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Determination of Phenol and Catechol Concentrations with Oxygen Probes Coated with Immobilized Enzymes or Immobilized Cells

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Abstract

The enzyme phenol 2-hydroxylase was immobilized on Sepharose and used in conjunction with an O_2 electrode for quantitating phenol. Similarly, catechol 1,2-oxygenase was used for quantitating catechol. A third probe was prepared by immobilization of *Trichosporon cutaneum* cells rather than purified phenol 2-hydroxylase for phenol quantitation. The whole cell system gave results comparable to the immobilized enzyme system.

Index Entries: Phenol, determinations with immobilized enzymes and cells; catechol, determination with immobilized enzymes and cells; immobilized enzymes and cells, determination of phenols and catechols with; enzymes, immobilized, in determinations of phenols and catechols; cells, immobilized, in determination of phenols and catechols; *Trichosporon cutaneum*.

The method is a "spin off" of our studies on an inductive pathway for the catabolism of phenols in yeast. The data presented here were obtained with the soil isolate *Trichosporon cutaneum* and with the enzymes purified from this yeast strain.

Let us have a quick glance at the catabolic pathway involved. The attack on the phenolic ring is initiated by phenol 2-hydroxylase (EC 1.14.13.7). The resulting *o*-diol is then cleaved by catechol 1,2-oxygenase (EC 1.13.1.1) to *cis,cis*-muconate if phenol has been the primary substrate, or to maleyl acetate if resorcinol has been hydroxylated to 1,2,4-trihydroxybenzene. The ring cleavage products undergo further enzymatic conversions to give β-ketoadipate, which is split into a C_2 and a C_4 fragment before being fed into the central metabolism of the cell (*1*, *2*). Of importance for my presentation are the first two enzymes of the

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pathway viz., phenol 2-hydroxylase and catechol 1,2-oxygenase. Both are oxygenases, i.e., they incorporate molecular oxygen into their substrates. Therefore, we use the phenol-dependent O_2 depletion as the basis of our assay. We have developed three different probes. Two are for determination of phenol. One is based on purified and immobilized phenol 2-hydroxylase, the other on whole immobilized cells of our organism. A third probe is based on the purified, immobilized catechol 1,2-oxygenase. It specifically measures catechol and does not respond to monohydric phenols.

In all three cases we use polargraphic oxygen electrodes. Several types proved suitable, but most of the work reported here was done with the Clark Oxygen Electrode, Model 4004, from Yellow Springs Instruments, Ohio, USA. The probe is fitted into a thermostated 2-mL chamber provided with magnetic stirring. The equipment also comprises an electronic device converting the time–oxygen depletion curve to its first derivative. Both curves are simultaneously recorded on a two-channel recorder. This is essential, because the principle of our assays is kinetic. It involves the initial reaction rate—in contrast to many other enzyme electrodes that instead measure the end point, i.e., the total conversion of a substrate. There are many advantages to the kinetic method when using oxygen probes. One of them is that rigorous protection of the sample from outside oxygen becomes superfluous. Further, as the oxygen concentration in the sample solution drops, the enzyme(s) may no longer be saturated with this substrate, thus causing deviations from linearity. Figure 1 shows the principle of our recording. On the left, the primary curves of O_2 consumption; on the right, the corresponding first derivatives. The height of the latter gives a much more rapid and more accurate readout of the electrode response, than the primary curves.

Varieties of the enzyme electrode for phenol were obtained by immobilizing phenol 2-hydroxylase in different ways. This enzyme is a rather large, NADPH-dependent flavoprotein with an unusually high number of essential SH groups (*3*) and with a sensitive amino group. This hampered the use of the many convenient coupling methods that employ nucleophilic groups on the enzyme, e.g., crosslinking with glutaraldehyde or coupling to CNBr-activated Sepharose. The enzyme was, however, successfully immobilized through its carboxy groups using carbodiimide as the coupling reagent, or by adsorption to weak anion exchangers (*4*, *5*). Best results were obtained with phenol hydroxylase covalently bound to AH-Sepharose 4B or to nylon nets, as well as with the enzyme adsorbed on ion exchangers. The response of such electrodes was linearly dependent on phenol concentration over the range 0.05–5 mg/L (*5*). Electrodes with soluble enzyme behind a dialysis membrane gave responses too low for practical use. Even lower were the responses of electrodes with the enzyme entrapped in polyacrylamide gels. This probably reflects differences in diffusional restrictions with respect to the three substrates of phenol hydroxylase—oxygen, phenol, and NADPH, which have different size and hence different diffusivity (*5*).

