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Purification and Characterization of a New Thermoalkali-Stable xylanase Produced from *Bacillus Amyloliquifaciens* NRRL B-14393 by Solid-State Fermentation of Water Hyacinth.

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ABSTRACT

Purification and characterization of crude xylanase produced by *Bacillus amyloliquifaciens* NRRL B-14393 grown on water hyacinth under solid-state fermentation were investigated. Xylanase was purified using DEAE-Sepharose and Sephadex G-100 columns which affected specific activity (1009.14 U/mg) and 18.67 purification fold. The pure xylanase has molecular weights of 34.679 and 29 KDa by gel filtration and SDS-polyacrylamide electrophoresis, respectively. The pure xylanase showed a maximal activity at pH 9.5 and 50°C. The enzyme showed a broad range of pH stability (pH 5.5–12). Xylanase retained its full activity up to 40˚C for 15 min. It was stable up to 60˚C with a thermal denaturing half- life of 90 min. The enzyme exhibited a high specificity for the birch wood xylan substrate. Xylanase had a K_m value of 2.94mg ml^{-1} and V_{max} of 10.92 µmole min^{-1} ml^{-1}.The enzyme was nearly completely inhibited by $MnSO_4$ and $HgCl_2$ at 1 and 10mM concentration. It consisted of 17 amino acids and rich in aspartic acid and tyrosine. These properties make this enzyme a potential candidate for future use in biotechnological applications particularly in the pulp and paper industry as well as large scale production of xylooligosaccharides.

Keywords: Xylanase; *Bacillus amyloliquifaciens*; Water hyacinth; Purification; Characterization; Solid state fermentation.

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INTRODUCTION

Lignocellulosic wastes are generated in large quantity through forestry, agricultural practices and industrial processes. These wastes are biodegradable and can be converted to valuable products, such as, biofuel, chemicals or cheap energy source for fermentation.

Lignocellulose consists of cellulose, hemicellulose and lignin [1]. Xylan is a component of hemicelluloses, being the major structural and second most abundant polysaccharide in plant cells, accounts for approximately 1/3rd of all renewable carbon on earth. It is a hetero polysaccharide with a homopolymeric chain of 1,4,β-d-xylosidic linkages with the backbone comprising of O-acetyl, α-l-arabinofuranosyl,α-,1,2-linked glucuronic or 4-O-methylglucuronic acid [2]. The complete hydrolysis of xylan requires endo-1,4-β-d-xylanases (EC3.2.1.8),β-d-xylosidases (EC3.2.1.37), α-l-arabinofuranosidases (EC3.2.1.55),α-d-glucuronidases (EC 3.2.1.139) and acetylxylanesterases (EC3.1.1.72) [2].

Now a days using microbes for the xylanase production has become popular because of their widespread application in industrial processes such as improving the digestibility of animal feed stocks, bio bleaching of pulp, textile industry, production of xylooligosaccharides, waste-water treatment, texture improvement in bakery products, clarification of juices and wine, debarking process and bioconversion of lingo cellulosic wastes into useful economical products such as ethanol, single-cell protein, sugar syrups, and liquid and gaseous fuels [3]. Xylan-degrading enzymes have been produced mainly by a wide variety of microorganisms that include bacteria, fungi and yeast [4-6]. However, the industrial demand for alkali-stable and thermo-stable xylanases has not yet been met adequately [4].

Solid State Fermentation (SSF) has gained renewed interest in recent years for the production of many enzymes due to lower operation costs and energy requirements [7]. Water hyacinth pose a big and increasing problem in Egypt, mechanical control alone might not be sufficient [8]. Among the consequent serious problems of invasion by water hyacinth is the vast range and rapid spread of the aquatic weeds in the Egyptian water bodies, particularly in the network of irrigation and drainage canals in the Nile Delta region [9]. It produces serious problems due to increased water loss and evaporation, retardation of water flow, interference with navigation, health hazards and alteration in the physicochemical characteristics of both water and hydro soil [10]. A more productive way to finally control the growth can be to make use of the plant by using it as a substrate for enzymes production [11-14].

To our knowledge, rare studies have been carried out on the production of xylanase enzyme from bacteria especially *Bacillus* in a pure form. Therefore, it is the objective of this study is the preparation of pure homogenous enzyme from *B. amyloliquefaciens* for further characterization, a simple reproducible method was established. Earlier we have reported the optimum conditions for the production of xylanase from this strain [14].

