0273-2289/97/620203—0159$12.00

Production and Partial Characterization of Extracellular Peroxidases Produced by *Streptomyces avermitilis* UAH30

ABDUL ROB,*[,1] MANUEL HERNANDEZ,[2]
ANDREW S. BALL,[1] MUNIR TUNCER,[1]
MARIA E. ARIAS,[2] AND MICHAEL T. WILSON[1]

[1]*Departments of Biological and Chemical Sciences, University of Essex, Wivenhoe Park, Colchester, UK; and* [2]*Departamento de Microbiologia y Parasitologia, Universidad de Alcala de Henares, Madrid, Spain*

Received September 8, 1995; Accepted November 28, 1995

ABSTRACT

The effect of a number of environmental parameters (pH, temperature, carbon and nitrogen ratio of nutrient) on the production of extracellular peroxidase enzymes by *Streptomyces avermitilis* UAH30 was examined. Maximum specific peroxidase activity (0.12 U/mg of protein) was obtained after 72 hours of 1 incubation at 45°C in a minimal salt medium (pH 7.5) containing 0.6% (w/v) yeast extract and 0.6% (w/v) xylan corresponding to a C:N ratio of 4 to 1. A study of the effect of incubation on peroxidase activity showed that the enzyme was stable and active for at least one hour after incubation at 50°C while at higher temperatures the stability and activity of the peroxidase was reduced such that at 60°C the peroxidase activity has a half life of 20 min while at 80°C the half life was reduced to 5 min. The activation energy for deactivation as a result of thermal denaturation of the enzyme was calculated to be 80 ± 7 kJ/mol. The optimum pH for the activity occurred between a pH range of 6.5–8.5 with pKa_1 and pKa_2 of 5.1 ± 0.1 and 9.7 ± 0.1, respectively. The K_m and V_{max} for the peroxidase activity were determined to be 1.45 m*M* and 0.31 unit per mg protein respectively using 2,4-dicholorophenol (2,4-DCP) as a substrate. Characterization of the peroxidase activity revealed activity against L,3–4 dihydroxyphenylalanine and guaiacol, while no inhibition of peroxidase activity could be detected with the haem inhibitors such as potassium cyanide and

*Author to whom all correspondence and reprint requests should be addressed.

sodium azide, suggesting the lack of haem component in the tertiary structure. Aspects of using the crude peroxidase preparation in the pulp and paper industry are discussed.

Index Entries: *Streptomyces avermitilis* UAH30; peroxidase; paper industry application; KCN; sodium azide.

INTRODUCTION

Peroxidases catalyze the oxidation of a number of substrates in the presence of hydrogen peroxide (H_2O_2) and use a wide range of substrates, according to their biological roles. For example, plant peroxidases, responsible for the polymerization of lignin, catalyze the oxidation of coniferyl alcohol to form a radical. Similarly, salivary peroxidases catalyze the oxidation of halide ions or SCN^- to produce halogens. Peroxidases are widely distributed intracellularly in biological systems (e.g., bacteria, plants, animals, and mammals) *(1–10)* signifying their importance in biological systems. Most of the peroxidases examined contain heme in their tertiary structure. However a number of intracellular peroxidases have been characterized that do not contain heme for example intracellular peroxidases from *Ascophilum nodosum (11)*, *Streptomyces aureofaciens (12)*, *Pseudomonas pyrrocina (13)*, and *Corallina officinalis (14)*.

In contrast, extracellular peroxidases have only been identified in a few specific microorganisms, including ligninolytic fungi such as *Phanerochaete chyrosporium (15)* and the detailed biochemistry of the role of these enzymes in the breakdown of lignocellulose is beginning to be resolved. Another group of organisms in which extracellular peroxidases have been identified are actinomycetes. These are a heterogeneous group of gram-positive bacteria, widely distributed in natural environments such as soil and compost that secrete a range of extracellular enzymes including peroxidases thought to be involved in the degradation of lignin *(16–20)*. However the role of these peroxidases in the lignin degradation is yet to be resolved. Recent work suggests that peroxidase enzymes produced by the thermophilic actinomycete, *Thermomonospora fusca* BD25, lack the characteristic absorption spectrum of heme-containing peroxidases *(21–23)*. Extracellular peroxidases have also been found to be produced by *Streptomyces avermitilis* UAH30. This strain has been isolated from lignocellulosic material and selected for the present study because of its ability to remove the color from paper-mill effluent obtained after semichemical alkaline pulping of wheat straw *(24)*.

This paper reports the optimum condition for the production of high levels of extracellular peroxidase enzymes by *Streptomyces avermitilis* UAH30 and the partial characterization of the peroxidase enzymes.

MATERIAL AND METHODS

Microorganism and Media

Streptomyces avermitilis UAH30 was grown on agar plates containing: glucose 10 g/L; asparagine 1g/L; yeast extract 0.5 g/L; K_2HPO_4 0.5 g/L; $MgSO_4$ 0.5 g/L and $FeSO_4$ 0.01 g/L *(24)*. A spore and hyphae suspension was used to inoculate 250-mL flasks containing 100 mL mineral salt medium (g/L) KH_2PO_4 1g/L; Na_2HPO_4 5.35 g/L; NaCl 0.2 g/L; $MgSO_4$ 0.2 g/L and $CaCl_2$ 0.05 g/L; trace elements solution 0.1% *(25)* supplemented with different concentrations of yeast extract ranging from 0–1.2% (w/v) and a fixed concentration of oat-spelt xylan, 0.6% (w/v).

Cultivation Conditions

The cultures were grown at 37°C over a period of 7 d with shaking at 150*g*. Samples were removed each day, and the activity determined using the standard assay procedure described below. The effects of different temperature (25–55°C) and pH (2.0–13.0) on the production of the extracellular peroxidase was also studied.

Determination of Enzyme Activity

Peroxidase activity was assayed using 5 m*M* 2,4-dicholorophenol (2,4-DCP) and 3.2 m*M* 4-aminoantipyrine in 100 m*M* potassium phosphate buffer pH 7 *(26)*. The reaction was initiated by the addition of 10 m*M* H_2O_2 and the absorbance monitored at 510 nm for one min at 50°C. One unit of enzyme activity is defined as the amount required for an increase in absorbance of 1.0 U/min. In addition, the specific activity is defined as the unit of enzyme activity per mg of protein (U/mg) *(30,31)*.

