Clostridium isatidis colonised carbon electrodes: voltammetric evidence for direct solid state redox processes

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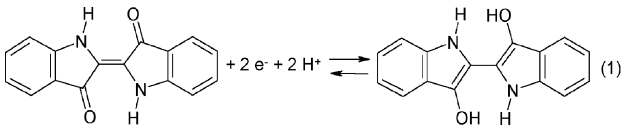
Bacteria are known to interact with their environment *via* oxidation and reduction processes driving or driven by their metabolism. These bacteriological processes are important in areas of corrosion, mining and waste treatment. However, molecular level details on how electrons are transferred between the cell and a solid reactant are not well known. In this study *in situ* electrochemical interrogation of redox processes in living bacteria probed with a graphite electrode yields unique mechanistic information on the reduction of solid indigo by the moderately thermophilic bacterium *Clostridium isatidis*. This reduction process yields the reduced form, leuco-indigo, which is an important intermediate in the dyeing industry. The process is shown to be a 'true' solid state process rather than involving solubilisation of indigo. This finding has important implications for the application of bacterial-driven reduction in indigo dyeing.

Introduction

The interest in chemical processes of technological relevance driven by bacteria,[1] is very high due to the range of problems which can be approached and the use of natural energy resources. Although the presence of bacteria poses a considerable problem in corrosion processes,[2] their use in organic synthesis, mining[3] and waste treatment[4] can be of considerable benefit. As a particular type of chemical process bacteria driven redox processes[5] are of fundamental importance.

It has recently been demonstrated that a moderate thermophile *Clostridium isatidis*, isolated from a reproduced ancient woad dye vat[6] and from a 10th century dye vat[7] can reduce indigo to leuco-indigo, the soluble intermediate in the indigo dyeing process. Reduction can occur in a nutrient-rich medium at 50 °C where water-soluble indigo carmine as well as the highly insoluble indigo have been demonstrated to be reduced to their leuco forms (eqn. (1)). Anaerobic fermentation by *C. isatidis* converts complex carbohydrates to give acidic and gaseous (CO_2, H_2) products.



C. isatidis cells are motile, slime-forming rods (0.3–0.6 μm × 1.8–9.1 μm). Fig. 1 shows *C. isatidis* reducing indigo dye particles. The mechanism of the observed reduction process is not easily understood, since indigo is very insoluble; reduction could proceed *via* a solubilisation process, with dye at low concentrations diffusing into the cell in the oxidised form, or *via* a direct extracellular pathway. However, electrochemical techniques can be employed to demonstrate the ability of the bacteria in a nutrient rich environment to directly cause redox processes at the surface of solid materials.

Experimental

Clostridium isatidis (type strain WV6) were maintained on Reinforced Clostridial Agar (Oxoid) and grown on Reinforced Clostridial Medium (RCM, 50 ml)[5] at 50 °C adjusted to pH 9 with NaOH. Indigo and indigo carmine were obtained from Aldrich. Electrochemical experiments were conducted with a PGSTAT 20 Autolab system (Eco Chemie, Netherlands). Electrodes used were a 4.9 mm diameter basal plane pyrolytic graphite disc ('Pyrocarbon', Le Carbone UK Ltd.), a Ag/AgCl (3M NaCl) reference (BAS), and a carbon rod counter electrode. The surface of the graphite working electrode was renewed prior to experiments by removing surface layers with fine carborundum polishing paper. The procedure employed for modifying the graphite electrode surface by mechanically attaching solid microcrystalline particles has been described recently.[8] The pH (Hanna checker pH probe, Aldrich) and the

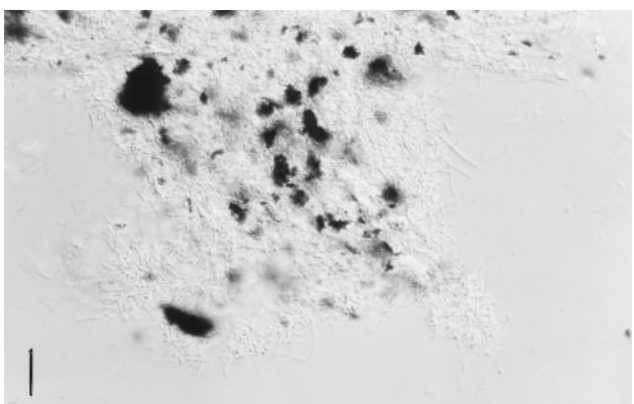


Fig. 1 Photomicrograph of *C. isatidis* growing at 50 °C in RCM (Reinforced Clostridial Medium) containing 0.01% (w/v) indigo after 24 h (bar 10 μm).

optical density were monitored *in situ* with a home built device based on a light emitting diode ($\lambda = 600$ nm) and a 1 cm path length. The optical probe was immersed separately from the working electrode and provided an approximate measure of the state of the bacterial culture during growth. All experiments were conducted in a closed thermostatted system at $T = 50\,^{\circ}C$.

