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Three-Step Purification and Characterization of Organic Solvent-Tolerant and Alkali-Thermo-Tolerant Xylanase from *Bacillus paramycoides* T4 [MN370035]

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Abstract: In the present study, an extracellular alkali-thermo-tolerant xylanase from *Bacillus paramycoides* was produced in the presence of an organic solvent. The enzyme was purified by ammonium sulphate precipitation, gel filtration, and ion exchange chromatography, with an overall recovery of 25.9%. The purified enzyme hada 70 kDa molecular weight (MW) confirmed by SDS-PAGE gel analysis. The maximum enzyme activity was reported at 55 °C and pH 7.0. Xylanase activity and stability were improved in the presence of 30% (*v*/*v*) n-dodecane, iso-octane, n-decane, and cyclohexane (7 days). The enzyme activity was improved by Co^{2+}, EDTA, and Triton-X-100 while vigorously repressed by Hg^{2+} and Cu^{2+}. The purified enzyme showed 1.473 mg/mL K_m and 654.017 µg/mL/min V_{max} values. The distinctive assets of the isolate verified the potential application in the field of biomass conversion into fuel and other industrial processes. Organic solvent-tolerant xylanases can be used for concurrent saccharification and bioethanol production, the amplification of intoxicating beverages, and the fermenting industry.

Keywords: *Bacillus paramycoides*; kinetic study; purification; thin layer chromatography; xylanase

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1. Introduction

Xylan is the most important renewable hemicellulose and is a complex polysaccharide of the plant cell wall. Because hardwoods contain 25–32% hemicelluloses, and softwood includes just 15–25% hemicelluloses, they constitute a considerable reservoir of utilizable biomass. Undoubtedly, hemicelluloses are the second most abundant renewable resource, only exceeded by cellulose. Xylan is a complex structure having β-1, 4-linked xylose residues in the backbone, to which short side-chains of O-acetyl, α-L-arabinofuranosyl, D-α-glucuronic, and phenolic acid residues are bound [1]. The structure of xylan varies,

ranging from linear polyxylose chains with 1–4 β-links to highly branched heteropolysaccharides. α-L-arabinofuranosyl (AFase) residues are monomeric or oligomeric side-chains on the xylose or galactose backbone in xylans, arabinoxylans, and arabinogalactans at the O-3 position, or sometimes both the O-2 and O-3 positions, whereas 1,5-linked arabinofuranosyl residues are monomeric or oligomeric side-chains on the backbone of arabinans [2]. Xylanases are glycosidases (E.C.3.2.1.8), which are important hemicellulose hydrolytic enzymes that degrade xylan, xylo-oligosaccharides, and xylose. Pre-bleaching of pulp with xylanases removes the hemicelluloses that attach to the pulp. In the pulp and paper sector, the cellulose-free xylanase activity at high temperatures and pH is gaining popularity as it eliminates the need for hazardous chlorinated chemicals, making the bleaching process more environmentally friendly [3,4]. As a result, xylanases from alkalophilic bacteria and actinobacteria have received a lot of attention [5]. The use of thermostable alkaline xylanases for enzyme-assisted pulp bleaching could drastically minimize the requirements for pH and temperature adjustments, resulting in significant technical and financial benefits [6]. Thermostable xylanases active at alkaline pH are of great interest for biotechnological applications such as the pre-bleaching of pulp, enhancing the degradation of animal nourish stocks, alteration in cereal-based materials, biodegradation of lignocellulosic biomass and agro-waste to fermentable goods, elucidation of fruit drinks, and degumming of plant twines [7]. The excellent organic-solvent-tolerant xylanases could be used for simultaneous saccharification and fermentation to produce bioethanol, alcoholic beverage clarity, and in the brewing sector and other industrial applications [8,9]. Organic solvents result in the structural flexibility of enzymes, which could be responsible for their high activity and stability. Xylanase has been produced by bacteria [10–16], fungi [17–25], actinobacteria [26], and yeast [27]. However, due to their sluggish generation time, the co-production of very viscous polymers, and the slow oxygen transport, a large-scale culture of fungus and actinobacteria is typically challenging [28]. *Bacillus* sp. is used in individual fermentations more commonly than other bacteria as it produces the majority of their enzymes. In this study, an attempt was made to purify a thermo-solvent-tolerant and alkaline xylanase produced by *Bacillus paramycoides* T4 upto homogeneity. Biochemical characterization and TLC were also performed for better evaluation of the xylanase enzyme activity and their sustainability for industrial application. To our knowledge, this is the first report describing the production of thermo-alkalophilic organic-solvent xylanase by *B. paramycoides* T4. The organic-solvent-tolerant and heavy-metal-resistant xylanases can have some novel properties; however, there are very few studies on this type of xylanase. Heavy-metal-resistant xylanase could apply to pharmaceutical industries. This strain also showed resistance properties with a surfactant, so it can be applied in the detergent industry. It has been accepted that the suitability of an enzyme for the industrial application depends upon the enzyme's homogeneity.

2. Results

This study involves the purification and characterization of organic solvent-tolerant xylanase from alkali-thermophilic *B. paramycoides* T4. Heavy metal tolerance in xylanase-producing bacteria has not been reported so far. The isolate T4 showed high tolerance for different heavy metals, suggesting that it may be used in the bioremediation of such metals from natural contaminated sites. The purified and biochemical characterized enzyme was significantly used for bio-ethanol production.

2.1. Heavy Metal Resistance Pattern

Various heavy metals under investigation had varying levels of toxicity on the isolate. Strain T4 was able to tolerate extremely high concentrations of several heavy metals, which were arranged in the following order of tolerance: chromium (3000 µg/mL), arsenic (2800 µg/mL), cesium (1500 µg/mL), and lead (950 µg/mL). It was likewise resistant to relatively high amounts of cobalt (325 µg/mL), nickel (300 µg/mL), and selenium (325 µg/mL), but sensitive to even lower mercury quantities (90 µg/mL) (Table 1).

Table 1. Heavy-metal-resistant pattern system.

S.N.	Metal Conc. (μg/mL)	Different Heavy Metals							
		Hg	Ni	Co	Se	Pb	Ce	As	Cr
1	50.0	+	+	+	+	+	+	+	+
	* 60.0	+							
	* 70.0	+							
2	* 80.0	+							
	* 90.0	+							
	100.0	−	+	+	+	+	+	+	+
3	150.0	−	+	+	+	+	+	+	+
4	200.0	−	+	+	+	+	+	+	+
5	250.0	−	+	+	+	+	+	+	+
6	300.0	−	+	+	+	+	+	+	+
	# 325.0	−		+	+				
7	350.0	−	−	−	−	+	+	+	+
8	400.0	−	−	−	−	+	+	+	+
9	500.0	−	−	−	−	+	+	+	+
10	600.0	−	−	−	−	+	+	+	+
11	700.0	−	−	−	−	+	+	+	+
12	800.0	−	−	−	−	+	+	+	+
13	900.0	−	−	−	−	+	+	+	+
	** 950.0					+			
14	1000.0	−	−	−	−	−	+	+	+
15	1500.0	−	−	−	−	−	+	+	+
16	2000.0	−	−	−	−	−	−	+	+
	2500.0	−	−	−	−	−	−	+	+
	*** 2600.0							+	
17	*** 2700.0							+	
	*** 2800.0							+	
	*** 2900.0							−	
18	3000.0	−	−	−	−	−	−	−	+
19	3500.0	−	−	−	−	−	−	−	−

Each value presented here is an average of triplicates of three independent trials. Symbols: (+) tolerance; (−) susceptibility; (*) 60 to 90 μg/mL concentrations were used only for Hg (mercury); (#) 325 μg/mL concentration was used only for Co (cobalt) and Se (selenium); (**) 950 μg/mL concentration wasused only for Pb (lead); (***) 2600 to 2900 μg/mL concentrations were used only for As (Arsenic). This concentration was used for further examinations.

