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ORIGINAL RESEARCH PAPER

OPTIMIZATION OF BIOSYNTHESIS CONDITIONS AND CATALITIC BEHAVIOR EVALUATION OF CELLULASE-FREE XYLANASE PRODUCED BY A NEW *STREPTOMYCES* SP. STRAIN

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Cellulase-free xylanase by *Streptomyces* sp.P12-137 was obtained by cultivation on the wheat bran as the sole carbon source. The effect of carbon and nitrogen sources and a ratio of them on the cellulase-free xylanase production was investigated. The new isolate *Streptomyces* sp. strain was able to grow in submerged system and to produce an increased level of xylanase. Wheat bran induced xylanase biosynthesis yield at a high level (9.27 UA/ml). For economical reasons cultivation was achieved on a cheap fermentative medium represented by agro-industrial wastes. The optima of the pH and temperature of the crude xylanase activity were 5.5 and 70°C, respectively.

Keywords: cellulose-free xylanase, *Streptomyces*, submerge fermentation, thermostable enzyme

Introduction

Agroindustrial residues represent one of the most energy-rich resources available on the planet and when they are not discharged or used, lead to environmental pollution (Heck *et al*., 2005). The hemicellulases unit is a big group of high molecular polysaccharides soluble in water. These are associated with cellulose and lignin from plant cell walls structure. The composition of hemicelluloses is represented by a branched heteropolymers of pentoses (xylose, arabinose), hexoses (mannose, galactose, glucose) and uronic acids (Dobrev *et al.*, 2007).

Xylan is the major hemicellulosic polysaccharide of food industry and agricultural wastes, where it comprises up to 20 – 35% dry weight (Senthilkumar *et al*., 2005). Xylan is composed of a xylose backborne, linked by β-1,4-xylosidic bonds, substitued with glucuronosyl, acetyl and arabinosyl residues (Senthilkumar *et al*., 2005). The total hydrolysis of xylan is obtained with the following enzymes:

xylanase: (endo-1,4-β-D-xylanase EC 3.2.1.8), β-D-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55), acetylxylan esterase (EC 3.1.1.72), α-L-glucuronosidase (EC 3.2.1.139), p-coumaric acidic esterase and ferulic acidic esterase (EC 3.1.1.73) (Dobrev *et al*., 2007). Among xylanolytic enzymes, exoxylanase, endoxylanase and β-xylosidase are the key enzymes that cleave the xylan backborne into lower xylooligomers and xylose units (Senthilkumar *et al*., 2005).

The industrial production of xylanase enzyme preparation is based on a microbial biosynthetis. Moulds strain of the species *Aspergillus* sp., *Trichoderma* sp. and bacterial strains of the species *Bacillus* sp., *Streptomyces* sp. are used for industrial production of xylanase (Dobrev *et al*., 2007). *Streptomycetes* are Gram-positive soil bacteria able to degrade many macromolecules such as proteins, cellulose, starch, lipids and chitin. For the degradation of cellulose, hemicellulose, and lignin that are abundant in plants, different strains of *Streptomyces* sp. have been studied and found to be good producers of xylanase and cellulase, but the enzymes are mainly thermolabile (Jang, 2003). *Streptomyces* sp. can produce thermostable and alkaline-stable cellulase-free xylanase. These microorganisms can grow easily at low cost and with simple substrates, such as wheat bran, can bagasse, corn cobs. The submerged cultivation of *Streptomyces* sp. xylanases producing strains is more used, allowing a higher degree of processes intensification and a better level of automation. Xylanase obtained from bacteries has attracted a great attention in the last decade because of the biotechnological potential of enzyme. Such applications require xylanase(s) with particular properties. The bio-bleaching of paper pulp requires a xylanase that remains active even above *p*H 9.0 and devoid of cellulase activity (Tseng *et al.*, 2002). Cellulase-free xylanases active at high temperature are gaining importance in pulp and paper technology as alternatives to the use of toxic chlorinated compounds (Sharma *et al*., 2009). Xylanases are being used, primarily, for the removal of the lignin–carbohydrate complex (LCC) that is generated in the kraft process and acts as physical barriers to the entry of bleaching chemicals. A prerequisite in the pulp and paper industry is the use of cellulase free xylanases that ensure minimal damage to the pulp fibres and also generate rayon grade or superior quality dissolving pulps (Gupta *et al*., 2000). Xylanases are produced on a industrial scale as food additives in poultry products, in flour for improving dough handing and for clarification of juice and wines (Liu *et al*., 2007).

