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Thermophilic xylanase isolated from the Xerophytic- *Cereus pterogonus* and *Opuntia vulgaris* plant sp.

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Abstract

A thermo stable xylanase was isolated and characterized from the cladodes of crude *Cereus pterogonus* and *Opuntia vulgaris* plant species. The enzyme was active in acidic pH 5.0 and temperature optimum for *Cereus pterogonus* was 60°C and 80°C, where as in the *Opuntia vulgaris* plant sp showed three temperature isoforms 50°C, 70°C and 90°C respectively. The metal ions 10mM Co^{2+}, Mn^{2+}, 5mM Ni^{2+} and 1mM Fe^{3+} showed enzyme activity at 60°C and 80°C for Cereus pterogonus plant sp, where as *Opuntia vulgaris* plant sp the maximum activity was observed on 50°C, 70°C and 90°C. The crude homogenate of both plant sp showed several protein bands on SDS-PAGE, but two activity bands on the zymogram analysis. The main aim of the present study was to check the xylanase activity on eukaryotic xerophytic plant sp, surprisingly the enzyme activity was observed in *Cereus pterogonus* and *Opuntia vulgaris* plant sp. This study may help protein engineers to design more efficient and acidophilic enzymes for use in different biotechnological processes. In future work the enzyme will purify and characterized for various industrial applications.

Introduction

Plant cell walls are the most abundant renewable source of fermentable sugars on earth (1) and are the major reservoir of fixed carbon in nature (2). Plant biomass comprises on average 23% lignin, 40% cellulose, and 33% hemicellulose by dry weight (3). Biomass is an alternative natural source for chemical and feed stocks with a replacement cycle short enough to meet the demand in the world fuel market (4). Complete degradation of the complex heteropolysaccharides requires the action of several main-chain and side-chain cleaving enzymes such as endoxylanase (endo-1,4-β-xylanase, E.C.3.2.1.8), β-xylosidase (xylan 1, 4-β-xylosidase, E.C. 3.2.1.37), α-glucuronidase (α-glucosiduronase, E.C. 3.2.1.139), α-arabinofuranosidase (α-L-arabinofuranosidase, E.C. 3.2.1.55) and acetylxylan esterase (E.C. 3.1.1.72) (5). The most abundant hemicellulosic polymers are xylans, made up of β-1,4-linked xylose units. Xylan constitutes about 20-40% of total plant biomass (6). Xylan represents an immense resource of biopolymers for practical applications accounting for 25-30% of the dry biomass of woody tissues of dicots and lignified

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tissues of monocots and occurs up to 50% in some tissues of cereal grains (7). Among the annual plants, hard woods and softwoods contain 20-25% and 7-12% xylan, respectively (8). Xylan has a high potential for degradation to useful end products. Endo-β-1, 4 xylanases catalyze the hydrolysis of the backbone of xylan to produce xylooligosaccahrides, which in turn can be converted to xylose by β-xylosidase (9). Currently, xylanases and cellulases together with pectinases account for 20% of the world enzyme market (10). One of the most important large-scale biotechnological applications of recent years is the use of xylanases as bleaching agents in pulp and paper industry (11). *Cereus pterogonus* and *Opuntia vulgaris* is a member of the family of xerophytic plants capable of growing in arid regions and has cellulose free thermo stable xylanase enzyme activity like that in microorganisms. Surprisingly there has been no report on the characterization of xylanases from plant sp. This work is therefore a first attempt at thermophilic xylanase identification from the eukaryotic, xerophytic *Cereus pterogonus* and *Opuntia vulgaris* plant sp.

Materials and Methods

Extraction of enzyme activity

Cereus pterogonus cladodes were weighed and chopped into small pieces after dethorning and removal of hard cuticular layer using a sharp edged razor blade and was homogenized in 0.05M citrate buffer, pH 5.0, containing 1mM PMSF to prepare a 20 % w/v tissue homogenate. The homogenate was filtered through a muslin cloth to remove cell debris. The filtrate was centrifuged at 10,000 x g for 20 minutes in a sorvall RC-5C refrigerated centrifuge using SS-34 rotor to obtain a clear supernatant that was used for enzyme activity.

