ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Purification and Characterization of a Xylanase from *Rhizopus oligosporus*

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ABSTRACT

Xylanases are promising eco-friendly candidate for many biotechnological and industrial fields including food, feed processing, biorefining and bio-intermediates production, biofuel, textile industries and paper and pulp industry as a biobleaching agent. An extracellular xylanase secreted by *Rhizopus oligosporus* produced under solid sate fermentation using wheat bran was purified and characterized. The freeze-dried crude filtrate was subjected to two-step purification using ion exchange chromatography by DEAE-Sepharose and gel filtration chromatography over Sephacryl S-200. The enzyme was purified to homogeneity and showed a monomeric single protein band over SDS-PAGE of a molecular mass of about 23 kDa. The purified xylanase revealed maximum activity at 40^{o}C and at pH 6.0 while remained active over a wide range of pH (4.5-8.0) and temperature (40–60^{o}C). The purified enzyme showed a significant activity towards birchwood, beechwood and oat spelt xylan with Km values of 8.0, 4.7 and 3.2 mg ml^{-1}, respectively. The xylanolytic activity was stimulated by Ni^{2+} and Na^{+} ions, while it is completely inhibited by Hg^{2+}.

Keywords: Xylanase, *Rhizopus oligosporus,* production, purification, characterization

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ISSN: 0975-8585

INTRODUCTION

Notwithstanding the fact that control recent advancement, enzymes are important organic polymers that catalyze particular biochemical reactions, changing over substrates into specific products [1]. Hemicellulose represents about 20–30% of lignocellulosic biomass which is the second most basic polysaccharide in nature [2]. Xylan is the fundamental part of hemicelluloses which is viewed as a noteworthy wellspring of renewable biomass[3]. Structurally xylan is a heterogeneous carbohydrate comprising of a spine of β-1, 4 connected D-xylopyranosyl units and branches consisting of O-acetyl, α-L-arabinofuranosyl and α-D-glucuronyl residues [4]. Xylan hydrolysis is an indispensable step towards the debasement of lignocellulosic matter in nature [5]. The transformation of xylan into useful products represents to a noteworthy part of the push to accomplish prudent reasonability of the lignocelluloses biomass preparing and to produce distinctive approaches to achieve deliver chemicals and renewable energy too [6]. Subsequently, there has been developing consideration towards xylanase production.

Xylanases are a class of proteins that degrade 1, 4-xylan, a direct polysaccharide mainly present as hemicellulose in plant cell dividers, into xylose [7]. Because of the auxiliary heterogeneity of xylan, complete degradation of this heteropolysaccharide needs the synergistic activity of various xylanolytic enzymes, for example, endo-xylanase, glucuronidase, xylosidase, esterase and arabinofuranosidase [1,5]. Xylanases are subdivided inside glycoside hydrolase (GH) families into 5, 7, 8, 10, 11 and 43 [8]. They differ in their structure, mechanism activity and substrate specificity [9]. In between these, the most vital one is endo-1,4-xylanase (1, 4-D-xylan xylohydrolase, EC 3.2.1.8), which is otherwise called xylanase, and which begins the debasement of xylan into various sizes of xylooligosaccharides and xylose [10].