In this paper are presented new isolates of thermotolerant microorganisms that can produce cellulase-free xylanase from agricultural waste materials. We describe the catalitic behavior (*p*H, temperature, enyzme:substrate ratio) of xylanases from *Streptomyces* sp. P12-137 and optimization of biosynthesis conditions (carbon and nitrogen sources, carbon: nitrogen ratio) for this strain.

Materials and Methods

Microorganism

In this study *Streptomyces* sp. P12-137 was used for the production of the cellulase-free xylanase enzyme. This strain was isolated from soil sample from

Galati region and then selected as a active producer of xylanase. Stock cultures were maintained on Gauze-agar medium at 4°C.

Filamentous bacterium cultivation and cellulase–free xylanase production in submerged fermentation system

The fermentative medium contained (in g/l): carbon source 10.0; YE 0.075; KH_2PO_4 15; K_2HPO_4 2.0; $(NH_4)_2SO_4$ 4.5; trace element solution 2.7 ml/l. Medium at *p*H 7.2 was sterilized in autoclave at 121^0C, 1.5 atm for 20 min. The trace element solution contained (in g/l): $ZnSO_4 \cdot 7H_2O$ 140; $MnSO_4 \cdot H_2O$ 160; $FeSO_4 \cdot 7H_2O$ 500; $CoCl_2 \cdot 2H_2O$ 200. Inoculum represented of 5 percent spore suspension was inoculated into 250 ml Erlenmeyer flask containing 100 ml of medium. Flasks were incubated for 5 days at 28°C in an orbitory shaker at 150 rpm.

After the incubation period, the biomass was filtered over a Whatman No.1 filter paper. The filtrate was centrifuged at 10000 rpm for 20 min at 4°C and the clear supernatant was used as enzyme source.

Enzyme assay

The xylanase activity was determined by measuring the release of reducing sugars from birchwood xylan (1.5% w/v) using the dinitrosalicylic acid method (Miller, 1959; Yin, 2006; Yin, 2007). Reaction mixture containing 4 ml of a solution 1.5% of birchwood xylan in acetate buffer (0.2M, *p*H 5.0) and 0.4 ml of the diluted crude enzyme, was incubated for 20 min at 50°C. Samples were collected at the beginning and the end of enzymatic reactions.The amount of reducing sugar in the reaction tubes was measured using the dinitrosalicylic acid (DNS) method. The absorbance was read at λ=535 nm using Jenway 610 UV/VIS spectrophotometer (Yin, 2007). One unit of xylanase was defined as the amount of enzyme required to released 1 μmol of the xylose from xylan in 1 minute under the assay condition (Miller, 1959). Enzyme assay was performed in triplicate.

The biosynthesis of cellulase–free xylanase by *Streptomyces* sp. P12-137 was tested in submerged system using various lignocellulosic substrates present in raw materials as carbon sources.

D(+)Xylose, D(-)Arabinose, Raffinose pentahydrate, D(+) Mannose and Xylitol were tested as carbon source on medium previously described with added agar. Strain was inoculated „in point". The plates were incubated at 28°C for 120 hours.

For selection of suitable substrate for enzyme production, the bacterial strain *Streptomyces* sp. P12-137 was grown in 250 ml Erlenmeyer flask containing 1 g of each various substrates (wheat bran, rice bran, wheat straw, corn cob, sawdust, CMC) in addition with mineral salt solution.The enzyme was extracted and assayed. The experiments were conducted in triplicate and the average values are raported.

Results and discussion

The effect of carbon and nitrogen sources on the cell-free xylanase biosynthesis

The growth diameters of the colonies were evaluated at the end of cultivation (Figure 1).

