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Assessing the effect of oxygen and microbial inhibitors to optimize ferricyanide-mediated BOD assay

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ABSTRACT

Methods for short-term BOD analysis (BOD_{st}) based on ferricyanide mediator reduction have succeeded in overcoming some problems associated with the standard BOD test analysis (BOD_5) such as long-term incubations (5 days), the need to dilute samples and low reproducibility. Here we present a bioassay where a *Klebsiella pneumoniae* environmental strain successfully reduces ferricyanide without de-aeration of the samples with linear BOD_5 ranges between 30 and 500 mg L^{-1} or 30 and 200 mg L^{-1}, using glucose-glutamic acid solution (GGA) or OECD standards respectively. We further propose a new assay termination solution that allows higher reproducibility and standardization of the cell-based assay, employing formaldehyde (22.7 g L^{-1}) or other compounds in order to stop ferricyanide reduction without affecting the amperometric detection and therefore replace the centrifugation step normally used to stop microbial-driven reactions in ferricyanide-mediated bioassays. These improvements led to an accurate determination of real municipal wastewater samples.

1. Introduction

BOD is widely used as an indicator of the amount of easily biodegradable organic matter in water samples. The standard method for evaluating BOD involves measurements of the amount of dissolved oxygen consumed after 5 days of incubation under specific conditions proposed by the *Standard methods for the examination of water and wastewater* [1] in a microbially inoculated sample (with either a commercial blend or an indigenous population).

A standard glucose-glutamic acid solution (GGA) is frequently used to verify the methodological protocols and the viability of indigenous populations. The known BOD_5 value of a GGA solution containing 150 mg L^{-1} of glutamic acid and 150 mg L^{-1} of glucose is 198 ± 31 mg L^{-1} (expressed as mg L^{-1} of consumed O_2). Synthetic sewage found in the *OECD 303A test guideline* [2] is also a standard employed to assess the biodegradability or the removal of substances in activated sludge systems.

However, the BOD_5 standard method presents several drawbacks: the method is time-consuming and depends heavily on the experience of operators to achieve reproducible and reliable results. Since it takes 5 days for BOD determination, this method is not suitable for process control and real-time monitoring. In the case of a pollution event in a natural ecosystem or an overload in a wastewater treatment plant, results would be known 5 days later [3] then leading to expensive and complicated remediation procedures.

Other problems with the method that have been reported are (1) the need to dilute samples, given that low solubility of oxygen in water (8.7 mg L^{-1} at 25 °C) quickly becomes the rate-limiting reagent (the linear range of the method is between 1 and 9 mg L^{-1} of O_2 or mg L^{-1} BOD_5); (2) the lack of stoichiometric validation [4]; and (3) temperature sensibility [5]. Despite these limitations, the BOD_5 test is used extensively and recognized by regulatory agencies.

Alternatively biological approaches applied in conjunction with chemical analysis can give more complete information about a potential biological impact produced by complex and polluted effluents. The most relevant biological techniques applied to environmental analysis can be classified as bioassays, immunoassays or biosensors according to the technical principles used [6].

Bioassays and biosensors are biological tools used for measuring a global biological effect on living organisms or their component

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parts, as well as for assessing global parameters such as BOD. The difference between both tools lies in the disposition of the biological recognition element that in the case of biosensors is immobilized on a transducer [6].

Karube proposed in 1977 a BOD biosensor based on immobilized whole cells and an oxygen electrode [5] to overcome the major problem (the 5 days determination time) of the aforementioned BOD_5 method. BOD biosensors usually measure the respiration rate of microorganisms at an oxygen electrode interface that correlates with biodegradable material [7].

However biosensors with immobilized microorganisms based on membrane entrapment can yield non-reproducible results given the vital role played by changes in their physiological state and the concentration on the membrane [8–17]. Biofilm-based systems also have been proposed, but problems related mainly to storage and preservation conditions remain [18].

Microbial sensors are perhaps best suited for rapid determination of BOD if the microbial system selected is capable of rapidly degrading a wide range of organic compounds. There are two main options that have been proposed: the use either of a microbial consortium or a unique strain with a broad range of degradation capacity. To achieve utilization of a broad range of organic compounds many different microorganisms could be mixed [5,7,12,13,19,20]; however, it has been shown that mixed community biosensors change their properties in time leading to non-reproducible results [7,13].

To avoid this limitation we sought to design and construct a microbial bioassay based on a unique strain, thus resulting in a more stable behavior, even though a single strain usually does not metabolize a broad range of organic compounds.

The genus *Klebsiella* is defined as a Gram negative, nonmotile, lactose-fermenting, usually encapsulated rod-shaped bacteria of the *Enterobacteriaceae* family, ubiquitous in nature [21–23]. Almost all *Klebsiella* strains grow readily in ordinary media and also in minimal medium, with ammonium ions or nitrate as sole nitrogen source and without a requirement of growth factors. Such bacteria have shown to utilize all the major sugars, aminoacids, and uronic acids derived from the hydrolysates of hemicellulosic and cellulosic materials [21,24]. Many environmental strains have been found involved in degradation of herbicide, pesticide, and several other toxic compounds [25–30]. Therefore *Klebsiella pneumoniae* and *Klebsiella oxytoca* are the biotypes more adequate for a bioassay design and also present less nutritional requirements [21].

Even though the vast majority of biosensors and bioassays for BOD_{st} determination are based on amperometric oxygen electrodes [8,9] (or recently, oxygen optrodes) [31–33] as transducer system, these approaches cannot overcome the main methodological drawback of low oxygen solubility in water.