On other hand, xylanase degrades xylan filaments to short chains of sugar deposits which have enormous applications in industry [11]. An assortment of microorganisms can deliver xylanases including yeasts, bacteria and fungi [8,12]. Filamentous fungi are industrially potential producers of xylanase due to higher yield contrasted with yeast and bacteria [13]. Xylanases are central biocatalysts in the biorefining of hemicelluloses and they have many industrial applications (e.g. paper, textile, enhancing bread quality, clarification of juice and liquefaction of fruits and vegetables**)** [14]. Xylanases are have important role of modern industries, which can be utilized in paper pulp bleaching, increasing the shine of mash and culminating the absorbability of animal feed [9]. Xylanase improves juice clarification and maceration, diminishes viscosity and increases extraction yield and filtration, process performance and product quality. Additionally it diminishes the substance of non-starch polysaccharides, accordingly decreasing the intestinal consistency and enhancing the usage of proteins and starch, Therefore, it enhances the digestibility of animal feed for better nourish usage and nutritive estimation of inadequately degradable [16]. They are likewise, of modern significance, which can be utilized for illumination of organic product juices, upgrading the nutritional wholesome properties of farming silage and grains nourish [16]. On other hand, the products of xylan hydrolysis have applications in wheat flour for enhancing dough handling and the nature of baked products or for extraction of coffee and plant oils [17]. Concern with xylanases has notably expanded because of their potential applications in the food and beverage industries, feedstock betterment and the quality improvement of lignocellulosic buildups [18]. Where, xylanases are utilized to change over the polymeric xylan into fermentable sugars for the generation of ethanol and xylitol from plant biomass [19]. It enhances flexibility and quality of the mixture, in this way permitting less demanding taking care of in baking food industry. Xylanases can likewise be utilized for tailor designing of medications and changing the properties of food [9]. Treatment with xylanase does not represent any ecological danger and along these lines gives a hint of something to look forward to environmentalists [20]. Specifically, xylanases give an option eco-friendly innovation for bleaching Kraft pulp, which offers additional points of interest over genuine chemical treatments [21].

MATERIALS AND METHODS

Microorganism

Rhizopus oligosporus NRRL 2710, from Northern Regional Research Laboratory, United States Department of Agriculture, Peoria Illinois, USA was used for enzyme.

Enzyme production under solid state fermentation conditions

Sterilized 10 g of wheat bran was moistened with 10 ml of fermentation medium and incubated at 30°C ± 2°C.

Composition of fermentation medium

The fermentation medium had the following composition (g/ l dist. H_2O), NaNO, 9, KCl, 0.52, KH_2PO_4, 1.52, $CoCl_2$, 6 H_2O , 0.008; $MgSO_4$, 7 H_2O, 0.02; $FeSO_4$ 7 H_2O, 0.01; Casein hydrolysate, 1 and Oat xylan, 6 [22].

Preparation of xylan

Purified xylan was prepared from sugar cane bagasse according to Chen and Anderson [23] as follows: 10 g of bagasse were mixed with 100 ml of 8% NaOH (w/v) and autoclaved at 120°C for 30 min. The final filtrates were mixed to make a volume of 100 ml. The filtrate pH was adjusted to 5.0 with HCl. 150 ml of 95% ethanol were added, and the mixture was allowed to stand at room temperature for 24 h. The precipitate was collected and washed with 70% ethanol to remove lignin and other soluble. The precipitate was dehydrated with 95% ethanol, and the ethanol was removed by filtration and vacuum drying at 50°C. The extracted xylan from bagass was used in the fermentation for enzyme production.

Harvesting and the Extraction of the enzyme

For solid substrate fermentation, 100 ml dist. Water was added to each 10g fermented material and after shaking for 2 h, the extracted medium was filtered and kept frozen until use.

Xylanase assay

Xylanase activity was measured according to the method of Saha [24] using the 0.1 ml of 1% (w/v) solution of Brichwood xylan incubated with 0.4 ml of the appropriately diluted culture supernatant or enzyme enrichment fraction in 50 mM acetate buffer (pH 5.5) for 30 min at 40°C The released reducing sugars were assayed using the DNS method [25]. One unit of xylanase activity was defined as the amount of the enzyme that liberated 1 µ mol of reducing sugar equivalent to xylose per min under the standard assay conditions. Protein was determined either by measuring the absorbance at 280 nm [26] or by the method of Bradford [27] using bovine serum albumin as a standard.