Assay for xylanase activity

Assay for xylanase activity was performed using 1% soluble oat spelt xylan (Sigma) as substrate in 0.05M citrate buffer, pH 5.0. The reaction mixture consisted of 1.8 ml of substrate and 0.2 ml of enzyme source. The mixture was incubated in a water bath at 50^{o}C for 5 min. The released reducing sugar was measured by the 3,5-dinitrosalicylic acid (DNSA) method (12). The reaction was stopped by adding 3 ml of DNSA acid reagent. A reddish brown color developed after placing the reaction tubes in a boiling water bath for 5 min. After cooling the reaction tubes to room temperature, the absorbance was measured at 540 nm with xylose as the standard. One unit (U) of xylanase activity was defined as the amount of enzyme that released 1 µmol xylose/min/ml under the stated conditions.

Determination of protein content

Protein content of the samples was measured by the method of Bradford (13) using bovine serum albumin as a standard.

Effect of pH and Temperature on xylanase activity

The optimal pH for xylanase activity was determined by assaying the enzyme activity in the pH range 3.0-11. Three different buffers (0.05M) were used for this study: Citrate buffer (pH 3.0-6.0) phosphate buffer (pH 6.0-8.0) and glycine-NaOH buffer (pH 8.0-11). The enzyme samples were incubated along with the substrate in the different pH buffers at room temperature for 10 minutes. The residual enzyme activity was measured as described earlier (12) similarly the optimum temperature for the xylanase was identified by assaying the enzyme activity at different temperatures in the range 30 -100^{o}C at intervals of 10^{o}C. The reaction mixture was incubated with substrate for 10 min at each temperature before assaying the enzyme activity as described earlier (12).

Effect of Metal ions on xylanase activity

Studies investigating the effect of metal ions on the xylanase enzyme activity were carried out employing divalent cations (Co^{2+}, Mn^{2+} etc.) in their chloride form and analysis for enzyme activity in 0.05 M Citrate buffer, pH 5.0 for 1 h employing a fixed amount of the enzyme. The metal ions were used at 1, 5, 10 mM concentration in independent reactions. The xylanase activity was also determined in the

absence of the metal ion to serve as a control. The enzyme activity was assayed as described earlier (12)

Determination of enzyme molecular mass

The molecular mass of the xylanase was estimated employing SDS-PAGE electrophoresis. SDS-PAGE (10%) was performed as described by Laemmli (14). Proteins bands were then visualized by staining with coomassie brilliant blue in methanol (10%) acetic acid (7%) and destaining the gel using the same solvent.

Generation of zymogram for xylanase activity

A zymogram for xylanase activity was developed using SDS-PAGE electrophoresis as described by Blanco and Pastor (15). Oat spelt xylan (0.2%) was included in the gel prior to polymerization. After electrophoresis, gels were soaked in 2.5% Triton X-100 for 30 min, washed in citrate buffer, pH 5.0 for 30 min, and incubated at 60^{o}C for 15 min in the same buffer. Gels were then stained with 0.1% Congo red for 15 min and washed with 1 M NaCl until xylanase active bands were visible. Immersion of the gels in 5% acetic acid yielded the background as dark blue.

Results and Discussion

In recent years the use of xylanase in various industries had increased significantly, since then researchers worldwide have focused their attention on thermophilic xylanase from newer sources, therefore the present study deals with thermophilic xylanase isolation from xerophytic *Cereus pterogonus* and *Opuntia vulgaris* plant sp. This study identified many advantages that included stability on storage, low cultivation cost, and very low risk of contamination as compared xylanase was isolated from microbial sp. The profile was measured standard assay procedure, both plant sp showed maximum enzyme activity at pH 5.0 only, making this an acidic xylanase (Fig.1a & 1b). Most fungal xylanases showed higher activity under slightly acidic conditions. Stable xylanases at acidic pH (pH 1.0–5.0) have been reported in fungi such as Aspergillus kawachii, Cryptococcus sp. S-2, and Penicillium sp. 40 (16, 17), but the number of acidophilic xylanases that have been reported thus far were much lower than those of alkaliphilic xylanases. In addition, the xylanase isolated from *Cereus pterogonus* and *Opuntia vulgaris* is the first acidophilic xylanase identified from a xerophytic

