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Review

Microbial biosensors

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Abstract

A microbial biosensor consists of a transducer in conjunction with immobilised viable or non-viable microbial cells. Non-viable cells obtained after permeabilisation or whole cells containing periplasmic enzymes have mostly been used as an economical substitute for enzymes. Viable cells make use of the respiratory and metabolic functions of the cell, the analyte to be monitored being either a substrate or an inhibitor of these processes. Bioluminescence-based microbial biosensors have also been developed using genetically engineered microorganisms constructed by fusing the *lux* gene with an inducible gene promoter for toxicity and bioavailability testing. In this review, some of the recent trends in microbial biosensors with reference to the advantages and limitations are been discussed. Some of the recent applications of microbial biosensors in environmental monitoring and for use in food, fermentation and allied fields have been reviewed. Prospective future microbial biosensor designs have also been identified.

Keywords: Microbial biosensors; Immobilised cells; Bioluminescence biosensors (cells); Permeabilised cells; Environmental biosensors; Food biosensors

1. Introduction

A biosensor is a device that detects, transmits and records information regarding a physiological or biochemical change. Technically, it is a probe that integrates a biological component with an electronic transducer thereby converting a biochemical signal into a quantifiable electrical response. Biosensors make use of a variety of transducers such as electrochemical, optical, acoustic and electronic (Turner et al., 1987; Blum and Coulet, 1991; Ivnitski et al., 1999). The function of a biosensor depends on the biochemical specificity of the biologically active material. The choice of the biological material will depend on a number of factors viz the specificity, storage, operational and environmental stability. Selection also depends on the analyte to be detected such as chemical compounds, antigens, microbes, hormones, nucleic acids or any subjective parameters like smell and taste. Enzymes, antibodies, DNA, receptors, organelles and microorganisms as well as animal and plant cells or tissues have been used as biological sensing elements. Some of the major attributes of a good biosensing system are its specificity, sensitivity, reliability, portability, (in most cases) ability to function even in optically opaque solutions, real-time analysis and simplicity of operation. A number of reviews and books dealing with these aspects (Turner et al., 1987; Blum and Coulet, 1991; Nikolelis et al., 1998; Ramsay, 1998; Mulchandani and Rogers, 1998; Rogers and Mulchandani, 1998; D'Souza, 2001) and a few reviews dealing specifically with microbial biosensors have appeared in the literature (Margineanu et al., 1985; Karube, 1990; Korpan and Elskaya, 1995; Corbisier et al., 1996; Nomura and Karube, 1996; Riedel, 1998; Arikawa et al., 1998; Simonian et al., 1998; Matrubutham and Sayler, 1998). The current review focuses on the use of microbial cells as biological sensing elements in biosensors and delineates some of the recent developments in that field.

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2. Use of microbial cells as biosensing elements

Microbes have a number of advantages as biological sensing materials in the fabrication of biosensors. They are present ubiquitously and are able to metabolise a wide range of chemical compounds. Microorganisms have a great capacity to adapt to adverse conditions and to develop the ability to degrade new molecules with time. Microbes are also amenable for genetic modifications through mutation or through recombinant DNA technology and serve as an economical source of intracellular enzymes.

Purified enzymes have been most commonly used in the construction of biosensors due to their high specific activities as well as high analytical specificity. Purified enzymes are, however, expensive and unstable, thus limiting their applications in the field of biosensors. Over 90% of the enzymes known to date are intracellular. In this respect, the utilisation of whole cells as a source of intracellular enzymes has been shown to be a better alternative to purified enzymes in various industrial processes (Bickerstaff, 1997; D'Souza, 1999). It avoids the lengthy and expensive operations of enzyme purification, preserves the enzyme in its natural environment and protects it from inactivation by external toxicants such as heavy metals. Whole cells also provide a multipurpose catalyst especially when the process requires the participation of a number of enzymes in sequence.

Whole cells have been used either in a viable or non-viable form. Viable cells are gaining considerable importance in the fabrication of biosensors (Burlage and Kuo, 1994; Riedel, 1998; Arikawa et al., 1998; Simonian et al., 1998). Viable microbes metabolise various organic compounds either anaerobically or aerobically resulting in various end products like ammonia, carbon dioxide, acids etc that can be monitored using a variety of transducers. Viable cells are mainly used when the overall substrate assimilation capacity of microorganisms is taken as an index of respiratory metabolic activity, as in the case of estimation of biological oxygen demand (BOD) or utilisation of other growth or metabolically related nutrients like vitamins, sugars, organic acids and nitrogenous compounds (Riedel, 1998). Another mechanism used for the viable microbial biosensor involves the inhibition of microbial respiration by the analyte of interest, like environmental pollutants (Arikawa et al., 1998).

The major limitation to the use of whole cells is the diffusion of substrate and products through the cell wall resulting in a slow response as compared to enzyme-based sensors (Rainina et al., 1996). One of the ways to obviate this problem is to use permeabilised cells. The cells can be permeabilised using physical (freezing and thawing), chemical (organic solvents/detergents) and enzymatic (lysozyme, papain) approaches (Felix, 1982; D'Souza 1989a; D'Souza, 1999; Patil and D'Souza, 1997). The most common technique uses organic solvents such as toluene, chloroform, ethanol and butanol or detergents like *N*-cetyl-*N*,*N*,*N*-trimethyl ammonium bromide (CTAB), Na-deoxycholate and digitonin (Patil and D'Souza, 1997). Such chemical treatment creates minute pores by removing some of the lipids from the cell membranes, thereby allowing for the free diffusion of small molecular weight substrates/products across the cell membrane while retaining most of the macromolecular compounds like the enzymes inside the cell. The permeabilisation process, however, renders the cell non-viable but can serve as an economical source of intracellular enzymes. They can be used for simple biosensor applications which do not require cofactor regeneration or metabolic respiration, viz glucose oxidase, β-galactosidase, amino acid oxidase, invertase etc (Mulchandani and Rogers, 1998; Svitel et al., 1998; D'Souza, 1999, 2001).

In the case of periplasmic enzymes such as invertase and catalase in yeast (D'Souza and Nadkarni, 1980; Svitel et al., 1998) and urease and phosphatases in bacteria (Kamath and D'Souza, 1992; Macaskie et al., 1992) whole cells can be used without permeabilisation. One of the recent advances is to engineer the cell to transport the intracellular enzyme and anchor it into the periplasmic space. Such an approach has been applied to obtain recombinant *Escherichia coli* cells with surface expressed oragnophosphorous hydrolase (OPH), an enzyme useful in the fabrication of biosensors for the detection of organophosphate compounds (Mulchandani et al., 1998a,b). These cells could degrade the organophosphates more efficiently (Mulchandani et al., 1998a,b) without the diffusional limitations otherwise observed in engineered cells expressing OPH intracellularly (Rainina et al., 1996). The above approach is an important development in the field of microbial biosensors as it provides a cell system with no membrane transport problems and at the same time will not affect the cellular structure and activity. This is in contrast to chemically permeabilised cells which result in loss of cell viability.