Purification of xylanase

Unless otherwise stated all steps were performed at 4-7°C. The crude supernatant extracellular xylanase was obtained by centrifugation at 15,000Xg for 15 min at 4°C and the supernatant was lypholized. The lypholized powder was dissolved in 3 ml of 50 mM acetate buffer, pH 5.5. It was loaded on a DEAE-Sepharose column (15 X 1.6 cm i.d.) which was previously equilibrated with 50 mM acetate buffer, pH 5.5. Proteins were eluted with a stepwise gradient of NaCl ranging from 0.0 to 0.5 M NaCl in the same buffer at a flow rate of 60 ml/h. Fractions exhibiting xylanase activity were eluted with 0.05 M NaCl. The DEAE-Sepharose fractions having the highest xylanase activities were combined, concentrated and loaded onto a Sephacryl S-200 column (100 X 1.6 cm i.d.) pre-equilibrated with 50 mM acetate buffer, pH 5.5, the fractions having the highest xylanase activity were pooled and used for enzyme characterization.

Molecular weight determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Discontinuous reducing 0.1% (w/v) SDS-PAGE was performed according to the procedure of Laemmli [28] using 15% and 5% (w/v) acrylamide resolving and stacking gels, respectively. Samples were treated with reducing 2-mercaptoethanol containing sample buffer and boiled for 5 min prior to application to the gel. Electrophoresis was run at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. Standards proteins were coresolved on gel and used to determine the subunit molecular weight. After electrophoresis, proteins in the gel were visualized by staining with Coomassie blue R-250.

Effect of temperature on xylanase activity and thermostability

The effect of temperature on xylanase activity was determined by incubating the enriched xylanase fraction in 50 mM sodium acetate buffer, pH 5.5 at various temperatures (20 to 70°C at 10°C intervals) for 30 min before assaying xylanase activity. The thermostability of xylanase was investigated by preincubating the enriched xylanase fraction at various temperatures (20 to 70°C at 10°C intervals) in the same buffer for the 30 min, cooling to 4°C followed by assaying the residual xylanase activity.

pH-optimum of xylanase

Incubating the enriched xylanase fraction in buffers of broadly similar salinity levels, but varying in pH from 3.0 to 9.0 was used to assess the pretreatment pH stability and the pH optima of xylanase. The buffers of 50 mM used were sodium citrate (pH 3.0 to 4.0), sodium acetate (pH 4.0 to 5.5), sodium phosphate (pH 5.5 to 8.0), Tris-HCl (pH 8.0 to 9.0). The enriched xylanase fraction was mixed with the above mentioned buffers plus the control (50 mM sodium acetate buffer, pH 5.5) and assayed for xylanase activity. For pH stability, the above xylanase-buffer mixtures were left for 30 min at room temperature and then adjusted back to 50 mM sodium acetate buffer, pH 5.5 and assayed for xylanase activity as described previously.

RESULTS AND DISCUSSION

Xylanase production

Xylanase was produced by *Rhizopus oligosporus* which was not reported earlier utilizing wheat bran under solid state fermentation. However, some xylanase producing strains have been beforehand reported by Paloheimo *et al*. [29] and Li *et al*. [30]. This study illustrated that the produced xylanase from *Rhizopus oligosporus* was lypholized, purified and characterized.

Purification of *R. oligosporus* xylanase

R. oligosporus xylanase was purified to the point of homogeneity as shows in (Table 1). Purification of xylanase has been carried out by other researchers utilizing different microbial strains [31,32]. The lypholized culture filtrate was applied onto DEAE-Sepharose column with different molarities of NaCl where only one single peak was eluted with 0.05 M NaCl (Fig.1) with a specific activity of 51.3 units/mg protein. The gel filtration chromatography was carried out for the pooled 0.05 M NaCl enzyme fraction using a column of Sephacryl S-200 where a single peak of *R. oligosporus* xylanase was eluted (Fig.2) with a specific activity of 96.7 units/mg protein. In the same line, Sharma *et al*. [33] reported that the purified alkaline xylanase from *Paenibacillus macquariensis* demonstrated one protein band on SDS-PAGE with a specific activity of 25.20 units/mg protein. While in another study of Ahmad *et al*. [34] was carried out to find xylanase from *A. niger* with the purification fold was figured to be 7.93, with 48.63% yield and a specific activity of 613.13 IU/mg.