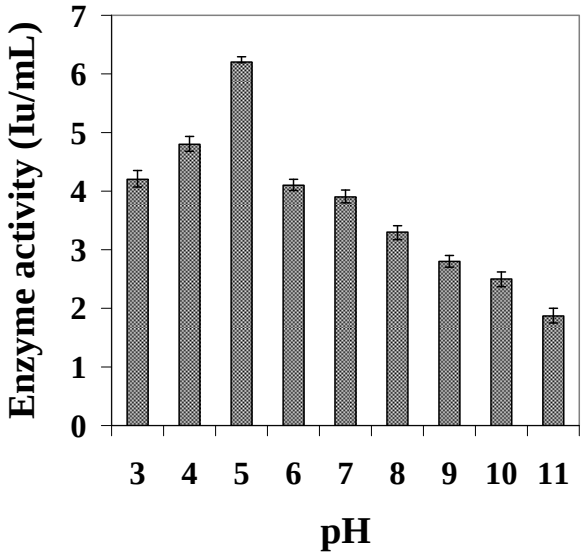

Fig.1 (a). Effect of pH profile of *Cereus pterogonus* xylanase enzyme activity from 10,000 x g crude homogenate. Values are the mean of three independent assays.

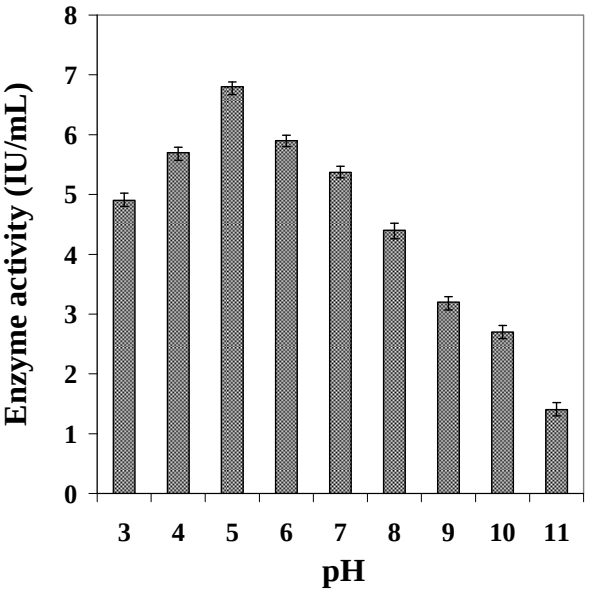

Fig 1 (b). Effect of pH profile of *Opuntia vulgaris* xylanase enzyme activity from 10,000 x g crude homogenate. Values are the mean of three independent assays.

plant sp.

The effect of temperature on the activity of *Cereus pterogonus* and *Opuntia vulgaris* xylanase was determined at pH 5. The temperature profile of *cereus pterogonus* xylanase enzyme showed maximum enzyme activity at 60°C and 80°C, where as *Opuntia vulgaris* xylanase enzyme showed maximum enzyme activity at 50°C, 70°C, and 90°C (Fig. 2a & 2b). From this results *Cereus pterogonus* plant have two temperature isoforms and *Opuntia vulgaris* have three temperature isoforms. Xylanase from the hyper thermophilic crenarchaeon sulfolobus solfataricus strain MT4 showed the optimum temperature 90°C (18). Winterhalter et al., reported xylanase isolated from *Thermotoga maritima* showed optimum temperature of 92 and 105^{0}C respectively, at acidic pH (19). Similarly, xylanase isolated from *Thermotoga neapolitana* had optimum temperature of 102°C at pH 5.5 (20). The xylanase isolated from Ceratocystis paradoxa, Aspergillus strain, Trichoderma reesei DB1 showing the maximum activity above 80°C (21, 22, 23).

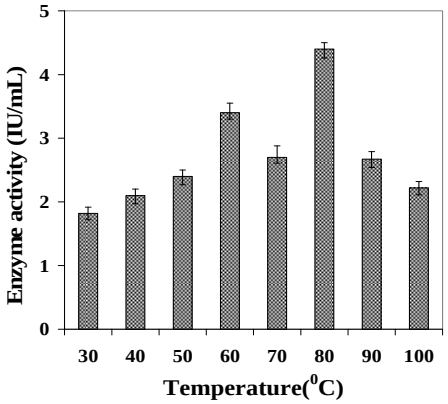

Fig 2 (a). Effect of temperature profile of *Cereus pterogonus* xylanase enzyme activity from 10,000 x g crude homogenate. Values are the mean of three independent assays.