These types of genetic approaches may have major significance in the future, especially for sensors like BOD wherein polymers such as protein, starch, lipid etc have to be broken down to monomers before they can be metabolised. Recently, genetically engineered cells have been obtained for expression of cellulase activity on the cell surface. Such modified whole cells have been shown to hydrolyse the cellulose from the media (Murai et al., 1997) and can replace the use of acid-induced breakdown of biological polymers (currently practiced) prior to biosensor analysis. Alternatively, studies from our laboratory and others have shown the possibility of introducing a variety of lytic and other enzymes onto a cell wall surface through chemical or biospecific affinity techniques (D'Souza, 1989b, 2001).

Another limitation in using whole cells is the low specificity as compared to biosensors containing pure enzymes. This is mainly due to the unwanted side reactions catalysed by other enzymes in a cell. Several approaches are being investigated to minimise such non-specific reactions. Permeabilisation of the cell empties it of most of the small molecular weight cofactors etc, thus minimizing the unwanted side reactions (D'Souza, 1989a). Thus a whole cell of yeast containing intracellular β-galactosidase converts lactose to ethanol and CO_2 whereas the same cell on permeabilisation converts lactose only to glucose and galactose due to the loss of cofactors from the cell (Rao et al., 1988; Joshi et al., 1989). Side reactions, which can occur due to the presence of other enzymes in a cell, can also be minimised by inactivating such enzymes either by physical (heat) or chemical means when non-viable cells are used (Godbole et al., 1983; Di Paolantonio and Rechnitz, 1983; D'Souza, 1989a; Riedel, 1998). Another approach that is of significance in viable cell-based biosensors is the blockage of unwanted metabolic pathways or transport systems. Thus, for the determination of glutamic acid in the presence of glucose by *Bacillus subtilis*, the glucose uptake carrier system of the cell was blocked using a thiol inhibitor like chloromercuribenzoate and also the glycolysis was reversibly inhibited by NaF (Riedel and Scheller, 1987). Developments in rDNA technology may help in the future in producing whole cells rich in the enzyme of interest and also engineered to have minimal amounts of enzymes that might catalyse side reactions.

Selection of an appropriate culture is essential as the specific microbial species used in biosensors have characteristic substrate spectra which may or may not correspond well with the spectrum of compounds present in the sample. Adaptation of a microbe for induction of desirable metabolic pathways and uptake systems by cultivation in medium containing appropriate substrates may often be desirable (Di Paolantonio and Rechnitz, 1982; Riedel et al., 1990; Fleschin et al., 1998). For the biochemical degradation of complex substrates such a mixtures of phenols, the use of activated sludge obtained from waste treatment plants can serve as an acclimatised mixed microbial consortium as compared to pure cultures (Joshi and D'Souza, 1999). An activated sludge-based biosensor for BOD estimation has been recently reported (Liu et al., 2000). Microorganisms that are adapted to metabolise the compound of interest in the waste have also been isolated from activated sludge from respective waste treatment plants. A few examples include the actinomycetes-like organism capable of biosensing a broad spectrum of halogenated hydrocarbons (Peter et al., 1997a) and the linear alkyl benzene sulfonate-degrading organism for the fabrication of a biosensor for anionic surfactants (Nomura et al., 1994). Most of the aerobic microbial biosensors need a continuous supply of oxygen. Synthetic oxygen carriers like perfluorodecaline have been investigated for the hyperoxygenation of microbial biosensor systems (Reshetilov et al., 1998).

A single cell may quite often not contain all the enzymes necessary for a sequential set of reactions. In such cases, mixed microbial cultures have shown promise. Thus *Gluconobacter oxydans* containing glucose oxidase has been used in conjunction with *Saccharomyces cerevisiae* cells containing periplasmic invertase or permeabilised *Kluyveromyces marxianus* cells containing intracellular β-galactosidase, in the fabrication of a sucrose and lactose biosensor, respectively (Svitel et al., 1998). Alternatively, studies from our laboratory have shown the possibility of introducing a deficient enzyme by directly binding it on its cell wall surface. These include binding of glucose oxidase using lectins (concanavaline-A) or polyethylenimine (PEI) on microbial cells induced for invertase or β-galactosidase (D'Souza, 1989b; D'Souza and Melo, 1991).

Microbial biosensors based on light emission from luminescent bacteria are being applied as a sensitive, rapid and non-invasive assay in several biological systems (Burlage and Kuo, 1994; Matrubutham and Sayler, 1998). Bioluminescent bacteria are found in nature, their habitat ranging from marine (*Vibrio fischeri*) to terrestrial (*Photorhabdus luminescens*) environments. Bioluminescent whole cell biosensors have also been developed using genetically engineered microorganisms (GEM) for the monitoring of organic, pesticide and heavy metal contamination. The microorganisms used in these biosensors are typically produced with a constructed plasmid in which genes that code for luciferase are placed under the control of a promoter that recognises the analyte of interest. When such microbes metabolise the organic pollutants, the genetic control mechanism also turns on the synthesis of luciferase, which produces light that can be detected by luminometers. Facile detection of transcriptional activation is achieved by the fusion of a promoter element to reporter genes. A useful reporter system responsible for light emission is made up of five structural genes, *luxCDABE*, of the bioluminescence operon derived from marine bacterium *V. fischeri*. The *luxCDE* genes encode an enzyme complex (fatty acid reductase, synthetase and transferase) that synthesises the substrate (a fatty aldehyde) for luciferase, using precursors from the fatty acid cycle. The *luxAB* genes encode the luciferase enzyme (Meighen, 1994).

One approach to environmental monitoring is to detect changes in gene expression patterns induced by adverse conditions. Bacterial strains that increase light production in the presence of specific chemicals have been constructed using bioluminescence genes (*lux*) as reporters of transcriptional responses. A typical example is the *Pseudomonas fluorescens* HK44, a *lux*-based

bioluminescent bioreporter that is capable of emitting light upon exposure to naphthalene, salicylate and other substituted analogues. (Heitzer et al., 1994; Ripp et al., 2000). A complementary approach, not requiring prior knowledge of expected contaminants, uses less-specific stress responses as general indicators of deleterious conditions. For example, a large variety of environmental stresses trigger the heat shock response (Georgopoulos et al., 1994). Accordingly, *E. coli* strains containing the heat shock promoter *grpE*, *dnaK* or *lon* fused to the *lux* reporter increase the bioluminescence in response to many chemicals (Van Dyk et al., 1994; BenIsrael et al., 1998).

Cellular organelles can be considered to be multifunctional biocatalysts, intermediate in complexity between whole cells and enzymes. Their functions have been exploited in the fabrication of biosensors. Electrons generated during the primary light-induced charge separation steps in chloroplast can be transferred to an electrode. Some of the chemicals like herbicides, heavy metals, etc generally inhibit the photosytem II-dependent electron flow. This property has prompted researchers to develop biosensors using cellular organelles like chloroplasts or thylakoid membranes (Loranger and Carpentier, 1994; Rouillon et al., 1995).

In addition to cells and cellular organelles, plant tissues have shown potential as biological recognition elements in the fabrication of tissue-based biosensors. Some examples include, sugar beet slice for tyrosine, squash mesocarp slice for glutamate and peel of cucumber or squash for L-ascorbate and phenol, respectively (Macholan, 1987). Potato (*Solanum tuberosum*) slices, a rich source of polyphenol oxidase, have been used in conjunction with an oxygen electrode for the determination of mono and polyphenols like atrazine (Mazzei et al., 1995). A mushroom tissue-based biosensor for inhibitor monitoring has also been reported. (Wang et al., 1996). Recently, cultured human hepatoblastoma Hep G2 cells have been investigated for the development of a rapid and sensitive bioassay device for the on-site evaluation of environmental waters (Shoji et al., 2000).

