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Preservation of immobilized bacterial cell-matrix by drying for direct use in microbial sensors

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A simple and effective method for the drying of immobilized bacterial cells to be used directly in a microbial biosensor for measurement of activity is reported. As a case example, plasmid-bearing cells of *Alcaligenes eutrophus* JMP 134, DSM 4058 were immobilized on various carriers and liquid-dried. The dried cell-matrix was used directly after rehydration/reactivation as the biological component of a biosensor for determining the concentration of xenobiotic compounds in the environment. Good viability results were obtained after long-term storage and cells exhibited no loss of plasmids responsible for the 2,4-dichlorophenoxyacetic acid (2,4-D) degradation. The activity of the cells for 2,4-D was proved using a respiration electrode. No time-consuming, repeated cell cultivation and harvesting was required, as the cells preserved from a single batch served as a continuous source for activity measurements. Many other microbial cultures can be preserved by this method and the cells preserved in the form of immobilized dried cell-matrix can be used directly to perform enzymatic tests, complex biochemical conversions and for production in the reactors. The dried cell-matrix can serve as a stable interchangeable component for a multipurpose biosensor.

Key words: Bacterial immobilized cells, liquid-drying, preservation, microbial biosensors, xenobiotics detection.

Biosensors are micro-detectors which were developed in the early 1960s. They have several applications and play an important role in the simple and quick determination of oxygen concentration, to monitor products of biochemical fermentations and toxic substances in food and the environment.

The biological component of a biosensor varies from microorganisms to enzymes and antibodies. The microbial biosensor consists of a transducer covered with a biological layer (Figure 1). For the preparation of an immobilized biological layer, a fresh bacterial inoculum, grown to a definite stage, is always required. This has short life and involves time-consuming cell cultivation and harvesting.

There are several applications and advantages of using immobilized cells (Moo-Young 1988). However, immobilized, active cells show short-term viability and stability because, unlike free-living cells, they cannot be sub-cultured regularly. Vacuum-drying helps cell binding (adsorption or entrapment) on surfaces and drying also reduces the cells metabolic rate and enzyme activity, so improving long-term viability. The loss of viability and stability after drying and after storage can be reduced to a minimum if drying is conducted under controlled conditions (Annear 1962; Webb 1967; Malik 1988a). Thus it is convenient and economical to prepare immobilized, dried microbial cell-matrix with good activity and viability which can be stored for long periods and can be used directly in a microbial sensor.

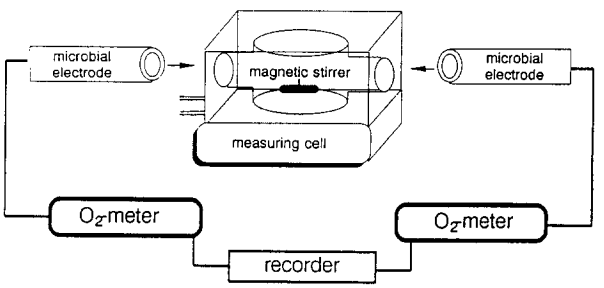

Figure 1. The system for measuring the respiration activity of cells, using a microbial biosensor.

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Alcaligenes eutrophus JMP 134 cells were used in the present study to form the biological layer. This is one of the most thoroughly studied microorganisms with respect to degradation of xenobiotics (Don & Pemberton 1981). In this strain the plasmid pJP4 codes for 2,4-dichlorophenoxyacetic acid (2,4-D) degradation (Don & Pemberton 1985). *Alcaligenes eutrophus* JMP 134 requires O_2 for the degradation of chlorinated derivatives of phenoxyacetates. Therefore, the concentration of these xenobiotics can be determined via the respiration activity of immobilized cells, using a microbial biosensor. This paper describes a method for the successful drying of *Alcaligenes eutrophus* JMP 134 in the form of an immobilized cell-matrix. The dried cell-matrices could be stored for long periods, gained activity after short-term rehydration and could therefore be used directly (without regrowing the bacteria) as the interchangeable biological components of a biosensor.

