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Production and partial characterization of extracellular peroxidase produced by *Streptomyces* sp. F6616 isolated in Turkey

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Abstract - *Streptomyces* sp. F6616 was found to produce higher levels of extracellular peroxidase activity (0.535 U/mL) without any inducers than other actinobacteria which are previously reported. Maximum specific peroxidase activity (6.21 U/mg of protein) was obtained after 72 h of incubation at 30 °C in a minimal salt medium (pH 8.0) containing (in wt/v) 0.6% yeast extract and 0.8% ball-milled wheat straw corresponding to a C:N ratio of 4.6:1. Characterization of the peroxidase revealed that the optimal temperature for the enzyme activity, using the standard 2,4-dichlorophenol (2,4-DCP) assay was 50 °C, when the enzyme reaction was performed at pH 8.0. A study of the effect of temperature on the stability of peroxidase over time, showed that the enzyme was stable at 50 °C, with a half-life of 145 min, while at higher temperature the stability and activity was reduced such that at 60 °C the half-life of the enzyme was 30 min. The optimum pH for the activity of the enzyme occurred between pH 9.0 and 10.0. The apparent K_m and V_{max} values for the peroxidase preparations were determined to be 1.52 mmol/L and 1.84 U/mg protein, respectively using 2,4-DCP as a substrate. Characterization of the peroxidase activity revealed activity against 2,4-DCP, L-3,4-dihydroxyphenylalanine (L-DOPA), 2,4,5-trichlorophenol and other chlorophenols in the presence of hydrogen peroxide. However, inhibition of peroxidase activity with the addition of potassium cyanide and sodium azide, suggested the presence of heme component in the tertiary structure of the enzyme.

Key words: lignocellulose; peroxidase; *Streptomyces*; 2,4-dichlorophenol; oat spelt xylan.

INTRODUCTION

The biological degradation of cellulose, hemicellulose, and lignin has attracted the interest of microbiologist and biotechnologist for many years. The diversity of cellulosic and lignicellulosic substrates has contributed to the difficulties found in enzymatic studies. Because the substrates are insoluble, both bacterial and fungal degradation have to occur exocellularly, either in association with the outer cell-envelope layer or extracellularly. Microorganisms have two types of extracellular enzymatic systems: the hydrolytic system, which produces hydrolases and is responsible for cellulose and hemicellulose degradation; and a unique oxidative and extracellular ligninolytic system, which depolymerises lignin (Perez *et al*., 2002).

Two major families of enzymes are involved in ligninolysis are lignin peroxidases and laccases. 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, have been found widely throughout plants, animals and microorganisms, signifying their important role in biological systems (Franssen, 1994; Rob *et al*., 1997). Most of the peroxidases examined contain heme in their tertiary structure (Martinez, 2002). However a number of intracellular lignin peroxidases have been characterized that do not contain heme, for example intracellular lignin peroxidases from *Ascophilum nodosum* (De Boer *et al*., 1986), *Streptomyces aureofaciens* (Krenn *et al*., 1988), *Pseudomonas pyrocina* (Wiesner *et al*., 1986)*, Corallina officinalis* (Yu and Whittaker, 1989) and *Serratia mercescens* (Burd *et al*., 1995). Although lignin peroxidases are primarily found intracellularly (Maliki and Zimmermann, 1992) in almost all organisms examined (Ball and Trigo, 1995), extracellular lignin peroxidases are less widespread with the exception of fungi such as *Phanerochaete chrysosporium* and *Pleurotus eryngii* (Orth *et al*., 1993; Vazquez-Duhalt *et al*., 1995; Martinez, 2002). Another group of organisms in which extracellular peroxidases have been identified are actinobacteria (Ball and Trigo, 1995; Mercer *et al*., 1996; Rob *et al*., 1996; Kang

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et al., 1999; Tuncer *et al*., 2004). Degradation of lignin and lignin-degrading enzymes has also been reported for actinobacteria, mainly from the *Thermomonospora fusca* BD25 (Trigo and Ball, 1994; Ball and Trigo, 1995; Rob *et al*., 1996), *Streptomyces avermitilis* UAH30 (Rob *et al*., 1997), *Streptomyces albus* ATCC 3005 (Antonopoluos *et al*., 2001), *Streptomyces sp.* UAH47 (Hernandez *et al*., 2001), and *Streptomyces* sp. F2621 (Tuncer *et al*., 2004).

Attention has been given to these organisms and their peroxidases due to their involvement in the degradation of the complex lignin polymer (Thompson *et al*., 1998) as was shown from early experiments with ^{14}C (Crawford and Crawford, 1976), lignin substructure model compounds (Glenn *et al*., 1983; Tien and Kirk, 1983; Ramachandra *et al*., 1988) and acid precipitable polymeric lignin (APPL) (Crawford *et al*., 1983). Since then, many reports have been written involving microbial peroxidases in the degradation of a wide-range of compounds including dyes (Ollikka *et al*., 1993; Goszczynski *et al*., 1994; Spadaro and Renganathan, 1994; Chivukala *et al*., 1995; Ball and Colton, 1996; Heinfling *et al*., 1998; Thakkar *et al*., 2006), molasses (Pazarlioglu *et al*., 2005), olive mill waste-waters (Sayadi and Ellouz, 1995), nitro aromatics (Vali *et al*., 1992a), dioxins (Vali *et al*. 1992b), herbicides (Shelton *et al*., 1989), chlorinated (Hammel and Tardone, 1988) and many other compounds as reviewed by Pasczynski and Crawford (1995).

Lignocelluloses in nature derive from wood, grass, agricultural residues, forestry wastes and municipal solid wastes. Several biological methods for lignocellulose recycling, based on the enzymology of cellulose-, hemicellulose- and lignin-degradation, have been suggested. Among them, compostage and their use as raw material for the production of ethanol as an alternative combustible seem to be the most economically feasible. Moreover, the general use of alternative, environmentally friendly technologies that introduce lignocellulose enzymes at different stages of pulp and paper manufacture as a pre-treatment to pulping (bio-pulping), bleaching (bio-bleaching), or wastewater treatment has allowed considerable electrical power savings and a reduction of pollutants in the waste water from these industries. In addition, pre-treatment of agricultural wastes with ligninolytic microorganisms enables their use as raw material for paper manufacturing (Perez *et al*., 2002).

The finding that peroxidases from fungi and bacteria have a wide range of potential biotechnological applications has caused an increased interest in finding new species that produce beneficial peroxidase activity (Orth *et al*., 1993; Mercer *et al*., 1996; Kang *et al*., 1999). Although peroxidases are ubiquitous, two main factors limit their exploitation: (a) the levels of enzyme production and (b) the instability of peroxidase activity under conditions such as high pH and temperatures, conditions generally encountered in industrial processes.