MATERIALS AND METHODS

Plant material:

Water hyacinth plant (WH) was collected in winter season from Mansoureya canal, Giza, Egypt. Its roots were cut off and the green parts (stem and leaves) were thoroughly washed with tap water, sliced, crushed in a mixer to small pieces 0.5-1 cm and store at 4°C till use. Then this substrate was used as a sole carbon source for the production of the enzyme.

Experimental organism:

Bacillus amyloliquifaciens NRRL B-14393 was obtained from Agricultural Research Service, Peoria, Illinois, USA. The strain was maintained on nutrient agar slant containing (3g/l beef extract, 5g/l peptone, 8g/l sodium chloride and 15g/l agar), then stored at 4 °C and sub-cultured monthly. Inoculum was developed by transferring a loopful of stock culture into a sterile nutrient medium and incubated at 37 °C on a shaker at 200 rpm for 24 h.

Production and extraction of xylanase:

Ten gram of fresh water hyacinth supplemented with 0.5 % w/w sucrose was placed in 100-mL Erlenmeyer flasks and autoclaved at 121 °C for 15 min, cooled and then sprayed with 7% of *B. amyloliquifaciens* inoculum. The inoculated medium with initial pH 6.0 and initial moisture level of 95% was then incubated statically for 24 h. at 35°C [14].

The enzyme was extracted from the fermented matter with 10-fold (v/w) distilled water by shaking (200 rpm) at 30°C for 60 min. [15]. The resultant slurry was filtered through a wet muslin cloth and centrifuged at 10,000xg for 15 min. Finally, the extracts were collected and considered as a source of crude enzyme.

Xylanase assay:

Xylanase activity was measured by incubating 1.0% (w/v) birch wood xylan in 0.05 M acetate buffer (pH 5.5) and an appreciable amount of diluted enzyme extract in a total volume of 0.3ml at 50°C for 60 min [5]. The release of reducing sugar was measured as xylose by Somogyi-Nelson method [16, 17] using xylose as a standard. One unit (U) of xylanase is defined as the amount of enzyme that releases 1 µmol xylose/min under the assay conditions. The specific activity is expressed as units per mg of protein.

Protein determination:

The absorbance at 280 nm was measured to monitor the protein content during chromatographic separation by using Agilant technologies-Cary100, UV-Vis spectrophotometer [18]. For specific activity determination, Protein was measured by Lowry et al. [19] method using a bovine serum albumin as a standard.

Purification of xylanase produced by *B. amyloliquifaciens:*

The whole optimized culture medium collected from several batches was extracted by 10-fold (v/w) distilled water. The resultant slurry was filtered through a wet muslin cloth and centrifuged at 10,000xg for15 min. Finally, the extracts were collected and considered as a source of crude enzyme.

Fractional precipitation by ammonium sulfate:

Attempts were done for the partial purification of xylanase by ammonium sulfate according to the method of Green and Hughes [20].Certain volume of the prepared crude enzyme was treated with different concentrations of ammonium sulfate (0-80%) of saturation, each fraction was obtained by centrifugation (13,000 xg, 4°C) for 15 min. The resulted precipitates were dissolved in an appropriate amount of distilled water and dialyzed exhaustively against distilled water for 2 days at 4 °C to get rid of the excess of ammonium sulfate. Undissolved protein was removed by centrifugation before enzyme assay. Enzyme activity and protein content were determined in each fraction.

Fractional precipitation by acetone:

This was carried out by using acetone as protein-precipitant. In this experiment, a suitable amount of crude enzyme was fractionated to four fractions by stepwise addition of the cold acetone to the levels of 30, 60, 70 and 80% and centrifugation was carried out for 15 min. at 4 °C and 13,000 xg. Each precipitate was then dissolved in 0.05 M acetate buffer, pH 5.5 and dialyzed against the same buffer.

Ion-exchange chromatography (DEAE-Sepharose):

Crude lyophilized enzyme was applied separately on the top of the column (22.5 x 2.5 cm) of pre-swollen DEAE-Sepharose equilibrated with 0.05 M acetate buffer pH 5.5 for 24h after packing the column. Elution was carried out using the same buffer with a rate 30 ml/h, then a linear gradient of NaCl (0.1-0.5 M) in the same buffer was eluted. Fractions of 10 ml were collected. Each fraction eluted by NaCl gradient was dialyzed against the elution buffer for 48 h at 4 °C. The active fractions were lyophilized and stored.

