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A new microbial biosensor for organic water pollution based on measurement of carbon dioxide production

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ABSTRACT

As aerobic respiration proceeds it consumes oxygen and produces carbon dioxide; and the relation between these two parameters, the respiratory quotient (RQ), is related to the type of substances being respired. Therefore, for a given water or wastewater origin, a more or less stable RQ is expected, making possible the estimation of BOD_5 by means of CO_2 production measurement. The microbial breathing intensity was continuously measured using a potentiometric CO_2 electrode as transducer. We named this new biosensor $BOD_{st}CO_2$. Proof of concept in this study was carried out with *Saccharomyces cerevisiae* or a BODSEED mixed microbial community as a biological recognition component. The effect of microbial load (0.5, 5, and 25 mg cm^{-2} membrane, dry weight) over the apparent linear range (up to 670 mg L^{-1} BOD_5, 5 mg cm^{-2} membrane), detection limit (ca. 1 mg L^{-1} BOD_5), stabilization time, reproducibility (typically better than 10%) and bio-membrane type (membrane or PVA hydrogel entrapment) were studied. When the Nernstian biosensor response was used for calibration, up to 20,000 mg L^{-1} glucose standard was measured without sample dilution. BOD calibrations were accomplished using the two more commonly used standard artificial wastewaters, GGA and OECD solutions. The results showed that the potentiometric CO_2 electrode was an useful transducer, allowing us to build, calibrate and characterize a BOD-like biosensor. Moreover, limitations present at oxygen amperometric electrode (customarily used as BOD biosensor-based transducer) such as oxygen low solubility and its reduction at the cathode were avoided.

1. Introduction

Environmental biosensors are a growing area of biosensor applications, despite their still small representation in the worldwide market, where clinical glucose biosensors constitute close to 90% of the total sales. The predicted increase of environmental biosensor sales is related to a still rapid increase in the worldwide population, and use of energy and natural resources. The facts mentioned, as well cultural and social changes around the word are demanding huge amounts of fresh water and producing enormous amounts of waste and wastewater. Consequently, natural ecosystems (freshwater ones are especially sensitive) are being exploited, suffering strong damage, sometimes close to their natural homeostatic and purification capacity, or are irreversibly damaged. The discharge of raw municipal and industrial wastewater into rivers, lakes, and marine environments is yet a common practice in underdeveloped countries.

There are several standard methods used to evaluate the degree of organic pollution in water, the biochemical oxygen demand (BOD) being one of the more useful. This assay is usually performed in BOD bottles, where the water samples, previously fully oxygenated, are incubated during 5 days (BOD_5) under specified conditions [1]. When the incubation period is finished, the remaining oxygen is measured, the difference between this amount and that initially present in the sample being the oxygen used by the microbial community to degrade (oxidize) organic substances (and in other metabolic processes, usually less oxygen-consuming, like nitrogen oxidation).

Another assay used to evaluate organic contamination that correlates to some extent to BOD_5 is the chemical oxygen demand (COD), in which the organic matter in the sample is completely oxidized with a strong chemical oxidant. If a high proportion of the organic matter present in the water is refractory (like humic acids, lignin or cellulose) the COD values are expected to be higher than the respective BOD_5 values; both values can be correlated when water or wastewater of similar composition are measured.

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The standardized BOD_5 measurement embraces several drawbacks, perhaps the more evident one being the long time delay between sample and data availability. This delay makes the data of no or poor value for optimizing, for example, wastewater purification facilities. The second problem is the size and set-up of the assay, standardized in 300 mL bottles. In order to minimize costs, analytical assays are evolving to mL or lower volume set-ups. If possible, FIA systems are preferred because of their superior reproducibility and high throughput. Another evident problem is related to the low oxygen solubility; when the amount of degradable organic matter in the sample is moderate or high, dilution with thoroughly oxygenated dilution water must be accomplished. The dilution could change the sample physical–chemical characteristics, leading to inaccurate results.

To obtain faster and more convenient BOD-like information, microbial BOD biosensors have been developed. They allow the determination of the so-called "short term BOD" (BOD_{st}), which can to some extent be correlated with conventional BOD_5. The first BOD_{st} biosensor was a thin layer of micro-organisms attached by a filtration membrane over an oxygen amperometric electrode [2–4]. Since this very first device, very different biosensor configurations and set-ups have been described, but almost all them make use of an amperometric (Clark-type) oxygen electrode as transducer. Low oxygen solubility in water (ca. 9 mg L^{-1} at 20 °C) and oxygen consumption (reduction at the cathode) are important concerns when using this type of electrode as a transducer.

Oxygen-sensitive optrodes based on oxygen quenching of a fluorescent dye, located on top of an optical fiber [5,6] have been described and used as biosensor transducers. Another interesting approach makes use of a recombinant bacteria, a luminescent *Escherichia coli* strain, used as a biorecognition element [7], in which the amount of light emitted by the attached bacteria is related to the total amount of available carbon sources in the sample, allowing BOD_{st} calculation. These light-based methods are still limited by low oxygen solubility, which defeats oxygen consumption problems related to the amperometric dissolved oxygen electrode.

To overcome low oxygen solubility limitations, the use of soluble mediators, which may replace and/or compete with oxygen as electron acceptors, has been proposed [8–10]. Ferricyanide (one of the most used soluble mediators) is able to function as an electron acceptor and be reduced to ferrocyanide by cellular metabolism, which in turn could be re-oxidized at the anode by means of amperometry or coulometry techniques. Although, this electrochemical set-up is prone to electroactive interference and electrode fouling; soluble mediators are generally expensive and must be disposed of as hazardous waste.