This paper reports the effects of environmental conditions on the production of high levels of extracellular peroxidase activity and the partial characterization of the crude enzyme produced by *Streptomyces* sp. F6616 isolated from soil in Turkey, indicating a number of key properties of the enzyme and investigates its biotechnological potential in the biological treatment of wheat straw and Kraft pulp.

MATERIALS AND METHODS

Microorganism and growth conditions. The isolation and identification of *Streptomyces* sp. F6616 were done according to Tuncer *et al*. (2004).

Liquid culture. For observation on the time course of extracellular peroxidase production, cultures were incubated in minimal salts-yeast extract medium, which described below and supplemented with ball-milled wheat straw (0.2% wt/v) at 30 °C for up to 10 days at 150 rev/min. However, for examination of the effects of incubation pH (5.0-11.0), temperature (25-50 °C) and different growth substrates as main carbon and energy source, cultures were incubated at 30 °C at 150 rev/min for 3-4 days, and samples were removed at 24 hour intervals from each flask for enzyme assays. The culture supernatant fluids were centrifuged at 10000 *x g* at 4 °C for 10 min and then used for enzyme assays.

For investigation of substrate concentration and C:N ratio effects on growth and extracellular peroxidase production, suspensions of sporulating growth were used to inoculate 100 mL shake flasks containing 20 mL of minimal salts-yeast extract nutrient medium (pH 8.0) based on that described by Ramachandra *et al*. (1988), supplemented either with different concentrations (in wt/v) of oat spelt xylan (Sigma) or ball-milled wheat straw, ranging from 0.0 to 1.2%. All other ingredients were kept fixed at the usual concentrations in both cases. The liquid medium consisting of (in g/L): yeast extract, 6.0; ammonium sulphate, 0.1; sodium chloride, 0.3; magnesium sulphate, 0.1; calcium carbonate, 0.02 and 1 mL of trace-elements solution, pH 8.0. The trace-elements solution contained (in g/L): ferrous sulphate, 1.0; zinc sulphate, 0.9; manganese sulphate, 0.2. In order to ensure aerobic conditions, the volume of the medium was restricted to 1/5 of the total volume of each flask. Elemental analysis showed that oat spelt xylan contained (in wt/v) 38.8% C, 0.08% N, while yeast extract contained (in wt/v) 39.4% C and 10.8% N.

Preparation of ball-milled wheat straw (BMWS). According to Tuncer and Ball (2002), wheat straw was chopped and milled in a rotating ball-milled apparatus (50 rpm) for 72 h. A fine powder of straw was obtained after sieving (500 µm pore size) the ball-milled wheat straw.

Harvesting of culture supernatants. Harvesting of culture supernatant fluids were done according to Tuncer *et al*. (1999) and Tuncer and Ball (2002, 2003).

Determination of enzyme activities. Peroxidase activity was assayed using 2,4-dichlorophenol (2,4-DCP) as the substrate. The reaction mixture (total volume 1 mL) contained equal volumes (0.2 mL) of potassium phosphate buffer (100 mmol/L, pH 8.0) 2,4-DCP (25 mmol/L), 4-aminoantipyrine (16 mmol/L), enzyme solution and H_2O_2 (50 mmol/L). The reaction was initiated with the addition of H_2O_2 and the reaction was monitored at 30 °C for 1 min at a wavelength of 510 nm (Ramachandra *et al*., 1988; Iqbal *et al*., 1994). One unit (U) of peroxidase activity was defined as the amount of enzyme required for an increase in absorbance of 1 unit per minute. In addition, the specific activity of peroxidase was defined as the unit of enzyme activity per mg of protein (U/mg). The absorbance value obtained at the end of the one-min incubation was corrected by subtracting a control absorbance value obtained by replacing hydrogen peroxide with water in the reaction mixture.

Protein estimation. The culture fluid protein concentrations and production of cell biomass as estimated by total cell protein were measured as described previously (Tuncer *et al*., 1999).

Purification of peroxidase from *Streptomyces* sp. F6616. The initial purification step for peroxidase was ultra-filtration of the cell-free extracellular liquid cultures. This was carried out using Amicon ultra-filtration cells (Millipore, Watford, UK) using 10 kDa molecular weight cut-off membranes pressurised to 0.21 MPa with nitrogen. The dialysed 45-fold crude concentrated preparation was then loaded onto a gel filtration column (1.5 id x 30 cm), which was packed with Sephadex G-75 (Sigma-Aldrich, Missouri, USA) column material according to the manufacturers instructions. The sample was eluted using potassium phosphate buffer (pH 7.0) at a flow rate of 20 mL/h (data not shown). Positive fractions for peroxidase activities were pooled and used for anion exchange chromatography.

Anion exchange chromatography was carried out using diethylaminoethyl-sepharose fast flow (Sigma-Aldrich) column material. The material was packed into a Pharmacia-XK50 column (5.0 id x 5.0 cm; Pharmacia, Milton Keynes, UK) according to the manufacturer's instructions. The pooled active fraction of gel filtration was loaded onto an anion exchange column and eluted with a NaCl gradient (0-1 mol/L) in Tris-HCl buffer (50 mmol/L, pH 8.5) at a flow rate of 0.5 mL/min (data not shown). Fractions showing peroxidase activity were concentrated by disposable concentration units (Centricon-10; Millipore) and the salt content of the sample was removed by washing with sample buffer. Enzyme activities were assayed at each step by standard 2,4-DCP assay method.

Thermostability and the effect of temperature on the activity of the peroxidase. The effect of temperature on the activity of peroxidase was monitored by measuring the peroxidase activity of enzyme solution at specific temperatures ranging from 30 to 80 °C using the standard 2,4-DCP assay method. The effect of temperature on the stability of peroxidase over time was determined by incubating the enzyme preparations in the absence of substrate over a period of 24 h at 50 and 60 °C. Activity of these samples was then measured at 30 °C using the standard 2,4-DCP assay method.

Effect of pH on the activity of the peroxidase. The activity of the peroxidase over a pH range of 3-12 was investigated using the 2,4-DCP assay method. However, the sodium phosphate buffer was replaced with universal buffer (Johnson and Lindsey, 1939) which contained (in g/L): 6.008, citric acid; 1.769, boric acid; 3.893, sodium dihydrogen phosphate and 5.26, diethylbarbituric acid. The pH was adjusted with sodium hydroxide or hydrochloric acid.

Effect of substrate concentration on the activity of the peroxidase. The effect of substrate concentration on the activity of extracellular peroxidase was determined using the standard 2,4-DCP assay procedure. However the substrate (2,4-DCP) concentration was varied from 0.2-12 mmol/L in the final reaction mixture. The other components (H_2O_2, 10 mmol/L; 4-aminoantipyrine, 3.2 mmol/L) of the reaction mixture were kept constant. The reaction was monitored for 1 min at 30 °C.

