

Full Length Research Paper

Effect of cultural conditions on xylanase production by *Streptomyces sp.* (strain Ib 24D) and its potential to utilize tomato pomace

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Accepted 17 February, 2005

The purpose of this study was to determine the effect of some cultural conditions on the xylanase enzyme production by *Streptomyces sp.* (strain Ib 24D) and to investigate its potential to produce xylanase utilizing tomato pomace as a substrate. Xylanase activity was detected using the dinitrosalicylic acid assay method. The crude enzyme was maximally active at pH 6.5 and 60°C. The maximal enzyme production was obtained when oat spelt xylan was used as a carbon source. When tomato pomace was used as carbon and nitrogen source, the maximal xylanase production was 1447 U/ml. SDS-PAGE and zymogram analysis revealed one band of 58 kDa with xylanolytic activity. The *Streptomyces sp.* (strain Ib 24D) was able to produce a considerable amount of xylanase with high levels of activity at a broad ranges of pH and temperature, in addition to its ability to utilize tomato pomace as a carbon and a nitrogen source.

Key words: Agricultural wastes, biodegradation, *Streptomyces sp.*, xylanase.

INTRODUCTION

Xylan is a major hemicellulose and considered with cellulose and chitin as being among the most abundant polysaccharides in nature. It is composed of a linear backbone of 1,4- β -linked-D-xylopyranosyl units that often has side chains of O-acetyl, arabinosyl and methylglucuronosyl substituents (Maheshwari et al., 2000; Blanco et al., 1997). Endo- β -1,4-xylanase (1,4- β -D-xylan xylanohydrolase: E.C. 3.2.1.8) is the main enzyme responsible for the cleavage of the linkages within the xylan backbone (Belfaquih et al., 2002).

Bacterial, fungal and actinomycete xylanases have attracted considerable research interest (Bastawde, 1992; Wong et al., 1988) because of their potential applications in recovery of fermentable sugars from hemicellulose,

biobleaching of pulp and paper industry and to other industrial applications (Kang et al., 1996; Kuhad and Singh, 1993).

This paper deals with strain Ib 24D that was isolated from soils in the Ajlun forests in north of Jordan and showed the highest activity among the other xylanase producing *Streptomyces* strains. In this laboratory study, the effect of different cultural conditions on the xylanase enzyme production by *Streptomyces sp.* (strain Ib 24D) was determined, in addition to its ability to utilize tomato pomace as a cheap substrate which could have an economic impact on using this enzyme in pulp and paper industry.

MATERIALS AND METHODS

Sample collection and processing

Five soil samples were collected from the Ibbin area of the Ajlun forest in the north of Jordan in October 2001. After removing

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approx. 3 cm of soil from the surface, samples were taken to a depth of 10 cm. Each soil sample was crushed, mixed thoroughly and sieved through a 2 mm sieve (Retsch, Haan, Germany) to get rid of large debris, and the sieved soil used for isolation of *Streptomyces*.

Enrichment and isolation of *Streptomyces* spp.

Streptomyces isolates were enriched by two methods, those of Williams (1972) and El -Nakeeb and Lechevalier (1963). A 1-g aliquot of each soil sample was dried at 45°C for 12 h in an oven (Supertek, India), CaCO₃ added, and the soil incubated at 26°C for 7 days in a water bath. All chemicals used were of analytical grade.

Isolation

Sub samples of 1 g were suspended in 100 ml sterile distilled water then incubated in an orbital shaker incubator (TEQ, Portugal) at 28°C with shaking at 140 rpm for 30 min. Mixtures were allowed to settle then serial dilutions up to 10⁻⁶ were prepared. From each dilution, 0.1 ml was taken and spread evenly over the surface of starch casein nitrate agar (SCNA) (El -Nakeeb and Lechevalier, 1963; Kuster and Williams, 1964) plates (in triplicates) with sterile L-shaped glass rod then incubated at 27°C for 10 days. Plated dilutions that gave 20-200 colonies were chosen for further isolation. Repeated streaking on SCNA plates purified bacterial colonies that showed *Streptomyces*-like appearance.

Screening for xylanase-producing *Streptomyces*

Purified isolates of *Streptomyces* spp. were cultured on oat spelt xylan agar medium (Nanmori et al., 1990) and incubated at 28°C for 4 days. The plates were then flooded with absolute ethanol and left for 16 h at room temperature to precipitate xylan. Colonies producing xylanase enzyme were surrounded clear zones against an opaque background of non-hydrolyzed media. Positive results were confirmed in a repeat test.

Characterization of the most active isolate

The *Streptomyces* isolate that showed the largest clear zone (1 cm, the distance from the edge of the colony to the rim of the clear zone) was selected for further investigation and characterization according to the guidelines of the International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966).

