

## Purification and Biochemical Characterization of Xylanases from *Bacillus Pumilus* and their Potential for Hydrolysis of Polysaccharides

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### Abstract

Extracellular xylanases free of cellulase produced by the alkalophilic bacteria *Bacillus pumilus* was purified to homogeneity throughout the precipitation with  $(\text{NH}_4)_2\text{SO}_4$ , Q-Sepharose chromatography and characterized. The purified xylanases were proteins, with molecular mass ~14 kDa (Xyl 1), ~ 35 kDa (Xyl 2) and ~ 60 kDa (Xyl 3) as determined by SDS-PAGE. The optimal temperature and pH for the action of the enzyme were at 50°C and 7 respectively. They exhibited thermal stability over a range of 20 to 40°C at pH-7 and has retained 85 % at 60°C. The activity strongly inhibited by 10 mM of  $\text{Hg}^{2+}$ , SDS and  $\text{Fe}^{2+}$ . The xylanase exhibited  $K_m$  and  $V_{max}$  values were 4.0 mg/ml, 5000  $\mu\text{mol}/\text{min}/\text{mg}$  protein (Xyl 1) as well as 3.5 mg/ml, 3448  $\mu\text{mol}/\text{min}/\text{mg}$  of protein (Xyl 2) for oat spelt xylan.

**Keywords:** Alkaline-stability; *Bacillus pumilus*; Purification; Solid-state cultivation; Endoxylanase; Hydrolysis

### Introduction

Hemicelluloses which act as the cementing material between cellulose and lignin, it consists of pectin, arabinoxylan and xylan. Xylan is the principal component with  $\beta$ -1, 4-linked-D-xylopyranosyl residues as backbone and different substitute groups in the side chain. Several enzymes are required for complete hydrolysis and assimilation of xylans, including  $\beta$ -endoxylanase,  $\beta$ -xylosidase, and xylose isomerase of which  $\beta$ -endoxylanase received the broader attention among these enzymes. Xylanases have potential applications in food, feed, chemical, pharmaceutical and paper industries. These enzymes produced by several bacterial and fungal species. There were reports related endoxylanase production by *Bacillus*, with different molecular masses [1]. Solid-state cultivation (SSC) on wheat bran as a substrate is an effective procedure for xylanase production. However, SSC has gained renewed interest in recent years for the production of many enzymes due to lower operation costs and energy requirements [2]. The thermotolerant alkalophilic bacteria *B. pumilus* has attracted attention as a rich source of xylanolytic enzymes. *B. pumilus* was reported previously as good producer of cellulase-free endoxylanase [3]. This paper focuses on the purification and biochemically characterize extracellular xylanases produced by *B. pumilus* grown on SSC using wheat bran. Until now there were no reports related to low molecular weight xylanases from this bacterial species by SSC. Of the three isoforms purified Xyl-1 and Xyl-2 exhibited high activity against hydrolysis of plant polysaccharides.

### Materials and Methods

#### Microorganism and culture conditions

*B. pumilus* was isolated from forest soil of Kerala and maintained at 4°C on Xylan agar slants [3]. For xylanase production *B. pumilus* was cultivated in 250 ml Erlenmeyer flasks containing 10 g of fresh wheat bran as solid substrate. It was moistened with basal medium of composition (g/l)  $\text{KH}_2\text{PO}_4$ , 2 and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.4, in the ratio 1: 2.5 and autoclaved at 15 lbs for 45 min. The cooled medium was inoculated with 18 h grown culture spores and incubated at 35°C. After fermentation process, the protein content of the solid-state culture flasks was extracted with distilled water. For extraction it was placed

on a rotary shaker (150 rpm) at room temperature for 1 h. Content filtered through a muslin cloth and filtrate was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant used as crude enzyme sources for further studies.

