Appl Biochem Biotechnol (2008) 151:653–664
DOI 10.1007/s12010-008-8280-6

Cephalosporins Determination with a Novel Microbial Biosensor Based on Permeabilized *Pseudomonas aeruginosa* Whole Cells

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Received: 13 December 2007 / Accepted: 7 May 2008 /
Published online: 13 June 2008

Abstract A new potentiometric microbial biosensor based on *Pseudomonas aeruginosa* was developed in this study for detecting the cephalosporin group of antibiotics. Preliminary results with the biosensor indicated that *P. aeruginosa* cells, when treated with lysozyme, showed more efficiency in detecting cephalosporin C in a wide concentration range of 0.1–11 mM with high sensitivity compared to the normal cells. Optimization of the three important biosensor design parameters permeabilized cell contents, quantities of gelatin, and glutaraldehyde resulted in high performance of the biosensor. The optimized values of the above parameters were cell contents 2.5 mg/cm^2, gelatin 8.5 mg/cm^2, and 0.25% glutaraldehyde. The assay conditions, namely phosphate buffer pH, ionic strength, and temperature, were optimized for best performance of the biosensor. The specificity test of the biosensor towards detecting different β-lactam antibiotics showed good response only for the cephalosporins. The operational and storage stability in detecting cephalosporin C indicated very good potential of the biosensor in detecting cephalosporins with high accuracy.

Keywords *Pseudomonas aeruginosa* · Microbial biosensor · Cephalosporins · β-Lactamase · Cephalosporinase · Permeabilized cells

Introduction

β-Lactam antibiotics are a broad class of antibiotics that contains a β-lactam ring in its molecular structure and includes cephalosporins and penicillin derivatives [1]. Cephalosporins have been reported to be the safest and the most frequently administered antibiotic among the β-lactam and other antibiotic groups and have an effective broad spectrum of

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bactericidal activity available to the clinician [2]. 7-Aminocephalosporanic acid, composed of a β-lactam ring fused with a dihydrothiazine ring, proceeds from the hydrolysis of biologically active cephalosporin C and forms the basis of many semisynthetic cephalosporins, which vary in their side chains [3].

The determination of cephalosporins is of great importance in several fields such as the fermentation industry, the clinical laboratory, and quality control practices. Conventional techniques for cephalosporin measurement include those involving colorimetric, titrimetric, or bioassay principles. Some of these techniques are ultraviolet-visible spectrophotometry [4–7], fluorimetry [8], chemiluminiscence [9], and atomic absorption spectrometry [7]. Detection of cephalosporins and other β-lactam antibiotics can also be carried out by chromatography [10–13] or capillary electrophoresis [14, 15]. However, the main drawbacks of these techniques are that these are usually tedious and time consuming and often require skilled personnel.

Compared to the above conventional analytical techniques, microbial biosensors have several advantages, such as simplicity and cheap construction, high regeneration potential, and selective and simple operation [16]. The use of whole cells, having a defined enzymatic activity, in the construction of biosensors for estimation of β-lactam antibiotics has already been reported [17–21]. Some systems are based on the hydrolysis of a β-lactam ring of the antibiotic by cephalosporinase or penicillinase to produce cephalosporic acid or penicilloic acid, respectively [18, 21, 22], according to the following scheme:

$$\text{Cephalosporin} \xrightarrow{\text{cephalospinase}} \text{Cephalosporic acid} + H^+ \quad \text{Penicillin} \xrightarrow{\text{penicillinase}} \text{Penicilloic acid} + H^+$$

These reactions were monitored by measuring with pH electrodes [18–22] or ion selective field effect transistor-based biosensors [23]. Analyses of β-lactam antibiotics using a microbial receptor protein-based biosensor [24], optical monitoring employing an enzyme reactor/flow injection system-based biosensor [25], fiber optic-based biosensor [26], and surface plasmon resonance-based biosensor [27, 28] on the inhibition of carboxypeptidase activity have also been reported. Hence, it is evident that the need for a simple, reliable, and specific determination method for the cephalosporin group of antibiotics has led to the development of many biological sensors. However, the main disadvantages of these microbial sensors include their low sensitivity, thus necessitating the use of large amounts of the microbial cells. This, in turn, adversely affects the performance of the biosensors. An alternative to overcome this problem is to increase the specific activity of the enzyme of interest and sensitivity. This can be easily achieved by the permeabilization of the bacteria using various methods. Until now, there are no reports available to develop an enzyme permeabilized microbial biosensor based on *Pseudomonas aeruginosa* for the determination of cephalosporins. In the present study, we report the development of a new potentiometric microbial biosensor based on permeabilized *P. aeruginosa* cells for selective and rapid determination of cephalosporins.