Results and discussion

Bacteria are known to be electrochemically active in the sense of being able to modify the redox state of their environment due to their metabolism. Fermentation products are formed such as hydrogen gas, which may act as a reversible redox system in conjunction with a platinum potentiometric sensor. This principle has been employed to monitor the presence of different types of bacteria qualitatively and quantitatively with platinum electrodes.[9] Most of the nutrients and products of metabolism do not form reversible redox systems, and do therefore not directly participate in establishing the potential measured by the electrode employed as sensor. The kinetics of the hydrogen/H^+ couple on platinum are reversible and therefore dominate the measured potential. However, at graphite electrodes this process is sufficiently slow and associated with a considerable overpotential, so that it cannot participate in stabilising the measured potential.

Fig. 2 shows a plot of the changes in pH (curve A), the equilibrium potential detected at a basal plane pyrolytic graphite electrode (curve B), and the optical density (curve C) monitored during the course of a growth experiment with *C. isatidis* in Reinforced Clostridial Medium (RCM) at 50 °C. After a 'lag phase' of approximately 6 h, during which residual oxygen is removed and growth of the bacteria commences, a decrease in pH is detected associated with the formation of protons by the now exponentially growing culture. After 10 h the proton concentration stabilises at pH 5.5 due to acid production, and the optical density ceases to increase.

The equilibrium potential measured at a basal plane pyrolytic graphite electrode (curve B) drops approximately 5–6 h after the growth experiment was initiated. At this stage the bacteria appear to be able to dominate the processes at the electrode and to establish reducing conditions. Removing the electrode from the RCM solution phase and re-immersion after cleaning the surface causes a delay of 10 to 30 min for the equilibrium potential to return. The potential stabilises at −0.5 V *vs.* Ag/AgCl before returning to a more positive value in the later stages of the experiment. From the approximately linear change in potential (curve B) and pH (curve A) between 8–9 h duration a change in equilibrium potential of *ca.* 60 mV per pH unit can be calculated. This correlation is indicative of a redox process involving the uptake of one proton per electron transferred or two protons per two electrons transferred, respectively (*vide infra*). The theoretically expected shift in the equilibrium potential at 50 °C is given by $(RT/F)\ln(10) = 64$ mV.

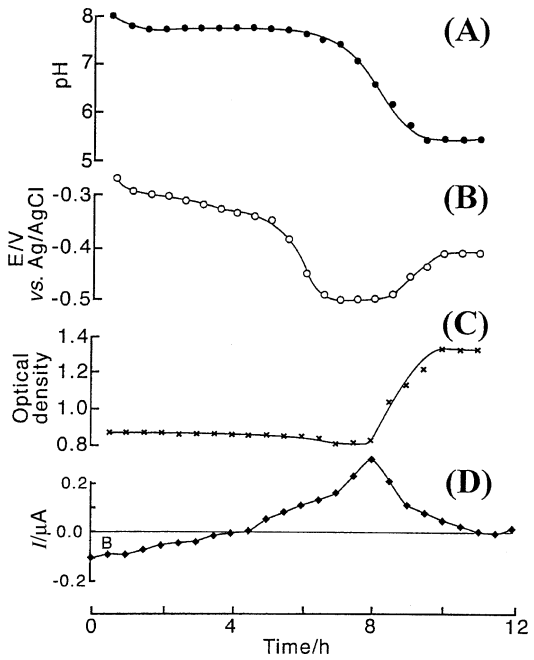


Fig. 2 Time dependence of (A) the pH, (B) the equilibrium potential measured at a 4.9 mm diameter basal plane pyrolytic graphite electrode and (C) the optical density monitored during the course of a growth experiment with *C. isatidis* in RCM at 50 °C. (D) Time dependence of the current observed at a 4.9 mm diameter basal plane pyrolytic graphite electrode held at a potential of −0.4 V *vs.* Ag/AgCl.

Cyclic voltammograms recorded during the course of the growth experiment yield further information on the nature of the bacterial redox process. Fig. 3 shows voltammograms recorded at the beginning (pH 7.8) and towards the end (pH 5.5) at a basal plane pyrolytic graphite electrode in RCM at 50 °C. Initially only very weak reduction and re-oxidation responses (Fig. 3A) are detected with $E_{p,\,red} = -0.52$ V *vs.* Ag/AgCl and $E_{p,\,ox} = -0.48$ V *vs.* Ag/AgCl, which during the course of the growth experiment increase accompanied by a considerable shift in potential. At pH 5.5 (Fig. 3B) the peak potentials for the reduction, $E_{p,\,red} = -0.40$ V *vs.* Ag/AgCl, and for the re-oxidation, $E_{p,\,ox} = -0.34$ V *vs.* Ag/AgCl, underwent a shift of approximately 60 mV per pH unit consistent with the equilibrium potential data (*vide supra*). Therefore it can be concluded that (i) the redox system detected by cyclic voltammetry is responsible for the equilibrium potential detected at a basal plane pyrolytic graphite electrode and associated with the presence of *C. isatidis* and (ii) the transfer of electrons in the reduction process is associated with the protonation of the product.