3. Immobilisation of biomaterials

The basic requirement of a biosensor is that the biological material should bring the physico-chemical changes in close proximity of a transducer. In this direction, immobilisation technology has played a major role (D'Souza, 2001). Immobilisation not only helps in forming the required close proximity between the biomaterial and the transducer, but also helps in stabilising it for reuse. The biological material has been immobilised directly on the transducer or in most cases, in membranes, which can subsequently be mounted on the transducer. Biomaterials can be immobilised either through adsorption, entrapment, covalent binding, cross-linking or a combination of all these techniques (D'Souza, 1989a, 1999; Bickerstaff, 1997). A number of techniques have been developed in our laboratory for the immobilisation of viable and non-viable cells as well as cell–enzyme conjugates (D'Souza, 1989a, 1990, 1999, 2001). Selection of a technique and/or support would depend on the nature of the biomaterial and the substrate and configuration of the transducer used. The choice of support and technique for the preparation of membranes has often been dictated by the low diffusional resistance of the membrane (Kumar et al., 1992). Gentle techniques need to be applied when viable cell preparations are to be used. Covalent binding, a commonly used technique for the immobilisation of enzymes and antibodies, has not been useful for the immobilisation of cells. One of the general problems with covalent binding is that the cells are exposed to potent reactive groups and other harsh reaction conditions thus affecting their viability. There may also be a loss in the structural integrity of the cell during continuous use, leading to loss of intracellular enzymes. Among others is the very low cell loading that is achieved as compared to entrapment and other techniques.

Cross-linking using bifunctional reagents like glutaraldehyde has been successfully used for the immobilisation of cells in various supports. Of these, proteinic supports such as gelatine (Deshpande et al., 1986; Svitel et al., 1998), albumin (Loranger and Carpentier, 1994) and hen egg white (Marolia and D'Souza, 1994, 1999) have been extensively used. Even though this technique obviates some of the limitations of covalent binding, the chemical cross-linking reagents used often affect the cell viability. Thus cross-linking technique will be useful in obtaining immobilised non-viable cell preparations containing active intracellular enzymes. Stable microbial preparations are often required for use under varied environmental factors. Cross-linking has been extensively used for the stabilisation of enzymes (Tyagi et al., 1999). It has also been used for the stabilisation of cellular organelles to osmotic shock (D'Souza, 1983), prevention of lysis of extremely halophilic cells in low salt or salt free environments (D'Souza et al., 1992) and the prevention of lysis of microbial cells by lytic enzymes present in the processing streams (D'Souza and Marolia, 1999).

Entrapment and adsorption techniques are more useful when viable cells are used. A common approach is to retain the cells in close proximity to the transducer surface using membranes like the dialysis membrane. In general, the outer membrane must be chemically and mechanically stable, with a thickness of 10–15 μm and a pore size of 0.1–1.0 μm. Especially suitable are the so-called nuclear pore trace membranes made of poly-

carbonate or polyphthalate (Riedel, 1998). Microbial cells have been immobilised by entrapment in a variety of synthetic or natural polymeric gels for use in industrial processing (Bickerstaff, 1997; D'Souza, 1999; Ramakrishna and Prakasham, 1999). Some of these entrapment techniques have been used for biosensors. The synthetic polymers used for microbial biosensor applications include polyacrylamide (Deshpande et al., 1987; Peter et al., 1996), polyurethane-based hydrogels (Koenig et al., 1997a), photo cross-linkable resins (Fukui et al., 1987; Yang et al., 1997) and polyvinyl alcohol (Tag et al., 2000). Polyvinyl alcohol is one of the most widely studied polymers, as it can form membranes, fibres, etc. Enzymes and cells have been immobilised in these membranes either by entrapment, covalent binding, cross-linking, freezing and thawing, γ-irradiation, photo cross-linking or entrapment followed by cross-linking (Uhlich et al., 1996). However, the major limitation of synthetic polymer is the possible loss of viability of the cell. This is more pronounced when polyacrylamide gels are used (D'Souza, 1989a; Peter et al., 1996). Photo cross-linkable polyvinyl alcohol bearing styrylpyridinium groups has been shown to entrap cellular organelles and cells under very mild conditions, for use in biosensors (Rouillon et al., 1995, 1999). Polyacrylonitrile membranes (Ulbricht and Papra, 1997) and albumin–poly(ethylene glycol) hydrogel (D'Urso and Fortier, 1996), which have shown promise in the immobilisation of enzymes may also gain importance in microbial biosensors. Albumin–poly(ethylene glycol) hydrogels, in view of their biocompatibility, may be useful in the fabrication of in vivo implantable biosensors (D'Urso and Fortier, 1996).

Natural polymers used for the entrapment of the cells include alginate (Nomura et al., 1994; Schmidt et al., 1996), carrageenan (Peter et al., 1996), low-melting agarose (Mulchandani et al., 1998b), chitosan (Peter et al., 1997b) etc. These polymers are known to be very useful in obtaining viable cell-immobilised systems. Among these, entrapment in alginate by ionotropic gelation using a variety of divalent and trivalent cations has found extensive use in immobilised cell technology (Smidsord and Skjak-Braek, 1990). The major limitations of Ca-alginate gels are their destabilisation and subsequent solubilisation by the Ca-chelators present in the processing solution or waste. A novel technique has been developed in our laboratory for stabilising the alginate membranes and beads towards Ca-chelators by reinforcing them with gamma ray polymerised polyacrylamide (Gupte and D'Souza, 1999). A number of other techniques for the stabilisation of Ca-alginate have also been proposed (Smidsord and Skjak-Braek, 1990). Major limitation of entrapment technique is the additional diffusional barrier offered by the entrapment materials, which can be minimised by increasing the porosity of the matrix using open pore entrapment techniques (SivaRaman et al., 1982; Miranda and D'Souza, 1988). A highly porous sponge type proteinic matrix has been developed in our laboratory that allows for the diffusion of even bacterial cells into the vicinity of the bound enzyme (Marolia and D'Souza, 1999).

Sensitivity of the enzyme sensors has been improved by combining various types of mediators with enzymes (Mulchandani and Rogers, 1998). In the case of whole cells, the mediator should be able to shuttle the electrons between redox centers of intracellular enzymes and the electrode surface. Possibility of constructing such sensors by combining the whole cells of *Aspergillus niger* containing glucose oxidase and a mediator like ferrocene by entrapment in carbon paste has been demonstrated (Katrlik et al., 1997).