Materials and Methods

Chemicals

Cephalosporins and other β-lactam antibiotics, gelatin (225 bloom), glutaraldehyde, lysozyme (chicken egg white), and all other chemicals were purchased from either Sigma Chemical, St. Louis, MO, USA, or Himedia, Mumbai, India.

Microorganism and Culture Conditions

The bacterial culture *P. aeruginosa* MTCC 647 was obtained from Microbial Type Culture Collection & Gene Bank, Institute of Microbial Technology, Chandigarh, India. The bacteria was grown and maintained in nutrient agar (pH 7.0) containing (g L^{-1}): beef extract 1.0, yeast extract 2.0, NaCl 5.0, peptone 5.0 and agar 15.0, pH 7.0 for 24 h at 37 °C. The organism was subcultured every 4 weeks and stored at 4±1 °C. To induce cephalosporinase enzyme production by the bacterium, *P. aeruginosa* cells were cultured in 50 ml of growth medium containing (g L^{-1}); glucose 10.0, yeast extract 3.0, $Na_2HPO_4{\cdot}2H_2O$ 3.0, $MgSO_4{\cdot}7H_2O$ 0.5, $CaCl_2$ 0.1, peptone 0.5, NaCl 0.5, and cephalosporin C 1.0 for 16 h at 37 °C under agitation (200 rpm) condition.

Construction, Assembly, and Cephalosporins Assay Procedure of the Microbial Biosensor

The biosensor consisted of a membrane holder (made of plastic and open at both ends) with a bioactive layer of *P. aeruginosa* cells and gelatin immobilized on to a cellulose acetate membrane (Type HA, 0.2 μm pore size, Sartorious, Germany) using glutaraldehyde as the fixing agent. Two types of bioactive layers were used in this assembly—lysozyme permeabilized cells and normal cells of *P. aeruginosa*. For permeabilization, the cells were treated with lysozyme and Na_2EDTA. The bacterial culture in its late log phase was centrifuged at 10,000 rpm for 10 min at 4 °C, washed with phosphate buffer (pH 7.0, 50 mM), and subsequently treated with 100 μg ml^{-1} of lysozyme and 1 μM Na_2EDTA for 10 min at 30 °C [29]. The lysozyme-treated cells were centrifuged and finally taken into 1.0 ml of phosphate buffer (pH 7.0; 50 mM) and lyophilized. Normal cells were prepared in a similar manner but without any treatment with the enzyme. Lyophilized cells, either permeabilized or normal, and gelatin in predetermined quantities were suspended in 210 μL phosphate buffer (pH 7.0, 50 mM) and spread over the cellulose acetate membrane, which was later dried at 4 °C for 30 min. For fixing the bioactive layer on the membrane, glutaraldehyde (0.25%, v/v) in phosphate buffer (pH 7.0, 50 mM) was used and allowed to react for 5 min. The membrane containing the immobilized bioactive layer and a flat surface pH electrode (S450CD model, Sensorex, Garden Grove, CA, USA) were held together by a membrane holder with the aid of parafilm. The entire microbial electrode assembly connected to a pH meter served as the biosensor in the study. Another pH-sensitive electrode connected to the same pH meter, via a different port, acted as the reference. Schematic diagram of the entire set up is shown in Fig. 1.

Prior to any measurements with the biosensor, both the electrodes were allowed to reach the same pH value in phosphate buffer (pH 7.0, 50 mM) solution free of cephalosporins. The functioning of the microbial biosensor can better be described on the basis of Fig. 2. The figure reveals that diffusion of cephalosporin from its medium occurs through the bioactive layer membrane having either lysozyme-treated or normal *P. aeruginosa* cells, where the cephalosporin is hydrolyzed, and the generated protons are presented at the flat surface of the pH bioelectrode. Due to this increase in protons, the bioelectrode records a reduction in pH value, whereas the reference electrode does not. Hence, the response of the system is measured as the difference in pH value between these two electrodes.

