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Cellulase-Free Thermostable Alkaline Xylanase from Thermophilic and Alkalophilic *Bacillus* sp. JB-99

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Abstract The characterization of a partially purified, cellulase-free, thermostable alkaline xylanase from thermoalkalophilic *Bacillus* sp. JB-99 was investigated. The xylanase production was the highest when birchwood xylan was added to a medium containing finely powdered rice bran, showing 4,826 IU ml^{-1} of activity for 15 h of incubation. The partially purified xylanase exhibited an optimum temperature and pH at 70°C and 10, respectively. The enzyme was stable at pH 5–11 at 50°C. The xylanase activity was strongly inhibited by Hg^{2+}, while dithiothreitol, cysteine, and β-mercaptoethanol enhanced the activity.

Key words: *Bacillus* sp., biobleaching, cellulase-free xylanase, thermoalkalophilic

Hemicelluloses are the second most abundant polysaccharides in nature. The major constituents of hemicelluloses are the hetero-1,4-β-D-xylan and hetero-1,4-β-D-mannans (galactoglucomannan and glucomannan). Xylans are present predominantly in hardwoods and constitute more than 30% of their dry weight [17]. Xylanase has a number of biotechnological applications including its use in the pulp and paper industry. In pulp bleaching, xylanase degrades the xylan by interrupting lignin-carbohydrate bonds in the fiber pores, thereby enhancing the free-flow of bleaching chemicals into the fiber. In particular, cellulase-free xylanase has been used extensively in the pulp and paper industries, wherein only xylan is removed from the cellulose without affecting its fiber length [1]. Unbleached pulp is usually alkaline in nature, and pulping processes occur at slightly high temperatures (50–60°C). The advantages of using thermoalkaline xylanase for pulp biobleaching include to reduce the need for high temperatures and pH adjustment significantly, thereby offering technical and economical advantages.

Xylanases from thermophilic microorganisms have high temperature and near-neutral pH for optimum conditions, whereas alkaline xylanases have low temperature optima. Previously, thermoalkalophilic *Bacillus* sp. secreting thermoalkaline xylanases have been isolated from soil [14, 16]. Various investigators have stressed the importance of isolating microbes using traditional screening methods for novel enzymes [3, 15]. However, reports on the isolation of microorganisms which can secrete extracellular, cellulase-free, and thermoalkaline xylanase are rare, and at present, there is no report on a *Bacillus* sp. capable of producing extracellular, cellulase-free, and thermostable alkaline xylanase. The objective of the current study was to evaluate the properties of a novel xylanase isolated from a new thermoalkalophilic isolate, *Bacillus* sp. JB-99.

The bacterial strain *Bacillus* sp. JB-99 used in the present investigation was isolated in our laboratory [9]. *Bacillus* sp. JB-99 was grown in the following medium consisting of (g/l): rice bran, 10.0; oat spelt xylan, 1.0; $NaNO_3$, 5.0; K_2HPO_4, 5.0; NaCl, 5.0; $MgSO_4 \cdot 7H_2O$, 0.4; $CaCl_2 \cdot 2H_2O$, 0.2; and sodium carbonate (g/l) 5.0, which was sterilized separately and added to the above medium just before inoculation. The enzyme production was carried out in Erlenmeyer flasks (250 ml capacity) containing 50 ml of the medium. The flasks were inoculated with a 3% (v/v) inoculum of a culture which was grown overnight and placed on an orbital shaker at 180 rpm and 50°C for 18 h.

The cells were removed by centrifugation at 12,000 ×g for 10 min and the clear supernatant was kept at 4°C until use. Solid ammonium sulfate was added to the supernatant (2,500 ml) at 0–4°C until 20% saturation, and kept standing overnight at 4°C. The precipitate was removed by centrifugation at 4°C and 10,000 ×g for 15 min. To the resultant supernatant, solid ammonium sulfate was added slowly up to 80%

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saturation and kept at 0°C for 6 h. The precipitate was collected by centrifugation, and the resulting pellet was then dissolved and dialyzed against glycine-NaOH buffer (pH 10, 25 mM). The dialyzed solution was then used for further investigations.

