Developing novel agarose beads in mustard oil and their use in fast detection of BOD of industrial waste waters

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Introduction

Immobilization of the biological elements is a crucial step in the construction of biosensors. Immobilization has been defined as "*the confinement or localization of viable microbial cells to a certain defined region of space in such a way as to exhibit hydrodynamic characteristics which differ from those of the surrounding environment*"(Webb 1996). The most important part of any biosensor is the selective membrane/layer, usually positioned on the sensor tip, which interacts with the sample so as to provide analytically useful information about a particular component i.e., the analyte in the sample. This membrane must be immobilized in a manner such that the selective chemical processes occurring at the membrane-sample boundary are coupled to a transducer using either electronic or optical means in order to sense the response. The immobilization should not harm the biological element and should ideally contribute to its stability. Stability is particularly important in continuous environmental monitoring *in situ*. Selection of support material and method of immobilization is made by weighing the various characteristics and required features of the enzyme/cell application against the properties/limitations/characteristics of the combined immobilization/support. A number of practical aspects should be considered before embarking on experimental work to ensure that the final immobilized enzyme and/or cell preparation is fit for the planned purpose or application and will operate at optimum effectiveness (Nunez et al. 1987).

The immobilized microorganisms can be entrapped within a support (Jamuna & Ramakrishna, 1996), they can be adsorbed on to a support (Balfanz & Rehm 1991; Anselmo & Novais 1992) or can be covalently linked to the support with the help of a cross-linking agent. Despite such a vast array of techniques available for cell immobilization, none has been found suitable/apt for the construction of immobilized microbial membrane to be used in a BOD biosensor. This is so because for use in a BOD biosensor, the cells must be immobilized in/on a membrane such that the immobilized cell preparation can be attached firmly on the tip of an oxygen electrode (transducer), without losing any of their metabolic potentials. Moreover, the immobilization support must be mechanically strong, should not swell in aqueous solutions and should have good diffusional characteristics. In addition, the techniques used for immobilization must impart a good interaction between the catalysts and the support without causing any physical harm to the immobilized entity as well as should allow minimum/negligible leaching of the catalysts from the support. To provide good reproducibility and sensing characteristics to the BOD biosensor, the immobilized microbial membrane must have a long shelf-life, high viability and stability and should allow the effective diffusion of substrates in/out of the membrane.

In view of this, an attempt was made to select a novel support for developing cell beads sensor in conjunction with a novel immobilization methodology, which helped immobilize the cells by conferring increased stability, viability and negligible leaching to the developed sensor. Few samples from beverage and dairy waste water showed BOD values comparable to the conventional method.

Material and methods

Three bacteria namely, *Enterobacter sakazaki, Pseudomonas aeruginosa* and *Aeromonas sobria* were used for the preparation of cell beads. For this, individual bacterial strains were inoculated in

50 ml nutrient broth with 0.01% tween-80. All the cultures were incubated at 37^{o}C. for 16-18 hours in an incubator shaker at 150 rpm. Optical density of all the cultures was maintained to 1.0. Cells were harvested by centrifuging the bacterial suspension at 8000 rpm for 20 minutes at 4^{o}C. The pellet obtained was suspended in 1.0-2.0 ml of 10-100 mM phosphate buffer, pH 6.8 and recentrifuged at 8000 rpm for 20 minutes at 4^{o}C. The resultant pellet of individual bacteria was mixed with 3% agarose and extruded drop wise in mustard oil kept at a temperature ranging between 4-8^{o}C. The cell beads were first washed with petroleum ether and then rewashed 3-5 times with 10-100 mM phosphate buffer, pH 6.5-7.5. The cell beads thus obtained were stored in 10-100 mM phosphate buffer, pH 6.5-7.5 at a temperature preferably at 4^{o}C. The beads obtained were spherical, porous and stable and were used for further study.

Appropriate amount of the prepared cell beads was added in stirred phosphate buffer solution. For measuring the response a commercially available `DO` probe was immersed in the system. The system was covered with parafilm to render it a closed system. The probe was attached with a multimeter (Keithley) to note the readings. An external polarization voltage of -0.65 volts was applied to the system to provide the reduction of oxygen at the cathode.

The immobilized cell beads were checked for their response in terms of change in current using GGA as a reference standard in BOD analysis. For this, the electrode was dipped in stirred phosphate buffer solution, 50mM, pH6.8 containing the immobilized microbial beads. After a stable current was obtained, known strength of GGA was injected into the reaction assembly. Consumption of oxygen by the microbial cells immobilized in beads caused a decrease in dissolved oxygen in the system. This was elucidated by a gradual decrease in current until a stable value was attained.

The selected immobilized microbial consortium was stored at different temperatures i.e., 4-37 ^{o}C. for stability and viability studies. The membrane was also studied for leaching studies which is an important parameter for long shelf life of the biosensor.

Waste water samples were collected from beverage and dairy industry and BOD load was determined with the developed sensor as well as with the conventional method.