Some authors have also proposed transducers not related to oxygen sensors, or with mediators replacing oxygen as electron acceptors [11]. They have proposed the measurement of surface photovoltage (related to surface pH), considering that the metabolism of microorganisms produces acidic substances such as hydrogen carbonate and organic acids, which could be related to BOD_5 concentrations.

More recently, a biosensor-like system based on the determination of CO_2 has been described [12,13]. Outgas from a fluidized bed reactor was analyzed by an infrared CO_2 detector, and CO_2 concentration related to BOD_5 values. As the equipment described in the aforementioned paper is based on the measurement of CO_2 production, the utilization of an infrared detector, the need of obtaining outgas, and the interference from water vapor made this system considerably complex.

Whereas the CO_2 potentiometric electrode was previously used as a transducer for glucose, pyruvate, lysine, glutamic acid and uric acid biosensors [14], to the best of our knowledge it has never before been proposed as a transducer for BOD or BOD-related water quality biosensors.

Selection of the microbial strain, group of strains or microbial community is also an important topic in the design of a new BOD_{st} biosensor. Some strains degrade a large array of organic compounds easily and rapidly, whereas others are more selective and/or show a slow metabolic rate. Some authors have used a single strain or a combination of two or more strains as biological recognition biosensor component. It has been shown that an increase in microbial membrane complexity (as number of species) usually diminishes the reproducibility between different microbial membranes, but increases the correlation between BOD_5 and BOD_{st} [15].

We first used a simple single-strain biosensor model (based on *Saccharomyces cerevisiae* entrapped membrane) to investigate the response and analytical possibilities of the CO_2 electrode as metabolic and BOD_{st} transducer. Later, a microbial blend of commercial origin, BODSEED capsules, used routinely to inoculate BOD_5 assays, was used as a biorecognition membrane. Similar commercial microbial blends have been used previously in amperometric BOD_{st} biosensors [16]. The biosensor was calibrated using GGA and OECD solutions, and used to calculate BOD_{st} values in simulated samples, and compared with BOD_5 values obtained by an independent laboratory.

We present here a BOD_{st} (st, short term) biosensor based on an alternative transduction principle, designated $BOD_{st}CO_2$, in which microbial cells are attached to the gas permeable membrane of a carbon dioxide ion-selective potentiometric electrode. In this way, instead of measuring one of the substrates of the respiration process, we measure one of its products. This approach allows us to overcome the problems associated with low oxygen solubility, as well to obtain some analytical advantages, which will be discussed later in this paper.

2. Materials and methods

2.1. Carbon dioxide electrode

The potentiometric electrode constructed in our laboratory is a modified Severinghaus electrode [17] based on a combined pH electrode (Orion), a silicon gas permeable membrane, and an electrolyte chamber filled with a buffer solution prepared by dissolving 1.17 g of sodium chloride and 0.42 g of sodium hydrogen carbonate in 1 L of distilled water. Schematics of this carbon dioxide sensor, as well as a theoretical discussion and calibration procedures can be found elsewhere [18,19]. An Orion pH-meter (inner impedance > 10^{12} Ω) was used to measure E (mV) or the instrumentation and measuring system described in Supplementary Material.

2.2. Micro-organisms, immobilization and biosensor assembly

Preliminary data on biosensor performance and immobilization procedure were carried out using commercial lyophilized baker's yeast *S. cerevisiae*, from a local provider (active dry yeast, SAF Argentine, Lesaffre group, Buenos Aires, Argentina), and used directly.

Further data were obtained by immobilization of a mixed microbial community (BODSEED capsules), obtained from Bio-Systems International, IL, USA. The BODSEED capsules contain ca. 0.5 g (dry weight) of a heterogeneous mix of aerobic bacteria and wheat bran.

Membrane entrapment immobilization (using an acetate filtration membrane, 47 mm diameter, 0.45 μm pore) and polyvinyl alcohol gel immobilization (PVA, MW 72,000, from Fluka) details are included in Supplementary Material.

2.3. Calibration solutions

BOD calibration was made using two commonly used standards, (1) a glucose–glutamic acid solution (GGA) composed of 150 mg of D-glucose (Merck) and 150 mg of glutamic acid (DIFCO) per liter,

with a measured BOD_5 of 198 ± 30 mg L^{-1} [1], or (2) a more complex artificial wastewater according to a recipe from the Organization for Economic Cooperation and Development (OECD), in g L^{-1}: peptone 15.0 (Difco), beef extract 11.0 (Britania), urea 3.0 (Merck), NaCl 0.7 (Merck), $CaCl_2 \cdot 2H_20$ 0.4 (Merck), K_2HPO_4 2.8 (Mallinckrodt) and $MgSO_4 \cdot 7H_2O$ 0.2 (Merck), with a measured BOD_5 value of 17 ± 1 g L^{-1} [20]. When necessary, more concentrated (X10 and X100) GGA solutions were prepared. Solutions were sterilized by filtration.

2.4. Experimental procedure

Unless otherwise stated, all the experiments were performed at 25 ± 1 °C, in 100 mM phosphate buffer, pH 7.0. A beaker with 50 mL of buffer was stirred (magnetically) and air was bubbled by a diffuser to maintain an almost constant (atmospherically related) bulk concentration of O_2 and CO_2. The biosensor was immersed in the beaker until a constant signal (mV) was obtained; then consecutive aliquots of calibration solution were added, increasing the BOD concentration, to obtain a calibration curve. Later, the electrode was immersed in fresh buffer until a constant (lower CO_2 concentration related) signal was obtained. Previous to the biosensor assembly, the CO_2 electrode was calibrated, and the resulting calibration curve used to relate mV to the CO_2 concentration, where appropriate. Detailed protocol to measure pH and NaCl effects are detailed in Supplementary Material.

