

PRODUCTION OF XYLANASES BY *STREPTOMYCES* SPECIES AND THEIR BLEACHING EFFECT ON RICE STRAW PULP

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Abstract. The number of potential applications of microbial xylanases in the pulp and paper industry is gradually increasing and several are approaching commercial use. This industry needs a xylanase, which is free of cellulase. Twenty isolated *Streptomyces* strains from Egyptian soils, which produce cellulase free-xylanase and are easily grown using a low-cost agriculture waste substrate, were investigated. The two most active strains have been identified as *Streptomyces albus* and *Streptomyces chromofuscus*. Their maximum xylanase activity was 13.25, 19.31 and 32.53, 43.01 on untreated rice straw pulp and pulp treated with TiO₂ in both *Streptomyces* species respectively. The enzyme activity increased when both isolates were grown on yeast extract. Optimum production of xylanase was recorded after five days of fermentation. Xylanase produced with *Streptomyces chromofuscus* showed higher bleaching activity than that from *Streptomyces albus*. The enzyme enhanced the liberation of reducing sugars, which improved pulp bleachability.

Key words: *Streptomyces* sp., cellulase-free xylanases, rice straw pulp, biobleaching.

Introduction

Xylan is widely distributed in plant cell walls and forms a main part of the hemicellulose fraction [29]. In some higher plants and agricultural wastes, xylan constitutes from 20-40% of the dry weight [5 and 19]. Xylan together with hemicellulose forms the second most abundant renewable polysaccharide in the biosphere. It has been estimated that 500 million tons of such materials could be annually available from the residues of major crops [5]. Effective extraction by enzymatic and microbiological processes of these materials is of great interest. Hemicellulase and endoxylanase enzymes have been extensively studied, since they hydrolyse polysaccharides in the pulp of woods [8]. An attractive application of this hydrolysis process is the removal of xylan from wood pulp for manufacturing of dissolved pulp.

Recently, the interest in cellulase-free xylanases has focused on pulping and bleaching processes, in which chlorine (Cl₂) and hypochlorine (ClO₂) for biobleaching can be reduced [25, 13 and 14]. Naturally occurring microbial strains capable of secreting xylanases free of cellulase activity would be attractive for such applications. The most important enzyme needed for enhancing the bleaching of pulp is endo-B-xylanase [10, 18 and 26]. Xylanases enhance the cleaving of reprecipitated xylan formed on the outer surfaces of the cellulose fibers after pulping [11]. This causes increased permeability of the pulp fibers to the bleaching chemicals and allows the passage of larger fragments of residual lignin out of the pulp.

Xylanases have been isolated from a wide range of microorganisms including fungi, actinomycetes and eubacteria. Actinomycetes enzymes, which are thermostable, are of particular interest. Most actinomycete strains secreting high activity xylanases free of substantial cellulase activity are thermotolerant: *Streptomyces* sp. [12], *Saccharomonospora* [20] and *Streptomyces roseiscleroticus* [6]. Treatment with xylanases can improve the chemical extraction of lignin from pulp. This would lead to a significant reduction in the amount of chemicals required for bleaching and, hence, in the levels of toxic chlorine compounds released into the environment.

The present paper deals with the production of xylanase enzymes from various xylan assimilating streptomyces strains as potent producers of xylanase. In addition, the nutritional requirement and optimum fermentation condition for maximum yield of xylanase have been studied. The effect of the produced xylanase on the bleachability of pulp was also investigated.

Material and methods

Strain isolation

The cultures were isolated from a soil sample containing decomposed rice straw pulp detritus on a basal medium containing (g/l: 2.0 KNO₃, 1.0 K₂HPO₄, 0.5 MgSO₄, 3.0 CaCO₃, 0.01 FeSO₄ and 20.0 agar). Plates were incubated at 28°C for seven days and single colonies were evaluated for growth. Streptomyces colonies were picked followed by purification and preliminary evaluation of the isolates in shake flasks. Two of the strains, which showed high activity of xylanase production, were taken for detailed investigation.

Characterisation of the two selected isolates

Characterisation of the active isolates of streptomyces was done through polyphasic characterisation. Morphological, physiological, chemotaxonomical and molecular identification were carried out according to various methods [22, 30, 9 and 15].

Culture media and conditions

The same previous basal medium was used. The pH was adjusted to 7.0-7.2 and the pulp substrate (2% w/v) was added prior to sterilisation by autoclave at 121°C for 15 min. Shake flask cultivations were carried out with 50 ml of medium in 250 ml flasks at 28°C and shaking at 200 rpm/min. Cultivation was done for 5 days after inoculation of 5 ml of a 5-day culture prepared on starch nitrate medium.