Xylanase activity assay

Assays for crude xylanase were performed using 0.5% soluble oat spelt xylan (Sigma) in 50 mM sodium phosphate buffer, pH 7.0. The reaction mixture was composed of 1.8 ml substrate and 0.2 ml crude enzyme. The mixture was incubated in a water bath at 60°C for 15 min. The released reducing sugar was measured by the 3,5-dinitrosalicylic acid (DNSA) method (Miller, 1959) in which the reaction was stopped by adding 3 ml of DNSA acid reagent. A reddish brown colour developed after placing the reaction tubes in a boiling water bath for 5 min. After cooling the reaction tubes to room temperature, the O.D. was measured at 575 nm with xylose as the standard, where one unit (U) of xylanase activity is defined as the amount of enzyme that releases 1 mol xylose/min/ml under the above mentioned conditions.

Effects of pH and temperature on xylanase activity

The range of buffers were used at 50 mM in preparing 0.5% (w/v) xylan solution for detection of xylanase activity was aceto-acetate buffer (pH 4, 5 and 6), phosphate buffer (pH 6.5 and 7), Tris buffer (pH 8 and 9). The reaction conditions were as mentioned before using the culture filtrate as the enzyme source. The pH value giving the highest enzyme activity was used in further enzyme assays.

The optimal temperature for enzyme activity was determined by performing the standard assay procedure at a range of temperature from 4 to 100°C was used to determine that is mentioned earlier. All further enzyme assays was performed at the optimum temperature.

Effect of various carbon sources on xylanase production

To detect the effect of various carbon-sources on xylanase production 250 ml Erlenmeyer flasks were prepared containing 100 ml of mineral salts medium supplemented with 0.2% (w/v) of one of the following carbon sources: glucose, xylose, arabinose, xylan, carboxymethyl cellulose (CMC), and 0.3% xylan, giving a total of 0.5% carbon source. Xylanase assays were performed daily.

Xylanase production in tomato pomace medium

Tomato pomace medium was prepared in 250 ml flasks as follows: 1 g of dried powdered tomato pomace was suspended in 100 ml distilled water, then supplemented with 0.1 ml trace salt solution (1 ml/L: FeSO₄. 7H₂O, 0.1 g; MnCl₂. 4H₂O, 0.1 g; ZnSO₄.7H₂O, 0.1 g; distilled water, 100 ml) (Shirling and Gottlieb, 1966) and adjusted to pH 7.5 prior to autoclaving. Each flask was inoculated with 0.5 ml spore suspension (10⁷ CFU/ml). Flasks were incubated and monitored for enzyme production as mentioned before.

Gel electrophoresis and zymogram of crude xylanase

Polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5% denaturing polyacrylamide gels by the method of Laemmli (1970). A modification was the incubation of the protein samples with sample buffer at 50°C for 10 min instead of 100 °C for 2-5 min (Blanco et al., 1997). The samples were electrophoresed on the gel using a mini protean II (BioRad) system, A broad range protein marker (Promega) being used for molecular weight determination. Proteins were stained with 0.1% (w/v) Coomassie blue R (Fluka) in 50% (v/v) methanol, 10% (v/v) acetic acid.

For the zymogram analysis, the crude enzyme samples were electrophoresed as above on SDS-PAGE containing xylan (0.1%). After running, the gel was washed four times for 30 min in 100 mM phosphate buffer (pH 7.0), the first two washes containing 25% (v/v) isopropyl alcohol, to remove SDS and renature protein in the gel. The gel was then incubated for 20 min at 37°C before soaking in Congo Red solution for 5 min at room temperature and washing with 1 M NaCl until excess dye was removed from the active band. The zymogram was prepared after soaking the gel in 0.5% acetic acid solution. The background turned dark blue, and clear zones were observed in the areas exposed to xylanase activity (Nakamura et al., 1993).

RESULTS

After isolation and screening for xylanase producing *Streptomyces* sp., one active strain (Ib 24D) showed a

broad degradative capability, breaking down of xylan in addition to CMC, pectin and starch (data not shown). Morphological and physiological characterization of this, the most active strain, revealed that it belonged to the grey colour series with a dark green substrate mycelium (reverse colour), did not produce diffusable and melanin pigments and had a rectiflexible (RF) sporophore arrangement.

