On-line enzyme activity determination using the stopped-flow technique: application to laccase activity in pulp mill waste-water treatment

Abstract An automated system for on-line measurement of enzyme activity is proposed. The system uses a flow injection manifold in the stopped-flow mode to measure initial reaction rates. The time during which the flow is halted is selected in such a way as to optimise the enzyme/substrate ratio for the correct determination of activity values. The proposed system was used to determine the activity of laccase produced by the fungus *Trametes versicolor* immobilised on nylon in a fixed-bed reactor used for treating pulp mill waste water.

Introduction

Biotechnology is increasingly being used in environmental applications with purposes such as decreasing the amount of waste resulting from production processes or treating such waste. The paper industry has long striven to find new and less polluting technologies. As a result, many industrial pulp and paper manufacturing processes currently rely on biotechnology. Some processes are therefore performed in the presence of enzymes in order to facilitate the subsequent extraction of lignin, the process known as biopulping (Reid 1991) or pulp bleaching, also known as biobleaching (Bajpai and Bajpai 1992).

The production of waste water, even when clean techniques are used, is virtually unavoidable. However, if biotechnological procedures are followed its environmental impact can be reduced. Pulp and paper mill waste water consists chiefly of lignin, which is a complex, heterogeneous aromatic biopolymer that protects plants from insect and microbial attacks and plays a prominent structural role. Some microorganisms have the ability to degrade lignin; the so-called ligninolytic fungi are particularly effective in this respect. The underlying lytic mechanism involves an intricate extracellular enzyme system. The most significant components of which are seemingly laccase (EC 1.10.3.2), manganese peroxidase (EC 1.11.1.7) and lignin peroxidase (EC 1.11.1.7). The low specificity of these enzymes makes them applicable to a variety of processes. Thus, laccase has been used for treating waste water from lignocellulose (Mehna et al. 1995; Roy-Arcand and Archibald 1991; Christine 1985), in biosensors for detecting phenol compounds (Zouari et al. 1994; Ghindilis et al. 1992), in phenol conversion reactions (Shuttleworth and Bollag 1986) and in antibiotic production (Agematu et al. 1993). This enzyme has also been the subject of molecular biochemical studies; its gene has been located and cloned in various microorganisms (Coll et al. 1993; German and Lerch 1985). The purification of laccase, and mechanistic, kinetic and stability studies have also been reported (Higuchi 1989; Rogalski et al. 1991).

All of the above processes, where enzyme activity has to be monitored or used as a process control parameter, call for automated activity tests. Conventional methods for determining enzyme activity, most of which rely on spectrophotometric techniques, escape automation and hence hinder process monitoring. One way of automating enzyme activity analyses is by use of flow-injection analysis (FIA), the assets of which in terms of time and reagent savings, and reproducibility, are well documented (Valcarcel and Luque de Castro 1984; Ruzicka and Hansen 1988).

This paper reports a stopped-flow injection analysis system, based on electronic dilution, for determining enzyme activity. The on-line automated system provides laccase activity fitting automatically to the desired
concentration range. The ensuing method was applied to pulp mill waste water, a substrate having a varying colour that interferes with activity measurements by a conventional FIA system.

**Materials and methods**

**Microorganism**

The fungal strain used in this work, *Trametes versicolor* ATCC 42530, was kept in petri dishes containing 2% agar/malt-extract and was subcultured periodically.

**Inoculum preparation**

A suspension of *T. versicolor* mycelium was obtained by inoculating four agar cubes of 1 cm side withdrawn from the fungus-growing zone in a petri dish, with 150 ml 2% malt extract in 250-ml conical flasks. The inocula were incubated at 23 °C with orbital agitation for 4–5 days. The resulting mycelium solution was milled and the resulting mycelium solution kept in sterile 0.8% saline at 4 °C.

**Immobilisation**

The fungus was immobilised on Scotch Brite nylon cubes of 0.5–1 cm side (Font et al. 1993). The cubes were previously washed several times and subsequently sterilised with distilled water in order to remove impurities. The growth medium used to immobilise the fungus contained 5 g glucose, 1.9 g NH₄Cl, 200 ml 80 mM 2,2-dimethylsuccinate buffer and 1.01 ml Kirk supplementary medium (Kirk et al. 1978). Before the culture began, 100 nylon cubes and 500 ml medium (pH = 4.5) were sterilised at 120 °C for 20 min in 1-l conical flasks. The sterilised medium was then inoculated with 2 ml mycelium solution and incubated at 23 °C with orbital agitation for 4 days.

