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Optimization of xylanase and peroxidase production from *Streptomyces sp.* K37

Mahmoud M. Nour El-Dein [1]
Abdou Eldyme A. Shereif [2]
Fathey A. Mansour [2]
Mohamed I. Abou-Dobara [1]
Andrew S. Ball [3]

Authors' addresses:
[1] Department of Botany,
Faculty of Sciences, Damietta,
Mansoura University, Egypt
[2] Department of Botany,
Faculty of Sciences, Mansoura,
Mansoura University, Egypt.
[3] Department of Biological Sciences,
Essex University, England.

Correspondence:
Mahmoud M. Nour El-Dein
Department of Botany, Faculty of Sciences, Damietta, Mansoura University, Egypt.
Tel.: +201094744952
e-mail: mnoureldein@yahoo.com

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ABSTRACT

The optimal conditions for the production of xylanase and peroxidase from *Streptomyces* sp. K37 were investigated. The production of xylanase and peroxidase increased during the growth phase of the cultures after 72 hours. This indicates that the productions of such enzymes are wholly growth associated in these organisms. The optimum pH for xylanase and peroxidase production from *Streptomyces* sp. K37 occurred at pH 8.0 and pH 7.0. The optimum temperature for xylanase and peroxidase production by *Streptomyces* sp. K37 occurred at 50°C and 40°C respectively. Oat spelt xylan was the best carbon source for the production of xylanase and peroxidase from *Streptomyces* sp. K37. The optimum concentration of ball milled straw for the production of xylanase from *Streptomyces* sp. K37 was 1.0%, while the concentration for the production of peroxidase was 0.20%. The optimal concentration of yeast extract and casamino acid for the production of xylanase and peroxidase from *Streptomyces* sp. K37 was found to be 0.2%.

Key words: Characterization, production, xylanase, peroxidase, *Streptomyces* sp. K37

Introduction

Xylans are one of the most abundant biopolymers, after cellulose, synthesised in the biosphere. In the cell wall of land plants, xylan is the most common hemicellulosic polysaccharide, representing more than 30% of the dry weight (Joseleau et al., 1992). Xylans are composed of 1,4-linked β-D-xylopyranosyl residues (Whistler & Richards, 1970). Xylans from land plants are heteropolysaccharides that constitute a large group of related hemicelluloses showing a great variability in their structures (Joseleau et al., 1992). This type of xylan contains different substituent groups in the backbone chain and in the side chain (Biely, 1985; Puls & Poutanen, 1989). The common substituent found on the backbone of xylan is acetyl, arabinosyl, and glucurunosyl residues (Whistler & Richards, 1970).

Lignin is considered to be the second most abundant natural polymer (after cellulose), making up 15 to 30% of the woody cells walls of gymnosperms (softwood) and angiosperms (hardwood) (Gold & Alic, 1993). It forms a matrix surrounding the cellulose.

Lignin is a phenylpropanoid polymer synthesised from the phenolic precursor's coniferyl, synapyl, and p-coumaryl alcohols (Freudenberg, 1968; Sarkanen & Ludwig, 1971). Free-radical condensation of these precursors, initiated by plant cell wall peroxidases, results in the formation of a heterogeneous, amorphous, optically inactive, random, and highly branched polymer with at least 12 different types of linkages such as aryl ether and carbon-carbon bonds connecting the aromatic nuclei (Gold & Alic, 1993). These linkages are not subject to enzymatic hydrolysis. The majority of bonds within a typical lignin structure consist of aryl glycerol β–aryl ether (β-O-4-) and diaryl propane (β–1) bonds (Crawford, 1981). This unique structure requiring depolymerisation by extracellular oxidative mechanisms account for the recalcitrance of lignin toward degradation by most microorganisms (Crawford, 1981; Eriksson et al., 1990; Gold et al., 1989; Kirk & Farrell, 1987).

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Yee et al. (1996) pointed out that since lignin also contains molecules of different chirality, lignin-degrading enzymes must be non-stereo selective (Crawford, 1981). Due to the irregular structure of the lignin, few organisms are able to degrade it. Sakakibara (1983) showed a schematic structural formula for lignin. Lignin is resistant to microbial degradation, although there are known lignin-degrading fungi, actinomycetes, and bacteria (Crawford 1981; Crawford & Crawford, 1984). This matrix retards the microbial depolymerisation of cellulose. The degradation of lignin represents a significant step in the global carbon cycle (Freudenberg, 1968; Sarkanen & Ludwig, 1971; Crawford, 1981). Also, lignin is an obstacle to the efficient utilisation of cellulose in a wide range of industrial processes.

Due to the variable composition and structure of xylans that can be obtained from different sources, the co-operative action of microbial enzymes that are excreted from the microorganism to affect the complete hydrolysis of the polysaccharide will be required. During the past five years some significant developments have given fresh impetus to the study of xylan-degrading enzymes (Coughlan & Hazlewood, 1993). These are the availability of recombinant DNA techniques for isolating single genes and expressing them in heterologous hosts devoid of hemicellulase activities, the introduction of biochemical techniques more suited to the analysis of complex mixtures of enzymes, and the commercial production of relevant synthetic and purified native substrates. A further important factor has been the general realisation that these enzymes have potentially valuable uses in the pulp and paper, textile and food industries and in agriculture.

For the complete hydrolysis of the xylan complex, number of enzymes is required. These enzymes work synergistically and co-operatively to convert xylan to its simple sugar. This complex enzyme system is common among bacteria (Woodward 1984; Wong et al., 1988; Coughlan & Hazlewood, 1993). The hydrolysis of xylan is carried out by a number of main chain and side chain cleaving enzymes. The main chain enzymes include endo-β-1,4-xylanases (β-1,4-D-xylanxylanohydrolase; EC 3.2.1.8), β-1,4-xylosidase (β-1,4-D-xyloside xylohydrolase; EC 3.2.1.37) and possibly exoxylanases (β-1,4-D-xylan xylohydrolase) while the side chain cleaving activities include α-L-arabinofuranosidases and α-D-glucuronodases as well as the esterases liberating acetyl, coumaroyl and feruloyl substituent's (Coughlan & Hazlewood, 1993).