Passive trapping of cells into the pores or adhesion onto the surfaces of cellulose or other synthetic membranes has been well documented (Mulchandani and Rogers, 1998). The major advantage is that the cells immobilised through adhesion are in direct contact with the liquid phase containing the substrate, even though the cell and the liquid phase are distinctly separate thus reducing or eliminating the mass transfer problems commonly associated with gel entrapment methods. A basic limitation of passive trapping or adhesion, however, is the possibility of cell wash out during continuous use. Novel techniques have been developed in our laboratory for immobilising viable or non-viable cells through adhesion on a variety of polymeric surfaces including glass, cotton fabric and synthetic polymeric membranes using PEI (D'Souza et al., 1986; D'Souza and Kamath, 1988; D'Souza, 1990; Kamath and D'Souza, 1992; Melo and D'Souza, 1999). The adhesion is found to be rapid and the cells adhere as a monolayer. The adhesion being very strong, the high ionic concentrations and extreme pH conditions which normally disrupt the ionic interactions fail to desorb the cells. Cells can be adhered by coating the cells, the supports or both with PEI (D'Souza et al., 1986; D'Souza and Kamath, 1988). Viability of the cell was not affected by this treatment. A variety of cells containing different enzymes including urease (Kamath and D'Souza, 1992) have been immobilised using this technique and studies are underway for use of such biofilms in the fabrication of microbial biosensors (D'Souza, 2001). Recently, Nandakumar and Mattiasson (1999a) have applied the PEI technique developed in our laboratory for the fabrication of a microbial biosensor for the on-line monitoring of phenolic compounds. Glucose oxidase bound on cheesecloth using PEI has been used in the fabrication of a glucose sensor in our laboratory (Kumar et al., 1992, 1994).

Biospecific reversible immobilisation using lectins has been used for the introduction of biological catalysts into analytical systems (Mattiasson, 1982). The basic

advantage is that when the enzyme activity goes below the practical limits, the bound enzyme can be easily eluted and the membranes or the transducer surface can be loaded with a fresh batch of the enzyme for reuse without significantly affecting the transducer or membrane characteristics. Reversible immobilisation through hydrophobic interaction has also shown promise (D'Souza and Deshpande, 2001). As microbial cell surfaces contain a number of biospecific affinity binding or hydrophobic sites, this approach can be used in the future for the reversible introduction of microbial cells on transducer surfaces (Margineanu et al., 1985; D'Souza, 1999).

4. Microbial biosensors for environmental applications

The major application of microbial biosensors is in the environmental field (Gaisford et al., 1991; Brooks, 1994; Rogers and Williams, 1995; Corbisier et al., 1996; Marco and Barcelo, 1996; Rogers and Koglin, 1997; Rogers, 1998; Nikolelis et al., 1998; Rogers and Gerlach, 1999; Bilitewski and Turner, 2000). Microbial biosensors have been developed for assaying BOD, a value related to total content of organic materials in wastewater. BOD sensors take advantage of the high reaction rates of microorganisms interfaced to electrodes to measure the oxygen depletion rates. Standard BOD assay requires 5 days compared to 15 min for a biosensor-based analysis (Marty et al., 1997). Ever since the first report of such a microbial sensor by Karube et al. (1977) was made, a large number of papers have appeared in this field and is perhaps, the most extensively investigated microbial biosensor. Few of the recent advances are summarised in Table 1. A variety of microbes as pure cultures or as a consortium have been developed (Liu et al., 2000). One of the major criteria in the selection of these microbes is that they should be able to utilise a very broad range of substrates as discussed above. The biosensor should also be stable to environmental adversaries such as heavy metal toxicity, salinity etc. Recent studies on a salt tolerant dimorphic (budding and mycelial) yeast *Arxula adeninivorans* LS3 has shown promise for BOD measurements even in salt water (Riedel et al., 1998; Tag et al., 1999; Chan et al., 1999). This biosensor has been applied for BOD estimations of real samples of coastal and island regions (Lehmann et al., 1999). Unlike the sensor containing budding yeast which can also be used to measure BOD in salt water up to 10% (w/v); the mycelial sensor was not influenced by NaCl even up to 30% (w/v) (Tag et al., 2000).

The first commercial BOD sensor was produced by the Japanese company Nisshin Electric in 1983 and a number of other commercial BOD biosensors based on viable microbial cells are being marketed by Aucoteam, GmbH, Berlin; Prufgeratewrk, Medingen GmbH, Dresden; and Dr Lange, GmbH, Berlin. Another BOD sensor based on a soil bacterium *Pseudomonas putida* capable of determining low BOD levels in river water and secondary effluents and exhibiting negligible response to interference by chloride and heavy metals has been reported (Chee et al., 1999). The instrument is commercially available through Central Kagaku Corp., Tokyo. The use of these devices has been incorporated into industrial standard methods in Japan (Rogers and Gerlach, 1999).

Other advances include the development of a disposable BOD sensor (Yang et al., 1996). Significant efforts have been made towards the development of a portable BOD biosensor system incorporating disposable electrodes. Miniature Clark-type oxygen electrode arrays were fabricated using thin film technology for mass production with assured quality (Yang et al., 1997). A microbial biosensor consisting of an oxygen microelectrode with microbial cells immobilised in polyvinyl alcohol has been fabricated for the measurement of bioavailable organic carbon in oxic sediments. The biosensor allows the estimation of available dissolved organic carbon in sediment profiles on a microscale (Neudoerfer and Meyer, 1997). Optical fibre (Preininger et al., 1994) and calorimetry (Weppen et al., 1991) based transducers have been used in BOD biosensors.

Microbial biosensors have been investigated for a variety of other environmental applications (Table 1). Halogenated hydrocarbons used as pesticides, foaming agents, flame-retardants, pharmaceuticals and intermediates in the polymer production are one of the largest group of environmental pollutants. Microbial bioassays using immobilised cells of *Rhodococcus* strain containing alkyl-halidohydrolase has been described by Hutter et al. (1995). The enzyme present in the cell liberates halogen ions from halogenated hydrocarbons. These studies were extended in the fabrication of a microbial sensor (Peter et al., 1996). The sensor can be stored in the dry form at 277 K for 1 week. Major disadvantage was the additional preincubation period of 30 min that is required before the electrode potential attains stability (Peter et al., 1996). More recently a gram-positive actinomycete-like organism, exhibiting a broad spectrum for the dehalogenation of halogenated hydrocarbons, has shown better promise and may have potential in the fabrication of a broad specificity biosensor for halogenated hydrocarbons (Peter et al., 1997a). The strain of *Xanthobacter autotrophicus* GJ 10, a nitrogen-fixing microbe can utilise 1,2-dichloroethane as the sole source of carbon and energy. The bacterium is known to form two halogenases. One is specific for the dehalogenation of halogenated alkanes, other converts halogenated carboxylic acids by hydrolytic cleavage into corresponding alcohol and halogen ion. The cells immobilised in chitosan beads have been used in a semi-

Table 1
Microbial biosensors for environmental applications[a]