Gel filtration chromatography (Sephadex G-100):

Sephadex G-100 was soaked in distilled water for two days. Fine particles must be removed by decantation. A glass column (66 x 1.2 cm) loaded with Sephadex G-100 was used and equilibrated with 0.05M acetate buffer pH 5.5 before use. The concentrated lyophilized fractions of enzyme (eluted from DEAE-Sepharose column) were eluted with the same buffer at a flow rate of 15ml/h.

Physico-chemical properties of the purified *B. amyloliquifaciens* xylanase:

Ultraviolet absorption spectrum:

The absorbance behavior of the enzyme was studied at 200 -300 nm in 0.05M acetate buffer, pH 5.5 using Agilant technologies- cary100, UV-Vis spectrophotometer.

Molecular weight determination:

a. By gel filtration:

The molecular weight was determined by gel filtration technique using Sephadex G-100 [21]. A glass column (66 x 1.2 cm) loaded with Sephadex G-100 was used and equilibrated with 0.05M acetate buffer pH 5.5. The column was calibrated with cytochrome C (12.27 KDa), trypsin (23 KDa), Ovalbumin (45 KDa) and bovine serum albumin (69 KDa). Dextran blue (2000 KDa) was used to determine the void volume (V_0). Protein solution of 0.2 ml volume containing 4-6 mg protein/ml were applied to the same column and developed using the same buffer at a flow rate of 15 ml/h, and fractions of 3 ml volume were collected. A calibration curve was constructed by plotting log molecular weight versus V/V_0, where V was the elution volume and V_0 was the void volume.

b. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis technique (PAGE) :

This was done according to the method described by Laemmli [22] using SCIE-PLAS vertical mini-gel unit, model TV-100.

Effect of pH on the activity and the stability:

Small aliquots of the enzyme was assayed with five buffering agents, namely citrate (pH 3.0-4.0), acetate (pH 4.5-5.5), phosphate (pH 6.0-7.5) , Tris-HCl (pH 8.0-10.5.) and glycine- NaOH (pH 11-12) at 0.05 M for each one, for recording pH profile under the standard assay conditions. To study the effect of pH on the stability, the purified enzyme was pre-incubated for 30 min. at 30 °C with the five previous buffering systems before testing the enzymatic activities at the standard assay conditions, then relative activity was calculated.

Effect of temperature on the activity and the stability:

The maximum activity of the pure enzyme was determined at different incubation temperatures ranged from 30-70°C. Thermostability was studied by pre incubating small aliquots of the purified enzyme at different temperatures; 30, 40, 50, 60 and 70 °C for varying time intervals; 15, 30, 45 and 60 min. The remaining enzyme activity was then assayed using the standard assay conditions.

Shelf life of xylanase:

The shelf life stability of xylanase was under refrigeration (4˚C) and freezing conditions (-20˚C). Enzyme samples were withdrawn at different time intervals up to 30 weeks to monitor residual xylanase activity under the standard assay conditions.

Effect of reaction time:

The purified enzymes were incubated with the substrate for different time intervals up to 180 min., then the reaction products (mg) were estimated and its amount was calculated per reaction mixture.

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Effect of enzyme concentration:

The enzyme activity was estimated at different concentrations of the purified enzyme with the substrate at the optimum temperature. The relation between enzyme concentration and reaction products was plotted.

Determination of substrate specificity:

The activity of the purified enzyme on several substrates was tested. When polysaccharides were used, the activity was determined by the release of reducing sugars. The synthetic substrates p-nitro phenyl derivatives were determined with liberated nitrophenol.

Determination of Michaelis constant (K_m):

The K_m value was determined according to the method of Line weaver-Burk [23], using birch wood xylan as a substrate.

Effect of metal ions and reagents on enzyme activity:

The effect of different metal ions and various chemicals on the activity of enzyme was studied. The chemicals which were used namely, KCl, $MgCL_2$, $CdSO_4$, $HgCl_2$, $CoCl_2$, $CuSO_4$, NaCl, $FeSO_4$, $CaCl_2$, $MnSO_4$, $ZnSO_4$, Pb-acetate, EDTA and p-Chloromercuri-benzoate (pcmb), pre incubated with the purified enzymes at 1mM and 10 mM for 30 min. at 30 °C. The relative activities were determined under standard assay protocol comparing with control without metal ions or compounds (100%).