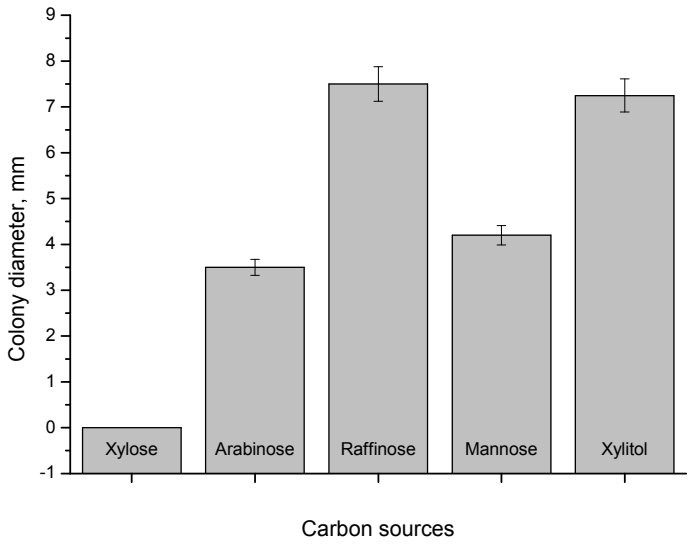

Figure 1. Glucanasic potential of *Streptomyces* sp.P12-137 strain by cultivation on media with hemicelluloses as single carbon sources *Streptomyces*. The bars are means of 3 different experiments ± SEM

Growth testing of selected strain *Streptomyces* sp.P12-137 on hemicellulosic carbon sources showed a good growth on raffinos, xylitol, arabinose, mannose as substrate and a reduced growth on xylose. Lack of development on xylose leads to the conclusion that a certain concentration is a limiting factor of growth of *Streptomyces* strain. Ghosh *et al.*(1993) reported that xylose, the ultimate breakdown product of xylan, serves as a good inducer of this enzyme.

Carbon source of different origins was used in the production medium for determining the highest yield of xylanase production. Results indicated the role of carbon compounds in maximizing the production of xylanase enzyme. The highest activity was obtained when wheat bran was used as the carbon source (Figure 2) while the activity was minimum with CMC. The universal suitability of wheat bran may be due to insolubility in water, and to the nature of cellulose and hemicellulose.

In order to determine the dynamics of the xylanase accumulation during growth on medium with xylan, the xylanase and CMCase activities in the growth medium were measured (Figure 3). A 100 ml basic medium of 10 g wheat bran as substrate was added in 250 ml flasks, autoclaved, inoculated with 72 h old culture and incubated as described above. The contents of the flasks were harvested and assayed at every 24 h intervals. Enzyme assay was performed in triplicate with analytical grade reagents.

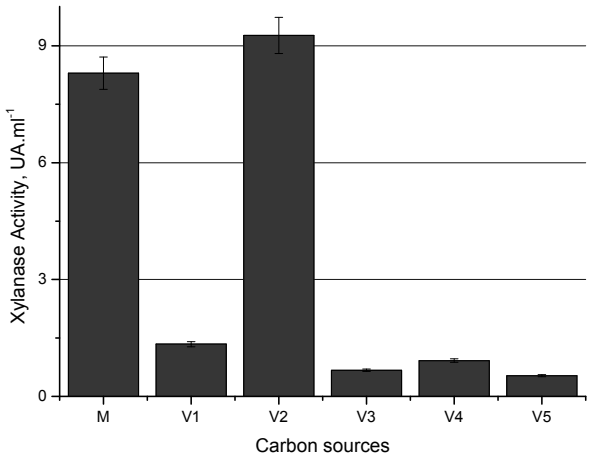

Figure 2. Effect of carbon sources on xylanase enzyme production by submerged cultivation at 28°C, 150 rpm, 120 hours (M – martor (xylan birchwood), V1 – corn cobs, V2 – wheat bran, V3 – wheat straw, V4 – sawdust, V5 – CMC)

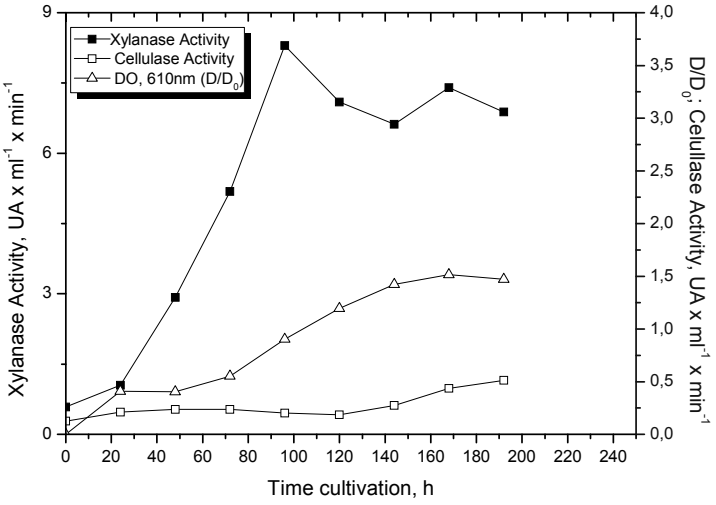

Figure 3. Growth of streptomycete and xylanase and CMCase biosynthesis by submerged cultivation on medium with xylan birchwood ((■) xylanase activity,(○) CMCase activity, (Δ) DO (D/D_0) at 610 nm)

