Bioaffinity layering of enzymes employing Concanavalin A
RESULTS

3.1 BIOAFFINITY LAYERING OF GLUCOSE OXIDASE ON SEPHAROSE PRECOUPLED WITH CON A

Cyanogen bromide activated Sepharose was incubated with appropriate amounts of Con A as detailed in the text in order to couple 1.7 mg protein per ml gel. Con A coupled to Sepharose was activated with metal ions and suspended in 0.1 M sodium acetate buffer, pH 5.2. For glucose oxidase immobilization, each ml of Con A-Sepharose was first incubated with the enzyme in buffer. After washing off the unbound enzyme with the same buffer, the Con A-Sepharose bound glucose oxidase was reincubated with a fresh sample of activated Con A, washed and again incubated with glucose oxidase as described earlier. These alternate incubation cycles with Con A and glucose oxidase were continued until preparations with required number of affinity layers were obtained. The theoretical and actual activity of matrix associated enzyme after the formation of various enzyme layers was determined as described in methods. Table 3.1 summarizes the results obtained during the affinity layering of glucose oxidase immobilized as three affinity layers on Sepharose. The Con A-Sepharose was highly effective in binding of glucose oxidase and a 15 fold increase in bound enzyme activity resulted after three incubations cycles with Con A and glucose oxidase.

An increase in effectiveness factor ‘η’ with layering suggested the arrangement of enzyme layers with most of the enzyme molecules retaining
Table 3.1

Bioaffinity layering of glucose oxidase performed with the help of Con A on CNBr Sepharose

<table>
<thead>
<tr>
<th>Layer number</th>
<th>Bound glucose oxidase (units/ml gel) (^b)</th>
<th>Effectiveness factor ((\eta)) (B/A)</th>
<th>Increase over layer I (Fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical (^c) (A)</td>
<td>Actual (^d) (B)</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>11</td>
<td>4</td>
<td>0.36</td>
</tr>
<tr>
<td>II</td>
<td>46</td>
<td>38</td>
<td>0.8</td>
</tr>
<tr>
<td>III</td>
<td>53</td>
<td>59</td>
<td>1.1</td>
</tr>
</tbody>
</table>

\(^a\) each value represents the mean of at least two independent experiments performed in duplicate. Variation did not exceed 9%.

\(^b\) glucose oxidase was assayed as described by Iqbal and Saleemuddin (1983a). One enzyme unit is the quantity of the enzyme that converts one micromole of substrate to product per minute under assay conditions at 37 °C.

\(^c\) determined by subtracting the number of units of enzyme remaining in the supernatant and washings after incubation from those added.

\(^d\) determined by assaying appropriate aliquots of the immobilized enzyme under assay conditions with continuous agitation.
catalytic activity. After six incubation of cells, the amount of glucose oxidase associated with the support was increased over 250 times more than that bound when the matrix was directly incubated with the enzyme (Fig. 3.1 A). The amount of Con A associated with the support also increased with layering and so did the ability of Con A molecules to bind glucose oxidase as evident from the layer-by-layer increase in the glucose oxidase-Con A ratios on the support. A remarkable 17.5 mg Con A and 19.0 mg glucose oxidase were found to be associated with one ml of Sepharose after seven incubation cycles (Fig. 3.1 B).

3.1.1 Effect of Glutaraldehyde Treatment

Treatment of affinity layered glucose oxidase preparations with 0.5% glutaraldehyde resulted in the loss of some enzyme activity. A decrease of 35% activity was observed in case of preparation having one layer of enzyme compared to only 2% loss in the catalytic activity of preparation comprising of three affinity layers of glucose oxidase (Table 3.2).

3.1.2 Properties of Bioaffinity Layered Glucose oxidase

3.1.2.1 Effect of pH

The effect of pH on the activity of soluble as well as bioaffinity layered glucose oxidase before and after crosslinking with glutaraldehyde was investigated in 50 mM sodium acetate/sodium phosphate buffers in various pH regions (Fig. 3.2 A, B). The pH optimum of the bioaffinity layered preparations remained unaltered at pH 6.0. The immobilized preparations were however found to retain far greater activity in the alkaline range compared to the native
Fig. 3.1 Bioaffinity layering induced increase in immobilized glucose oxidase (A) and Con A & glucose oxidase (B) on CNBr-Sepharose.

One ml of CNBr-Sepharose was incubated with Con A and glucose oxidase in alternate fashion as described in the text till the formation of seven affinity layers. After each binding cycle, the matrix was washed thoroughly with 0.1 M sodium acetate buffer, pH 5.2 to remove the unbound protein. The matrix bound enzyme (■) and Con A (●) was determined after each layer.
Table 3.2

Effect of glutaraldehyde treatment on the activity of bioaffinity layered glucose oxidase

<table>
<thead>
<tr>
<th>Layer number</th>
<th>Glucose oxidase (% activity retained)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>I</td>
<td>100</td>
</tr>
<tr>
<td>II</td>
<td>100</td>
</tr>
<tr>
<td>III</td>
<td>100</td>
</tr>
</tbody>
</table>

Appropriate units of glucose oxidase immobilized as affinity layers were treated with 0.5% glutaraldehyde for 2 hrs in 0.1 M sodium phosphate buffer, pH 6.1. The remaining groups were blocked by 0.1M glycine for 1 hr. and after washing with the buffer, the residual activity determined and expressed as percent activity retained.
Fig. 3.2  pH-activity profiles of soluble and bioaffinity layered glucose oxidase preparations prior to (A) and after treatment with glutaraldehyde (B).

Native glucose oxidase (■) and immobilized preparations bearing one ( • ) two ( ▲ ) or three layers ( ▼ ) of enzyme were incubated in appropriate buffers for 15 min and assayed at the indicated pH. The buffers used were 50 mM sodium acetate (pH 4.0, 5.0) and sodium phosphate (pH 6.0-8.0). The preparations were incubated with 0.5% glutaraldehyde for 2 hrs at 10°C.
glucose oxidase. Crosslinking with glutaraldehyde appeared to further enhance the activity retention in alkaline range.

3.1.2.2 Effect of Temperature

3.1.2.2.1 Activity at various temperatures

The temperature-activity profiles of native, bioaffinity layered glucose oxidase as well as bioaffinity layered and crosslinked enzyme preparations were compared (Fig. 3.3 A, B). Both native and bioaffinity layered preparations were optimally active at 40°C (Fig. 3.3 A) but there was observed a shift in the optimum to 50°C after treatment with glutaraldehyde (Fig. 3.3 B). A layer-by-layer increase in the fraction of the activity retained at high temperatures was exhibited both by crosslinked and uncrosslinked preparations although the stabilization appeared clearly more marked in the later.