β-1,4-endoxylanase (β-1,4-D-xylanxylanohydrolase; EC 3.2.1.8) cleaves the internal glycosidic linkages of the heteroxylan backbone, resulting in a decreased degree of polymerisation (DP) of the substrate. During the early course of hydrolysis of xylan, the main products formed are xylooligosaccharides. As hydrolysis proceeds, these oligosaccharides will be further hydrolysed to xylotriose, xylobiose, and xylose (Dekker & Richards 1976, Wong et al. 1988, Debeire-Gosselin et al. 1992 a, 1992b). β-1,4-linkages between unsubstituted xylose residues and residues carrying monomeric glucuronosyl side-chains which can be cleaved by a novel enzyme, glucuronoxylanase (Nishitani & Nevins, 1991).

β-D-xylosidase (β-1,4-D-xyloside xylohydrolase; EC 3.2.1.37) are exoglycosidases that hydrolyse short xylooligosaccharides and xylobiose from the non-reducing end to liberate xylose (Wong et al., 1988). B-xylosidase is an important enzyme when complete hydrolysis of xylan is required (Biely, 1993). True β-xylosidases are able to cleave artificial substrates like p-nitrophenyl β-D-xyloside (Coughlan & Hazelwood, 1993). An important role of β-xylosidase seems to be relieving the end product inhibition of endoxylanase (Sunna & Antranikian, 1997).

Ferulic and p-coumaric acid are linked to xylan by ester bonds (Bacon et al., 1975). Ferulic acid esterase (EC 3.1.1.-) cleaves the ester linkages between arabinose side chains and ferulic acids in xylan. Similarly, p-coumaric esterase (EC 3.1.1.-) cleaves the ester linkage between arabinose and p-coumaric acid (Müller-Harvey et al., 1986; Mackenzie et al., 1987).

Microbial xylan-degrading enzyme systems differ markedly with respect to whether they exist as free entities or as components of complexes (Hazlewood & Gilbert, 1993). Some microbial hemicellulases are secreted freely and do not associate with either the cell envelope or with other secreted proteins (Hazlewood et al., 1992).

Sharma and Kumar (2013) pointed out that microbial endo-1,4-β- xylanases are the preferred catalysts for xylan hydrolysis due to their high specificity, mild reaction conditions, negligible substrate loss, and side product generations.

Sunna and Antranikian (1997) identified the presence of a structure analogous to the cellosome, the xylanosome; as was reported in *Butyrivibrio fibrisolvens* H17c (Lin & Thomson, 1991). The extracellular complex B (Cβ) from *B. fibrisolvens* exists as a multisubunit protein aggregate. The complex has a

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molecular weight higher than 669 kDa, and is composed of 11 protein bands with xylanase activity and 3 bands showing endoglucanase activity. The presence of xylanosomes in the anaerobic bacterium *Thermoanaerobacterium saccharolyticum* B6A-RI was reported by Zeikus et al., (1991). In this bacterium, xylanase exists as a high molecular-weight complex, and enzyme activity is mainly cell-associated when the organism is grown on insoluble xylan. In the anaerobic thermophile *Clostridium thermocellum*, at least one well-characterised xylanase is a component of the highly-ordered multiprotein aggregate, termed the cellosome, which in early culture mediates the attachment of cells to cellulosic substrates (Grépinet et al., 1988; Morag et al., 1990).

The thermophilic actinomycete *Thermomonospora fusca* possess a complex multicomponent xylan-degrading system that consists of endoxylanase, β-xylosidase, α-arabinofuranosidase, and acetyl esterase activities (Bachmann & McCarthy, 1991). The cooperative action between these enzymes has been studied.

Wong et al. (1988) noted that β-xylanases can be classified into two families according to their molecular weight in combination with their isoelectric point (pI). They have stated that one of the two families consists of high molecular weight enzymes with low pI, while the other consists of low molecular weight enzymes with high pI. This classification of β-xylanases according to Wong et al. (1988) was found to correspond with the classification of glycanases on the basis of hydrophobic cluster analysis and similarities in primary structures (Gilkes et al., 1991; Shareck et al., 1991). The high molecular weight β-xylanases belong to the F-family of glycanases while the low molecular weight β-xylanases belong to the G-family. Shareck et al. (1991) purified and sequenced three β-xylanases from *Streptomyces lividans* when growing this organism on xylan.

Furthermore, there is another classification of xylanases depending on the mode of action of the enzyme, i.e. the nature of the end product of the enzyme. Also β-xylanases can be classified into two groups according to the α–L-arabinofuranosidase activity of β-xylanases. These two groups include arabinose-liberating β-xylanases and arabinose non-liberating β-xylanases (Dekker & Richards 1976; Reilly, 1981). The existence of arabinose liberating xylanases supports the suggestion that there could be genes encoding enzymes with two catalytic domains. One of these is responsible for β-xylanase and the other for α–L-arabinofuranosidase activity (Matte & Forsberg, 1992).

The production of xylanases is common in nature. It has been reported to be found in some soil inhabiting organisms including bacteria, actinomycetes, fungi, rumen and marine bacteria, protozoa, insects and seeds of some terrestrial plants. Xylanases have been reported to occur in some thermophilic bacteria, actinomycetes and fungi, *Streptomyces thermoviolaceus* OPC-520 (Mackenzie et al., 1987); *Nocardia* spp., *Saccharmonospora viridis* (Ball & McCarthy 1988, 1989); *Streptomyces lividans* 66 (Yasui et al., 1988; Hernandez-Coronado et al., 1997); *Thermomonospora fusca* BD25 (Bachmann & McCarthy, 1989; Irwin et al., 1994); *Streptomyces chartreusis* GS 901 (Tsujibo et al., 1992); *Streptomyces chattanoogensis* CECT 3336 (Kluepfel et al., 1990); *Streptomyces* strains (Grabski & Jeffries, 1991; Grabski et al., 1993); *Thermomonospora fusca* (Berens et al., 1996); *Streptomyces sp*. strain B-12-2 (Senior et al., 1992, Biely et al. 1993); *Streptomyces sp*. strain S38 (Elegir et al. 1994); *Streptomyces lividans* (Abd EL-Nasser & Foda 1995); *Streptomyces spp*. (Lopez-Fernandez et al., 1998); *Streptomyces cyaneus* SN32 (Ninawe et al., 2008); *Thermomonospora curvata* (Tuncer, 1999); *Microtetraspora flexuosa* SIIX (Matsuo et al., 2000), *Streptomyces roseiscleroticus* (Georis et al., 2000).