At the end of the growth phase, 15% of the total xylanase activity was detected in the extracellular fraction. The maximal xylanase activity detected in the extracellular fraction was 8.3 UA/ml·min, and xylanase production correlated

reasonably well with the cell growth phase. For celullase activity, the maximum was reached after 192 hours at value 1.15 UA/ml/min.

Maintaining the carbon sources at optimized condition, nitrogen sources of varying nature were tested. The basic medium was used for studying the effect of various nitrogen compounds: peptone (1%), urea (0.2%), KNO_3 (0.5%), $(NH_4)_2SO_4$ (0.5%). The broth was distributed into various flasks of each nitrogen source. Cultures in triplicates were incubated at already standardized parameters. The strain showed maximum activity in the presence of KNO_3. An equal xylanase activity was recorded for peptone, urea, $(NH_4)_2SO_4$ (Figure 4).

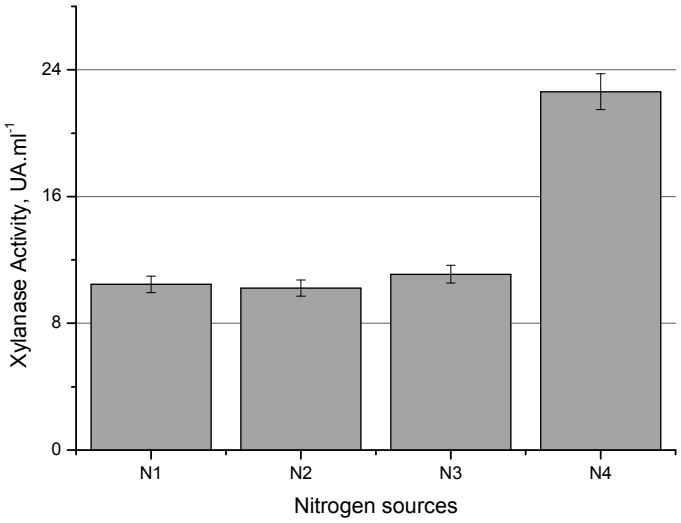

Figure 4. Effect of nitrogen sources on xylanase production by submerged cultivation of *Streptomyces* sp.P12-137: N1-peptone, N2-urea, N3-$(NH_4)_2SO_4$, N4-KNO_3.The bars are means of 3 different experiments ± SEM

A maximum xylanase activity performed with two strains of streptomycetes using KNO_3 as a nitrogen source was reported of Rifat (2005). This is due to easy accessibility of inorganic compound water soluble and richly represented in the soil resulting from decomposition of plant biomass. Bakri *et al*. (2008) reported also that sodium nitrate, potassium nitrate, yeast extract and peptone induce the xylanase synthesis.

It was investigated the relationship between carbon source (wheat bran) and nitrogen source (KNO_3) on xylanase activity. Report C:N tested in submerged fermentation system was 1:10, 1:5, 1:2.5, 1:1 and 2:1 respectively (Figure 5).