Substrate specificity of the peroxidase. The activity of the extracellular peroxidase against various substrates, such as 4-chlorophenol; 2,4-DCP; 2,6-dichlorophenol (2,6-DCP); 2,4,5-thrichlorophenol; 2,4,6-thrichlorophenol; L-3,4-dihydroxyphenylalanine (L-DOPA); veratryl alcohol; dye azure B and guaiacol, was studied as described below.

Peroxidase activity was assayed by measuring the formation of the dopachrome pigment from L-DOPA. Under the conditions described for the 2,4-DCP assay but in the absence of 4-aminoantipyrine, with absorbance measured at 470 nm (Deobald and Crawford, 1987). Reactivity of the enzyme against veratryl alcohol and dye Azure B were also measured according to Archibald (1992) and the appearance of the products were measured at 310 nm and 651 nm, respectively. Also, the reactivity of the peroxidase against guaiacol was measured. The reaction was initiated with 200 μL of 50 mmol/L H_2O_2 (final volume 1 mL) and the changes in absorbance measured at 485 nm (Fukumori *et al*., 1985).

Inhibition studies. Inhibition studies on the extracellular peroxidase activity were performed with partially purified enzyme samples. A diluted solution of commercial horseradish peroxidase (HRP) (Sigma) was used as a control heme-peroxidase. The peroxidase activity was measured by standard 2,4-DCP assay method with a range of concentrations of sodium azide (0-100 mmol/L in final reaction mixture). The experiment was also repeated with using potassium cyanide in the concentration range of 0-20 mmol/L in final reaction mixture.

Treatment of Kraft pulp (KP), ball-milled wheat straw (BMWS) and oat spelt xylan (OSX) with *Streptomyces* sp. F6616 enzymes. The effect of extracellular lignocellulose-degrading enzymes of *Streptomyces* sp. F6616 on eucalyptus KP (Kappa no. 17.7), BMWS and OSX were done according to Tuncer and Ball (2002). Culture supernatants were harvested, concentrated (45-fold) and dialysed as described earlier. The crude concentrated dialyzed supernatant-enzyme preparations were incubated with potassium phosphate buffer (100 mmol/L, pH 8.0) containing eucalyptus KP, BMWS and OSX (10 mg/mL) in the presence of sodium azide (0.03% wt/v) and hydrogen peroxide (50 mmol/L). The samples were incubated in small flask at 30 °C at 150 rev/min for up to 24 h. Endo-xylanase (1,4-β-D-xylan xylanohydrolase, E.C.3.2.1.8), peroxidase and α-L-arabinofuranosidase activities (per gram of dry substrate) in the flasks containing concentrated culture supernatants were approximately 378 U, 4.4 U and, 1.1 U, respectively. Sample supernatants were examined for the release of reducing sugars (as xylose equivalents) and lignin-equivalents, which were estimated by Folin phenol reagent (A_{750}) (Lowry *et al*., 1951). Lignin-equivalents were calculated from a standard curve of *p*-coumaric acid as a lignin component. Percent hydrolysis of each substrate was calculated as:

Xylan (100% xylan)

$$\text{Percent hydrolysis} = \frac{\mu\text{mol of reducing sugar as xylose}}{\mu\text{mol of substrate as xylose x 100}}$$

Straw (30% xylan)

$$\text{Percent hydrolysis} = \frac{\mu\text{mol of reducing sugar as xylose}}{\mu\text{mol of substrate as xylose x 30}}$$

Kraft pulp (20% xylan)

$$\text{Percent hydrolysis} = \frac{\mu\text{mol of reducing sugar as xylose}}{\mu\text{mol of substrate as xylose x 20}}$$

RESULTS AND DISCUSSION

In this paper first, the effects of environmental conditions on the production of extracellular peroxidase activity by *Streptomyces* sp. F6616 was investigated and discussed and then partial biochemical characterization given. Finally, the potential use of extracellular peroxidase in the delignification of Kraft pulp and wheat straw is also assessed.

The effect of cultivation time, carbon sources and C:N ratio on peroxidase production

Streptomyces sp. F6616 was capable of growth in basal salts-yeast extract medium containing either BMWS or OSX. However, production of extracellular peroxidase by *Streptomyces* sp. F6616 was found to vary between the different carbon sources and concentrations. The production of extracellular peroxidase increased significantly during the growth phase of culture (1-3 d). This period falling within the range frequently reported for actinobacteria (2 d to 3 d), including *Streptomyces thermoviolaceus* (Iqbal *et al*., 1994), *Thermomonospora fusca* BD25 (Rob *et al*., 1996; Tuncer *et al*., 1999), *Streptomyces avermitilis* UAH30 (Rob *et al*., 1997), *Streptomyces viridosporus* T7A (Zerbini *et al*., 1999), and *Streptomyces albus* ATCC 3005 (Antonopoulos *et al*., 2001). After the first 3 d of incubation, activity of peroxidase declined until 10 d of incubation. A typical growth curve and peroxidase production by *Streptomyces* sp. F6616 in basal salts-yeast extract media containing 0.2% (wt/v) xylan is shown in Fig. 1. Similar growth curves and enzyme production patterns were observed for *Streptomyces* sp. F6616 grown in all carbon sources used (data not shown).

The highest production of extracellular peroxidase was found when *Streptomyces* sp. F6616 was grown in a media containing either OSX (0.441 U/mL) (Fig. 2A) or BMWS (0.535 U/mL) (Fig. 2B). When the effect of the concentration of the two basic nutrients (xylan or wheat straw and yeast extract) was investigated, maximal peroxidase activity (0.535 U/mL) was obtained when 0.8% (wt/v) wheat straw and 0.6% (wt/v) yeast extract was supplied in the culture medium, corresponding to a C:N ratios of around 4.6:1 (Fig. 2B). The maximal specific peroxidase activity reported here (6.21 U/mg) without any chemical inducer is almost 2-fold higher than that reported for *S. albus* ATCC 3005; 3.42 U/mg (Antonopoulos *et al*., 2001), 21-fold higher than that reported for *S. viridosporus,* 0.300 U/mg (Ramachandra *et al*., 1988), 52-fold higher than that reported for *S. avermitilis* UAH30; 0.120 U/mg (Rob *et al*., 1997), and 155-fold higher than that reported for *T. fusca* BD25 (Rob *et al*. 1996) and *S. thermoviolaceus* (Iqbal *et al*., 1994), both of which showed a maximal specific peroxidase activity of 0.040 U/mg. Thus, under the condition described above, sufficient *Streptomyces* sp. F6616 peroxidase can be produced to enable partial characterization. To our knowledge this is the most active peroxidase producing actinobacteria reported.

Xylan was also a good inducing substrate that resulted in the maximum production of peroxidase (0.441 U/mL) (Fig. 2) with the highest activity of peroxidase was detected on 0.6% (wt/v) xylan and 0.6% (wt/v) yeast extract was supplied in the culture medium, which corresponded to C:N ratio of 6.8:1 (Fig. 2A).