Peroxidases catalyse the oxidation of a wide range of substrates including organic and inorganic compounds. They are widely found in plants, animals and microorganisms. Peroxidases have been detected intracellulary in bacteria (van Pée &Lingens, 1984, 1985), plants (Chibbar & Van Huystee 1984; Welinder 1985), oviparous animals (Wever et al., 1980) and mammals (Moldoveanu et al., 1982). Therefore, it can be concluded that peroxidases play an important role in biological systems. Extracellular peroxidases have also been described (Rob et al., 1996, 1997). van Pée et al. (1987) studied a non-heme bromoperoxidase from *Streptomyces aureofaciens*. Extracellular peroxidases have also been described in other actinomycetes (Ramachandra et al., 1988; Winter et al., 1991; Godden et al., 1992). They are secreted in multiple forms by a wide range of actinomycetes (Ball et al., 1990; Trigo & Ball, 1994). It is expected that extracellular peroxidases have improved stability over their intracellular counterparts, particularly those from thermophiles (Iqbal et al., 1994), and thus have potential for application in diagnostic kits (Mercer et al., 1996).

Intracellular peroxidases of *Streptomycetes* have also been specifically studied in relation to their role in the biosynthesis of halogenated antibiotics (van Pée et al., 1987).

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An important difference between lignin peroxidase (LiP) and manganese peroxidase (MnP) is in the nature of the reducing substrate (Kirk & Farrell 1987; Gold et al., 1989; Schoemaker, 1990; Gold & Alic, 1993). LiP catalyses the oxidation of non-phenolic lignin model compounds such as veratryl alcohol to veratryl aldehyde. Therefore the unique feature of this enzyme is that it is able to oxidise aromatic compounds with redox potential beyond that of horseradish peroxidase (HRP), while the oxidation of lignin and other phenols by MnP is dependent on free manganese ion (Glenn & Gold, 1985; Paszczyński et al., 1986; Gold et al., 1989).

Streptomyces viridosporus T7A produces a variety of enzymes that play a role in lignin degradation (Yee et al., 1996). These extracellular enzymes include various peroxidases, cellulases, esterases, and endoglucanases (Crawford et al., 1983, Borgmeyer & Crawford, 1985; Ramachandra et al., 1987; Adhi et al., 1989). One of the isoforms of peroxidase (AliP-P3) produced by *Streptomyces viridosporus* T7A catalyses the degradation of lignin model compounds by oxidative cleavage of Cα–Cβ and Cα-carbonyl bonds and is the only isoform detectable with an enzyme assay using 2,4-dichlorophenol as the substrate (Ramachandra et al., 1988). Several peroxidases have been isolated, sequenced and characterised (Banci, 1997).

One of the most important applications of the peroxidase is the degradation of the harmful compounds (like chlorophenol) that can cause many problems to plant, animals, and humans. In addition to that, peroxidases play an important part in the degradation of lignin and lignocellulosic materials. Tuncer (1999) suggested that the ligninolytic activity could be used in a biotechnological application including treatment of bleach plant effluent (Michel Jr et al., 1991) and the bioremediation of aromatic pollutants such as olive mill waste, a crucial problem in some Mediterranean countries (Sayadi & Ellouz, 1995).

The lignin peroxidase (LiP) of *Streptomyces viridosporus* T7A is known to degrade lignin and various phenolic compounds (Yee et al., 1996). Therefore, *Streptomyces* may be used to degrade or mineralise recalcitrant waste compounds such as azo dyes (Paszczyński et al. 1992) and pesticides (Gauger et al., 1986, Paszczyński et al., 1992).

Xylanases can be used in different biotechnological applications. One of the most recent important applications is the use of xylanases in prebleaching of Kraft pulps for the paper industry. The bleaching of pulps depends on the removal of lignin from pulps and this essentially requires the use of toxic chemicals including chlorine and hypochlorite. The use of xylanases in pulp bleaching and the formation of toxic organic chlorines have attracted public attention in the last few years (Viikari et al., 1991, 1994; Coughlan & Hazlewood, 1993). The principle for the use of xylanases is that xylan hydrolysis facilitates the release of lignin and this minimises the need for chlorine and other toxic chemicals. The use of xylanases in pulp bleaching results in reduction the kappa number and increase of the brightness of the pulp. There are important aims for the process (Viikari et al., 1991, 1994; Yang et al., 1992; Daneault et al., 1994).

Most of these applications will not require the presence of the purified enzymes. This is an important economic factor as the costs associated with purification of enzymes are high. However, in the case of pulp pre-bleaching a cellulases free xylanase is required. The presence of cellulases with xylanase in the process would destroy the cellulose fibres and that led to destroying of the pulp. Therefore, cellulases-free preparations are required. This may involve enzyme purification or the use of cellulases free strains such as *Streptomyces sp*. CH-M-1035 (Flores et al., 1997).

The aim of this work is to optimize the conditions for xylanase and peroxidase production from *Streptomyces sp*. K37.

Materials and Methods

Growth of isolates in liquid cultures

The organisms were grown in conical flasks containing Sorensen medium (Sorensen, 1957) supplied with oat spelt xylan (1.0% (w/v) and yeast extract (0.0-1.2% (w/v)). Oat spelt xylan in some cases was replaced by different carbon sources or wheat straw. The pH of the medium was adjusted to 7.5 using NaOH and HCl (1.0 M) prior to sterilization. Flasks were inoculated and incubated at 50°C with shaking at 150 rev min^{-1} for 48-72 hours.

