Microbial-Based Electrochemical Bioassay for Water-Quality Analysis. One Step Towards a Disposable Biosensor System

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Abstract

A ferricyanide-mediated short term biochemical oxygen demand (BOD_{st}) approach has been reported to overcome low oxygen solubility in water limitation that contributes to long incubation time (5 days) in the standard BOD test (BOD_5), internationally employed as a water quality parameter. BOD test is also used to determine organic pollution in natural waters and the total degradable organic load in wastewaters.

Here we present results obtained using free bacterial cells, in a batch system. Our results show that one of the bacterial strains tested reduces ferricyanide efficiently and is able to oxidize several carbon sources. These results are going to be used in the design of a microbial biosensor that, proficient for BOD_{st} determination, will improve and facilitate active intervention in environmental monitoring. The development of rapid methods to be used in-situ, based in disposable electrochemical strips, is our ultimate goal.

Keywords: BOD, Ferricyanide, Wastewater, Microbial, Biosensor.

1.- Introduction

Organic pollution in wastewaters is commonly determined through a standard method called 5-days biochemical oxygen demand assay (BOD_5) [1] that correlates biodegradable organic matter in samples with dissolved oxygen consumed by microorganisms, after 5 days incubation. But oxygen low solubility (8.7 mg/l at 25°C) in water quickly becomes the rate-limiting reagent in microbial aerobic catabolism of organic matter [2], making difficult an active intervention for environmental monitoring and/or process control.

The complex combination of different size and type of biodegradable biomolecules present in waste water has different capacities of being biodegraded, therefore complicating its direct quantification. Measuring consumed O_2, a biodegradable organic matter co-substrate, is a good estimation to the required quantification.

However BOD_5 test presents practical difficulties, as low oxygen solubility above many others like a lack of stoichiometric validation, dilutions requirements and temperature sensibility [3].

The use of a ferricyanide-mediated rapid BOD approach to overcome the oxygen limitation problems has been reported [4]. O_2 was replaced by ferricyanide ion (with higher solubility) as alternative electron acceptor in the biochemical reaction, allowing the use of increased bacterial concentration and greatly decreased incubation times required to microbial oxidize significant amounts of organic substrate [4]. The reduced soluble mediator accumulation (ferrocyanide in this work) is proportional to the organic matter degraded [7, 8, 9, 10].

Microbial biosensors have been developed to determine the BOD value (or a related parameter, called BOD_{st}) using microbial cells with broad substrate range. To achieve a broadest substrate range, many

different microorganisms should be mixed, however it has been shown that mixed populations biosensors changes its properties in time and yields non-reproducible results [5].

We report the results obtained using a bacterial strain isolated from a commercial lyophilized product and the optimization of the electrochemical method based in ferricyanide respiration.

2.- Materials and Methods

2.1.- Solutions and Culture Media

BO365 strain was cultured in LB liquid medium until the required absorbance was reached. LB liquid medium contained bacto triptone (10 g/l); NaCl (10 g/l) and yeast extract (5 g/l).

Escherichia coli minimum media (EMM) was used as buffer in all electrochemical cells. It was prepared with Na_2HPO_4 (6 g/l); KH_2PO_4 (3 g/l); NH_4Cl (1 g/l); NaCl (0.5 g/l); $MgSO_4 \cdot 7H_2O$ (0.12 g/l) and $CaCl_2 \cdot 2H_2O$ (0.01 g/l) at 7.0 pH units.

Glucose solution was made in distilled water and sterilized; sucrose, lactose, L-glutamic acid, succinate; D-fructose and glycil-glycine solutions were made in EMM and not sterilized; these solutions were employed immediately after its preparation. Sucrose, lactose, L-glutamic, succinate, D-fructose were employed at 2.8 mM final concentration, glycil-glycine was employed at 1.6 mM final concentration.

Potassium ferricyanide solution was prepared in EMM.

BOD standard solution (150 mg glucose/l and 150 mg L-glutamic acid/l) was prepared in EMM, without sterilization and employed immediately after its preparation. This solution has a known value of 198 $\pm$ 31 mg BOD_5/l or mg O_2/l. All chemicals used in this work were analytical reagent grade, with exception of lactose and D-fructose that were microbiological grade.