2.2. Purification of Xylanase

The ammonium sulfate precipitation method was used to concentrate the raw/crude enzyme extract initially. The results showed that 80% ammonium sulfate precipitation reported maximum activity with a 19.54 mg/mL protein content. The enzyme fraction had 904.59 U/mg of specific activity with a 68.8% recovery rate. This fraction showed a 3.6-fold purification after the process (Table 2).

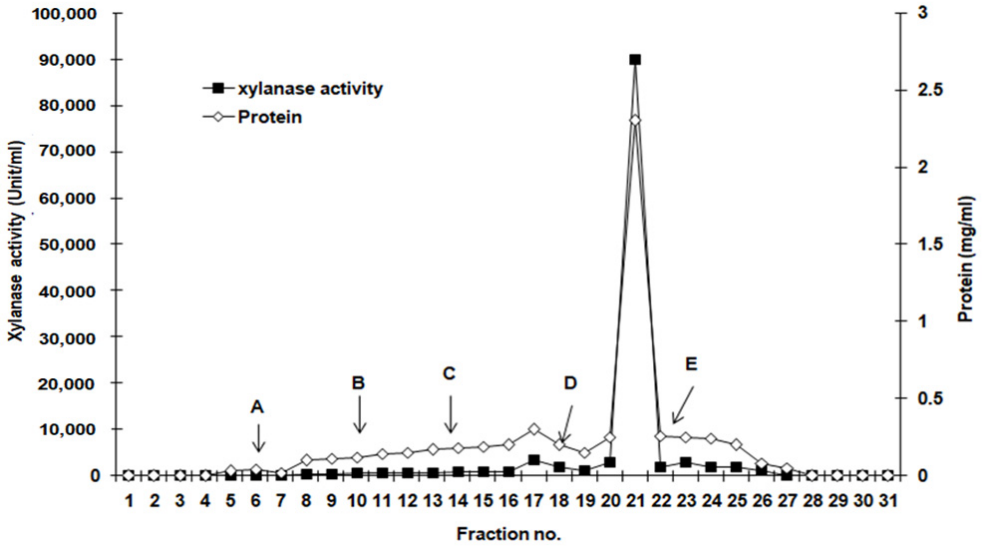
Table 2. Summary of purification of xylanase from *Bacillus paramycoides* T4.

Purification Steps	Total Activity (U)	Total Protein (mg)	Specific Activity (U/mg Protein)	Yield (%)	Purification Fold
Crude	25687.5	104.5	245.81	100	1.0
Ammonium sulfate	17674.5	19.54	904.59	68.8	3.6
Q-Sepharose	12123.4	4.79	2530.98	47.2	10.3
Sephadex G-75	9898.7	2.98	3321.71	38.5	13.51

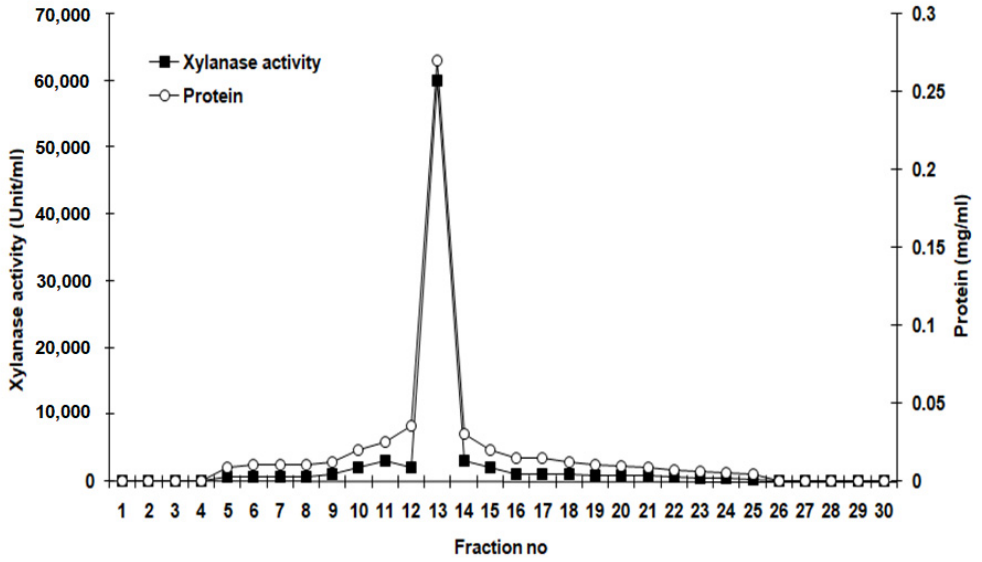
Each value presented here is an average of triplicates of three independent trials. The mean standard deviation for each value is <±5.0%.

The concentrated enzyme (1 mL) was loaded onto the Q-sepharose column and almost all the xylanase activity was detected in the unbound fraction. There was no xylanase activity in the fraction, while 1.32 mg/mL of protein was estimated. The xylanase activity

was detected in the fraction released by the addition of 0.5 M NaCl, and an anion-exchange chromatography of xylanase resulted in one prominent peak at the 21st fraction (Figure 1a).



(a)



(b)

Figure 1. (**a**) Purification profile of extracellular xylanase from *Bacillus paramycoides* T4 on an anion-exchange column (Q-Sepharose) equilibrated with sodium phosphate buffer (100 mM, pH 7.0). The xylanase was eluted with a gradient of sodium chloride (0.1 M–0.5 M) in sodium phosphate buffer (100 mM, pH 7.0). A—0.1 M, B—0.2 M, C—0.3 M, D—0.4 M, and E—0.5 M. (**b**) Further purification of active fraction from ion exchange chromatography xylanase purification profile using Sephadex G-75 gel filtration chromatography. Sodium phosphate buffer was used to equilibrate the column (100mM, pH 7.0). The same buffer was used to load and elute the sample.

Further purification of the active fraction was performed by size-exclusion chromatography on a Sephadex G-75 column. Figure 1b represents the fractionation chart of xylanase on the Sephadex G-75 column with one protein peak that overlapped the xylanase activity. The purification technique yielded a purity factor of 13.51 and a final recovery of 38.5% of the enzyme, with a specific activity of 3321.71 U/mg (Table 2).

2.3. Electrophoretic Analysis

A single band on SDS-PAGE was the confirmation of the pure enzyme of the isolate, and its molecular weight was determined as approximately 70 kDa (Figure 2).

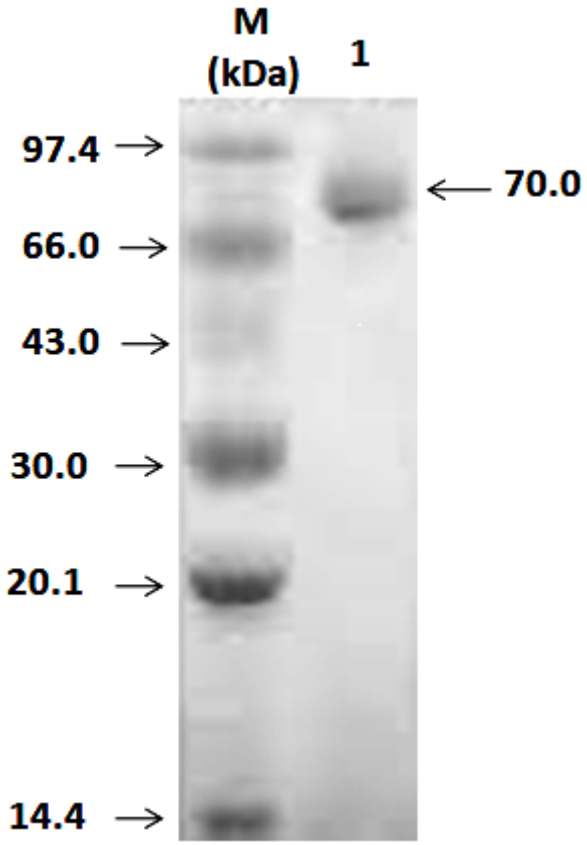


Figure 2. SDS-PAGE of xylanase of *Bacillus paramycoides* T4 obtained during purification steps.Lane 1: marker proteins; lane 2: purified enzyme obtained after gel-filtration chromatography. Molecular weights were presented in the form of kDa.