Effect of Various Design Parameters and Assay Conditions on the Performance of the Microbial Biosensor

The effect of the main factors known to influence the biosensor response, namely, the permeability of the cells, the amount of bacterial cells, gelatin, and glutaraldehyde

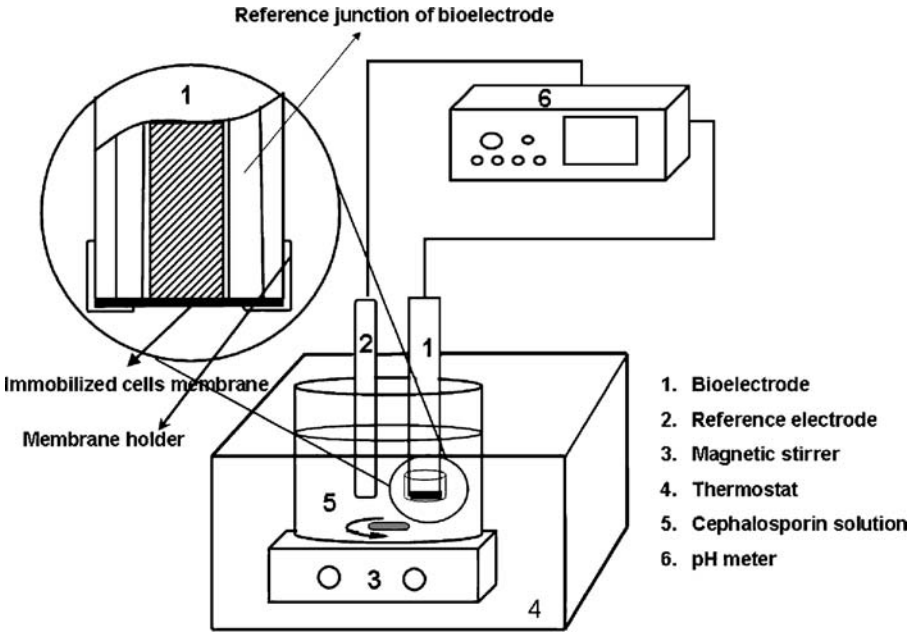

Fig. 1 Schematic diagram of the biosensor set up used in the study. *In circle*, cross sectional view of the bioelectrode

concentration, were studied by varying their levels, one at a time. The cells were permeabilized with lysozyme and Na_2EDTA, as mentioned earlier, for different time periods: 5 to 90 min, and tested for their efficiency in detecting cephalosporin C. The effects of the other three factors were then studied in the biosensor assembly containing immobilized lysozyme-treated cells. The amount of permeabilized cells in the bioactive layer was varied from 1.5 to 4.5 mg cm^{-2} of the membrane area, and gelatin was added in the range of 4.5 to 12.5 mg cm^{-2}. Glutaraldehyde concentration was varied from 0.1% to 0.5% (v/v). The response of biosensor in this study was recorded by a change in pH value

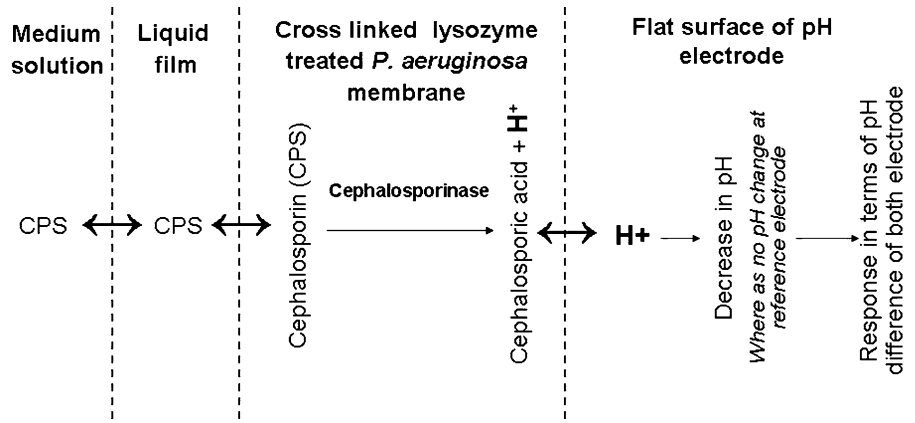

Fig. 2 Mechanism of action of the bioelectrode

due to the conversion of cephalosporin C in the reaction mixture by the action of cephalosporinase. The sensitivity of the response was defined by the slope of a calibration curve obtained by plotting a graph of changes in pH vs cephalosporin C concentration.