Sodium azide (in distilled water), ethanol and iodine povidone final concentrations employed were 0.2 %, 19 % and 0.5 % respectively.

2.2.- BO365 Isolation

BO365 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 (LB broth in 20% glycerol). This strain was preliminary identified as *Klebsiella pneumoniae* employing the test API® 20 E (Biomérieux) with a 97.5 % of certainty.

2.3.- Cultivation and Preparation of Bacterial Solutions

1 mL of BO365 inoculate (cryopreserved) was seeded in 300 ml LB medium and incubated during 20 $\pm$ 2 hours at 30°C, up to 0.9 $\pm$ 0.1 absorbance units at λ=600 nm (stationary phase). Cultures were centrifuged in a micro centrifuge (Cavour, VT 1216) at 13.500 rpm and re-suspended in EMM. Final bacterial suspensions had 18 $\pm$ 2 absorbance units (corresponding to 2.35 * 10^8 CFU/ml). In some experiments (indicated in text) before obtaining the final suspensions, the cells were washed twice.

When required, a lyophilized BODseed capsule was cultured in LB medium. After two hours in shaker at 29°C it was left 30 minutes without shaking to allow decantation and the supernatant trespassed to fresh LB medium and incubated for 22 $\pm$ 1 hours at 30 °C without shaking, up to 1.4 $\pm$ 0.1 absorbance units at λ=600 nm. Cultures were centrifuged at 13.500 rpm, washed twice and suspended in EMM. The final bacterial suspensions had 18 $\pm$ 2 absorbance units.

2.4.- Electrochemical Cells and Electrodes

Chronoamperometric studies were done using a standard three electrode system, which was employed in all experiments, using a saturated Ag/AgCl reference electrode (RE) and a stainless steel wire counterelectrode (CE). REs employed were lab-made and tested against a reference commercial saturated Ag/AgCl before used (ΔE below $\pm$ 20 mV and stability were considered as usability criteria).

Several materials were tested as working electrodes (WE) (Au or Pt wire and carbon rod or ink by screen-printing); after preliminary tests, a 0,196 mm^2 Pt electrode was employed. +500 mV vs. RE was applied in all experiments reported here, adequate to measure limiting currents produced by ferrocyanide oxidation.

The WE was manually polished during 1 minute with alumina (0.1 μm) in a moist cloth. Between measuring each well, the electrodes were washed with a 1:1 water-ethanol solution and finally distilled water.

3.- Results and Discussion

3.1.- Ferricyanide Reduction Driven by BO365 Strain

A BO365 stationary culture was centrifuged and re-suspended in EMM. The final concentrations of the samples in each well are 19 mM ferricyanide, 3.9 bacterial absorbance units (approximately 9.8 * 10^8 CFU/ml) and 1.25 g/l glucose.

Samples final volumes were 2 ml; control samples contained EMM (2 ml with glucose); 19 mM ferricyanide final concentration plus EMM (with glucose); EMM with glucose and 3.9 absorbance units; or supernatant (LB media from the centrifuged bacterial culture) plus 19 mM ferricyanide.

For this preliminary experiment the WE employed was a Pt, with a geometrical area of 19.6 mm^2. +500 mV was applied to oxidize the ferrocyanide produced by bacterial ferricyanide reducing activity.

Significant currents were registered when bacteria were incubated in presence of ferricyanide and glucose after 20 minutes or 80 minutes of incubation at 37°C, 5.40 and 10.80 μA respectively, after applying 5 seconds the potential or 3.50 and 7.20 μA, applying for 20 seconds the potential indicated.

Currents obtained in all control samples after 20 and 80 minutes incubation were less than 0.7 and 1 μA, respectively.

3.2 Bacteria Concentration Effect

Duplicate samples containing ferricyanide 19 mM, 0.25 g/l glucose and between 2.35 * 10^8 CFU/ml to 2.35 * 10^2 CFU/ml bacterial final concentration were assayed by chronoamperometry with a Pt WE (19.6 mm^2), the results are shown in Figure 1.

Samples were incubated five hours and measurements were done before incubating and after two and five hour incubation.