Protein Estimation

Supernatant protein estimation was performed spectrophotometrically at 595 nm using the Bio-Rad assay method *(27)*. The total concentration of the peroxidases present in the concentrated supernatant was estimated from a calibration curve constructed using albumin in the range of 0–25 microgram protein per assay.

Thermostability and the Effect of Temperature on the Activity of the Enzyme

The effect of temperature on the stability of the extracellular peroxidase produced by *S. avermitillis* UAH30 was studied by incubating the enzyme for 10 min in the absence of the substrate over a range of temperature (e.g., 30°C to 90°C). The activity was measured at 50°C using the standard 2,4-DCP assay. Similarly the effect of time on the stability of the enzyme was determined by incubating the enzyme in the absence of substrate over a period of

one hour at various temperatures in the range 50–80°C. Again the activity was measured at 50°C using the standard 2,4-DCP assay. Finally, the effect of temperature on the activity was monitored by measuring the activity using the 2,4-DCP assay at specific temperature ranging from 35°C to 75°C.

Effect of pH on the Activity

The activity of the peroxidase over a pH range of 3.0–12.0 was investigated using the standard assay. However the potassium phosphate buffer was replaced with universal buffer *(33)* which contained (g/L): 6.008, citric acid; 1.769, boric acid; 3.893, KH_2PO_4; 5.26, diethylbarituric acid. The pH was adjusted with 0.1 *M* NaOH or HCl.

Effect of Substrate Concentration

The effect of substrate concentration on the activity of extracellular peroxidase produced by *Streptomyces avermitilis* UAH30 was determined using the standard assay procedure. However the substrate (2,4-DCP) concentration was varied from 1–8 m*M* in the final reaction mixture. The other components [(hydrogen peroxide 10.0 mM HP and 4-amino antipyrine (3.2m*M*)] of the reaction mixture were kept constant. The reaction was monitored for one minute at 50°C.

Substrate Specificity

The activity of the peroxidase against various substrates such as 2,4-dicholorophenol (24-DCP), L,3–4, dihydroxyphenylalanine (L-DOPA), veratryl alcohol, dye azure B, and guaiacol, was studied. Peroxidase activity was assayed by measuring the formation of the dopachrome pigment from L-DOPA under the conditions described for 2,4-DCP assay with absorbance measured at 470 nm *(10)*. For veratryl alcohol, the reaction mixture consisted of 400 µL of 100 m*M* potassium phosphate buffer (pH 7.0), 200 µL veratryl alcohol (2.0 m*M*) and 200 µL of the enzyme, and the reaction was initiated with 200 µL H_2O_2 (50 m*M*); the appearance of the product, veratryl aldehyde was measured at 310 nm *(28)*. The oxidation of the dye azure B was measured by simply replacing veratryl alcohol with the dye at a concentration of 32 µ*M* *(28)*. The reactivity of the enzymes against guaiacol was measured with an assay reaction mixture that consisted of 400 µL of 100m*M* potassium phosphate buffer (pH 7.0), 200 µL guaiacol (20.0 m*M*) and 200 µL of the enzyme. The reaction was initiated with 200 µL H_2O_2 (50 m*M*), and the changes in absorbance measured at 485 nm *(29)*.

Inhibition Studies

Inhibition studies on the peroxidase produced by *S. avermitilis* were performed with crude supernatant samples. A diluted solution of commercial horseradish peroxidase (Sigma, Dorset, UK) was used as a control

haem-peroxidase. The 2,4-DCP peroxidase activity was measured in a total volume of 1 mL containing, 200 µL enzymatic solution, 5 m*M* 2,4-DCP, 3.2 m*M* 4-aminoantipyrine, and a range of concentrations of sodium azide (5–100 m*M*) in TRIS-HCL buffer pH 7. The reaction was initiated by addition of 200 µL of 50 m*M* H_2O_2 and monitored at 510 nm. The experiment was repeated using potassium cyanide in the concentration range 1–10 m*M*.

RESULTS

Production of Peroxidases

Effect of Yeast Concentration and Incubation Time

Extracellular peroxidases were produced by growing *S. avermitilus* in a liquid medium in which the main carbon and nitrogen sources were xylan and yeast extract, respectively. To optimize the production of extracellular peroxidases, the effects of carbon to nitrogen ratio of the growth medium on enzyme production were investigated by varying the yeast extract concentrations while maintaining a fixed carbon concentration. Maximum peroxidase activity was detected in a minimal salts medium containing 0.6% (w/v) xylan and 0.6 % (w/v) yeast extract (Fig. 1A). Maximum peroxidase activity for *S. avermitilis* growing in this medium occurred after 72 h growth (Fig. 1B), corresponding to the late exponential phase of growth (data not shown).

Effect of pH

The effect of pH on the production of extracellular peroxidases by *S. avermitilis* UAH30 was investigated by adjusting the pH of the growth medium in a pH range of 2.0–12.0. The optimum pH for peroxidase production occurred in the pH range of 7–8.0 (Fig. 2A). This corresponded to the maximum growth yield for *S. avermitilis*.

Effect of Temperature

The effect of temperature on the production of extracellular peroxidases by *S. avermitilis* UAH30 was investigated by growing the organisms in a temperature range of 25–55°C. From the analysis of the results it is evident that the optimum temperature for peroxidase production occurred between 37–45°C (Fig. 2B).