3.1.2.2.2 Thermal stability at 60°C

Native, bioaffinity layered glucose oxidase as well as bioaffinity layered and crosslinked preparations were investigated for the ability to resist inactivation induced by incubation at 60°C (Fig. 3.4 A, B). Immobilization as bioaffinity layers improved the stability of the immobilized preparations which exhibited a layer-by-layer increase in the fraction of the activity retained after incubations for various durations at 60°C. The glutaraldehyde treatment further improved the stability of the preparations. The preparation with three affinity layers of glucose oxidase preincubated at 60°C for 2 hrs exhibited 50% activity,
Fig. 3.3 Temperature-activity profiles of soluble and bioaffinity layered glucose oxidase preparations prior to (A) and after glutaraldehyde treatment (B).

The activity of soluble and affinity layered glucose oxidase preparation was determined by incubating the enzyme preparations as indicated. Details are given in methods. Soluble and various immobilized preparations are indicated by symbols as in fig. legend 3.2.
Fig. 3.4  Thermal stability of soluble and bioaffinity layered glucose oxidase prior to (A) and after glutaraldehyde treatment (B).

For the determination of stability at 60°C, soluble and various immobilized preparations of glucose oxidase were incubated in 0.1 M sodium phosphate buffer pH 6.1 for indicated durations, rapidly cooled in ice for 1 hr and assayed for the residual activity. Aliquots of comparable activity of native enzyme not exposed to 60°C were taken as 100 for the calculation of percent activity in heat treated samples. Soluble and various immobilized preparations are represented by symbols as detailed in Legend to fig. 3.2.
which was increased to 80% after crosslinking. The soluble enzyme retained only 20% of initial under the same conditions.

3.1.2.3 Agitational Effects

The effect of agitation at different speeds on the enzyme activity was investigated in order to study the mechanical stability of the affinity layered preparations. No activity loss was detectable when bioaffinity layered glucose oxidase preparations containing one, two or three affinity layers were continuously agitated up to 1 hr at 700 & 1400 rpm on a rotary shaker indicating the strong association of the enzyme with support in preparations containing single and multiple affinity layers (Fig. 3.5).

3.2 BIOAFFINITY LAYERING OF INVERTASE

Con A–Sepharose was prepared according to the procedure described by Porath et al. (1967) to yield a preparation with 1.7 mg Con A per ml of Sepharose. The preparation obtained thus was incubated alternately with invertase and Con A in 0.1 M sodium acetate buffer, pH 5.2 to assemble three layers of invertase. A layer-by-layer increase in activity was observed and the amount of enzyme immobilized increased by 18 fold after formation of three affinity layers (Table 3.3). A gradual increase in the effectiveness factor was also seen with layering indicating the good accessibility of the enzyme associated with the support.
Fig. 3.5 Mechanical stability of affinity layered glucose oxidase preparations.

Aliquots of Sepharose matrix with one (●, ◦), two (▲, △) or three (▼, ▽) affinity layered glucose oxidase in 50 mM sodium phosphate buffer, pH 6.1 were mechanically agitated on a rotary shaker (Eppendorf Thermomix, 5437); at 700 rpm (filled symbols) or 1400 rpm (hollow symbols) washed with buffer containing 0.15 M NaCl and bound glucose oxidase activity determined. Activity of equal aliquots of the respective affinity layered preparation not subjected to agitation was taken as 100 for the calculation of percent activity in the agitated samples.
Table 3.3

Bioaffinity layering of invertase performed with the help of Con A on CNBr Sepharose

<table>
<thead>
<tr>
<th>Layer number</th>
<th>Bound Invertase (units/ml gel)</th>
<th>Effectiveness factor (η) (B/A)</th>
<th>Increase over layer I (Fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical (A)</td>
<td>Actual (B)</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>245</td>
<td>55</td>
<td>0.22</td>
</tr>
<tr>
<td>II</td>
<td>913</td>
<td>349</td>
<td>0.38</td>
</tr>
<tr>
<td>III</td>
<td>1495</td>
<td>973</td>
<td>0.65</td>
</tr>
</tbody>
</table>

Each value represents the mean of at least two independent experiments performed in duplicate. Variation did not exceed 9%.

Invertase was assayed as described by Bernfeld (1955). One enzyme unit is the quantity of the enzyme that converts one micromole of substrate to product per minute under assay conditions at 37 °C.

determined by subtracting the number of units of enzyme remaining in the supernatant and washings after incubation from those added.

determined by assaying appropriate aliquots of the immobilized enzyme under assay conditions with continuous agitation.
3.2.1 Properties of Bioaffinity Layered Invertase

3.2.1.1 Effect of pH

Activity profiles of native and bioaffinity layered invertase preparations containing one, two or three enzyme layers were investigated in 0.2 M sodium acetate or sodium phosphate buffers of various pH at 37°C (Fig. 3.6). Optimum pH values for preparations with two or three layers were same as that of the native enzyme however the preparation with one enzyme layer showed a shift of pH optimum to 4.0.

3.2.1.2 Effect of temperature

3.2.1.2.1 Activity at various temperatures

Temperature-activity profiles for native invertase and its bioaffinity layered preparations were investigated in 0.2 M sodium acetate buffer, pH 4.9 at indicated temperatures as shown in fig. 3.7. Native invertase showed optimum activity at 50°C and the preparation with one enzyme layer showed a comparable behaviour. In contrast, preparations with two or three enzyme layers showed an upper shift in temperature optima from 50°C to 60°C. Further more these preparations retained greater fraction of maximum activity over a wide range between 30°C and 60°C.

3.2.1.2.2 Thermal stability at 60°C

Preincubation of native and bioaffinity layered invertase preparations at 60°C resulted in almost complete inactivation of the native enzyme after 30 min.
Appropriate units of soluble and affinity layered invertase preparation were incubated in 0.2 M sodium acetate (pH 3.0-5.0) and 0.2 M sodium phosphate (pH 6.0-8.0) for 10 min and the activity determined under standard assay conditions. Activity profiles of soluble (■), affinity layered invertase with one (●), two (▲) and three layers (▼) are shown.
Fig. 3.7 Temperature activity profiles of native and bioaffinity layered invertase preparation.

Aliquots of soluble and bioaffinity layered invertase preparations were incubated at indicated temperatures for 10 min and assayed for enzyme activity under standard condition of pH and substrate concentration. Soluble and various immobilized preparations are represented by symbols as detailed in Legend to fig. 3.6.
In contrast, the immobilized preparations retained considerable enzyme activity even after 2 hrs of incubation at 60°C. The increase in thermal stability was layer dependent with the preparation and that bearing three enzyme layers retained 30% activity after 90 min of preincubation at 60°C (Fig. 3.8).

