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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

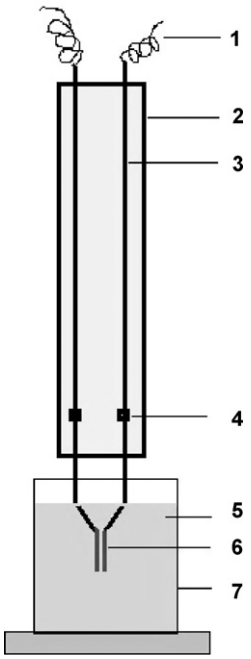

Fig. 1. Schematic diagram of Pt-twin wire electrode. The components shown are: (1) copper wire contacts connecting to bipotentiostat, (2) glass body filled with epoxy, (3) copper wire, (4) solder (contact), (5) PSS–PAni or other buffer solutions, (6) Pt wires converging at a gap of 100 μm (which is the site of formation of dark green PSS–PAni) and (7) glass beaker of 5 ml capacity.

(vs. SCE). Control polymers were synthesized in absence of microbial cells for standardisation of the polymerization method.

2.7. Sensor measurement

An AFRDE4 bipotentiostat (PINE instrument Co., USA) was used to measure the resistance of the sensor devices. Resistance measurement was performed by keeping one of the wires at a fixed potential of V_g (gate potential) and other (V_d) at $V_g + 20$ mV (Sukeerthi and Contractor, 1999). Drain voltage V_d was maintained at 20 mV and the current flowing as a result of the potential difference was 'I_g' (gate current). Measurement of resistance was carried out in 25 mM phosphate buffer of pH 7.4. The sensor response of the polymer was measured in terms of ($\delta r/r_0$), where 'r_0' is the resistance of the polymer in buffer, 'r' is the resistance of the polymer in different concentration of the analyte and $\delta r = r - r_0$. The batch-wise variation of the sensor response could be minimized by representing the sensor response in terms of $\delta r/r_0$ (Kanungo et al., 2003).

A higher biomass loading in the polymer will supposedly produce larger sensor response. To ascertain this fact, sensor response was recorded for different amounts of biomass loading, numerically: 10, 15, 30, 40 and 60 mg/ml polymerization mixture containing approximately 10, 15, 30, 40 and 60 U activities respectively, along with fixed urea concentration of 100 mM. Sensor selectivity towards urea was demonstrated by exposing the devise prepared with 60 mg/ml biomass loading to different concentrations of glucose. Control experiments were performed in absence of the bacterial loading. The sensor was tested for stability and shelf-life by keeping the devise both in dry state as well as in 25 mM, pH 7 phosphate buffer.

2.8. Measurement of enzyme activity on the polymer-bacteria film

The polymer deposited on a Pt twin wire electrode from a biomass with 40 U of urease activity was scratched out and dispersed in 25 mM phosphate buffer (pH 7). Enzyme activity in the dispersion was measured according to the procedure described in Section 2.3. The control experiment was carried out in absence of bacteria.

2.9. Microscopy

The PSS–PAni polymer was prepared by electrodeposition with biomass (40 mg/ml) on a Pt foil (1×2 cm^2). The polymer was dried in vacuum overnight and a thin layer of gold was sputtered on to it to avoid charging of polymer. The bacterial cells entrapped in the polymer were visualized under a JEOL JSM 6400 scanning electron microscope.

Bacterial viability in the polymer was ascertained by live–dead fluorescent staining using fluorescein diacetate and propidium iodides. PSS–PAni was synthesized at pH 0.5 (in 0.5 M H_2SO_4) and 4.0 (in 50 mM phthalate buffer) along with biomass. The polymer granules were scratched out from Pt surface and suspended in pH 0.5 (0.5 M H_2SO_4) and 4.0 (50 mM phthalate buffer) for 1 h at room temperature. Cells were stained thereafter as per the procedure described earlier (Schupp and Erlandsen, 1987). A smear of stained bacterial cells with polymer granules was spread on glass slides. Free bacterial cells with similar treatment were stained for comparison. The slides were visualized under fluorescent microscope (model Axioscop 40 from Carl Zeiss) at 100× resolution with 480 nm excitation wavelength.