Material and Methods

Organism and Growth Conditions

Alcaligenes eutrophus DSM 4058, strain JMP 134 (Don & Pemberton 1981) was kindly provided by K. N. Timmis, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany. The strain was grown in a minimal medium used by Don & Pemberton (1985) containing (g/l): K_2HPO_4, 1.6; KH_2PO_4, 0.4; $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 0.05; $FeSO_4.7H_2O$, 0.01. The medium was sterilized at 121°C for 15 min. The 2,4-D was added as the only source of carbon to give 1 mM. For this purpose, 2,4-D was dissolved in a minimum of ethanol and filter-sterilized. The cells were cultivated at 30°C for just 10 h because the microbial activity towards the degradation of 2,4-D starts to decline after this time.

Preparation of Protective Agents and Other Materials

Solutions of three protective agents, 5% (w/v) *meso*-inositol, 5% (w/v) *meso*-inositol with 1% (w/v) neutral activated charcoal, and 5% (w/v) *meso*-inositol with 5% (w/v) gelatin, were prepared in distilled water, autoclaved at 115°C for 13 min and stored at 4°C.

Three types of membrane filters were used: 0.2 μm pore cellulose mixed ester (reference no. 401 706; Schleicher & Schuell, 3354 Dassel, Germany); 0.2 μm pore cellulose acetate (Sartorius, 3400 Göttingen, Germany); and 0.4 μm pore capillary membrane (Oxyphen GmbH, Dresden, Germany). The membranes were cut into small discs of such a size that they covered the face of the oxygen electrode. The membrane filters were autoclaved at 115°C for 13 min in glass Petri dishes lined with filter paper.

For the preparation of gelatin discs, plates were prepared with paraffin wax bases. Solid paraffin wax (5 to 7 g) was sterilized and poured while molten (70 to 80°C) into sterile Petri dishes, covered and allowed to solidify.

Preparation of Cell Suspension for Liquid-drying

A thick cell suspension was prepared in an appropriate protective medium. The cells were harvested by aseptic centrifugation for 30 min at 4000 × ***g*** and the pellet was suspended in various protective agents to yield a heavy cell suspension (at least 10^8 cells ml^{-1}).

To each membrane disc about 25 μl (1 drop from a Pasteur pipette) of cell suspension were applied aseptically. The homogenized cell suspension in gelatin was briefly maintained at about 45°C and from this 10 to 15 drops (each about 30 μl) from a Pasteur pipette (or a dropping pipette) were dropped separately onto solidified paraffin in a Petri dish.

The Petri dishes containing membranes and gelatin drops were quickly placed into a metallic jar maintained at 20°C in a water bath (Malik 1990a) and then, after 20 to 30 min of equilibration, they were subjected to liquid-drying under vacuum.

Liquid-drying Procedure

Liquid-drying (L-drying) was done in two stages according to Malik (1990a). Using a centrifugal freeze-drying machine (Edwards Freeze-dryer Modulyo), the primary drying was achieved in two stages without external freezing or involving the use of the centrifugal head. When the condenser temperature fell below −50°C, the metallic anaerobic jar with evacuation tube was connected to the freeze-drying chamber, the vacuum pump was switched on and the air inlet valve was closed slowly and partially to achieve a mild vacuum. (If available a vacuum controller can be attached between the anaerobic jar or vacuum pump and the freeze-drying machine to achieve the correct vacuum.) First-step drying was for about 2 h at 10 to 40 mbar and second-step drying was at 0.1 to 0.05 mbar vacuum for about 1 h. The temperature was maintained at about 20°C during drying. At the end of the drying period the vacuum was replaced with sterile air (or, preferably, N_2 gas). The dried gelatin discs and membranes could easily be picked up with sterile pointed forceps and were transfered into a sterile, screw-cap (gas-tight), glass vial for storage at 4–9°C or, preferably, at −30°C. For prolonged storage, the dried bacterial cell preparations were sealed under vacuum as described previously (Malik 1988a, 1990a).