Analyte	Microorganism	Transducer/immobilisation	Detection limit	Reference
BOD	*Trichosporum cutaneum*	Miniature oxygen electrode (UV cross-linking resin (ENT-3400)	0.2–18 mg/l	Yang et al. (1996)
BOD	*T. cutaneum*	Miniature oxygen electrode array (photo cross-linkable resin)	<32 mg/l	Yang et al. (1997)
BOD	*T. cutaneum*	Oxygen electrode (entrapment)	10–70 mg/l	Marty et al. (1997)
BOD	*Ps. putida*	Oxygen electrode (adsorption on porous nitro cellulose membrane)	>0.5 mg/l	Chee et al. (1999)
BOD	Activated sludge (mixed microbial consortium)	Oxygen electrode/flow injection system (entrapped in dialysis membrane)	>3.5 mg/l	Liu et al. (2000)
BOD	Salt tolerant mycelial yeast *A. adeninivorans* LS3	Oxygen electrode (PVA)	2.61–524 mg/l	Tag et al. (2000)
Bioavailable organic carbon in oxic sediments	Yeast cells	Oxygen electrode (PVA)	Microscale	Neudoerfer and Meyer (1997)
Aniomic surfactants (linear alky benzene sulfonates (LAS)	LAS degrading bacteria isolated from activated sludge	Oxygen electrode, (reactor type sensor, ca-alginate)	<6 mg/l	Nomura et al. (1994)
Acrylamide; acrylic acid	*Brevibacterium* sp.	Oxygen electrode (free cells)	0.01–0.075 and 0.01–0.1 g/l	Ignatov et al. (1997)
Phenolic compounds	*Ps. putida*	Oxygen electrode (reactor with cells adsorbed on PEI glass)	100 μM	Nandakumar and Mattiasson (1999a)
Nitrite	*Nitrobacter vulgaris* DSM10236	Oxygen electrode (adsorption on Whatman paper)	>10 μM	Reshetilov et al. (2000)
Cyanide	*S. cerevisiae*	Oxygen electrode (PVA)	0.15–15 nM	Ikebukuro et al. (1996)
Chlorophenols	*Rhodococcus* sp.; *Trichosporon beigelii*	Oxygen electrode (PVA)	0.004–0.04 and 0.002–0.04 mM	Riedel et al. (1993, 1995)
3-Chloro-benzoate	*Ps. putida*	Oxygen electrode (PVA)	40–200 μM	Riedel et al. (1991)
Chlorinated and brominated hydrocarbons (1-chlorobutane and ethylenebromide)	*Rhodococcus* sp. DSM 6344	Ion selective electrodes (alginate)	0.22 and 0.04 mg/l	Peter et al. (1996)
Polycyclic aromatic hydrocarbons (Naphthalene)	*Sphingomonas yanoikuyae* B1 or *Ps. fluorescens* WW4	Oxygen electrode (polyurethane based hydrogel)	0.01–3.0 mg/l	Koenig et al. (1996, 1997a)
Organophosphate nerve agents (paraxon, methyl parathion, diazinon)	GEM[b] *E. coli* (organophosphorous hydrolase)	Potentiometric (adsorption on electrode surface)	0.055–1.8, 0.06–0.91 and 0.46–8.5 mM	Mulchandani et al. (1998a)
Organophosphate nerve agents (paraxon, parathion, coumaphos)	GEM[b] *E. coli* (organophosphorous hydrolase)	Fiber-optic (agarose)	0.0–0.6, 0.0–0.03 and 0.0–0.075 mM	Mulchandani et al. (1998b)
Pollutants such as diuron and mercuric chloride	*Synechococcus* sp. PCC 7942	Photoelectrochemical (photo cross linkable PVA bearing styrylpyridium group)	0.2 and 0.06 μM	Rouillon et al. (1999)
Herbicides[c] (diuron and atrazine)	Chloroplast/thylakoid membranes	Pt-electrode in microelectrochemical cell (photo cross linkable PVA bearing styrylpyridium group	2×10^{-5} and 2×10^{-4} mM	Rouillon et al. (1995)
Mono and polyphenols[c] (atrazine)	Potato (*S. tuberosum*) slices (polyphenol oxidase inhibition)	Oxygen electrode (tissue slice sandwitched between membranes)	20–130 μM	Mazzei et al. (1995)

[a] Polyvinyl alcohol.
[b] Genetically engineered microbes.
[c] Tissues or cellular organelle based.

continuous system for the detection of halogenated short-chain hydrocarbons in water samples (Peter et al., 1997b).

Polycyclic aromatic hydrocarbons (PAH) are carcinogenic compounds which are ubiquitous and especially found around contaminated areas of closed-down gas works and cooking plants. Naphthalene being highly water soluble has been found in contaminated soils. Amperometric biosensors for naphthalene were developed using either *Sphingomonas* sp. B1 or *Ps. fluorescens* WW4 cells immobilised within a polyurethane-based hydrogel. These were tested in a flow-through system and a stirred cell (batch method) and were shown to be equally suited for the quantification of naphthalene in aqueous solutions. The sensors had an operational lifetime of up to 20 days (Koenig et al., 1996). A similar biosensor has also been made using immobilised *Sphingomonas yanoikuyae* B1 (previously *Sphingomonas* sp B1) for the detection of naphthalene and phenanthrene (Koenig et al., 1997a). Other microbial sensors for xenobiotics include phenol, chlorinated phenols, polychlorinated biphenyls, benzene, 3-chlorobenzoate, etc (see Table 1) (Riedel, 1998).

Acrylamide, acrylic acid and acrylonitrile are widely used in the chemical industry for the production of various polymers, fibres and resins. Microbial assays, which may have potential in the fabrication of biosensors, have been reported for their quantification in the wastewaters. The approach makes use of the total respiratory activity of *Brevibacterium* sp. for acrylamide and acrylic acid when both the compounds are present in the waste. In the absence of acrylamide, cells show respiratory activity towards acrylic acid alone (Ignatov et al., 1997). These cells do not possess respiratory activity towards acrylonitrile, thus they do not interfere in the assay. Acrylonitrile, however, could be separately measured using *Pseudomonas pseudoalcaligenes* (Ignatov et al., 1996, 1997).

Organophosphorous compounds widely used as pesticides, insecticides and chemical warfare agents have created public concern because of their widespread use and toxicity. A variety of enzyme sensors based on acetyl choline esterase (Palchetti et al., 1997) and OPH (Mulchandani et al., 1999) have been reported. Biosensors based on genetically modified microbial cells with surface expressed OPH have been recently used in the construction of potentiometric (Mulchandani et al., 1998a) as well as a fibre-optic based microbial biosensor (Mulchandani et al., 1998b).

Nitroaromatic compounds (nitrophenols, picric acid, trinitrotoluene and similar compounds) represent a wide-spread group of xenobiotics present in wastes of chemical armament plants as well as civil factories manufacturing dyes, pesticides and other chemicals. One of the major microbial degradative products of nitroaromatics is nitrite and this is used to monitor concentrations of such pollutants. *Nitrobacter* sp. possessing high nitrite oxido reductases has been described (Sundermeyer-Klinger et al., 1984). Among them, the mixotrophs have been characterised by high selectivity and sensitivity to nitrite due to the induction of nitrite oxidoreductase during mixotrophic cultivation. In contrast to obligate autotrophs, the mixotrophic bacteria will not be liable to repression by organic compounds in the waste sample. In this respect, the mixotrophic strain of *N. vulgaris* has shown promise in the development of a biosensor possessing high selectivity with a lower limit of detection of 10 μM (Reshetilov et al., 2000).