The effect of cultivation pH and temperature on peroxidase production

The maximal production of extracellular peroxidase occurred in the pH of 8.0 (Fig. 3A). This also corresponded to the maximum growth yield for *Streptomyces* sp. F6616

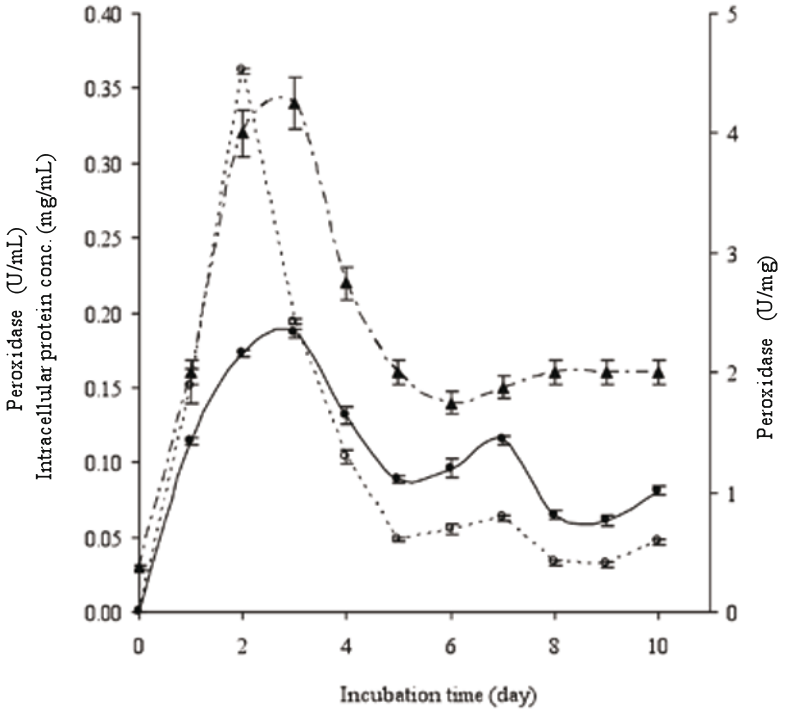

FIG. 1 - Effect of cultivation time on the extracellular peroxidase activity (-•-), specific activity (-o-) and biomass protein (-▲-) production in basal salts-yeast extract medium supplemented with 0.2% (wt/v) oat spelt xylan. After inoculation, cultures were incubated with shaking at 150 rev/min at 30 °C. The data are presented as means ± SEM for triplicate measurements.

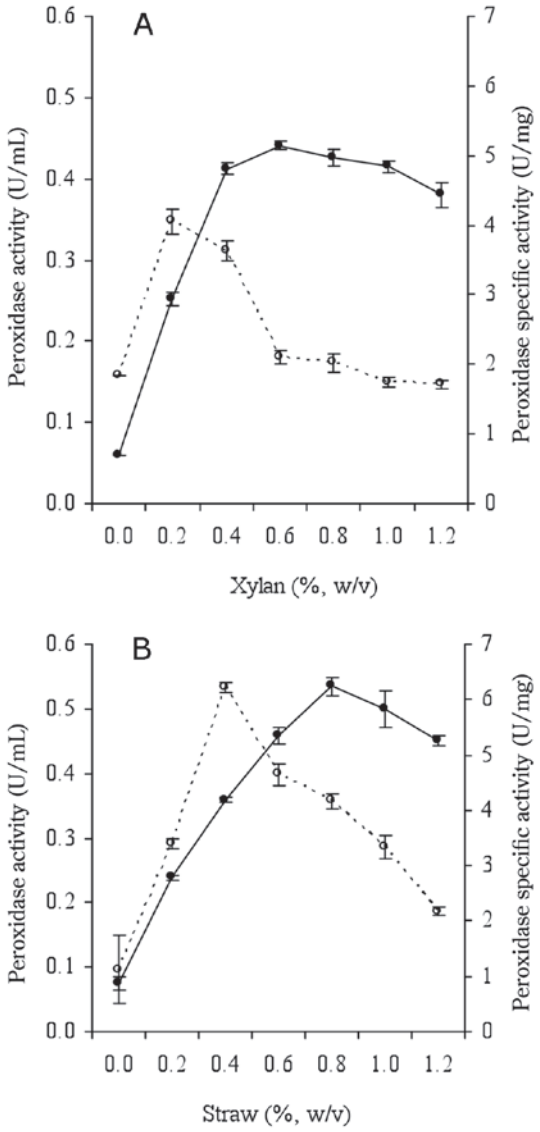

FIG. 2 - Effects of oat spelt xylan (A) and ball-milled wheat straw (B) concentrations (0-1.2%, wt/v) on the production of extracellular peroxidase (-•-) activity and specific activity (-o-) in basal salts-yeast extract medium. The cultures were harvested after 72 h of incubation and cell-free supernatants were assayed for peroxidase activities. The data are presented as means ± SEM for triplicate measurements.

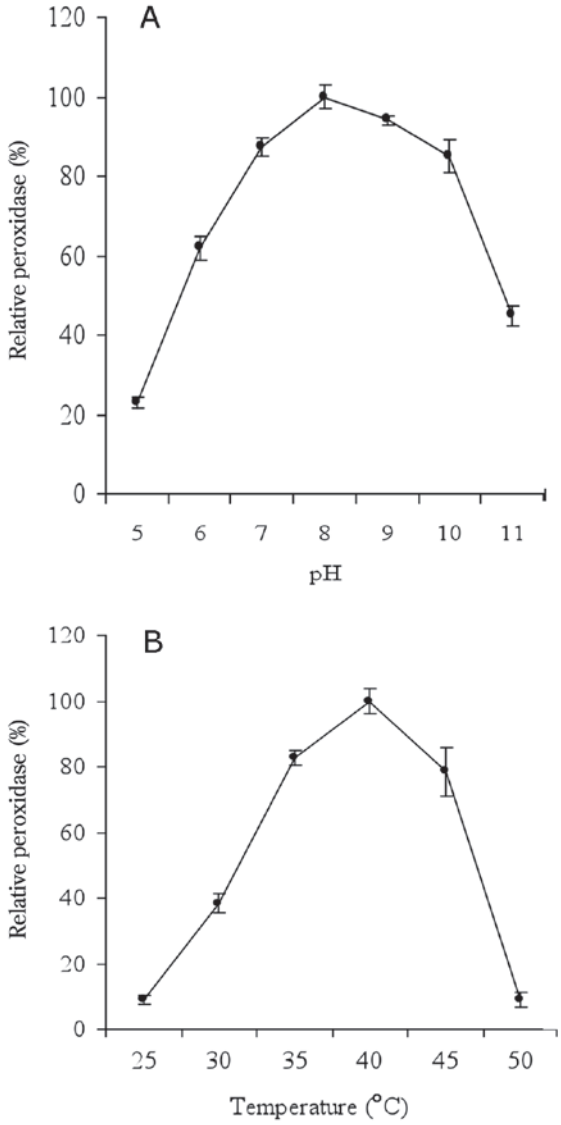

FIG. 3 - The effect of cultivation pH (A) and temperature (B) on the production of the extracellular peroxidase activity by *Streptomyces* sp. F6616. The data are presented as means ± SEM for triplicate measurements.