3.2.1.3 Effect of substrate concentration

When invertase catalyzed sucrose hydrolysis was measured as a function of substrate concentration at 37°C in 0.2 M sodium acetate buffer pH 4.9, a typical Michaelis-Menten behavior was observed both in case of soluble and bioaffinity layered preparations containing one, two or three enzyme layers (Fig. 3.9). The Line Weaver-Burk plot indicated $K_m$ values for native and affinity layered preparations with one, two and three invertase layers as 25 mM, 36 mM, 25 mM and 83 mM indicating a small increase in case of the three layered preparations. An increased $V_{\text{max}}$ was observed after affinity immobilization of invertase with preparation containing three enzyme layers having a $V_{\text{max}}$ of 7.1 μmoles/10 min. compared to 3.3 μmoles/10 min for the native counterpart.

3.3 CON-A MEDIATED BIOAFFINITY LAYERING OF β-GALACTOSIDASE ON SEPHAROSE SUPPORT

In an attempt to assemble layers of β-galactosidase on Sepharose, a support with 1.7 mg Con A/ml gel was incubated with β-galactosidase and Con A alternately in 0.1 M sodium acetate buffer pH 5.2 for required duration as mentioned in the text in order to assemble three layers of enzyme. Data obtained
Fig. 3.8  Effect of pre-incubation at 60°C on thermal inactivation of soluble and affinity layered invertase preparations.

Soluble and bioaffinity layered invertase preparations in 0.2 M sodium acetate buffer, pH 4.9 were incubated at 60°C for indicated time duration, cooled in ice for 1 hr and residual activity determined under standard assay condition. Activity of equal aliquot of soluble enzyme not subjected to heat inactivation was taken as 100 for calculation of percent activity. Soluble and various immobilized preparations are represented by symbols as detailed in Legend to fig. 3.6.
Fig. 3.9  Line Weaver Burk plots for sucrose hydrolysis by soluble and affinity layered invertase preparations.

Comparable quantities of soluble and affinity layered invertase preparations were incubated in a series of tubes with standard assays mixture containing varying concentration of substrate. After 10 min incubation at 37°C, the activity in all the preparations determined. Preparations used were soluble invertase (■), or those with one (●), two (▲) or three (▼) layers.
by determining the theoretical and actual activity of enzyme is summarized in Table 3.4. A layer-by-layer increase in the activity immobilized was also observed and enzyme units bound per ml gel could be increased from 16.0 to 105.0 - an over 6 fold rise.

Considering the specificity of interaction of Con A with glycoenzymes and with a view to cut down the cost of the immobilization, bioaffinity layering of \( \beta \)-galactosidase was also attempted using Jack bean extract in place of pure Con A. Experimental details are given in the text. Table 3.5 indicates that effective layering of the enzyme on the support was indeed possible. A 5.4-fold increase in activity on the support resulted after the assembly of three layers of \( \beta \)-galactosidase with the help of extract.

### 3.3.1 Elution of \( \beta \)-Galactosidase Immobilized on Con A-Sepharose

\( \beta \)-galactosidase assembled as three affinity layers on Con A-Sepharose either employing Con A or Jack bean extract was treated with 0.2 M glycine-HCl buffer, pH 2.5; 0.15 M NaCl for 15 min. The supernatant obtained after centrifugation were analyzed on 8.75% SDS PAGE after extensive dialysis against 0.1 M sodium acetate buffer pH 5.2. Figure 3.10 A shows that affinity layering of \( \beta \)-galactosidase performed using Jack bean extract results in immobilization of Con A on the support, with other proteins being removed.
Table 3.4

Bioaffinity layering of β-galactosidase performed using Con A on CNBr Sepharose

<table>
<thead>
<tr>
<th>Layer number</th>
<th>Bound β-galactosidase (units/ml gel)^b</th>
<th>Effectiveness factor (η) (B/A)</th>
<th>Increase over layer I (Fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical ^c (A)</td>
<td>Actual ^d (B)</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>17.0</td>
<td>16.5</td>
<td>0.97</td>
</tr>
<tr>
<td>II</td>
<td>96</td>
<td>94</td>
<td>0.98</td>
</tr>
<tr>
<td>III</td>
<td>105</td>
<td>105</td>
<td>1.0</td>
</tr>
</tbody>
</table>

^ each value represents the mean of at least two independent experiments performed in duplicate. Variation did not exceed 9%.

^b β-galactosidase was assayed as described by Khare and Gupta (1988 b). One enzyme unit is the quantity of the enzyme that converts one micromole of substrate to product per minute under assay conditions at 37 °C.

c determined by subtracting the number of units of enzyme remaining in the supernatant and washings after incubation from those added.

d determined by assaying appropriate aliquots of the immobilized enzyme under assay conditions with continuous agitation.
Table 3.5

Bioaffinity layering of β-galactosidase performed using Jack bean extract on CNBr Sepharose

<table>
<thead>
<tr>
<th>Layer number</th>
<th>Bound β-galactosidase (units/ml gel)</th>
<th>Effectiveness factor (η) (B/A)</th>
<th>Increase over layer I (Fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical (A)</td>
<td>Actual (B)</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5.9</td>
<td>5.0</td>
<td>0.86</td>
</tr>
<tr>
<td>II</td>
<td>15</td>
<td>20</td>
<td>1.0</td>
</tr>
<tr>
<td>III</td>
<td>14.4</td>
<td>27</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*a* each value represents the mean of at least three independent experiments performed in duplicate. Variation did not exceed 9%.

*b* β-galactosidase was assayed as described by Khare and Gupta (1988b). One enzyme unit is the quantity of the enzyme that converts one micromole of substrate to product per minute under assay conditions at 37 °C.

*c* determined by subtracting the number of units of enzyme remaining in the supernatant and washings after incubation from those added.

*d* determined by assaying appropriate aliquots of the immobilized enzyme under assay conditions with continuous agitation.

---

^ each value represents the mean of at least three independent experiments performed in duplicate. Variation did not exceed 9%.

^ β-galactosidase was assayed as described by Khare and Gupta (1988b). One enzyme unit is the quantity of the enzyme that converts one micromole of substrate to product per minute under assay conditions at 37 °C.

^ determined by substracting the number of units of enzyme remaining in the supernatant and washings after incubation from those added.

^ determined by assaying appropriate aliquots of the immobilized enzyme under assay conditions with continuous agitation.
Fig. 3.10 SDS-PAGE of Concanavalin A, Jack bean extract, β-galactosidase, and fraction eluted from the support after affinity layering of the enzyme of Con A-Sepharose using jack bean extract (A) or pure Con A (B).