**Waste water**

The effluent used in this study was a hemp black liquor obtained from a soda pulping mill (Celesa, Tortosa, Spain).

**Laccase production**

Cultures were placed in a fixed-bed reactor containing 500 ml medium and 300 cubes. The composition of the culture medium was 52 ml waste water, 5 g glucose, 1.9 g NH₄Cl, 200 ml 80 mM dimethylsuccinate buffer and 1.01 ml Kirk supplementary medium (Kirk et al. 1978). The pH was adjusted to 4.5 and the solution sterilised at 120 °C for 20 min.

When the experimental validation was performed, the enzyme evolution was monitored on-line under the above-described conditions, but with 300 ml culture medium.

**Enzyme test**

Laccase activity was measured by using a modified version of the method of Ptaszczyński (Paszczynski et al. 1988) for the determination of manganese peroxidase (Kaal et al. 1993). 2,6-Dimethoxyphenol is oxidised by laccase, even in the absence of a cofactor. On the other hand, oxidation by manganese peroxidase requires the presence of H₂O₂ as cofactor and catalytically active Mn²⁺. This avoids any potential interference from manganese peroxidase and other peroxidases with the enzyme activity determination.

The reaction mixture used to determine laccase activity by the conventional method consisted of 66.6 mM sodium malonate at pH 4.5, 1.3 mM 2,6-dimethoxyphenol and up to 500 μl sample in an overall volume of 750 μl. Absorbance changes at 468 nm and 30 °C were monitored for 2 min. On the basis of a molar absorption coefficient of 10 000 M⁻¹ cm⁻¹ for 2,6-dimethoxyphenol and the slope of the analytical curve, an activity unit (AU) was defined in terms of the number of micromoles of 2,6-dimethoxyphenol converted l⁻¹ min⁻¹.

**Apparatus**

The carrier and substrate were propelled by a Gilson Minipuls 2 peristaltic pump. An ATI/Unicam UV/visible 8625 series spectrophotometer, equipped with an RS-232C port and an analog output for absorbance between 0 and 2 units, monitored absorbance changes. The flow cell used had an inner volume of 30 μl and a light pathlength of 10 mm. The sample was injected by means of an Omnifit automatic valve equipped with a TTL-compatible signal input that enabled computerised control of its operation.

**Computer hardware and software**

Data were acquired and the FIA system controlled via a personal computer equipped with a PC-MultiLab PCL-711B A/D converter.

The system was controlled and monitored with the aid of software developed in Microsoft QuickBasic 4.5 language for DOS. The software includes various menus that facilitate altering its performance in response to system modifications.

**FIA manifold**

Teflon tubing (internal diameter 0.80 mm) was used throughout the system. The FIA manifold used, depicted in Fig. 1, comprised two channels. In order to ensure the presence of excess substrate at the centre of the sample zone, the substrate stream was merged with the carrier stream on injection of the sample. The carrier and substrate were propelled at a flow rate of 1.4 ml/min each. The injected sample volume was 90 μl. After the two streams were merged, the sample travelled along a reactor of 90 cm before it reached the detector.

The carrier was 50 mM malonate buffer at pH 4.5 and the substrate a solution containing 10 mM 2,6-dimethoxyphenol in 100 mM malonate buffer at the same pH as the carrier. The acid pH of the 2,6-dimethoxyphenol solution delayed its photochemical oxidation and hence increased its stability.

**Fig. 1** Analysis system manifold. L = 90 cm, O₁ = O₂ = 1.4 ml/min

*DMF* 2,6-dimethoxyphenol, TTL transistor-transistor logic
Results

Automating conventional FIA enzyme assays (i.e. those based on measurements of the height of a peak resulting from the formation of a product of the substrate in the presence of the enzyme) is only possible with samples in which the enzyme is in an accurately known medium where no other component interferes with the detection of the product and the concentration of the components does not change throughout the process.

The effluent used in this work was dark brown in colour, owing to its high content in lignin derivatives. These compounds absorb at 468 nm, the wavelength for the enzyme test. If the effluent colour did not change throughout the process, FIA enzyme analyses could be performed by measuring peak height, since the interference would always be of the same magnitude. However, because one of the purposes of treating this type of wastewater is to reduce the effluent colour, the sample colour will change during treatment. Therefore, determining enzyme activity in samples from industrial effluents, which may be subject to interference from composition variability, entails using a more sophisticated FIA methodology.