Table 1: Purification scheme of *R. oligosporus* xylanase

Purification steps	Total activity* (Units)	Total protein	Specific Activity (Units/mg protein)	Fold Purification	Recovery (%)
Freeze-dried, culture filtrate	244	32.2	7.577	1.0	100
0.05 M Na Cl DEAE-Sepharose fraction	200	3.9	51.3	6.7	82
Sephacryl S-200 fraction	170	1.76	96.6	12.7	70

*One enzyme unit was defined as one μmole xylose liberated per minute under standard assay conditions.

Molecular weight determination (SDS-PAGE)

The molecular weight of *R. oligosporus* xylanase was found to be 23 kDa by Sephacryl S-200 and affirmed by SDS-PAGE as a single subunit (Fig.3). The molecular weight of *R. oligosporus* xylanase less than those previously reported with molecular weights of 29 kDa for xylanase from *Hypocrea lixii* SS1 [1] and molecular weight 30 kDa for xylanase from *Asperguils niger* [34]. A high molecular weight of 205 kDa was reported for xylanase from *Paenibacillus macerans* [35].

ISSN: 0975-8585

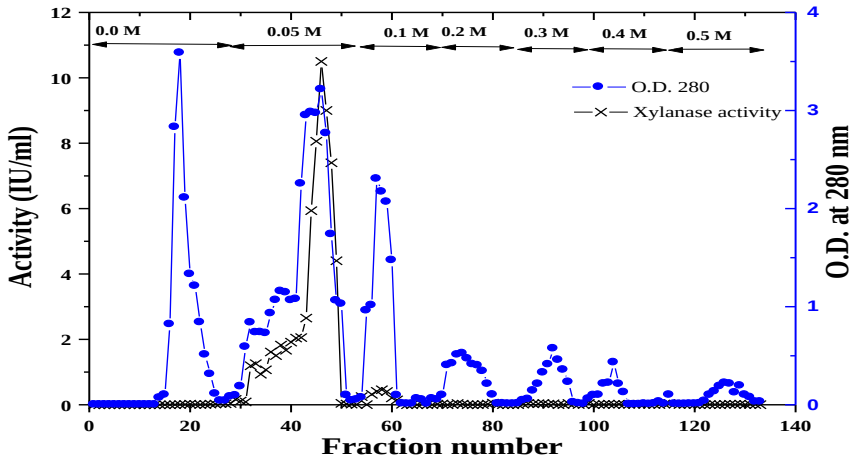

Fig 1: A typical elution profile for the chromatography of *Rhizopus oligosporus* xylanase on DEAE-Sepharose column (10 cm × 1.6 cm) previously equlibrated with 50 mM sodium acetate buffer, pH 5.5 at a flow rate of 30 ml/h and 3 ml fractions.

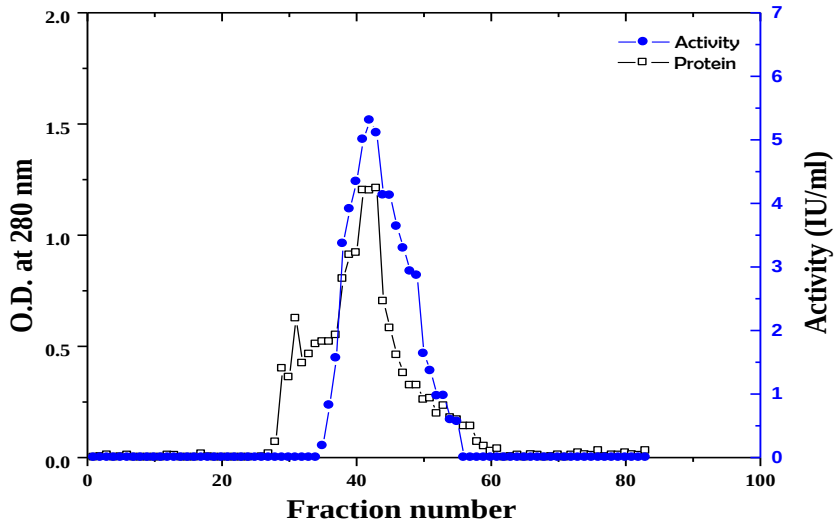

Fig 2: A Typical elution profile for *R. oligosporus* 0.05 M NaCl xylanase pooled fraction on Sephacryl S-200 column.