To overcome the abovementioned drawback a ferricyanide-mediated BOD_{st} technique has been reported as an approach in which O_2 was replaced by potassium hexacyanoferrate (III) (ferricyanide) [34]. Ferrocyanide, the soluble reduced mediator, is accumulated while organic compounds are microbially degraded producing carbon dioxide, as shown in Eq. (1) [19].

$$\{CH_2O\} + H_2O + 4\,Fe(CN)_6^{3-} \rightarrow CO_2 + 4\,Fe(CN)_6^{4-} + 4\,H^+ \qquad (1)$$

The ferrocyanide quantification, conducted by amperometry or coulometry, has been used as an indirect method to determine BOD_5 values [10,11,19,20,35,36].

MICREDOX®, a ferricyanide-mediated BOD_{st} assay based on coulometric measurements in which *Proteus vulgaris* is incubated for 1 h, has improved the linear range of detection (between 18.8 and 150 mg L^{-1} BOD_5) without the need of sample dilution. Although some advances have been made using PVA-immobilized bacteria, the preceding assay cannot yet be transformed easily into a portable instrument, given the need of de-aerate samples [11].

Furthermore the centrifugation step necessary to stop the microbial reaction has not yet been avoided in ferricyanide-mediated methods where free living bacteria are used as catalysts [19,20,36–38]. The development of a solution to this problem is critical since current measurements are necessarily consecutive (for a simple non-multiplexed potentiostat) and, if bacteria activity is not stopped, ferricyanide reduction continues while samples are being measured. So there is a real need to standardize time incubations, i.e. stop the biochemical reaction at the same time in all the simultaneously assayed samples, before the measurement is done.

Despite the fact that centrifugation is the method commonly used to stop microbial ferricyanide reduction it is not a practical resource for an autonomous portable device. We then present a ferricyanide mediated BOD assay where the critical issue resolved is standardization of incubation times in all samples by using a bacteria growth inhibitor to stop microbial ferricyanide reduction.

Many compounds usually employed as disinfectants involve a relative lack of selective toxicity [39]. If the inhibitor compound is effective in small quantities, the employment of a disinfectant may be the solution to avoid centrifugation. So we assayed ordinary compounds with known inhibitory properties in bacterial metabolism and low cost, such as formaldehyde, iodine povidone, sodium hypochlorite, sodium azide, chloroxylenol, ethanol, isopropanol, thymol, H_2O_2 and NaOH [39–41].

There is another drawback in the ferricyanide-mediated BOD technique related to the effect that the cyanide ions released by ferricyanide may cause in the performance of the bioassay. Ferricyanide could disrupt electron transfer to itself due to the liberation of free cyanide anions. In this regard, Liu has proved that ferricyanide may produce growth inhibition, respiration decrease and morphological changes in *E. coli* and that those effects were not reversible depending on the ferricyanide concentrations used [42,43].

We present here improvements to the ferricyanide-mediated approach with a cell-based bioassay in which *K. pneumoniae* (referred to here as $BOD_{K.\ pneumoniae}$) has been successfully used to determine the BOD values of 4 municipal wastewater and 2 synthetic samples; and for the first time we propose the use of formaldehyde as an inhibitor to successfully stop the microbial ferricyanide reduction (leading to the standardization of incubation times) without affecting the amperometric technique.

2. Materials and methods

2.1. Solutions and culture media

The LB broth contained (g L^{-1}) bacto triptone (10), NaCl (10), and yeast extract (5). For agar plate count, 1.5% (w/v) of agar was added.

The minimum medium (MM) contained (g L^{-1}) Na_2HPO_4 (6), KH_2PO_4 (3), NH_4Cl (1), NaCl (0.5), $MgSO_4{\cdot}7H_2O$ (0.12), and $CaCl_2{\cdot}2H_2O$ (0.01), with pH adjusted to 7. The potassium ferricyanide stock solution (100 mmol L^{-1}) was prepared in MM.

The D-glucose, D-fructose, L-glutamic acid, lactose, succinic acid, sucrose and GGA solutions were made in MM and sterilized by membrane filtration (0.22 μm).

The OECD synthetic wastewater contained (g L^{-1}) peptone (15), meat extract (11), urea (3.0), NaCl (0.7), $CaCl_2$ anhydrous (0.3), $MgSO_4{\cdot}7H_2O$ (0.2), and $K_2HPO_4{\cdot}3H_2O$ (3.7) with a measured BOD_5 value of 17,000 mg L^{-1}.

The synthetic sample Sim-1 final concentration in well (mg L^{-1}) was: peptone (11), meat extract (150) in MM. A 1:2 dilution of Sim-1 (Sim-2) was also assayed.

The inhibitor compounds employed and the corresponding stock solution concentrations were: chloroxylenol (48 g L^{-1} from

ESPADOL® formulation), ethanol (96% v/v), formaldehyde (40% w/v), H_2O_2 (12% w/w), iodine povidone (100 g L^{-1} from Pervinox® formulation), isopropanol (100%), NaOH (400 g L^{-1}), sodium azide (20 g L^{-1}), sodium hypochlorite (55 g L^{-1}), and thymol (500 g L^{-1} solution in ethanol 96%).

The solutions were prepared with double osmosis water, unless otherwise indicated.

2.2. Isolation, identification, and maintenance of *K. pneumoniae*

A *K. pneumoniae* strain was isolated from a lyophilized product (BODSEED capsules), commercialized by Bio-systems International (1238 E. Inman Parkway Beloit, WI 53511), and maintained in cryopreserved cultures at −75 °C (20% v/v of glycerol in LB broth).