The enzyme assays were carried out by measuring the amount of reducing sugar liberated from oat spelt xylan using the 3,5-dinitrosalycylic acid (DNS) method [13]. The reaction mixture contained 0.5 ml of 1% (w/v) oat spelt xylan in glycine-NaOH buffer (pH 10, 25 mM) and 0.5 ml of appropriately diluted enzyme. The reaction was conducted at 70°C for 10 min, and 1.0 ml of DNS was then added to arrest the reaction, followed by boiling for 5 min. Absorbance was measured at 540 nm and the xylanase activity was expressed in International Units (IU). One IU was defined as 1 μmole of xylose liberated per min per ml under the standard experimental conditions.

The cellulase activity was also measured by the DNS method. The spent medium and partially purified enzyme were both tested for cellulase activity. The oat spelt xylan was replaced with 1% (w/v) carboxymethylcellulose as the substrate, while the other assay conditions were similar to those described above.

The xylanase activity was measured at temperatures ranging from 30°C to 80°C. The enzyme activities were also tested at pHs ranging from 5 to 12. The temperature stability of the enzyme was studied by incubating the enzyme in the absence of a substrate at different temperatures ranging from 30°C to 80°C for 30 min at pH 10. The residual activity was determined under standard assay conditions. In order to determine the pH stability of the enzyme, the dialyzed enzyme was incubated at different pHs ranging from pH 5 to 12 for 4 h in the absence of a substrate. The samples were withdrawn, and the residual activity was determined under standard assay conditions.

The dialyzed enzyme solution was preincubated in the presence of *N*-bromosuccinamide (10 mM), urea (1 M), ethylenediaminetetraacetic acid (EDTA) (10 mM), dithiothreitol (DTT) (100 mM), L-cysteine (100 mM), and β-mercaptoethanol (100 mM) in glycine-NaOH buffer (pH 10, 25 mM) at 45°C for 2 h in the absence of a substrate. The dialyzed enzyme was preincubated in the presence of metal ions (10 mM) such as Ca^{2+}, Mg^{2+}, Hg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}, Fe^{2+}, Ni^{2+}, and Mn^{2+} in glycine-NaOH buffer at 45°C for 2 h in the absence of a substrate, and the residual activity was subsequently measured under standard assay conditions.

The thermoalkalophilic strain JB-99 was isolated from an enrichment culture containing oat spelt xylan. Strain JB-99 was selected from other isolates on the basis of the largest clearance zone exhibited. The strain grew in the pH range of 5–11 and temperature range of 25–65°C. The strain exhibited maximal growth at pH 10.0 and 50°C. Various investigators have reported on the isolation of *Bacillus* sp. from different habitats: A thermoalkalophilic

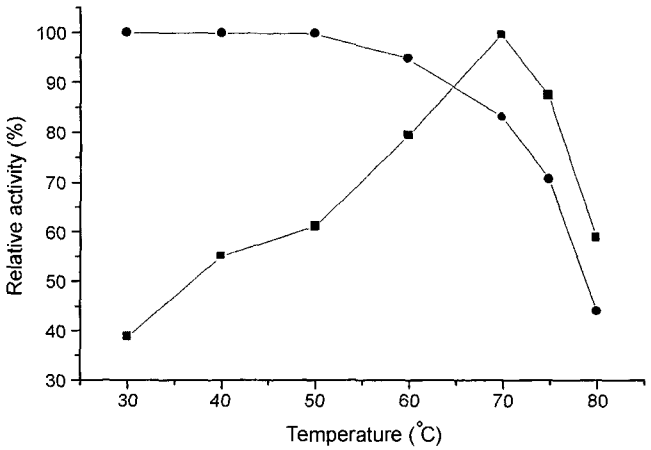

Fig. 1. Effect of temperature on the activity (■) and stability (●) of xylanase.

Bacillus sp. was previously isolated from a hot spring in Portugal [2], and an alkalophilic *Bacillus* sp. was from soil containing coconut fiber debris [1].

The new isolate, *Bacillus* sp. JB-99, was also found to grow in a chemically defined medium. In the current study, the xylanase secretion was growth associated and the maximum amount of cellulase-free xylanase (4,826 IU) was produced within less than 15 h in a shake flask culture (data not shown). Accordingly, the current findings revealed higher activity compared to earlier reports [8, 7, 6, 18].