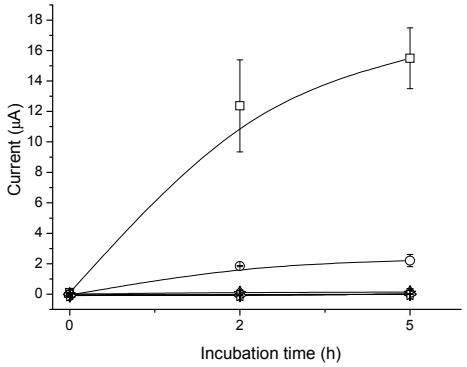

*Figure 1: Different bacterial concentrations were assayed by chronoamperometry employing a Pt WE (19.6 mm^2). Bacteria concentration assayed were: 2.35 * 10^8 CFU/ml (□); 2.35 * 10^7 CFU/ml (○); 2.35 * 10^6 CFU/ml (△); 2.35 * 10^5 CFU/ml (▽); 2.35 * 10^4 CFU/ml (◇); 2.35 * 10^3 CFU/ml (◁) and 2.35 * 10^2 CFU/ml (▷).*

These results shown a direct relation between bacteria concentration and currents (Figure 1); higher currents are obtained with the higher bacteria concentration assayed, 2.35 * 10^8 CFU/ml. This is consistent with the assumption that ferrycianide respirometry is a good approximation to the bacteria metabolism study and shows how may this technique improve the BOD determination time comparing it to the traditional BOD_5 test.

In this experiment it can also be seen that after 5 hours incubation there is still a considerable current registered even when the slope decrease and so BO365 strain would be a good candidate to our microbial biosensor. We assume this decreasing slope is related to the glucose consume and so, in this experiment the limiting parameter could be the glucose concentration.

3.3.- Selection of the Working Electrode

The selection of a working electrode (WE) with suitable currents and good reproducibility (small SD) was made in samples with a suspension of the BO365 strain as biocatalyst and ferricyanide as a soluble mediator (plus glucose).

Pt WEs of 19.6 mm^2 and 0.196 mm^2; Au, 3.1 mm^2; carbon rod, 0.385 mm^2 and carbon ink 4.0 mm^2 (made by screen-printing) were studied. Pt, Au and carbon rod electrodes were made in our laboratory.

Comparing normalized currents *per* area we found that a 0.196 mm^2 platinum and a 3.1 mm^2 gold WEs were the best materials to employ in our research.

3.4.- Searching for a Convenient Biocide Substance

In order to standardize the incubation time, we search a substance able to stop the microbial driven ferricyanide reduction, without affecting the amperometric detection. Duplicate assays containing 2.35 * 10^8 CFU/ml, 19 mM ferricyanide and 0.75 g/l glucose (final concentrations) were measured with a three electrode system with a Pt 0.196 mm^2 WE.

As control two wells containing bacteria and ferricyanide were incubated without the addition of any biocide or biostatic substance.

After 2.7 hours incubation time, currents were registered (considered time 0) and immediately sodium azide, ethanol or iodine povidone were added in duplicate samples (0.2; 19; or 0.5 % final concentration respectively).

Currents were registered 15 minutes after the addition of each compound (corresponding to 0.25 hours incubation) and after 0.9, 1.9 and 2.9 hours incubation time (Figure 2).

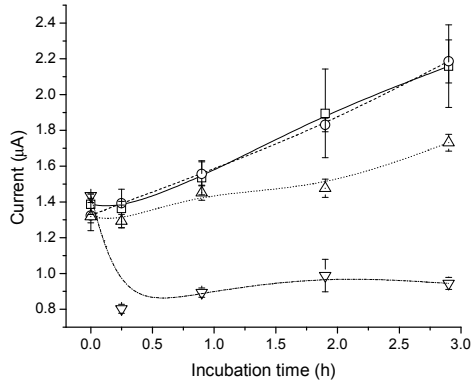

Figure 2: Chronoamperometry using a Pt WE (0,196 mm^2). Control (□); sodium azide (○); iodine povidone (△); and ethanol (▽).

The ethanol volume used was high, so probably this is the main reason why the current decrease; after that, it remains almost invariable.

Sodium azide, commonly employed as a preserver compound [6], has not been useful to our needs; the currents obtained were no significantly different to those obtained with control samples, perhaps a higher concentration is needed.