Preparation of ball milled straw

Wheat straw was cut into small pieces and milled in a rotating ball-milled apparatus (The Pascal Engineering Co. Ltd., Sussex, England). A fine powder of straw was obtained.

Preparation of inoculum

A suspension of spores and hyphal fragments from pure colonies of the organisms from Petri dishes were used to inoculate flasks containing Sorensen medium (Sorensen, 1957). The pre-culture was incubated at 50°C at 150 rev min^{-1} for 24-48 hours. Production flasks were then inoculated with an aliquot (1 ml) of this culture.

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Preparation of the crude enzymes

After the incubation period, cultures were harvested by centrifugation at 12,000g for 10 minutes at 4°C using a Sorval® RC 5B Plus (Du Pont Company, Sorval® Instruments, Delaware, USA). Culture supernatants were used in assaying for extracellular enzyme activities (xylanase, cellulase and peroxidase). The remaining supernatants were used for the determination of intracellular protein. This was stored at –20°C for several weeks

Determination of intracellular protein

Harvested pellets were washed three times with distilled water. Washed pellets were centrifuged, washed and dissolved in 20 ml of NaOH (1M), and boiled for 20 minutes. Boiled pellets were allowed to cool and then centrifuged. Dilution of the clarified solution was used to determine the intracellular protein concentration of the organisms using the Bio-Rad protein assay (Bradford, 1976). In this assay, 800 µL of protein solution was mixed with 200 µL of Bio-Rad protein reagent and incubated at room temperature. After 5 minutes, the optical density at 595 nm was recorded using a micro plate reader, (Dias micro-plate reader Dynatech Laboratories Inc. USA and BioLinx 2.10™ software). Protein concentrations were determined from a standard curve with bovine serum albumin (BSA) ranging from 0 to 25 µg ml^{-1} .

Xylanase and cellulase activities determined using the Method of Miller (1959)

Xylanase and cellulase activities were assayed by the estimation of reducing sugar released from oat spelt xylan and carboxymethylcellulose respectively. The reaction mixture contained the following: 200 µL of 1.0% oat spelt xylan or carboxymethylcellulose dissolved in 0.1 M potassium phosphate buffer (pH 7.0) and an aliquot of diluted culture supernatant or purified enzymes preparation. The mixture was incubated at 50°C for 20 minutes. The determination of reducing sugar released during the incubation mixture was detected by the dinitrosalicylic acid method of Miller (1959). Determination of the concentration of the reducing sugar was performed spectrophotometrically at 550 nm using the dinitrosalicylic acid (DNS) for the determination of reducing sugar. 200 µL of dinitrosalicylic acid reagent was added to 200 µL of the clarified reaction mixture and standards. After mixing, the mixtures were boiled for 5 minutes. After cooling under tap water, the optical density of the coloured product at 550 nm was taken using a Microplate reader. D-xylose and D-glucose standard solutions were used to construct standard curves. Calibration curves constructed using D-xylose and D-glucose standards in the range of 0-5 µM ml^{-1} were used. One unit of enzyme activity was defined as the amount of enzyme that released 1 µM of reducing sugar (expressed as xylose equivalents) per millilitre per minute under assay conditions. Enzyme and substrate controls were included routinely.

Peroxidase assay

This method was adapted from the assay developed by Ramachandra et al., (1987, 1988). Peroxidase was assayed using 2,4-dichlorophenol (2,4-DCP) as substrate. The final reaction (1 ml) mixture contained potassium phosphate buffer (200 µL, 0.1 M, pH 7.0); 16 mM 4-aminoantipyrine (200 µL); 25 mM 2,4- dichlorophenol (200 µL); culture supernatant (200 µL) and 50 mM hydrogen peroxide (200 µL). The reaction was initiated by the addition of hydrogen peroxide which was added last. Samples were then incubated at 50°C for 1.0 minute. The increase in absorbance as a result of the oxidation of 4-aminoantipyrine was measured at 510 nm. This assay procedure was scaled down to a total volume of 200 µL in a microplate reader. One unit of enzyme activity was defined as the amount of enzyme required for an increase an absorbance of 1.0 unit ml^{-1}.

Environmental factors affecting xylanase and peroxidase production by Streptomyces sp. K37

Effect of incubation periods

Streptomyces sp. K37 was grown in liquid oat spelt xylan medium (Sorensen, 1957) supplemented with 2 g L^{-1} yeast-extract as a basal medium. The medium was supplied with 10.0 g L^{-1} oat spelt xylan. This medium was adjusted to pH 7.5 using 1 N NaOH and 1 N HCl, distributed into 100 ml conical flask containing 50 ml. Following inoculation with 1.0 ml of spore suspension, flasks were incubated at 50°C at 150 rev min^{-1}. After 24, 48, 72, 96 and 120 hours, cell free extracts were obtained by centrifuging the bacterial filtrates at 12,000 g for 10 minutes at 4 °C. The cell free extracts were used for estimation the xylanase and peroxidase activities.

Effect of different initial pH

For studying the effect of initial pH on xylanase and peroxidase production, *Streptomyces* sp. K37 was grown at 150 rev. min^{-1} in liquid Sorensen medium supplemented with 2 g L^{-1} yeast extract and 10.0 g L^{-1} oat spelt xylan.

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The pH was adjusted at 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 using 1N NaOH or 1N HCl. After 72 hours incubation, the final pH was recorded, xylanase and peroxidase activities were determined as before.

Effect of different temperatures

Streptomyces sp. K37 was grown in liquid Sorensen medium supplied with 2 g L^{-1} yeast extract and 10.0 g L^{-1} Oat spelt xylan. Incubation was carried out at 30, 40, 50 and 6°C respectively and at 150 rev min^{-1} for 72 hours. The supernatant was used for determination of xylanase and peroxidase activities.

Effect of different carbon sources

In this experiment, two types of carbon sources were used:

Type 1 - Oat spelt xylan, starch, phenylalanine, glycerol, cellulose and carboxymethylcellulose were added separately (1.0 %) (w/v) to the liquid Sorensen medium;

Type 2- Cellobiose and xylose (1.0%) (w/v) were added following sterilization by filtration through millipore filter (0.45 µm).