3. Results and discussion

3.1. S. cerevisiae (yeast) experiments

The assembled biosensor was dipped into buffer until a stable readout (signal change < 0.5 mV h^{-1}) was obtained. Depending on the membrane microbial load, typically 2–6 h were necessary; faster stabilization was related to lower microbial load. Three yeast cell concentrations were used, 0.5, 5, and 25 mg (dry weight) per cm^2 of microbial membrane.

3.1.1. Effect of microbial membrane load on response and sensitivity

Following the stabilization time (3.1), the biosensor was removed from the stabilization solution (buffer) and immersed in fresh buffer. In this starved situation, the production of CO_2 is minimal, corresponding to the basal microbial respiration. Later, successive additions of a glucose standard allowed us to elaborate response curves, and estimate the possible lineal dynamic calibration range for these sensors and their sensitivity. Figs. 1 and 2 show the data obtained when the potentiometric electrode response (mV) is used directly.

Fig. 1 shows data obtained with 7 different biosensors to study inter-biosensor reproducibility. From the figure it is evident that the response shows saturation kinetics, good reproducibility, and a very wide concentration area in which the biosensor response increases with glucose concentration; this response range goes from 20 to 20,000 mg L^{-1}. Such extended dynamic range is related to the Nernstian response of the potentiometric CO_2 electrode. An apparent linear response was observed at lower concentrations, ca. 10–700, 20–1050, and 30–670 mg L^{-1} of glucose for 0.5, 5, and 25 mg cm^{-2} membrane biosensor. Also, better reproducibility (RSD = 6.7%, $n = 2$) was obtained with 5 mg membrane in this low-concentration range. At high concentration (10,000 mg L^{-1}), a RSD = 2.3% was obtained (5 mg membrane, $n = 2$).

In Fig. 2 the same data as in Fig. 1 were re-plotted. The increase in the basal (plain buffer related) mV readout when higher microbial load is used at the biosensor is evident; average values of

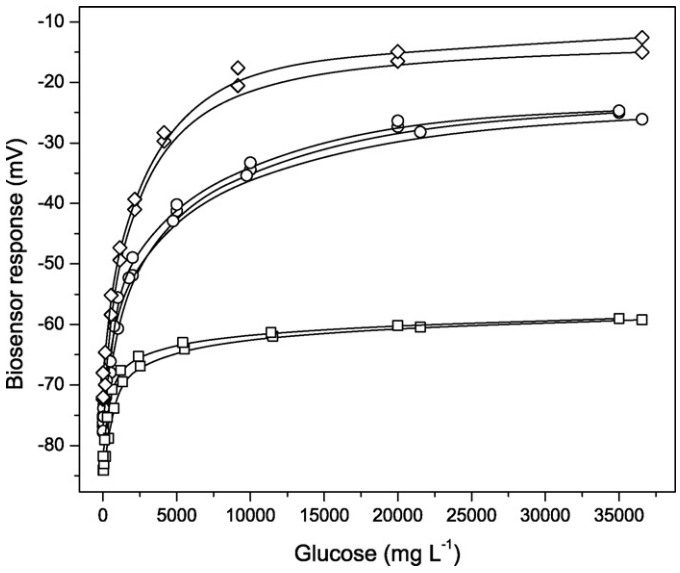

Fig. 1. Extended calibration curves, *S. cerevisiae* biosensor (membrane entrapment), spiked with glucose aliquots. Microbial membrane loads were 0.5 (□), 5 (○), and 25 (◊) mg cm^{-2}.

−83.0, −76.5 and −70.1 were obtained for biosensors at 0.5, 5, and 25 mg cm^{-2} membrane. The basal mV values are related to an increase in basal metabolic CO_2 production when a higher cellular biomass was used. In these log representations better lineal range (ca. 1000–27,000 mg L^{-1}) and good averaged sensitivity were obtained (ca. 1.2 μV mg^{-1} L) when 5 mg cm^{-2} membrane was used. Although, given the Nernstian response of potentiometric CO_2 electrode, sensitivity deteriorates as concentration increases, a factor to be considered in future biosensor design.

When the mV data were transformed to CO_2 molar concentration [19] by using a calibration curve and plotted (Fig. 3), a more realistic picture of the respiration change at the different biosensor configurations is given. At 30,000 mg L^{-1} of glucose, the CO_2 concentration measured (average) was ca. 0.13, 4.4, and 14.6 mM L^{-1}, indicating a direct relationship between microbial membrane load and CO_2 production. The ratio between membrane microbial load

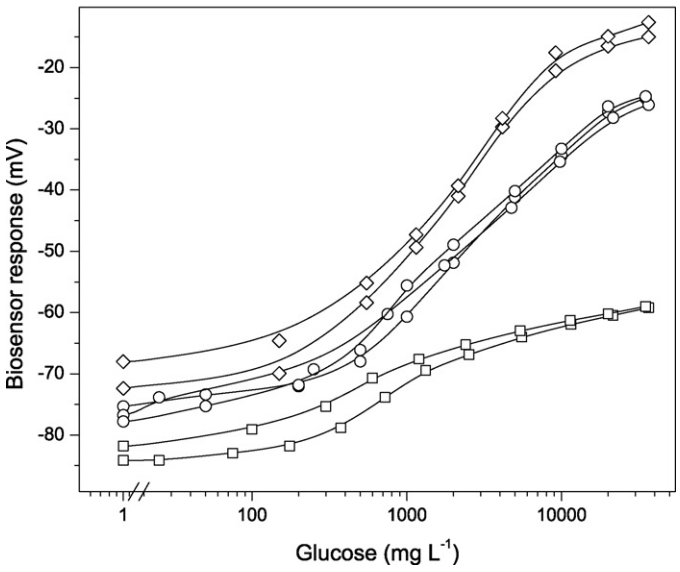

Fig. 2. Extended calibration curves, *S. cerevisiae* biosensor (membrane entrapment), spiked with glucose aliquots. Microbial membrane loads were 0.5 (□), 5 (○), and 25 (◊) mg cm^{-2}.