Characterization

Thermostability and the Effect of Temperature on the Activity of the Enzyme

The effect of temperature on the stability of the extracellular peroxidase produced by *S. avermitillis* UAH30 was studied by incubating the enzyme for a fixed period of time in the absence of the substrate at a number of temperatures. Fig. 3 shows that between 30 and 50°C the enzyme is stable and

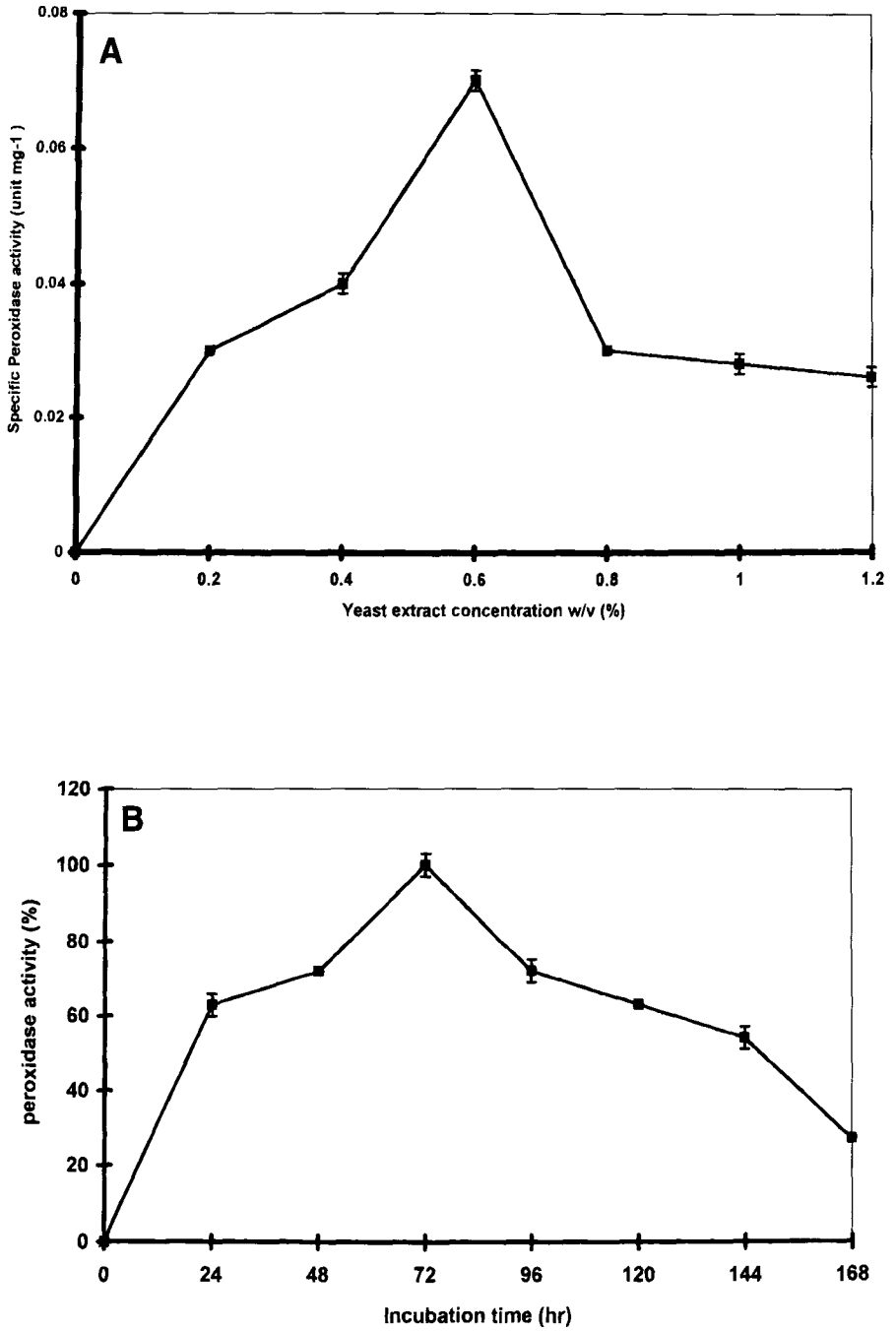

Fig. 1. Production of specific extracellular peroxidase activity by *S. avermitilis* UAH30. After inoculation, cultures were incubated with shaking (150*g*) at 37°C. **(A)** Effect of yeast extract concentration (0–1.2%, w/v) on peroxidase production. The cultures were harvested after 24 h incubation and the 2,4-DCP assay was used to determine the peroxidase activity. **(B)** Effect of incubation time on specific peroxidase production in basal mineral medium supplemented with 0.6% (w/v) yeast extract. The enzyme activity is expressed relative to the maximal value (0.12 U/mg). The data are presented as means ± SEM for triplicate measurements.