Amino acid analysis:

Amino acid composition was carried out as described by Millipore and Cooperative [24].The dried sample was dissolved in 1 ml of dilution buffer/eppendorf, then injected into full automated amino acid analyzer, eppendorf LC 3000.

RESULTS AND DISCUSSION

Partial purification of the crude xylanase produced by *B. amyloliquefaciens* was carried out by fractional precipitation with acetone and ammonium sulfate. The results revealed that ammonium sulfate was not suitable precipitating agent for the purification of the enzyme. Also fractional precipitation with acetone revealed its unsuitability as a precipitating agent due to the poor yield obtained relative to the crude enzyme.

It was preferable to load the concentrated culture filtrate directly into anion exchange chromatographic column DEAE-Sepharose giving specific activity of 251.15 U/mg protein, 4.6 purification fold and 95.14 % yield (Table 1). Further purification steps were carried out using Sephadex G-100 led to obtain one peak (Figure1) having xylanase enzyme with sp. activity (1009.14 U/mg protein), 18.67 purification fold and 49.30% recovery (Table 1) which also indicates its purity and also it means that *B. amyloliquifaciens* NRRL B-14393 elaborates only one type of endo-1, 4-β-xylanase with regard to the mode of attack on xylan.

Table 1: Typical *B. amyloliquifaciens* xylanase enzyme purification

Purification steps	Activity (Units)	Protein (mg)	Specific activity (U/mg)	Purification fold	Yield (٪)
Crude extract	7165	132.57	54.04	1.00	100
DEAE –Sepharose Buffer fraction	6816.90	27.14	251.15	4.60	95.14
Sephadex G-100	3531.96	3.49	1009.14	18.67	49.30

-Each value is the average of three to seven experiments for different batches.

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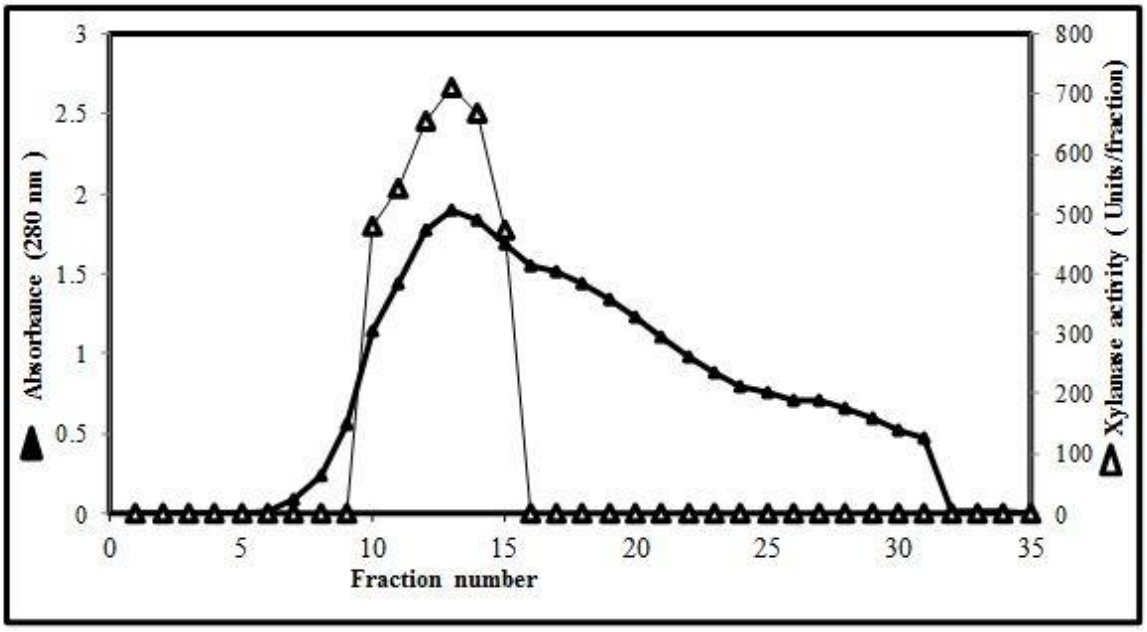

Figure1: A typical elution profile for *B. amyloliquifaciens* xylanase on Sephadex G-100

It can be seen from the purification steps, that *B. amyloliquefaciens* xylanase have higher purification fold (18.67) than that obtained by Prakash et al. [25] and Prakash et al. [26] (10.6 and 11.8 purification fold respectively). Also, the specific activity of the purified enzyme was higher than xylanase from *B. lichenformis* as detected by Bajaj and Manhas [27] (48.78 U/mg) but it was lower than the enzyme isolated from *P. macerans* IIPSP3 (4170 U/mg) as indicated by Dheeran et al. [28].