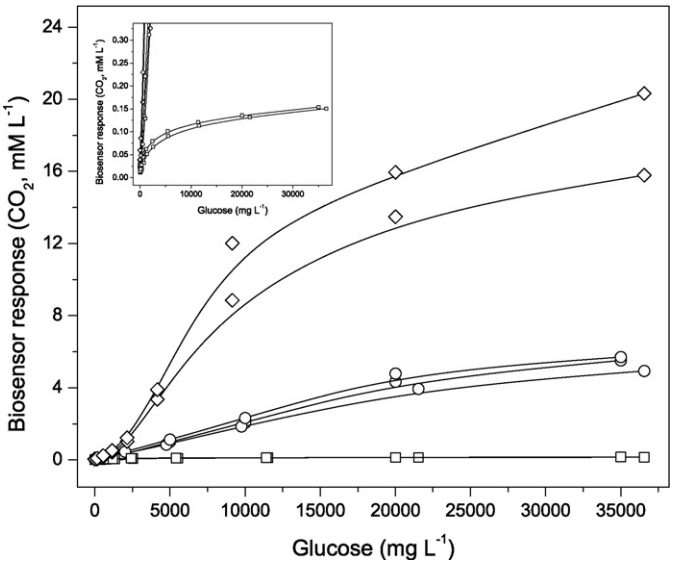

Fig. 3. Extended calibration curves, *S. cerevisiae* biosensor (membrane entrapment), spiked with glucose aliquots. Microbial membrane loads were 0.5 (□), 5 (○), and 25 (◊) mg cm^{-2}. mV electrode response values were converted to [CO_2] mM, by means of a calibration curve. Inset shows detail of Fig. 3 at low CO_2 concentration.

and CO_2 (mM) measured was calculated, resulting in values of 3.85, 1.36, and 1.7 mg L mM^{-1} for 0.5, 5, and 25 mg cm^{-2} membrane biosensors.

3.1.2. pH effect

In Fig. 4 we present the data obtained when mV values were adequately (calibration curve) transformed to CO_2 concentration, to calculate calibration curve slopes. Given that basal carbon dioxide measured by the biosensor at each pH was different (and so the basal mV values), the mV values were not chosen for this comparison. The change in pH solution value is expected to influence the biosensor response at two levels. First, the metabolism of membrane-entrapped yeast will change, as *S. cerevisae* grows better in slightly acidic media (pH 5), which agrees with the data obtained, where calibration slopes are steeper at acidic pHs, and gentler at alkaline values (Fig. 4). Second, the pH value affects the carbon dioxide equilibrium, given that carbonate species and carbon dioxide concentration depends on solution pH. In acidic solutions, equilibrium is displaced to carbonic acid and dissolved CO_2 formation, being at pH = 5 more than 90% of the total carbonate species [19]. Therefore, the CO_2 produced by the microbial layer remains mainly as dissolved CO_2 at acidic pHs, a species able to cross the silicone CO_2-electrode membrane, generating a higher signal. Combinations of these two effects are observed in our data (Fig. 4). A recent work using a salt tolerant strain of *S. cerevisiae* and optical detection (a redox dye indicator, 2,6-dichlorophenolindophenol) found an optimal pH of about 7 [21]. Perhaps in this experimental set-up the pH value obtained could be related also to the pH influence over reduction kinetics values for the dye used in this work.

Acidic pHs also produce better calibration curves, with a slope standard error on the order of 4–5%, reaching a maximum at pH 9–10, where they show unacceptable values of around 18%.

3.1.3. Effect of NaCl addition

At almost all the NaCl concentration assayed the effect was modest, with the exception of the more elevated concentration used. A steeper slope and minor slope standard error (typically 7% respect to the slope value) was found between 100 and 500 mM NaCl (Fig. 5). At higher concentrations important increases of slope standard errors were observed (up to 15%). These results show, as expected, a deleterious effect over *S. cerevisiae* yeast metabolism (evident at the higher NaCl concentration used), given that the industrial strain used is not expected to be halotolerant. In a recent work [21], using an halotolerant *S. cerevisiae* strain, increasing responses were obtained up to 1.4 M NaCl

3.2. BODSEED experiments

The metabolic activity (carbon dioxide production) of BODSEED cells, when compared with *S. cerevisiae* ones, was significantly lower. Because of that, 10 mg (dry weight) per cm^2 of membrane was selected for the following experiments.

3.2.1. Stabilization and typical response of a newly assembled microbial membrane

When the microbial membrane is joined to the CO_2 electrode gas permeable membrane, the level of CO_2 increased from its atmospheric level (ca. −80 mV, Fig. 6), reaching a maximum (ca. −74 mV). Following that, the respiration rate slowed, mainly because of the progressive exhausting of intracellular reserves.