Panel A: Lane a – Con A, lane b – Jack bean extract, lane c – fraction eluted with 0.2 M glycine-HCl buffer, pH 2.5

Panel B: Lane a – Con A, lane b – β-galactosidase, lane c – fraction eluted with 0.2 M glycine-HCl buffer, pH 2.5.

20 to 45 μg of protein was loaded in each well.
during washing. Eluates of preparations obtained using pure Con A were run as control (Fig. 3.10 B).

3.4 BIOAFFINITY LAYERING OF GLUCOAMYLASE ON CON A-SEPHAROSE

Bioaffinity layers of glucoamylase were assembled on Con A Sepharose preparation as described by Porath et al. (1967). A preparation with 1.7 mg Con A per ml of Sepharose was alternately incubated with glucoamylase and Con A in 0.1 M sodium acetate buffer pH 5.2 as described earlier to assemble three layers on enzyme on the support. The data obtained is summarized in Table 3.6. A layer-by-layer increase in the enzyme activity associated with the support as has been observed in case of glucose oxidase, invertase and β-galactosidase. The effectiveness factor ‘η’ also gradually increased with layering.

3.5 MEASUREMENTS USING SENSORS WITH BIOAFFINITY LAYERED GLUCOSE OXIDASE

3.5.1 Calibration Curves of a Bioaffinity Layered Glucose Sensor

Con A-Sepharose was incubated with glucose oxidase and Con A alternately for the assembly of Con A and glucose oxidase layers on Sepharose by affinity immobilization as described earlier. One ml of preparations obtained thus were packed in a flow through cartridge and integrated into a FIA system equipped with a Clark oxygen electrode (Fig. 2.1A, B). The activity in the enzyme layers was determined using glucose standards by measuring the consumption of O₂ in terms of the change in voltage. Calibration curves from cartridges
Table 3.6

Bioaffinity layering of amyloglucosidase performed using Con A on CNBr Sepharose\(^a\)

<table>
<thead>
<tr>
<th>Layer number</th>
<th>Bound amyloglucosidase (units/ml gel)(^b)</th>
<th>Effectiveness factor ((\eta)) (B/A)</th>
<th>Increase over layer I (Fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical(^c) (A)</td>
<td>Actual(^d) (B)</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1.66</td>
<td>0.94</td>
<td>0.56</td>
</tr>
<tr>
<td>II</td>
<td>3.82</td>
<td>2.10</td>
<td>0.55</td>
</tr>
<tr>
<td>III</td>
<td>5.86</td>
<td>6.16</td>
<td>1.0</td>
</tr>
</tbody>
</table>

\(^a\) each value represents the mean of at least two independent experiments performed in duplicate. Variation did not exceed 9%.

\(^b\) amyloglucosidase was assayed as described by Rao et al. (1981a). One enzyme unit is the quantity of the enzyme that converts one micromole of substrate to product per minute under assay conditions at 37 °C.

\(^c\) determined by subtracting the number of units of enzyme remaining in the supernatant and washings after incubation from those added.

\(^d\) determined by assaying appropriate aliquots of the immobilized enzyme under assay conditions with continuous agitation.
containing up to six affinity layers were plotted in fig. 3.11. As can be seen the sensitivity of the analyses increased consistently with the formation of successive layers, with the electrode signal remaining almost linear till 1.0 g/l glucose. The formation of more than five bioaffinity layers of enzyme on Sepharose matrix was ineffective in further enhancing the signal of the glucose sensor.

3.5.2 Reloadable Nature of the Sensor

Flow through cartridges containing Con A-Sepharose bearing one layer of glucose oxidase were treated with 0.2 M glycine-HCl, pH 2.5; 0.15 M NaCl or 0.5 M methyl α-D-mano.pyranoside for indicated time durations in order to study the reversibility of affinity immobilization. After washing the cartridges with the carrier buffer (50 mM potassium phosphate buffer, pH 7.0; 3.0 g/l NaCl), the cartridges were integrated into the FIA system and electrode signal measured. Almost complete elution was observed on treatment with glycine-HCl buffer, pH 2.5 as indicated by the loss of the signal after 15 mins of acid treatment (Fig. 3.12 A). However, incubation with manoside even for 15 hrs resulted in only partial elution of the enzyme from the support (Fig. 3.12 B). For reloading, the Con A-Sepharose was incubated with fresh glucose oxidase as detailed earlier in the text. After washing off the unbound enzyme, signal was recorded from the cartridge integrated in FIA by an oxygen electrode. The elution with glycine-HCl buffer and reloading was repeated twice on the same Con A-Sepharose support. Figure 3.12 A shows that it was indeed possible to bind fresh enzyme after elution on the support with full restoration of the electrode signal.
Fig. 3.11 Calibration curves of a bioaffinity layered glucose oxidase sensor.

Flow cartridges (Mobitec) containing one ml of Sepharose with one to six enzyme layers were integrated into a FIA system equipped with a Clark oxygen electrode. Electrode signal from the glucose sensor was measured continuously over a range of glucose standard in 50 mM potassium phosphate buffer, pH 7.0; 0.1 M NaCl. Each value represents an average of three experiments with a variation less than 5%. Preparation used were those containing one ( ■ ), two ( ● ), three ( ▲ ), four ( ▼ ), five ( ♦ ), and six ( X ) affinity layers.
Fig. 3.12 Elution of glucose oxidase immobilized on Con A-Sepharose and reloading with 0.2 M glycine HCl, pH 2.5 (A) or with 0.5 M methylamanoside (B)

0.2 M glycine-HCl, pH 2.5 or 0.5 M mannoside was passed through enzyme cartridges bearing Con A bound glucose oxidase for indicated time durations. After washing the cartridges with 50 mm phosphate buffer, pH 7.0, residual activity was measured. For reloading glucose oxidase (2 mg/ml) was passed through the cartridge for 2 hrs at a flow rate of 0.5 ml/min.

Panel A: Calibration curve prepared prior to (■), after elution with acid pH (●), after first (▲) and second (▼) reloading.