The extraction of nucleic acids, the PCR amplification, and the 16S rRNA sequencing process were performed by Macrogen Inc. (Seoul, South Korea). Universal primers (518 F: 5′-CCAGCAGCCGCGGTAATACG-3′ and 800R: 5′-ACCAGGGTATCTAATCC-3′) were used for PCR amplification and sequencing processes, and the nucleotide sequence data were obtained using the Big Dye™ terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the ABI 3730 XL analyzer (Applied Biosystems).

The sequence obtained was compared with the sequences of reference species of bacteria contained in genomic database banks, using the BLAST sequence alignment tool (http://www.ncbi.nlm.nih.gov/blastresulting). The isolated strain presented a similarity level of 98% with *K. pneumoniae* strain K30 and *K. pneumoniae* strain K8 (GenBank accession numbers EU661377 and EU661374), both originally isolated from rhizosphere [44].

2.3. Preparation and cultivation of *K. pneumoniae* and BODSEED consortium

The *K. pneumoniae* cryopreserved culture was inoculated in LB broth and incubated at 29 ± 1 °C without agitation until a late exponential phase was reached (OD_{600} of 1 ± 0.1), corresponding to 2.4×10^8 CFU mL^{-1}. Such cultures were centrifuged at 14,000 × *g* for 30 s, or at 900 × *g* for 10 min, and re-suspended in MM, unless otherwise stated. The final optical density (OD) of those bacterial suspensions was adjusted to an OD_{600} of 18 (4.2×10^9 CFU mL^{-1}).

The lyophilized BODSEED capsules were cultured in LB medium. After 2 h in a shaker at 29 ± 1 °C, the solids (wheat bran) were allowed to settle for 30 min and the supernatant transferred to a fresh LB medium and incubated at 29 ± 1 °C until an OD_{600} of 1 ± 0.1 was reached. The culture was centrifuged in a microcentrifuge at 14,000 × *g* for 30 s, and it was washed twice and then re-suspended in MM. The BODSEED consortium final suspension was also adjusted to an OD_{600} of 18 (1.1×10^{10} UFC mL^{-1}).

2.4. Electrodes: fabrication and manipulation

A standard three-electrode system was employed in chronoamperometric assays, using a 0.196 mm^2 Pt electrode as working electrode (WE), a saturated Ag/AgCl as reference electrode (RE), and a stainless steel wire as counter electrode (CE). REs were lab-made weekly and its potential verified against a commercial saturated Ag/AgCl electrode before use.

Electrodes were washed with distilled water before each measure. Afterwards the WE was polished manually for 2 min with alumina (1 and 0.3 μm). Finally, all electrodes were thoroughly rinsed with distilled water and used in the next measure.

2.5. Chronoamperometric measurements and procedures

Measurements proceeded under static conditions and amperometric currents were registered continuously in samples placed in 12 plastic well plates, with 2 mL final volume in each well. All incubations were done at 37 °C unless otherwise stated.

The multi-well plate was manually shaken and left still for 1 min (quiet time). Then a +500 mV potential step versus Ag/AgCl RE was applied for 10 s (instantaneous limiting current value was used as analytical data) to each sample measured.

2.6. General procedure of sample preparation

The base levels of ferricyanide reduction were obtained from wells where no bacteria were added (blank); and endogenous metabolism was obtained from wells where no carbon source was supplied (assayed for each bacterium concentration studied). The total metabolism was measured in samples where a carbon source such as glucose was added to the medium. All the samples were prepared in MM.

Unless otherwise stated, the data we present here are the denominated exogenous metabolism (exogenous = total − endogenous).

2.7. Effect of inhibitors in current production and microbial growth

A sample batch (44 mL) containing 6.25 g L^{-1} of ferricyanide, 5 g L^{-1} of glucose and an OD_{600} of 1 of *K. pneumoniae*, was incubated for 75 min. Afterwards, 2 mL of this batch were added sequentially in wells ($n = 4$) containing either an inhibitor or none (positive control, PC), then PC controls were measured, and all wells incubated at 37 °C. Two more measurements of all the samples were done after 120, and 180 min of incubation.

The inhibitors and final concentrations used in each well were chloroxylenol (2.4 g L^{-1}), ethanol (9.6% v/v), formaldehyde (2% w/v), H_2O_2 (1.2% w/w), iodine povidone (5 or 0.5 g L^{-1}), isopropanol (10% v/v), NaOH (2 g L^{-1}), sodium azide (0.02 g L^{-1}), sodium hypochlorite (5.5 g L^{-1}), or thymol (5.25 g L^{-1}). Volumes added in each well were 10% or less than the control sample volume.

Another sample batch (24 mL) containing 6.25 g L^{-1} of ferricyanide, 5 g L^{-1} of glucose, and an OD_{600} of 1 of *K. pneumoniae*, was incubated for 1 h. Then 2 mL of this batch were added sequentially to wells ($n = 4$) containing only ethanol, formaldehyde, iodine povidone (0.05 g L^{-1}), isopropanol, NaOH, or thymol (and also a PC control); PC controls were measured and all samples incubated at 37 °C. After 3 more hours, wells were measured sequentially and stored at 4 °C to avoid evaporation. Three more measurements were made at 25, 49 and 74 h.

After measuring the samples with inhibitors at 25 and 74 h of incubation, 100 μL of each well sample were cultured in LB agar plates for 5 days at 37 °C (referred to here as A25 samples and A74 samples, respectively) to assess viability.