2.4. Biochemical Characterization of the Purified Enzyme

2.4.1. Effect of Temperature on Enzyme Activity and Stability

The effect of temperature on enzyme activity and stability was studied by varying the temperature range from 35 to 105 °C. The purified enzyme showed its activity in wide-ranging temperatures (40–95 °C) with the maximum activity at 55 °C (Figure 3). From the result, it was clear that the enzyme activity was 150.23% at 55 °C and thereafter the activity decreased gradually with the increase in the temperature (Figure 3). Interestingly, the enzyme retained 100 and 80% activities even at 90 and 100 °C temperatures, respectively.

The thermal stability profile of the enzyme showed initial retention of 100% activity in the wide temperature range (45–90 °C) throughout the incubation period (60 min). However, with a further rise in temperature, there was a gradual decline in enzyme stability (20–21%) upto 105 °C (Figure 3). Above 105 °C, a sharp decrease in enzyme stability was observed (data not shown) (Figure 3).

2.4.2. Effect of pH on Enzyme Activity and Stability

The observations for the effect of pH and buffer system on the activity suggested that the composition of buffer noticeably affected the enzyme activity. The enzyme was active in the broad range of pH 5.0–9.0 and had optimum activity at pH 7.0 (148.3%) followed by pH 7.5 (129.1%) with the sodium phosphate buffer system. The minimum activity was observed at pH 4.0 (sodium acetate, 100 mM) and pH 10.0 (Tris-HCl, 100 mM), which was only 32.0 and 35.0%, respectively, when compared to the control (Figure 4). From pH 4.0,

the increase in pH value increased the enzyme activity, which was maximum at pH 7.0; however, at the same pH of different buffer systems, the activity was different.

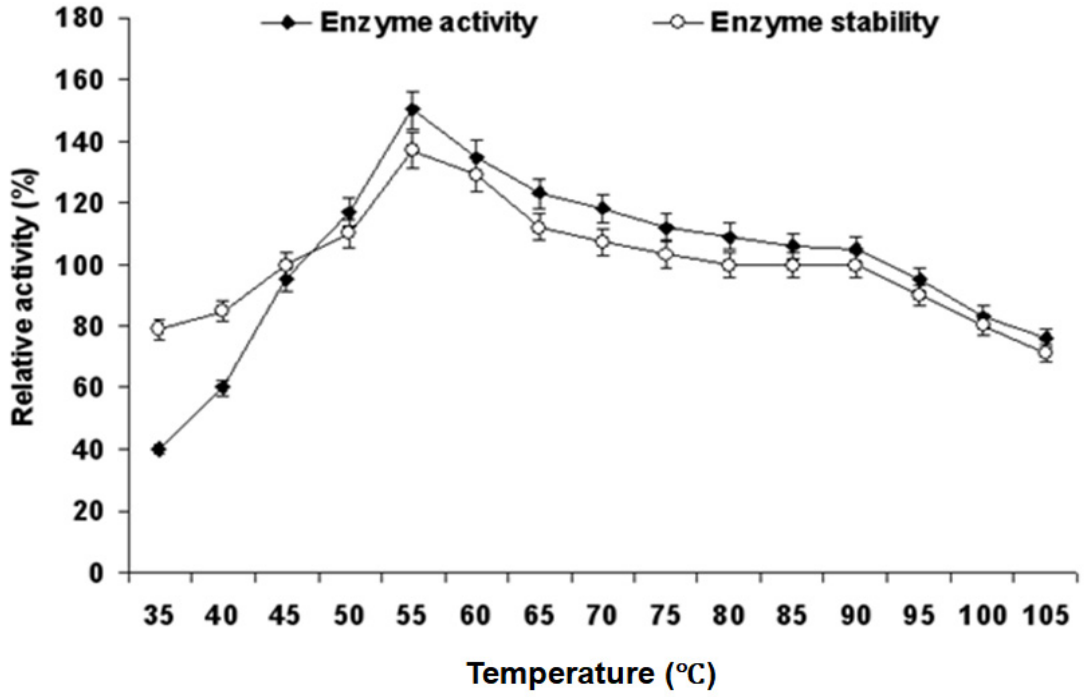


Figure 3. Effect of temperature on enzyme activity and stability. The reaction mixture was incubated at various temperatures (35–105 °C) for enzyme activity. For enzyme stability, the enzyme was pre-incubated at respective temperatures for 1 h, and the reaction was carried out as per the standard test method.

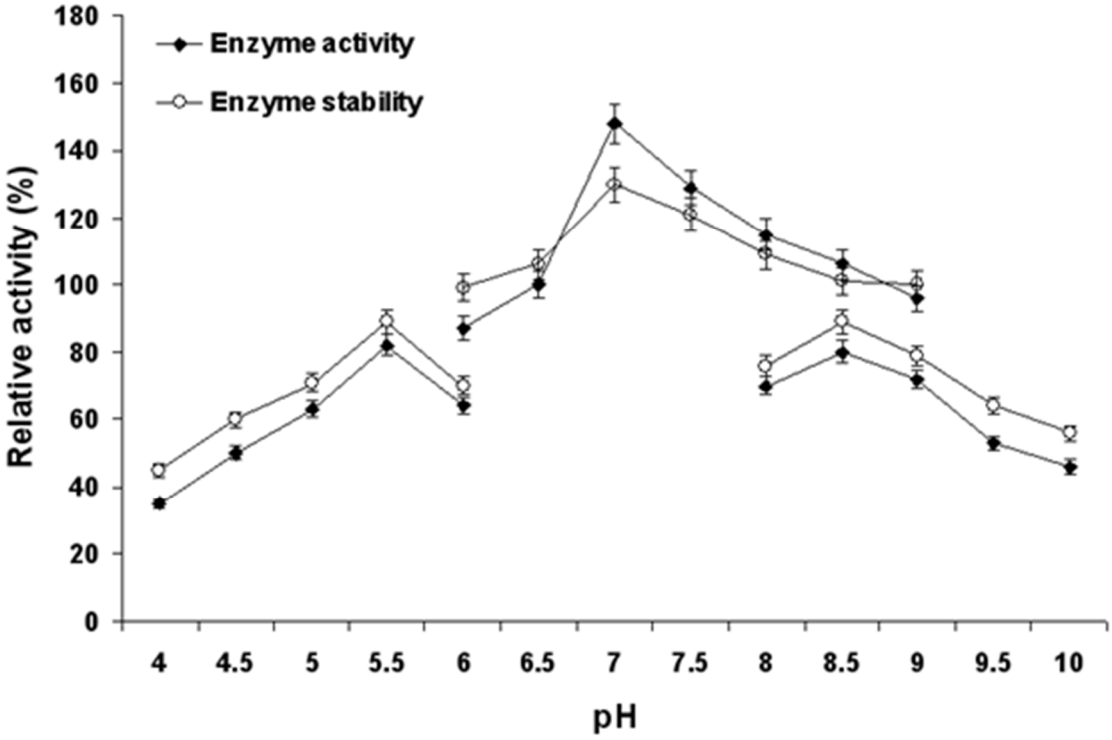


Figure 4. The impact of pH on enzyme activity and stability. For enzyme activity, the reaction was measured at the appropriate pH, and for stability, the enzyme was pre-incubated for 1 h at 55 °C with

buffers (100 mM, in a 1:1 ratio) of various pH (sodium acetate (pH 4.0–6.0), sodium phosphate (pH 6.0–8.0), and Tris-HCl (pH 8.0–10.0) and then measured using a standard assay method. The enzyme assay performed at pH 7.0 (sodium phosphate buffer) and 55 °C was served as control (100%). For enzyme stability, the residual activities were compared with enzyme activity measured at pH 7 (sodium phosphate buffer) and 55 °C (100%).