The pH was adjusted to 7.5 using 1N NaOH or 1N HCl. Flasks were inoculated and incubated at 50°C for 72 hours at 150 rev min^{-1}. After incubation of *Streptomyces* sp. K37, the supernatant was used for estimation of xylanase and peroxidase activities.

Effect of different concentrations of ball-milled straw

Streptomyces sp. K37 was cultivated using Sorensen medium fortified by different concentrations of rice straw (0.00, 0.25, 0.50, 0.75, 1.00 and 1.25% (w/v). The pH was adjusted to 7.5 by 1N NaOH and 1N HCl. Flasks were inoculated, incubated at 50°C and at 150 rev min^{-1}. After 72 hours, cell free extracts were obtained by harvesting the cultures by centrifugation at 12,000 g for 10 minutes at 4°C. Cultures filtrates were used for estimation of xylanase and peroxidase activities.

Effect of different concentrations of yeast extract and casamino acid

Streptomyces sp. K37 was grown in Sorensen medium supplemented with different concentrations (0.0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 g L^{-1}) of yeast extract and casamino acid. Cultures were incubated at 50°C at 150 rev min^{-1} for 72 hours and pH 7.5.

Results

Growth and production of xylanase and peroxidase of Streptomyces sp. K37.

Growth and production of xylanase and peroxidase of *Streptomyces* sp. K37 in Sorensen medium containing oat spelt xylan (1.0%) is shown in Figure 1. The production of xylanase and peroxidase increased significantly during the growth of the organism, with the maximum production of both enzymes detected after 72 hours (4.337 U/mg intracellular protein ml^{-1} and 0.537 U/mg intracellular protein ml^{-1} for xylanase and peroxidase respectively). Intracellular protein, used as a measure of cell biomass (1.459 U/mg intracellular protein ml^{-1}) increased gradually and reached its maximum level after 72 hours of incubation. After 72 hours, the amount of the intracellular protein decreased until it reached a minimum level after 120 hours (0.163 U/mg intracellular protein ml^{-1}).

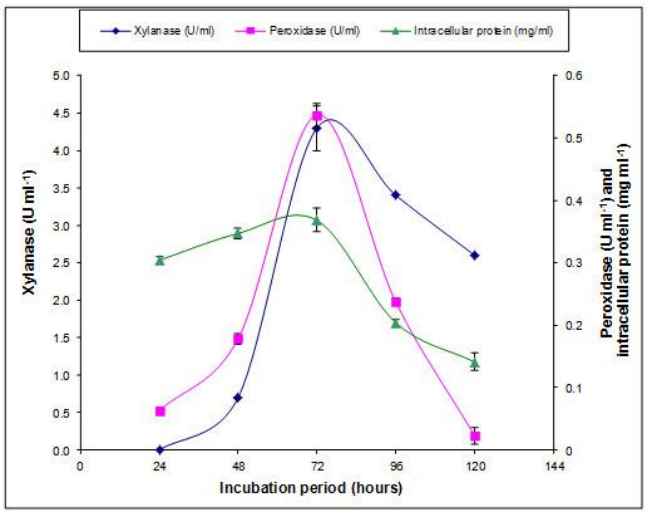

Figure 1. *Growth (▲), production of xylanase (◆) and peroxidase (■) by Streptomyces sp. K37 grown in Sorensen medium containing 1.0% oat spelt xylan. Data are represented as means of three replicates with standard errors.*

Effect of pH on the production of xylanase and peroxidase

Maximum xylanase production occurred in the pH range of 6.0 and 8.0. Below pH 6.0, the activity of xylanase production increased with increasing pH until it reached the maximum production at pH 8.0 (8.813 U/mg intracellular protein ml^{-1}). Above pH 8.0, xylanase yield decrease and

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retained only 4.15% of the activity at pH 9.0. Below pH 7.0, peroxidase production increased with increasing of the pH of the medium and maximum peroxidase production occurred at pH 7.0 (0.927 U/mg intracellular protein ml^{-1}). Above this pH, the yield of the enzyme decreased to 40% of its maximal activity at pH 8.0 and was completely inhibited at pH 10.0 (Figure 2).

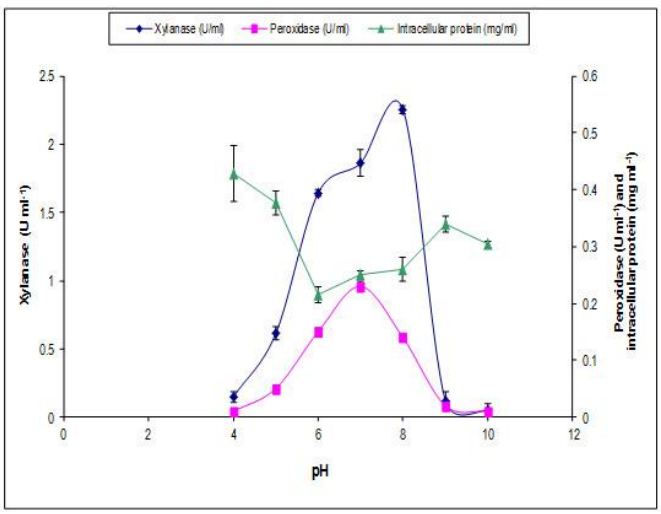

Figure 2. *Effect of pH on growth (▲) and production of xylanase (◆) and peroxidase (■) by Streptomyces sp. K37 grown in Sorensen medium containing 1.0% (w/v) oat spelt xylan. Data are represented as means of three replicates with standard errors.*

Effect of temperature on the production of xylanase and peroxidase

The optimum temperatures for the production of xylanase and peroxidase activities were 50°C and 40°C respectively. Peroxidase activity was reduced by 67.87% at 50°C. At 60°C, no xylanase and peroxidase could be detected (Figure 3).

