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Entrapment of live microbial cells in electropolymerized polyaniline and their use as urea biosensor

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ABSTRACT

The lyophilized biomass of bacterium *Brevibacterium ammoniagenes* was immobilized in polystyrene sulphonate–polyaniline (PSS–PANI) conducting polymer on a Pt twin wire electrode by potentiostatic electropolymerization. The bacterial cells retained their viability as well as urease activity under entrapped state, as confirmed with bacterial live–dead fluorescent assay and enzymatic assays. The entrapped cells were visualized using scanning electron microscope. The immobilized cells were used as a source of unpurified urease to develop a conductometric urea biosensor. The catalytic action of urease in the sensor released ammonia, thereby causing an increase in the pH of the microenvironment. The pH dependant change in the resistivity of the polymer was used as the basis of sensing mechanism. The sensor response was linear over a range of 0–75 mM urea with a sensitivity of 0.125 mM^{-1}. The sensor could be reused for 12–15 independent measurements and was quite stable in dry as well as buffered storage condition at 4 °C for at least 7 days.

1. Introduction

Immobilization of whole cells improves the stability of the enzyme by retaining them in their natural surrounding and decreasing the cost for lengthy and expensive procedures for extraction and purification of the enzymes (D'Souza, 2001a; D'Souza, 1999). Cell bound enzymes are more tolerant to environmental perturbations like pH, temperature, heavy metal poisoning denaturation and inactivation (D'Souza, 1989). Immobilized biomass can also act like self-proliferating biocatalyst within the matrix (D'Souza, 1989). One of the limitations of using whole cells in sensor is the low sensitivity and specificity because of permeability barrier and unwanted side reactions catalysed by other enzymes within the cell. These interferences can be minimized by cell permeabilization that leads to essential cofactor loss or heat inactivation (D'Souza, 2001a,b). Another strategy is to use recombinant DNA technology or controlled expression of the gene of interest for maximum yield of desired enzyme. The latter requires cultivation of microorganism in specific medium containing appropriate substances for controlled gene expression (Di Paolantonio and Rechnitz, 1982; Fleschin et al., 1998; Riedel et al., 1990). Thus microbial urease synthesis is repressed in presence of nitrogen rich compounds including ammonia and urea whereas derepressed under nitrogen starvation conditions (Harry and Robert, 1989).

Choice of a suitable immobilization matrix is an important parameter for biosensor. Conducting polymers have emerged as an immobilization matrix that can also serve as a transducer (Contractor et al., 1994; Gerard et al., 2002; Hoa et al., 1992; Sukeerthi and Contractor, 1998). They can be used for immobilization of pure enzyme (Hoa et al., 1992), aptamers (Liao et al., 2008), nanoparticles (Fredj et al., 2008) as well as microbial cells (Palmqvist et al., 1994). Polyaniline (PAni) is one such widely studied polymer. It is a stable dark-green amorphous substance with melting point above 300 °C and is insoluble in water, bases and mineral acid solutions (Bacon and Adams, 1968). It finds use in energy storage elements in capacitors and batteries (Trindal et al., 1991), light emitting diodes (Grem et al., 1992) and memory storage devices (Tseng et al., 2005). It has been used as a suitable matrix in potentiometric (Arkady et al., 1996), amperometric (Samuel et al., 1996) and conductometric (Hoa et al., 1992; Sangodkar et al., 1996; Sukeerthi and Contractor, 1999) biosensors. Few examples of such biosensors include those for detecting glucose (Setti et al., 2005), glutamate (Rahman et al., 2005a), choline (Rahman et al., 2004), lactate (Chaubey et al., 2003), inorganic phosphate (Rahman et al., 2005b) and urea (Luo and Do, 2004). PAni can be synthesized by chemical oxidation or electrodeposition methods in fibrillar morphology making it possible to entrap biomolecules (Arkady et al., 1996). Electrodeposition is one of the safest ways of immobilization of biomass as invasive techniques like cross linking, covalent binding, radiation polymerization etc., can often lead to enzyme

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deactivation (D'Souza, 1986). Also, application of PAni in biosensor holds certain advantages compared to known systems like its high sensitivity towards pH change and possibility of enzyme immobilization in a pH sensitive matrix (Arkady et al., 1996; Hoa et al., 1992).

Few attempts have been made in the past to adsorb animal or microbial cells on preformed polyaniline surface (Wong et al., 1994). These methods possessed large disadvantage of cell desorption and unsuitability in reuse or prolonged use of the biosensor. The electrodeposition kinetics and electronic conductivity of PAni are highly dependent on their electrochemical state and pH. The polymerization is poor above pH 3. On the other side pH below 3 is unsuitable for the enzyme stability of urease, which makes it essential to select a system that permits the polymerization of aniline at a higher pH, suitable for the enzyme stability.

Polyanionic detergents such as polystyrene sulphonate (PSS) and polyvinyl sulphonate (PVS) have been reported as the accelerators of the rate of polymerization of aniline by suitably aligning the cationic monomers for the formation of polymer (Hyodo et al., 1991; Michaelson et al., 1992). PSS or PVS–PAni system is conducting at neutral pH (Austrian et al., 1991; Mafe et al., 1993) and can be synthesized at pH 4 (Kanungo et al., 2003; Kuramoto et al., 1990; Liu et al., 1999). Also, the response from biosensor incorporating this type of matrix is less affected by buffer capacitance (Arkady et al., 1996). Therefore PSS–PAni is supposed to be suitable matrix for microbial immobilization and construction of biosensor transducer.

