N HCl and rinsed thoroughly in deionized water. The graphite felt is then coated with platinum/carbon powder/Nafion to use as the cathode. The platinum/carbon powder/Nafion mixture is prepared by mixing 5/24 weight parts of the Nafion ionomer (5%, Dupont Co.) dissolved in iso-propanol (1 g Nafion in 5 g solvent) with a suspension of 1 weight part of platinum powder (2 nm diameters) and 1.5 weight parts of Vulcan XC72 carbon (E-TEK). The mixture is suspended in *iso*-propanol at a ratio of 300 mL solvent to 1 g mixture and sonicated for 30 min. The fully mixed slurry is then sprayed onto the cleaned graphite felt at a ratio of 0.28 mg platinum per cm² surface using a spray gun.

4.3. MFC Assembly and Installation

An electrical connector is fixed to each polyacrylic cell using an epoxy, then platinum wires are soldered to the electrical connectors. Two graphite felt sheets are put in the chamber of the polyacrylic sheet, and the platinum wires in each chamber are sandwiched by the two graphite felt sheets, creating a physical contact between the platinum wires and the graphite felt sheets. Platinum-coated graphite is used for the cathode. The fuel cell is then assembled. A silicon rubber gasket and Nafion membrane are placed on the anode

polyacrylic sheet containing the graphite felt sheets. The second silicon rubber gasket and cathode polyacrylic sheet are then placed on the membrane, and the cell is securely fastened using several screw bolts and nuts. The assembled MFC is then installed in an incubator (35 °C). The anode and cathode are connected through a voltmeter with a resistance of 10 Ω to monitor the current under close circuit conditions. The current is calculated as $I = V/R_{load}$, where V is the potential drop across R_{load} .

4.4. Enrichment

The anode compartment of the MFC is loaded with freshly collected sludge from a sewage works using a syringe and kept for 24 h. The wastewater to be monitored or artificial wastewater is fed into the anode continuously using a peristaltic pump for enrichment, while the cathode compartment is fed with air-saturated tap water. The feeding rate of the air-saturated water should be determined to maintain the dissolved oxygen concentration in the cathode compartment at a level higher than 2 mg/L, the critical oxygen concentration for the platinum-coated electrode to avoid oxygen limitation. The wastewater is purged with oxygen-free nitrogen in a pretreatment tank before feeding. Within 3 weeks of the enrichment process, a stable current is generated, and the MFC considered as enriched with electrochemically active bacteria. The enriched MFC can then be used as a BOD sensor. The response time can be manipulated by varying the sample feeding rate, which should be optimized to minimize the response time.

4.5. BOD Measurement

4.5.1. Continuous Operation

A sample is diluted using a concentrated phosphate buffer to 50 mM (pH 7.0), and purged with oxygen-free nitrogen at a rate of 10 mL/min for 10 min before being fed to the MFC. Azide or cyanide is also added to a sample containing nitrate to a final concentration of 1 mM. The temperature should be kept constant at 35°C to avoid temperature shock. A calibration curve is prepared using the sample to be analyzed. The BOD value of the sample is determined using the conventional method, and the current recorded from the MFC fed with the sample diluted to within the detection range, which is 20–200 mg/L for a C-type MFC. The MFC is continuously fed with the diluted sample and the current recorded, which is then converted to the BOD value from the calibration curve. A typical calibration curve is shown in Fig. 6.

4.5.2. Batch Operation

A sample is treated as above before being fed to the MFC. A predetermined volume of the sample is injected into the anode compartment for each measurement using a syringe or peristaltic pump. The current generated after adding the sample is recorded in a personal computer until the current drops to a base line. The peak current or coulomb is then used to obtain the BOD values from a predetermined calibration curve in a similar way to above. For values higher than the upper detection limit of the peak current method,

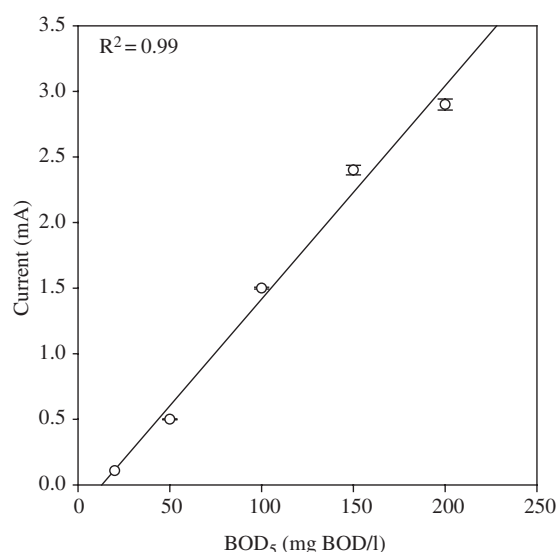


Figure 6. Typical calibration curve for BOD determination using C-type MFC as BOD sensor.

the BOD value can be obtained from the coulomb, which is calculated by integrating the current over the time in the batch operation.

5. CONCLUSION

An MFC is a microbial device that converts chemical energy into electricity through microbial activity. A fuel cell-type electrochemical device can be used to enrich electrochemically active microbes with different nutritional characteristics. Thus, since the current generated from an MFC is directly proportional to the strength of the wastewater used, MFCs enriched in this way can be used as a BOD sensor. This novel BOD sensor is also free from the problems encountered with other BOD sensors. Accordingly, an MFC-type BOD sensor offers considerable advantages, including minimal maintenance requirements and a long operational stability, which is an important factor for biosensors. For example, if the fuel (wastewater) supply is not limited, the operational stability can be maintained up to 5 years, which is much longer than previously reported BOD biosensors. Various factors have been identified that influence the performance of MFCs as BOD sensors, and modifications to the fuel cell structure have been made to overcome these problems. The decrease in current generation due to oxygen and nitrate can be avoided with the use of respiratory terminal oxidase inhibitors, such as cyanide and azide, without compromising the MFC performance. Molecular biological methods have shown that the bacterial population in an MFC differs from that in an inoculum and another MFC enriched with a different fuel.

GLOSSARY

16S rDNA A ribosome is an organelle in all living cells responsible for protein synthesis. A bacterial ribosome consists of 5S rRNA, 16S rRNA, and 23S rRNA with a large

number of proteins. The genes for rRNAs are very well conserved with some variable sequences. The sequence of the 16S rRNA gene is an important index in bacterial phylogeny.

Copiotroph Microorganism that grows in an environment with greater nutritional opportunities.

Coulomb yield The theoretical coulomb is calculated based on the COD consumed in an MFC. One mg COD is equivalent to 12 C. The coulomb yield is known as the fraction of the actual coulomb obtained over the theoretical coulomb, and this value can be used to evaluate the performance of an MFC-type BOD sensor. The difference between the theoretical and actual coulomb indicates the amount of electrons consumed to reduce an alternative electron acceptor, such as oxygen and nitrate. Reliable BOD values can be obtained from MFCs with a high coulomb yield.

Denaturing gradient gel electrophoresis (DGGE) A molecular biological technique used to differentiate the nucleotide sequences of short DNA segments.

Electrochemically active bacteria (EAB) Bacteria whose cells can exchange electrons with an electrode. This activity can be determined using cyclic voltammetry or a fuel cell-type electrochemical device, as described in the text.

Enrichment culture A microbiological technique used to select microbes with certain characteristics from a natural mixed population.

Oligotroph Microorganism that grows optimally in an environment with low levels of nutrients.

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