Effect of carbon sources on the production of xylanase and peroxidase

The best carbon source for the enzyme activity was found to be oat spelt xylan (10.265 U/mg intracellular protein ml^{-1} and 1.52 U/mg intracellular protein ml^{-1} for xylanase and peroxidase, respectively). With regard to xylanase, phenylalanine, xylose, starch and carboxymethylcellulose were found to be good sources for the production of xylanase, with activity varying from 8.974 U/mg intracellular protein ml^{-1} to 5.668 U/mg intracellular protein ml^{-1}, while the growth on cellulose, glycerol and cellobiose resulted lower activity ranging from 3.347 U/mg intracellular protein ml^{-1} to 2.804 U/mg intracellular protein ml^{-1}. Conversely, carboxymethylcellulose, glycerol and starch were found to be good substrates for the production of peroxidase with activity varying from 1.184 U/mg intracellular protein ml^{-1} to 1.063 U/mg intracellular protein ml^{-1}, while the organism produced low peroxidase activity when it grew on phenylalanine, xylose, cellobiose and cellulose, with activity varying from 0.423 U/mg intracellular protein ml^{-1} to 0.027 U/mg intracellular protein ml^{-1} (Figure 4a and 4b).

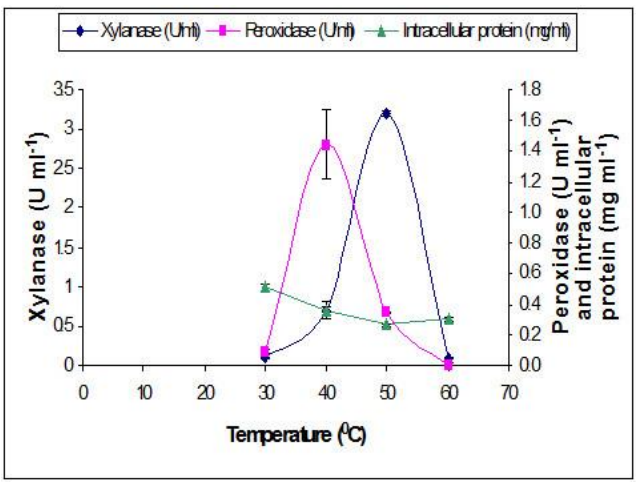

Figure 3. *Effect of temperature on the production of xylanase (◆), intracellular protein (▲) and peroxidase (■) by Streptomyces sp. K37 grown in Sorensen medium containing 1.0% (w/v) oat spelt xylan. Data are represented as means of three replicates with standard errors.*

Effect of ball milled straw on the production of xylanase and peroxidase

The effect of different concentrations of ball milled straw on the production of xylanase and peroxidase was investigated (Figure 5). Straw was found to be a good source for xylanase production and activity increased gradually with the optimum concentration for production occurring at a ball milled straw concentration of 1.0% (w/v) (6.598 U/mg intracellular protein ml^{-1}). Above this concentration, xylanase activity declined until it reached a minimum value at a ball milled straw concentration of 1.5% (w/v). However, the maximum peroxidase activity (0.109 U/mg intracellular protein ml^{-1}) occurred at a concentration of 0.25%, after this, the production decreased and reaches the minimum activity at 1.5% (0.019 U/mg intracellular protein ml^{-1}).

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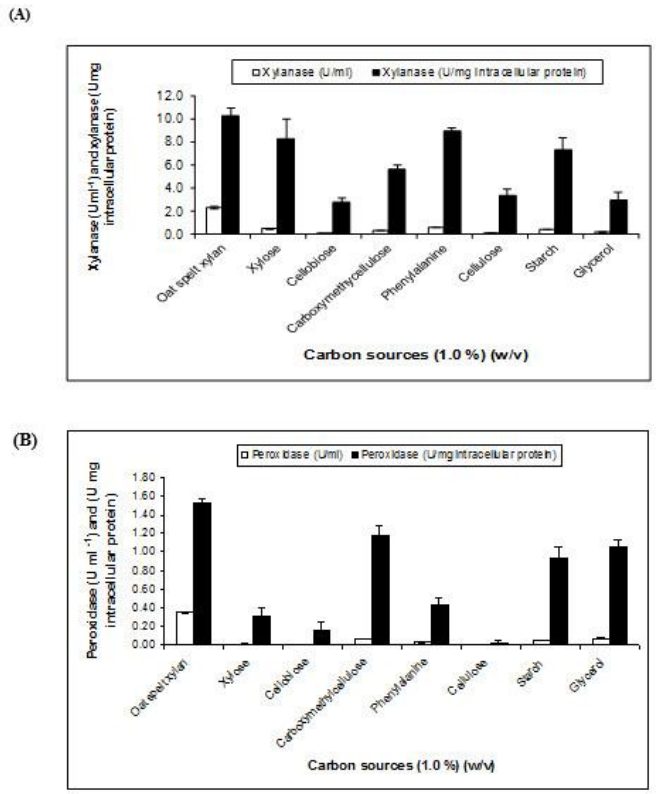

Figure 4. *Effect of carbon sources on the production of xylanase (A) and peroxidase (B) by Streptomyces sp. K37 grown in Sorensen medium. Data are represented as means of three replicates with standard errors.*

Effect of yeast extract and casamino acid on the production of xylanase and peroxidase

The effect of different concentrations of yeast extract and casamino acid on the production of xylanase and peroxidase by *Streptomyces* sp. K37 was also investigated (Figure 5). The highest xylanase production (10.265 U/mg intracellular protein ml^{-1} and 11.798 U/mg intracellular protein ml^{-1}) and peroxidase production (0.892 U/mg intracellular protein ml^{-1} and 2.315 U/mg intracellular protein ml^{-1}) were recorded in media supplemented with 0.2% yeast extract and casamino acid respectively. Above this concentration, xylanase and peroxidase production decreased significantly and reached the minimum level at a concentration of 1.2% (3.111 U/mg intracellular protein ml^{-1} and 2.142 U/mg intracellular protein ml^{-1} for xylanase and 0.230 U/mg intracellular protein ml^{-1} and 0.218 U/mg intracellular protein ml^{-1} for peroxidase on yeast extract and casamino acids, respectively).