Long-term Storage

Generally, an accelerated storage test is used to predict long-term storage capacity of the dried microbial cells. In the present study, the accelerated storage test was conducted by keeping the dried cells in parallel at 37°C and 45°C for a few weeks.

Estimation of Viability

Survival recoveries were checked before L-drying, immediately after L-drying and after storage. For comparison, the viable cell counts were calculated and the percentage survival determined. For the estimation of viability counts, a known volume (30 μl) of cell suspension was liquid-dried and serial decimal dilutions were prepared in the appropriate liquid media. Plating and counting were by standard methods.

Reactivation of Dried Cell-matrix and Activity Testing

The ability of *Alcaligenes eutrophus* JMP 134 cells to degrade 2,4-D before and after L-drying was tested. The activity test for 2,4-D was based on the increase in respiration of cells after the addition of 2,4-D, measured according to Riedel *et al.* (1985) using a respiration electrode (Figure 1). Two oxygen electrodes fit into the measuring cell. For activity testing, a 0.1 M solution of 2,4-D was used. The solubility of 2,4-D in water was achieved by dissolving it first in 0.1 M NaOH solution.

The dried bacterial cells were first rehydrated in two different media to regain activity. For reactivation 'Solution A' containing nutrient broth with 1 mM 2,4-D diluted (1:1) with 0.1 M potassium phosphate buffer (pH 7.0) and 'Solution B' containing minimal medium with 1 mM 2,4-D diluted (1:1) with 0.1 M potassium phosphate buffer (pH 7.0) were used. The membrane discs containing dried cell-matrix (for comparative activity testing cells in gelatin were also dried on membranes) were placed in a sterile

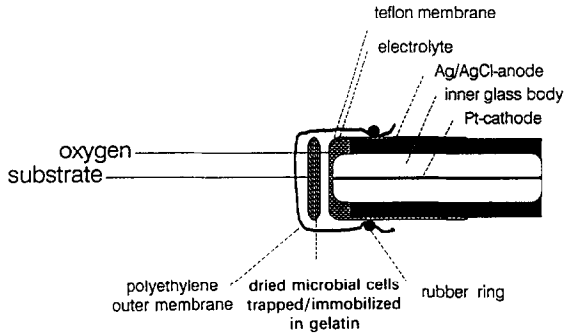

Figure 2. Schematic diagram of a microbial electrode using a dried cell-matrix.

Petri dish (lined with a filter paper). A drop of Solution A or B was applied to each membrane disc and these were then incubated at 20°C for 6, 12 or 24 h. At the end of each incubation period, the soaked cells on membranes were placed on a capillary membrane (pore diameter 0.4 μm; Oxyphen GmbH, Dresden, Germany) which was placed directly on the polyethylene membrane of an oxygen electrode. The cells dried directly in gelatin discs could also conveniently be placed on membrane discs for rehydration and reactivation (Figure 2). The outer membrane was held in place using a rubber ring (Figure 2). The microbial biosensor was placed in a measuring cell containing 3.0 ml of minimal medium with 1 mM 2,4-D diluted (1:1) with 0.1 M potassium phosphate buffer (pH 7.0) for 2, 4, 6, 8 or 10 h at room temperature in order to determine the optimal time (Figure 1). At the end of each incubation, the measuring cell was washed two or three times with 0.1 M phosphate buffer (pH 7.0) and the activity test was started by adding 0.1 ml of 0.1 M 2,4-D to the measuring cell containing 3.0 ml of 0.1 M potassium phosphate buffer. Immediately after the addition of 2,4-D a decrease in the oxygen concentration was observed. The acceleration of respiration directly after the addition of the substrate was measured within 12 s as an initial rate of current change per minute (Riedel *et al.* 1988). The change in current was followed at −600 mV vs Ag/AgCl (Figures 1 and 2). For a detailed description of this method see Riedel *et al.* (1985).