The crude xylanase from the strain Ib 24D was active over a pH range of 5- 8 and was most active at pH 6-7, with maximum activity at pH 6.5 At pH 9 and pH 4, the

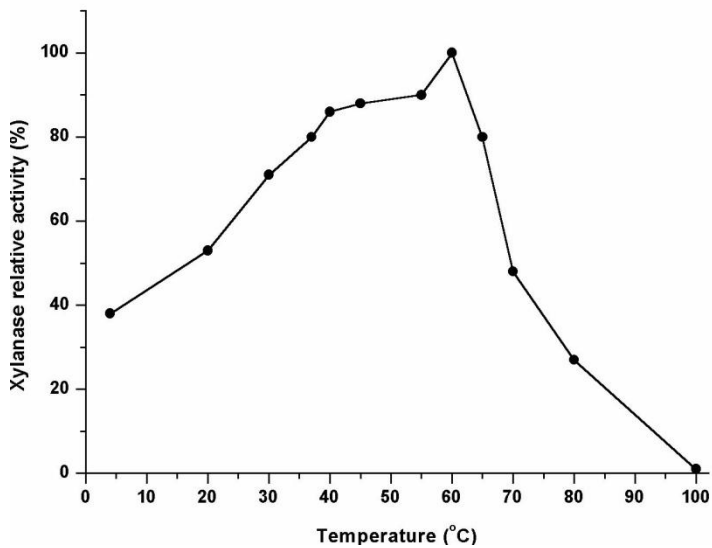


Figure 1. Effect of temperature on xylanase activity. Enzyme activities are compared to the highest value, considered as 100%.

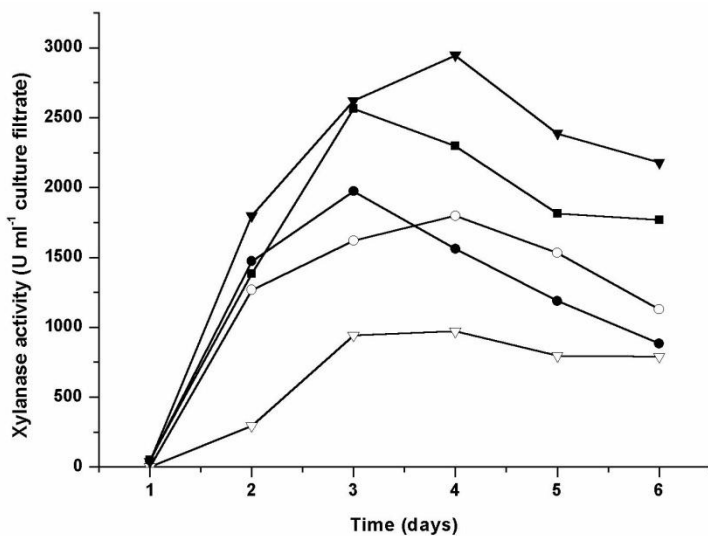


Figure 2. Effect of different carbon sources (0.2%) plus 0.3% xylan: (▽) glucose, (○) arabinose, (◻) xylose, (◼) CMC and (◊) xylan on xylanase production. Enzyme activities are compared to the highest, considered as 100%.

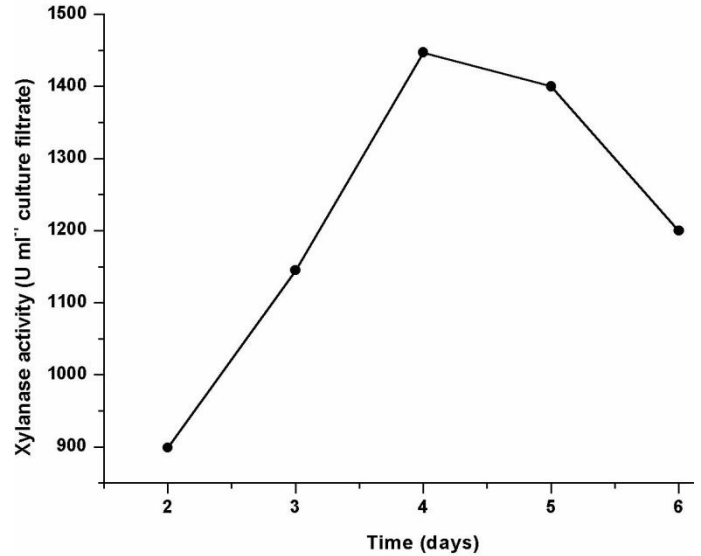


Figure 3. Production of xylanase in tomato pomace medium.

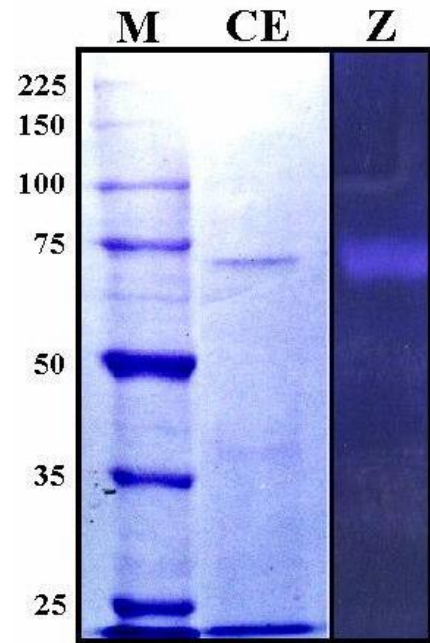


Figure 4. 12.5% SDS-PAGE and zymogram analysis of crude xylanase from the active *Streptomyces* (Ib 24D) strain. M = broad range protein marker (kda); CE = crude enzyme; Z = zymogram. Arrow indicates the active enzyme band.

relative activity was approximately 55% and 35%, respectively. Xylanase activity was present over the temperature range 30-65°C, with the maximum activity at 60°C. At 70°C (Figure 1) the relative xylanase activity was approximately 50% of the maximum.