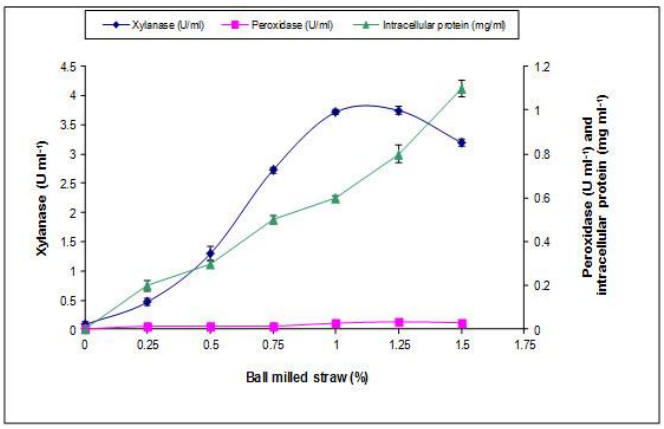

Figure 5. *Effect of ball milled straw on the production of xylanase (◆), peroxidase (■) and intracellular protein (▲) by Streptomyces sp. K37 grown in Sorensen medium supplied with 0.2% yeast extract. Data are represented as means of three replicates with standard errors.*

Discussion

Effect of incubation period on xylanase and peroxidase production

Streptomyces sp. K 37 produced high levels of xylanase and peroxidase when grown in Sorensen medium containing xylan as the main carbon source. Xylanase production from *Streptomyces* sp. K37 (1.06 U/ml^{-1}) and peroxidase production from *Streptomyces* sp. K37 were higher than that reported for *Streptomyces cyaneus* (0.15 U/ml^{-1}) (Wang et al., 1993), *Streptomyces badius* 252 (1.0 U/ml^{-1}), *Amycolata autotrophica* DSM 43099 (0.4 U/ml^{-1}) (Ball & McCarthy, 1989) and *Thermomonospora fusca* BD25 (0.52 U/ml^{-1}) (Tuncer, 1999).

Xylanase and peroxidase production by *Streptomyces* sp. K37, increased during the growth of the cultures in Sorensen medium with the maximum production detected after 72 hours incubation. After this period, the activities of the enzymes decreased. The observed peaking and troughing of the production of extracellular enzymes can be attributed to: (1) The differences in the timing of induction of separate components of the xylanase system; (2) The products of action of one component inducing the synthesis of another; (3) Differential inhibition by products of substrate hydrolysis; (4) Differential inactivation by proteases, or variation in the pH during cultivation conditions (Tuohy & Coughlan, 1992; Wang et al., 1993).

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A decrease in cellular protein was observed after 72 hours of growth of *Streptomyces* sp. K37. This probably resulted from cellular lysis, an observation previously reported by Godden et al. (1989).

These results indicate that the production of extracellular enzymes by *Streptomyces* sp. K37 was growth associated and this is in agreement with other actinomycetes (Ball & McCarthy, 1989; Trigo & Ball, 1994; Tuncer, 1999).

The production of peroxidase during growth of *Streptomyces* sp. K37 was similar to *Streptomyces avermitilis* UAH30. Rob et al. (1997) reported that the maximum specific peroxidase activity was obtained after 72 hours. In contrast, peroxidase production from *Streptomyces* sp. K37 was different from that reported by Iqbal et al., (1994) who showed that the maximum production of peroxidase from *Streptomyces thermoviolaceus* was obtained after 50 hours. Lodha et al., (1991) found that lignin peroxidase from *Streptomyces viridosporous* T7A was a non-growth associated, secondary metabolite with maximum lignin peroxidase activity occurring after 36 hours.

Production of extracellular peroxidase is common among actinomycetes and the enzyme plays an important role in the oxidation of a number of aromatic compounds and in the degradation of lignin. Peroxidase of *Streptomyces viridosporus* catalysed C-C bond cleavage of phenolic and nonphenolic arylglycerol-β-aryl ether lignin model compounds (Ramachandra et al., 1988). The maximal production of peroxidase by *Streptomyces* sp. K37 (0.35 U ml^{-1}) was similar to that reported for *Streptomyces viridosporus* T7A (0.24 U/ml^{-1}) (Ramachandra et al., 1987), but higher than that reported for *Streptomyces thermoviolaceus* (0.04 U/mg) (Iqbal et al. 1994) and *Streptomyces avermitilis* (0.12 U/mg) (Rob et al., 1997).

Effect of pH on xylanase and peroxidase production

Xylanase production was maximal at pH 8.0 for *Streptomyces* sp. K37 and the optimal production of peroxidase by *Streptomyces* sp. K37 was found to be at pH 7.0. These results are in accordance with the work that has been reported for *Streptomyces griseoflavus* and *Streptomyces violarus* (Abd EL-Nasser & Foda, 1995), for *Thermomonospora fusca* (Tuncer, 1999), for *Streptomyces badius* (Adhi et al., 1989) and for peroxidase from *Streptomyces avermitilis* UAH30 (pH 7.5) (Rob et al., 1997) and from *Streptomyces viridosporus* T7A (Lodha et al., 1991) (pH 6.0).

When xylanase activity was expressed as U/mg intracellular protein, the comparative ability of the organism to produce xylanase was not changed, indicating that the results did not merely reflect differences in growth yield. This is an important point often overlooked when xylanase activities are reported (Ball & McCarthy, 1989). The authors also showed that xylanase production of *Streptomyces* strain EC1 and *Amycolata autotrophica* was growth associated in these organisms.

Effect of temperature

The optimal temperature for xylanase production occurred at 50°C for *Streptomyces* sp. K37 and the optimal production of peroxidase by *Streptomyces* sp. K37 was found to occur at 40°C. This is in accordance with the results obtained by Tuncer, (1999) and McCarthy (1987). The results of peroxidase production is in agreement with that observed by Rob et al. (1997) and Lodha et al, (1991) who reported that the maximum production of peroxidase from *Streptomyces avermitilis* UAH30 and *Streptomyces viridosporus* T7A occurred at 45°C.

Effect of carbon sources

The best carbon source for xylanase production by *Streptomyces* sp. K37 was found to be birchwood xylan and oat spelt xylan. Low levels of xylanases were detected when the organisms were grown on xylose, starch, arabinose, sucrose, glucose, cellobiose, glycerol, phenylalanine, carboxymethylcellulose, and cellulose.