Many investigators obtained pure xylanase preparations from different microorganisms by different methods including ammonium sulfate, followed by ion-exchange chromatography, then gel filtration chromatography or vice versa was investigated [28,29].

A two-step chromatography procedure consisting of gel filtration and anion exchange without any additional pre-purification steps yielded 21.8 purification fold to apparent homogeneity with 9.6% recovery from *Scytalidium thermophilum* xylanase as demonstrated by Didem et al. [30]. The absorption spectrum indicated the presence of a peak at 209 nm for purified xylanase. This, together with the absence of absorbance of spectra in the visible region, suggests the absence of chromophore and nucleotides [31].

The molecular weight (M.wt.) of pure *B. amyloliquefaciens* xylanase enzyme determined by both SDS-PAGE and gel filtration technique was found to be 29 and 34.679 KDa, respectively (Figure 2). Sharma et al. [32] reported that the M.wt. of *P. macquariensis* xylanase was identical (31 KDa) by using either SDS-PAGE or gel filtration. On the other hand, *Chaetomium* sp. xylanase recorded a molecular mass of approx. 25.1 KDa on SDS-PAGE and by gel filtration of 22 KDa [29]. Also, these values are similar to those established by several investigators using different microorganisms [33, 34]. While extracellular xylanase of the *Streptomyces* sp. CS624 had M.wt. equal to 40 kDa [35]. While, Shrinivas et al. [36] disagreed with our results that the M.wt. of *Bacillus* sp JB 99 xylanase was 20 kDa. Sometimes different xylanases were produced in the culture filtrate of one strain have different M.wts. like the strain *B. Pumilus* which have xylanases (I, II, III) as mentioned by Poorna et al. [37] where xylanase I was 14 kDa, xylanase II 35 kDa and that of xylanase III was 60 kDa. Contrarily, Dheeran et al. [28] reported a M.wt., 205 kDa for xylanase from *P. macerans*.

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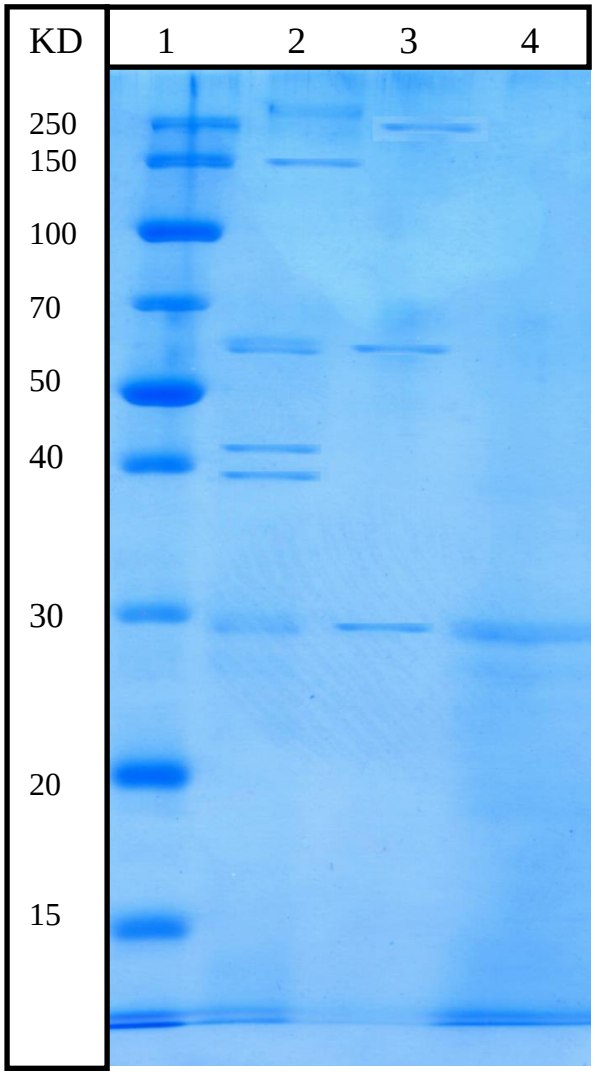

Figure 2: SDS-polyacrylamide gel electrophoresis of xylanase
Lane 1: Standard M.wt., **Lane 2:** Crude enzyme., **Lane 3:** Fraction DEAE-Sepharose., **Lane 4:** Fraction Sephadex G-100

The appearance of single protein band in SDS gel electrophoresis (Figure2) and near values of the M.wt. by both SDS gel electrophoresis and gel filtration suggested that the xylanase was monomeric [29].