The enzyme exhibited considerable stability with all the buffers tested. However, maximum stability (100%) was recorded in the enzyme sample incubated with buffers having pH 6.0–9.0. The enzyme was almost unstable at pH 4.0; however, a marginal decrease in the stability was observed at pH 9.5 and 10.0 with Tris-HCl buffer (Figure 4).

2.4.3. Substrate Specificity

The mechanism actions of the purified xylanase for different substrates were examined. Xylanase showed maximum activity in the presence of birchwood xylan followed by oat speltxylan and p-nitrophenyl xylopyranoside. When Avicel, carboxymethyl cellulose, cellobiose, and starch were utilized as substrates, the enzyme showed no activity (Table 3). Even when the enzyme concentration was six times higher than in the standard assay and the incubation period was 30 min rather than 10 min, purified xylanase was not active on Avicel, carboxymethyl cellulose, cellobiose, or starch (data not shown).

Table 3. Substrate specificity of purified enzyme.

Substrate (1%)	Xylanase Activity (U/mg Protein)
Birchwood xylan	675.50 ± 1.2
Oat spelt xylan	498.2 ± 0.9
Cellobiose	0.0 ± 0.0
Carboxy methyl cellulose (CMC)	0.0 ± 0.0
p-nitrophenyl xylopyronoside	96.0 ± 0.7
Avicel	0.0 ± 0.0
Starch	0.0 ± 0.0

The mean and standard error values are represented by each value.

2.4.4. Kinetic Analysis

Different concentrations of birchwood xylan (0.25 to 4.0 mg/mL) were used to study the enzyme kinetics of purified enzyme. The Lineweaver–Burk plots revealed that the K_m and V_{max} for birchwood xylan were 1.473 mg/mL and 654.017 g/mL/min, respectively (Figure 5).

2.4.5. Effect of Metal ions on Activity and Stability

B. paramycoides T4 xylanase was activated by 10 mM Co^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{2+} but was inhibited by all other metal ions to a variable extent. The results indicate that xylanase had the highest relative activity (284.3, 256.7, 245.1, 234.5, and 229.4%) and stability (234.7, 216.1, 213.5, 205, and 206.5%) in the presence of Co^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{2+} ions, respectively. Metal ions such as Ni^{2+}, Mn^{2+}, and Na^{2+} all decreased xylanase activity marginally (Table 4). In the presence of Cu^{2+} and Hg^{2+}, xylanase activity was highly repressed.

2.4.6. Effect of Inhibitors and Surfactants on Enzyme Stability

The data suggested that the enzyme activity was induced by all the inhibitors investigated. When the purified enzyme was mixed with EDTA, PMSF, dithiothreitol (DTT), urea, and β-mercaptoethanol, it retained its activity at 145.5, 105, 123.9, 104, and 171% of the original activity at a concentration of 10 mM, respectively (Table 5).

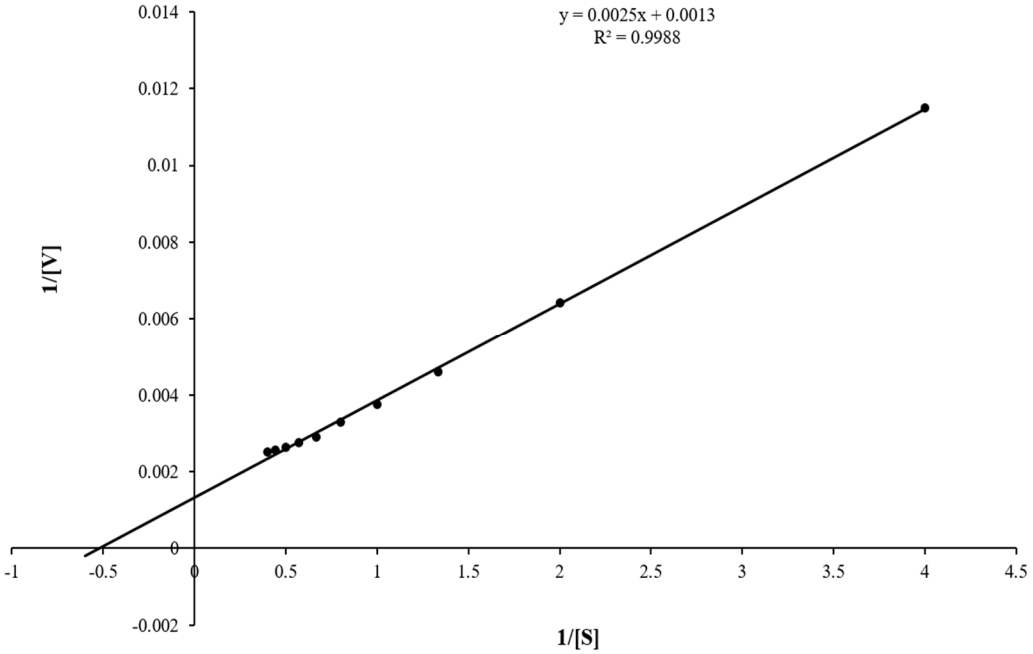


Figure 5. Lineweaver–Burk plot of purified xylanase from *Bacillus paramycoides* T4.

Table 4. Effect of metal ions on enzyme activity and stability.

Metal Ions	Concentration (mM)	Residual Activity (%)	
		Activity	Stability
Control		100.0	100.0
$CaCl_2$	5	206.4	195.7
	10	256.7	216.1
$NiCl_2$	5	131.8	119.1
	10	150.4	127.5
$FeSO_4$	5	192.6	186.8
	10	229.4	206.5
$MgCl_2$	5	215.9	195.6
	10	245.1	213.5
$CuSO_4$	5	67.4	59.5
	10	56.7	49.5
$HgCl_2$	5	46	52
	10	25	32
$MnCl_2$	5	88.9	83.9
	10	96.9	90.2
NaCl	5	156.2	141.3
	10	181.9	165.9
$ZnSO_4$	5	209.9	188.9
	10	234.5	205
$CoCl_2$	5	227.4	209.8
	10	284.3	234.7

Enzyme activity was measured directly at 55 °C in the presence of metal ions in the reaction mixture, and for stability, the enzyme was pre-incubated with various metal ions at 55 °C for 1 h before being tested using the standard assay method. The enzyme activity without metal was considered as a control (100%) against which residual activity (%) was calculated. The control for the metallic ions was measured against the buffer. For all of the values, the mean standard deviation is ≥5.0 percent.

