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Preliminary Studies for the Use of Bioluminescent Bacteria in the Development of Wireless Biosensors for Environmental Monitoring

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In the last decades the European countries increased the policies related to environmental pollution, in order to improve water quality and people health.
To date, environmental monitoring is mainly based on “spot-sampling”, or on the assessment of certain biomarkers in specific contexts. Research in recent years, however, has revealed several limitations of these methods, such as the inability of a detailed evaluation over time, and the difficulty in determining the bioavailable portion of pollutants, which is the effectively toxic fraction.
To overcome such drawbacks bio-based monitoring methods, which include assays based on whole cells, are under development. Whole cell systems are suitable for environmental monitoring, where the source and nature of the pollutants cannot be predicted, for this reason these biosensors can act as a Biological Early Warning System or BEWS, which will subsequently be supported by more specific actions and analysis.
Microorganisms represent a good opportunity for low cost, long life, pH and temperature adaptable, in addition to the ability to detect a broad spectrum of chemicals, an ability which can be further improved by using genetic engineering.
In this work preliminary tests were made using a *Vibrio fischeri* strain, in order to obtain a useful long-term biosensing element coupled to wireless devices to create a wireless BEWS network, making feasible the idea of a continuous environmental monitoring.

1. Introduction

Starting from “The Stockholm Convention on Persistent Organic Pollutants” (http://www.pops.int/), European countries focused their attention on the limitation of the environmental pollution, throughout the establishment of monitoring policies as the Waterframe directive (2000/60/CE). Nowadays, environmental monitoring is mainly based on combining sampling and laboratory analyses, in a lengthy and expensive way, that precluded a detailed evaluation over time, limited by the few samples, and the complexity in determining the bioavailable fraction of pollutants (Allan et al.2006).
During last years, biosensors and biological assays became much more involved in the environmental monitoring thanks to their ability to get over previous limitations, and open to new possibilities of environmental protection. A promising class of biosensors

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is the one using complete cell systems (whole cell): microorganisms like bacteria, algae, filamentous fungi and yeasts, as well as more sophisticated systems such as eukaryotic animal cells (Rodriguez-Mozaz et al. 2006) that can potentially be interfaced with a wide range of transducers, including optical, electrochemical, and piezoelectric ones (Rogers 2006). This type of system is able to respond to the presence of various organic compounds such as xenobiotics (e.g. alkanes, phenolic compounds, chlorinated compounds, aromatics), metals (e.g. Cd, Cr, Co, Ni, Cu, Hg, Zn), radiation or pH changes, in all environment (soil, water and air) (Gu et al. 2004). Many devices have been developed from wild-type cells or recombinant organisms, genetically modified by fusion of a regulatory element (promoter) and a reporter gene (Gu et al. 2004, Rogers 2006). Both approaches utilize a mechanism by which a specific cellular response varies in the presence of the analyte.

One of the most important advantages in using whole-cell systems, compared to traditional analytical methods, is to get an indication of the mere bioavailable fraction, that often coincides with the real concentration of pollutants capable of giving a toxic effect (Allan et al.2006, Rodriguez-Mozaz et al. 2006). It may be, for example, the case of metal ions: some fraction will never be bioavailable and toxic, but complexed by chelating agents or adsorbed on other materials. For some heavy metals the inhibition of enzymes such as alkaline phosphatase or urease has been exploited, but also recombinant bioluminescent bacteria has been used for the determination of cadmium, zinc, mercury and chromium in sediments, as well as nitrates in drinking water (Rodriguez-Mozaz et al. 2006).

Usually whole cell system could be seen as a on-line BEWS, that continuously controls the overall quality of natural ecosystems, and is able to alerts authorities to get more specific actions and analysis, or to speed up interventions in case of emergency (Gu et al. 2004). Whole cell systems are particularly useful in any case the source and nature of the pollutants cannot be predicted. Furthermore, microorganisms represent a good opportunity for low cost, long life, pH and temperature adaptable, in addition to the ability to detect a broad spectrum of chemicals, an ability which can be further improved by using genetic engineering (Mulchandani and Rogers 1998).

For on-line analysis, it is necessary to use organisms or microorganisms as compatible as possible with the field and the matrix chosen: for this reason, if it’s required to investigate the health of a watercourse, it must be essential to rely on an aquatic organism, usually native. In this preliminary study, the behaviour of a *Vibrio fischeri* culture was studied according to typical physical chemical parameters of the environmental matrix (seawater), in order to evaluate differences in the biological response. An indicative scheme of the final biosensor is shown in Figure 1.