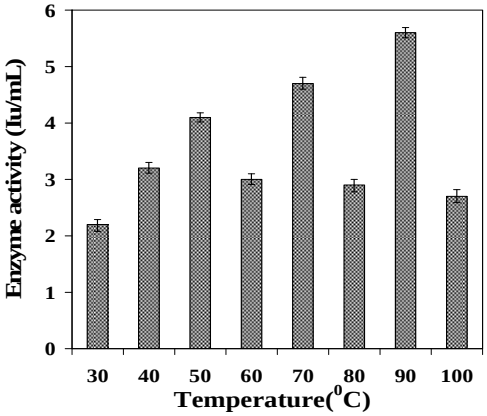

Fig 2 (b). Effect of temperature profile of *Opuntia vulgaris* xylanase enzyme activity from 10,000 x g crude homogenate. Values are the mean of three independent assays.

The effects of different divalent cations at different concentrations (1mM, 5mM and 10mM) on the xylanase enzyme activity from *Cereus pterogonus* and *Opuntia vulgaris* was showed in figure (3 & 4 a-d). Results showed that maximum enzyme activity was observed on 10mM Co^{2+} and Mn^{2+} treated enzyme solutions of Cereus pterogonus 60°C, 80°C and 50°C, 70°C and 90°C for *Opuntia vulgaris*. The metal ion 5mM Ni^{2+} and 1mM Fe^{3+} showed maximum enzyme activity on 60°C, 80°C for *cereus pterogonus* plant sp, where as *Opuntia vulgaris* showed maximum enzyme activity on 50°C, 70°C and 90°C respectively. Further work using more metal ions, their analogues and combinations there of would be necessary to ascertain the mode of action of xylanase. However, our results are similar to those reported by others, for example, Kim et.al. (24).

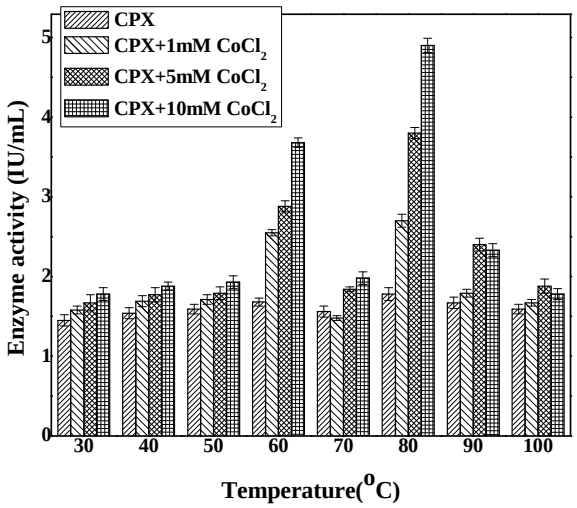

Fig 3 (a). Effect of $CoCl_2$ on *Cereus pterogonus* xylanase enzyme activity using 10,000 x g supernatant at different temperatures. Values are the mean of three independent assays.

SDS-PAGE of the crude *Cereus pterogonus* and *Opuntia vulgaris* xylanase enzyme showed that several protein band (Fig.5a) The zymogram for xylanase activity for these two plants sp showed two activity bands (fig.5b).

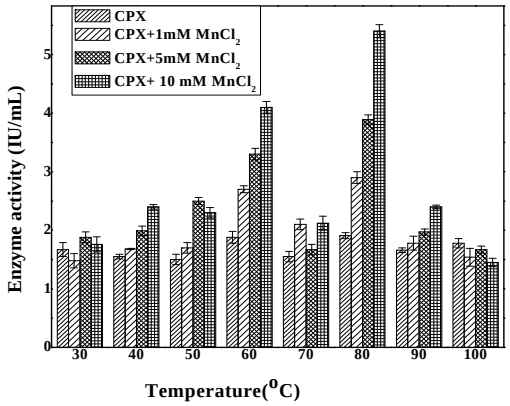

Fig 3 (b). Effect of $MnCl_2$ on *Cereus pterogonus* xylanase enzyme activity using 10,000 x g supernatant at different temperatures. Values are the mean of three independent assays.

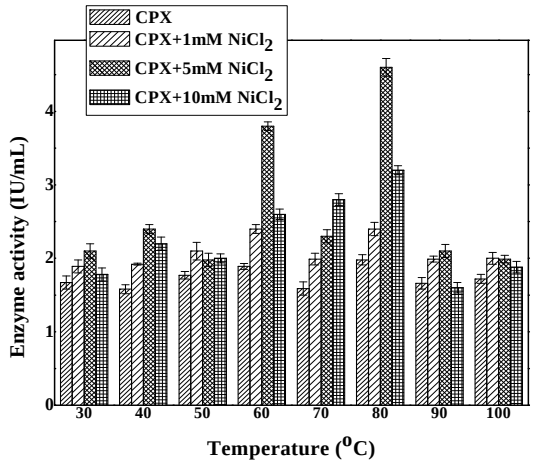

Fig 3 (c). Effect of $NiCl_2$ on *Cereus pterogonus* xylanase enzyme activity using 10,000 x g supernatant at different temperatures. Values are the mean of three independent assays.