2.8. Effect of different carbon sources on current production

An OD_{600} of 1 of *K. pneumoniae* and 6.25 g L^{-1} of ferricyanide was assayed either with different glucose concentrations or different carbon sources ($n = 3$), such as fructose, L-glutamic acid, lactose, succinic acid, or sucrose at 2.8 mmol L^{-1} (molar concentration equivalent to the 0.5 g L^{-1} glucose concentration). In these assays bacteria pellets were washed twice with MM before use, and 0.5 g L^{-1} of iodine povidone was added after 2 h incubation.

2.9. Construction of calibration curves using standard solutions

An OD_{600} of 1 of *K. pneumoniae* and 6.25 g L^{-1} of ferricyanide was used to construct calibration curves with GGA or OECD standard solutions ($n = 4$). Bacteria pellets were washed twice with MM before use.

GGA concentrations from 0 to 1.5 g L^{-1} were assayed after 2 h incubation, and 0.5 g L^{-1} of iodine povidone was added to stop the microbially mediated ferricyanide reduction.

OECD was assayed between a range of 30 and 800 mg L^{-1} BOD_5 concentrations after 2 h incubation. In this case, ferricyanide reduction was first stopped by centrifugation (under 14,000 × *g* for 30 s, and pellets were kept under–20 °C for 10 min, and then under 4 °C). After measurements were done, samples were recomposed and 2 mL of each sample was added to a well with formaldehyde (2% w/v) where currents were measured again.

2.10. Preparation of de-aerated samples and incubation conditions

When the de-aerated condition (condition A) was assayed, each sample was sparged with O_2-free N_2 for 10 min. The multi-well plates were placed immediately in a container also flushed with N_2 for 10 min, and sealed. Samples were incubated up to 1.5 h and formaldehyde was added afterwards.

2.11. Equipment

OD measurements were done with a Kontron Uvikon 710 Spectrophotometer (Zurich, Switzerland). Bacterial pellets were obtained with a Cavour VT 1216 Microcentrifuge (Buenos Aires, Argentina), at 14,000 × *g*, or a Sorvall RC-5B Centrifuge, GMI Inc. (Minnesota, USA) at 900 × *g*.

Chronoamperometric measurements were done with an ISKRA Polarograph MA 5440 (Kranj, Yugoslavia); continuous data acquisition was done by means of a Fluke-289 (Fluke Corporation, WA, USA) connected to an analog ISKRA output and to a PC computer via a USB bus.

2.12. Determination of BOD_5

Four municipal wastewater samples taken from different parts of the Buenos Aires sewer system, and two synthetic samples (Sim-1 and Sim-2) were assayed for the traditional BOD_5 test. The analysis was made under the *5210B Standard* determination [45] by AySA, Aguas y Saneamientos Argentino (Buenos Aires, Argentina).

2.13. $BOD_{K.\ pneumoniae}$ measurement of real wastewater and synthetic samples

Municipal wastewater samples were filtered at 0.2 μm before incubation with an OD_{600} of 1 of *K. pneumoniae* and 6.25 g L^{-1} of ferricyanide for 2 h ($n = 4$), followed by formaldehyde addition. These samples were maintained under −20 °C for a week afterwards. Sim-1 and Sim-2 were assayed under the same conditions.

Base levels of ferricyanide reduction in the real samples were measured in wells where no *K. pneumoniae* was added.

The $BOD_{K.\ pneumoniae}$ value of the samples was calculated against an OECD calibration curve made the same day.

3. Results and discussion

3.1. Search for a bacteria growth inhibitor

To standardize incubation times of sequentially measured samples, we searched for a substance to stop bacterial ferricyanide reduction with no electrochemical interference. The mechanism of action of the inhibitors was not within the scope of our research.

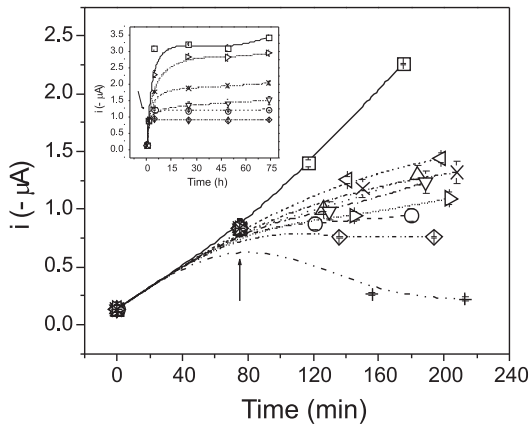

Fig. 1. Effect of inhibitors in ferricyanide microbial reduction. Amperometric measurements where control (□); ethanol (△); formaldehyde (◊); iodine povidone 5 g L^{-1} (◁); iodine povidone 0.5 g L^{-1} (▷); isopropanol (▽); NaOH (×); sodium hypochlorite (+), or thymol (○) was assayed along 3 h after their addition. The inset shows an amperometric assay where control, formaldehyde, iodine povidone (0.5 g L^{-1}), isopropanol, NaOH or thymol was assayed along 74 h (measured once a day). The arrows indicate time of addition of the inhibitors. Values represent the mean ± $SD_{(n-1)}$ of the total metabolism ($n = 4$).

When sodium hypochlorite was added as inhibitor, currents decayed significantly (Student's *t* test, independent populations at the 0.05 level, $n = 4$). In contrast, when H_2O_2 was used (data not shown) currents increased significantly up to 6 μA 45 min after its addition. Thus, none of these compounds fulfilled our need for stopping the reduction of ferricyanide without affecting the amperometric technique (Fig. 1).

Sodium azide, commonly employed as a growth inhibitor of Gram negative bacteria, and chloroxylenol were previously assayed, but none was adequate for our purpose. Sodium azide could not stop ferricyanide reduction, and chloroxylenol interfered with the measurements (possibly pine oil present in the ESPADOL® formulation interfered) (data not shown).