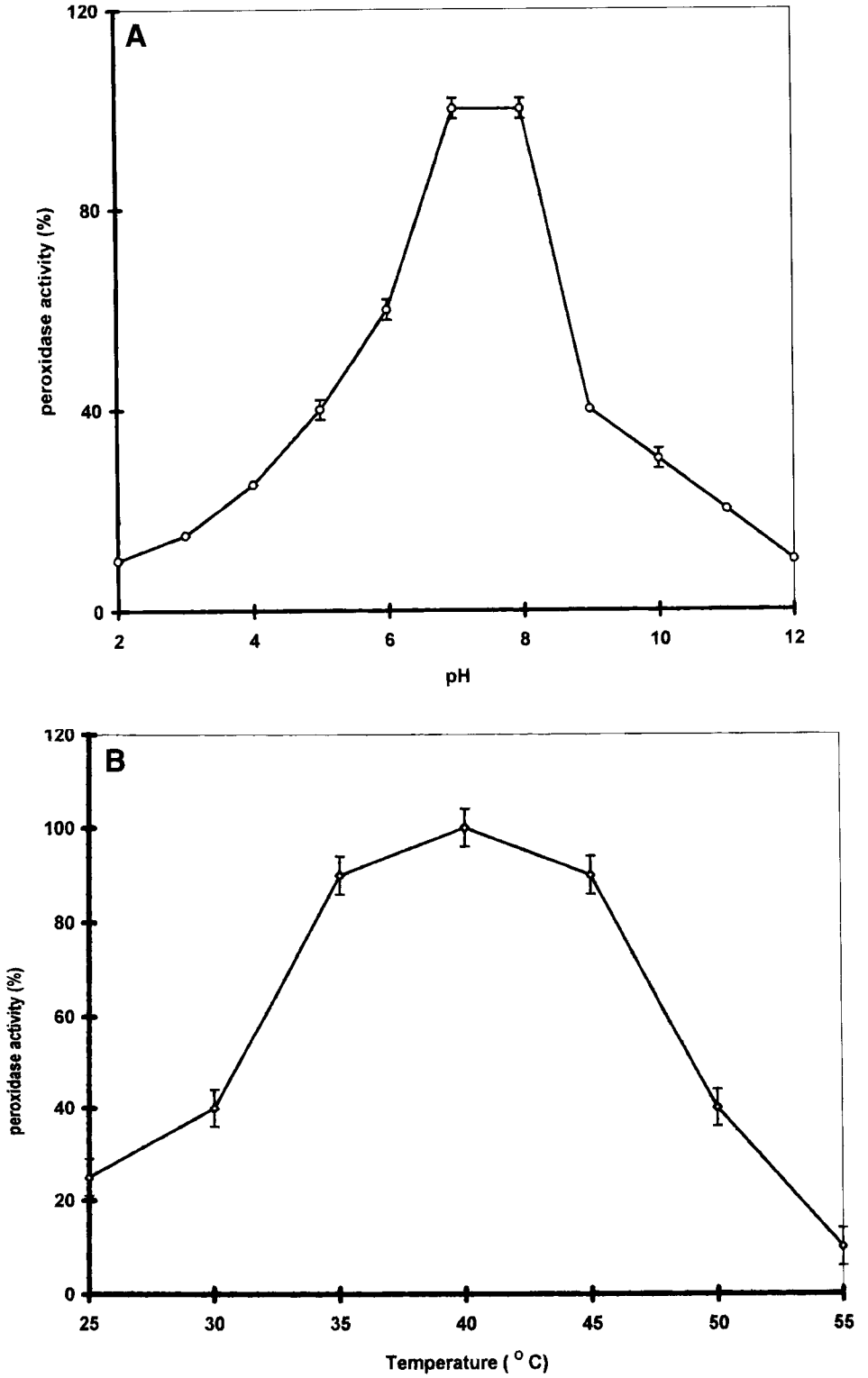

Fig. 2. The effect of cultivation pH **(A)** and temperature **(B)** on the production of the extracellular peroxidase activity by *S. avermitilis* UAH30. The activity is expressed relative to the maximal value (0.07 U/mg). The data are presented as means ± SEM for triplicate measurements.

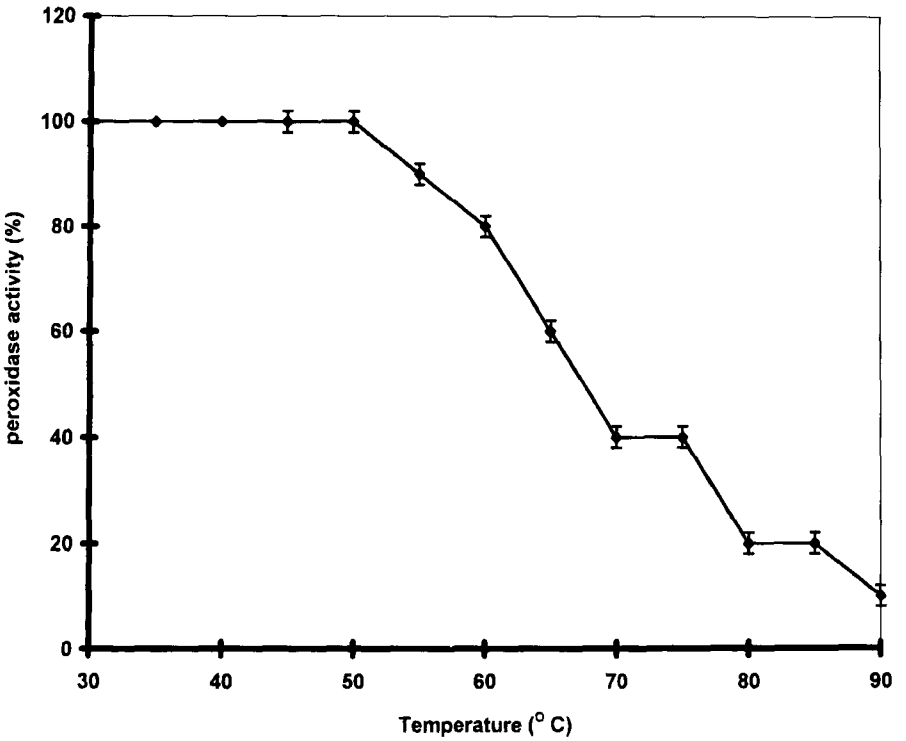

Fig. 3. The effect of temperature on the stability of extracellular peroxidase produced by *S. avermitilis* UAH30. The enzyme supernatant was incubated for 10 min at each of the temperatures prior to the peroxidase assay using the 2,4-DCP method at 50°C. The enzyme activity is expressed relative to the maximal value (0.07 U/mg). The data are presented as means ± SEM for triplicate measurements.

loses no activity over a 10 min period. The effect of time on the stability of the enzyme was also examined by incubating the enzyme in the absence of substrate for a period of one hour over a range of temperatures from 50–80°C. From the analysis of the results it was evident that the enzyme was stable and active for one hour at 50°C whereas at higher temperatures the stability of the peroxidase was significantly reduced. The activation energy for deactivation as a result of thermal denaturation of the enzyme was calculated to be 80.0 ± 7 kJ/mol. This value was determined from a linear fit to the data collected between 50–80°C. There is some evidence however, that the Arrhenius plot was biphasic. Finally, the effect of temperature on the activity was monitored by measuring the activity using the 2,4-DCP assay at specific temperatures ranging from 35 to 75°C. From the analysis of the results (Fig. 4) it is evident that the activity showed a broad maximum around 55°C.

pH Studies

The activity of the peroxidase over a pH range of 3.0–12.0 was investigated using the standard assay. The optimum pH for the activity, as determined under these assay conditions was found to occur between a