Assay of Cephalosporinase Activity

Cephalosporinase activity was measured by incubating the bacterial cells (normal or lysozyme-treated) in a reaction mixture containing cephalosporin C at 5 mM in phosphate buffer (pH 7.0; 50 mM). The change in cephalosporin C concentration was measured by hydroxylamine method [30]. The optimum assay condition for recording the biosensor response was obtained by varying the levels of pH, temperature, and concentration of the buffer. The ranges of levels of the parameters were buffer pH 6.75–7.5, buffer strength 10–100 mM, and temperature 25 to 45 °C.

Characterization of the Microbial Biosensor

Further characterization of the biosensor, in terms of detection limits, response time, substrate specificity, and interference due to cosubstrate, storage stability, and accuracy of detection were investigated at the optimum design and assay conditions of the biosensor. Different substrates such as cephalosporin C, cephalotaxime, cephalexin, cephaloridine, penicillin G, and amoxicillin were used in the substrate specificity test.

All the above experiments with the biosensor were conducted in triplicate, and data shown are the average of three measurements. The variation in the measurements from these triplicates was found to be within ±5%.

Results and Discussion

Results on the effect of permeabilization of *P. aeruginosa* cells using lysozyme on its cephalosporinase activity were obtained using the free bacterial cells, and are shown in Fig. 3. The figure shows that permeabilization enhanced the enzyme activity up to 6.5 times compared to the normal cells. Na_2EDTA acted as a chelating agent by sequestering divalent cations that contribute to the stability of the outer membrane, thus creating a hydrophobic pathway for channeling of certain substances [31]. EDTA has been reported to potentiate

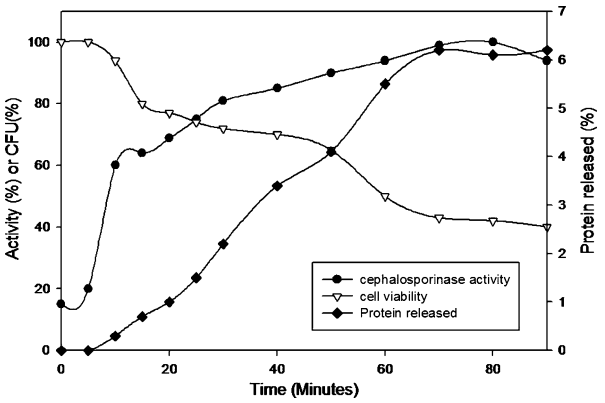

Fig. 3 Effect of permeabilization of *P. aeruginosa* cells with lysozyme on cephalosporinase activity (%), cell viability in colony-forming units (%), and amount of protein released (%)

the activity of bacterial cell wall degrading agents (e.g., lysozyme, nisin) [32]. The time reveals that the maximum increase in enzyme activity occurred between 5 and 10 min of incubation. The figure also reveals that the viability and protein content of the biomass were conserved only within the 10 min of incubation. Based on these results, optimum permeabilization time of the cells was found to be 10 min for use in the bioactive layer of the biosensor assembly. Chao and Lee [19] developed a microbial biosensor based on β-lacatamase using cells that were premeabilized by chemical and mechanical methods, but a serious decline in steady responses was observed due to the loss of enzyme activity during the cell permeabilization. Such loss in enzyme activity during cell permeabilization was not at all observed in the present study.

Effect of Various Design Factors on the Biosensor Performance

For assessing the performance of the biosensor using normal or permeabilized *P. aeruginosa* cells, 2.5 mg cm^{-2} quantity of cells was used in preparing the bioactive layer. In line with our previous results on cell permeabilization, the lysozyme-treated cells showed more sensitivity of the biosensor in detecting cephalosporin C in the reaction mixture. Sensitivity of the bioelectrode with lysozyme-treated cells showed 76.3 (pH difference/mM of cephalosporin C$\times 10^3$) compared to only 30.3 (pH difference/mM of cephalosporin C$\times 10^3$) obtained using the normal cells (data not shown).

Figure 4 presents the effect of permeabilized biomass content in bioactive layer of the biosensor on its sensitivity. The optimum response was achieved using a biomass content of 2.5 mg cm^{-2} and viability of the lysozyme-treated cells was found to be 1.07×10^5 colony-forming units per square centimeter through the standard plate count method [33]. Galindo et al. [20] observed a similar result using the same concentration of cells in their biosensor for detecting penicillin G by a β-lactamase-producing recombinant strain of *Escherichia coli*.