(0.08 mg protein/mL of culture). At pH 5.0 and 11.0, the productions of extracellular peroxidase (0.01 U/mL and 0.18 U/mL, respectively) were significantly decreased to 23 and 45%, respectively, of the maximal production.

The effect of temperature on the production of extracellular peroxidase by *Streptomyces* sp. F6616 was also investigated by growing the organism in a temperature range of 25 to 50 °C. From the analysis of the results, it is evident that the optimum temperature for the production of extracellular peroxidase (0.39 U/mL) occurred at 40 °C (Fig. 3B). However, at incubation temperature of 25 °C and 50 °C, the productions of extracellular peroxidase (0.04 U/mL) were significantly decreased to 9% of the maximal production.

Partial purification of peroxidase

The specific activity of peroxidase (6.22 U/mg of protein) in crude culture supernatant increased after gel filtration to 14.48 U/mg of protein. Pooled active fraction was applied to anion exchange matrix and eluted around 300 mmol/L NaCl with a specific activity of 56.96 U/mg of protein. The final purification factor for peroxidase was about 9-fold and overall yield was approximately 27%. The homogeneity of the enzyme was checked by native and SDS-PAGE; both yielded multiple protein bands (data not shown).

The effect of temperature and pH on peroxidase activity

The effects of temperature on the activity and stability of peroxidase were monitored by measuring the activity using

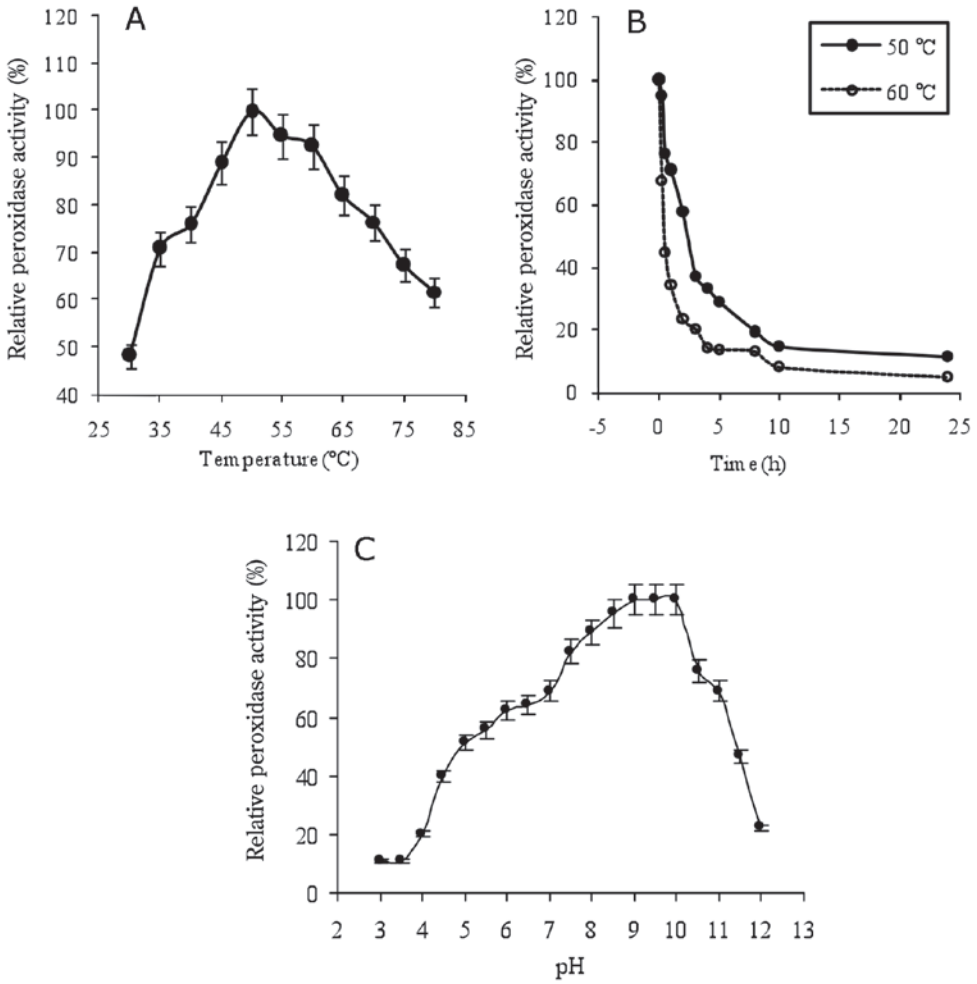

FIG. 4 - The effects of temperature (A), the stability over time at 50 °C (-•-) and 60 °C (-o-) (B), and the effect of pH (C) on the activity of the extracellular peroxidase produced by *Streptomyces* sp. F6616. The enzyme activity is expressed relative to the maximal value (0.535 U/mL) produced by *Streptomyces* sp. F6616. The reaction was monitored at each one of the specified temperatures and pH using the 2,4-DCP assay. The data are presented as means ± SEM for triplicate measurements.

the standard 2,4-DCP assay method. Under the conditions applied, maximal peroxidase activity (0.535 U/mL) was obtained when the incubation temperature was set at 50 °C (Fig. 4A). At the incubation temperature of 30 °C, which was the lowest temperature examined, the activity of the enzyme was 48.3% of the maximal value; at 80 °C, the highest temperature examined, activity was found to be 61.9% of the maximal activity (Fig. 4A). When the effect of temperature on the stability of peroxidase over time was examined, at 50 °C the peroxidase activity showed a half-life of 145 min which was reduced to 30 min at 60 °C (Fig. 4B). After 60 min of incubation at 50 °C, peroxidase activity was reduced by only 29%, while for the same period of time the respective activity reduction at 60 °C was 66% of the maximal value (Fig. 4B).

Thus, the optimal temperature for the peroxidase activity was estimated to be 50 °C when the assay was performed at pH 8.0 with a half-life of 145 min. Similarly in the case of *S. albus* ATCC 3005 optimum temperature at pH 7.2 was found to be 53 °C (Antonopoulos *et al*., 2001) while the peroxidase from *S. viridosporus* T7A the optimum temperature was found to be 50 °C when the assay was carried out at pH 5.5 (Lodha *et al*., 1991). In the case of *T. fusca* BD25 (Rob *et al*., 1996) and *S. avermitilis* UAH30 (Rob *et al*., 1997), the optimal incubation temperature for performing the 2,4-DCP assay was found to be 60 °C, justifying the thermophilic nature of the organisms. However, at higher temperatures, the stability of the *Streptomyces* sp. F6616 peroxidase was reduced, with the enzyme having a half-life of 30 min at 60 °C. This value is higher than the thermostability of peroxidase produced by *S. avermitilis* UAH30 (Rob *et al*., 1997) and *S. albus* ATCC 3005 (Antonopoulos *et al*., 2001), those shows half-lives of 20 min and 13 min at 60 °C, respectively.