#### Xylanase purification

The purification steps were all performed at room temperature since previous experiments showed no difference in enzyme stability at 4 or 25°C. Firstly, proteins were precipitated between 20 and 70% of ammonium sulphate saturation and centrifuged at 10,000 $\times$ g for 20 min. The pellet was resuspended in 50 mM sodium phosphate buffer, pH 7 and dialyzed for 18 h against this buffer. Q-Sepharose F column (2.5  $\times$  12 cm) which was pre-equilibrated with 10 mM Tris - HCl buffer (pH 8.0). The column was washed with 200 ml of the respective buffers at a flow rate of 3.0 ml/min followed by the sample elution with 5-bed-volume of NaCl salt in gradient (0- 1.0 M) in the equilibrating buffers. The proteins were eluted with a NaCl gradient (0 - 1.0 M) in the same buffer. The active xylanases fractions were collected and concentrated by lyophilisation. One milliliter fractions were collected and used to measure absorbance at 280 nm and xylanase activity. Three peaks with xylanase activity were eluted and pooled, dialyzed against sodium phosphate buffer and used for biochemical characterization.

#### Enzyme assay and protein determination

Xylanase activity was assayed using 0.5 % (w/v) of oat spelt xylan as substrate. Reaction mixtures contained 0.1 ml enzyme and 0.9 ml substrate in 100 mM sodium phosphate buffer pH 7. The mixture was

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incubated at 50°C, and after 10 min the released reducing sugars were estimated with 3-5- dinitrosalicylic acid (DNS), using xylose as standard [2]. One unit (U) of xylanase activity defined as the amount of enzyme that released 1µmol of reducing sugar equivalent to xylose / minute, under the standard assay conditions. Endoxylanase production by SSC expressed as U/g DBB (dry bacterial bran). Total soluble protein was estimated using bovine serum albumin as standard and also by direct method where the absorbance taken at 280 nm, the free Tyrosine (Tyr) and Tryptophan (Trp), uric acid, and bilirubin interfere at 280 nm [4].

### Polyacrylamide gel electrophoresis

Electrophoresis of proteins, PAGE and Non-denaturing SDS-PAGE was carried out using a 12% gel [5]. Molecular mass markers used were: phosphorylase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and  $\alpha$  - lactalbumin (14.4 kDa). Proteins were stained with silver (SDS-PAGE) or Coomassie Blue (PAGE). Xylanase activity in PAGE gels was detected by overlaying on the gel with 2 % agarose and 0.5 % oat spelt xylan dissolved in 100 mM sodium phosphate buffer, pH 7 and incubated for 30 min at 50°C.

### Characterization of xylanase

Optimal temperature for endoxylanase activity was determined between 30-65°C. Thermal stability estimated by incubation the enzyme at different temperatures ranging 25-65°C for 90 min and measuring the residual activity. Optimal pH was determined by using different buffers ranging between 5 and 11 (citrate phosphate buffer, pH 5 to 7, phosphate buffer 6 to 8 and Glycine - NaOH buffer, 9 to 11 at 100 mM). pH stability determined by incubating equal volume of enzyme solution with different buffers ranging between 5 and 11 at 50°C for 90 min residual activity estimated. The effect of various compounds on xylanase activity was determined by incubating the purified enzyme in the presence of 10 mM in 100 mM sodium phosphate buffer pH 7 of each compound for 30 min before the reaction with the substrate. Residual activity was expressed as the percentage of the activity observed by the standard assay described above.

### Kinetic parameters

The effect of substrate concentration, ranging from 2.0 to 20 mgmL<sup>-1</sup>, on xylanase activity was evaluated under optimal assay conditions (50°C and pH 7.0). Xylan presents low solubility, so higher concentrations could not be used. The kinetic parameters of Michaelis-Menten constant,  $K_m$ , and maximal reaction velocity,  $V_{max}$ , were estimated by linear regression according to the Lineweaver and Burk double-reciprocal plot [6].

### Hydrolysis of plant polymeric substrate

In order to assess the enzyme ability to hydrolyze several different agro-industrial residues, reaction mixtures containing purified enzyme solution and 50 mg dry weight of selected agro-industrial residues (rice bran, rice straw, wheat bran, oat spelt xylan, birch wood xylan, beech wood xylan, carboxymethyl cellulose and filter paper) suspended in 100 mM sodium phosphate buffer pH 7.0 were incubated for 1 h at 60°C and 100 rpm. The reaction was stopped by placing the mixture in boiling water for 5 min, and then centrifuged at 8000 x g for 10 min. Reducing sugars liberated by hydrolysis of these substrates were quantified by the dinitrosalicylic acid method.