Next, in order to evaluate the optimum levels of gelatin and glutaraldehyde, their levels were varied one at a time by keeping the biomass concentration at its optimal level (2.5 mg cm^{-2}). It is well established that, while glutaraldehyde fixes microbial cells to a support by cross linking various functional groups of the cells, gelatin acts as a stabilizing agent by reacting with its surface proteins [34]. Both these immobilizing materials have

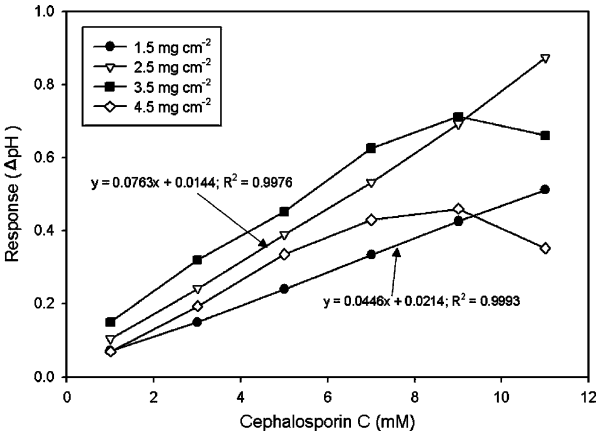

Fig. 4 Effect of permeabilized *P. aeruginosa* cells content on the biosensor response

been used successfully for the design of other biosensors, as well [35, 36]. The effects of gelatin and glutaraldehyde on the sensitivity of the microbial biosensor were carried out. The optimum levels of 0.25% (v/v) and 8.5 mg cm^{-2} were thus obtained for glutaraldehyde and gelatin, respectively (data not shown). Akyilmaz et al. [36] found the optimum levels of gelatin and glutaraldehyde for immobilizing an active layer of *Saccharomyces cerevisiae* cells for the development of a biosensor to be 8.43 mg cm^{-2} and 0.25% respectively. The authors also observed that higher concentrations of gelatin than 12.5 mg cm^{-2} lead to diffusion problems affecting their biosensor response [34–36]. A similar observation was also found in this investigation with the biosensor.

Effect of Various Factors on Assay Conditions Employing the Biosensor

Since the biosensor response (ΔpH) solely depends on the cephalosporinase activity of *P. aeruginosa* cells, the factors pH, ionic strength, and temperature of enzyme assay medium play a vital role on the biosensor response. Therefore, the effects of these factors were studied by varying one variable at a time. Before optimization, the values of pH, ionic strength, and temperature selected for operating the biosensor were 7.0, 50 mM, and 37 °C, which resulted in a maximum sensitivity value of 76.3 (pH difference/mM of cephalosporin $C \times 10^3$). This maximum sensitivity value decreased 3.2 times when pH was changed to 7.5, with the other factor remaining the same. Figure 5 shows the effect of ionic strength on the sensitivity of the biosensor. Although a maximum response was achieved with 10 mM phosphate buffer, it was observed that, at this concentration, the linear relationship between cephalosporin C concentration and biosensor response was less accurate with a determination coefficient (R^2) value of 0.8892 compared to those obtained using other concentrations of the buffer. Hence, a next higher buffer concentration of 25 mM was chosen to be the optimum ionic strength providing good sensitivity of the system. The role of temperature was also found on the sensitivity of biosensor by keeping other factors at their optimal levels (pH 7.0, ionic strength 25 mM). Between the range 35 °C and 40 °C, the sensitivity did not vary much but decreased sharply beyond 40 °C and up to 45 °C. The decrease in sensitivity was observed to be 4.1-fold at 45 °C, and at 25 °C, it was 2.07-fold. From these experiments, the optimum assay conditions for recording the biosensor response were identified to be pH 7.0, buffer strength 25 mM, and temperature 37 °C.