The ability of cyanide to inhibit *S. cerevisiae* respiration has been utilised in developing a flow-type cyanide sensor (Ikebukuro et al., 1996). The sensor was stable for about 16 days. A microbial sensor for measuring inhibitors and substrates for nitrification in wastewater has been reported by Koenig et al. (1997b). For the determination of phytotoxicity, isolated chloroplasts or photosynthetic membranes have been used. Major limitation of this approach, however, is the difficulty in their isolation. These problems have now been obviated by the use of photosynthetic cyanobacterium such as *Synechococcus* sp. (Rouillon et al., 1999). A microbial assay system based on photoelectrochemical cell for detecting pollutant-induced effects on the activity of the cyanobacterium immobilised in polyvinylalcohol-bearing styrylpyridinium groups has shown promise. The measurements achieved with diuron and mercuric chloride has indicated future prospects of this bioassay for the detection of pollutants inhibiting photosynthetic electron flow (Rouillon et al., 1999).

5. Applications of microbial biosensors in food, fermentation and allied fields

In recent years, the demand for quick and specific analytical tools for food and fermentation analysis has increased and is still expanding. Both industry and government health agencies require a wide array of different analytical methods in the quality assurance of food materials. Analysis is needed for monitoring nutritional parameters, food additives, food contaminants, microbial counts, shelf life assessment and other olfactory characteristics like smell and odour. A variety of sensors based on enzymes and antibodies (Wagner and Schmid, 1990; Ramsay, 1998; Mulchandani and Rogers, 1998; Rogers and Mulchandani, 1998) as well as electronic noses (Pavlou and Turner, 2000; Magan and Evans, 2000) have been reported. Microbial biosensors have also shown potential in food analysis (Mulchandani and Rogers, 1998). A few typical examples have been summarised in Table 2 with special reference to current developments.

Table 2
Applications of microbial biosensors in food, fermentation and allied fields

Analyte	Microorganism	Transducer/immobilisation	Detection limit	Reference
Alcohol	*Candida vini*	Oxygen electrode (porous acetyl cellulose filter)	2×10^{-2} –2×10^{-1} mM	Mascini et al. (1989)
Glucose	*A. niger* (glucose oxidase)	Oxygen electrode (entrapment in dialysis membrane)	>1.75 mM	Katrlik et al. (1996)
Glucose, sucrose, lactose	*G. oxydans* (D-glucose dehydrogenase),*S. cerevisiae* (invertase), *K. marxianus* (β-galactosidase)	Oxygen electrode (gelatine)	up to 0–0.8 mM	Svitel et al. (1998)
Sugars (glucose)	Psychrophilic *D. radiodurans*	Oxygen electrode (agarose)	0.03–0.55 mM	Nandakumar and Mattiasson (1999b)
Short chain fatty acids in milk (butyric acid)	*A. nicotianae* (acyl-CoA oxidase)	Oxygen electrode (Polyvinyl alcohol)	0.11–1.7 mM	Ukeda et al. (1992a,b)
Short chain fatty acids in milk (butyric acid)	*A. nicotianae* (acyl-CoA oxidase)	Oxygen electrode (Ca-alginate)	9.5–165.5 μM	Schmidt et al. (1996)
Phosphate	*Chlorella vulgaris*	Oxygen electrode (polycarbonate membrane)	8–70 mM	Matsunaga et al. (1984)
CO_2	CO_2 utilizing autotropic bacteria (*Pseudomonas*)	Oxygen electrode (bound on cellulose nitrate membrane)	0.2–5 mM	Suzuki and Karube (1987)
Vitamin B-6	*S. uvarum*	Oxygen electrode (adsorption on cellulose nitrate membrane)	0.5–2.5 ng/ml	Endo et al. (1995)
Vitamin B-12	*E. coli*	Oxygen electrode (trapped in porous acetyl cellulose membrane)	$5–25\times10^{-9}$ mM	Karube et al. (1987)
Peptides (aspartame)	*B. subtilis*	Oxygen electrode (filter paper strip and dialysis membrane)	0.07–0.6 mM	Renneberg et al. (1985)
Phenylalanine	*P. vulgaris* (Phenylalanine deaminase)	Amperometric oxygen electrode (Ca-alginate)	2.5×10^{-2}–2.5 mM	Liu et al. (1996)
Pyruvate	*Streptococcus faecium* (Pyruvate dehydrogenase complex)	CO_2 gas sensing electrode (direct immobilisation on sensor membrane)	0.22–32 mM	Di Paolantonio and Rechnitz (1983)
Tyrosine	*A. phenologenes* (Tyrosine-phenol lyase)	NH_3 gas sensing electrode (direct immobilisation on sensor membrane)	8.2×10^{-2}–1.0 mM	Di Paolantonio and Rechnitz (1982)
Enalapril maleate (angiotensin)	*B. subtilis*	Oxygen electrode	–	Fleschin et al. (1998)

Monitoring the quality of milk is an important parameter because present methods involve bulk collection and prolonged storage. Rancidity or off-flavour in milk and milk products is caused by the liberation of short-chain fatty acids (C_4–C_{12}). Unlike other microorganisms, *Arthrobacter nicotianae* has been shown to possess enzymes of the β-oxidation pathway with a high specificity towards short-chain fatty acids. These cells have been used in conjunction with an oxygen electrode for the fabrication of a microbial sensor in flow injection analysis of short-chain free fatty acids in milk (Ukeda et al., 1992a,b, 1994). Schmidt et al. (1996) have reported a microbial biosensor based on thick film technology using *A. nicotianae*, immobilised in Ca-alginate, directly on the oxygen electrode surface. The sensor was used in a batch system for the determination of free fatty acids in milk. No sample pretreatment was necessary and the sensor exhibited a response time of only 3 min.

A number of reports are available on microbial biosensors for amino acids such as tyrosine (*Aeromonas phenologenes*), tryptophan (*Ps. fluorescens*) and glutamic acid (*B. subtilis*). (Riedel, 1998; Simonian et al., 1998). Determination of phenylalanine is needed not only for the process control of the phenylalanine fermentation but also for the neonatal diagnosis and dietary management of hyperphenylalaninaemia. A microbial biosensor based on *Proteus vulgaris* cells immobilised in Ca-alginate on an amperometric oxygen electrode has been reported (Liu et al., 1996). Phenylalanine deaminase present in the cell oxidises phenylalanine to phenylacetic acid.

Microbial sensors have been developed for the determination of vitamins such as vitamin B-12 (*E.coli* L15) and ascorbic acid (*Enterobacter agglomerans*) (Riedel, 1998). Such biosensor systems, for rapid determination of vitamin B-6, based on the respiratory activity of an immobilised yeast (*Saccharomyces uvarum*) and Clark-type oxygen electrode has been reported. (Endo et al., 1995). It provided rapid and simple determinations of vitamin B-6 in marine products at ng/ml levels (cf Table 1) with good correlation to traditional microbial assay values. Another use of microbial biosensor in the food

industry is for the determination of sulphite in various foods with the help of *Thiobacillus thiooxidans*. This microbial sensor has been investigated for the determination of total sulphite in 40 kinds of foods including dried vegetables (Matsumoto et al., 1996a,b). Some of the environmental microbial biosensors discussed above can also find applications in the determination of pesticides, insecticides, heavy metals and other chemical contaminants present in agricultural products or processed foods. Microbial sensors for the determination of sugars such as glucose, sucrose and lactose and other compounds like iron (*T. ferrooxidans*), ammonia (*B. subtilis*), acetic acid (*Trichosporon brassicae*), pyruvate, phosphate, peptide sweetener-aspartame, alcohol, etc have also been reported (cf Table 1 and Riedel, 1998).