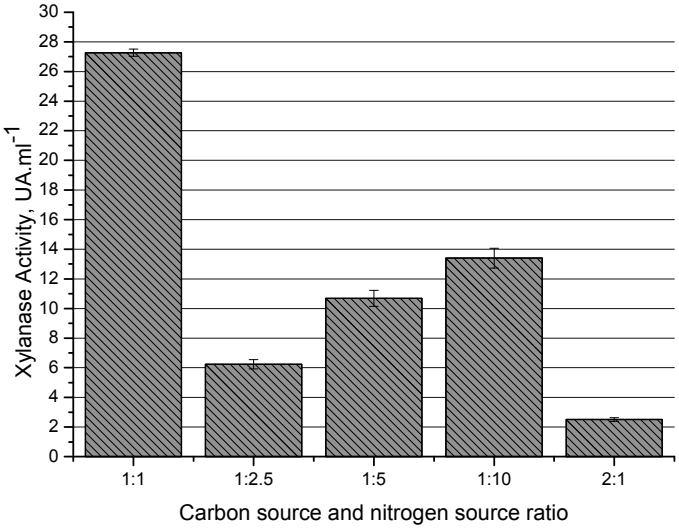

Figure 5. Effect of C:N ratio on xylanase enzyme production

It can be seen from figure 5 that the xylanase activity varied significantly with the ratio C:N of substrate within range 2.52 – 27.27 UA/ml. The lowest value (2.52 UA/ml) was obtained when using a larger quantity of carbon source and reduced nitrogen source. The highest value (27.27 UA/ml) was achieved with an equal ratio 1:1 between the source of carbon and nitrogen, where it concluded that the nitrogen source influences positively the production of xylanase.

Evaluation of catalic behavior (pH, temperature, enyzme: substrate ratio) of cell-free xylanase produced by streptomycetes

The rate of enzymatic reaction speed depends on the pH of reaction environment. The effect of *p*H on xylanase activity was determined under the standard conditions for the xylanase assay (50°C for 20 min). Xylanases activities were assayed at 50° C to determine the effect of pH on its activity over the *p*H 3.0 – 8.0 range; 200 mM acetate buffer for *p*H 3.0 – 6.0; 200 mM phosphate buffer for *p*H 7.0 – 8.0.
Figure 6. shows the activity of *Streptomyces* sp.P12-137 xylanase at different pH values. The optimum *p*H values of the xylanase were *p*H 5.5. Alkaline *p*H had inhibitory effect on the enzyme production. Maheswari and Chandra (2000) reported an optimum pH value of 5.5 and 30 – 40% activity retained in the *p*H range 4.5 – 6.0. Ninave *et al.* (2008) suggested a value of 6.0 for optimum *p*H and 60–65°C for temperature.

The temperature affects positively the rate of reaction to achieve an optimum reaction value then decreases rapidly. Higher temperatures inactivate rapidly, first reversible and then irreversible, enzymes. Low temperature is a fully reversible inhibition, enzymes can be preserved even at low temperatures. Temperature

dependence of the *Streptomyces* P12-137 strain xylanase activity was studied at different temperatures. The influence of temperature on the xylanase activity produced by *Streptomyces sp.*P12-137 was studied by incubating the reaction mixture at different temperatures, ranging from 30 to 80° C and *p*H 5.5 for 20 minutes.

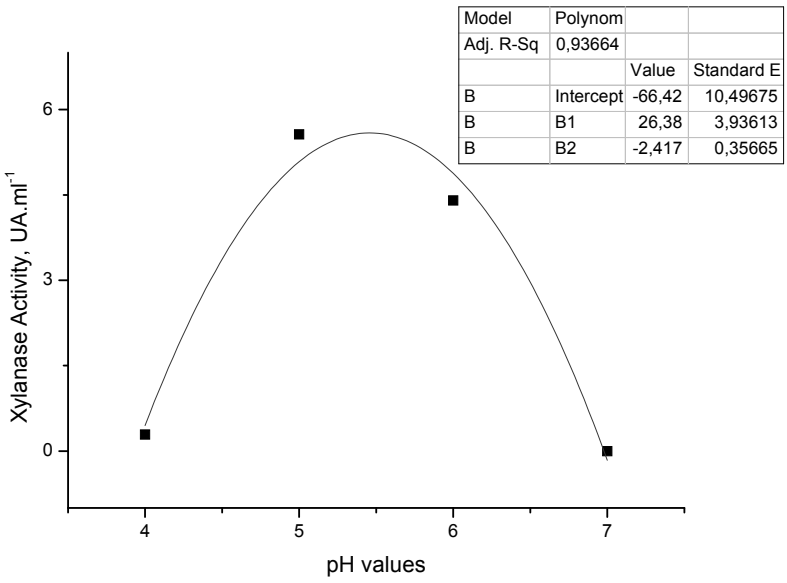

Figure 6. Effect of *p*H on the activity of *Streptomyces* sp.P12-137 xylanase

Maximum activity values for xylanase activity were obtained at 50°C, *p*H 5.5 after 20 minutes of incubation. The enzyme had an optimum temperature at 70°C (Figure 7). Maheswari and Chandra (2000) reported an optimum temperature at 70°C for a xylanase produced by a new strain *Streptomyces cuspidosporus* in the presence of a 10% w/v wheat bran. Significant termostability has been reported for xylanase from *Streptomyces* T7 (50°C, *p*H 5.0 for 6 days) (Beg, 2000; Keskar, 1992).