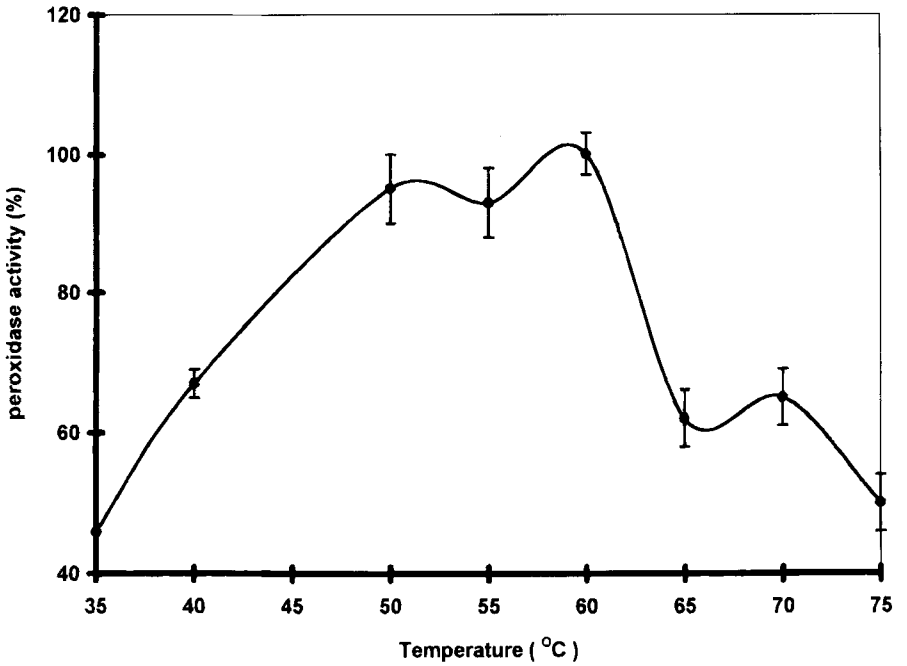

Fig. 4. The effect of temperature on the activity of peroxidase. The reaction was monitored at each of the specified temperatures using the 2,4-DCP assay. The enzyme activity is expressed relative to the maximal value (0.07 U/mg). The data are presented as means ±SEM for triplicate measurements.

pH of 6.5–8.5. The pH dependence conformed to a standard model for the pH dependence of the enzyme activity based on two transitions with pKa values of 5.1 ± 0.1 and 9.7 ± 0.1, respectively (Fig. 5A).

Effect of Substrate Concentration

The K_m value for an enzyme depends on the particular substrate used for the kinetic measurement and also on the environmental conditions such as pH, temperature and ionic strength. The effect of 2,4-DCP concentration at a pH of 7.0 and at a temperature of 50°C on the activity of peroxidase was investigated. The enzyme gave a typical Michaelis-Menten type response, with K_m and V_{max} of 1.45 m*M* and 0.31 unit per mg protein respectively at 50°C (Fig 5B).

Substrate Specificity

The extracellular peroxidase produced by *S. avermitilis* showed peroxidase activity against the substrates 2,4-DCP, L-DOPA and guaiacol (Table 1) but no activity was detected in the presence of veratryl alcohol or the dye azure B. However, the control, haem specific horse radish peroxidase showed positive activity against all of these substrates (Table 1).

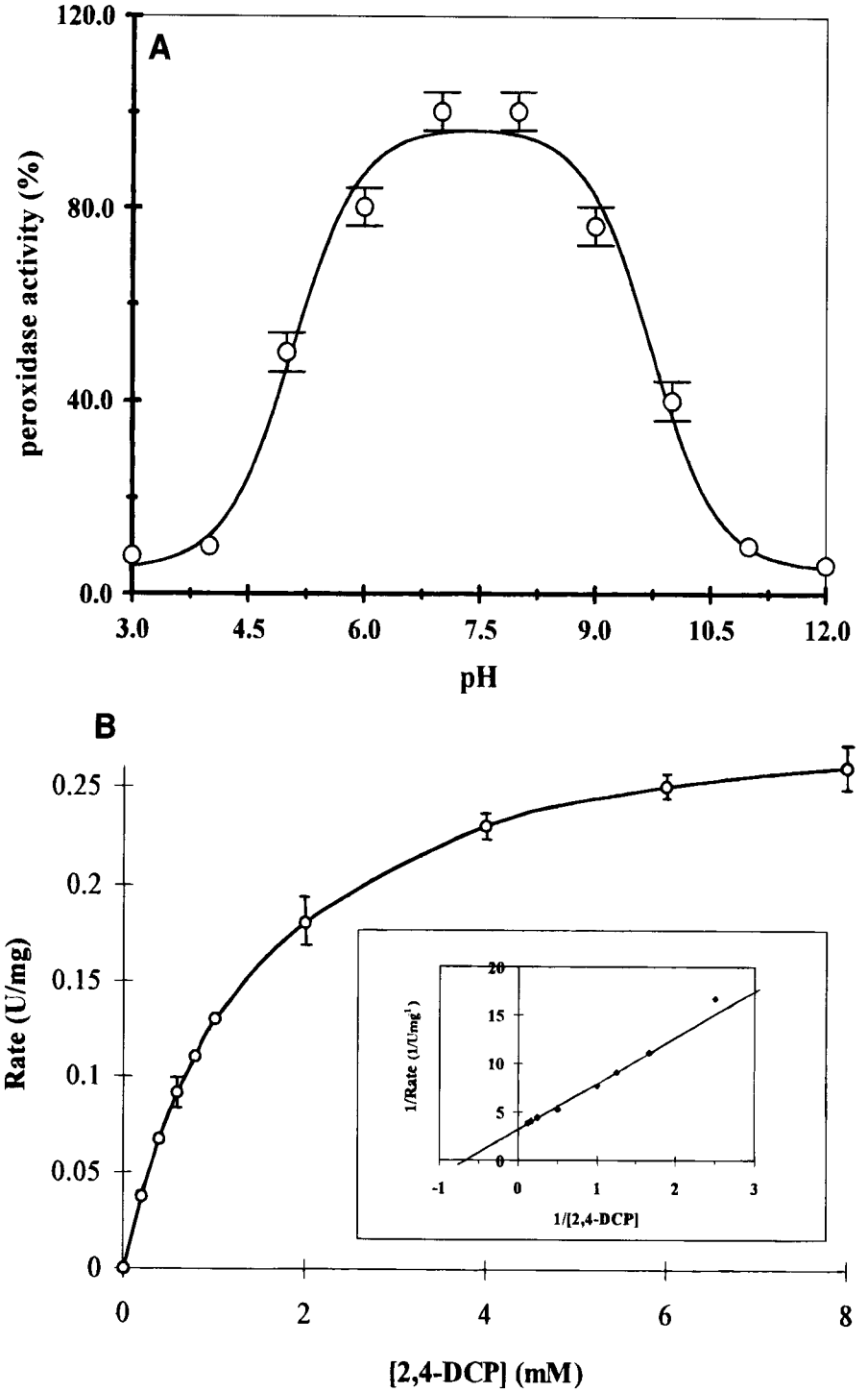

Fig. 5. **(A)** The effect of pH on the activity of extracellular peroxidase produced by *S. avermitilis* UAH30. The solid line represents a fit of experimental data to a model comprising two pH transitions. The enzyme activity is expressed relative to the maximal value (0.07 U/mg). **(B)** The effect of substrate concentration on the activity of the peroxidase. The reaction was monitored at 50°C and at pH 7.0 (100 m*M* potassium phosphate buffer). Also shown is the Lineweaver-Burk plot. The data are presented as means ± SEM for triplicate measurements.