Abd EL-Nasser & Foda, (1995) found that lactose gave high levels of xylanase in cultures of *Streptomyces griseoflavus* and *Streptomyces violarus* while glucose utilisation resulted in complete suppression of enzyme formation. The endoxylanase activity from *Thermoanaerobacterium saccharolyticum* B6A-RI was catabolite repressed by glucose (Shao et al., 1995).

In this work, xylanases of *Streptomyces* sp. K37 were induced by growth of the organisms on a medium containing xylan. This phenomenon has been observed for other actinomycetes (McCarthy et al., 1985, Mackenzie et al., 1987, Holtz et al., 1991, Tuncer, 1999). Induction by xylan may be attributed to the role of xylobiose which, like cellobiose, is known to be potent inducer of xylanolytic enzymes in many microorganisms (Rho et al., 1982). Wang et al., (1992) characterised three xylanases from *Streptomyces cyaneus* and explained the induction mechanism of secretion for these enzymes. They stated that polymeric xylan is

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unlikely to penetrate the cells and the immediate inducers played an important role in the induction process. The same authors pointed out that the presence of xylan degradation products could be fortuitous, due to the presence of other cells in the environment that are already xylanolytic. They described an alternative hypothesis that the cell itself was able to sense the presence of xylan via such inducers and this may be as a result of the activity of xylanase that is synthesised constitutively.

The best carbon source for the optimal peroxidase production by *Streptomyces* sp. K37 was found to be oat spelt xylan and the organism was able to produce peroxidase when grown on xylose, carboxymethylcellulose, starch, cellulose, glycerol and phenylalanine. Lodha et al. (1991) reported that the production of peroxidase from *Streptomyces viridosporus* T7A was maximal when yeast extract was used as a carbon source and the production was reduced by 83% when yeast extract was replaced by carboxymethylcellulose.

Effect of ball milled straw on the production of xylanase and peroxidase

Ball milled straws were found to be good inducers of xylanase production. *Streptomyces sp*. K37 produced xylanases when they were grown in a medium containing straw. The optimum production for xylanase by *Streptomyces sp*. K37 production was found to occur in the range of concentrations between 1.25% to 1.50% ball milled straw. A similar observation was recorded by Wang et al. (1993) who reported that *Streptomyces cyaneus* produced nearly twice as much xylanase activity when grown on ball milled straw than when grown on xylan. Godden et al. (1989) reported that *Streptomyces sp*. isolated from compost produced higher levels of xylanase activity when grown on ball milled straw than xylan. Zimmermann et al. (1988) reported that the highest xylanase activities were produced by *Streptomyces* strains and the thermophile *Saccharomonospora viridis* when grown on ball milled straw. Furthermore, ground wheat straw was found to be a very good substrate for the production of xylanase by *Actinomadura* SIIx, producing a 1.5-fold increase in that activity levels compared with those produced during growth on xylan (Holtz et al. 1991). Similar results were recorded by Abd EL-Nasser & Foda, (1995) who reported that several agricultural by-products, including wheat bran, wheat straw and corn cobs, yielded higher xylanase activities than those produced on pure xylan by *Streptomyces griseoflavus* and *Streptomyces violarus*.

The production of the enzymes described in this work, during the growth of *Streptomycetes* on agricultural by-products containing medium can be explained by the partial solubility of the components of agricultural material in water under the effect of high temperature and pressure of autoclaving. Part of the substrate becomes soluble and such material contains low molecular weight components as well as lignin carbohydrate derivatives. These components support the initial growth of organisms and the production of the enzymes.

Streptomyces sp. K37 produced low levels of peroxidase when grown in a medium containing ball milled straw as the main carbon source. This can be explained by the fact that the utilisation of xylan for peroxidase production proceeds much faster than the utilisation of ball milled straw, resulting in greater peroxidase production, during growth on xylan.

Streptomyces sp. K37 produced low levels of xylanase and peroxidase when grown in high concentrations of xylan and ball milled straw. This can be attributed to end product inhibition of simple sugars such as xylose, glucose and other oligosaccharides (Tuncer, 1999).

The production of microbial xylanases from cellulosic agro-industrial residues is of particularly interest because these residues are abundant, cheap and non-toxic, in addition to being underutilized (Stutzenberger, 1994; Patel & Ray, 1994).

Effect of nitrogen sources. Effect of yeast extract and casamino acid

Streptomyces sp. K37 produced high levels of xylanase and peroxidase when the medium was supplemented with 0.2% yeast extract and casamino acid, but high concentrations led to a decrease of xylanase and peroxidase production. This is in agreement with Yee et al. (1996) and Korus et al. (1991) who reported the highest production of lignin peroxidase from *Streptomyces viridosporous* T7A occurred using 0.3% (w/v) yeast extract, whereas higher concentrations inhibited peroxidase production, perhaps as a consequence of high concentrations of toxic compounds which have an inhibitory effect on growth and peroxidase production.

Streptomyces sp. K37 produced different levels of xylanase and peroxidase with varying C:N ratios and this may be explained as a different types of induction of enzymes. This may suggest different mechanisms of production of extracellular enzymes of *Streptomyces* sp. K37 when grown with different C:N ratios. *Streptomyces* sp. K37

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produced high levels of extracellular enzymes when the C:N ratio was optimal and as the result of changing this ratio the organism produced low levels of extracellular enzymes. These results are in accordance with reported by Fernandez et al. (1995) and Tuncer (1999), who obtained different xylan degrading enzyme activities from *Streptomyces chattanoogensis* UAH 23 and *Thermomonospora fusca* BD25 when grown in media containing different C:N ratios, (4:1 and 1.3:1).

Two general areas of exploitation can be envisaged as a result of lignocellulose degradation: (i) production of biomass that can be used as a source of cheap single-cell protein or for mediating a secondary process; and (ii) release of monomers from polymeric plant material that can be used by a second organism for the growth and production of commercial biochemical's (Edwards, 1993).

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