Further, the approximately linear dependency of the observed peak current on the potential scan rate[10] ranging from 5 to 500 mV s^{-1} suggests that the redox active material is confined to the electrode surface rather than dissolved in solution. Data from voltammetric experiments are summarised in Table 1. Removing the electrode from the RCM solution phase, polishing and re-immersing it causes the voltammetric response to disappear. Only after a period of 10 to 30 min can the same voltammetric response be detected again. Therefore it is proposed that bacteria in the RCM environment at 50 °C are able to adhere to the graphite electrode surface and the 'bio-layer' formed in this way contains a redox system responsible for the electrochemical activity.

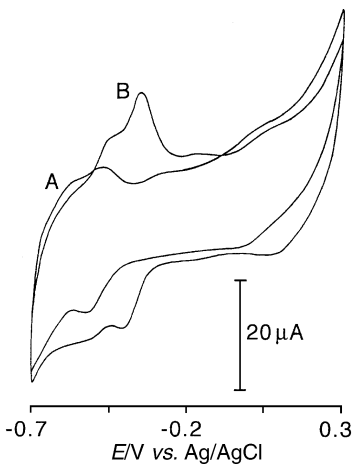


Fig. 3 Cyclic voltammograms recorded at a 4.9 mm diameter basal plane pyrolytic graphite electrode immersed in RCM at 50 °C (A: pH 7.8, B: pH 5.5, scan rate 0.1 V s^{-1}).

Table 1 Cyclic voltammetric data for *C. isatidis* in RCM after 8 h growth recorded at a permanently immersed 4.9 mm diameter basal plane pyrolytic graphite electrode at $T = 50\,^{\circ}C$

Scan rate /mV s^{-1}	$E_{p,red}$[a]/V *vs.* Ag/AgCl	$I_{p,red}/\mu A$	$E_{p,ox}$/V *vs.* Ag/AgCl	$I_{p,ox}/\mu A$	$E_{1/2}$[b]/V
20	−0.60	5.0	−0.40	3.3	−0.50
50	−0.60	12	−0.39	9.1	−0.50
100	−0.60	21	−0.37	16	−0.49

[a] Peak potentials correspond to a complex voltammetric wave composed of more than one signal. [b] The apparent half wave potential corresponds to the average of the peak potentials $E_{1/2} = 0.5\,(E_p^{red} + E_p^{ox})$.

The redox chemistry of solid indigo particles in aqueous media has been studied recently[11] and two types of pH-dependent reduction processes have been identified: (i) a surface confined reduction associated with the protonation of the product (eqn. (1)) and (ii) a bulk reduction process accompanied by cation insertion. Indigo in the form of solid particles mechanically attached[8] to the surface of a basal plane pyrolytic graphite electrode and immersed in RCM at 50 °C shows very similar characteristics with a reduction response at $E_{1/2} = -0.47$ V *vs.* Ag/AgCl at pH 7.8. This reduction response is *ca.* 30 mV positive compared to the redox couple detected in the presence of *C. isatidis* with the same 60 mV shift per pH unit. The resulting driving force for the reduction of indigo by the bacteria is therefore sufficient to bring about reduction to the leuco form. In chronopotentiometric experiments the equilibrium potential reached in the presence of bacteria depends on the presence of indigo mechanically attached as a solid to the electrode surface. In a growth experiment after 6 h the equilibrium potential detected is −0.5 V *vs.* Ag/AgCl. In the presence of indigo the potential stabilises at −0.44 V *vs.* Ag/AgCl consistent with the redox couple indigo/leuco-indigo 'pinning' the potential of the graphite electrode due to reduction by the bacteria.

Finally, a chronoamperometric experiment was employed to study the ability of the bacteria to reduce insoluble materials. Fig. 2D shows the change in the current detected at a basal plane pyrolytic graphite permanently held at a potential of −0.4 V *vs.* Ag/AgCl and immersed in RCM. It can be seen that after 8 h the change in optical density was accompanied by an anodic current response. This is most readily interpreted in terms of bacteria being able to couple electrochemically to the 'insoluble' solid electrode surface and to pass electrons into the electrode in a manner similar to that with which they would interact with other insoluble materials.

The solid state redox process identified here requires an electron transfer from the bacterial cell interior to a solid external electron acceptor. We note that despite the technological importance of the phenomenon[2–4] no biochemical mechanism is currently available (see for example ref. 12) to account for such a transfer.

Conclusions

It has been demonstrated that bacteria can directly interact with insoluble organic compounds suspended in aqueous solution and a process such as the reduction of indigo to leuco-indigo can be driven in the absence of an added redox mediator. Important implications of our present finding are that (i) electrochemical measurements can give a direct measure for the driving force and rate of bacterial redox processes, (ii) the ability of *C. isatidis* to reduce is strongly pH-dependent and (iii) a bacterial-driven reduction for indigo dyeing in the absence of redox mediator requires direct contact between bacteria and the solid indigo.

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