Because conventional analyses for enzyme activity determination rely on measurements of the formation of a product or the disappearance of the substrate, the slope of the analytical curve is related to the enzyme activity. This method avoids the interference of compounds absorbing at the working wavelength. On the other hand, the manual implementation of this technique is labour-intensive and involves preparing a number of dilutions spanning the linear concentration range. Furthermore, only an automatic method allows the use of this parameter for on-line process control.

Conventional enzyme activity tests can be performed in an automated FIA system by using the stopped-flow technique (Olsen et al. 1982; Worsfold et al. 1981; Hansen 1989; Christian and Ruzicka 1992; Ruzicka 1992; Hansen and Jensen 1993; Hansen et al. 1994), a technique that involves halting the flow after a pre-set time (stop time) following the appearance of the peak maximum. After the flow is halted, the reaction is continuously monitored via the detector signal. According to the well-known Michaelis-Menten equation, provided that a large enough substrate excess is used to ensure that the substrate concentration remains virtually constant throughout the FIA peak, the slope obtained is a function of the enzyme activity.

Tests performed with samples of variable colour revealed that the peak height was not directly related to the enzyme activity. Figure 2a illustrates two injections of enzyme of different activity and sample colour (interference). As can be seen, the peak height was not proportional to the slope (enzyme activity) obtained after the flow was stopped: the smaller peak was that corresponding to the higher enzyme activity and lighter sample colour.

Because enzyme activity changes during treatment, it would be interesting to devise some procedure for working in different activity ranges. In order to operate at very low enzyme-to-substrate ratios, two different procedures can be used. One involves the inclusion of a dilution stream (Valero et al. 1990), which would complicate the manifold exceedingly. The other is to use the electronic dilution technique (Olsen et al. 1982; Worsfold et al. 1981; Hansen 1989). This technique is based on the concentration gradient arising in the sample zone as a result of the dispersion of the sample within the carrier stream (Fig. 3). This concept is used to stop the flow at variable times, measuring a different enzyme/substrate ratio at each different stop time.

To show the application of the electronic dilution, the analytical slopes for a given sample, obtained at increasing stop times, are plotted on top of each other in Fig. 2b. As can be seen, increasing the stop time results in a decreasing slope, that is, there is a decrease in the enzyme/substrate ratio.

The joint use of the two above-described FIA methods allows the flow to be stopped at the most appropriate time to ensure the highest sensitivity. In addition, this strategy avoids the interference of the substrate colour with activity measurements. As a result, it can be
used for determining enzyme activity in samples containing variable concentrations of compounds potentially interfering with activity measurements.

The reactor used to process pulp mill waste water was a fixed-bed reactor loaded with *T. versicolor* immobilised on nylon cubes. Samples were withdrawn at different process times corresponding to a variable enzyme activity.

Initially the conventional test for the determination of laccase activity was implemented to find the linear section of the slope range for the test. Figure 4 shows the results obtained for an initial activity of 1400 AU tested at different dilutions. The analytical error was determined from the results obtained for 12 samples of variable enzyme activity that were measured in triplicate, and was found to be 4%.

To test the FIA system, altogether 14 samples of enzyme activity ranging between 3 AU and 3500 AU were assayed at different stop times. The flow was stopped automatically at various times (19, 21, 24, 26, 28, 30 and 32 s) following injection. In this way, the activity was determined immediately prior to the FIA analysis. Since each sample was processed at different stop times and with replicate measurements, in all, 254 injections were carried out.

The initial total assay time was set to 95 s. The FIA curves obtained were used to determine the slope at each stop time. With the purpose of optimising the analysis length, different data intervals were studied. Slope measurements were made within different ranges between 3 s after stopping and +10, +20, +40 and +60 s. For each measurement range, a correlation curve between the FIA slope, at a given stop time, and the corresponding enzyme activity was obtained. In each case, the parameters for the correlation curve (slope, intercept and regression coefficient, *r*), as well as the relative standard deviation (RSD), were determined. In order to find the optimum range for calculating the slope, the means obtained for the RSD and regression coefficient for the different stop times and measurement range studied were analysed. The results are shown in Table 1. Note that the number of injections is variable because, for high enzyme activities and large measurement ranges, the absorbance exceeds the maximum output of the spectrophotometer.

Table 2 gives the fitting parameters for each curve obtained at a variable stop time with the measurement range of +20 s, as well as their regression coefficients and RSD. As can be seen, all correlations were quite linear throughout the large activity range studied.