In the work described herein, studies were conducted on a novel entrapment method of gram-positive bacterium *Brevibacterium ammoniagenes* in PSS–PAni polymer matrix by electropolymerization. It has been demonstrated that bacterial cells can be effectively entrapped in the polymer and be kept alive under certain conditions. Further, a urea biosensor was developed using the immobilized whole cells, which was also a source for urease. The cells were grown in selective medium for enhanced urease expression. The catalytic action of urease in the sensor assembly over substrate urea liberated ammonia, causing an increase in the pH of the microenvironment and a simultaneous increase in the resistance of the PSS–PAni. The change in resistivity of the sensor (Contractor et al., 1994) was used for calibration of the urea biosensor.

2. Materials and methods

2.1. Chemicals and materials

Nutrient broth was purchased from Himedia Ltd (Mumbai, India). Freshly distilled aniline (Merck Ltd, Mumbai) was used for preparing monomer solution; sulphuric acid used was MOS grade with 99.9% purity (SD fine chemical chemicals, Mumbai). Polystyrene sulphonate was bought from Aldrich; urea, phthalic anhydride and Tris were obtained from Sisco research laboratories (Mumbai). Propidium iodide (PI) and fluorescein diacetate were purchased from Fluka. Infinity™ urea (Nitrogen) reagent (powder) was from Thermo Scientific Inc. All other chemicals used were of analytical reagent grade and were used without further purification.

2.2. Microbial culture

A soil isolate of *Brevibacterium ammoniagenes*, which was urease positive and glucose oxidase negative gram-positive bacteria, was cultured in nutrient broth under aerobic condition. Inoculum (5%) from overnight grown culture was transferred to urea broth containing 0.1% NaCl, 0.05% yeast extract, 0.2% sucrose, 0.2% dipotassium hydrogen phosphate and 2% filter sterilized urea. The culture from different intervals of growth period were harvested by centrifugation at 6000 g for 20 min, washed with saline (0.85% NaCl) twice and resuspended in 50 mM Tris-HNO_3 buffer of pH 7.4.

2.3. Analytical methods

Urease was assayed for 15 min in 1 ml reaction volume with 100 μL of 1% (w/v) lyophilized bacterial suspension; 250 μL 5 mM Tris-HNO_3 buffer pH 7.4, 500 μL of 1 M urea. To stop the reaction 100 μL of 100 mM iodoacetic acid was added. The ammonia liberated was estimated colorimetrically by the phenol-hypochloride method (Weatherburn, 1967). Protein concentration in the culture suspension was estimated using method suggested by Lowry (Lowry et al., 1951).

2.4. Lyophilization of microbial biomass

A large scale culture (5 L) of *Brevibacterium ammoniagenes* grown in urea broth to late log phase was harvested by centrifuged at 6000 g for 20 min and washed twice with saline to remove media component and ammonia. The biomass was lyophilized in a food freeze-drier lyophilizer (model-BARC) after freeze-drying with liquid nitrogen. The lyophilized biomass was checked for urease activity and stored at −20 °C in small vials.

2.5. Characterization of bacterial urease

The bacterial whole cell urease was characterized to get optimum enzyme activity. Variation of enzyme activity at different growth phases and optimum temperature for enzyme (studied for 8, 22, 30, 39, 65 and 85 °C in saline of pH 5.7) were evaluated. Optimum pH of enzyme was determined for a range of 1–10 by adjusting the pH of saline with NaOH or HCl. The kinetic parameters for native urease (urease located in cell biomass) such as K_m and V_{max} were determined from Lineweaver–Burk double reciprocal plot (Lehninger, 1975; Mao et al., 2002).

2.6. Polymer synthesis

The sensor devises were fabricated on a Pt twin wire working electrode as per method described earlier (Gholamian et al., 1986) (Fig. 1). A potentiostat from EG&G Princeton (model EG&G 362) was used for polymerization purpose. An electrochemical quartz crystal microbalance cum potentiostat (from CH instruments, model 405 coupled to a computer for data visualization) was used for electrode cleaning, cyclic voltammetry and polymerization.

Pt electrodes were first soaked in chromic acid overnight and then washed with dilute HCl and deionised water several times. These were further cleaned electrochemically in 0.5 M H_2SO_4 by cycling between potentials −0.5 and +1.6 V vs. standard calomel electrode (SCE) prior to use in polymerization (Sukeerthi and Contractor, 1999).

The polymerization was carried out electrochemically on a three-electrode system with a Pt foil (1 cm^2 area) as counter and SCE as the reference electrodes. The polymerization mixture constituted of 0.1 M aniline, 50 mM PSS and 20 mg lyophilized biomass dispersed in 2 ml of phthalate buffer (50 mM, pH 4). Two different procedures were used in polymerization of PSS–PAni system: potentiostatic and potentiodynamic. In potentiostatic mode, immobilization of bacterial cells was carried out by applying a constant potential of +1.2 V (vs. SCE) to the working electrode for 60–150 min till a polymer bridge was formed between the Pt twin wires. Under potentiodynamic situation, polymer was formed from the similar constituents while cycling the potential between −0.2 V and +0.8 V