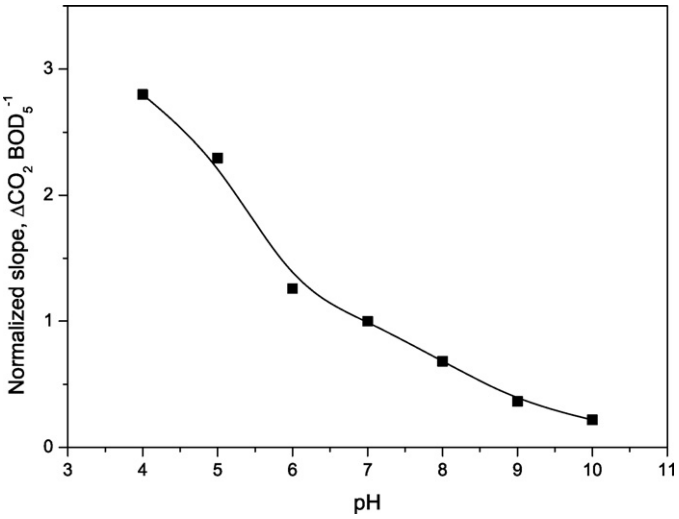

Fig. 4. Effects of pH on *S. cerevisiae* biosensor sensitivity, (membrane entrapment, 5 mg cm^{-1}). Calibration GGA curves were obtained, each point is the normalized calibration slope, with respect to the value obtained at pH 7.

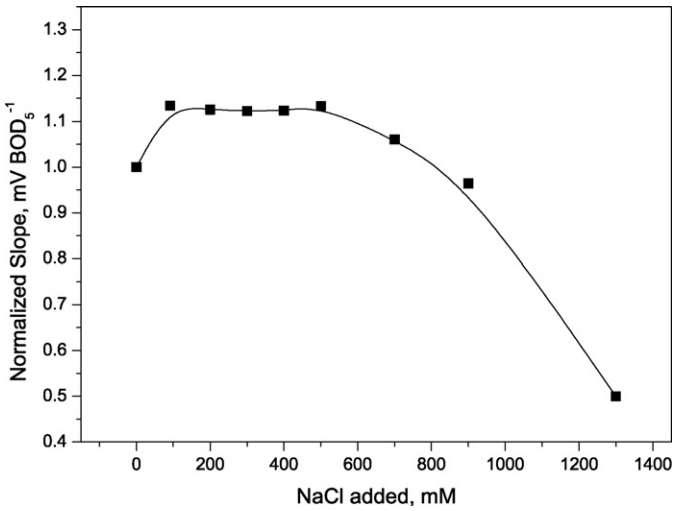

Fig. 5. Effects of NaCl addition on *S. cerevisiae* biosensor sensitivity, (membrane entrapment, 5 mg cm^{-1}). Calibration GGA curves were obtained, each point is the normalized calibration slope, with respect to the value obtained at 0 NaCl addition.

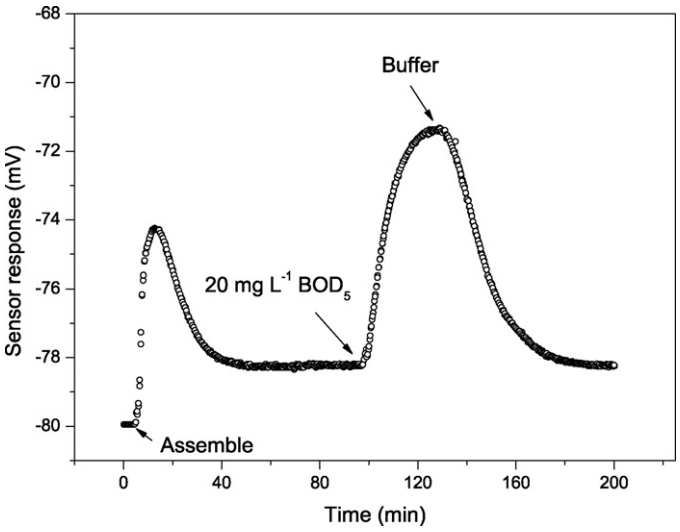

Fig. 6. Stabilization and typical response of a BODSEED based biosensor, PVA entrapment, 10 mg cm^{-2} microbial membrane.

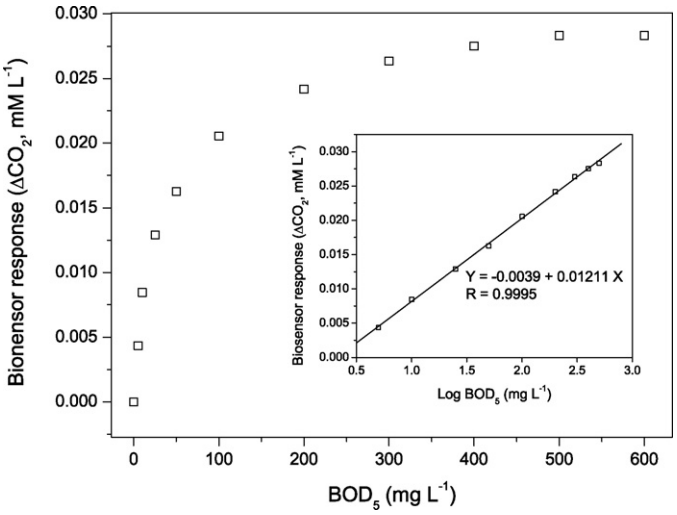

Fig. 7. Response of BODSEED based biosensor, PVA entrapment, 10 mg cm^{-2} of microbial membrane. Inset shows the logarithmic calibration curve.