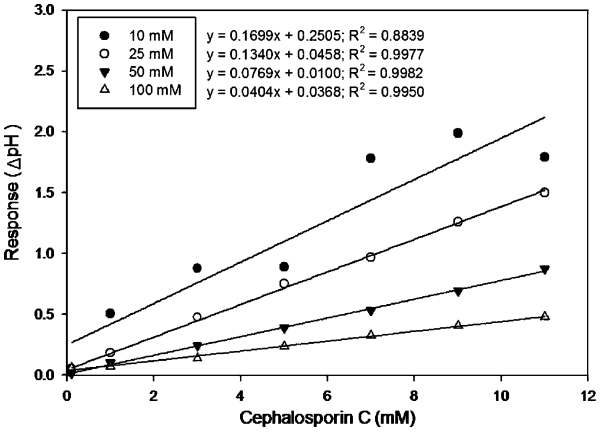

Fig. 5 Calibration curves obtained with the biosensor using different buffer concentrations (pH=7.0; temperature=37 °C)

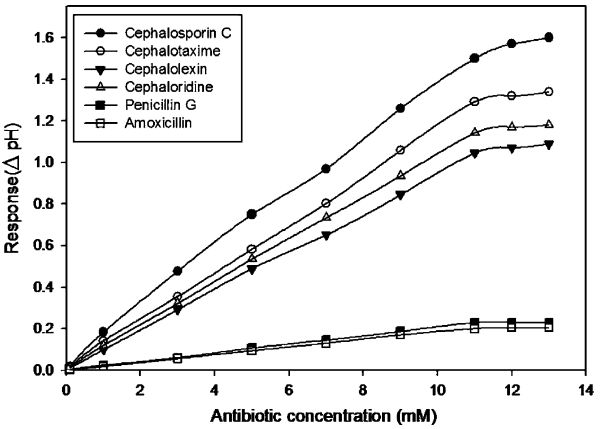

Fig. 6 Calibration curves obtained for different β-lactam antibiotics under the optimized assay conditions

Characterization of the Microbial Biosensor

Detection Limits of Cephalosporins, Response Time, and Storage Stability of the Biosensor

Based on the previously obtained optimum values of the biosensor for its design and assay conditions, a linear relationship between the biosensor response (ΔpH) and concentration of β-lactam antibiotics was achieved in the range of 0.1 to 11 mM, with a R^2 value of 0.99. At concentrations below 0.1 mM or above 11 mM, the biosensor response was insignificant and/or showed a nonlinear pattern (Fig. 6). Gracia et al. [21] reported cephalosporins detection limit of 0.3–4.4 mM using a protein-engineered β-lactamase. The typical response time of the biosensor in this study was found to be between 3.5 and 11 min depending on the type of cephalosporins used. The stability of the biosensor was steady for 7 days.

The response time profile of the microbial biosensor to detect cephalosporins at 5 mM concentration is presented in Fig. 7, which indicates that the system took 3 min for the response to get stabilized, with major fluctuations occurring between 20 and 160 s. The response was stable even after 4 min. Based on these observations, a 3-min time period was taken to be an optimum response time of the biosensor.

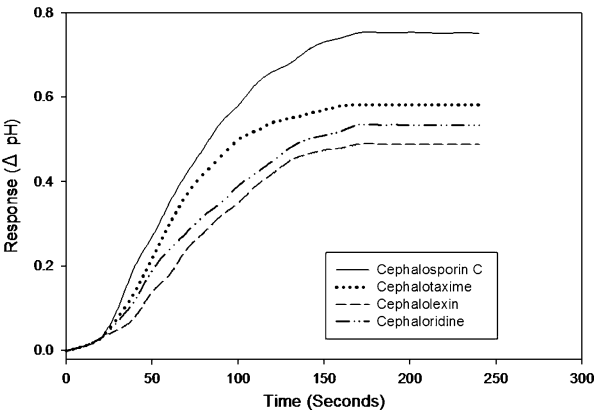

Fig. 7 Response time curves for cephalosorins at 5 mM concentration

To test the stability of the biosensor, the detection limits and the response time of the biosensor were also tested over a period of 14 days. It was observed that the biosensor was highly stable up to a period of 8 days, and at the end of the 14-day period, the microbial biosensor lost 40% of its initial activity. Moreover, an increase in the biosensor response time was observed after 8 days of its operation. Similar effects on storage stability and response time were observed by Hewetson et al. [37] with a penicillin bioelectrode. The above results on detection limits of β-lactam antibiotics, response time, and storage stability of the microbial-based biosensor are consistent with those of Matasumoto et al. [18], Chao and Lee [19], Galindo et al. [20], and Gustavsson et al. [27, 28].

Substrate Specificity and Interference due to other Antibiotics

To test the versatility of the biosensor in detecting a range of cephalosporin antibiotics, its responses with the various antibiotics were compared using the previously optimized design and assay conditions. Five other β-lactam antibiotics in addition to cephalosporin C were tested. Figure 6 depicts, in terms of response and sensitivity, the system in this study. As observed earlier with cephalosporin C, the biosensor showed very good response with other cephalosporin antibiotics, as well. It was observed that the biosensor showed negligible sensitivity when penicillin G or amoxicillin was present in the assay mixture.