Xylanase assay

The activity of xylanase was measured by determining the release of reducing sugars from soluble xylan. The reaction mixture consisted of 5 ml of xylan solution (3% as anhydroxylose), 4 ml of 0.1 M phosphate buffer (pH 6.5) and 1 ml of enzyme solution. The reaction was run at 40°C for 30 min. Increase of reducing sugars in the mixture was determined as xylose by the method of Somogyi [17]. One unit of xylanase is defined as the amount of enzyme, which is capable of liberating 1MM equivalent of xylose in one minute. Cellulase activity was also determined using the same procedure in addition to 5 mg/ml cellulose.

Pulping

Rice straw was cut into 3-4 cm then extracted by a mixture of methanol and benzene (1:1) for 6 hour to remove waxes and resins. The residual material was air dried. Soft wood soda pulps were prepared by cooking these dried materials under the following conditions: total alkali 12% and cooking temperature 160oC for 1 hour.

Treatment of the pulp with TiO₂

Pulp was impregnated in water, TiO₂ was added as fine powder and the bulk was beaten up to So SoR in a Jorko mill beaker at 150 r.p.m.

Pulp properties

Kappa number, degree of brightness and degree of polymerisation were carried out according to the method of Casey [3]. Kappa number is used to describe the degree of delignification obtained in the chemical and/or enzymatic process. Degree of brightness (%), which means the whiteness of the tested paper, was measured by using a Hunter Lab colour/ Difference Meter D25-2 at wavelength 457 nm. Degree of polymarisation (DP) is a function of average length of the cellulose chains and of fiber length. It is one of the significant factors of cellulose sample strength.

Scanning electron microscope

Pulp samples were coated by thin layer of gold and investigated by Jeol JXA-840 Å Electron Microscope.

Results and discussion

Strain characterisation

Twenty Streptomyces isolates from Egyptian soils were screened for their xylanolytic activity. The most two active isolates have been taxonomically characterised. Based on the polyphasic characterisation (morphological, physiological, chemotaxonomical and 16S rDNA sequences), the selected two isolates could be identified as Sterptomyces albus and Streptomyces chromofuscus.

Xylanase production by Streptomyces sp.

Xylanase production using Sterptomyces albus and Streptomyces chromofuscus was improved with untreated and treated rice straw pulp as substrate. As shown in *Table 1*, xylanase was produced on treated pulp with a maximum activity of 43.01 u/ml. Streptomyces chromofuscus exhibited a higher xylanase activity using either untreated or treated pulp as substrate rather than Sterptomyces albus (*Table 1*).

Table 1. Xylanase production by Streptomyces species growing on pulp at 2% w/v after 5 days

Substrate	Maximum xylanase activity (u/ml)	
	<i>Streptomyces albus</i>	<i>Streptomyces chromofuscus</i>
Untreated pulp	13.25	19.31
Treated pulp withTiO ₂	32.53	43.01

Cellulase activity was not detected in the culture supernatants of both cases. The activates of 19.31 or 13.25 u/ml on untreated pulp were comparable or slightly lower than those reported for most other mesophilic actinomycetes that produce cellulase-free

xylanase. Antonopoulos et al. [1] reported 12 u/ml of xylanase in a 5 day culture of *Streptomyces albus*. Similar low levels of cellulase-free xylanase produced from treated crude agricultural waste have been reported. Growth of xylanase was inducible by treated rice straw pulp in both *Streptomyces* species.

Effect of cultural conditions on xylanase production

Effect of various carbon sources on xylanase production

The activity response of *Streptomyces albus* and *Streptomyces chromofuscus* grown on different carbohydrate sources is shown in *Table 2*. The highest xylanase production was obtained with 20.0 gram xylan as sole carbon sources for both *Streptomyces* strains. Glucose, xylose, maltose, sucrose, dextrin and cellulose could simulate enzyme activity, although they support the growth of the *Streptomyces* species. These results obtained are in agreement with those reported by Techapun et al. [23 and 24].