Formaldehyde and thymol were the compounds that, after 73 h of their addition, still maintained the analytical signal almost unaltered (Fig. 1). No significant current differences (at the 0.05 level) from the values registered in control wells (PC) at $t = 60$ min were obtained (inset in Fig. 1). Isopropanol maintained measured currents near the 60 min PC values, but a high dispersion of the measurements was observed (Fig. 1). Iodine povidone seems to inhibit bacteria metabolism for a couple of hours after its addition, but yields no reproducible results if SDs and currents are compared within Fig. 1. However, in several experiments it was used as inhibitor because measurements were done immediately after its addition.

As the purpose of this experiment was to stop bacterial reduction of ferricyanide, we searched for bacterial growth in agar plates. No bacterial growth was detected in the A25 samples (agar cultures after 25 h of the addition of inhibitors) from wells where formaldehyde, NaOH, or thymol was added. Only wells with formaldehyde were found non-contaminated in A74 samples after 5 days incubation.

3.2. Effects of ferricyanide concentration on current production and *K. pneumoniae* OD

Ferricyanide concentrations usually employed in ferricyanide-mediated BOD studies are between 40 and 55 mmol L^{-1}, since in that range currents are almost independent of the mediator

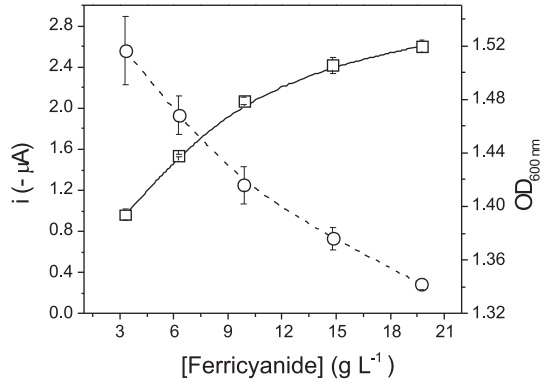

Fig. 2. Effect of ferricyanide concentrations (3.3; 6.25; 9.9; 14.8 and 19.8 g L^{-1}) in current production (□ and solid line) and OD (○ and dashed line) after 2 h incubation. Chronoamperometry assayed with an initial OD_{600} of 1 of *K. pneumoniae*, and 5 g L^{-1} of glucose; iodine povidone was added afterwards. Values represent the mean ± $SD_{(n-1)}$ (n = 2).

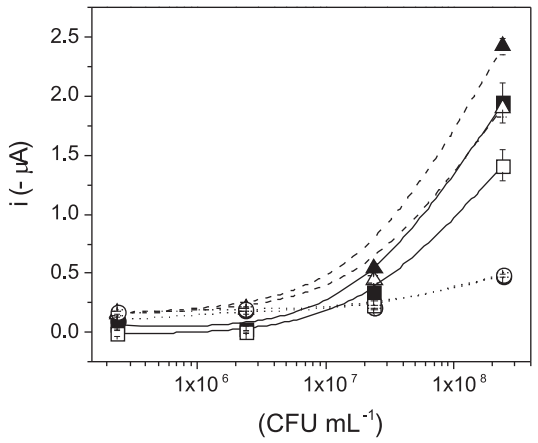

Fig. 3. Effects of bacteria concentrations and de-aeration in current production. Chronoamperometric assay where different bacteria concentrations were incubated with 6.25 g L^{-1} of ferricyanide and 5 g L^{-1} of glucose under two conditions, A (△, □, ○) or B (▲, ■, ●) for 1.5 h; formaldehyde was added afterwards. Total (△, ▲ and dashed line), exogenous (□, ■ and solid line), and endogenous (○, ● and dotted line) metabolisms have been included and values represent the mean ± $SD_{(n-1)}$ (n = 4).

concentration [11,20,35–37] and the reproducibility of the assay improves. Therefore, we studied the effect of 3.3, 6.25, 9.9, 14.8 and 19.8 g L^{-1} ferricyanide concentrations in the current production of an initial *K. pneumoniae* OD_{600} of 1, after 2 h incubation and the addition of iodine povidone (Fig. 2).

As can be seen, at low ferricyanide concentrations the current increases in a linear way (up to 10 g L^{-1}), but at higher ferricyanide concentrations it seems to be reaching a plateau. Thus, the currents seem to became independent at ferricyanide concentrations higher than 19.8 g L^{-1} (60 mmol L^{-1}) employing *K. pneumoniae* as biocatalyst, despite the results obtained using other bacterial strains [7,16,17].

But ferricyanide can be harmful to bacteria. Liu has seen adverse effects of different ferricyanide concentrations in cell growth, morphology and metabolism (by measuring dissolved oxygen) of *E. coli* at different incubation times [41]. So the OD has been measured after incubation and chronoamperometric measurements of the samples previously described in this section. The possible adverse effects of ferricyanide have been assayed only after 2 h incubation because it is the time that has been generally used for our assays (Fig. 2).

Significant lower OD can be seen in samples where the *Klebsiella* strain was incubated with higher ferricyanide concentrations, the OD differences being statistically significant between 3.3 and 9.9 g L^{-1} (Student's t test, n = 2) and non significant between 3.3 and 6.25 g L^{-1}. These results show some adverse effects on microbial growth at high ferricyanide concentrations since the initial bacteria concentration was an OD_{600} of 1 ± 0.1.

3.3. Effect of bacteria concentration and samples de-aeration on current production

The usual procedure for ferricyanide-mediated BOD assays includes a de-aeration step sparging the samples with N_2 before incubation [10,11,19,20,34,37,46,47]. In none of these works an explanation about the need to de-aerate the samples is given. Maybe the de-aeration of the samples is a standardized step since Kaláb found that a significant increase of signal can be obtained using *Paracoccus denitrificans* when competitive flux of electrons to oxygen was blocked using cyanide [48].