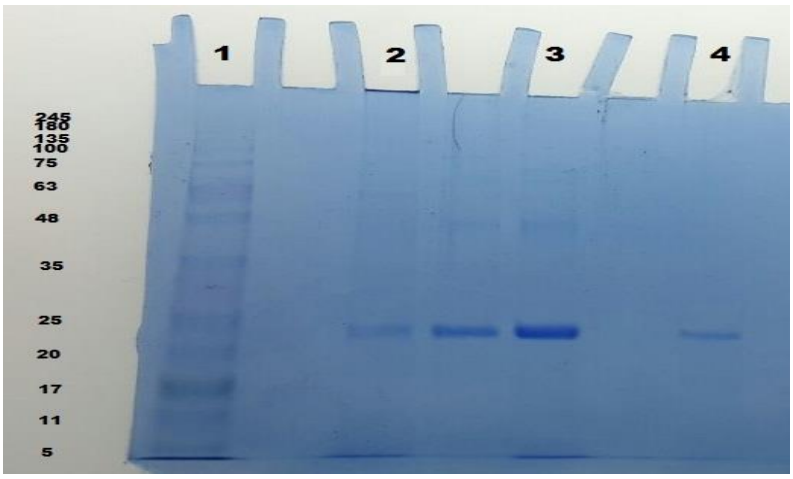

Fig 3: SDS-PAGE for molecular weight determination of *R. oligosporus* xylanase
1- Standard proteins. 2- Crude enzyme 3- activity on ion exchange
4-activity on gel filtration

ISSN: 0975-8585

Substrate specificity

The activity of the purified xylanase towards different substrates was assessed. Table (2) shows that the *R. oligosporus* xylanase herein was active on birchwood xylan (main substrate for assay) as 100%. Relative activity was increased to 126.6 and 129.2% toward beechwood xylan and oat spelt xylan, respectively. The enzyme was moderately active on glycogen but it has a slight activity on starch, CM-cellulose and pectin. This result was similar to xylanase from *T. inhamatum* [6], showing the inclination of the catalysts for branched and heterogeneous xylan.

Table 2: Relative activities of the purified xylanase towards different substrates

Substrate	% Relative activity
Birchwood xylan	100
Oat spelt xylan	129.2
Beechwood xylan	126.6
Pectin	13.2
Glycogen	40.8
Starch	23.2
CM-cellulose	16.3

K_m value

Determination of the apparent K_m values of *R. oligosporus* xylanase for birchwood, beechwood and oat spelt were refined through studies relating substrate concentrations to the responses speeds (Fig.4). The K_m value for oat spelt equal to 3.2 mg/ml with Vmax of 1.7 IU ml^{-1} min^{-1}, K_m value for beechwood were 4.7 mg/ml with Vmax of 1.8 IU ml^{-1} min^{-1} while for birchwood K_m value equal to 8 mg/ml with Vmax of 2.2 IU ml^{-1} min^{-1}. While Silva *et al*. [6] showed that *T. inhamatum* xylanase K_m value for oat spelt was 14.5 mg/ml and for birchwood K_m value was 1.6 mg/ml.

The K_m values showed that *R. oligosporus* xylanase had higher affinity for oat spelt, beechwood than for birchwood. This result is similar to *Penicillium capsulatum* xylanase which was able to hydrolyze oat spelt substrate more efficient than birchwood [36].