Panel B: Symbols representing signal from cartridge before (■) and after 15 hrs of mannoside treatment (●).
3.5.3 Online Monitoring of Medium Glucose Concentration During *Saccharomyces cerevisiae* Cultivation

*S. cerevisiae* was cultivated on a semi-synthetic medium as described earlier in a 5.0 l bioreactor. The starting medium glucose concentration was 30 g/l. A glucose sensitive biosensor bearing six affinity layers of glucose oxidase integrated into a FIA system was used to monitor the cultivation process in terms of glucose concentration. Cell-free samples from culture fluid were pumped into the FIA system using an *in situ* sampling probe (Scheper et al., 1996) for continuous glucose measurement with the biosensor. Culture fluids samples were also taken out every hour through the rubber septum for the off-line analysis using a spectrophotometer. At indicated time, the neural network control for the maintenance of glucose concentration was made operative. Figure 3.13 shows that glucose concentration quantitated using affinity layered enzyme cartridge correspond closely with those obtained during off-line spectrophotometric analyses. The neural network control maintained the glucose concentration at 10 g/l during the cultivation process.

3.6 BIOAFFINITY LAYERING OF GLUCOSE OXIDASE AND PEROXIDASE ON pH-SENSITIVE FIELD EFFECT TRANSISTORS

A FIA system comprising of peristaltic pumps, injector valve and selector valve was used to assemble affinity layers of enzyme and Con A on the gate of the pH-FET integrated into the flow system via a flow through cell (Fig. 2.2). The surface of the gate of pH-FET was coated with a layer of glutaraldehyde crosslinked Con A/HSA or starch as described in the methods. The enzyme
S. cerevisiae was cultivated on a semi-synthetic Schatzman medium containing 30 g/l glucose in a 5.0 l bioreactor (Biostat B. Braun, Germany). Cell-free samples were withdrawn from the culture medium through an in situ sampler using a peristaltic pump and injected into the FIA system equipped with oxygen electrode and enzyme cartridge bearing 6 affinity layers of glucose oxidase as the glucose sensor. Glucose was measured on-line over the entire cultivation continuously (—) with discontinuous off-line glucose measurement done spectrometrically (●). At times indicated by the arrow, neural network control for maintenance of medium glucose concentration was brought into operation.
solutions, washing buffer and Con A solution were passed through the cell in required order to assemble layers of enzyme and Con A on the pH-FET. Calibration curves were prepared at the end of each enzyme binding cycles.

3.6.1 Calibration Curves of a Bioaffinity Layered Glucose oxidase pH FET

A glucose sensitive pH-FET with bioaffinity layered glucose oxidase membrane integrated into a FIA system was used to prepare calibration curves using different concentration of glucose. The initial rate of oxidation of glucose was measured as the change in output voltage caused by the production of protons during the enzymatic reaction (Fig. 3.14). As evident there was a layer-by-layer increase in the output signal till the formation over 20 layers, after which the signal registered no further increase. While the rise in signal with each additional enzyme layer was evident over the entire range of glucose concentrations investigated, the response deviated more and more from linearity after formation of multiple layers and at higher glucose concentration.

Signal response from the multiple layered glucose-FET plotted as a function of number of glucose oxidase layers in fig. 3.15. As evident the signal response increased for each glucose concentration with layer number but sloped off remarkably after the formation of 20 layers. The rise in signal with formation of additional layers was slow initially, rose significantly after the formation of about 10 layers but sloped off after 15 layers.

The calibration curve of a glucose-FET comprising of 25 layers of glucose oxidase prepared with low concentration of glucose is shown in fig. 3.16. The
Fig. 3.14 Calibration curves of bioaffinity layered glucose oxidase-FET.

Calibration curves of glucose oxidation prepared using the glucose oxidase pH-FET as a function of number of bioaffinity layers (N). From bottom to top: N = 1, 2, 3, 4, ..., 25. Glucose standards were prepared in 20 mM potassium phosphate buffer, pH 7.0, 0.1 M NaCl and mixed with 0.1 M potassium ferricyanide prior to analysis. For details see text.
Fig. 3.15 Signal response of glucose oxidase-FET as a function of layer number.

Signal response of a glucose oxidase-FET plotted as a function of the number of enzyme layers at various concentration of glucose. Measuring conditions: 20 mM potassium phosphate buffer, pH 7.0, 0.1 M NaCl, 25°C. Glucose concentration used were 2.5 g/l (■), 5.0 g/l (●) 7.5 g/l (▲) and 10 g/l (▼).
Fig. 3.16 Calibration curve of a glucose oxidase-FET comprising of 25 affinity layers of the enzyme. See text for details.
response was completely linear over the glucose concentration of 0.0-1.0 g/l and concentration as low as 0.25 g/l glucose could be conveniently measured with the multilayered pH-FET.

3.6.1.1 Elution and reloading of glucose oxidase on a bioaffinity layered glucose-FET

The calibration curve of a glucose-FET with one layer of enzyme associated with Con A on basic starch membrane was prepared followed by elution of the bound enzyme with 0.2 M glycine-HCl buffer, pH 2.5; 0.15 M NaCl. After determining the residual activity of enzyme membrane, glucose oxidase was reloaded on FET as seven affinity layers. Figure 3.17 shows that glucose oxidase associated with FET could be readily and completely eluted by the acid pH treatment and not only was it possible to rebind glucose oxidase but the enzyme could also be assembled as multiple layers on the support with the help of Con A.

3.6.1.2 Monitoring of medium glucose during cultivation of S.cerevisiae

The glucose biosensor bearing glucose oxidase assembled as six affinity layers on the pH-FET was tested for the on-line monitoring of fed-batch cultivation of S.cerevisiae. Yeast cells were cultivated in a 3.0 l bioreactor on a semi-synthetic Schatzmann medium (Schatzmann, 1975). An in situ (Scheper et al., 1996) probe was used for on-line sampling and the culture medium after appropriate dilution with the co-substrate was injected into the FIA system integrated with glucose-FET and glucose concentration measured over the entire cultivation process. For off-line analysis, small aliquots of the cultivation media
Fig. 3.17 Elution and reloading of bioaffinity layered glucose oxidase-FET.

Calibration curves were prepared using pH-FET with single layer of glucose oxidase (■). The enzyme was eluted with 0.2 M glycine-HCl buffer pH 2.5, 0.15 M NaCl and signal response recorded at various glucose concentration (●). For reloading Con A and glucose oxidase solutions were passed through the FET as described in the text to build 7 layers of enzyme after which the calibration were prepared (▲). The 7 layer preparation was subsequently eluted with the glycine HCl buffer (▼).
was removed hourly and analyzed by YSI glucose analyzer. Excellent agreement was observed between the measurements made with the FET and those with YSI device over a period of 22 hrs measurements during which medium glucose concentration decreased from 30 g/l to 0.5 g/l (Fig. 3.18).

3.6.2 Bioaffinity Layering of Peroxidase on pH-FET

The calibration curves prepared after the formation of various layers of peroxidase mediated by Con A on the starch/glutaraldehyde membrane of a pH-FET are shown in fig. 3.19. The electrode signal indicated an increase with layering till the formation of seven affinity layers. The sensitivity of the FET also increased remarkably and H$_2$O$_2$ concentration below 1.0 mM could be effectively measured.