Enzyme electrodes for catechol were obtained using catechol 1,2-oxygenase covalently coupled to CNBr-activated Sepharose or crosslinked with glutaraldehyde (*6*). Such electrodes gave specific responses linearly dependent on catechol concentrations over the range 0–20 mg/L with a lower limit of sensitivity

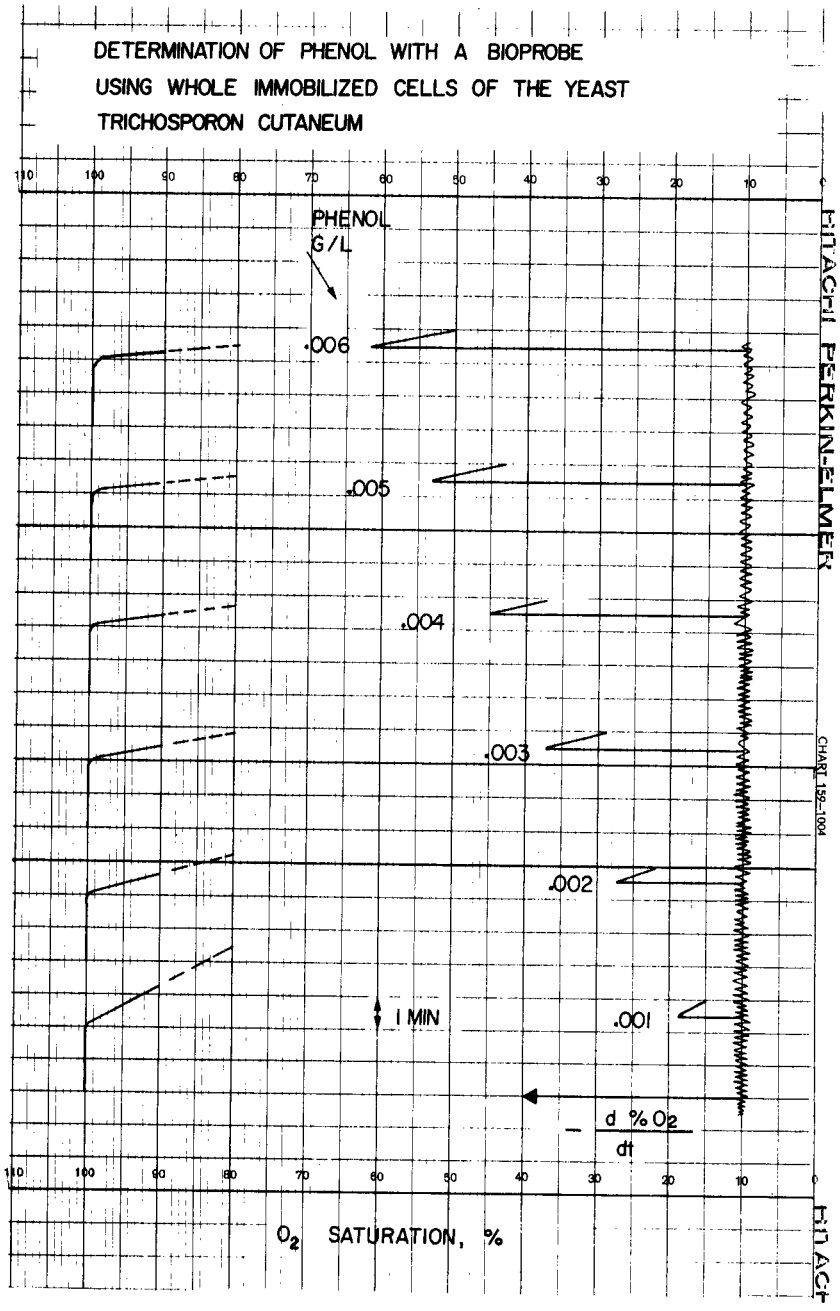

Fig. 1. Determination of phenol with a bioprobe using whole immobilized cells of the yeast *Trichosporon cutaneum*.

at about 0.5 mg/L. The response of the electrodes was practically unaffected by the presence of high amounts of biological materials, e.g., urine (50%), blood serum (25%), or liver extract (75 g fresh weight/L).