In order to validate the proposed system, the activity of the enzyme acting in the treatment of waste water with *T. versicolor* fungi was monitored. The proposed FIA system was used to monitor the laccase activity online throughout the process. Samples were obtained in an automatic manner since the injection valve was located off the reactor recycling line. No filtering was

<table>
<thead>
<tr>
<th>Range</th>
<th>RSD (%)</th>
<th><em>r</em></th>
<th>Injections</th>
</tr>
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<tbody>
<tr>
<td>+60 s</td>
<td>4.43</td>
<td>0.9857</td>
<td>186</td>
</tr>
<tr>
<td>+40 s</td>
<td>4.44</td>
<td>0.9827</td>
<td>206</td>
</tr>
<tr>
<td>+20 s</td>
<td>4.13</td>
<td>0.9751</td>
<td>232</td>
</tr>
<tr>
<td>+10 s</td>
<td>6.02</td>
<td>0.9655</td>
<td>254</td>
</tr>
</tbody>
</table>

Table 2 Parameters obtained by least-square regression for the different stop times (*t*), where *A* denotes intercept, *B* denotes slope, *r* denotes regression coefficient and RSD denotes the relative standard error

<table>
<thead>
<tr>
<th><em>t</em></th>
<th><em>A</em></th>
<th><em>B</em></th>
<th><em>r</em></th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>2.09 × 10⁻³</td>
<td>1.56 × 10⁻⁵</td>
<td>0.9912</td>
<td>5.13</td>
</tr>
<tr>
<td>21</td>
<td>1.27 × 10⁻³</td>
<td>1.36 × 10⁻⁵</td>
<td>0.9836</td>
<td>3.61</td>
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<tr>
<td>24</td>
<td>1.12 × 10⁻³</td>
<td>9.20 × 10⁻⁶</td>
<td>0.9835</td>
<td>4.70</td>
</tr>
<tr>
<td>26</td>
<td>5.31 × 10⁻⁴</td>
<td>7.53 × 10⁻⁶</td>
<td>0.9842</td>
<td>3.59</td>
</tr>
<tr>
<td>28</td>
<td>7.33 × 10⁻⁴</td>
<td>5.61 × 10⁻⁶</td>
<td>0.9821</td>
<td>3.37</td>
</tr>
<tr>
<td>30</td>
<td>6.45 × 10⁻⁴</td>
<td>4.19 × 10⁻⁶</td>
<td>0.9629</td>
<td>3.82</td>
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<td>32</td>
<td>8.23 × 10⁻⁴</td>
<td>2.82 × 10⁻⁶</td>
<td>0.9385</td>
<td>4.69</td>
</tr>
</tbody>
</table>
required because the fungus was used in an immobilised form, so the samples were biomass-free. The sampling frequency used in this validation was two samples per day, although a greater sampling frequency could be obtained (Fig. 5). This sampling frequency is adequate to monitor the enzymatic activity evolution. The software used calculated the laccase activity from the correlation curves previously obtained.

The computer used to control the FIA system also acquired the data from the oxygen probe furnished in the reactor. Figure 6 illustrates the monitoring of a black liquor treatment process through the variation of the enzyme activity with the glucose and dissolved oxygen concentrations.

Discussion

The analysis of the conventional test for laccase activity measurement showed that there was a linear region between the slope values $5.5 \times 10^{-3}$ and $2.5 \times 10^{-3}$. In performing the activity test, the sample must be diluted to the extent needed to bring the slope within such a linear range. For the sample studied, this entailed dilution between 1:18 and 1:50 (v/v).

The examination of the parameters obtained for each correlation curve indicated that the best measurement range was between 3 s after stopping and +20 s. This resulted in the lowest RSD (Table 1). In addition, the activity value obtained in this range was more representative of the initial rate measurement than those ending at +40 and +60 s, which, however, gave a better regression coefficient ($r$). The selected measurement range was then 3–20 s. Under these conditions a maximum sampling frequency of 1 analysis/140 s could be achieved. In the selected measurement range, all correlation curves were quite linear throughout the large activity range studied (Table 2).

Monitoring an enzyme activity during a treatment process does not necessarily entail performing analyses at every stop time investigated. The flow can be stopped after a short time (19–21 s) when little enzyme activity (0–700 AU) is expected or after a longer time (30–32 s) when the activity is expected to exceed 750 AU or even reach 3500 AU. The stop time can then be selected automatically in terms of the activity obtained in the previous analysis.

The validation of the proposed FIA system shows this method to be a good alternative to the conventional enzymatic assays, allowing automation and on-line measuring of enzymatic activity.

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