Although the literature is quite replete with studies on β-lactamase-based enzymatic and microbial electrodes for detecting penicillin and its derivatives [17, 19, 20, 38–40], only a few reports concerning cephalosporin detection by such biosensors are available [18, 21]. Matasumoto et al. [18] constructed a cephalosporinase-based microbial flow-type sensor having *Citrobacter freundii* that showed good specificity towards cephalosporins but with limited detection range and response time. Gracia et al. [21] reported a protein-engineered β-lactamase microbial biosensor, constructed by site-directed mutagenesis in *E. coli* (E104M/G238S), to have a broad specificity towards detecting cephalosporins of first, second, and third generations, but the biosensor showed limited detection ranges with these antibiotics.

In order to check the interference of a cosubstrate, another β-lactam antibiotic, on the performance of the biosensor, the assay medium contained a mixture of two β-lactam antibiotics at equal concentrations. The results of this experiment are presented in Table 1. In this study, the biosensor response only with cephalosporin C was assumed to be 100% and compared with the results obtained in presence of another antibiotic. The biosensor would not be able to distinguish largely between cephalosporins. Nevertheless, the biosensor was almost insensitive to the noncephalosporin group of antibiotics like penicillin and amoxicillin, as indicated by the responses that were very close to cephalosporin C in the medium. Also, when the assay mixture consisted of two cephalosporins other than cephalosporin C, a similar good response was observed.

In order to determine the accuracy of the biosensor in cephalosporins detection, different concentrations (3, 5, 8, and 10 mM) of cephalosporin C were examined using five replicates at each concentration (Table 2). In this test, the amount of the antibiotic detected by the biosensor was also verified by the hydroxylamine method. From the error estimates of standard deviation and percent coefficient of variation, it can be said that determinations through the biosensor were highly precise and accurate.

The overall results of this detailed study showed that permeabilized cells of *P. aeruginosa* could effectively be integrated in a biosensor set up by proper design and its operation in detecting cephalosporin group of antibiotics with high levels of accuracy and precision. The method is recommended for the routine determination of the selected antibiotics in pure and in pharmaceutical preparations as an alternative to the already existing methods.

Table 1 Substrate specificity and interference due to a cosubstrate on the performance of the microbial biosensor.

Substrate[a]	Response (%)
Cephalosporin C	100
Cephalotaxime	82
Cephalexin	69
Cephaloridine	77
Penicillin G	12
Amoxicillin	7
Cephalosporin C + Cephalotaxime	173
Cephalosporin C + Cephalexin	164
Cephalosporin C + Cephaloridine	171
Cephalosporin C + Penicillin G	101
Cephalosporin C + Amoxicillin	100
Cephalotaxime + Cephalexin	150
Cephaloridine + Cephalexin	142
Cephalotaxime + Cephaloridine	157
Penicillin G + Amoxicillin	12

[a] Concentration: 5 mM each

Conclusion

A novel biosensor based on lysozyme-pretreated *P. aeruginosa* whole cells was constructed and evaluated for its performance in detecting cephalosporin C and similar groups of antibiotics. Among the various design parameters, biomass content, glutaraldehyde, and gelatin were optimized for best performance in terms of sensitivity of the biosensor. Similarly, assay conditions, i.e., pH, temperature, and buffer strength, to yield good sensitivity of the biosensor in detecting cephalosporin C were optimized. Based on the optimized design and assay conditions, further characterization of the biosensor, namely, substrate versatility, specificity, storage stability, and accuracy of the system, was performed. The results of various investigations in this study showed very high potential of the novel biosensor in detecting a wide spectrum of cephalosporin group of antibiotics.

Table 2 Accuracy of the microbial biosensor in cephalosporin C determination.

Cephalosporin C concentration (actual) (mM)	Concentration determined by		Accuracy of estimation using the biosensor in relation with the actual (n=5)	
	Hydroxylamine method	Microbial biosensor	Standard deviation (SD)	Coefficient of variation (CV, %)
3.0	3.12	2.91	±0.0832	1.67
5.0	5.09	4.92	±0.0926	1.87
8.0	8.16	7.87	±0.0732	1.54
10.0	10.12	9.89	±0.0985	1.92

Average of five replicates

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