A third variety of an "enzyme probe" was obtained with whole immobilized cells of *Trichosporon cutaneum* induced for phenol degradation. Preparation of

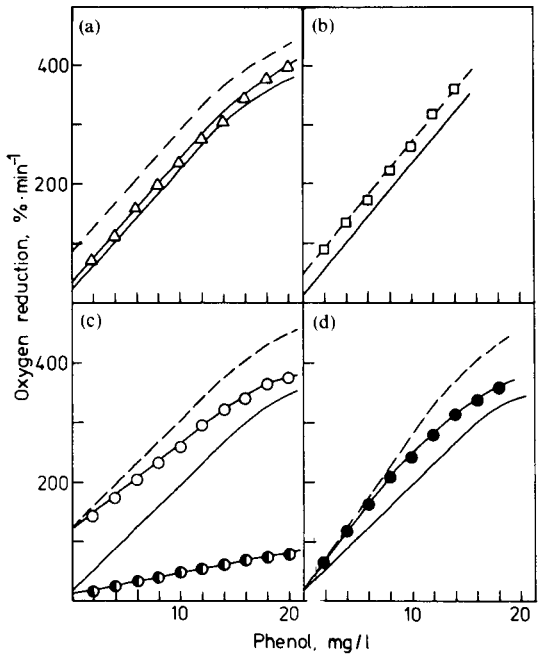

Fig. 2. The effect of phenolic and non phenolic oxygen-consuming compounds on the response of bioprobe for phenol (Clark oxygen electrode, coated with cell paste of phenol-induced *T. cutaneum:* (———) phenol alone; (---) calculated curve if response to phenol is added to that of other sample components. (a) (Δ) Phenol in presence of 200 mg glucose/L. (b) (□) Phenol in presence of 456 mg/L (anhydrous) sodium acetate. (c) Mixture of eight phenol derivitaves including cresols, chlorophenols, resorcinol, and catechol in equimolar amounts (◑); phenol in the presence of 36 mg/L of the same mixture (○). (d) Mixture of the eight phenol derivatives together with an equimolar amount of phenol (●). Reproduced from *Biotechnology and Bioengineering* **21,** 671 (1979).

such "bioprobes" is extremely simple. The organism can be grown in shake flasks or on agar plates or slants. A tiny amount of cells is applied to the surface of the oxygen probe and kept in place by a piece of dialysis tubing. The time-consuming purification of enzymes is avoided, as is also enzyme immobilization. No reduced cofactor (NADPH) needs to be added during assay. The sensitivity to phenol is comparable to that of an enzyme electrode. Interference from phenol analogs and nonphenolic oxygen-consuming components in the sample is negligible. This is shown in Fig. 2 (cf. ref. 7), which also illustrates how the presence of such substances can be detected and evaluated by comparing the slopes and the intercepts of the dose–response curves in buffer alone with those in the sample solutions (7).

All three varieties of enzyme electrodes gave very stable and reproducible responses. After an initial "run in" of about 5–10 assays, at least 150–200 subsequent assays were carried out without any sign of deterioration. The electrodes with purified and immobilized enzymes were stored for several months in refrigerator with repeated intermittent use. The bioprobe with whole immobilized cells proved unaffected by storage for 8–10 days at room temperature with repeated use. Longer storage times were not tried because of the extreme ease of preparing fresh

bioprobes. The induced cells of the organism can be stored frozen as a cell paste for more than a year without loss of activity.

The high sensitivity and selectivity of the bioprobe stems from the fact that, in cells induced to degrade phenol, phenol hydroxylase constitutes as much as 2% of all cellular proteins. This opens a fascinating perspective on the use of whole immobilized cells in enzyme electrodes, especially when the enzyme involved is of inductive character.

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