The results in Figure 3a showed that the optimum pH for xylanase activity was 9.5 and showed 94.30% of its activity at pH 9.0 these results resemble to some extent those reported by Kumar and Satyanarayana, [38] and Prakash et al. [25] using *B. halodurans* KR-1 and *Chromohalobacter* sp. TPSV 101,respectively. While, pH values higher than these values were obtained with *Actinomadura* sp. (pH10) and showed about 90% of its optimal activity at pH 11 [39]. On the other hand, Li et al. [40], Poorna [37] and Kallel et al. [4] stated that the maximum activity of xylanase from different microorganisms was achieved at lower pH values ranging from 3 to 8. Xylanase of *B. amyloliquefaciens* NRRL B-14393 was found to be stable at pH 5.5-12.0 (Figure 3b), which is similar to that for xylanase from *B. pumilus* SV-85S ,*B. lichenformis* and *P. macquariensis*as determined by Nagar et al. [3] , Bajaj and Manhas [27] and Sharma et al. [32] ,respectively. Thus, the enzyme is more stable in alkaline conditions than that of other bacterial xylan-degrading enzymes [4,41]. The higher activity and stability of the enzyme in alkaline conditions have advantages in paper and pulp industries and in xylooligosaccharides generation.

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The optimum temperature for xylanase activity was found to be 50 °C (Figure 3c) which is consistent with optimum temperature of different microorganisms indicated by Yin et al. [42] and Kallel et al. [4] and contradict with those mentioned by Dhiman et al. [33], Bajaj and Manhas [27] and Prakash et al. [25], who determined the maximum activity of xylanases at temperature range from 60 to 80 °C.

On studying the heat stability of *B. amyloliquifaciens* xylanase in the absence of xylan substrate, the results showed that full enzyme activity (100%) was retained up to 40˚C for 15 min. and more than 80% of the activity remained till 90 min. Whereas, 10.3% only of the activity was lost on heating at 50˚C for 15 min., even after 90 min. a slight decrease of 22.0% was detected. The enzyme was stable up to 60˚C and retained 60.0% of its activity till 60 min. incubation. However, an appreciable half-life of the enzyme was observed up to 60˚C for a period of 90 min. (Figure 3d). Complete inhibition of the enzyme activity was reached at 70˚C after 15 min. This result coincide with that of xylanase from *S. rameus* [40] and considerably different from those of *Bacillus* sp. strain JB-99, different species of *B. halodurans, Actinomadura sp. and B. majavensis* UEB-FK indicated by Shrinivas et al. [36], Kumar and Satyanarayana [38], Prakash et al. [25] and Kallel et al. [4]. This satisfactory heat stability and alkaline pH suggest that the *B. amyloliquifaciens* NRRL B-14393 xylanase enzyme could be employed constructively in an industrial or agrochemical context [27,38].