The effect of temperature on the enzyme activity and stability is shown in Fig. 1. The enzyme exhibited its activity at temperatures between 30–80°C. Under standard assay conditions at pH 10.0, the optimum temperature was 70°C, at which the enzyme retained up to 85% activity after 30 min of heat treatment. The results from the current study are in agreement with those reported by other investigators [1, 14, 2]. Xylanase from alkalophilic fungus *Cephalosporium* having pH optima at 8.0 has been reported.

The xylanase was inactive at pH below 5.0. As shown in Fig. 2, the enzyme activity at pH above 10.0 decreased rapidly in comparison with that observed at the optimal pH. Similarly, the enzyme retained 100% of its activity up to 50°C, whereas it lost 10% and 20% of its original activity at 60°C and 70°C, respectively. The xylanase from *Bacillus* sp. retains 100% of its activity at 60°C after 6 h of incubation [2]. The spent medium or partially purified xylanse did not exhibit any amount of cellulase activity. Applications of xylanase free of cellulase activity are useful in the pulp and paper industries.

In the presence of 10 mM *N*-bromosuccinamide, the enzyme retained 85% residual activity; however, various microbial strains have earlier been reported to become inactive in the presence of *N*-bromosuccinamide [10]. The enzyme activity was completely inhibited by 10 mM Hg^{2+},

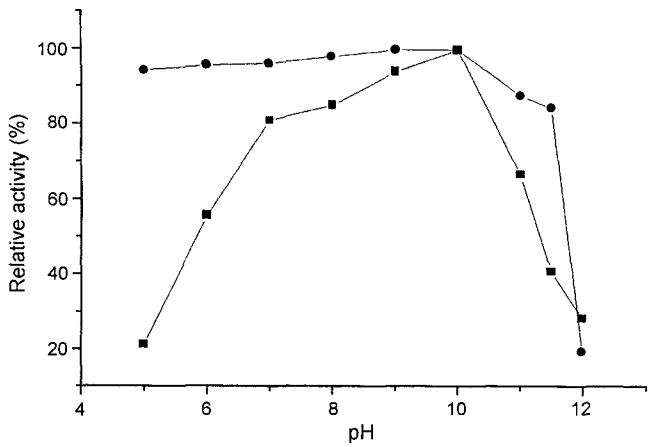

Fig. 2. Effect of pH on the activity (■) and stability (●) of xylanase.

indicating that the tryptophan residue appears to play a role in the catalytic processes of the xylanase reaction. Significant inhibitory effects were also observed in the presence of 10 mM Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, and Mn^{2+}. Similar results were also reported by earlier investigators [14, 4, 12, 11].

The effects of thiol compounds, like β-mercaptoethanol, dithiothreitol, and L-cysteine, on xylanase activity are shown in Table 1. These compounds strongly enhanced the xylanase activity. Earlier investigators reported similar observations regarding xylanase activity [5]. Based on these characteristics, the newly isolated thermoalkalophilic *Bacillus* sp. JB-99 was found to be a novel strain for the production of cellulase-free, thermostable, alkaline xylanase, which could extensively be used in the pulp and paper industries.

Table 1. Effect of inhibitors, metal ions, and activators on *Bacillus* sp. JB-99 xylanase activity.

Inhibitors/Metal ions	Concentration (mM)	Residual activity (%)
N-Bromosuccinamide	10	85.0
Urea	100	80.0
EDTA	10	42.0
$CaCl_2$	10	39.0
$MgSO_4$	10	70.67
$MgCl_2$	10	77.44
$HgCl_2$	10	0.0
$CuSO_4$	10	73.68
$ZnSO_4$	10	87.96
$CoCl_2$	10	54.88
$FeCl_3$	10	88.72
$FeSO_4$	10	42.10
$NiCl_2$	10	114.28
$MnCl_2$	10	27.86
$MnSO_4$	10	24.0
L-Cysteine hydrochloride	100	289.47
DTT	100	240.0
β-Mercaptoethanol	100	297.0

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