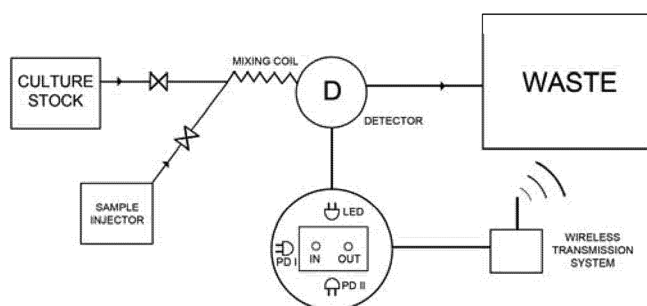

Figure 1: Scheme of the biosensor system.

2. Materials and Methods

Vibrio fischeri NRRL B-1117 was selected because it has a well-characterized sensitivity to a wide range of toxicants, and to date is used as biological ecotoxicological biomarker. Culture were grown into 500 ml Erlenmeyer flasks on Seawater medium (DSMZ medium 246) at 20°C, on a rotary shaker at 120 rpm. Different methods of inoculum were performed: A, a single colony, B, 0.5ml (OD620nm=2,5), C, D and E, 5ml (OD620nm=0,8), F, 0.5ml (OD620nm=2,8), G, 20ml (OD620nm=2,8), added to medium to a final volume of 200ml. Glucose solution used in fed-batch was 100g/L. Cell growth was monitored by measuring the optical density (OD) at 620 nm (HP 8452A Diode Array Spectrophotometer). pH was recorded with a Crison pHmeter.

3. Results and Discussion

Environmental temperature changes along with seasons and this influences the growth and the behaviour of a microbial culture. In order to take advantage of the bioluminescent reaction to detect pollutants in the environment, a steady-state culture must be obtained, and the influence of physical-chemical parameters on the growth must be known.
Mediterranean sea temperature oscillates between 15° and 21° C during winter and summer respectively (Zenetos et al.2002). In this preliminary study, to evaluate growth conditions related to the possible application field, 20° C was chosen as starting temperature. In different batch trials conducted at 20°C, the exponential phase was reached in less than 20 hours, with an OD value around 3-3,5: is possible to see very reproducible OD trend in different test with the same inoculum.
Different methods of inoculum influences the slope of the exponential growth phase, as expected, but also the pH trend. In Figure 2 two different trials have been reported: A, inoculated with a single colony, and B, inoculated with 0,5ml from tube precultures. Although each culture start from the same medium with identical pH (7.40), after 17 hours of culture, OD values of batch B is almost double respect to A, and pH is higher

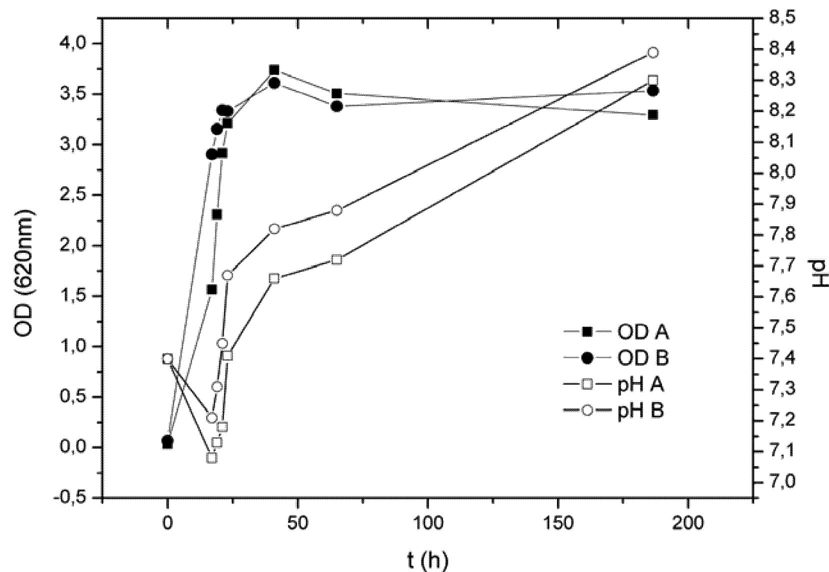

Figure 2: OD and pH of cultures A and B

(7.21 for B and 7,08 for A). This gap is maintained during the whole trial, with a maximum ΔpH after 24 hours of culture. These differences between culture A and B could be due to the method of inoculum: probably a single colony requires an adaptive lag phase respect to a liquid inoculum taken from a preculture step. This hypothesis was confirmed in other tests: a type B inoculum with a OD value almost three times lower showed similar slope during the exponential phase. Concerning pH, it is reduced during the early growth phase (15-20 hours), then it increases along with biomass growth until the end of the trial, but with a slower rate during the stationary phase. This kind of behaviour have been seen in all tested repetitions, possibly due to nitrogen up-take, or to organic acid excreted by bacteria, or both mechanism. As stated before, these consideration could not be easily verified in a complex medium.

The bioluminescent emission were visually seen during fermentation for 25-30 hours, starting from an OD value of 2.4, reached during the exponential growth phase: it became rapidly bright as soon as the quorum sensing mechanism activate the expression of the Lux operon, with a later slow decline during the stationary phase.