Chloroxylenol was also tested in a preview experiment, but it was not useful to our needs. We deduce that pine oil present in ESPADOL®, product used as a chloroxylenol source, produced electrochemical interference.

No one of the assayed substances fulfills or requirements, more studies with other substances or concentrations are needed. Anyway, in some experiments iodine povidone was used to inhibit ferricyanide bio-reduction. As the currents increases after an hour after addition, care was taken to standardize measurement intervals.

3.5.- Response to Different Organic Compounds

Duplicate samples were assayed containing different glucose concentrations (0; 0.05; 0.1; 0.25; 0.5; and 0.75 g/l). Samples also contained ferricyanide (19 mM) BO365 bacteria 2.35 * 10^8 CFU/ml. Measurements were done after 2 hours incubation and subsequent iodine povidone addition.

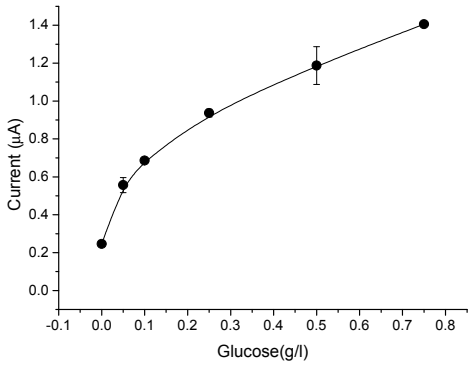

Figure 3: Effects of incubation with different glucose concentrations. Chronoamperometric results, Pt WE (0.196 mm^2).

As it is observed (Figure 3) currents registered increase when the glucose final concentration is higher.

Duplicated samples containing sucrose, lactose, fructose, succinic acid or L-glutamic acid were also assayed in equal concentration than glucose was (2.8 mM). Samples also contained microbial cells, 2.35 * 10^8 CFU/ml of BO365, and ferricyanide (19 mM). Glycil-glycine was assayed at different concentration (1.6 mM).

After two hours incubation, iodine povidone was added and electrochemical response was registered by chronoamperometry.

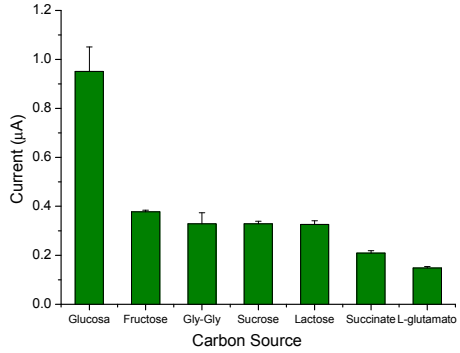

Figure 4: Currents obtained with a Pt WE (0.196 mm^2) after two hours incubation with each carbon source (it has been subtracted the endogenous current values).

BO365 strain produced significant current values with each carbon source, but there was a clear preference (higher currents) for glucose (Figure 4).

3.6.- Single Strain or Mixed Population as Biocatalyst

As GGA is a standard solution used in the BOD_5 assay, its effect in samples seeded either with BO365 strain or BODseed population was tested, to compare current production.

Duplicate samples containing 19 mM ferricyanide, concentrations of 0; 0.01; 0.05; 0.075; 0.01; 0.15; 0.5; 0.75; 1; and 1.5 g/l GGA) and a 2.35 *10^8 CFU/ml were assayed. After two hours incubation and iodine povidone addition, +500 mV vs Ag/AgCl were applied and currents recorded.

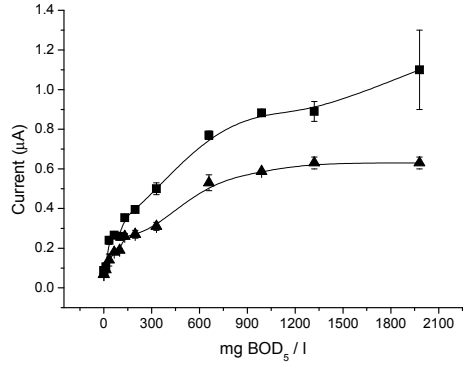

Figure 5: BO365 strain (■) or BODseed population (▲) incubation in presence of different GGA concentrations. Chronoamperometry with a Pt WE (0.196 mm^2).