It is interesting to note the contrast with *S. cerevisiae* microbial membranes, in which the stabilization time was quite long (data not shown), at least 4 h in buffer was required to reach a stable baseline. *S. cerevisiae* is well known to have abundant intracellular reserves, the gradual consumption of reserves, denoted by the gradual decrease in CO_2 production, providing an irregular baseline up to 6 h from membrane entrapment.

Also, a typical response of the biosensor is shown in Fig. 6, when GGA was spiked into the batch, reaching a final concentration of 20 mg L^{-1} BOD_5. The increase in microbial respiratory activity causes a new CO_2 equilibrium, which was achieved in ca. 30 min. The response and recuperation time at this PVA membrane entrapment based sensor was slower than that obtained from our cellulose acetate membrane entrapment biosensor (10–15 min), which was similar to that achieved with BOD_{st} biosensors based on oxygen amperometric electrodes [15]. Slower diffusion of substrates and products of respiratory metabolism through the PVA gel could be the reason for this sluggish response time.

3.2.2. Limit of detection, linear range, and sensitivity of BODSEED biosensors

Fig. 7 shows the biosensor response from 5 to 600 mg L^{-1} BOD_5, where saturation kinetics and logarithmic response (related to Nernstian CO_2 electrode response) are evident. The inset shows the linear range obtained when the data are plotted using the log of BOD. Using this transformation, a linear relationship is obtained from 5 to 500 mg L^{-1} BOD_5. For three different sensors, the RSD of the sensors responses was approximately 12.5% at 50 mg L^{-1} BOD_5. The ΔCO_2 (M) measured in a similar experiment for 1 mg L^{-1} BOD_5 GGA standard was 9.2×10^{-7}, with a RSD = 14.4% (n = 6), which can be considered a realistic detection limit for the proposed method. In a previous work using BODSEED as biological recognition element [16], under a similar experimental conditions a calibration range of 5–50 mg L^{-1} BOD_5 was obtained.

A comparison between calibration with GGA and OECD calibration solutions was made, great sensitivity is obtained with GGA solution, and data obtained is presented in Supplementary Material.

3.2.3. Measurement of real samples and comparison with standard BOD_5 method

Four waste-water samples (Waste-1 to Waste-4) were taken at different parts of the Buenos Aires sewer system. Samples were measured using the standard BOD_5 method at an independent laboratory. A subsample was also measured using the $BOD_{st}CO_2$ biosensor. To do that, 25 mL of homogenized (stirred) sample was simply diluted with 25 mL of phosphate buffer, 100 mM, pH 7, bubbled with air, and maintained at 30 °C for 15 min; after that, the biosensor was introduced into the diluted sample. The mV response was registered and the data obtained after 15 min incubation were used as analytical signals. Calibrations with GGA standards were made every 4 measurements, samples measured in duplicate. The same procedure was used with two simulated samples composed of beef extract (150 mg L^{-1}) and peptone (11 mg L^{-1}) prepared with distilled water (Sim-1) and a 1:2 dilution (Sim-2). $BOD_{st}CO_2$ RSD values were lower than 15%.

Table 1
Comparison between the $BOD_{st}CO_2$ values and those determined by the BOD_5 standard method. Calibration was made using GGA standards.

Sample	BOD (mg L^{-1}) BOD_5	$BOD_{st}CO_2$	Percentage deviation $BOD_{st}CO_2$ vs. BOD_5
Waste-1	145	258	+78
Waste-2	120	188	+56
Waste-3	130	210	+61
Waste-4	120	83	−31
Sim-1	175	220	+26
Sim-2	85	102	+20

The results are presented at Table 1, where the agreement between both methods is evident. Low reproducibility and higher values (with respect to BOD_5 Standard Method) obtained when waste-water was measured need to be investigated further. We observed a diminution of the measured values (lower BOD concentration) when a prepared (diluted and air-bubbled) sample was measured consecutively. These results are compatible with the known effect that volatile acids like sulfide species have as an interference with the CO_2 potentiometric electrode. Sulfide species are normally present in waste-water and highly polluted rivers, so probably during sample processing and bubbling an important part of these volatile acids could be purged from the sample.

4. Conclusions

A new BOD_{st} biosensor, based on measurement of CO_2 production is described and characterized. The utilization of CO_2 measurement instead of O_2 has several analytical advantages; the proposed system is not limited by the low oxygen solubility in

water, allowing a greatly extended dynamic range of measured BOD_{st}, without need of dilutions. Problems related to low solubility of O_2 are solved without using of a soluble mediator. The proposed biosensor is simple and does not use dangerous substances (as some soluble mediators do). Also, the potentiometric electrode does not consume its analyte, as the amperometric oxygen electrode does, which could be a source of error, especially at low O_2 concentrations. The calibration solution used is and important factor in order to reach good agreement between BOD_5 and $BOD_{st}CO_2$, and needed to be investigated further, given the high influence over the produced data [22]. The developed biosensor presented here have rapid response time (less than 15 min), when compared to other new BOD sensing principles, as microbial fuel cell biosensors [23], where sluggish response time was reported, from 4.5 to 14 h, or with recently reported BOD biosensor based in more commonly used amperometric oxygen electrode, with response time of 90 min [24].

The CO_2-based biosensor presented allows direct determination of the so-designated carbonaceous biochemical demand ($CBOD_5$), whereas O_2 consumption related to other biochemical and non-biochemical processes is not observed. Oxygen in water samples is also used to oxidize reduced forms of nitrogen (nitrogenous demand) and to oxidize inorganic material such as ferrous iron; both are considered sources of error in BOD_5 measurements. To prevent the measurement of nitrogenous demand, a nitrification inhibitor, like 2-chloro-6-(trichloromethyl) pyridine, is usually added to BOD samples [1]. In the approach proposed here this reagent might no longer be necessary.