In pharmaceuticals and medicine, the microbial based sensors have been studied for the estimation of steroids such as cholesterol (*Nocardia erythropolis*), androstendione (*N. erythropolis*), testosterone (*N. erythropolis*); antibiotics such as nystatin (*S. cerevisiae*) and other compounds like gonadotropin releasing hormone (*B. subtilis*), urea (nitrifying bacteria), uric acid (*Altenaria tennis*), creatinine (*Nitrobacter* sp.) and iron (II and III) (*T. ferrooxidans*) (Riedel, 1998). Enalapril maleate (EMa) belongs to a new class of antihypertensive agents known as angiotensin converting enzyme (acetylcholine esterase) inhibitors. A microbial biosensor for EMa using induced *B. subtilis* cells has been recently developed (Fleschin et al., 1998). This biosensor measures the acceleration of respiration during specific metabolic pathways of this drug and has been applied, with good results, for the determination of the active ingredient in the pharmaceutical tablet formulations.

6. Applications of bioluminescence-based biosensors

An important attribute of a biosensor is the real-time process monitoring. This is necessary for monitoring fermentors and wastewater biotreatment plants (WWBP) (Scheper and Lammers, 1994). Prompt warning of upsets in WWBP are important because they circumvent the cost and time involved in reactivating or reinitiating the WWBP after shut down. A frequent cause of upsets is the inflow of highly toxic materials, including heavy metals and organic chemicals, which exhibit toxicity to biota in a WWBP. Development of an early warning system would be useful in solving these problems. The bioluminescent reporter has advantageous properties such as rapid response, excellent sensitivity, large dynamic range and non-invasive continuous measurements amenable to automated data collection with minimal manipulations. Accordingly, a microbial assay of metabolic death by loss of bioluminescence from the marine microorganism *Photobacterium phosphoreum* has been commercialised as the Microtox system (Munkittrick et al., 1991). Quantification using bioluminescent microbial biosensors, which are based on heat shock gene-bioluminescence gene fusions has been proposed to be most suitable to face these challenges (Van Dyk et al., 1994; Gu et al., 1996; Rupani et al., 1996).

The *lux* gene reporters have been shown to be useful in understanding plant pathogenesis, rhizosphere bacterial colonisation, mutagenesis and dynamics of gene induction and expression (Burlage and Kuo, 1994; Matrubutham and Sayler, 1998). A few of the recent developments have been summarised in Table 3. Monitoring of the PAH-like naphthalene and salicylate using the genetically modified *Ps. fluorescens* HK44 has been extensively studied (Heitzer et al., 1994; Webb et al., 1997). This strain harbours the bioluminescent reporter plasmid pUTK21 that contains a *nahG-luxCDABE* fusion in a salicylate inducible operon. The use of *Ps. fluorescens* HK44 as a real time reporter has been demonstrated in both online and in situ studies (Heitzer et al., 1994; Ripp et al., 2000). In order to develop HK44 biosensor, its characteristics have been studied using a packed bed reactor with alginate-immobilised *Ps. fluorescens* HK44. Using this system *Ps. fluorescens* HK44 could be well characterised for modelling, which should be helpful in biosensor design (Webb et al., 1997). In October 1996, the US Environmental Protection Agency approved the release of HK44 into contaminated soil ecosystems as a means of monitoring PAH bioavailability during a long-term bioremediation process (Sayler et al., 1999). Procedures are being developed to enumerate HK44 cell numbers in soil under long-term (over 2 years) field conditions (Ripp et al., 2000).

Bioluminescent microbial biosensors have been extensively investigated for the detection of heavy metals like Hg^{2+} (Selifonova et al., 1993)Zn^{2+} (Erbe et al., 1996), chromate–copper-arsenate (Cai and DuBow, 1997), Ni^{2+} and chromate (Peitzsch et al., 1998), antimonite and arsenite (Ramanathan et al., 1997) and Cd^{2+} and Pb^{2+} (Tauriainen et al., 1998). In recent years, the scientific validity for using total metal concentrations that are measured using chemical methods, as a basis for metal limits in soil, has been questioned. This is mainly due to the fact that a considerable amount of metal will be in a bound form rendering it non-available for biological processes. Hence luminescence-based microbial biosensors that are relevant to soil ecosystems have been developed (Paton et al., 1997; BenIsrael et al., 1998) and have shown to be good predictors of the bioavailable fraction of metals in soils (McGrath et al., 1999). Recently, Preston et al. (2000), assessed the toxicity of heavy metals like Zn, Cu, and Cd, alone and in combination, using two luminescent microbial biosensors from different ecological niches:

Escherichia coli HB101 pUCD607 and *Ps. fluorescens* 10586 pUCD607. Significant synergistic interactions occurred between the toxic effects of Zn and Cu and Zn and Cd combinations (Preston et al., 2000).

Comparative assessment of the toxicity of a paper mill effluent containing heavy metals like Cd and organic toxicant such as pentachlorophenol by respirometry and luminescent bacterial assay has been studied by Brown et al. (1996). The luminescent assay using genetically modified (luminescence-marked) terrestrial bacteria was found to have greater sensitivity and reproducibility than the respirometric analysis, although both techniques were sensitive to the presence of pollutants. The toxicity of two common organotin pollutants and their initial breakdown products (tributyltin, dibutyltin, triphenyltin and diphenyltin) were assessed in extracted soil solutions using two different bioluminescent microbial biosensors: Microtox and *lux*-modified *P. fluorescens* pUCD 607 (Bundy et al., 1997). The two biosensors showed different response patterns, with Microtox being more sensitive to the triorganotins and *P. fluorescens* being more sensitive to the diorganotins.

Biocides are used in industry to prevent microbial growth in cooling towers and cutting fluids. Their concentration must be carefully monitored to assure that effective levels are maintained. The response of bioluminescent strain of *E. coli*, to several biocides has been studied and was found to be rapid and sensitive to concentrations of biocides in parts per million (Fabricant et al., 1995). Bioluminescence has also been used for the detection of GEM released in the environment (Shaw et al., 1992). Solvents, crop protection chemicals etc have also been monitored based on *E. coli* heat shock promoters, fused with *lux* genes (Van Dyk et al., 1994; Kobatake et al., 1995; Gu et al., 1996; Rupani et al., 1996).

A eukaryotic microbial biosensor for cytotoxicity analysis has been recently reported. The yeast *S. cerevisiae* was genetically modified to express firefly luciferase, generating a bioluminescent yeast strain. This strain senses chemicals known to be toxic to eukaryotes in samples assessed as non-toxic by prokaryotic biosensors (Hollis et al., 2000).

A number of methods described in the literature to monitor cellular metabolism in bacterial cultures (Scheper and Lammers, 1994) are laborious and often monitor either growth, viability or metabolism of microbial cultures. Bioluminescence is therefore gaining considerable importance (Unge et al., 1999; Marincs, 2000). In a typical study involving growth of *E. coli* using the *lux* phenotype as an indicator, it was observed that light emission increased rapidly up to late log phase of growth. However, towards the end of cultivation, light emission of the cultures decreased to undetectable levels, but the colony forming units were not affected. This indicated that despite the cellular metabolic activity being completely absent, cells retained their ability to be cultured (Marincs, 2000).