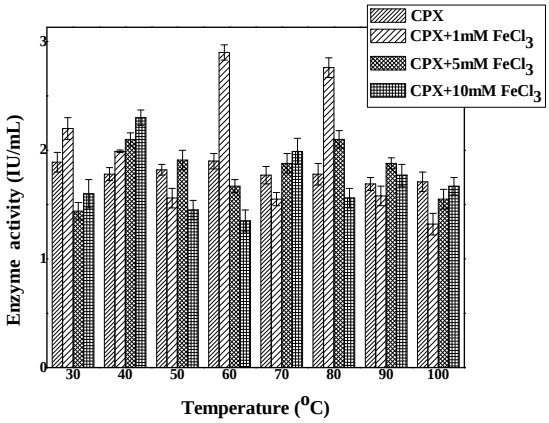

Fig 3 (d). Effect of $FeCl_3$ on *Cereus pterogonus* xylanase enzyme activity using 10,000 x g supernatant at different temperatures. Values are the mean of three independent assays.

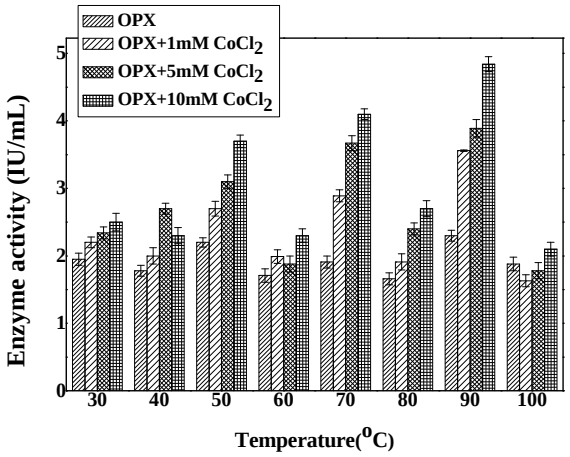

Fig 4 (a). Effect of $CoCl_2$ on *Opuntia vulgaris* xylanase enzyme activity using 10,000 x g supernatant at different temperatures. Values are the mean of three independent assays.

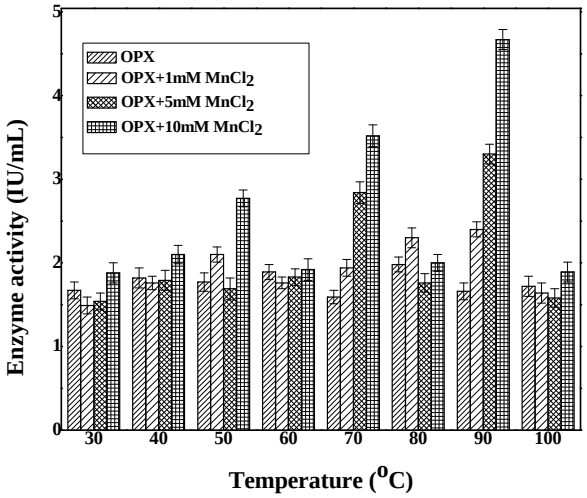

Fig 4 (b). Effect of $MnCl_2$ on *Opuntia vulgaris* xylanase enzyme activity using 10,000 x g supernatant at different temperatures. Values are the mean of three independent assays.

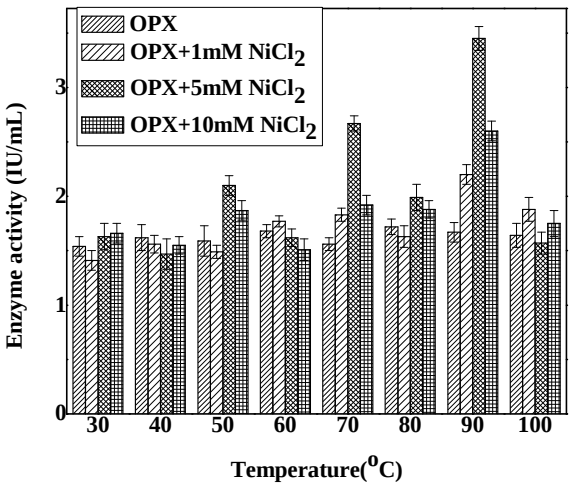

Fig 4(c). Effect of $NiCl_2$ on *Opuntia vulgaris* xylanase enzyme activity using 10,000 x g supernatant at different temperatures. Values are the mean of three independent assays.