Table 2. Effect of various carbon sources on xylanase production from *Streptomyces* species after 5 days in case of untreated sample

Carbon sources 2%	Maximum enzyme activity u/ml	
	<i>Streptomyces albus</i>	<i>Streptomyces chromofuscus</i>
Xylan	49.92	60.49
Cellulose	15.45	16.45
Dextrin	16.31	18.71
Sucrose	12.22	15.52
Maltose	12.23	15.42
Xylose	10.05	12.31
Glucose	9.01	10.26

Effect of organic and inorganic nitrogen sources on xylanase production

The addition of organic and inorganic nitrogen sources on enzyme production was also studied. The nitrogen in the basal medium was replaced by various nitrogen sources with equimolar amounts. Concerning the inorganic nitrogen sources used as shown in *Table 3*, a slight increase in enzyme activity was observed with KNO₃ in both *Streptomyces* species. In case of organic nitrogen sources, a maximum increase was observed with yeast extract rather than with peptone and casein (*Table 3*). In addition, yeast extract showed a maximum production comparable with different inorganic nitrogen sources. Thus, it could be concluded that yeast extract is beneficial for enzyme production, which is in accordance with the results of Balakrishnan et al. [2].

Effect of growth period on xylanase production

The results presented in *Figs. (1 and 2)* showed that xylanase was maximally produced at the 5th day of fermentation in both *Streptomyces* species. Xylanase production followed the growth curve of the strains. Maximum enzyme activity was observed at the end of the logarithmic phase and start of the stationary phase followed by decrease in activity with lag phase.

Table 3. Effect of different nitrogen sources on xylanase production from *Streptomyces* species after 5 days in case of treated sample

Nitrogen sources	Maximum enzyme activity u/ml	
	<i>Streptomyces albus</i>	<i>Streptomyces chromofuscus</i>
Inorganic nitrogen source		
KNO ₃	78.78	89.27
NaNO ₃	74.49	84.26
NH ₄ NO ₃	69.50	80.45
(NH ₄) ₂ SO ₄	76.56	87.31
NH ₄ H ₂ PO ₄	76.61	86.52
Organic nitrogen source		
Peptone	63.31	74.14
Casein	40.02	51.23
Yeast extract	79.21	90.01

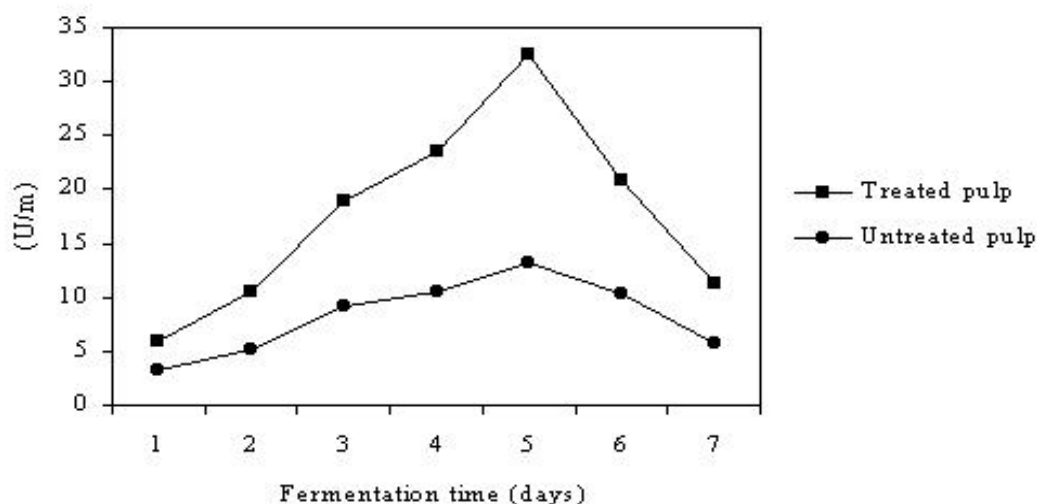


Figure 1. The effect of fermentation time (days) on xylanase production by *Streptomyces albus*

Hydrolysis of pulp

It is clear from Table 4 that hydrolysis of rice straw soft wood pulp with xylanase liberated reducing sugar from *Streptomyces chromofuscus* was more effective than from *Streptomyces albus*. The level of reducing sugars greatly increased with *Streptomyces albus* from 132 to 140 mg/ml and from 149 to 170 mg/ml with *Streptomyces chromofuscus*. Moreover, pulp treated with TiO₂ enhanced hydrolysis, as a result increasing reducing sugar liberation and brightness, which is in accordance with the results of Nagieb et al. [16]. This means that xylanase treatment improved bleachability and did not affect the quality of the pulp. Such results are better than those of previously reported enzymes by some workers [7 and 27].