The activity of peroxidase over the pH range 3.0 to 12.0 showed that maximal peroxidase activity (0.535 U/mL) was found to occur between pH 9.0 and 10.0 (Fig. 4C). However, at both pH 7.0 and 11.0, the activity detected was 68.9% of the maximal value, while at pH 3.0 the activity was 11.1% and decreased with decreasing pH (Fig. 4C).

The use of endo-xylanases in bleaching pulps has stimulated the search for enzymes with alkaline optima. The extracellular endo-xylanases, cellulases, and peroxidases produced by actinobacteria, generally exhibit a pH optima between 5.0 and 8.0 (McCarthy *et al*., 1985; Wilson, 1992; Trigo and Ball, 1994). Optimum pH for peroxidase activity produced by *Streptomyces* sp. F6616 was found to occur relatively higher pH range (9.0 to 10.0). This is significantly higher than the optimum pH range observed for *S. viridosporus* (5.5 to 7.5) (Zerbini *et al*., 1999),

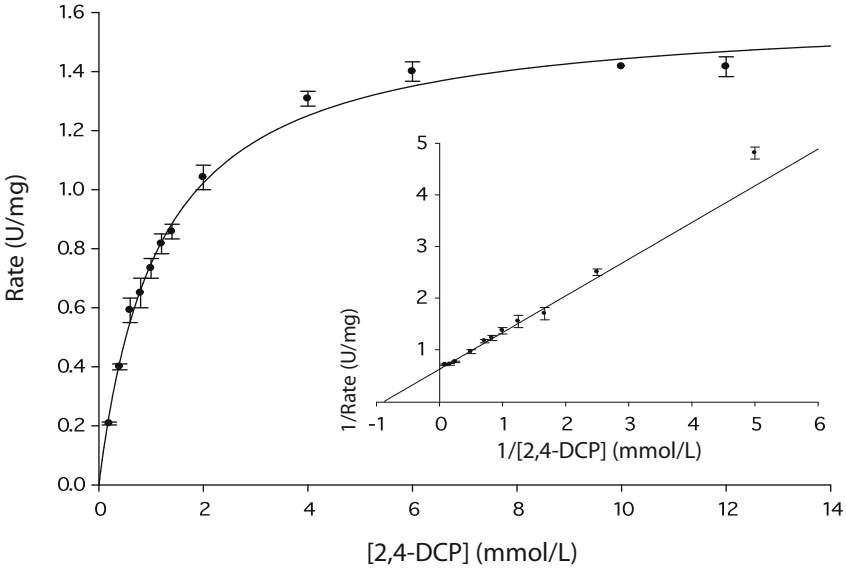

FIG. 5 - The effect of substrate (2.4-dichlorophenol) concentration on the activity of the peroxidase. The reaction was monitored at 30 °C and at pH 8.0 (100 mmol/L potassium phosphate buffer). Also shown is the Lineweaver-Burk plot. The data are presented as means ± SEM for triplicate measurements.

T. fusca BD25 (6.0 to 8.0) (Rob *et al*., 1996), *S. thermoviolaceus* (6.5 to 7.0) (Deobald and Crawford, 1987) and *S. avermitilis* UAH30 (6.0 to 8.5) (Rob *et al*., 1997), suggesting that this enzyme may be useful for the treatment of alkaline effluents.

Effect of substrate concentration and substrate specificity

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 8.0 and at a temperature of 30 °C on the activity of peroxidase was investigated. The enzyme gave a typical Michaelis-Menten type response, with K_m and V_{max} of 1.52 mmol/L and 1.84 U/mg of protein, respectively at 30 °C (Fig. 5).

Extracellular peroxidase activity produced by *Streptomyces sp.* F6616 showed activity against seven out of nine substrates examined, namely L-DOPA, 4-chlorophenol, 2,4-DCP, 2,6-DCP, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol and dye Azure B; no activity was detected against veratryl alcohol and guaicol (Table 1). Under the substrate concentrations applied, highest activity was obtained against L-DOPA, 2,4-DCP and 2,4,5-trichlorophenol, while lowest activity was obtained when dye Azure B was examined. However, the control, heme specific horseradish peroxidase showed activity against all of these substrates (Table 1).

Assessment of the activity of the enzyme against a range of chlorophenol substrates showed that the enzyme was able to oxidize successfully all chlorophenols, with 2,4-DCP and 2,4,5-trichlorophenol being the most reactive substrates (Table 1). The catalysis for all reacting chlorophenols proceeded with the formation of a pink/red colour due to coupling with the colour-forming reagent 4-aminoantipyrine. This observation is in accordance with the suggestion of Spiker *et al*. (1992) that the oxidation of phenols containing one or no carbon in the *para* position

TABLE 1 - Substrate specificity of extracellular *Streptomyces* sp. F6616 peroxidase

Substrate	Final substrate concentration (mmol/L)	Monitoring wavelength (nm)	SP*	HRP*
L-DOPA**	5.0	470	+	+
Veratryl alcohol	2.0	310	–	+
Azure dye B	0.032	651	+	+
Guaiacol	4.0	485	–	+
4-Chlorophenol	4.0	510	+	+
2,4-Dichlorophenol	5.0	510	+	+
2,6-Dichlorophenol	4.0	510	+	+
2,4,5-Trichlorophenol	4.0	510	+	+
2,4,6-Trichlorophenol	4.0	510	+	+

* SP: extracellular peroxidase produced by *Streptomyces sp*. F6616, HRP: horse radish peroxidase used as a control heme containing protein; positive peroxidase activity is denoted as +, no significant peroxidase activity detected is denoted as –. All measurements were carried out at 30 °C.

** L-DOPA: L-3,4-dihydroxyphenylalanine.

results in coupling with 4-aminoantipyrine to form a pink/red colour. The suggestion was also true for L-DOPA. Thus, *Streptomyces* sp. F6616 peroxidase can be used for bio-treatment of several waste waters such as bleach plant effluents or other waste water containing lignin-like polymers, as is the case of dye-industry effluents and olive-oil-mill waste-waters.