Results and Discussion

In microbial biosensors used for activity measurements, microorganisms are required which have been grown to a stage showing optimum activities (Riedel *et al.* 1988). For harvesting, time-consuming centrifugation and washing have always been required to prepare and immobilize the microbial cells to be used as a biological layer. It is thus desirable to have an effective method of microbial cell storage which maintains cell activity (stability) and viability for long periods so that the preserved immobilized cell-matrix can conveniently be used as a ready source for activity measurements.

Living microbial cells are immobilized on various agents which form a matrix and bind the cells together. The cells are first immobilized in a viscous substance which is then coated on carriers, creating a semi-porous membrane. Immobilization of microbial cells in gelatin or gelatin-like material has been used successfully to increase cell density. However, the living immobilized bacterial cell-matrix cannot be maintained for long without loss of good activity, viability and stability. By drying the immbolized cells, their metabolism can be reduced and enzyme activities can be minimized. This creates dormancy which helps to achieve long-term survival of the immobilized dried cells (Malik 1988a,b; 1990a).

Lyophilization is generally used for long-term storage of microorganisms. Although it has many advantages, freeze-drying of several plasmid bearing strains and Gram-negative bacteria resulted in loss of viability and stability, even in the presence of effective protective additives (Malik 1988a,b). Microorganisms which are sensitive to freezing and freeze-drying can be dried directly from the liquid phase without freezing, under controlled conditions and in the presence of protective agents (Malik 1990a, 1992). A new method of liquid-drying developed by Malik (1990a) has been successfully applied to the preservation of a collection of fragile microorganisms. This method was used, with some modifications, to dry immobilized bacterial cell-matrix on various carrier membranes. A dried cell matrix of *Alcaligenes eutrophus* JMP 134 resulted when cells suspended in 5% (w/v) *meso*-inositol with or without 5% (w/v) gelatin were dropped on membranes and then subjected to liquid-drying. This dried mass could easily be stored for long periods and good activity towards 2,4-D degradation after drying and long-term storage was observed. The dried cell-matrix of *Alcaligenes eutrophus* JMP 134 was ready to be placed on the oxygen electrode for activity measurements (Figure 2) immediately after reactivation.

By using a paraffin base, it was possible to prepare small gelatin discs without the use of other carrier membranes. The dried gelatin discs and membranes were easily picked up with sterile pointed forceps and were transfered into sterile vials for storage. For prolonged storage it is recommended that the dried cells are stored at low temperature under vacuum.

The survival recoveries of *Alcaligenes eutrophus* JMP 134 after L-drying are shown in Table 1. All dried preparations proved viable after L-drying and during storage. No mutation or loss of microbial activities were observed. The best viability and activity resulted when the cells were dried in gelatin discs. Fairly good long-term storage of the dried bacterial cells is predicted, based on the results of an accelerated storage test at 37°C and 45°C (Malik 1988a,b). The activities of immobilized cells during drying and after storage are shown in Table 2. The results indicate that the L-dried cells retained most of their activity after preservation. The reactivation procedure and the rehydration media have a great influence on the activation of the dried cell preparations (Malik 1990b); in the present study

Table 1. Survival of *Alcaligenes eutrophus* JMP 134 cells after drying and storage.

Immobilization	Viability counts (c.f.u.)					
			Storage at:			
	Drying		37°C		45°C	
	Before	After	1 week	3 weeks	1 week	3 weeks
Gelatin discs	1×10^9	5×10^8	5×10^7	1×10^7	1×10^7	5×10^6
Cellulose acetate membranes	1×10^9	3×10^8	5×10^6	5×10^4	5×10^4	5×10^3
Cellulose acetate ester membranes	5×10^7	5×10^6	NT	NT	NT	NT

NT—Not tested.