Results and Discussion

In the present study, cell beads sensor was developed by immobilizing the formulated microbial consortium on a suitable support i.e., agarose under a hydrophobic condition, useful for instant BOD estimation. The support used for the immobilization conferred high stability, viability and negligible leaching to the biocatalyst i.e., microorganisms. This could be attributed to the fact that the biocatalysts immobilized in hydrophilic supports under hydrophobic conditions confer least leaching to the immobilized biocatalyst.

The immobilized cell beads were checked for their response in terms of change in current using GGA as a reference standard in BOD analysis. The steady state indicated that the consumption of oxygen by the immobilized cell beads and the diffusion of oxygen from the solution to the beads is in equilibrium. As observed in the present course of study, the change in current is linearly related to GGA standard over the range of 30-300 mg/l (figure 1).

The selected immobilized microbial consortium when stored at different temperatures i.e., 4-37^{o}C for stability and viability studies (table 1), showed that the cell beads stored at 4^{o}C were more stable than the cell beads stored at other temperatures. In addition, the cell beads stored at 4^{o}C were more

viable in comparison to beads stored at temperature higher than 4°C. Negligible leaching was observed in case of beads prepared in the above mentioned manner.

S. No.	Time Days	Stability		Viability		Stability & Viability	Leach-ing*
		4°C	pH 6.8	4°C	pH 6.8	pH 6.8 /4°C Current Change (ΔI)	
1	0	++	++	+++	+++	1190	0.000
2	15	+++	+++	+++	+++	1192	0.008
3	30	+++	+++	+++	+++	1195	0.017
4	45	+++	+++	+++	+++	1194	0.045
5	60	+++	+++	+++	+++	1192	0.096
6	90	+	+	++	++	1195	0.0125
7	120	++	++	+	+	1198	0.130
8	150	+++	+++	+++	+++	1196	0.132
9	180	+++	+++	+++	+++	1196	0.141

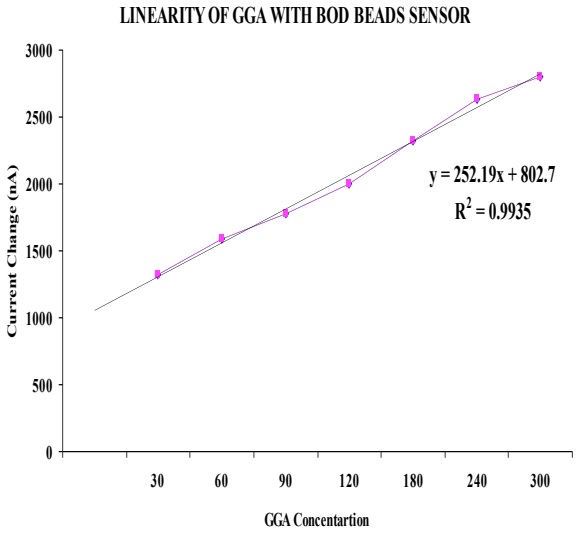

Figure1: Linearity of GGA with BOD bead sensor

* Optical density of storage buffer at 625nm
\+ 70-50% (Stability)
++ 90-70% (Stability)
+++ 100-90% (Stability)
\+ Poor Growth
++ Good Growth
+++ Excellent Growth

Table 1: Stability and viability of beads

Characterization of microbial membranes used for the estimation of BOD with a biosensor was reported for the first time by Galindo et al. (1992). A mixture of *Citrobacter* sp. and *Enterobacter* sp. was immobilized in Millipore filters. The sensor response was best when 0.5 mg cells were immobilized per cm^2 of membrane at 30°C, pH 7.0 using 0.05 M phosphate buffer. Shelf-life of these membranes was 20 days without appreciable loss of their response characteristics.

There is no report for the development of cell beads sensor using agarose as a support , for instant BOD estimation. On the contrary, there is only one report for the development of cell beads sensor for instant BOD estimation using supports other than agarose, such as kappa-carrageenan under hydrophilic conditions (Su et al. 1986). The limitations of such developed sensor is that it is used only for the BOD estimation of glucose-glutamic acid (GGA) covening a very low range i.e., up to a concentration of 24 mg/l.

Conclusions

The prepared immobilized cell beads when used in conjunction with an electronic device are capable of determining the BOD load of waste-waters instantly i.e., within 30-45 minutes as compared to the conventional method which gives BOD values within 3-5 days. The formulated selected microbial consortium of cell beads acts in a synergistic way and is capable of assimilating a vast array of organic compounds present in different types of industrial effluents. The method of gelling agarose in cold mustard oil, described is novel and cost effective. The support used for immobilization i.e. agarose is non-toxic to micro-organisms. Moreover, agarose is not utilizable by the microbial consortium in use, thereby not adding to BOD of the sample in any way. The prepared immobilized cell beads have long stability, viability and negligible leaching as compared to cell beads prepared using other supports. The immobilized microbial beads described in the invention do not act as barrier for the diffusion of oxygen through them, thereby aiding in instant BOD estimation.

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