3. Results and discussions

3.1. Characterization of bacterial urease

Brevibacterium ammoniagenes cultured with 5% inoculum had a generation time of 3 h with stationary phase starting after 17 h. As microbial urease expression is enhanced by nitrogen starvation (Kaltwasser et al., 1972), it was necessary to find conditions optimum for expression of urease. Highest urease expression was found at late log or early stationary phases where the culture media become nitrogen limited as a result of media depletion (Fig. 2). Therefore, biomass was harvested from this phase for all experiments.

Enzyme activity, protein content and specific activity of urease in lyophilized biomass were calculated under these conditions as 1000 U g^{-1} dry weight (±26.1), 341.3 mg g^{-1} (±7.4) and 2.9 U/mg protein (±0.013). The optimum temperature (65 °C) and pH (5.0–10.0) for *Brevibacterium ammoniagenes* cellular urease were identical to purified enzyme from similar source (Hirofumi et al., 1984). The bacterial urease was stable for more than 6 h at room temperature (25 °C) when kept in 25–50 mM phosphate buffer of

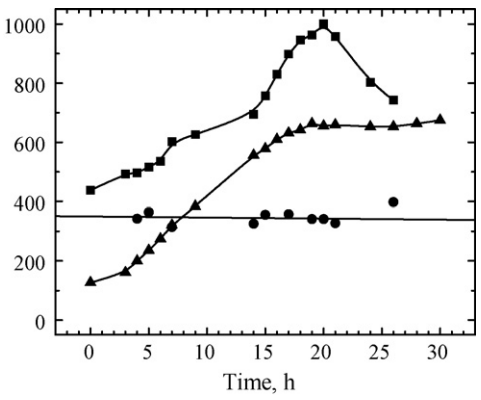

Fig. 2. Bacterial urease activity (U g^{-1}) (■) and protein content (mg g^{-1}) (●) in dry biomass as well as biomass yield (mg L^{-1} culture broth) (▲), against culture period (h) with 5% inoculum of *Brevibacterium ammoniagenes*.

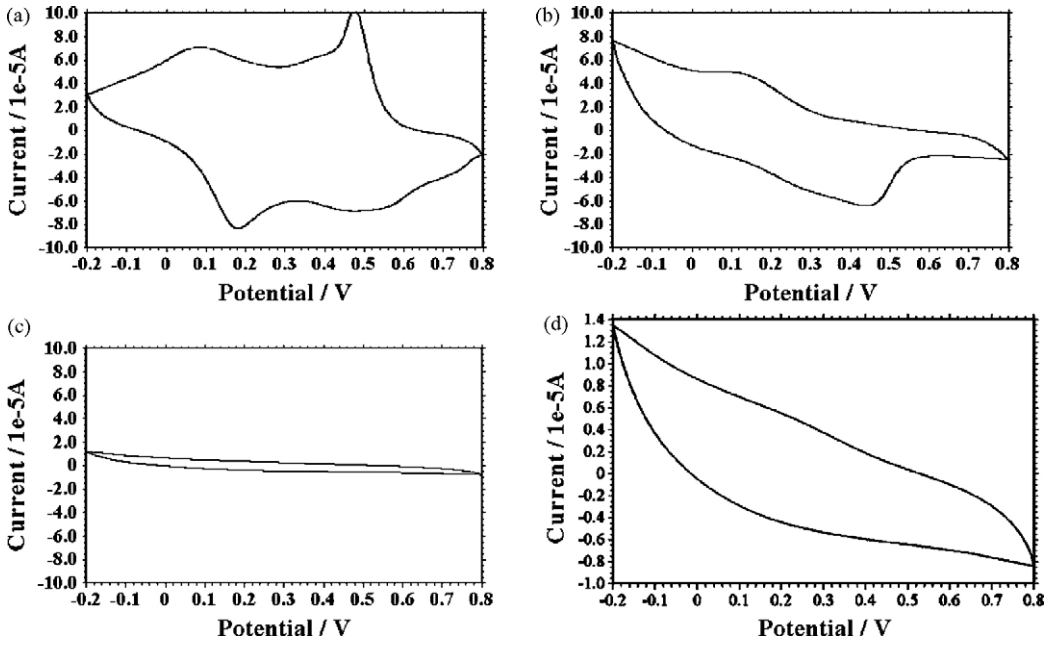

Fig. 3. CV of biomass loaded PSS–PAni (vs. SCE) at (a) pH 1.4, (b) pH 4.0, (c) pH 7.5 and (d) rescaled figure for (c) pH 7.5.