Table 3
Luminescence-based genetically engineered microbial biosensors

Application	Microorganism	Reference
Monitoring toxicity of compounds to eukaryotes	*S. cerevisiae* was genetically modified to express firefly luciferase	Hollis et al. (2000)
On-line monitoring of microbial growth	*E. coli* engineered for constitutive bioluminescence	Marincs (2000)
Toxicity of Zn, Cu and Cd, alone or in combination	*E.coli* HB101 and *Ps. fluorescens* 10586 genetically modified with *luxCDABE*	Preston et al. (2000)
Polycyclic aromatic hydrocarbons	*Ps. fluorescens* HK44 genetically modified with *luxCDABE*	Webb et al. (1997), Sayler et al. (1999), Ripp et al. (2000)
Ecotoxicity assessment of organotins and their initial breakdown products (tributyltin, dibutyltin, triphenyltin and diphenyltin)	Microtox and *luxCDABE* modified *Ps. fluorescens*	Bundy et al. (1997)
Ethanol as a model toxicant	*E. coli* TV1061, harboring the plasmid pGrpELux5	Gu et al. (1996), Rupani et al. (1996)
Monitoring of biocides	Bioluminescent strain of *E. coli* produced by recombinant DNA technology	Fabricant et al. (1995)
Metals, solvents, crop protection chemicals etc	*E. coli* heat shock promoters, *dnaK* and *grpE* were fused with *lux* genes of *V. fischeri*	Van Dyk et al. (1994)
Identifying constraints to bioremediation of BTEX-contaminated sites[a]	*luxCDABE* modified *Ps. fluorescens*	Sousa et al. (1998)
Assessment of the toxicity of metals in soils amended with sewage sludge	*luxCDABE* modified *Ps. fluorescens*	McGrath et al. (1999)

[a] Benzene, toluene, ethylbenzene,xylene.

7. Future microbial biosensor design strategies

As the biosensor technology starts moving from the proof-of-concept stage to field testing under realistic processes or waste monitoring conditions, the need for stable biological materials will become important. Biomaterials that are stable and functional in highly acidic, alkaline, hydrophobic, saline, oxidising, low and high temperature environments as well as immune to toxic substrates in the processing stream will play an important role (Gibson, 1999; D'Souza, 2001). A varied population of microrganisms like the thermophiles, alkalophiles, halophiles, psychrophiles, metallophiles, osmophiles, etc will gain importance in the successful applications of microbial sensors in realistic conditions. Selective screening methods for discovering the rare genera and species of microbes containing novel analytically useful enzymes are essential (Ogawa and Shimizu, 1999). Equally important is the need for an integrated approach to combine classical microbiology with developments in modern biotechnology (Srinivasan, 1994). Some of these aspects have been recently reviewed (D'Souza, 2001).

Extreme thermophiles may be required for in situ monitoring of fermentors and other high temperature processes (Rella et al., 1996; Jeffries et al., 1997; Arnold, 1998). Currently there is also an interest in cold active enzymes obtained from psychrophilic organisms exhibiting high activities even at low temperatures (Gerday et al., 2000). Basic advantage of such enzymes/organisms is the possibility of carrying out process monitoring even under chilled storage conditions normally practised in food and allied industries. Recently, a biosensor based on a psychrophilic strain of *Deinococcus* has been reported for the measurement of sugars (Nandakumar and Mattiasson, 1999b).

One of the major limitations in the use of microbial biosensors, especially in waste monitoring, is the heavy metal toxicity. Metal resistant organisms would gain importance for such microbial biosensor applications. One such organism is *Ralstonia* sp. CH34, formerly known as *Alcaligenes eutrophus*, a gram-negative bacterium which possesses a remarkable set of resistance determinants through inducible ion efflux systems, allowing it to survive in extreme environments that are heavily contaminated with toxic metal ions (Nies, 2000). Since all metal resistance determinants in this bacterium are inducible, their regulatory systems can be used to develop biosensors in the future that measure the biologically important concentrations of heavy metals as well as organic contaminants in a metal contaminated environment (Corbisier et al., 1996; Peitzsch et al., 1998; Nies, 2000). Several approaches have been developed to improve the heavy-metal biosorption capacity of microorganisms by cell wall modifications. Genetic techniques have been used to either over express proteins (metallothioneins) with high affinity for metals on the surface of microorganisms (Pazirandeh et al., 1995) or to introduce metal-binding epitopes into outer membrane proteins like OmpC (Cruz et al., 2000) for use in bioremediation. Essentially, such modified organisms prevent the entry of toxic heavy metals by keeping them adsorbed on the cell surface. Similar approaches could be useful in engineering metal resistant strains in the future for biosensor applications.

Deinococcus radiodurans strains highly resistant to radiations have been reported. Recently, different gene clusters have been used to engineer *D. radiodurans* for treatment of mixed radioactive wastes, to detoxify both mercury and toluene (Brim et al., 2000). These expression systems could provide models to guide future *D. radiodurans* engineering efforts aimed at integrating several remediation functions into a single host. In addition to their use in the remediation of contaminated sites, such cells may gain importance in biosensors for monitoring of organic pollutants in radiation-contaminated waste. Extremely halophilic (D'Souza et al., 1997) and halotolerant strains (Tag et al., 2000) will gain importance in the monitoring of saline environments. A large number of bioprocesses in future will be based on the use of organic solvents. In this direction, cross-linked enzyme crystals stable in organic solvents have shown promise (St. Clair and Navia, 1992). Microbial cells that are stable under these conditions may be required in the future for the fabrication of microbial sensors for use in organic solvents. The *lux* biosensors will gain importance in rapid monitoring of the overall toxicity subjected on a microorganism during a process (Sousa et al., 1998; McGrath et al., 1999).

Some of the basic limitations of microbial biosensors as compared to enzyme sensors has been their slow response and low sensitivity and detection limits. Theoretical models for enzyme-based biosensors with non-reactive transducer predict that enzyme electrode sensitivity and detection limit can be improved by either lowering the K_M or increasing the bimolecular rate constant. The advancement in enzyme engineering has made these goals possible through approaches like site directed mutagenesis (Lai et al., 1996). Slow response of microbial sensors as compared to enzyme sensors has also been attributed to diffusional problems associated with the cell membranes. Possibility of genetically engineering the cell to express the enzymes of interest on cell surface can overcome this problem (Mulchandani et al., 1998a). Thus, it can be foreseen that microbial biosensors of higher sensitivity and lower detection limit will be realisable in the future.

Biosensors have been miniaturised extensively in the recent years. Keeping in line with such developments, microbial cells with high enzyme activities may be required. This is essential especially when microbial

cells are used as substitutes to enzyme based sensors. Genetic engineering (rDNA technology) can help in the overproduction of enzymes of interest in microbial cells. Genetic engineering (metabolic engineering) may also be applied in enhancing the specificities of microbial sensors by activating certain pathways of metabolism and cellular uptake while switching off the undesirables ones. From a commercial point of view, development of stable, immobilised whole cell preparations with better storage and operational stability will be desirable.

There are interesting possibilities within the field of biosensors. Given the existing advances in biological sciences coupled with advances in various other scientific and engineering disciplines, it is imminent that many analytical applications will be replaced by biosensors. A fruitful fusion between biological sciences and other disciplines will help to realise the full potential of this technology in the future.

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