Significant differences in current values were observed at almost all the GGA concentrations assayed, being higher those obtained with BO365 isolated strain (Figure 5).

3.7.- Effect of Incubation Time

Duplicate samples containing GGA (0; 0.01; 0.05; 0.15; 0.5 and 1 g/l) and 2.35 * 10^8 CFU/ml of BO365 (19 mM ferricyanide) was assayed by chronoamperometry. Currents were registered continuously during 30 seconds after incubating 0, 30, 60, 90 and 120 minutes each sample.

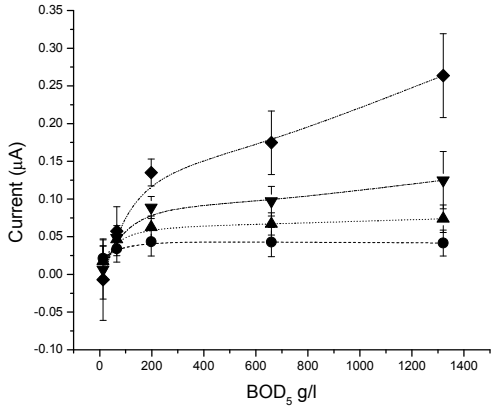

Figure 6: GGA was used as BOD calibration standard, BO365 strain was used. Chronoamperometry, Pt WE (0.196 mm^2). Currents were registered at incubation times of 30.333 (●); 60.333 (▲); 90.333 (▼) and 120.333 minutes (◆). Values registered when bacteria was incubated without GGA have been subtracted.

There are clear and significant differences between currents obtained at time 0 and at 30 minutes incubation time even employing low GGA concentration such as 0.05 g/l (corresponding to a 66 g/l DBO_5 concentration) (Figure 6).

Conclusions

BO365 strain (*Klebsiella pneumoniae*) shows good qualities to become the biocatalyst in a ferricyanide-based biosensor for BOD_{st} determination. It is able to metabolize different carbon sources, producing interesting current after incubating with ferricyanide and grows fast in a simple media. Furthermore, it is known that encapsulated bacteria (as this one) are resistant to a number of toxic agents, which are prone to produce interference in BOD measurements. It also has been showed that iodine povidone could be an adequate compound to standardize incubation times, is a common used and non-hazardous substance; more studies are required to optimize the use of this biocide.

In a previously published work [5] it is noted that BOD biosensors based in a microbial population are not stable enough (in terms of weeks) and so it is required the construction and design of a microbial biosensor based in a unique strain that enables a more stable behavior. Even though, is hard to find a single strain able to metabolize a broad range of compounds. We are wiling to continue assaying BO365 strain with other carbon sources and more complex substances (biopolymers) in order to design adequately our microbial biosensor for BOD_{st} determination.

Theoretically, the complete oxidation of the exogenous GGA standard substrate would yield 1.62 C; experimentally, a charge of 0.93 C has been obtained when *Proteus vulgaris* was incubated for 60 minutes in presence of a 0.15 g/l GGA solution employing bulk electrolysis [11].

The maximum charge produced by BO365 incubated 120 minutes in presence of 0.15 g/l GGA was 4.82 μC, charge value was calculated by integrating current values obtained within 10 and 30 seconds after applying +500 mV vs. Ag/AgCl. The charge value obtained when our strain was incubated with the same concentration and after 60 minutes incubation was only 2.24 μC. As it can be seen, our values are o fraction of the ones obtained by Pasco [11], but the techniques employed in both are essentially different.

In Ertl's work [12], values obtained after a 10 minutes incubation time of *E. coli* JM105 ($2 * 10^5$ CFU/sample) in 100 mM ferricyanide employing chronocoulometric measurements was 111 μC, integrating currents obtained during 2 minutes and 22 μC integrating currents obtained during 20 seconds. For this experiment a Pt WE ($2.7 * 10^{-4}$ mm^2) had been employed and the volume sample was 300 μl with a 10 mM succinate final concentration.

To compare with this last work, we have calculated a charge value of 0.13 μC in samples with a 1 g/l GGA final concentration for BO365 without incubation and 1.29 μC after 30 minutes incubation (charge values were calculated as explained before).

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