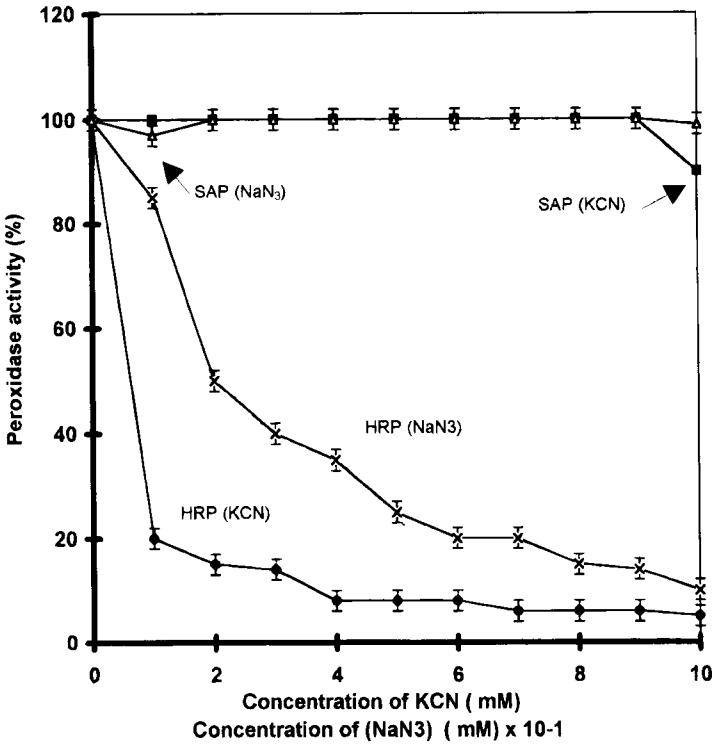

Fig. 6. The effect of sodium azide (NaN_3) and potassium cyanide (KCN) on the activity of the extracellular peroxidase produced by *S. avermitilis* UAH30 (SAP). The activity was measured using the standard 2,4-DCP assay method. All measurements were carried out at 50°C. Horseradish peroxidase (HRP) was used as a control heme-containing protein. The enzyme activity is expressed relative to the maximal value (SAP = 0.07 U/mg; HRP = 0.5 U/mg). The data are presented as means ± SEM for triplicate measurements.

Inhibition Studies with KCN and Sodium Azide

No inhibition of the *S. avermitilis* peroxidase by sodium azide (up to 100 m*M*) was detected; however, when the same experiment was repeated using horseradish peroxidase, the classical heme protein inhibition response was observed (Fig. 6). Similarly, there was no inhibition up to 9 m*M* KCN for the *S. avermitilis* peroxidase while a substantial inhibition was noticed for the horse radish peroxidase activity immediately after addition of 1 m*M* KCN (Fig. 6).

DISCUSSION

Pulp and paper mill effluents have been recognized as environmental hazards for many years. The chemical composition of such effluents depends on the nature of the feedstocks, as well as the treatment procedure. The dark brown color of these effluents is mainly due to their high contents of oxidized and partially degraded lignin. Reducing the color

Table 1
Substrate Specificity of Extracellular *S. avermitilis* UAH30 Peroxidase (SAP)

Substrate	Substrate concentration (mM)	Monitoring wavelength (nm)	SAP	HRP
24-DCP[a]	5.0	510	+	+
L-DOPA[a]	5.0	470	+	+
Guaiacol	4.0	485	+	+
Veratryl alcohol[a]	2.0	310	–	+
Azure dye B[a]	0.032	310	–	+

[a]Positive peroxidase activity is denoted as +, no significant peroxidase activity detected is denoted as –. All measurements were carried out at 50°C. Horse radish peroxidase (HRP) was used as a control haem containing protein. Substrate molecular structures are indicated in Fig. 7.

before the effluents are discharged into natural water is an important objective. Initial results suggest that the extracellular peroxidases produced by *S. avermitilis* may have an important role in the paper industry for biobleaching (*24*). However, for full exploitation of this enzyme for biotechnological application, the first requirement will be to understand its biochemistry. In this paper, the optimum conditions of production are first discussed and partial biochemical characterization given.

The production of extracellular peroxidases is a common trait among actinomycetes. The role of these enzymes in the degradation of lignin has yet to be established. One of the reasons for the lack of information regarding actinomycete peroxidases is the difficulty in detecting extracellular peroxidase activity in the culture supernatant without further concentration *(19–21)*. The extracellular peroxidase activity of *S. avermitilis* was produced in a liquid medium, in which the main carbon source was xylan and the nitrogen source was yeast extract. Under these experimental conditions sufficient peroxidase was produced to detect using the 2,4-DCP substrate specific assay without further concentration of the extracellular supernatant. However, for optimisation of the peroxidase production, the effect of different yeast extract concentrations was investigated. Also, the effect of pH and temperature on the production of the peroxidase was studied. From Fig. 1A, B, and 2A, B, it is concluded that the maximum level of peroxidase activity (0.12 U/mg^{-1}) was found after 72 h of incubation on 0.6 % (w/v) yeast extract and 0.6% (w/v) xylan (corresponding to a C:N ratio of 4 to 1) at an optimum pH range of 7.0–8.0 and a temperature range of 37–45°C. The maximum level of extracellular peroxidase reported above, produced by *S. avermitilis* when grown in oat spelt xylan, is similar to that reported for *S. virodosporus* peroxidase (0.14 U/mg) *(31)*. However, the maximum level of *S. avermitilis* peroxidase is at least three fold more than