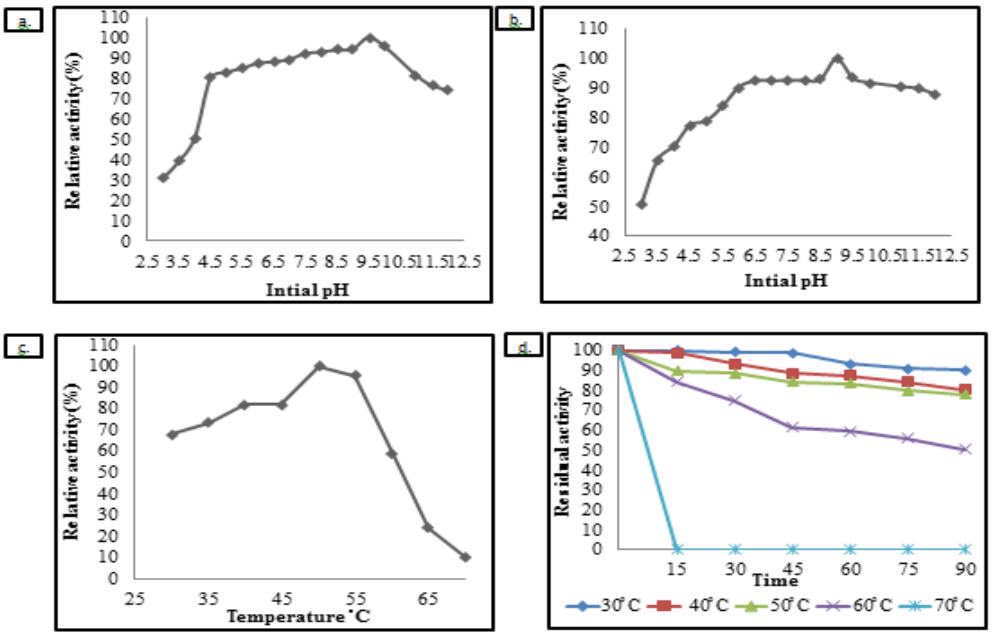

Figure 3: Effect of the pH and temperature on the activity and stability of *B. amyloliquefaciens* xylanase.

(a) Optimum pH, (b) pH stability, (c) Optimum temperature, (d) Thermostability.

B. amyloliquifaciens xylanase retained 64% of its activity up to seven months at 4˚C, while it retained 53.5% of its activity up to five months at -20˚C. However, Purified *S. cyaneus* SN32 xylanase was capable of retaining full activity after incubation at 4˚C for 20 weeks [43].

The amount of reducing sugars formed from xylan hydrolysis by the *B. amyloliquifaciens* xylanase in the reaction mixture increased with increasing time till 150 min.(0.71 mg/reaction mixture), followed by decrease in hydrolysis rate after 180 min. (0.65mg/reaction mixture). Whereas, Fathy et al. [31] recorded that the rate of xylan hydrolysis by *P. ostreatus* xylanase increased with increasing time till 120 min., beyond this period, the rate of hydrolysis decreases sharply with time. The activity of xylanase enzyme showed a linear function of enzyme concentration up to 0.25 mg protein/reaction mixture, beyond this concentration

inhibition occurred. It could be concluded that the enzyme at higher concentration was inactivated which in accordance to the results reported by Fathy et al. [31].

The enzyme is most active on birch wood xylan (100%) and less active on beech wood xylan (35.7%) followed by oat spelt xylan (24.9%) and the enzyme activity towards soluble starch was negligible. No activity on p-Nitrophenyl-β-xylopyranoside, p-Nitrophenyl-β-glucopyranoside, carboxymethylcellulose, cellobiose, dextrin and chitosan was observed (Table 2). So, it can be suggested that the purified *B. amyloliquifaciens* NRRL B-14393 xylanase is a true xylan degrading enzyme as it did not show any ability to liberate reducing glucooligosaccharide from carboxymethylcellulose [39]. The specificity of this enzyme in this respect was very similar to that of xylanases of *Actinomadura* sp. strain Cpt20 and *Bacillus* sp. YJ6 [39, 42] ,respectively. This phenomenon indicated that the substrate binding domain of xylanase had very high affinity for xylans from softwood (birch wood and beech wood) and hardwood (oat spelt). This might be due to the differences in xylan polymer structures. The binding of xylanase to xylans from birch wood, beech wood, and oat spelt might be due to reactive group exposure on the surface that can much more easily bind even the non- proteins [42].

Table 2: Relative activities of purified xylanase from *B. amyloliquefaciens* towards different substrates

Substrate	Concentration	Relative activity (%)
Birch wood xylan	1%	100.00
Beech wood xylan	1%	35.71
Oat spelt xylan	1%	24.96
Soluble starch	1%	0.19
Chitosan	1%	Nil
Carboxymethylcellulose	1%	Nil
Dextrin	1%	Nil
Cellobiose	5mM	Nil
*p-Nitrophenyl-β-xylopyranoside	5mM	Nil
*p-Nitrophenyl-β-glucopyranoside	5mM	Nil

-Data are means of triplicate tests.
-All substrates were determined with reducing assay with xylose or glucose as reference.
*Determined with liberated nitrophenol.

From these results, it can be concluded that the enzyme is neither cellulase nor xylosidase but true xylanase. The strict substrate specificity of *B. amyloliquefaciens* also indicates that this enzyme is a member of GH11 family [44].