From Table 5, it is clear that the enzyme was significantly stable with nonionic surfactants such as Tween-40, Tween-60, Tween-80, Triton-X-100, and detergent SDS. Conversely, at higher concentrations (1.0% *v/v*), these substances vaguely repressed the xylanase activity with 93.6, 95, 97.2, 115.8, and 98.4% of residual activity. Therefore, xylanase of

B. paramycoides T4 was resistant with 1% SDS (95%) and could be applicable as an efficient stabilizer in detergents.

Table 5. Effect of different inhibitors on enzyme stability.

Reagents	Conc. (%)	Residual Activity (%)	Surfactants	Conc. (%)	Residual Activity (%)
Control		100.0	Control		100.0
β–mercaptoethanol	0.1 (%)	180.9	Tween-20	0.1	116.7
	1.0 (%)	171.0		1.0	93.6
EDTA	5 mM	160.9	Tween-40	0.1	123.8
	10 mM	145.5		1.0	95.0
Urea	5 mM	118.0	Tween-60	0.1	119.8
	10 mM	104.0		1.0	97.2
PMSF	5 mM	123.9	Triton-X-100	0.1	131.5
	10 mM	105.0		1.0	115.8
DTT	5 mM	157.3	SDS	0.1	108
	10 mM	123.9		1.0	98.4

The enzyme was pre-incubated for 1 h at 55 °C with various inhibitors and surfactant agents before being analyzed using a standard test method. Without incubation with surfactants and commercial detergents, the enzyme activity was assumed to be 100%. For all of the values, the mean standard deviation is ≥5.0 percent.

2.4.7. Effect of Organic Solvents on Xylanase Stability

The influence of several organic solvents (30%, *v/v*) on xylanase stability was also tested for 7 days, and the results are mentioned in Table 6. The xylanase of *B. paramycoides* T4 in the present study revealed significant stability with both hydrophobic (log $p > 2.0$) and hydrophilic (log $p < 2.0$) organic solvents. Most hydrophobic organic solvents tended to increase enzyme stability, whereas hydrophilic solvents tended to reduce enzyme stability (Table 6). Other solvents, aside from benzene, propanol, and ethanol, were shown to increase xylanase activity. The xylanase activity increased to 300.9, 177.5, 279.3, 137, 250.2, 231, 239.3, 119.9, 129.8, and 140% after incubation with n-dodecane, iso-octane, n-decane, xylene, toluene, n-hexane, n-butanol, acetone, methanol, and cyclohexane, respectively. Benzene, ethanol, and propanol all lowered the xylanase activity slightly, with residual activities of 86.9%, 81.5, and 83.9%, respectively (Table 6).

Table 6. Stability of xylanase in presence of various organic solvents.

Organic Solvents (30%)	log *p*	Residual Activity (%)							
		1 h	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Butanol	−0.80	112.7	239.3	214.2	206.4	172.6	132.3	117	99
Methanol	−0.76	106.7	129.8	141.2	128.4	120.1	111.2	104.1	98.6
Iso-propanol	−0.28	92	109.6	100	94.9	91	91	87.7	83.9
Ethanol	−0.24	99	109.7	104.5	101.7	99.8	95.5	90.3	81.5
Acetone	−0.23	109.4	119.9	112.9	105.1	102	99	93	89
Benzene	2.13	100	119	124	106	109	98	90	86.9
Toluene	2.5	110.9	250.2	220.4	189.5	140.9	119.1	100.2	100
Iso-octane	2.9	117.3	177.5	146.9	132.6	120.8	112.4	105.6	101.2
Xylene	3.1	92	120	137	123	107	100	95	89
Cyclohexane	3.3	95	121	140	131.9	120.9	113	100	90.9
Hexane	3.6	128	231	214.9	204.6	187.7	149.7	124	100
n-decane	5.6	115.9	219.8	279.7	248.2	206.5	167.9	127	99.0
n-dodecane	6.0	121.2	237.7	300.9	269.4	229.8	190.5	153.0	110.0
Control		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

The enzyme was pre-incubated with various organic solvents at a concentration of 30% (*v/v*) at 55 °C for various periods before being analyzed using a standard assay technique. The enzyme activity without any organic solvent was considered as control (100%) against which residual activity (%) was calculated. Each value shown here is the average of three separate trials in duplicate. For all of the values, the mean standard deviation is ≥5.0%.

2.5. Analysis of Hydrolytic Products

In this experiment, xylotriose and xylotetraose were found as the main products after hydrolysis (4 h of incubation) of birchwood xylanby xylanase of *B. paramycoides*; nevertheless, a small amount of xylobiosewas also produced (Figure 6). From the above results, it is clear that this xylanase is an endoxylanase because it hydrolyzed the substrate and produced xylooligosaccharides, although it is not suitable to work on the resulting oligosaccharides to form xylose.

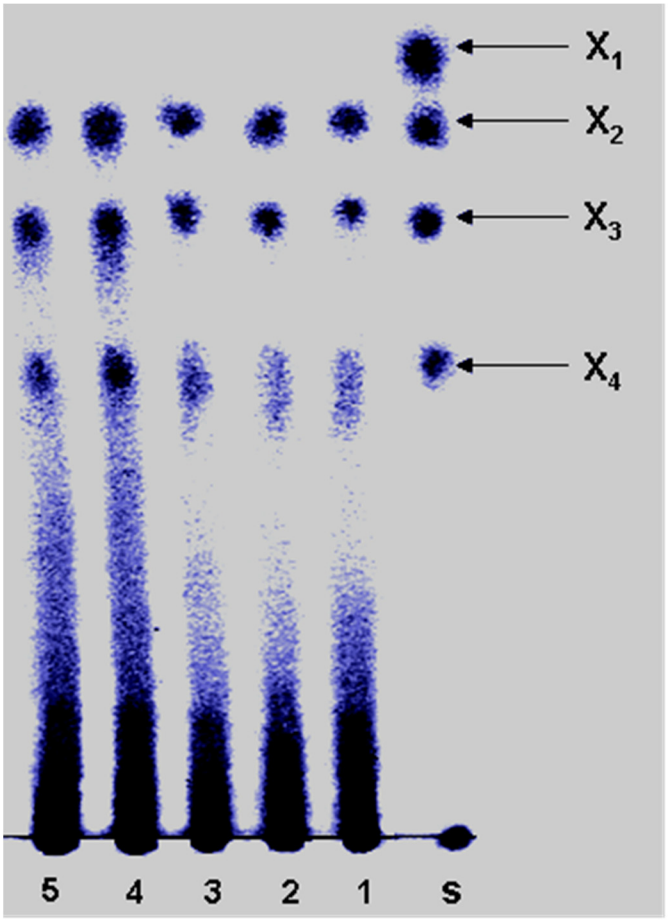


Figure 6. TLC study of birchwood xylan hydrolysis products produced by *B. paramycoides* xylanase.Lane S: a mixture of xylose (X_1), xylobiose (X_2), xylotriose (X_3), and xylotetraose (X_4); Lane 1: birchwood xylan hydrolysate (for 1 h); Lane 2: for 2 h; Lane 3: 3 h; Lane 4: for 12 h; Lane 5: 24 h.

3. Discussion

This study comprises the three-step purification and characterization of organic solvent-tolerant xylanase from alkali-thermophilic *B. paramycoides* T4. This is the first report on organic-solvent-tolerant xylanase from *B. paramycoides*. Furthermore, heavy metal tolerance in xylanase-producing bacteria has not been studied. The isolate T4 showed high tolerance for chromium, arsenic, lead, and cesium (Table 1), suggesting that it may be used in the bioremediation of such metals from natural contaminated sites. Xylanase-producing organisms showing heavy metal tolerance could be applicable for the management of multi-metal-polluted sludge produced from wastewater treatment. Pharmaceutical and metal industries increase a load of antibiotics/disinfectants and heavy metals, which generate discriminating stress for the survival of microorganisms in a polluted atmosphere. Hence, in various harassed situations, microbial cells acquire resistance/tolerances by the variation in the genetic framework also by mutation or the transfer of resistant genes among the microbial cell.