## Results and Discussion

### Purification of endoxylanase

High levels of xylanolytic activity (about 159.96 U/ml), but less than 0.0017 U/ml of cellulolytic activities was exhibited by crude filtrates of cultures from *B. pumilus* grown on wheat bran [3]. The xylanase from *B. pumilus* precipitated using ammonium sulphate (0-70 % saturation) to yield an active pellet. The active pellet was dialyzed and used as starting material for further purification using Q- Sepharose fast flow column. The profile of elution from this culture filtrate exhibited three peaks showing xylanase activities (Figure 1), these fractions were designated as Xyl 1 and Xyl 2 and Xyl 3. A summary of a representative purification protocol is shown in Table 1.

The three purified enzyme preparations appeared homogeneous, since they migrated as a single protein band under non-denaturing

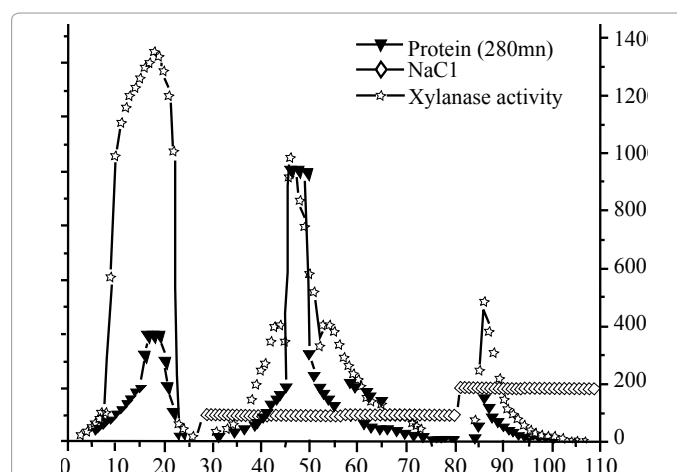


Figure 1: Elution profile of xylanase activity from culture filtrate of *B. pumilus* grown by SSC on wheat bran. Endoxylanase activity, protein absorbance at 280 nm, and NaCl gradient from Q- Sepharose Fast Flow column pre-equilibrated with 10 mM Tris HCl buffer (pH 8.0). The peaks indicate the pools of fractions 1, 2 and 3 used for biochemical characterization of the xylanases.

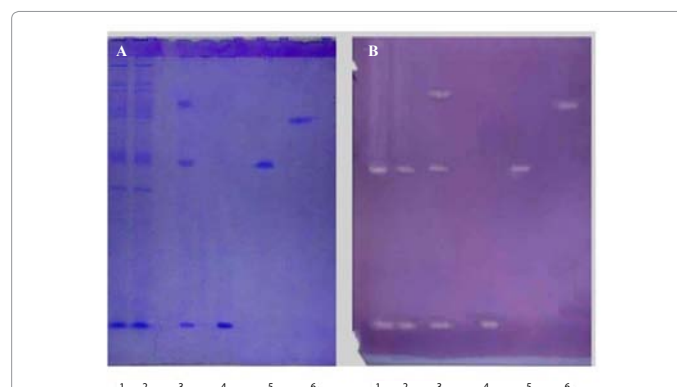


Figure 2: Native PAGE and zymogram analysis of endoxylanases from the extracellular culture filtrate of wheat bran cultivated with *B. pumilus*. (A) Native gel stained with Coomassie brilliant blue R-250-stained gel. (B) Zymogram analysis of gel incorporated with oat spelt xylan and stained with Congo red. Zymogram prepared by overlaying the activity gel on the Native - PAGE and incubating for 30min at 50°C. Both active gel and zymogram the lane as follows: Lane 1-crude extract; lane 2- (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fractions; lane 3- purified endoxylanase fraction pooled from Q- Sepharose column; lane 4- Xyl 1; Lane 5- Xyl 2 and Lane 6- Xyl 3.