3.6.2.1 Operational stability of peroxidase-FET

The stability of the peroxidase-FET against repeated use was investigated using a system bearing an enzyme layer immobilized on the Con A membrane associated with the starch layer on the FET surface. Signal from pH-FET was continuously monitored over a period of 15 hrs using 1.0 mM H$_2$O$_2$ as standard. For each measurement the injection time was 30s and a single total analysis took 7 min. As shown in fig. 3.20, the signal response towards H$_2$O$_2$ measurement did not show remarkable variation over the period investigated with standard deviation observed being 0.4128±0.0243.
Fig. 3.18 On-line monitoring of glucose concentration during \textit{S.cerevisae} cultivation.

The yeast cells were cultivated on a semi synthetic medium with 30 g/l glucose in 3.0 l bioreactor, pH 4.0, temperature 30°C with a stirring speed of 600 rpm. Samples were taken out through an \textit{in situ} sampling device and analysed using a bioaffinity layered glucose oxidase-FET with 6 layer of enzymes integrated in FIA system. Prior to cultivation analysis, the pH-FET was calibrated with glucose standards : 8, 16, 24 and 32 g/l. Values obtained during cultivation (—) were compared with off-line analyses made by YSI glucose analyser (▼).
Fig. 3.19 Calibration curves of bioaffinity layered peroxidase-FET measured after various enzyme binding cycles.

H₂O₂ standards were prepared in buffer and mixed with 0.1 M potassium fericyanide prior to analysis in the FIA system. Carrier buffer used was 20 mM potassium phosphate pH 7.0, 0.1 M NaCl, at the temperature of 25°C. Calibration curve prepared after one (■), three (●), five (▲) and seven (▼) affinity layers of peroxidase are shown in figure.
Fig. 3.20 Long term stability of peroxidase-FET

Long term stability of a peroxidase-FET having a single layer of peroxidase and Con A on basic starch/glutaraldehyde membrane. The measurements were made with 20 mM potassium phosphate buffer 0.1 M NaCl at pH 7.0, 25°C, 1 mM H₂O₂ standard, time of injection was 30s and analysis time 7 min.
3.7 DISCUSSION

The phenomenal growth in the area of biosensor is related to the need of rapid, reliable, reproducible, and sensitive analyses for many fields including food, medicine, research and biotechnological processes. The design of a biosensor involves combining of a biological component that generates signal after sensing the analyte and an optical, thermal, electrical or electrochemical element for transducing the signal generated. Of the various procedures available for coupling the biological element on the electrode surface, those leading to stable and where possible reversible binding are advantageous. Ideally enzyme immobilization strategies for most biosensor applications should ideally associate large amounts of enzyme with the support (Birnbaum, 1994; Alvarez-Kaza and Bilitewski, 1993).

Covalent coupling of enzyme through their amino acid side chain groups continues to remain the most popular method of enzyme immobilization but such procedures are however generally not effective for glycoenzyme immobilization due to the masking of the side chain groups by the oligosaccharide chains (Liberatore et al., 1976; Pollock et al., 1978). Among the alternative immobilization approaches in which the enzyme glycosyl groups form the point of contact between the enzyme and the support (Hsiao and Royer, 1979; Woodward and Wiseman, 1978; Chaga, 1994), those employing the lectin Con A appear more promising (Saleemuddin and Husain, 1991). While the amount of glycoenzyme that can be immobilized on supports can be raised remarkably by using matrices precoupled with Con A, they are limited by the amount of the lectin precoupled to the support.
In order to further increase the amount of the enzyme immobilized on such supports, a simple technique which we prefer to call as bioaffinity layering was investigated. Figure 3.21 shows the diagrammatic representation of the bioaffinity layering of glycoenzymes on matrix precoupled with Con A. The first step involves a simple incubation of glycoenzyme with Con A-Sepharose as is routinely done during lectin affinity purification or immobilization. After washing off the unbound enzyme with the buffer, Con A-Sepharose with the bound enzyme is reincubated with activated Con A. Excess Con A is similarly washed off and the support reincubated with the glycoenzyme. The alternate incubations with the activated Con A and glycoenzyme, separated by thorough washing steps were continued as desired to assemble layers of enzyme.

It was observed that layering resulted in marked increase in the amount of glucose oxidase associated with Con A-Sepharose and over 250 times more enzyme than that immobilized when the matrix was directly incubated with the enzyme could be bound after seven incubation cycles. The amount of Con A associated with the support also increased so did the effectiveness of the Con A molecules to bind glycoenzyme as evident from the layer-by-layer increase in the glycoenzyme: lectin ratio on the support. After seven incubations a remarkable 17.5 mg Con A and 19.0 mg glucose oxidase was immobilized on the support (Fig 3.1A, B).

The successful affinity layering of glucose oxidase (Table 3.1), invertase (Table 3.3), β-galactosidase (Table 3.4) and amyloglucosidase (Table 3.6) on Con A-Sepharose clearly suggests that the amount of all glycoenzymes having affinity for Con A (Saleemuddin and Husain, 1991) can be raised on supports.
Fig. 3.21 Schematic representation of bioaffinity layering of Con A and glycoenzyme on Con A-Sepharose.
remarkably using this strategy. The quantities of invertase, amyloglucosidase, β-galactosidase could be successfully raised to 18, 7, 6.3 fold after three binding cycles of the respective enzymes on the support precoupled to Con A. Glucose oxidase activity increased 15 fold under these conditions. Evidently the variation in increase of enzyme associated with the support by affinity layering may be influenced by the extent and nature of glycosylation. Invertase which is extensively glycosylated (Gascon et al., 1968) apparently has a far greater possibility of interacting with Con A and being incorporated in bioaffinity layers. The effectiveness factor ‘l’ (ratio of actual to theoretical activity) (Muller and Zwing, 1982) also increased with successive formation of affinity layers with all enzymes till the formation of at least three affinity layers (Table 3.1, 3.3, 3.4, 3.6). Most of the bound enzyme appears to be accessible for acting on substrates after the formation of three affinity layers. Incubation of the CNBr activated Sepharose with Con A results in the binding of lectin not only at the surface but also in the interior of the beads due to the high exclusion limit of the support. Glycoenzyme bound on Con-A Sepharose may initially be associated both with the lectin molecules located at the surface and those in interior of the Sepharose beads, with the latter being less accessible to the substrate. Increasingly smaller fractions of the glycoenzyme molecule incubated with the Con A support are expected to have access to the interior during the formation of successive affinity layers and binding may occur predominantly at the surface. In view of large molecular dimension of both Con A and glycoenzymes and the limited thickness of the bioaffinity layers, the structural network formed at the surface may be quite porous facilitating excellent accessibility to the substrate. Several other investigators have observed higher accessibility of glycoenzymes for their sub-
strates when they were immobilized on Con A matrices (Hsiao and Royer, 1979; Woodward and Wiseman, 1978), but the \( \eta \) values are strongly influenced by the amount of lectin associated with the support and the amount of enzyme bound thereon (Saleemuddin and Husain, 1991). Despite a low \( \eta \) value for the first layer, nearly all activity bound appears to be expressed after the formation of three layers. This effect may be related to the binding of a far greater amount of glycoenzyme in the second and third layer.