Obviously, the potentiometric Severinghaus CO_2 electrode used as transducer in this work is not suitable for miniaturization and cannot be made disposable, limiting its possible practical use. But a miniaturized potentiometric CO_2 transducer with a μm-sized tip diameter has been described [25], and could be the basis for smaller and lower-cost biosensors.

Depending on the microbial material (*Saccharomyces* or BODSED) and microbial membrane load, logarithmic (Nernstian-like) and linear responses were observed at different substrate concentrations (glucose, GGA or OECD solution).

By choosing between logarithmic and linear parts of the response curve, the CO_2 electrode based $BOD_{st}CO_2$ biosensor electrode allows construction of an extended calibration curve, without need of sample dilution, up to 500 mg L^{-1} BOD_5 (Fig. 7, BODSEED), better than almost all previously described BOD_{st} systems [15]. The limit of detection using GGA as standard is about 1 mg L^{-1} BOD_5, comparable with other previously published devices. Moreover, non-linear curves from 1 to 27 g L^{-1} glucose were obtained (Nernstian-like response), that show the real and amazing possibilities of our $BOD_{st}CO_2$ for direct measurement of highly polluted samples.

In our biosensor (as in almost all the described previously BOD_{st} systems), the experimental numerical value of BOD_{st} is highly dependent upon the water/wastewater organic material composition. Specifically, the amount of CO_2 produced is related to the type of organic substances being metabolically oxidized. The respiratory quotient (RQ) has been defined as the ratio of CO_2 eliminated to O_2 consumed by a system ($RQ = \Delta CO_2\ (-\Delta O_2)^{-1}$); polysaccharides, proteins, and saturated lipids have a RQ close to 1, 0.8, and 0.7, respectively [26].

Depending of how close the RQ is to 1, greater similarity between BOD_5 and BOD_{st} is expected with the $BOD_{st}CO_2$ biosensor. Stoichiometric values calculated from water with a mix of organic substances (40% protein, 40% carbohydrate, 15% lipid, and 5% nucleic acid) were reported to produce a RQ of 0.89 [27]. Moreover, previous work has shown that CO_2 production is directly related to BOD_5, as expected if RQ is close to 1 [13] when a small fluidized bed reactor was used.

Given the effect pH has on the biosensor response, sample pH must be regulated, or eventually the GGA calibration must be made at sample pH. For best sensibility, pH 5 or 4 could be chosen with our system. Also, the useful pH range, considering an acceptable slope standard error up to 10%, was between 4 and 7. Our results are different from those cited above using another *S. cerevisiae* strain [28], where pH 8 was determined as the optimum. In that work, alginate capsules of 2.2 mm were used, probably the internal pH and the bulk measured pH being different. Also, the apparent incongruence between our data could be explained by metabolic differences between the two strains used.

Volatile sulfur-containing substances, detected by the odor characteristic of waste-water samples we measure can be an important interference; some sample treatment protocols need to be studied to measure accurately these kinds of samples.

The coincidence between the BOD values obtained using the standard BOD_5 method and any method based on a rapid biosensor is a crucial issue for validation and practical use. Large differences are sometimes obtained between the two methods, deviation percentages as high as 60% having been reported recently [24]. Optimizations of calibration procedures and measurement protocols are underway to improve our deviation percentages in real samples, on the order of 20% or better.

Recent work has shown high reproducibility, lower response time (3–5 min) and good agreement with BOD_5 standard method by using an amperometric oxygen-based biosensor as detector in a FIA system [29]. We believe the performance of our $BOD_{st}CO_2$ could be improved in such systems, and work is underway to achieve this goal.

When PVA was used as immobilization matrix, we found a sluggish response time (ca. 80 min/assay, including recovery time) probably related to the PVA-biomembrane thickness of 1 mm. Other authors show that using PVA based membranes results in better response times [30] by use of a very thin microbial membrane (ca. 5 μm).

Studies are underway to use this biosensor as detector in a FIA system, and to correlate BOD_5 and $BOD_{st}CO_2$ values when real and simulated samples are measured. We expect that in such a system the reproducibility, recovery time, and number of samples/h, as well other analytical parameters, would show noteworthy improvement.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.snb.2010.04.039.

References

[1] Standard Methods for the Examination of Water and Wastewater, 20th ed., American Public Health Association, 1998.
[2] I. Karube, T. Matsunaga, S. Mitsuda, S. Suzuki, Microbial electrode BOD sensors, Biotechnol. Bioeng. 19 (1977) 1535–1547.
[3] I. Karube, T. Matsunaga, H. Susuki, A new microbial electrode for BOD estimation, J. Solid Phase Biochem. 2 (1977) 97–107.
[4] I. Karube, S. Mitsuda, T. Matsunaga, S. Suzuki, A rapid method for estimation of BOD by using immobilized microbial cells, J. Ferment. Technol. 55 (1977) 243–248.
[5] G.-J. Chee, Y. Nomura, K. Ikebukuro, I. Karube, Optical fiber biosensor for the determination of low biochemical oxygen demand, Biosens. Bioelectron. 15 (2000) 371–376.