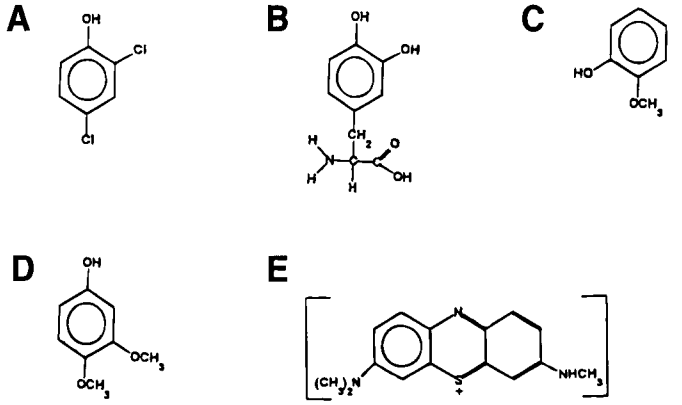

Fig. 7. Chemical structures of substrates: **(A),** 2,4-DCP; **(B),** L-DOPA; **(C),** Guaiacol; **(D),** Veratryl alcohol; **(E),** Azure dye B.

that reported for *S. thermoviolaceus* peroxidase (0.04 U/mg) *(30)* and *T. fusca* BD25 peroxidase (0.038 U/mg) *(23)*. Thus, under the condition described above, sufficient *S. avermitilis* peroxidase can be produced to enable partial characterization.

The effect of temperature on the stability of the extracellular peroxidase was studied by incubating the enzyme for a fixed period of time in the absence of substrate over a range of temperatures. The enzyme was stable upto 50°C (Fig. 3). Also the enzyme showed resistance to denaturation at 60, 70, and 80°C with a half-life of 20, 10, and 5 min respectively (data not shown) demonstrating the thermophilic nature of the enzyme. However, the stability of this enzyme is clearly lower than the reported thermophilic peroxidase isolated from *S. thermoviolaceus* (half life at 70°C is 70 min.) *(30)*. Nevertheless, the stability of the *S. avermitilis* peroxidase is significantly higher than the nonthermophilic extracellular esterase isolated from lignocellulose-degrading actinomycete *Streptomyces virodosporus* T7A (enzyme unstable above 40°C) *(35)*.

The extracellular xylanases, cellulases, and peroxidases produced by actinomycetes, generally exhibit a pH optima between 5.0 to 8.0 *(19–21)*. The pH optima for the peroxidases from *S. virodosporus* has been reported by Lodha *et al. (34)* to be similar, around 5.5–7.5. The peroxidase activity of the *S. avermitillis* studied here conformed to this general pattern exhibiting broadly similar pH optimum in the range of 6.5–8.8.

Potassium cyanide and sodium azide have been used for inhibition study of the extracellular haem containing lignin peroxidase by Ramachandra *et al. (31)*. In contrast, Burd *et al. (32)* found that the halogenating activity of the purified chloroperoxidase from *Serratia marcescens* was not inhibited by sodium azide and showed no absorption bands in the visible region of the spectrum suggesting that the enzyme did not contain heme as a prosthetic group. In order to determine whether the active site of

the *S. avermitilis* peroxidase contains a heme prosthetic group, attempts were made to react the enzyme with the two heme-specific reagents, sodium azide and KCN at a range of concentrations.

Figure 6 shows that there was no inhibition by the sodium azide (NaN_3) up to 100 m*M* for the *S. avermitilis* peroxidase, however when the same experiment was repeated using haem containing, horseradish peroxidase, inhibition was apparent and 90% of the activity was lost at 100 m*M* sodium azide concentration. Likewise there was no inhibition of the *S. avermitilis* peroxidase by potassium cyanide (KCN) up to 9.0 m*M* but a substantial inhibition up to 80% was noticed for the horseradish peroxidase immediately after addition of 1 m*M* KCN and almost 90% inhibition occurred at 10 m*M* KCN (Fig. 6).

Initial results from an inhibition study with a haem-specific reagent such as 2,2'-azino-di-[3-ethyl-benzthiazoline-6-sulphonic acid] (ABTS) *(30)* suggested that the extracellular peroxidase enzymes produced by *Streptomyces avermitilis* do not contain a heme group in their tertiary structures (data not shown). Thus the ABTS results and the results of the inhibition study with sodium azide and potassium cyanide strongly suggest that the extracellular peroxidase produced by *S. avermitilis* lack the protoporphyrin IX prosthetic group at the active site of the enzyme. However, full confirmation of the nature of the active site will require spectral analysis of the enzyme. In this regard, it is perhaps similar to the nonheme extracellular peroxidase produced by *T. fusca* BD25 *(23)*. In addition, the substrate specificity is also similar to that of the *T. fusca* BD25 but different from that of the heme-containing peroxidase isolated from horseradish *(22–23)*.

The temperature stability of the nonheme peroxidase produced by *S. avermitilis* UAH30, and its activity over a broad pH range makes this enzyme suitable for industrial application and preferable to the more labile heme containing peroxidase from *Chrysonilia sitophila* (TFB-27441 strain) (half-life of 9 h at 28°C) *(36–37)*. One potential large-scale application for peroxidases is the biological delignification of paper pulp. To date few suitable peroxidases have been available. The cost of production of the extracellular enzyme may out weigh its usefulness. A number of studies have been carried out with horseradish peroxidase from very crude and inexpensive sources *(38–41)*. Simple phenols and substituted phenols such as methoxyphenol and cresol have been removed in a matter of minutes with the conversion reaching almost 100%. The suitability of *S. avermitilis* UAH30 peroxidase for removal of simple and substituted phenols and delignification of paper pulp is currently being investigated.

CONCLUSIONS

This paper examined the effect of a number of environmental parameters, such as pH, temperature, and carbon source on the production of extracellular peroxidase enzymes by *Streptomyces avermitilis* UAH30.

From the analysis of the results, it was found that the maximum peroxidase activity occurred after 72 h of incubation at 45°C in a minimal salt medium at pH 7.5 containing 0.6% (w/v) yeast extract and 0.6% (w/v) xylan. Also the effect of incubation on peroxidase activity showed that the enzyme was stable and active for at least one hour after incubation at 50°C whereas at higher temperatures the stability and activity of the peroxidase was significantly reduced with the activation energy for deactivation as a result of thermal denaturation of 80 ± 7 kJ mol. The optimum pH for the activity was found to be between a pH range of 6.5–8.5 with pKa_1 and pKa_2 of 5.1 ± 0.1 and 9.7 ± 0.1, respectively.