The chromatography results showed that a 41.1-fold purification and 25.9% final recovery of the enzyme with 101,007.14 U/mg of specific activity was achieved during the purification process (Table 2). Sanghi et al. [29] reported that purified xylanase of *Bacillus subtilis* achieved a 10.5-fold purification factor with 43.05% recovery after gel filtration chromatography. From Figure 3, it is clear that the enzyme was purified up to homogeneity and had a molecular weight of approximately 70 kDa, confirmed by the presence of a single band on SDS-PAGE. Our observations are in good agreement with the study of Kamble and Jadhav [30] who also purified xylanase from an alkalophilic *Cellulosimicrobium* sp. MTCC10645 with a molecular weight of 78 kDa [30], but different from *Bacillus pumilus* xylanase (60 kDa) [31].

The xylanase of strain T4 was completely stable in the broad temperature range of 45–90 °C during 1 h of incubation, while, with a further temperature increase at every 5 °C, there was a gradual decrease in enzyme stability ranging between 20–21% and 105 °C. The enzyme retained 90, 80, and 71% activities at 95, 100, and 105 °C, respectively (Figure 4). Similarly, alkalophilic xylanase of *Bacillus subtilis*, *B. subtilis* ASH, and *Caldicoprobacter algeriensis* TH7C1^T showed a 100% enzyme activity and stability at 55 °C [14,29,32,33]. In another study, Kamble and Jadhav [30] also reported the xylanase from *Cellulosimicrobium* sp. MTCC 10645 showed optimum activity at 50 °C with a gradual decrease in enzyme activity at 80 °C. The xylanase of strain T4 is more thermostable than the xylanase studied by several other researchers. Purified xylanases from *Bacillus* sp. and *Cellulosimicrobium cellulans* CKMX1 have been reported to have an optimal temperature of 50–55 °C [4,13,34]. The activity pattern of pure enzymes is confirmed by the fact that increasing temperature increases the rate of an enzymatic reaction, but at temperatures around the optimal value, enzyme denaturation occurs, resulting in inactivation. However, some xylanases showed higher-temperature optima [35–39]. Its industrial application would require significant enzyme stability at higher temperatures. Hence, from the result, it is clear that the xylanase of strain T4 is thermostable and could be used for numerous industrial and biotechnological applications.

The effect of pH on enzyme activity and stability was examined by evaluating the enzyme activity at different pH values. From the result, it is clear that maximum activity resulted at pH 7.0. However, in the case of stability, it showed its best result at pH 7.0–8.0 (Figure 5). Similar results were also found in *Bacillus* sp. NTU-06 [40], *Bacillus* sp. [41], *Cellulosimicrobiumcellulans* CKMX1 [4], and *Streptomyces olivaceus* MSU3 [16]. It is well established that purified xylanases are usually steady over a broad range of pH from 6.0 to 11.0 [14,33,39,41]. The enzymes that remained stable in alkaline environments had fewer acidic residues and more arginines [42].

The enzyme showed maximum activity in the presence of birchwood xylan followed by oat speltxylan and p-nitrophenyl xylopyranoside. The enzyme reported zero activity when Avicel, CMC, cellobiose, and starch were used as substrates (Table 3). Similar to our results, several works have also been reported where purified xylanase of *Bacillus cereus*, *Bacillus arseniciselenatis*, *Trichoderma inhamatum*, and *Caldicoprobacteralgeriensis* were active on birchwood xylan and oat spelt xylan while reporting zero activity for glycans such as starch, carboxymethyl cellulose, cellobiose, and Avicel [14,23,30,43,44].

The K_m and V_{max} for birchwood xylan were recorded as 1.473 mg/mL and 654.017 g/mL/min, respectively (Figure 6). The K_m for birchwood xylan was reported to be 2.3 mg/mL, while the V_{max} for protein from *Aspergillus versicolor* was 233.1 µmol/mg/min [45]. Other similar studies, conducted by Wang et al. [40] and Boonchuay et al. [46], reported a comparatively higher value of K_m (3.45 mg/mL and 37.6 uM) and lower values of V_{max}(387.3 µmol/min/mg and 303 U/mg) from *Bacillus* sp. and *Streptomyces* spp. using birchwood xylan as a substrate. When the K_m value is low, it indicates that the enzyme has a high affinity for the substrate [44].

Xylanase had the highest relative activityand stability in the presence of Co^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{2+} ions (Table 4). Mamoo et al. [47] and Lv et al. [48] have also reported that Co^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+},and Fe^{2+} ions highly activate the xylanase activity. The stimu-

latory effects of metal ions on enzyme activity could be owing to a change in the enzyme's structure [37,38]. Others such as Ni^{2+}, Mn^{2+}, and Na^{2+} all decreased xylanase activity marginally while Cu^{2+} and Hg^{2+} repressed the activity highly. Analogous results were found from *Bacillus subtilis* [29], *Bacillus halodurans* PPKS-2 [49], *Simplicillium obclavatum* [50], *Trichoderma inhamatum* [23], *Streptomyces* sp. [46], and *Bacillus sp.* ASX42 [39]. Hg^{2+} ion suppression of xylanase activity has been suggested to be owing to its interaction with sulphydrylgroups of a cysteine residue in or near the enzyme active site [51]. Cu^{2+} ions may block xylanase due to conflict between exogenous cations and protein-associated cations, resulting in lower metalloenzyme activity.

Different inhibitors such as EDTA, PMSF, dithiothreitol (DTT), urea, and β-mercaptoethanol enhanced the enzyme activity (Table 5). Similarly, Lv et al. [48] and Hwang et al. [52] also reported that microbial community EMSD5 and *Paenibacillus* sp. retained full activity in the presence of EDTA, DTT, PMSF, and β-mercaptoethanol. Contrastingly, some other works reported that EDTA [38,53] and DTT [49] slightly inhibit the xylanase activity of *Bacillus* sp. In the presence of EDTA, the enzyme's activity is inhibited, demonstrating that xylanase contains any form of metal ion in its active site. The substantial increase in enzyme activity in the presence of thiol group-reducing chemicals such as β-mercaptoethanol can be explained by the prevention of sulfhydryl group oxidation [39,54].

The nonionic surfactants such as Tween-40, Tween-60, Tween-80, Triton-X-100, and detergent SDS stimulate the enzyme activity (Table 5). Similarly, Lv et al. [48] also reported that alkaliphilic xylanase from a microbial community EMSD5 exhibited a stimulatory effect on enzyme activity and stability in the presence of nonionic detergents such as Triton X-100 and Tween-20 and was slightly inhibited in the presence of SDS at a 0.1% concentration. Meanwhile, xylanase from alkalophilic bacteria *Bacillus pumilus*, *Trichoderma inhamatum*, and *Paecilomyces variotii* lost their total activity in the presence of SDS [23,31,38]. In another study, xylanase of *Simplicillium obclavatum* showed maximum enzyme activity in the presence of SDS while inhibiting its activity in the presence of Triton X-100 [43]. Therefore, xylanase of *B. paramycoides* T4 was resistant with 1% SDS (95%) and could be applicable as an efficient stabilizer in detergents.