Thus, in order to simplify the procedures usually employed in this method we studied currents produced in samples containing 6.25 g L^{-1} of ferricyanide, 5 g L^{-1} of glucose and different bacteria concentrations under two different conditions (samples sparged with N_2, condition A, or not, condition B) (n = 4) (Fig. 3). Maybe if bacteria concentration is high enough to consume all the oxygen present in a sample in a few minutes, there would be no need to de-aerate samples.

Significant differences between conditions A and B were found at the highest bacteria concentrations with higher currents measured in the condition B. Differences between both conditions were found in the exogenous metabolisms, but not found in the endogenous metabolism (Fig. 3). These results are different from those obtained by Liu [49] employing *E. coli*.

So, in our bioassay there is no need to de-aerate samples. Oxygen as final electron may be the second option if the molar ferricyanide/oxygen ratio used is about 80/1 (considering water O_2 saturation at 37 °C, 6.9 mg L^{-1}). The significant higher currents obtained in the condition B may be due to some physiological state problem resulting from the de-aeration procedure rather than to an effect of the presence or not of oxygen in the sample.

3.4. Response to different organic compounds

BOD determination is used to estimate the amount of oxygen that is utilized as microorganisms oxidize biodegradable organic substances in water samples. BOD assays should, therefore, employ microorganisms with the ability to degrade a wide range of organic substances and detect a product or sub product of their metabolism (either oxygen or ferrocyanide). But in short-term methods based on a single strain, BOD values could be lower than those obtained in the BOD_5 test, given that currents obtained are proportional to the greater or lesser degradability of the compound.

So an OD_{600} of 1 of *K. pneumoniae* and 6.25 g L^{-1} of ferricyanide have been assayed with different simple carbon sources. 0.5 g L^{-1} of iodine povidone was added after 2 h incubation (see Section 2.8 for more details).

The glucose calibration assay was made in order to determine on what glucose concentration currents are dependent. As expected, currents increased linearly at lower concentrations (Fig. 4) up to 0.25 g L^{-1}, and then the slope starts to decrease.

Other carbon sources were then assayed at a concentration of 2.8 mmol L^{-1}. As expected, the highest currents were obtained using glucose as the carbon source; and utilization of other monosaccharides gave higher currents than employment of disaccharides. Utilization of disaccharides gave higher currents than degradation of an amino acid. Also succinic acid gave higher currents (24%, also relative to glucose) than the degradation of glutamic acid, but lower than currents from disaccharides.

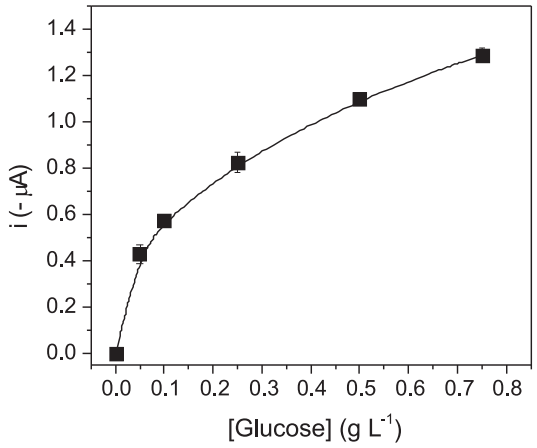

Fig. 4. Glucose calibration curve with an OD_{600} of 1 of *K. pneumoniae* and 6.25 g L^{-1} ferricyanide. Chronoamperometric measurements were done after 2 h incubation and addition of iodine povidone. Values represent the mean ± $SD_{(n-1)}$ (n = 3).

Table 1 compares normalized values relative to glucose using different methods for BOD determination, including the bioassay $BOD_{K.\ pneumoniae}$ presented in this work (values obtained with glucose were considered the 100% of the signal). In particular for $BOD_{K.\ pneumoniae}$, current value from the degradation of glucose at 0.5 g L^{-1} concentration (presented in Fig. 4) was considered as 100% of the signal.

It can be seen that any of the rapid methods of BOD determination adjust exactly with the results obtained in the BOD_5 method probably due to the short time determination, even when a consortium is used as the microbial system [5,10]

3.5. Comparison between *K. pneumoniae* and BODSEED population as biocatalysts

As GGA is a standard solution used in the BOD_5 assay, we studied the effect of different concentrations of GGA in samples seeded either with *K. pneumoniae* or a BODSEED consortium. The calibration curve data are shown in Fig. 5a.

The linear range of the sensor is defined as the substrate range that gives a signal directly proportional to the concentration [9,13], so a wider linear range between substrate concentration and currents would lead to the need of less dilutions of the samples to determine. The linear range observed with the *Klebsiella* strain (Fig. 5b) was higher than the one obtained with the BODSEED consortium, i.e. from 30 to 500 mg L^{-1} BOD_5 with *K. pneumoniae* (r^2 = 0.9926) and from 6.6 to 66 mg L^{-1} BOD_5 with the consortia (r^2 = 0.9806).

Even when the sensitivity of the assay either using a bacteria consortium or the single strain *K. pneumoniae* was similar (2.3 or 2.1 nA L mg^{-1}, respectively), the wider linear range obtained using *K. pneumoniae* provides a bioassay that can estimate BOD values without the need of extensive dilutions as the BOD_5 standard method.