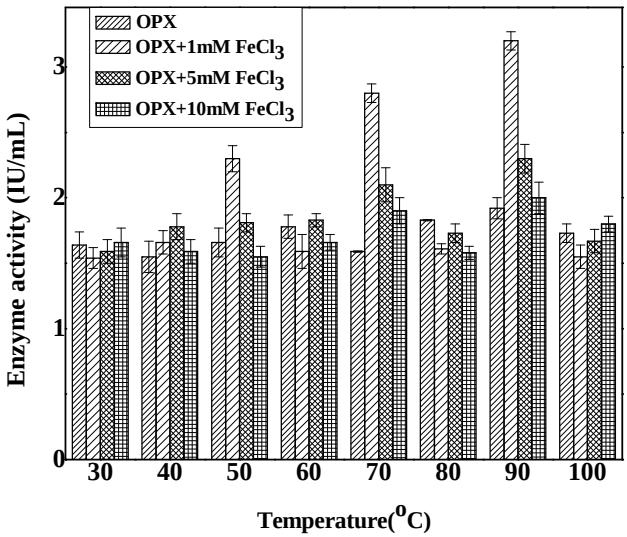

Fig 4 (d). Effect of $FeCl_3$ on *Opuntia vulgaris* xylanase enzyme activity using 10,000 x g supernatant at different temperatures. Values are the mean of three independent assays.

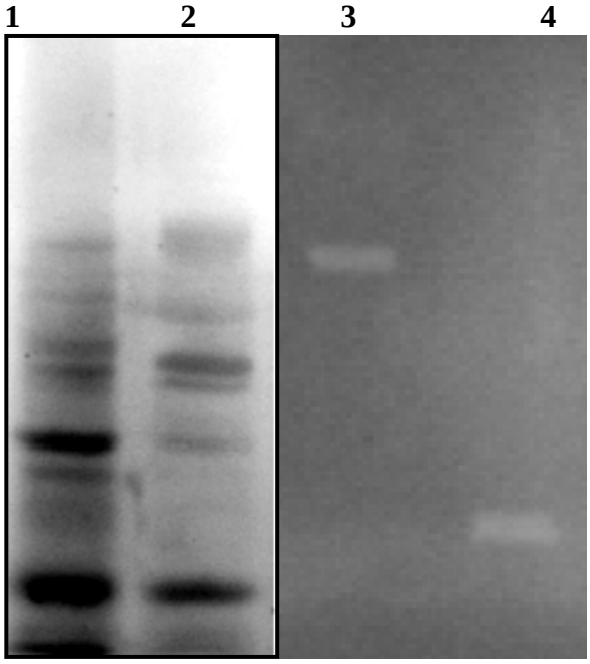

Fig.5. SDS-PAGE and Activity stain gel assay for xylanase activity

A – SDS-PAGE (7.5% gel) of *Cereus pterogonus* and *Opuntia vulgaris* cladode crude homogenate.

B – Native PAGE (7.5% gel) of the xylanase enzyme activity in *Cereus pterogonus* and *Opuntia vulgaris* cladode crude homogenate supernatant stained for xylanase activity activity.

Lane 1 and 3 – Protein and enzyme activity profile of *Cereus pterogonus* crude homogenate.

Lane 2 and 4 - Protein and enzyme activity profile of *Opuntia vulgaris* crude homogenate.

The main aim of the present study was to check the xylanase activity on eukaryotic xerophytic plant sp, surprisingly the enzyme activity was observed in *Cereus pterogonus* and *Opuntia vulgaris* plant sp. The enzyme was highly active on acidic pH, hyper thermophilic conditions and more stable for metal ion as a cofactor. These data can provide useful information on stability of enzymes. The *Cereus pterogonus* and *Opuntia vulgaris* -derived xylanase can be used as a good model protein for investigation of the molecular basis of acidophilicity of thermostable enzymes. This study may help protein engineers to design more efficient and acidophilic enzymes for use in different biotechnological processes. These data will also add information on *Cereus pterogonus* and *Opuntia vulgaris* derived xylanase for further comparative studies, as such data are limited on this useful plant sp. In future studies this enzyme will purified, and characterized from these two plant sp give more information.

Acknowledgement

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