The ability to withstand organic solvents could be a paramount property of xylanases as several industrial and biotechnological applications are carried out in the presence of solvents. For instance, ethanol-tolerant xylanases are employed in bioethanol production; solvent-tolerant xylanases are used in the bioremediation of solvent-contaminated industrial wastewaters; solvent- and surfactant-tolerant xylanases are used in the deinking of recycled paper; solvent tolerance makes it easier to selectively precipitate, recover, and use enzymes [55–58], as well as in the bioconversion of lignocellulose [59]. The xylanase under the current study revealed significant stability with hydrophobic ($\log p > 2.0$) and hydrophilic ($\log p < 2.0$) organic solvents. Generally, the enzyme stability was improved by various hydrophobic organic solvents, whereas the stability declined with hydrophilic organic solvents (Table 6). It was observed that the xylanase activity was increased by n-dodecane, iso-octane, n-decane, xylene, toluene, n-hexane, n-butanol, acetone, methanol, and cyclohexane except for benzene, propanol, and ethanol, which decreased the xylanase activity. The improved stability of xylanase in nonpolar solvents may be due to hydrophobic solvents' failure to extract the necessary water linked with the protein, allowing the enzyme to maintain its active conformation, which is required for the process. Similarly, Eltaweel et al. [60] also stated that the protease enzyme showed maximum stability from the hydrophobic solvent due to similar reasons discussed above. The reduced xylanase activity in hydrophilic solvents, on the other hand, could be owing to these solvents stripping the critical water layer around the enzyme, which is required for enzyme catalysis [61,62]. Alkaline cellulase of *Bacillus halodurans* CAS 1 showed enhanced activity by various organic solvents (25%, *v/v*) reported by Annamalai et al. [63]. Organic solvents may boost enzyme activity because residues of the carried-over nonpolar hydrophobic solvent provide an interface, retaining the enzyme in an open conformation and resulting in accelerated activation, according to Zaks and Klibanov [64]. As a result of our findings, it is clear

that *B. paramycoides* T4 xylanase is exceptionally stable in the presence of a wide range of hydrophilic and hydrophobic organic solvents.

In this experiment, it is clear that this xylanase is an endoxylanase because it hydrolyzed the substrate and produced xylo-oligosaccharides, although it is not suitable for work on the resulting oligosaccharides to form xylose (Figure 6). A similar result has been reported from endoxylanase of alkalophilic *Bacillus* sp. no. C-125 by high-pressure liquid chromatography (HPLC) analysis [65]. The thin-layer chromatography analysis for hydrolytic products of birchwood xylan by the xylanase of *Cellulosimicrobium* sp. reported that xylan was degraded to several intermediate products (xylobiose and xylotriose) without a significant accumulation of xylose [30]. Similarly, Lv et al. [48] and Amel et al. [14] also reported that xylooligosaccharides were the main hydrolytic products when xylanase of microbial community EMSD5 and *Caldicoprobacter algeriensis* reacted with birchwood xylan.

From the above study, it is clear that the purified xylanase from the *B. paramycoides* T4 showed some different and novel properties with heavy metal, surfactant, and organic solvent when compared with other *Bacillus* strains reported earlier [11,32,35,66]. The pure enzyme also showed high temperature and pH activity and stability [12,36,66], which could be applicable for different industrial applications.

4. Materials and Methods

4.1. Materials

All analytical-grade reagents and media components were purchased from Hi-Media (Mumbai, India) and Merk (Mumbai, India). Column chromatography materials and a protein ladder for electrophoresis were procured from Sigma-Aldrich Pvt. Ltd., St. Louis, MO, USA.

4.2. Microorganisms

The strains of *B. paramycoides* T4, isolated from the soil sample of different sites in Varanasi, were used in this study. The *B. paramycoides* T4 culture was maintained on xylan agar slants at 4 °C and sub-cultured monthly.

4.3. Inoculum Preparation

The mother culture was prepared by inoculating one full loop of the 24 h grown culture of *B. paramycoides* T4 (MN370035) from the xylan agar plate into a 50 mL xylan broth and incubated at 55 ± 1 °C overnight to achieve the active exponential phase. A suitable amount of cell suspension was used to inoculate the test flasks.

4.4. Enzyme Production

The culture was grown in a 150 mL Erlenmeyer flask containing a 50 mL basal medium containing 1% birchwood xylan and 0.05% ammonium sulfate for xylanase production. The pH of the medium was adjusted to 7.0 before sterilization. The flasks were inoculated and incubated at 55 ± 1 °C for 24–48 h. The crude enzyme was filtered and centrifuged at 10,000 rpm for 10 min at 4 °C, and an enzyme assay was carried out. Enzyme activity was measured by the Nelson–Somogyi method [67,68]. One unit of enzyme activity is defined as 1 mg of the reducing end group (glucose) released per minute at 55 ± 1 °C.

4.5. Heavy Metal Resistance Studies on Efficient Bacterial Strain T4

In this experiment, 0.1 mL of bacterial culture of 1.0 OD (A_{620} nm; 1cm cuvette) was spread on nutrient agar plates, supplemented with various concentrations (µg/mL) of the various heavy metals such as Pb (0.0–1000 µg/mL), As (0.0–3000 µg/mL), Cr (0.0–3200 µg/mL), Ce (0.0–2400 µg/mL), Hg (0.0–150 µg/mL), Se (0.0–350 µg/mL), Ni (0.0–350 µg/mL), and Co (0.0–350 µg/mL). Lead acetate [$Pb(CH_3COO)_2$], sodium arsenate [Na_2HAsO_4], potassium dichromate [$K_2Cr_2O_7$], cesium chloride [CeCl], mercuric chloride [$HgCl_2$], selenium sulfide [SeS], nickel chloride [$NiCl_2$], and cobaltous chloride [$CoCl_2$] were some of the metal salts employed. Bacterial growth was reported from 24 to 72 h at 55 ± 1 °C.

4.6. Extraction and Purification of Xylanase

A three-step purification process was used to purify the thermo-tolerant xylanase produced by the *B. paramycoides*. All the purification steps were performed at temperatures between 0 and 4 °C unless otherwise stated.

4.6.1. Enzyme Extraction

To achieve raw enzyme, 24 h old isolates (xylan broth containing 1.0%; xylan (Birchwood), 0.5%; ammonium sulfate) were centrifuged at 10,000 rpm for 15 min. After centrifugation, bacterial cells were removed and the supernatant was used as the raw/crude enzyme for different enzyme activities.

4.6.2. Ammonium Sulfate Precipitation

The culture supernatant of *B. paramycoides* was subjected to cooling centrifugation at 10,000 rpm for 15 min at 4 °C. The supernatant containing xylanase and other soluble proteins was treated to ammonium sulfate precipitation for partial purification, and the pellets containing cells and other debris were discarded. Saturation was achieved in the range of 30 to 80%. The ammonium sulfate was slowly added to the supernatant, which was then gently agitated for 60 min with a magnetic stirrer. The pellets were collected after 60 min by centrifugation at 12,000 rpm for 15 min at 4 °C, and the pellets of different fractions were re-suspended separately in a small amount of 100 mM phosphate buffer (2 mL, pH 7.0). By placing the re-suspended pellets in a nitrocellulose dialysis membrane with a molecular weight cut-off value of 10kDa, they were dialyzed against the phosphate buffer (pH7.0) to remove the salt. The buffer was stirred continuously for 10–12 h and the buffer was changed regularly. The dialyzed sample's xylanase activity and protein content were measured using different methods.

4.6.3. Ion exchange Chromatography

The dialyzed material was put into a Q-Sepharose (15 × 70 mm) column (Sigma-Aldrich, St. Louis, MO, USA) equilibrated with sodium phosphate buffer (100 mM, pH 7.0). For 2 h at 4 °C, the required enzyme fraction was allowed to bind to the matrix. The unbound fraction was collected and the enzyme activity and protein content were determined. At a flow rate of 1.0 mL/min, the bound fractions were eluted with a linear gradient of NaCl (0.1–0.5 M, 10 mL each) in the same buffer.