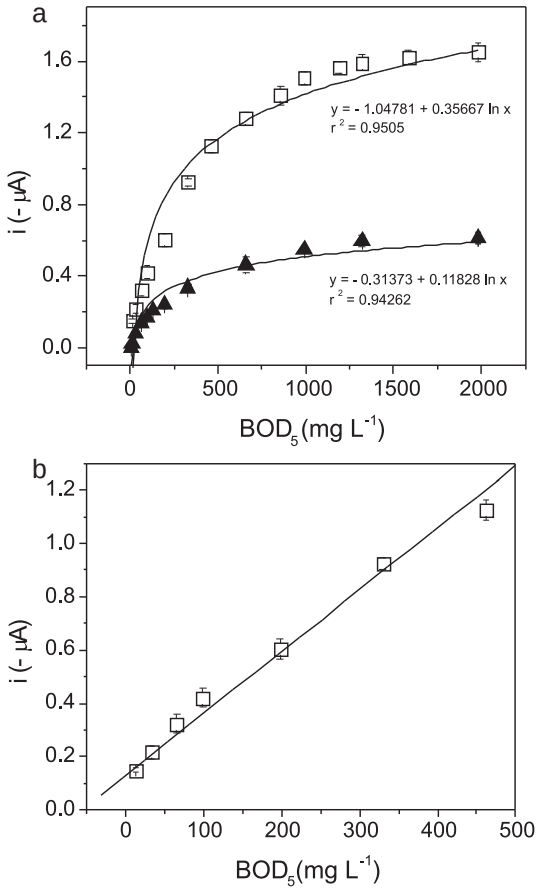

Fig. 5. (a) GGA calibration curve using 6.25 g L^{-1} ferricyanide and an OD_{600} of 1 either of *K. pneumoniae* (□) or BODSEED consortium (▲). (b) Linear range of the GGA calibration curve using *K. pneumoniae* as biocatalyst. Chronoamperometric assay made after 2 h incubation and addition of 0.5 g L^{-1} of iodine povidone. Values represent the mean ± $SD_{(n-1)}$ (n = 4).

3.6. Calibration of the bioassay using OECD as standard solution

The common GGA standard solution for BOD_5 calibration, however, is not often suitable for BOD determination in industrial or natural wastewater samples that usually present a variety of substances with a wide range of molecular weights.

Even though we did not find interferences between formaldehyde and glucose, wastewater samples have an unknown and often complex composition, and some interference may appear not only with real samples, but also with an OECD solution. So we made a calibration curve with samples containing OECD at different concentrations, and measured them after centrifugation and after the addition of formaldehyde (Fig. 6a).

No significant differences were found in measurements made after the use of formaldehyde and those obtained after centrifugation (Student's t test, n = 4) so no interferences were found by using

Table 1
Relative BOD values of different organic compounds (glucose considered as 100% of the signal) measured with different BOD_{st} methods and BOD_5.

Organic compound	$BOD_{K.\ pneumoniae}$	BOD_{SM} [50]	BOD_{SPV} [51]	BOD_S [5]	BOD_{FC} [10]	BOD_5 [35]
Glucose	100	100	100	100	100	100
Fructose	42	23	110	75	58.5	111
Sucrose	32.5	4.5	60.6	50	64.6	97.7
Lactose	32	1.3	6.1	5.6	3.1	91.4
Succinic acid	24	–	–	–	–	–
L-Glutamic acid	16	38	61	97.2	24.6	100

Table 2
Comparison between the $BOD_{K.\ pneumoniae}$ and BOD_5 values of 4 real wastewater and 2 synthetic samples. Calibration was made using the OECD standard. $BOD_{K.\ pneumoniae}$ values reported are the mean ± $SD_{(n-1)}$ and %RSD is calculated as 100 × SD/mean of these values (n = 4).

Sample	BOD_5 ($mg\,L^{-1}$)	$BOD_{K.\ pneumoniae}$ ($mg\,L^{-1}$)	%RSD (100 × SD/mean)	$BOD_{K.\ pneumoniae}/BOD_5$
Wastewater 1	145	167 ± 13	7.78	1.15
Wastewater 2	120	151 ± 14	9.27	1.26
Wastewater 3	130	119 ± 12	10.08	0.92
Wastewater 4	120	116 ± 12	10.34	0.97
Sim-1	175	43 ± 8	18.6	
Sim-2	85	24 ± 6	25	