This implies that the coloured substance (s) in the crude pulp is probably bound to cellulose by xylan rather than by mannan. The degree of brightness in the case of *Streptomyces albus* increased from 45.1 to 47.2 (untreated) and to 49.1% (treated). A remarkable increase in brightness is observed in case of *Streptomyces chromofuscus* from 45.1 to 50.2 (untreated) and to 54.2% (treated). The Kappa no. in treated pulp

decreased 4 points in the case of *Streptomyces albus* and dropped 7 points in the case of *Streptomyces chromofuscus*.

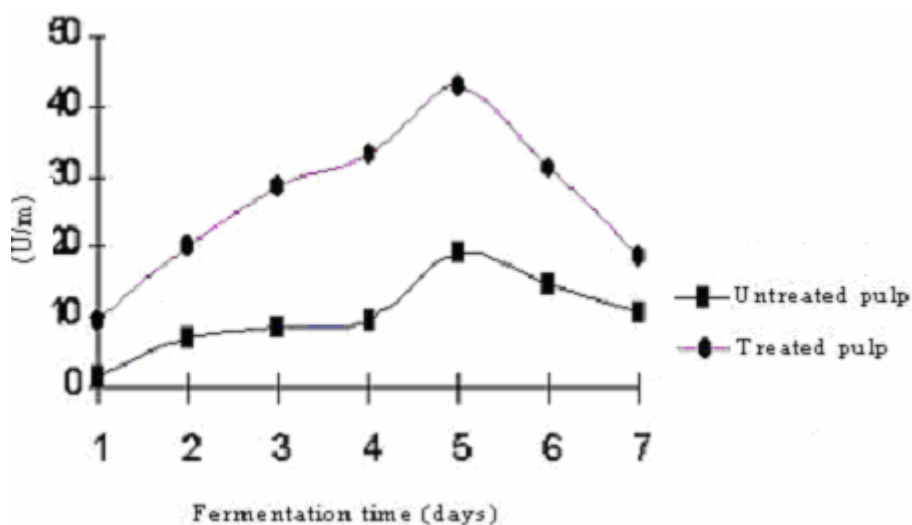


Figure 2. The effect of fermentation time (days) on xylanase production by *Streptomyces chromofuscus*

Table 4. Hydrolysis of soft wood rice straw pulp treated and untreated by *Streptomyces* species

Strain	Samples	Degree of polymerization	Kappa No.	Degree of brightness	Reduced sugar mg/ml
	Blank	960	17.2	45.1	-
<i>Streptomyces albus</i>	Untreated	970	15.2	47.2	132
	Treated	990	13.2	49.1	140
<i>Streptomyces chromofuscus</i>	Untreated	995	12.5	50.2	149
	Treated	1110	10.5	54.2	170

The prepared enzyme had no cellulase activity and showed a noticeable increase in the degree of polymerization (DP). This may be due to the selective degradation of low molecular mass fractions of xylan [4]. The DP increases from 960 to 970 to 990 and from 960 to 995 to 1110 in the untreated and treated samples of *Streptomyces albus* and *Streptomyces chromofuscus* respectively (Table 4).

Reducing sugars were released in the treated samples, especially *Streptomyces chromofuscus* (up to 170 mg/ml).

Examination with the scanning electron microscope (SEM)

In order to understand the effect of xylanase treatment on enhanced bleachability, the fiber surface morphology of the treated and untreated pulps without and with both *Streptomyces* species were studied by SEM. Significant changes on the fiber surface of the xylanase treated pulp were indicated by SEM as a result of xylan hydrolysis, while no surface effect was observed in untreated pulps (Fig. 4 and 5). Comparing hard sheets made from untreated and treated pulp, the SEM of the later shows that the white colour of TiO₂ has permeated into fibers of pulp, and has unified the bands strongly together (Fig. 3 a and b). In addition, the hard sheets made from pulp treated and untreated with

Streptomyces albus reveals that the fibers treated with TiO₂ exhibit a cleaner surface, apparent highly flexibility and conformability and a fiber with a vemar kable peeling effect rather than the control. Morphological changes such as holes, cracks, flakes, filaments and peeling are caused by enzyme treatment. These cracks and holes allow the diffusion of the larger lining macromolecules as reported by Wang et al. [28].