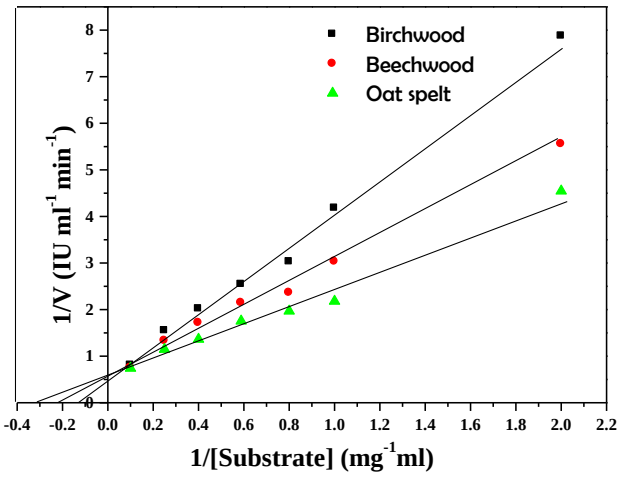

Fig 4: Lineweaver- Burk plot of *Rhizopus oligosporus* xylanase fraction with specific substrate (Birchwood, Beechwood and Oat spelt) at concentrations ranging from 0.5 mg to10 mg.

ISSN: 0975-8585

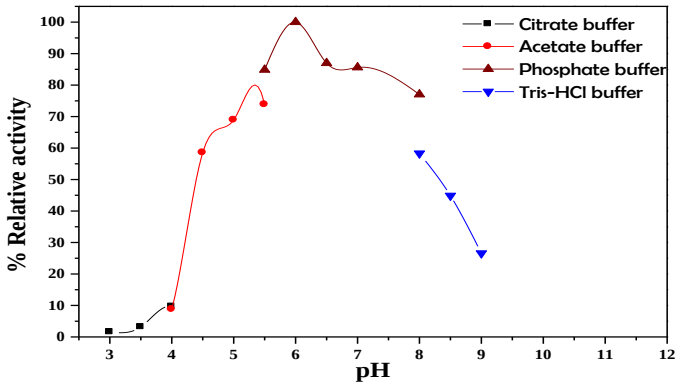

Fig 5: Effect of pH on activity of *R. oligosporus* Xylanase

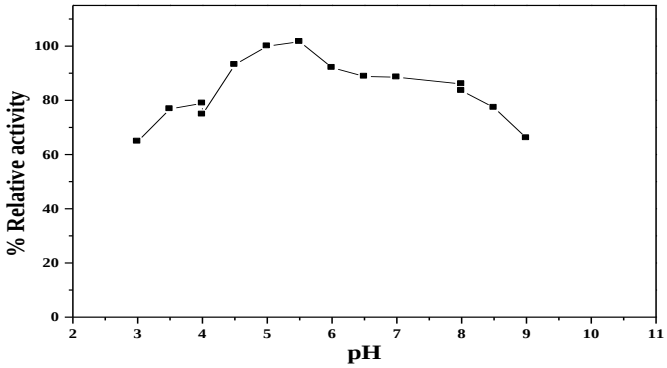

Fig 6: pH stability of *R. oligosporus* xylanase

pH of *R. oligosporus* xylanase

The activity of *R. oligosporus* xylanase was subject to pH. pH optimum of *R. oligosporus* xylanase was at pH 6.0 (Fig.5). Similar findings were also showed by Pithadiya *et al*. [15] who found that maximum xylanase production from *Bacillus circulans* was at pH 6.0. While, Lin *et al*. [37] illustrated that an optimum pH 6.5 for *Thermomyces lanuginosus*-SSBP xylanase. Also, Blanco *et al*. [38] showed a maximum activity at pH 5.5 for xylanase from a *Bacillus* species strain BP-23. This acidic xylanase is more beneficial in the textile industry than the acidic and alkaline ones because of less utilization of energy and crude material. In this way, the expense of production can be definitely diminished which thus will make it more acceptable in the market. On other hand, Irfan *et al*. [39] found an optimum pH 8.0 for production of *Bacillus* sp xylanase. However, an optimum pH 9.0 for xylanase from *Bacillus halodurans* strain KR-1and *Bacillus* sp. strain 41M were reported [33,40], respectively.