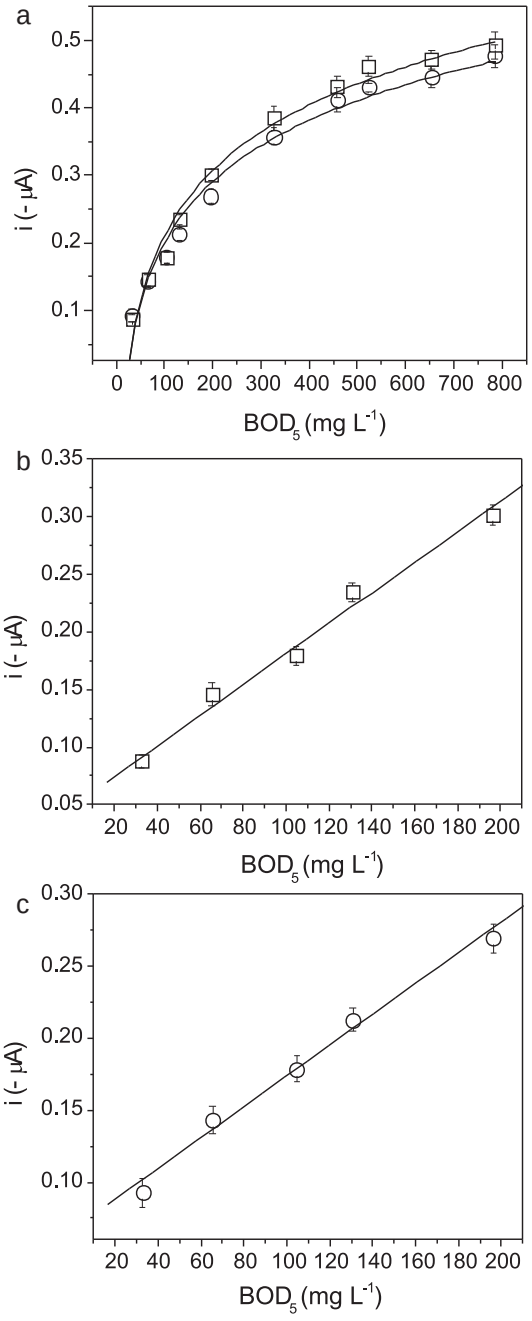

Fig. 6. (a) OECD calibration curve using 6.25 $g\,L^{-1}$ ferricyanide and an OD_{600} of 1 of *K. pneumoniae*. Measurements were made either after centrifugation of the samples (□) or after the addition of formaldehyde (○). (b) Linear range of the OECD calibration curve after centrifugation. (c) Linear range of the OECD calibration curve after addition of formaldehyde. Chronoamperometric assay made after 2 h incubation. Values represent the mean ± $SD_{(n-1)}$ (n = 4).

formaldehyde if OECD is the carbon source employed for calibration of the bioassay.

The linear range found in both cases (either after centrifugation, Fig. 6b or after the addition of formaldehyde, Fig. 6c) was between 30 and 200 $mg\,L^{-1}$ BOD_5 (with an r^2 = 0.9891 and r^2 = 0.9868 respectively). These results are comparable to linearity ranges found in the literature about biosensors [7]. If we consider the linear range obtained when the data were plotted as i vs. ln (BOD_5), the linear relationship was from 30 to 450 $mg\,L^{-1}$ BOD_5 (apparent linear range with r^2 = 0.991, data not shown).

Despite all, differences between measurements after centrifugation and after addition of formaldehyde were found not statistically significant (Student's t test, n = 4). Therefore the use of formaldehyde is an excellent choice to replace the centrifugation step in ferricyanide-mediated determinations.

3.7. Application of the bioassay to BOD determination in real wastewater samples

BOD determinations of 4 municipal wastewater samples and 2 synthetic samples (Sim-1 and Sim-2) were made, and the BOD_5 test was used as the standard method for comparison (Table 2).

In order to seek possible toxic effects of the samples two dilutions of each sample were measured (dilutions 1:4 and 1:8). BOD values obtained for samples 1 and 2 after dilution correction were similar in each case. In samples 3 and 4 significant lower currents were obtained in the lower dilution (around 25% of difference), meaning that the sample may have a toxic effect in the metabolism of the bacteria.

The BOD values obtained with our bioassay were accurate for the determination of the 4 wastewater samples assayed. A relation between the values obtained with the $BOD_{K.\ pneumoniae}$ and the BOD_5 assays was calculated as $BOD_{K.\ pneumoniae}/BOD_5$ (Table 2). Curiously, the samples that presented toxic effects also presented a better relation with the BOD_5 values (samples 3 and 4). The RSD% of each real sample was around 10% (Table 2).

Given that the *Standard Methods* [1] suggest using samples on the collecting day, real samples were measured again before disposal (seven days after the collecting day). In the second measurement the BOD values of samples 1 and 3 decreased about 15% and in sample 2 decreased 35%. The BOD value of sample 4 remained in the same value (117 ± 13 $mg\,L^{-1}$). These results show that the composition of some samples may change in time, even at low temperatures (−20 °C).

4. Conclusions

K. pneumoniae has proven to be a nutritionally versatile candidate for a ferricyanide-mediated bioassay with no need of samples de-aeration, simplifying the methodology usually employed and leading to the development of a field bioassay.

The linear range achieved with the *K. pneumoniae* strain has been from 30 to 500 $mg\,L^{-1}$ BOD_5 value (r^2 = 0.9926) when a GGA standard was used, and from 30 to 200 $mg\,L^{-1}$ BOD_5 value (r^2 = 0.9868)

when OECD was used as the standard solution (and an apparent linear range between 30 and 450 mg L^{-1} BOD_5). This linear range found with *K. pneumoniae* represents a big improvement over the linear range of the standard method (between 1 and 9 mg L^{-1} BOD_5).

The linear range obtained with the $BOD_{K.\ pneumoniae}$ when the OECD standard is used is comparable to those obtained by methods previously reported, which are between 200 and 300 mg L^{-1} BOD_5, despite the use of microbial consortia [20,52] or single strains [15,16,18,48]. However, the apparent linear range obtained with OECD is comparable to the one found by Chiappini [53].

The use of formaldehyde to finish the reduction of ferricyanide not only gave reproducible results with no electrochemical interference, but currents were non-significantly different to those from the control (PC) for up to 73 h after its addition. No microbial growth was detected during that time. No interferences were found when the OECD standard was used, including those with real wastewater samples. So we have succeeded in avoiding centrifugation towards a field bioassay and a higher reproducibility has been achieved (around a 10% RSD while for the BOD_5 a 20% RSD is accepted).

Finally, BOD values of 4 municipal wastewater samples and 2 artificial samples have been successfully determined by our bioassay. Good agreement has been achieved, calculated as $BOD_{K.\ pneumoniae}/BOD_5$, between $BOD_{K.\ pneumoniae}$ and BOD_5 values for wastewater samples.

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