Inhibition studies with KCN and sodium azide

Potassium cyanide and sodium azide have been used for inhibition study of the extracellular heme containing peroxidase by Ramachandra *et al*. (1988). In contrast, Burd *et al*. (1995) 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 *Streptomyces* sp. F6616 peroxidase contain a heme prosthetic group, attempts were made to react the enzyme with the two heme-specific reagents, KCN and sodium azide by adding the appropriate concentrations (0-20 mmol/L and 0-100 mmol/L in final reaction mixture, respectively) of these inhibitors to the reaction mixture. Also the same experiment was repeated using horseradish peroxidase as a control and the classical heme protein inhibition response was observed (Fig. 6).

The presence of 1 mmol/L KCN in the reaction mixture of peroxidase preparation resulted in approximately 60% reduction in activity, while the peroxidase retained full activity in the presence of 1 mmol/L NaN_3. However, addition of 10 mmol/L NaN_3 to the reaction mixture resulted in approximately 36% reduction in activity. In contrast, presence of 10 mmol/L KCN resulted in 90% reduction in peroxidase activity.

Figure 6 shows that inhibitions were apparent with potassium cyanide and sodium azide for the *Streptomyces* sp. F6616 peroxidase. Also heme containing horseradish peroxidase inhibition was apparent and 90% and 63% of the activities were lost at 10 mmol/L KCN and 100 mmol/L NaN_3 concentrations, respectively. Initial results from inhibition studies with potassium cyanide and sodium azide suggested that the extracellular peroxidase produced by *Streptomyces* sp. F6616 may contain a heme group in its tertiary structure.

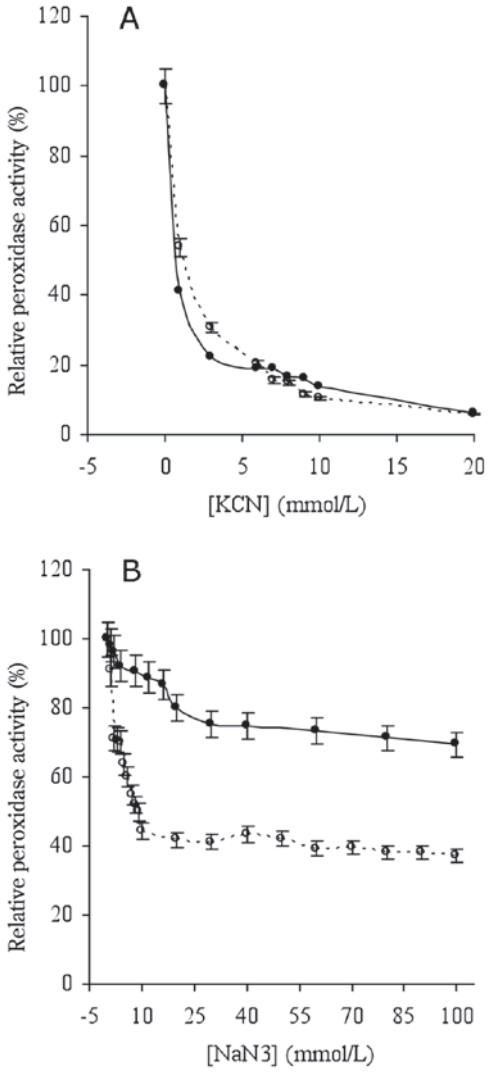

FIG. 6 - The effect of potassium cyanide (KCN) (A) and sodium azide (NaN_3) (B) on the activity of the extracellular peroxidase produced by *Streptomyces* sp. F6616 (SP) (-•-). The activity was measured using the standard 2,4-dichlorophenol assay method. All measurements were carried out at 30 °C. Horseradish peroxidase (HRP) (-o-) was used as a control heme-containing protein. The enzyme activity is expressed relative to the maximal value (SP = 0.75 U/mg, HRP = 5.03 U/mg). The data are presented as means ± SEM for triplicate measurements.

Release of reducing sugars and lignin equivalents from KP, BMWS and OSX

To assess the potential biotechnological application of lignocellulose-degrading enzymes including peroxidase from *Streptomyces* sp. F6616, the ability of the cell-free supernatant fluid to release reducing sugars and aromatic compounds from KP, BMWS and OSX was assessed. The results of colorimetric analysis of enzyme-treated KP, BMWS and OSX are presented in Fig. 7. At 30 °C, reducing sugars released from KP, BMWS and OSX were linear up to 10, 10 and 6 h, respectively, and about 29–36% of these activities remained up to 24 h of incubation (Fig. 7A). Reducing sugar concentrations from KP, BMWS and OSX at the end of the linear degradation periods (10, 10 and 6 h, respectively) were detected as 0.58, 0.56 and 3.56 mg/mL, corresponding to 5.8, 5.6 and 35.6% (wt/v) hydrolysis of the total substrate used, respectively. Overall, a higher percentage hydrolysis of OSX (55.9%) occurred after 24-h incubation. The hydrolysation of KP and BMWS were approximately 6-fold less than OSX hydrolysation under the same conditions (see Fig. 7A). The total percentage hydrolysis of available hemicellulose in KP and BMWS (43% and 26.3%, respectively) were also less then total percentage hydrolysis of OSX.

The release of aromatic compounds from KP and BMWS by *Streptomyces* sp. F6616 extracellular supernatant fluid showed a corresponding increase with the release of reducing sugars (Fig. 7B). Most of the aromatics from KP and BMWS were released within 4 and 3 h of incubation periods, respectively. The concentration of released aromatics from KP and BMWS were 15.3 and 22.3 μg/mL after 4 and 3 h (linear up to 4 and 3 h) of incubation at 30 °C, respectively. The release of aromatics from KP and BMWS reached 29 and 75.8 μg/mL after 24 h of incubation, respectively. The samples from KP and BMWS, which were