pH 7.4. The urease was stable for more than 1 month when kept in 25–50 mM of phosphate or tris-HNO_3 buffer of pH 7.4 at 4 °C.

These conditions were appropriate for biosensor measurements at room temperature. The kinetic parameters K_m and V_{max} were calculated for cellular urease from Lineweaver–Burk double reciprocal plot as 40 m moles L^{-1} urea and 1050 micromoles urea hydrolyzed min^{-1} mg^{-1} of protein. A K_m value of 40 mM as against 3–5 mM urea for purified urease from Jack beans suggests a low affinity of bacterial urease towards urea and therefore, any biosensor developed with this enzyme source would be suitable in higher range of urea concentration (Hirofumi et al., 1984).

3.2. Polymerization

Electrode cleanliness remains an important parameter in electrochemistry. Pt surface is often poisoned by atmospheric oxygen when stored dry. Therefore, it was necessary to clean it prior to use by cycling the potential between −0.5 and 1.6 V 3–4 times. For polymerization of PSS–PAni two different polymerization approaches were followed: potentiostatic and potentiodynamic. Both the approaches have some advantages or disadvantages on their own (Kanungo et al., 2003; Kanungo et al., 2002; Sukeerthi and Contractor, 1998; Sukeerthi and Contractor, 1999). Potentiodynamic polymerization yields the polymer with cycles of oxidation and reduction of the monomers. However, the average time taken to bridge the gap between Pt twin wires was 120 min as compared to 60 min using potentiostatic approach. Therefore, potentiostatic approach seemed to be favourable to avoid any possible enzyme inactivation during polymerization and to get the polymer quickly. Therefore, potentiostatic approach was followed in all other experiments. However, a variation in the polymerization period from 60 to 150 min was observed using this technique when higher amounts

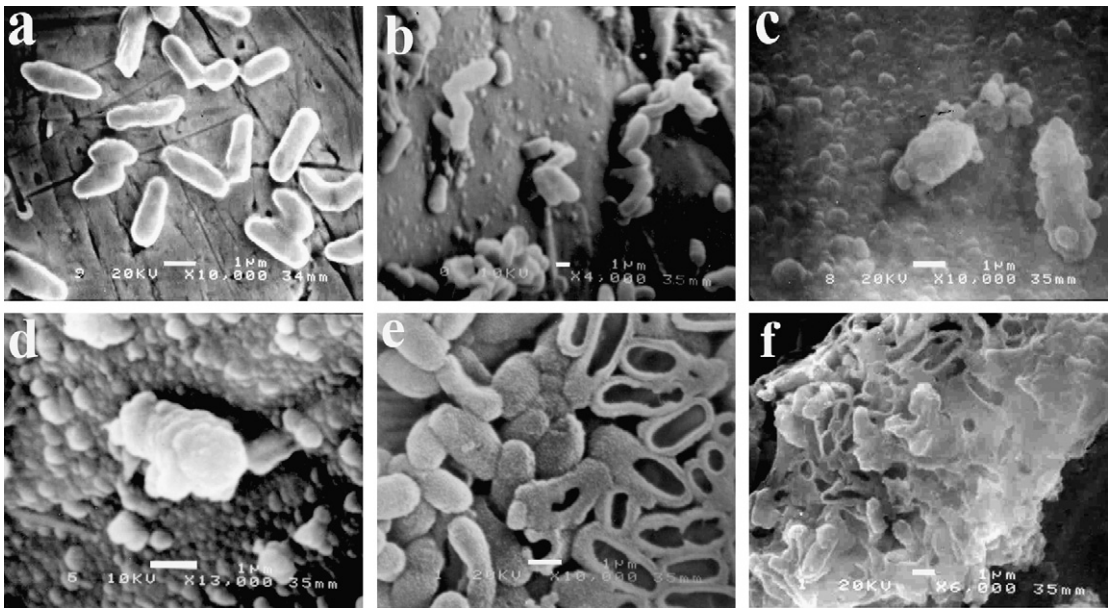

Fig. 4. Scanning electron micrographs of *Brevibacterium ammoniagenes*entrapped in PSS–PAni polymer.