pH stability of *R. oligosporus* xylanase

R. oligosporus xylanase was incubated at 4^{o}C for 30 min at different pHs ranged from 3.0-9.0. The enzyme showed stability at pH 5.5 and 6.0 (Fig. 6). The residual activities were estimated under standard assay conditions. pH ranged from 4.5-8.0 was the most favorable for stability of *R. oligosporus* xylanase activity activity for 30 minutes. This result is in accordance with Gowdhaman *et al*. [41] who found that xylanase enzyme from *B. aerophilus* had an optimum pH for activity at 4.0 and it was stable at pH range 2.0-6.0. Xylanase with low pH optimum and good stability under acidic conditions are appropriate for food industry due to extremely low pH prevailing in the alimentary system. While in comparable study of Goswami and Rawat [42], they reported a 100% xylanase activity at pH 8.0 by *Bacillus arseniciselenatis*. While Sunny *et al*.

[43] found maximum xylanase stability from *Bacillus sp*. at pH 9.0 indicating that the enzyme was alkali stable as well as moderately acid stable.

Effect of temperature on *R. oligosporus* xylanase activity and thermostability:

Fig. (7) portray the relation between various temperatures and relative activity of *R. oligosporus* xylanase. Xylanase activity was directly proportional to increment in the temperature even to 40°C where it recorded its most optimum activity before decreasing obviously at 50°C with about 18% activity at 70°C. The ideal temperature at 40°C was like that showed of *B. Megaterium* xylanase by Irfan *et al*. [39] and from *Bacillus halodurans* by Araki *et al*. [44].While the optimum temperature was at 45°C for *A. ficuum* AF-98 xylanase and *T. reesei* xylanase [45,46]. Comparable discoveries were furthermore reported by extreme enzyme activity from various *Bacillus species* has temperature-optima of 40–60°C [47,48]. While Monisha *et al*. [49] and Pithadiya *et al*. [15] found that *Bacillus circulans* was given greatest xylanase production at 30°C. Slightly higher optimum temperatures have been accounted for different xylanases, by strains of *Bacillus sp*. which gave most amazing yield of xylanase at optimal temperature of 45°C and 55°C [50]. In like manner Kavita *et al*. [51] reported an optimum temperature of 55°C for the purified of *Aspergillus nidulans* KK-99 xylanase. However, Dheeran *et al*. [35] found that *Paenibacillus macerans* xylanase has a wide range of enzyme activity at temperature from 40°C to 90°C and revealed an optimum activity at 60°C.

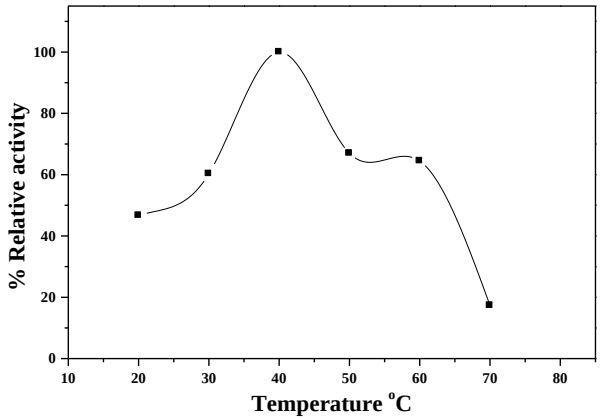

Fig 7: **Effect of temperature on *R. oligosporus* xylanase**

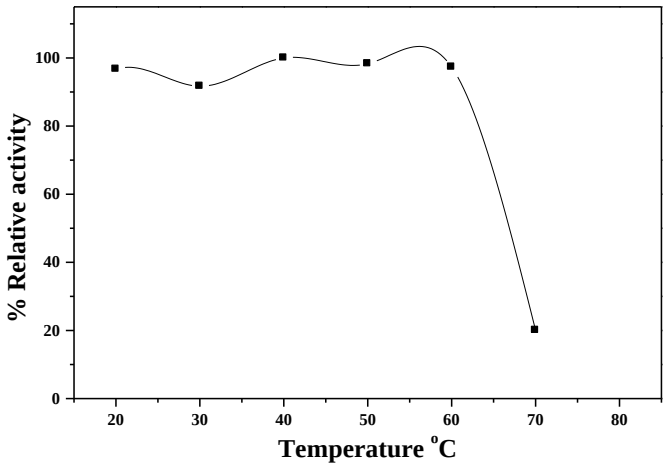

Fig 8: Thermal stability of *R. oligosporus* xylanase

Thermal stability

Thermal stability of *R. oligosporus* xylanase was estimated by keeping the protein at various temperatures extending from 20 to 70°C for 30 minutes followed by cooling in an ice bath. The residual