Purification steps	Fraction (ml)*	Total protein (mg)**	Total activity (Units)	Sp. activity (U mg <sup>-1</sup> protein)	Purification (fold)	Yield (%)
Crude	200	148	31992	216.1	1	100
Ultra filtration	110	75.2	22394.4	297.79	1.3	70
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> Precipitate	80	22.8	13436.64	589.32	2.7	42
DEAE –Sephrose Xyl 1	20	2.01	1357.1	675.14	3.12	4.2
xyl 2	10	4.28	980.8	229.15	1.06	3.1
Xyl 3	6	0.81	470.28	580.59	2.68	1.46

\*, \*\* values represents the mean of the values from three independent experiments, with a standard deviation of  $\pm 5\%$

**Table 1:** Summary of purification of xyl 1, 2 and 3 from *bacillus pumilus*.

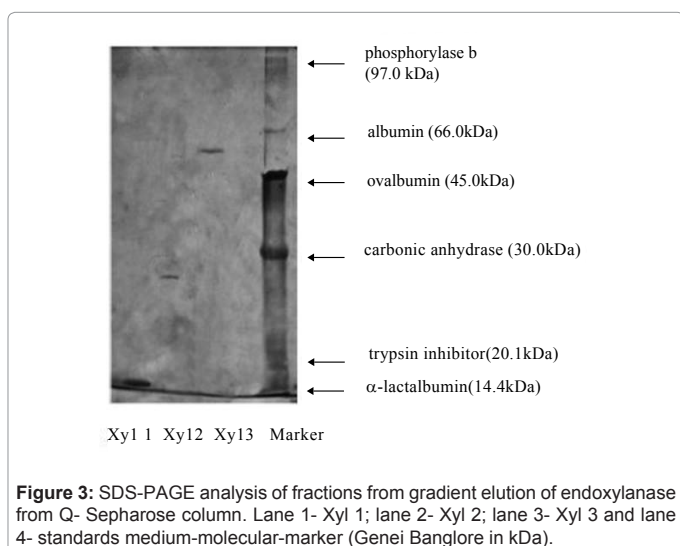
condition (PAGE). The xylanases were also assayed for activity on the gel, after PAGE, and the activities were synchronized with the protein band (Figure 2 A and B). The purified xylanases preparation was represented in the form of a single band on SDS - PAGE (Figure 3). The molecular mass of the purified xylanases was estimated to be approximately 14, 35 and 60 kDa for Xyl 1, Xyl 2 and Xyl 3 respectively, using SDS-PAGE. Purification and characterization are the most important fundamentals for thriving biotechnological application of enzymes. Purification of xylanase from microorganisms normally needs precipitation of the active protein followed by two to three chromatographic steps. Xylanase with molecular mass of 40 kDa purified to homogeneity by (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub> fractionation and anion-exchange chromatography from thermo alkalophilic *Bacillus* sp. strain TAR-I [7]. Xylanase II from *A. versicolor* was purified 28 - fold with DEAE- Sephadex and HPLC GF-510 gel filtration [8]. Crude xylanase (80 %) precipitated by acetone from wild strain of *A. nidulans* was subjected to SDS-PAGE, which revealed the occurrence of four isoxylanases with molecular weights of 50, 43, 20 and 18 kDa [9]. Only few reports were there related to low molecular weight xylanase that has application in paper pulp industry, due to its high penetration in the Kraft pulp that help in the easy removal of reprecipitated xylan. In the present study ammonium sulphate precipitated xylanase *B. pumilus* was purified to homogeneity following single step anion exchange chromatography. Ion exchange chromatography can easily scale up in comparison to other filtration or precipitation techniques related to procedure. Current procedure intended for high through put purification of xylanases for commercial application.