of biomass were loaded in the polymerization mixture. The variation was possibly due to kinetic hindrance in polymerization with increased viscosity of the polymerization mixture.

The PSS–PAni polymer was obtained with entrapped biomass using potentiostatic technique. The polymer prepared using 40 U enzyme activity in polymerization mixture actually contained 3.1 U (SD 0.14, $n = 5$) specific activity of entrapped enzyme (0.45 mg protein content) when examined after scratching out the polymer from Pt surface. This corresponded to about 1.3 mg (3.2%) biomass entrapment. Cyclic voltammograms for the biomass loaded PSS–PAni (vs. SCE) were obtained at pH 1.4 (0.1 M HCl with 0.1 M KCl), 4.0 (25 mM acetate buffer and 0.1 M KCl) and 7.4 (25 mM phosphate buffer with 0.1 M KCl) (Fig. 3a–c). Although the cathodic peak current, characteristic to PAni seems to be reduced at pH 7.4, some previous work in this regard suggested it to be sufficient (Fig. 3d) to detect changes in an enzymatic reaction near physiological pH (Kanungo et al., 2003).

3.3. Microscopy

Although, the work presented in this manuscript happens to be first report of entrapment of microbial whole cell in conducting polymer, it was necessary to corroborate the entrapment microscopically. Electron micrographs of PSS–PAni granules containing bacterial cells are shown in Fig. 4. These micrographs were obtained with single sample by simply changing the observation field. The Micrograph of *Brevibacterium ammoniagenes* adsorbed on Pt surface can be seen in Fig. 4a. The bacterium has the length of 1.5–2.0 μm and breadth as 0.5–1.0 μm. It also shows bacilli-cocci transition in the stationary phase. Fig. 4b shows formation of small polymer granules alongside the adsorbed bacteria. It could be easily clearer in Fig. 4c and d that PSS–PAni has started growing on the bacterial cell surface with an overall increase in the cellular dimensions. Fig. 4e shows cells completely coated by polymer and a few gaps carved alongside, which could be a result of desorption of cells during polymerization. Fig. 4f shows picture of a large polymer granule at a lower resolution. These results clearly show the bacterial entrapment within the polymer rather than mere adsorption.

The bacterial viability was checked using live–dead fluorescent staining with FDA and PI. Both free as well as immobilized cells were stained for comparison. In case of free cells, at the most favourable condition of pH 7, the population contained more number of viable cells (green) compared to dead cells (red) (See supplement). The number decreased drastically at pH 4 while hardly any live cells can be seen at pH 0.5. The trend was similar in case of immobilized cells, where viable cells existed only at pH 4 and not at 0.5. This justifies the necessity to synthesize PSS–PAni at a pH higher than its most suitable acidic condition.

3.4. Sensor response

The sensor response were measured at three gate potentials, $V_g = -0.2$, 0, and 0.2 V vs. SCE. The response was found highest at 0 V. Therefore, the results of this potential are reported here. Control experiments with sensors prepared in absence of enzyme and with glucose showed negligible response, thereby showing sensor specificity towards urea (Fig. 5).

The response from the sensors with different amounts of bacterial loading (10, 15, 30, 40 and 60 U) in the polymerization mixture was linear and saturated at certain urea concentration (Fig. 5). The response time of the biosensor was 3 min. Also, there was an increase in the highest linear response from 40 to 75 mm for 10–60 U of bacterial enzyme loading. Fig. 6 shows the highest linear response vs. the number of urease enzyme units added in the polymerization mixture.