Table 2. Activities of immobilized cells during drying and storage.[a]

Immobilization in	Activity values (nA/min)[b]					
	Drying		Storage at:			
	Before	After	37°C (days)		45°C (days)	
			10	20	10	20
Gelatin discs	1300	410	335	376	223	250

[a] Cells of *Alcaligenes eutrophus* JMP 134 were immobilized in gelatin and liquid-dried to achieve gelatin discs with trapped cells for direct use in a microbial biosensor.
[b] Average activity values from three repeated experiments. Activities were measured directly (without reactivation) before and after liquid-drying and after storage at 37°C and 45°C for 10 and 20 days (used as accelerated storage test). For more details see Material and Methods.

almost 80% of cell activity appeared to be lost directly after drying when the dried cells were not reactivated before use (Table 2). However, higher activity values were observed after reactivation in Solution A for about 12 h (Table 3). It is therefore recommended that the dried cell preparations should be briefly rehydrated before activity measurement to convert them from the reduced metabolic state, the 'dormant phase', to the 'active phase'. However, no prolonged incubation is required to get new growth. Figure 3 shows a typical response curve for the microbial sensor and the increase in activity values after short reactivation. The activity values during the first 6 h of incubation were very low and irregular, perhaps because no steady state had been reached. After incubation for 24 h there was no detectable respiration activity left in any sample.

There were some variation in the activity values measured before and after preservation or after prolonged storage, probably because the number of cells used was not constant. If too much pressure was applied when the cells were fixed to the cathode of the oxygen electrode, the cells

Table 3. Reactivation of immobilized dried cells in two different solutions.

Reactivation time (h)	Activity values (nA/min)*					
	Solution A			Solution B		
	G	CAE	CA	G	CAE	CA
6	701	300	165	165	361	232
12	998	735	913	139	152	–

* Average activity values of cells dried in or on: G—gelatin discs; CAE—cellulose acetate ester membranes; CA—cellulose acetate membranes. After 24 h of incubation there was no respiration activity in any sample. No attempt was made to optimize the conditions and media to achieve maximum activities.

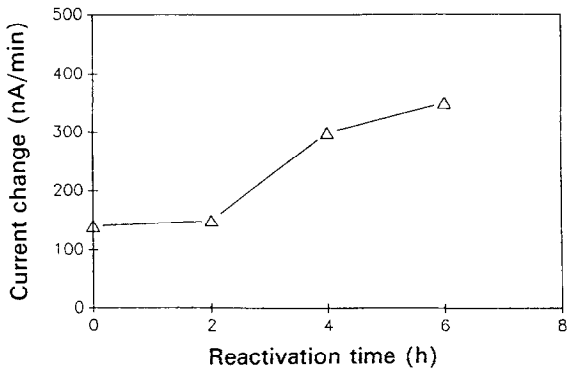

Figure 3. Activity, in terms of electrode current, of the dried microbial cells immobilized in gelatin after various periods of reactivation.

were pushed to the sides and if too little pressure was applied only a few cells remained in contact because of the round head of the electrode. This problem could be solved by drying a uniform, thick bacterial layer immobilized on carriers shaped to fit the electrodes (Figure 2).

The presence of activated charcoal during L-drying and in the reactivation medium has several advantages (Malik 1990b). In the present study, the activated charcoal initially used during L-drying improved survival recoveries. However, it was eventually omitted because it caused disturbance in the measurement of respiration activity due to its high absorption capacity.

The method described here is simple and effective. It is useful for the provision of a ready-to-use inoculum (cell mass), from the same batch, which is viable over periods of months or years and which can be used directly for activity measurements. The dense microbial cell suspension can be stored as a dried cell-matrix of suitable size and used when required. Thus the dried cell-matrix on membranes or in the form of gelatine discs served as a stable interchangeable biological component for a biosensor. Similar immobilized dried microbial cell-matrices could be used to perform enzymatic tests and complex biochemical conversions and to test the product from reactor-scale fermentations.

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