The molecular weights of purified xylanases of *B. pumilus* were observed to be 14, 35 and 60 kDa and are within the range of molecular weight for xylanases i.e. 11-85 kDa [10, 11]. Comparative analysis of molecular weight of Xyl 1 and its pI- 8 (data not shown) of purified xylanases from *B. pumilus* indicated that it follows dichotomous pattern and fall in Group 1 xylanases of low molecular and basic protein based on classification suggested by Wong [10]. Two xylanases from *B. subtilis* purified by native PAGE and homogenization extraction analyzed using HPLC. Both xylanase were endo acting, designated xyl I and xyl II (activity range pH 5.0 to 9.0 at 50°C with optimum activities at pH - 7 and 50°C) [4]. *A. versicolor* produces a xylanolytic complex with two components, the minor component designated xylanase II (5 days in 1 % wheat bran) a monomeric glycoprotein with molecular mass of 32 kDa with 14.1 % of carbohydrate content. Both xylanase isolated reported to have optimum activity at pH 6 - 7 and temperature 55°C [8].

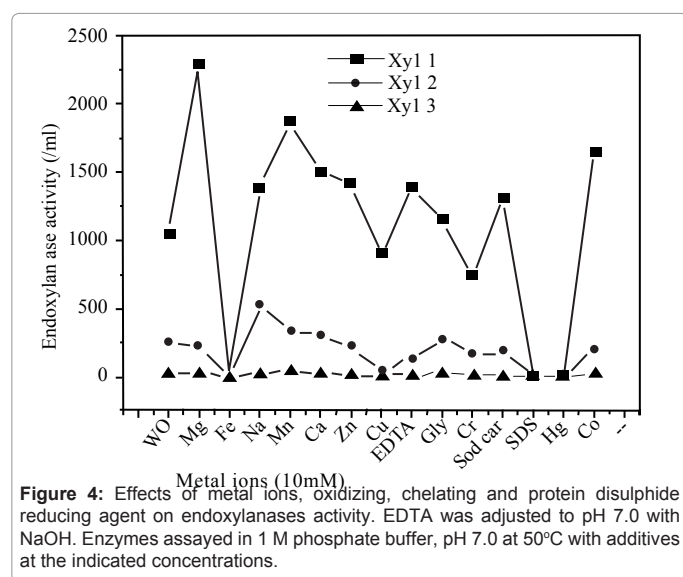
### Characterization of xylanases

Important external factors that influence enzyme activity were temperature and pH. The purified xylanase, Xyl 1 and 2 exhibited maximum activity at temperature 50°C and retained 93 % of activity at 55°C where as Xyl 3 active at 55°C at pH 7. Temperature and pH stability of the enzyme are very important factors when we intend to study the industrial importance of the enzyme. The isoforms were stable at 20 to 40°C and retained 90 % activity at 50°C and 85 % at 60°C. The purified xylanase Xyl 1 was active at pH 7 in phosphate buffer and Xyl 2 and 3 at pH 6 with citrate phosphate and phosphate buffer respectively. Xyl 1 also exhibited activity at pH 10 and 11 while Xyl 2 and 3 were inactive at this pH. Xyl 1 is stable at pH 7 and retained 90 % at pH 6, 8 and 9 in addition to it has retained 82 % at pH 10 and 11. Xyl 2 stable at pH 6, 7 and 8, retained 85 % activity at 9 and 10 additionally 80 % activity at pH 5 and 11. Xyl 3 is steady at pH 7 and retained 62 % activity at pH 8, inactive at pH 11 and very low activity at other pHs. Xylanase from *Streptomyces*. sp AMT- 3 was active over a range of temperature 55 - 60°C and pH - 6 [12]. Endoxylanase from *Rhizopus oryzae* have optimum pH and temperature at 4.5 and 55°C, respectively [13]. Xylanases (Xyl Ia and Ib) from *Myceliophthora* sp. IMI 387099 were optimally active at 75°C and at pH 6.0 and stable at pH 9.2 at 60°C for 2 h, but varying at pH 6.0 and above 50°C [14]. Difference in pH and temperature tolerance for xylanase excreted might be due to the effect of different enzymes mixtures excreted, and / or the post - translation modifications in xylanase excretion process, such as glycosylation, that improve stability in more extreme pH and temperature conditions [15].