[6] N.-Y. Kwok, S. Dong, W. Lo, K.-Y. Wong, An optical biosensor for multi-sample determination of biochemical oxygen demand (BOD), Sens. Actuators B 110 (2005) 289–298.
[7] T. Sakaguchi, K. Kitagawa, T. Ando, Y. Murakami, Y. Morita, A. Yamamura, K. Yokoyama, E. Tamiya, A rapid BOD sensing system using luminescent recombinants of *Escherichia coli*, Biosens. Bioelectron. 19 (2003) 115–121.
[8] N. Yoshida, K. Yano, T. Morita, S.J. McNiven, H. Nakamura, I. Karube, A mediator-type biosensor as a new approach to biochemical oxygen demand estimation, Analyst 125 (2000) 2280–2284.
[9] K. Catterall, K. Morris, C. Gladman, H. Zhao, N. Pasco, R. John, The use of microorganisms with broad range substrate utilization for the ferricyanide-mediated rapid determination of biochemical oxygen demand, Talanta 55 (2001) 1187–1194.
[10] S.P. Trosok, B.T. Driscoll, J.H.T. Luong, Mediated microbial biosensor using a novel yeast strain for wastewater BOD measurement, Appl. Microbiol. Biotechnol. 56 (2001) 550–554.
[11] Y. Murakami, T. Kikuchi, A. Yamamura, T. Sakaguchi, K. Yokoyama, Y. Ito, M. Takiue, H. Uchida, T. Katsube, E. Tamiya, An organic pollution sensor based on surface photovoltage, Sens. Actuators B 53 (1998) 163–172.
[12] E. Vaiopoulou, P. Melidis, E. Kampragou, A. Aivasidis, On-line load monitoring of wastewaters with a respirographic microbial sensor, Biosens. Bioelectron. 21 (2005) 365–371.
[13] P. Melidis, E. Vaiopoulou, A. Aivasidis, Development and implementation of microbial sensors for efficient process control in wastewater treatment plants, Bioprocess Biosyst. Eng. 31 (2008) 277–282.
[14] K. Riedel, R. Renneberg, U. Wollenberger, G. Kaiser, F.W. Scheller, Microbial sensors: fundamentals and application for process control, J. Chem. Technol. Biotechnol. 44 (1989) 85–106.
[15] J. Liu, B. Mattiasson, Microbial BOD sensors for wastewater analysis, Water Res. 36 (2002) 3786–3802.
[16] T.C. Tan, C. Wu, BOD sensors using multi-species living or thermally killed cells of BODSEED microbial culture, Sens. Actuators B 54 (1999) 252–260.
[17] J.W. Severinghaus, A.F. Bradley, Electrodes for blood P_{O2} and P_{CO2} determination, J. Appl. Physiol. 13 (1958) 515–520.
[18] E. Cortón, S. Kocmur, L. Haim, L.A. Galagovsky, Potentiometric determination of CO_2 concentration in the gaseous phase: applications in different laboratories activities, J. Chem. Educ. 77 (2000) 1188–1191.
[19] S. Kocmur, E. Cortón, L. Haim, G.A. Locascio, L.A. Galagovsky, CO_2 – potentiometric determination and electrode construction. A hands-on approach, J. Chem. Educ. 76 (1999) 1253–1255.
[20] Organization for Economic Cooperation and Development (OECD), OECD Guidel., Testing Chem. 209 (1991) 1.
[21] H. Nakamura, Y. Mogi, H. Hattori, Y. Kita, D. Hattori, A. Yoshimura, I. Karube, Absorption-based highly sensitive and reproducible biochemical oxygen demand measurement method for seawater using salt-tolerant yeast *Saccharomyces cerevisiae*, Anal. Chim. Acta 620 (2008) 127–133.
[22] S. Velling, T. Tenno, Different calibration methods of a microbial BOD sensor for analysis of municipal wastewaters, Sens. Actuators B 141 (2009) 233–238.
[23] M. Di Lorenzo, T.P. Curtis, I.M. Head, K. Scott, A single-chamber microbial fuel cell as a biosensor for wastewaters, Water Res. 43 (2009) 3145–3154.
[24] P. Dhall, A. Kumar, A. Joshi, T.K. Saxsena, A. Manoharan, S.D. Makhijani, R. Kumar, Quick and reliable estimation of BOD load of beverage industrial wastewater by developing BOD biosensor, Sens. Actuators B 133 (2008) 478–483.
[25] S. Hanstein, D. de Beer, H.H. Felle, Miniaturised carbon dioxide sensor designed for measurements within plant leaves, Sens. Actuators B 81 (2001) 107–114.
[26] P.A. del Giorgio, P.J. le, B. Williams, Respiration in Aquatic Ecosystems, Oxford University Press, 2005.
[27] P.J.L. Williams, J.E. Robertson, Overall planktonic oxygen and carbon dioxide metabolisms: the problem of reconciling observations and calculations of photosynthetic quotients, J. Plankton Res. 13 (1991) S153–S169.
[28] K.S. Seo, K.H. Choo, H.N. Chang, J.K. Park, A flow injection analysis system with encapsulated high-density *Saccharomyces cerevisiae* cells for rapid determination of biochemical oxygen demand, Appl. Microbiol. Biotechnol. 83 (2009) 217–223.
[29] S. Kara, B. Keskinler, E. Erhan, A novel microbial BOD biosensor developed by the immobilization of *P. Syringae* in micro-cellular polymers, J. Chem. Technol. Biotechnol. 84 (2009) 511–518.
[30] L. Liu, L. Shang, S. Guo, D. Li, C. Liu, Li Qi, S. Dong, Organic–inorganic hybrid material for the cells immobilization: long-term viability mechanism and application in BOD sensors, Biosens. Bioelectron. 25 (2009) 523–526.

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