4.6.4. Gel filtration Chromatography

The active fractions were collected and dialyzed against sodium phosphate buffer (pH 7.0) and were further purified by gel-filtration chromatography. The Sephadex G-75 column (1.5 × 40 cm) was equilibrated with sodium phosphate buffer (100 mM, pH 7.0) and 1 mL of concentrated sample was applied to the column. The flow rate was adjusted to 5–6 mL/h and a fraction of 2 mL each was collected.

4.7. Enzyme Assay and Protein Estimation

Xylanase was assayed by measuring the reducing sugar released by the reaction on xylan. A xylanase assay was performed by Nelson [67] and Somogyi [68] methods. Enzyme activity was defined as the amount of enzyme that liberates 1.0 µg of glucose/min/mL.

Quantitative estimation of protein content was performed by the method of Lowry et al. [69] using bovine serum albumin (BSA) as a standard and expressed as mg/mL. The protein content of individual fractions of purification steps was monitored by measuring the relative activity at 280 nm.

4.8. Polyacrylamide Gel Electrophoresis

The active fraction with the highest specific activity was electrophoresed in a 12.5% polyacrylamide gel using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), according to the Laemmli method [70]. SDS-PAGE was used to determine the

approximate molecular weight of the xylanase by comparing it to molecular mass markers such as lysozyme (14.3 kDa), β-lactoglobulin (20 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa), and phosphorylase B (97.4 kDa)

4.9. Biochemical Characterization of the Purified Enzyme

4.9.1. Determination of Optimum Temperature and Stability

The optimum temperature of the enzyme was determined by incubating the reaction mixture in sodium phosphate buffer (100 mM; pH 7.0) at various temperatures (30–105 °C) and measuring xylanase activity. The enzyme was pre-incubated for 1.0 at various temperatures (35–105 °C) to determine the thermal stability, and the residual activity was determined under standard assay conditions [67,68]. The non-heated enzyme was considered as control (100%).

4.9.2. Determination of Optimum pH and Stability

The effect of pH on purified xylanase was calculated by assaying its activity in the pH range of 4.0–10.0 using xylan as a substrate. The following buffers (100 mM), viz., sodium acetate (pH 4.0–6.0), sodium phosphate (pH 6.0–8.0), and Tris-HCl (pH 8.0–10.0) were applied for the analysis. The enzyme was diluted in de-ionized water to create appropriate enzyme units and pre-incubated with different buffers (in a 1:1 ratio) at 55 °C for 1 h to ensure pH stability [67,68]. The residual activity was measured under standard assay conditions.

4.9.3. Substrate Specificity

The purified xylanase substrate specificity was tested using 1% (*w/v*) birchwood xylan, oat spelt xylan, cellobiose, starch, carboxymethyl cellulose (CMC), p-nitrophenyl xylopyranoside, and Avicel as substrates. Substrates were prepared in sodium phosphate buffer (50 mM, pH 7.0) and the activity was assayed by following the standard method.

4.9.4. Kinetic Analysis (Determination of V_{max} and K_m Values)

The purified enzyme was used for kinetic analysis toward determining the K_m and V_{max} values with birchwood xylan as a substrate by following the method of Lineweaver and Burke [71]. The xylanase was incubated with different concentrations of substrate (2.5–4.0 mg/mL) and the activity was assayed by the standard assay method. The Lineweaver–Burk double reciprocal plot was used to represent the Michaelis–Menten equation. The calculation of both V_{max} and K_m was performed through nonlinear adjustment by using Microsoft Excel (MS Office) with the "Solver" add-in.

4.9.5. Effect of Metal Ions on Activity and Stability

The $FeSO_4$, $CaCl_2$, $CoCl_2$, NaCl, $MgCl_2$, $MnCl_2$, $ZnSO_4$, $CuSO_4$, $HgCl_2$, and $NiCl_2$ were used to investigate the effects of different metal ions (5 mM and 10 mM) on enzyme activity. To investigate metal ion stability, the enzyme was incubated with various metals at 55 °C for 1 h before being analyzed under normal assay conditions. The enzyme activity measured without metal ions was taken as a control (100%).

4.9.6. Effect of Inhibitors on Xylanase Activity

To characterize the enzyme, the effects of EDTA, β-mercaptoethanol, phenylmethylsulfonyl fluoride (PMSF), and urea as inhibitors on xylanase activity were investigated at concentrations of 5 mM and 10 mM. The purified enzyme was pre-incubated for 1 h at 55 °C with these enzyme inhibitors, and the residual activity (%) was calculated using standard assay procedures. The enzyme activity without any inhibitor was considered 100% (control).

4.9.7. Effect of Surfactants on Enzyme Stability

The xylanase sample was incubated with surfactants, viz., Tween-40, Tween-60, Tween-80, Triton-X-100, and SDS (0.1 and 1.0%, *v/v*) for 1 h at 55 °C, and then the residual activity (%) was examined under standard assay conditions. The enzyme activity without any surfactants was taken as a control (100%).

4.9.8. Effect of Organic Solvent on Xylanase Stability

The effect of various organic solvents having log *p* values ranging from 0.21 to 6.6 on enzyme stability was investigated by following the method of Ogino et al. [72]. The supernatant having maximum xylanase activity was filtered with nitrocellulose film (pore size of 0.22 µm) and mixed with different organic solvents, viz., n-dodecane, n-decane, iso-octane, xylene, n-hexane, n-butanol, cyclohexane, acetone, toluene, benzene, ethanol, methanol, and propanol at 30% (*v/v*) for 7 days in capped culture tubes at optimal temperature. The residual xylanase activity was measured against the control (solvent-free).

4.10. Analysis of Hydrolytic Products

Enzymatic hydrolysis products of birchwood xylan were distinguished by thin-layer chromatography (TLC). Chromatography (TLC plates, 0.25 mm layers of silica gel) was carried out with a mobile phase containing a solvent system of n-butanol, acetone, and H_2O (1:8:1 *v/v*). Sugar compounds were identified by spurtingethanol sulfuric acid mixture (1:1 *v/v*), followed by heating at 100 °C for 5min. D-xylose (X_1), xylobiose (X_2), xylotriose (X_3), and xylotetraose (X_4) were consideredstandard sugars.

4.11. Statistical Analyses

Each experiment with the required controls was performed in triplicate and the data are presented as the mean ± standard deviation (SD). The significance of differences between means was tested by analysis of variance (ANOVA) and Duncan's multiple means tests (DMMTs) on the parametric or arc-sine square-root-transformed data using the SPSS software, where avalue of less than 0.05 was considered as significant.

5. Conclusions

The present study is the first report of thermo-solvent-tolerant xylanase from a thermotolerant *Bacillus paramycoides* T4 isolate. Increased xylanase activity in the presence of several solvents with higher hydrophobicity (log P4.5) is specific to this strain. The isolate's tolerance to a variety of solvents and heavy metals makes it suitable for use in stressful situations. The thermo-alkaline character of xylanase is abundantly demonstrated by its activity in a wide pH and temperature range of 4.0–10.0 and 30–105 °C, respectively. Because of its stability at high temperatures and in mild alkaline environments, xylanase could be used in industry.

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Data Availability Statement: NCBI (National Center for Biotechnology Information) is the name of the repository where our data were deposited, and the dataset DOI is https://www.ncbi.nlm.nih.gov/nuccore/MN370035.1 (accessed on 24 May 2022).

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