The effects of potential inhibitors or activators on purified xylanases were shown in Figure 4. The results demonstrated some differences between Xyl 1, Xyl 2 and Xyl 3. Although both enzymes suffered inhibition by almost all the ions tested, activity of Xyl 1 was



**Figure 3:** SDS-PAGE analysis of fractions from gradient elution of endoxylanase from Q- Sepharose column. Lane 1- Xyl 1; lane 2- Xyl 2; lane 3- Xyl 3 and lane 4- standards medium-molecular-marker (Genei Bangalore in kDa).



**Figure 4:** Effects of metal ions, oxidizing, chelating and protein disulphide reducing agent on endoxylanases activity. EDTA was adjusted to pH 7.0 with NaOH. Enzymes assayed in 1 M phosphate buffer, pH 7.0 at 50°C with additives at the indicated concentrations.

more sensitive than Xyl 2 and 3. Addition of Hg<sup>2+</sup> drastically inhibited xylanase activities in all three, suggesting the existence of thiol groups at the active site of the enzymes. Hg<sup>2+</sup> is known to react with protein sulphhydryl groups as well as histidine and tryptophan residues as well as presences of cysteine residue in or close to the active site of the enzyme [16]. Addition of Fe<sup>2+</sup>, SDS also completely inhibited xylanase activity. EDTA did not considerably affect the activities, signifying that metal ions were not required for enzyme activities. The enzyme activities were stimulated by addition of Mg<sup>2+</sup> in Xyl 1 and Mn<sup>2+</sup> in other two fractions [17]. These ions seem to affect enzyme activity by altering the conformation of the polynucleotide complexes.

Xylanase from *Bacillus* sp. SSP-0 completely inhibited by Hg<sup>2+</sup> [18] and in *Bacillus subtilis* Mn<sup>2+</sup>, ions enhanced xylanolytic activities to 2.7-fold whereas Fe<sup>3+</sup> completely inhibited [4]. Xylanase II from *A. versicolor* show thermo inactivation at 50°C with a biphasic curve, the ions Hg<sup>2+</sup>, Cu<sup>2+</sup> and the detergent SDS were strong inhibitors, while Mn<sup>2+</sup> ions and dithiothreitol were stimulators of the enzyme activity [8]. *B. circulans* BL53 exhibited considerable decrease in activity in the presence of Cu<sup>2+</sup>, Zn<sup>2+</sup>, Ba<sup>2+</sup>, and EDTA, while Ca<sup>3+</sup>, Mn<sup>2+</sup> and Mg<sup>2+</sup> produced discrete inhibitions. These effects might preclude the use of this enzyme in industrial processes where these chemicals are present in relevant concentrations [19]. Stimulatory effects, obtained with Fe<sup>2+</sup> and Co<sup>2+</sup> suggesting that the xylanases from *B. coagulans* are metalloproteins, probably with Co<sup>2+</sup> or Fe<sup>2+</sup> at the active site [20]. Xylanases (Xyl Ia and Ib) from thermophilic, *Myceliophthora* sp. IMI 387099, Mg<sup>2+</sup>, Zn<sup>2+</sup>, Ca<sup>2+</sup>, Co<sup>2+</sup> and DTT increased activity by 1.5 to 3.0-folds, while SDS and NBS completely inhibited [14]. Characterization of the xylanase from *Bacillus* sp. YJ6, [21] with a mol. wt of 19 kDa, and optimal pH 5.0 as well as temperature at 50°C. This purified enzyme was stable at pH 5.0-9.0 or <50°C. It was inhibited by Cu<sup>2+</sup>, Fe<sup>3+</sup>, Hg<sup>2+</sup>, phenylmethyl sulfonyl fluoride (PMSF), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), N-ethylmaleimide (NEM), and leupeptin but activated by K<sup>+</sup>, Na<sup>+</sup>, Co<sup>2+</sup>, Mg<sup>2+</sup>, β-mercaptoethanol (β-ME), and glutathione (GSH). The purified xylanase had high specificity to beechwood, birchwood, and oat spelt xylans [21].