As indicated in Figure 2, the maximum enzyme activity was obtained when xylan 0.5% and CMC 0.2% + 0.3% xylan were used as carbon sources at day 4. The activity increased from 2593 U/ml culture filtrate (88%) at day 3 to 2947 U/ml culture filtrate (100%) at day 4. However, the xylanase production appeared to be suppressed in the presence of xylose, arabinose and glucose, with the greatest activities being 1975, 1798 and 973 U/ml culture filtrate at days 3 for xylose and 4 for both arabinose and glucose, respectively.

When tomato pomace was used as a carbon and nitrogen source, data indicated that the highest xylanase activity recorded being 1447 U/ml culture filtrate at day 4 (Figure 3).

When the crude xylanase from the culture filtrate of strain Ib 24D was electrophoresed, only one distinctive band exhibited a xylanolytic activity in the zymogram. This corresponded to a molecular weight of 58 kDa (Figure 4)

DISCUSSION

Xylanase from the active *Streptomyces sp.* (strain Ib 24D) had high activity at pH 5-8, with a maximum activity at pH 6.5. This result agrees with the finding of Antonopoulos et al. (2001) who reported a xylanase from *S. albus* ATCC 3005 with a pH optimum of 6.5. The broad pH range for strain Ib 24D xylanase activity agrees with the finding of Belfaqui et al. (2002), who stated that the xylanase produced by *S. achromogenes* strain 5028 (S1) exhibited high activity from pH 4.5 to 8.5.

The presence of high xylanase activity (>70%) over the temperature range 30-65°C, with a maximum at 60°C agrees with Belfaqui et al. (2002), who reported that the optimum temperature for xylanase activity in *S. achromogenes* 5028 (S1), *S. longisporus* ruber 4- 167 (S2) and *Streptomyces sp.* 8812 (S3) was between 60° and 65°C.

The finding that when glucose plus xylan were used as a carbon source, the activity was reduced by 67% of that with xylan alone might suggest catabolite repression of glucose. But experiments at molecular level would be required to explain this. Similar findings were reported by Ruiz-Arribas et al. (1997), who studied the xylanase produced by *S. halstedii* JM8. When xylose and xylan together were used as carbon source, the resultant high relative xylanase activity suggested that xylanase production was not subject to xylose repression (Srivastava and Srivastava, 1993). The slight reduction in activity when CMC was used with xylan as carbon source could be attributed to the production of CMCase, which would have hydrolyzed CMC to cellobiose, which has a repression effect on xylanase production (Ruiz-Arribas et al., 1997).

As noted in the results, the active *Streptomyces* strain Ib 24D is able to grow in tomato pomace medium and

show considerable xylanase activity. The maximum activity (1447 U/ml) compares with a maximum of 1900 U/ml after 3 days by *Bacillus sp* (Al-Oukily, 2000). Nascimento et al. (2003) have observed high endo- -1,4-xylanase titres (116 U/ml) when *S. malaysiensis* was grown in a larchwood medium. Kansoh et al. (2001) reported the utilization of a range of agricultural wastes, including corn cobs, wheat bran, peanut shells, sawdust, wheat straw and sugar cane bagasse and production of xylanolytic enzymes on these by a *Streptomyces* isolate. The presented data indicate that the *Streptomyces sp.*, strain Ib 24D is able to utilize tomato pomace, although the levels of xylanase activity in tomato pomace medium were lower than those in media containing CMC and xylan carbon sources and mineral salts. This could be attributed to the fact that tomato pomace contains simple sugars, which could result in the reduction or repression of xylanolytic enzyme production.

The single band exhibiting a xylanolytic activity (endoxylanase) revealed by zymogram analysis indicates that this enzyme (58 kDa) is relatively large compared with others. Poutanen (1988) reported that the molecular weight of endoxylanases from several *Streptomyces spp.* was in the range 25-50 kDa, and Tsujibo et al. (1992) determined that of an endoxylanase from *S. thermoviolaceus* to be 54 kDa.

Further work is recommended to purify and characterize the xylanase from *Streptomyces sp.*, strain Ib 24D and study the effect of this enzyme on other agricultural wastes. Assessment of the properties of this enzyme in biobleaching of pulp and paper is also recommended.

ACKNOWLEDGMENT

This research was financed by Jordan University of Science and Technology, grant No. 65/2002.

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