Attempts were also made to crosslink the bioaffinity layered glucose oxidase preparations using glutaraldehyde. As observed earlier (Husain et al., 1992), a decrease in catalytic activity of enzyme was obtained after crosslinking (Table 3.2) although the loss in activity of the preparations containing multiple layers was less marked. This may be related to the inaccessibility of some of the aminogroups of enzyme in the preparations bearing multiple enzyme layers.

Bioaffinity layering did not alter the pH-activity curves of the immobilized glucose oxidase preparations and there was no shift in the pH optimum of the enzyme when assembled as layers on the support even after cross-linking with glutaraldehyde suggesting no significant alteration in the microenvironment around the active site. However a significant broadening of the curves on the alkaline side was observed with the cross-linked preparations, which appeared to increase layer-by-layer (Fig. 3.2 A, B).

More interesting is the enhancement in the thermal stability of the affinity layered preparations. Affinity layering of glucose oxidase on Con A-Sepharose as one, two, or three affinity layers also showed considerable stabi-
lization against temperature above 40°C. The increase in stability was layer dependent and more pronounced after crosslinking the preparations with glutaraldehyde (Fig. 3.3 A, B). As is evident from fig. 3.4 A, B the fraction of the activity retained after exposure to 60 °C also increased layer-by-layer. Which was further improved on crosslinking the preparations. Glutaraldehyde treatment may improve the ability of the preparations to retain activity at higher temperatures by creating intra- and intermolecular crosslinks that restricts the unfolding of the enzyme molecules (Iqbal and Saleemuddin, 1983a; 1983b). Furthermore the possibility of the desorption of the enzyme from the support may be reduced as the result of crosslinking. Glutaraldehyde treatment however leads to covalent coupling of the enzyme to the support and thus making the process irreversible.

It has been observed that thermal stability of several enzymes can be raised by creating a number of covalent / non-covalent connections between the enzyme and the insoluble support (Monsan and Combes, 1988). It is very likely that Con A supports with multiple layers have increasing fraction of enzyme molecule reacting with more than one Con A molecule as in case of Con A-glycoenzyme flocculates that exhibit remarkable high degree of stability compared to the preparations obtained by binding to matrices with pre immobilized lectin (Husian et al., 1985; Ahmad et al., 1973; Bishayee and Bacchawat, 1974). The flocculates have however small particle dimensions, pack very compactly and need additional immobilization strategies for possible use in column reactors (Husain et al., 1985). Bioaffinity layering appears to offer the combination of high stability of glycoenzyme-Con A flocculates along with good flow properties.
of beaded support. The ability of Con A molecules to form an “affinity sandwich” has previously been used to bind glycoenzymes to Sephadex (Husain and Saleemuddin, 1989).

Enhancement in the mechanical stability of the glucose oxidase preparations assembled as affinity layers was also remarkable (Fig. 3.5). This is not surprising in view of the high affinity of Con A for glycoenzymes and long term operational stability of Con A support associated glycoenzymes (Husain et al., 1985; Montero and Romeu, 1993). With more and more molecules of enzyme interacting with several lectin molecules in the affinity layered preparations, the high mechanical stability of the affinity layered preparations is not surprising.

That the improvement in the performance of enzymes resulting from bioaffinity layering is not restricted to glucose oxidase is evident from the studies conducted on other glycoenzymes invertase and β-galactosidase. Invertase has been immobilized on a variety of supports including charcoal (Nelson and Griffin, 1916), Con A matrices (Hsaio and Royer, 1979; Iqbal and Saleemuddin, 1983; Husain et al., 1985), and on immuno-supports (Jafri et al., 1993, 1995).

No significant alteration in the pH and temperature-activity profiles of the native and bioaffinity layered invertase preparations was observed (Fig 3.6, 3.7) although the layering enhanced the resistance of the immobilized preparations against thermal inactivation. The thermal stability also increased layer-by-layer (Fig 3.8).

There was an increase in $K_m$ and $V_{max}$ for the preparation with three enzyme layers compared to the native enzyme (Fig 3.9). That the invertase immo-
bilization as multiple layers does not involve marked decrease in accessibility or major alteration in enzyme conformation as evident from the insignificant increase in $K_m$ values. The large dimension of Con A appears to contribute to the porosity of the enzyme-lectin network as discussed earlier.

In an earlier study from this laboratory it has been shown that Jack bean extract can be conveniently substituted for pure Con A in the preparation of insoluble Con A-glycoenzyme complexes or for immobilization on supports (Husain and Saleemuddin, 1986; 1989). Table 3.5 clearly indicates that $\beta$-galactosidase can also be assembled as bioaffinity layers using Jack bean extract in place of pure Con A. On dissociation of the glycoenzyme-Con A molecules from the support bearing three layers of $\beta$-galactosidase by 0.2 M glycine-HCl buffer, pH 2.5, it was observed that layering resulted in the affinity binding of mostly the Con A molecules from the extract due to their affinity for glycoenzyme. Faint bands corresponding to $\beta$-galactosidase were also visible. The poor staining of $\beta$-galactosidase may be related to its glycoprotein nature (Fig. 3.10 B). It is thus clear that use of crude Con A can conveniently cut down the cost of bioaffinity layering.

Immobilization strategies that result in the association of large amounts of enzyme with the support have the potential of increasing the sensitivity and stability of various enzyme based sensing devices. The potential of bioaffinity layering in improving the performance of a glucose oxidase based biosensor was also investigated. Affinity layered glucose oxidase preparations were packed in flow through cells and integrated in a FIA system coupled to an oxygen electrode (Fig. 2.1). The sensor signal was measured in terms of voltage using the
oxygen electrode by measuring the decrease in partial pressure of oxygen, which is directly related to the rate of oxidation of glucose catalyzed by glucose oxidase. The calibration curves are shown in fig 3.11. The sensitivity of the analyses increased consistently with the formation of the successive layers, although the signal slopped off after the formation of more than five layers. One possible reason for the observed behavior could be the limitation of the dissolved oxygen available for the oxidation of glucose (Mattiasson and Borrebeack, 1978). Using a preparation containing six layers of glucose oxidase, it was possible to measure accurately concentration of glucose as low as 0.02%.