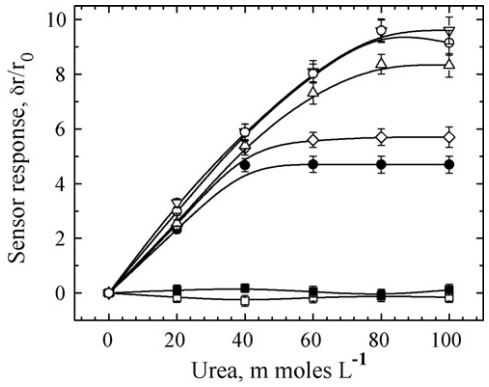

Fig. 5. Sensor response: (□) Control response from urease free sensor with up to 100 mM urea; (■) control experiment with glucose instead of urea; response from sensors constructed with biomass having (●) 10 U; (◊) 15 U; (△) 30 U; (▽) 40 U and (○) 60 U of urease loaded in the polymerization mixture. Error bar represents standard deviation between triplicate experiments.

The highest sensitivity (defined as the ratio of the highest linear response in $\delta r/r_0$ to the corresponding concentration of the analyte) of the biosensor (0.125/mM) was found in case of 40 U bacterial enzyme loading. Therefore, this amount of biomass was used during polymerization in further studies such as sensor accuracy, shelf-life and stability.

The biosensor accuracy (Mulchandani et al., 2005) was established by comparing the urea concentrations determined by the biosensor vis-à-vis Infinity™ blood urea nitrogen (BUN) reagent method (Jha et al., 2008). A slope of 0.94 with regression coefficient of 0.995 between the two methods demonstrated the excellent accuracy of the biosensor (see supplement). The sensitivity of biosensor (0.125 mM) was numerically better compared to Infinity reagent method (0.01 Δabsorbance/mM) while its response time was slower (3 min) compared to latter (90 s) (Giorgio and Jensen, 1998).

Stability and shelf-life of the sensor are important factors for evaluation of sensor performance in practical applications. The sensor response was found quite stable for 12–15 independent measurements. However, measurement of sensor response was permissible only in the ascending order of analyte concentration. Reversal of sensor response to baseline was possible by keeping it in 25 mM phosphate buffer for 2 h at room temperature under stationary condition. The biosensor tested for shelf-life by keeping it in dry state and in buffered condition at 4 °C, maintained 90–100% of its original response till 7 days or 12–15 measurements, provided the bridging between the Pt twin wires remained intact.

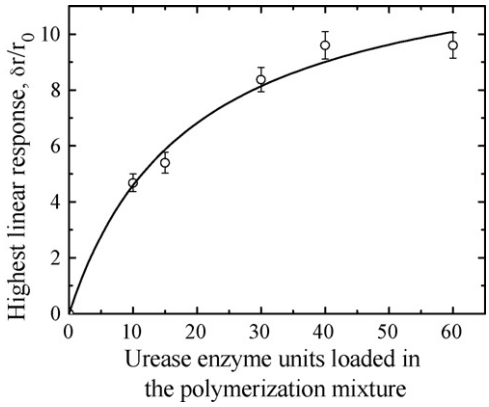

Fig. 6. Highest linear response of sensor with varying bacterial urease loading. A fixed urea concentration of 100 mM was used. Error bar represents standard deviation between triplicate experiments.

4. Conclusion

In this first demonstration of entrapment of microbial cells in conducting polymer PSS-–PAni by electropolymerization, the *Brevibacterium ammoniagenes* biomass was selectively prepared to contain optimum urease activity and was used as a source of unpurified urease. The most significant finding of this research was that cells could be kept viable within the polymer as well as they retained functionality in terms of enzyme activity. As a proof of concept, the immobilized bacterial cells were used with PSS–PAni in development of a biosensor for urea which produced reasonable performance. On the basis of these results, we predict that electropolymerization is one of the safest techniques for immobilization of live whole cells. Also, by combining the advantages of conducting polymer, this immobilization technique will aid the studies on bioMEMS, nanowires, and other miniaturized devices (Nomura and Karube, 1996; Ratner and Bryant, 2004) where whole cells and sometimes even single cell (Pamir et al., 2008), are needed to be immobilized directly over the micro-electrode.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.01.024.

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