The relationship between substrate concentration and enzyme concentration affects the enzymatic reaction rate. The influence of the enzyme-substrate ratio on the xylan birchwood hydrolisis reaction on crude extract enzyme was achieved by varying [E]/[S], such: 1:1, 1:2, 1:4, 1:6, 1:8, 1:10. The reaction was achieved at 70°C and *p*H 5.5 (200 mM acetate buffer). Optimum ratio is the value at which the xylanase has optimum activity. Enzyme productivity heavily depends on this report. A xylanase activity of 19.95 UA/ml was obtained at 1:1 enzyme substrate ratio (Figure 8). Optimum ratio [E]/[S] corresponds to a minimum amount of enzyme and to maximum amount of substrate. The 1:10 ratio meets this criterion.

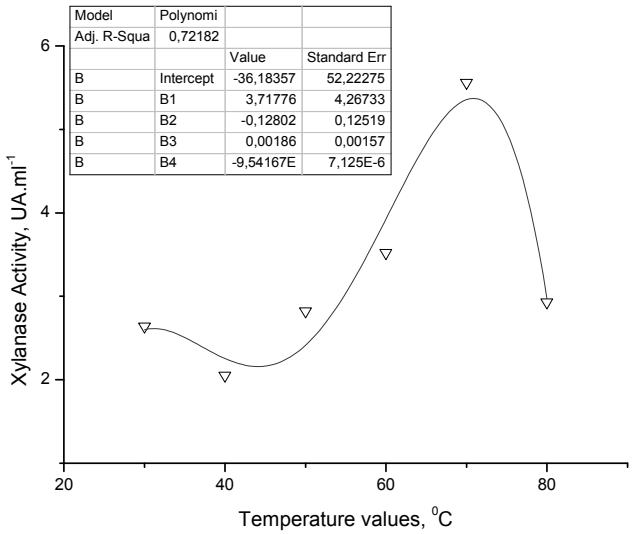

Figure 7. Effect of temperature on the activity of *Streptomyces* sp.P12-137 xylanase

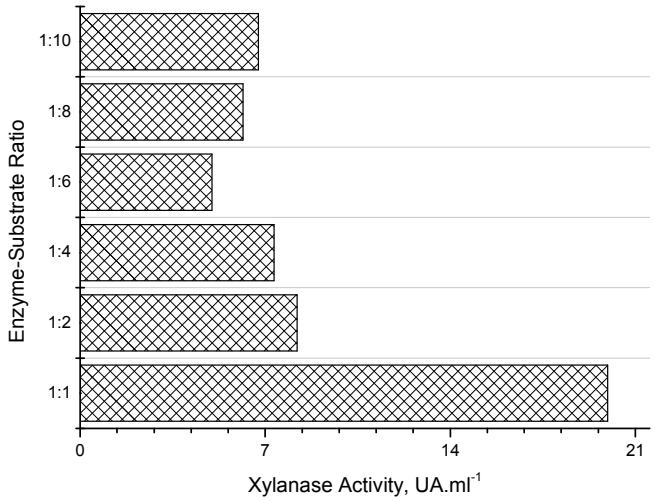

Figure 8. Effect of enzyme-substrate ratio on xylanase production

Conclusions

The results obtained from submerged culture indicate that significant improvement of xylanase production by *Streptomyces* sp. P12-137 strain could be obtained by a selective use of nutrients and growth conditions. Since xylan is an expensive substrate for commercial scale xylanase production, the possibility of using

agricultural residues for xylanase production was investigated in this report. Wheat straw (1% by mass per volume) could be used as a less expensive substrate for efficient xylanase production (9.27 UA/ml). This observation is interesting due to the low cost of these carbon sources. Xylanase obtained by *Streptomyces* sp.P12-137 is a thermostable enzyme (optimum temperatura 70°C), with optimum xylanase activity at *p*H 5.5 so it can be used in biobleaching process of pulp and paper industry. The results obtained are in good agreement with those reported by various researchers in recent years on xylanases produced by streptomycetes.

Acknowledgements

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