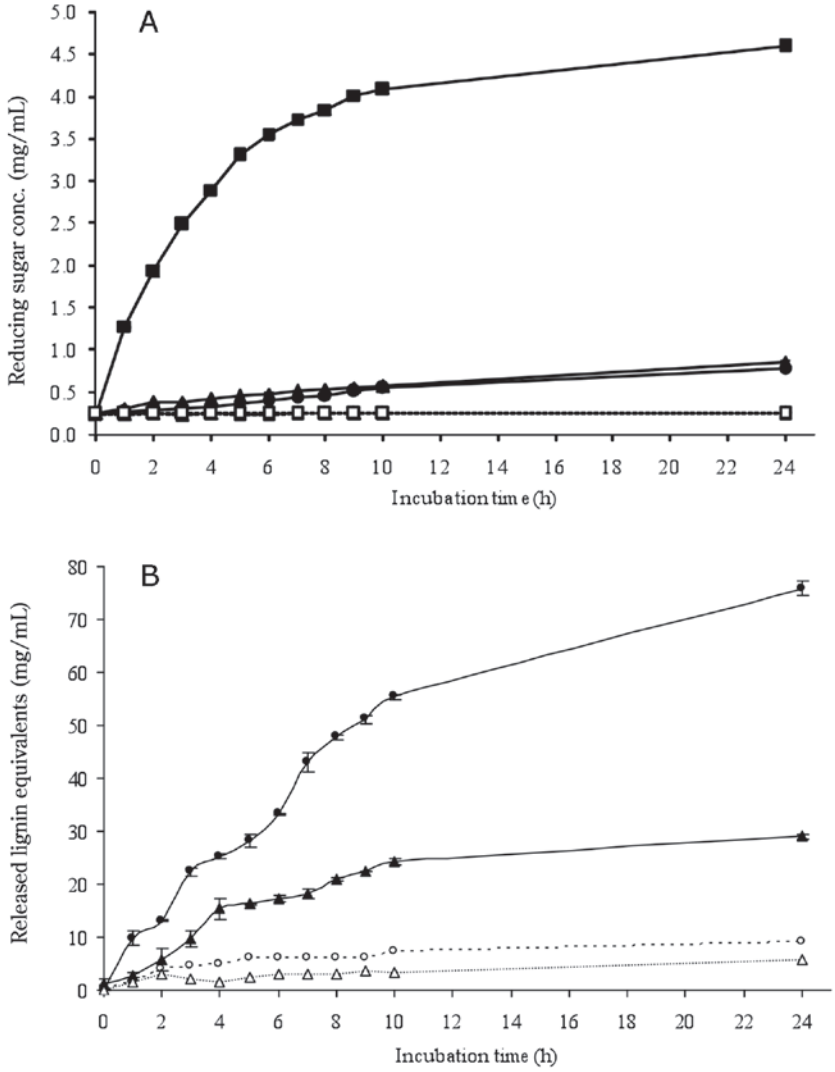

FIG. 7 - Release of reducing sugars (A) and aromatic equivalents (B) from Kraft pulp (KP, -▲-), ball-milled wheat straw (BMWS, -●-), and oat spelt xylan (OSX, -■-) with crude concentrated and dialysed supernatant enzymes of *Streptomyces* sp. F6616. Samples were incubated with shaking at 30 °C, 150 rev/min up to 24 h. Control treatments [KP (-Δ-), BMS (-o-) and OSX (-□-)] with enzyme preparations inactivated by boiling for 30 min were used as blanks. Data are presented as mean of three replicates of the same sample with SEs.

treated with inactivated enzyme preparations, released only trace amounts of aromatic compounds (see Fig. 7B).

The main use for enzymes participating in the hydrolysis of lignocellulose would be for the conversion of polymeric and oligomeric substances from lignocelluloses into monomeric sugars. Lignocellulose derived glucose and xylose by the enzymatic actions of *Streptomyces* sp. F6616, can also be used as substrates for microorganisms for the conversion into protein-rich cattle fodder. Thus, extracellular supernatant fluid enzymes of *Streptomyces* sp. F6616 could be useful for the conversion of lignocellulosic materials into monomeric sugars and consequently could be used for the production of microbial protein and/or ethanol or xylitol (which is used as a sweetener) using suitable microorganisms. Obtaining ethanol as alternative fuel using cellulose and lignocellulosic residues as a raw material offers a feedstock lover in price than starch. The transformation of lignocellulose into ethanol is completed in two steps: (a) hydrolysis of polymer, delignification to liberate cellulose and hemicellulose from their complex with lignin, and depolymerisation of carbohydrate polymer to produce free sugars, and (b) fermentation to ethanol using pentoses and hexoses liberated in the first step. In conventional ethanol production processes, lignins present in the raw materials and releasing fermentable sugars are eliminated by chemical and/or thermic pre-treatment followed by enzymatic/acidic hydrolysis. However, biological treatments have been proposed either to replace the physicochemical treatment for detoxification or specific removal of inhibitors prior to fermentation. Ligninolytic *Streptomyces* sp. F6616 can achieve delignification. The main advantages of biological delignification include mild reaction conditions, higher product yields, fewer side reactions and less energy demand.

The obvious interpretation of the lower hydrolysation rate and reducing sugar release patterns from KP and BMWS when compared to OSX is that the most readily accessible hemicelluloses are hydrolyzed initially at high rates. Once these materials are removed, lignin forms a barrier, denying the enzyme's access to the remaining

polysaccharides. Similar results have been reported for enzyme preparations from *T. curvata* MT 815 and *T. fusca* MT 816 (McCarthy *et al*., 1985) incubated with oat spelt xylan; *T. fusca* BD25 (Tuncer and Ball, 2002) and *Streptomyces* sp. F2621 (Tuncer *et al*., 2004) incubated with lignocellulose for 24 h.

Cellulase free endo-xylanase preparations are favoured by the paper industry for applications in bio-bleaching of Kraft pulps (Biely, 1985; Viikari *et al*., 1991). No production of cellulolytic activity during the growth phase exhibited by *Streptomyces* sp. F6616 makes it potentially suitable for use in the pulp and paper industry as the deleterious effect of cellulases on pulp viscosity is avoided.

In summary, this paper has identified *Streptomyces* sp. F6616 as a new and high peroxidase producing actinobacteria. Since any biotechnological process is likely to be based on crude extracts such as ethanol production, it is important to increase any particular enzymes activity in culture supernatant fluid through changes in the C:N ratio, carbon source and concentration. From a plot of the Lineweaver-Burk equation, the K_m and V_{max} for the crude peroxidase activity were determined to be 1.52 mmol/L and 1.82 U per mg protein, respectively using 2,4-DCP as substrate. Results showed that inhibition of peroxidase activity with the addition of heme inhibitors such as potassium cyanide (up to 20 mmol/L) and sodium azide (up to 100 mmol/L), suggesting the present of heme component in the tertiary structure.

Also results indicated the involvement of *Streptomyces* sp. F6616 peroxidase in the delignification and dechlorination of a range of chlorophenols, suggesting the use of the enzyme in the treatment of effluents from chemically bleached pulp. 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 feed-stocks, as well as the treatment procedures. Pulping processes release coloured compounds such as residual lignins, carbohydrate degradation products, and extractives (lipophilic compounds) into the effluent streams. Reducing the colour before the effluents are discharged into natural water is an important objective. The discharge of these coloured wastes is not only problem of aesthetics, but it has also been proven that chlorolignins are toxic and mutagenic (Ali and Sreekrishnan, 2001). Physical and chemical processes to remove the dark colour of effluents are expensive and do not solve the problem because the lignins persist, although in different form. An alternative treatment is the use of ligninolytic microorganisms such as *Streptomyces* sp. F6616.

In conclusion, our results suggest that *Streptomyces* sp. F6616 peroxidase can be used for bio-treatment of several waste waters such as bleach plant effluents or other waste water containing lignin-like polymers, as is the case of dye-industry effluents and olive-oil-mill waste-waters. Most importantly, *Streptomyces* sp. F6616 peroxidase is suitable for the treatment of alkaline pH effluents such as from Kraft pulping process and textile industry where known peroxidases from other species would not be applicable.

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