Studies were also undertaken to determine the regenerability of the support for which attempts were made to elute the adsorbed enzyme either by inducing conformational changes using acidic solution like 0.2 M glycine-HCl buffer, pH 2.5 (Mattiasson and Borrebeack, 1978) or by using methyl mannoside for which Con A has higher affinity (Bittiger and Schenebli, 1976). The results obtained shown in fig. 3.12 A, B indicated that elution with acidic pH was more effective in removing the bound glucose oxidase. Glucose oxidase binds very strongly to Con A supports. Complete elution with mannoside necessitates exceptionally long incubations (Mislovicova et al., 1995). This also indicated that the covalently attached Con A layer was not inactivated by the acid pH under the conditions used and bind fresh enzyme readily (Fig. 3.12 A).

With the wide applicability of the detection of glucose in the clinical investigation, and easy and ready availability of glucose oxidase, the enzyme has been a part of research since a long time. Though extensive work has been done with sensors employing enzyme cartridges (Kurtz and Crouch, 1991; Male
and Luong, 1993), ENFETs have received less attention. In view of the applicability of bioaffinity layering in increasing the sensitivity of glucose biosensor employing enzyme cartridge, attempts were also made to investigate the usefulness of the strategy in improving the performance of pH-FETs. The FET with covalently attached Con A or starch layer was integrated in a FIA system via a flow through cell. The alternate layer on enzyme and Con A were deposited with the help of an automated FIA setup as described in fig. 2.2. The system was programmed to carry out required number of binding cycles involving Con A and enzyme and calibration curves were prepared at the end of each cycle. The rate of oxidation of glucose was measured as the change in pH brought about by the enzymatic reaction. The calibration curves of a pH-FET with glucose oxidase assembled up to 25 layers are shown in fig. 3.14 and 3.15. A layer-by-layer increase in signal was observed till the formation of 20 layers, after which there was no more rise in the signal. With the FET with 25 layers of glucose oxidase attached on the basic starch membrane, the sensitivity was enhanced and glucose as low as 0.25 g/l could be conveniently measured (Fig. 3.16). While the rise in the signal with each additional layer was evident over entire range of glucose concentration investigated, the sensor response deviated more and more from linearity after the formation of multiple layers and at higher glucose concentration. The possible explanation for the observed non-linearity include, limitation of the diffusion of protons in the multiple layered preparation and/or limitation of the available oxygen for glucose oxidation.

Potassium ferricyanide was used as the oxidizing co-substrate in place of oxygen as the substitution of oxygen with ferricyanide as the electron acceptor in the enzymatic oxidation of glucose leads to substantial increase in ENFET
response as in this case three $H^+$ are produced instead of only one formed when oxygen is the acceptor (Fig. 3.22). Also, the biosensor dynamic range is extended as the limitation of the reaction rate due to low concentration of dissolved oxygen can be reduced by adjustment of the concentration of alternative electron acceptor (Shulga et al., 1994; Shulga and Gibson, 1994).

The bioaffinity layered glucose sensor was also used for the on-line monitoring the fed-batch cultivation process. *S.cerevisiae* was cultivated in semi-synthetic medium (Schatzmann, 1975) containing 30g/l glucose in a bioreactor and the medium glucose was monitored glucose sensor with glucose oxidase immobilized in the form of affinity layers. Enzyme cartridge bearing six layers of glucose oxidase assembled on Con A-Sepharose was integrated in a FIA system equipped with an oxygen electrode was used to monitor the cultivation of yeast in 5.0 l reactor. The results obtained are shown in fig. 3.13 indicated good agreement with the off-line analysis made using spectrophotometer.

A glucose oxidase-FET with glucose oxidase assembled as six layers was also tested for the on-line analysis of a fed-batch cultivation *S. cerevisiae* in 3.0 l fermentor. An *in situ* probe was used for on-line sampling (Scheper et al., 1996) and the culture medium after appropriate dilution with buffer and co-substrate was injected in to the FIA system. Excellent agreement was observed between the measurements made with the FET and those with the YSI device over a period of 22 hrs (Fig. 3.18). Culture processes are complex systems in which activity and state of the biological component is extremely sensitive to changes in the physiochemical environment of the bioreactor. To be able to describe the status of a bioprocess, on-line continuous monitoring of many vari-
Fig. 3.22 Schematic representation of the reaction-diffusion processes with in the enzyme layer immobilized on FET surface using $O_2$ and potassium ferricyanide as co-substrate leading to the generation of pH-FET response (Shulga et al., 1994).
ables are needed. FIA is an on-line method in combination with long term stabil-
ity and high reproducibility of enzyme cartridge or pH-FETs is qualified to con-
tinuously provide this form of process monitoring and control. A completely au-
tomated FIA system offers round the clock surveillance in addition to easy han-
dling and a 10-20% lower price per analysis than conventional at-line or off-line
test kits (Weigel, 1996).

Sensitivity of $H_2O_2$ measurements using a peroxidase-FET could be simi-
larly increased significantly by increasing the amount of peroxidase bound on
the FET surface by affinity layering (Fig. 3.16). There was an increase in the
sensitivity of the system with layering however the rise became undetectable
after the formation of seven layers. The sensitivity of the system was increased
and $H_2O_2$ as low as 1.0 mM could be detected. The saturation in the increase in
signal response after the formation of fewer layers than those observed in case
of glucose oxidase-FET may be related in addition to factor discussed above, to
the lower affinity of some of the isoenzymes peroxidase to Con A and hence
binding of fewer enzyme molecules (Mattiasson and Borrebeack, 1978).

Figure 3.20 shows the result of the stability test of the system to multiple
analysis when the $H_2O_2$ was continuously monitored for over 15 hrs. A variation
of only 5.9% was observed when 16 samples were analyzed continuously. This
implies that, during measurements, decrease in the sensor sensitivity is negli-
gible and also the leakage of the immobilized enzyme from the membrane may
be very low.

The mild conditions required for the immobilization of the enzyme, high
actively and stability of the immobilized preparations and regenerability of the
adsorbent are the main features of the method reported here. The multiple bioaffinity layering technique offers a remarkably simple opportunity to increase dramatically and reversibly the amount of glycoenzyme exhibiting substantial affinity for Con A immobilized on various supports. Since the Con A molecules are expected to behave predominantly as dimers between pH 5.0-6.0, used in the study (Bittiger and Schnebli, 1976).