Other trials, conducted at 30°C, showed an intermittent emission of light and slower growth, but achieving the same maximum OD values (data not shown). Probably because the optimal temperature is lower than 30°C.

Bioluminescence is temperature, pH, oxygen and nutrients dependent, as reported (Pooley et al. 2004, Scheerer et al. 2006, Waters and Lloyd 1985). To maintain a stable emission of light, one promising methods imply the use of nutrient fed-batch, especially for the carbon source (Pooley et al. 2004). On the basis of previously made batch tests (e.g. A and B, Figure 2), in the following runs the temperature was controlled, on the other hand pH was left uncontrolled and ranges between 7.08 and 8.40, which is by far included into the recommended 6.0 to 9.0 range (Scheerer et al. 2006).

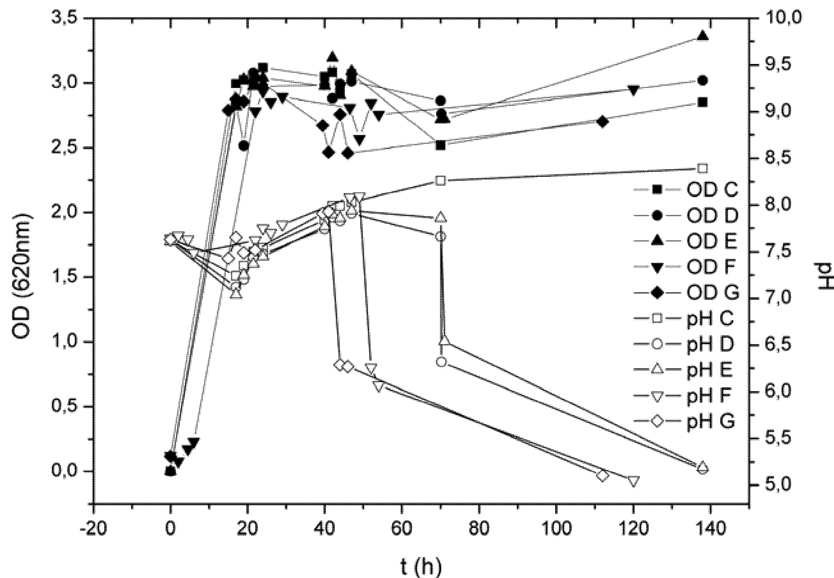

Figure 3: OD and pH of cultures C, D, E and F.

The effect of nutrient fed-batch was investigated, while the oxygen concentration was left uncontrolled, and its effect will be further investigated.

To verify the recovery of bioluminescence, a concentrate glucose solution was added when the culture reached a dim light emission. Very rapidly (a few minutes) in all culture (D, E, F, G) the emission increased to previous intensities and it was maintained through at least a few hours, while the control culture (C) wasn't able to recover the bioluminescent emission (Figure 3). Almost suddenly, after the glucose addition, pH dropped to acid values, probably because of the low pH of glucose solution. Despite this effect of acidification on the chemical level, there was a further decline in pH during the following hours of fermentation. It seems reasonable that this further acidification is due to glucose consumption (Figure 3). Ruby and Nealson showed that, growing *V. fischeri* on media containing glucose as carbon source, pyruvate tends to accumulate with a pH drop (Ruby and Nealson 1977). It is realistic that also in this case acidification is related to the glucose consumption, used mainly as energetic compound and not for bacterial growth, as shown by a little increase in the measured OD values. This observation is reinforced by the fact that the bioluminescence, which dissipates a large part of cellular energy, is recovered almost immediately in correspondence of the addition of nutrients.

Light emission was reduced a few hours after the feeding, maybe because the carbon source was depleted or was lower than a critical concentration. Oxygen, too, may have been limiting during the tests because its concentration in the flask was not controlled.

Furthermore, pH came close to acid values, unfavourable to light emission (Scheerer et al. 2006). Probably light emission was affected by all these parameters.

4. Conclusions

During these preliminary tests some bioluminescence controlling parameter of *V. fischeri* were investigated and some consideration on its growth were made.
First of all, the temperature of 20°C was examined with the growth and light emission expected, differently from 30°C that allow growth but not a stable bioluminescence over time. Moving forward from this considerations, in the next future other temperature values will be investigated with a useful comparison with environmental data.
Secondly, a precise and reproducible pH trend was seen, although with different preculture step a slight divergence was recorded.
Moreover, in order to obtain a stable bioluminescent light emission, a glucose fed-batch was investigated. This approach seemed to be effective, with a very rapid recovery of luminescence, but the adding of a not buffered solution dropped the medium pH. For this reason, to assess the effect of the recovery of the luminescence, and also the nutrients consumption, a buffered minimal medium can be used: nitrogen and carbon sources should be monitored and a useful correlation with bioluminescence and pH should be made. At the same time, oxygen influence, that wasn't controlled during tests, must be investigated too.

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