activities were estimated using the standard assay reaction mixture. The average of two assay experiments was taken for each point (Fig.8). The protein activity held more than 90% of its maximum activity from 40 to 60°C, while at 70°C the *R. oligosporus* xylanase activity was held only 20% of its maximum activity. In a comparable study xylanase enzyme was stable in a wide range of temperatures (40-90°C) and illustrated great thermal stability at 60°C with half life of 2 hours and could hold 80% enzyme activity up to 3 hours [42].

Effect of different metal ions on activity of *R. oligosporus* xylanase

Results regarding the effect of metal ions on xylanase activity were summarized in Table (3). It was found that Al^{+3}, Co^{2+}, Ba^{2+} and K^{+} has a mild inhibitory effects on xylanase activity; while, Ni^{2+} and Na^{+} ions had a weak stimulatory effects where, both Ni^{2+} and Na^{+} ions in 10 mM concentration elevated the activity to 103.5%. On other hand, the enzyme activity decreased to 75.7, 80, 83.5 and 85% in presence of 5 mM of Ca^{2+}, Mg^{2+}, Cu^{2+} and Zn^{2+}, respectively. Inkyung and Jaiesoon [52] adduce a report about stimulation of *Paenibacillus sp.* strain K1J1 xylanase by Ca^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, and Mn^{2+}. In a comparable study of Lee *et al*. [53], presence of Ca^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, and Mn^{2+} revealed a stimulation to *B. licheniformis* xylanase. While, Collins *et al*. [10] showed inhibition of *Pseudoalteromonas haloplanktis* xylanase in the presence of Cu^{2+} and Zn^{2+}. In the present study, *R. oligosporus* xylanase activity was completely inhibited in the presence of Hg^{+2} ions. The destruction of the xylanolytic ability by Hg^{2+} ions may be because of its interaction with sulphydryl groups, proposing that there is an essential cysteine residue in or near to the substrate binding site of the enzyme. Hg^{2+} has formerly been reported to completely inhibit the enzymatic activity of xylanase from many different sources *B. circulans, Paenibacillus macquariensis and T. inhamatum* [6,33,54].

Table 3: Effect of different metal ions on activity of *R. oligosporus* xylanase

	% Remaining activity	
	5 mM	10 mM
Non	100	100
Al^{3+}	54.0	81.9
Ba^{2+}	61.7	88.1
Ca^{2+}	75.7	94.3
Co^{2+}	57.2	80.7
Cu^{2+}	83.5	99.6
K^{+}	69.6	98.5
Mg^{2+}	80	101.8
Zn^{2+}	85	107.1
Ni^{2+}	90	103.5
Na^{+}	101	103.5
Hg^{2+}	0	0

CONCLUSION

Xylanase is a standout amongst the most critical enzymes used in agriculture and industry. It is produced by various microorganisms, such as yeast, bacteria and fungi. The present study focused on production of xylanase by *Rhizobus oligosporus* under solid state fermentation. The produced xylanase was purified and characterized. Additionally, activity of the purified xylanase enzyme toward different metals has been also determined. This manuscript presents the first report about the purification and properties of xylanase from *R. oligosporus* by a simple and inexpensive procedure. Furthermore, a comparison of the results indicates possible employment of such enzymes in some industrial processes. Since thermostability, acid conditions and wide-ranging pH stability are required properties for many industrial applications of xylanases, such as for animal feed, juice, wine, paper and textile industries in order to meet various industrial needs under the harsh environmental conditions.

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