From a plot of the Lineweaver-Burk equation, the K_m and V_{max} for the peroxidase activity were determined to be 1.45 m*M* and 0.31 U per mg protein respectively using 2,4-DCP as substrate. Characterization of the peroxidase activity revealed activity against 2,4-DCP, L,3–4 dihydroxyphenylalanine (L-DOPA) and guaiacol, although no inhibition of peroxidase activity could be detected with the heme inhibitors such as potassium cyanide (up to 10 m*M*) and sodium azide (up to 100 m*M*), suggesting the lack of heme component in the tertiary structure. Work on the purification of this enzyme is in progress.

ACKNOWLEDGMENTS

The major part of this work was carried out using a research grant awarded to ASB and MTW by the Biotechnology and Biological Sciences Research Councils (BBSRC) (UK). MH is very grateful to British Council for the funding provided to carryout some of the work described in this report. MT greatly acknowledges the studentship provided by the Turkish government.

REFERENCES

1. Van-Pee, K. H and Lingens, F. (1984), *FEBS Lett.* **173,** 5–8.
2. Welinder, K. G. (1985), *Eur. J. Biochem.* **151,** 447–450.
3. Itho, N., Izumi, Y., and Yamanda, H. (1986), *J. Biol. Chem.* **261,** 5194–5200.
4. Van-Pee, K. H., and Lingens, F. J. (1985), *J. Bacteriol.* **161,** 1171–1175.
5. Zeiner, R., Van-pee, K. H., and Lingens, F. (1988), *J. Gen. Microbiol.* **134,** 3141–3149.
6. Berger, R. G., Drawert, F., Kinzkofer, A., Kunz, C., and Radola, B. J. (1985), *Plant Physiol.* **77,** 211–214.
7. Chibbar, R. N. and Van-Huystee, R. B. (1984), *Plant Physiol.* **75,** 956–958.
8. Deits, T. M. and Shapiro, B. M. (1991), *Peroxidases in Biology & Chemistry* CRC **1,** pp 237–258.
9. Wever, R., Hamers, M. N., Weening, R. S., and Roos, D. (1980), *Eur. J. Biochem.* **108,** 491–495.
10. Deobald, L. A. and Crawford, D. L. (1987), *Appl. Microbiol. Biotechnol.* **26,** 158–163.
11. De Boer, E., Van Kooyk, Y., Tromp, M. G. M., Plat, H., and Wever, R. (1986), *Biochim. Biophys. Acta* **869,** 48–53.
12. Krenn, B. E., Plat, H., and Wever, R. (1988), *Biochim. Biophys. Acta* **952,** 252–260.
13. Wiesner, W., Van-Pee, K. H., and Lingens, F. (1986), *FEBS lett.* **209,** 321–424.

14. Yu, H. and Whittaker, J. W. (1989), *Biochim. Biophys. Commun.* **160,** 87–92.
15. Vazquez-Duhalt, R., Westlake, D. W. S., and Fedorak, P. M. (1995), *Appl. Microbiol. Biotechnol* **42,** 675–681.
16. Ball, A. S., Godden, B., Helvenstein, P., Penninekx, M. J., and McCarthy, A. J. (1990), *Appl. Environ. Microbiol* **56,** 3017–3022.
17. Ball, A. S. and McCarthy, A. J. (1988), *J. Gen. Microbiol.* **134,** 2139–2147.
18. McCarthy, A. J. and Williams, S. T. (1992), *Gene* **115,** 189–192.
19. McCarthy, A. J., Peace, W., and Broda P. (1985), *App. Mrobiol. Technol.* **21,** 238–244.
20. Wilson, D. B. (1992), *Crit. Rev. Biotechnol.* **12,** 45–63.
21. Trigo, C. and Ball, A. S. (1994), *Appl. Microbiol. Biotechnol.* **41,** 366–372.
22. Ball, A. S. and Trigo, C. (1995), *Biochem. Soc. Trans.* **23,** 272–276.
23. Rob, A., Ball, A. S., Tuncer, M., and Wilson, M. T. (1995), *Biochem. Soc. Trans.* **23,** 507.
24. Hernandez, M., Rodriguez, J., Soliveri, J., Copa, J. L., Perez, M. I., and Arias, M. E. (1994), *Appl. Environ. Microbiol.* **60,** 3909–3913.
25. Ramachandra, M., Crawford, D. L., and Pometto, A. L. (1987), *Appl. Environ. Microbiol.* **53,** 2754–2760.
26. Crawford, D. L. (1978), *Appl. Environ. Microbiol.* **35,** 1041–1045.
27. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.
28. Archibald, F. S. (1992), *Appl. Environ. Microbiol.* **58,** 3110–3116.
29. Fukumori, Y., Fujiwara, T., Okada-Takahashi, Y., Mukohata, Y., and Yamanaka T. (1985), *J. Biochem.* **98,** 1055–1061.
30. Iqbal, M., Mercer, D. K., Miller, P. G. G., and McCarthy, A. J. (1994), *Microbiology* **140,** 1457–1465.
31. Ramachandra, M., Crawford, D. L., and Hertel, G. (1988), *Appl. Environ. Microbiol.* **54,** 3057–3063.
32. Burd, W., Yourkevich, O., Voskoboev, A. J., and Van-Pee, K. H. (1995), *FEMS Microbiol. Lett.* **129,** 255–260.
33. Johnson, W. C. and Lindsey, A. J. (1939), *Analyst* **64,** 958–993.
34. Lodha, S. J., Korus, R. A., and Crawford, D. L. (1991), *Appl. Biochem. Biotechnol.* **28,** 411–420.
35. Donnelly, P. K. and Crawford, D. L. (1988), *Appl. Environ. Microbiol.* **54,** 2237–2244.
36. Duran, N., Ferrer, I., and Rodriquez, J. (1987), *Appl. Biochem. Biotechnol.* **16,** 157–167.
37. Ferrer, I., Esposito, E., and Duran, N. (1992), *Enzym. Microb. Technol* **14,** 402–406.
38. Klyachko, N. L. and Klibanov, A. M. (1992), *Appl. Biochem. Biotechnol.* **37,** 53–68.
39. Tatsumi, K., Ichikawa, H., and Wada, S. (1994), *Water Science and Tech* **30,** 79–86.
40. Klibanov, A. M. and Morris, E. D. (1981), *Enzyme Microb. Technol.* **3,** 119–122.
41. Klibanov, A. M., Tu, T. M., and Scott, K. P. (1983), *Science* **221,** 259–261.