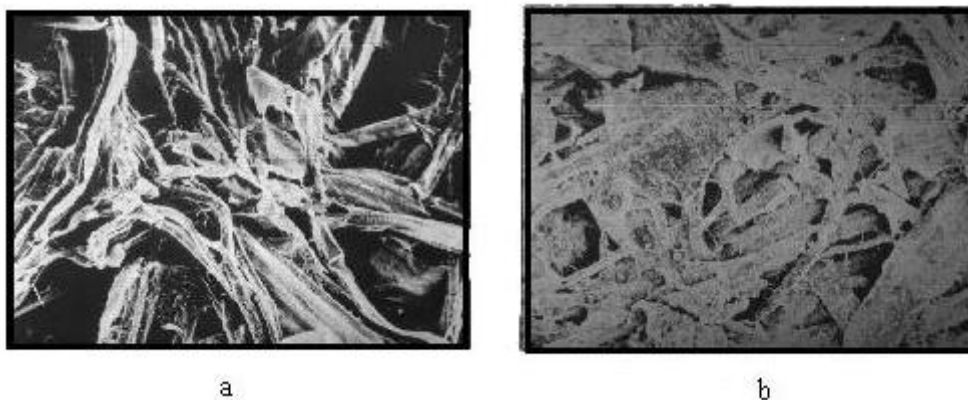


Figure 3. Photograph of SEM of hard sheets for untreated (a) and treated (b) pulp



Figure 4. Photograph of SEM of hard sheets for untreated (a) and treated (b) pulp after treatments with xylanase produced by *Streptomyces albus*

Xylanase treatment improves accessibility of bleaching chemicals to the pulp and decreasing diffusion resistance to outward movement of the degraded lignin fragments from cell wall. SEM of hard sheets treatment with *Streptomyces chromofuscus* show cell wall swelling softening and collapse of the cell structure (Figs. 5. a and b). Sachs et al. [21] came to the same observation. It is clear that hard sheet treated *Streptomyces chromofuscus* showing better apparent compressibility and bending as compared to treated by *Streptomyces albus*. This characteristic meets one of the required properties of the source of xylanase for the bleaching processes.

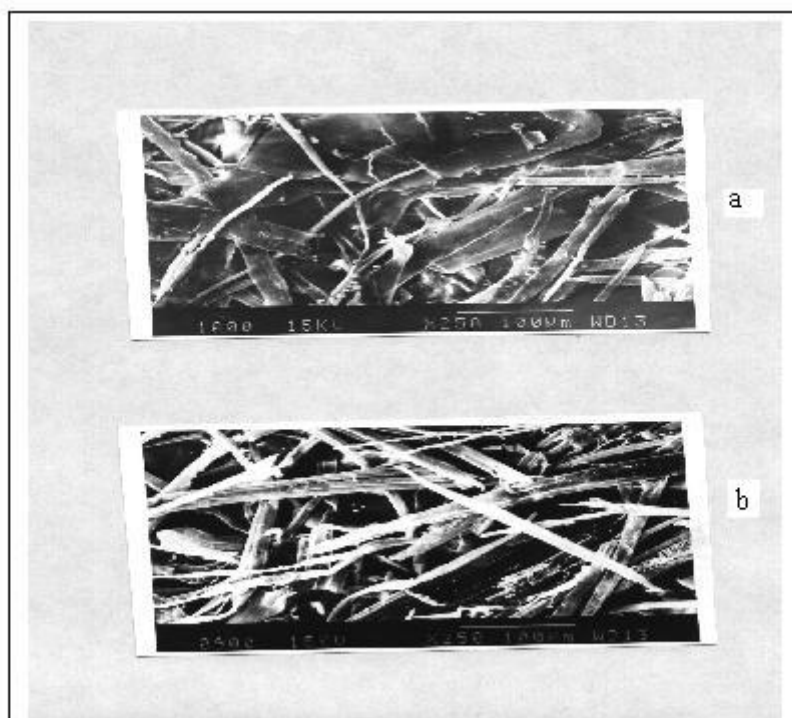


Figure 5. Photograph of SEM of hard sheets for untreated (a) and treated (b) pulp after treatments with xylanase produced by *Streptomyces chromofuscus*

In conclusion, the present study focuses on the production of cellulase free xylanase from *Streptomyces* species. However, not only the production level of xylanase, but also the ability to hyper produce enzyme on low cost agricultural residues such as rice straw. Xylanase produced by *Streptomyces chromofuscus* seems to be more efficient as compared with that of *Streptomyces albus* xylanase. So, the use of xylanase enzyme as an effective bio-reagents to achieve biobleaching in place of toxic chlorine compounds conventionally used today, is should be encouraged.

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