### Enzyme kinetics

The xylanases exhibited *Km* and *Vmax* values of 4.0 mg/ ml, 5000

μmol/ min/ mg protein for Xyl 1 and 3.5 mg/ ml and 3448 μmol/ min/ mg of protein for Xyl 2. *Vmax* / *Km* for Xyl 1 - 1250 and Xyl 2 - 985. Xyl 1 activity exhibited a higher *Vmax* than Xyl 2, and showed more affinity for the substrate. Because of the high *Km* the endoxylanases could not be saturated with substrate, and thus it is not known if substrate inhibition kinetics also occurs with the substrate. Xylanase from *Trichoderma* sp. reported to have similar high specific activity with wide range of *Km* (0.5 - 12.5 mg/ ml) and *Vmax* values (4,025 U / mg protein). These differences could be to a certain extent, attributed to different xylans or temperatures used in the xylanase assay and methods used in determination of sugar concentration [22]. Similar *Km* for birch wood xylan reported as 2.3 mg/ ml while *Vmax* was 233.1 μmol/ mg/ min of protein from *A. versicolor* [8].

Xylanase from *Bacillus* .sp has *Km* value ranging from 3-8 mg of Oat spelt xylan. Only few reports were there related to *Bacillus* sp. with low *Km* values, *B. sterothermophilus* (1.63 mg/ ml) [24]. High *Km* reported for *T. longibracchiatum* (10.14 mg/ ml) [23] Low *Km* of 0.7 mg/ ml and *Vmax* of 2420 nkat/ mg with birch wood xylan as substrate for *Bacillus* sp. Strain SPS- 0 [17]. Various strains of *B. pumilus* have different *Km* and *Vmax* values for the same substrate. The strains 5<sub>2</sub> and 5<sub>14</sub> (8.9 and 1.1 mg/ml) require low substrate concentration to reach the *Vmax* (178.57 and 112.36 μmol/ ml/min) for catalysis, where as 13<sub>a</sub> and 4<sub>a</sub> require high substrate (*Km* 33.3 and 71.4 mg/ ml) concentration to reach *Vmax* (1666.67 and 1428.57 μmol /ml /min). This shows that these strains have high catalytic power and could use for higher technology efficiencies [24].

### Substrate specificity

Purified enzyme fractions were subjected to hydrolysis experiment on several agro industrial by-products, and results explained in Table 2. Both Xyl 1 and Xyl 2 were mainly active on oat spelt xylan. The isoforms also hydrolysis natural sources like; wheat bran and rice bran. These sugar conversion abilities are very important since inexpensive agro industrial by-products, rich in cellulose and hemicelluloses, are the main potential substrates for the production of useful biomolecules, including chemicals and organic solvents. The increasing interest in biotechnological processes employing lignocellulosic residues is quite justifiable because these materials are cheap, renewable and a widespread source of sugars [18].

### Conclusion

The biochemical property like optimum of temperature, pH and molecular mass of all isoforms were in the range of other *Bacillus* xylanases. According to these properties, the xylanases produced by *Bacillus pumilus* fit into the categories xylanases proposed by Wong and coworkers [11]. Xylanases from *Bacillus*, *Aspergillus*, *Clostridium*,

Substrates	Xyl 1	Xyl 2	Xyl 3
Oat spelt Xylan	100	100	100
Birch wood xylan	98	95	83
Beech wood xylan	82	70	0
Carboxymethyl cellulose	0	0	0
Wheat bran	95	81	0
Rice bran	90	52	0

**Table 2:** Relative activities of purified xyl 1, 2 and 3 from *b. pumilus* towards different substrates.

*Streptomyces* and *Trichoderma*, species suggested having multiplicity. The existence of a evolutionarily conserved relationship between their molecular weight and *pI*, in which low MW (< 22.000) xylanases are basic proteins whereas high MW (> 43.000) xylanases are acidic proteins. Many commercially available fungal xylanases are acidic, but that from bacteria such as *Bacillus* are more promising. Reducing sugars were released from all the agro industrial residues, making this enzyme a promising candidate with application in the recovery of fermentable sugars from hemicelluloses. The characteristics of xylanase from *B. pumilus* enhance its industrial potential and can effectively used in clarification of juice and baking industry, in paper and pulp industry, as well as feed industry.

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