The Michaelis constant K_m value of *B. amyloliquifaciens* xylanase, was equal to 2.94mg/ ml and V_{max} was 10.92 µmole /min/ml (Figure 4). The rate of hydrolysis of xylan decreased in the higher range of substrate concentration, presumably because of substrate inhibition. These results resemble to some extent those for different microbial origin xylanases: *C. cellulans* CKMX1 (2.64 mg/ml) [34], *B. mojavensis* UEB-FK (3.85 mg/ml) [4] and *Bacillus* sp. JB 99 (4.8 mg/ml) [36]. The lower K_m value for xylanase was recorded in *Chromohalobacter* sp. (0.2 mg/ml) as mentioned by Prakash et al. [25]. While the higher K_m value for xylanase was recorded in *B. stearothermophilus* (14.29 mg/ml) [33].

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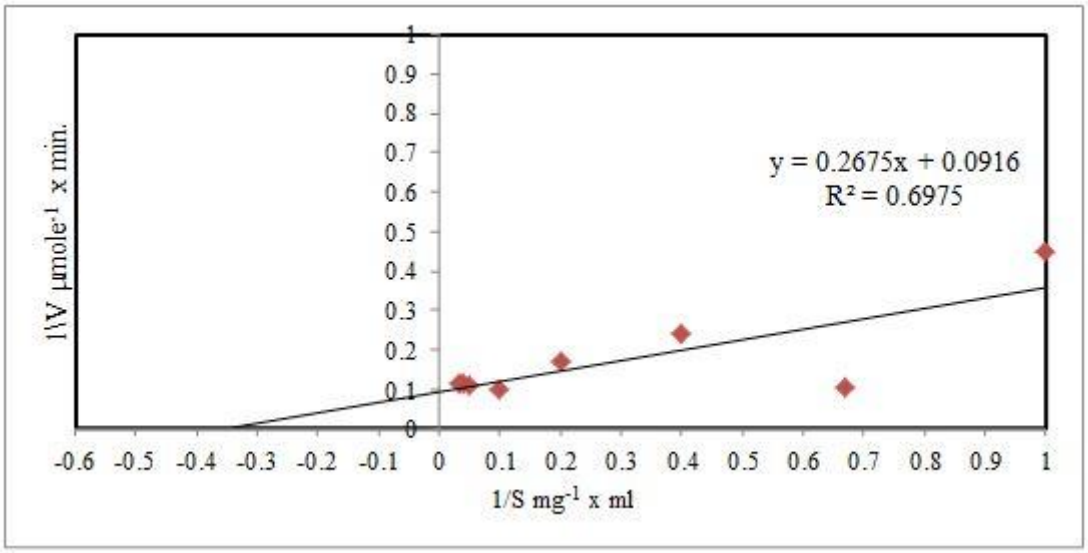

Figure 4: Linweaver-Burk plot for birch wood xylan hydrolysis by *B. amyloliquifaciens* xylanase

The results in Table 3 shown that the enzyme was completely inhibited by treatment with 10mM $MgCl_2$ and $HgCl_2$ suggested reaction with thiol groups. Also, NaCl, KCl, $CaCl_2$, $MnSO_4$, $FeSO_4$ and EDTA at concentration of 1&10mM, had moderate inhibitory effect on the enzyme activity. Similar effect by $CdSO_4$, $CuSO_4$, $ZnSO_4$, $Pb(CH_3COO)_2$, and p-Chloromercuribenzoate at only 1mM concentration was also observed. $CdSO_4$, $ZnSO_4$, $CuSO_4$, $Pb(CH_3COO)_2$ and p-Chloromercuribenzoate at 10mM concentration which had higher inhibitory effects on xylanase activity which can be attributed to the oxidizing nature of these metal ions. While, $CoCl_2$ had slightly inhibitory effect. These results are similar with xylanase produced from *B. halodurans* [38]. Xylanase from *Bacillus* sp. YJ6 was highly activated by K^+, Na^+, and Co^{+2} as reported by Yin et al. [42]. While, tremendous enhancement in activity was caused by Fe^{+2} ,Mn^{+2} ,Co^{+2},K^+ and Mg^{++} ,while Zn^{++} did it moderately and Ca^{++} did not show any effect [27].

In our results xylanase activity was inhibited by chelating reagents like EDTA and by disulfide-reducing agents p-Chloromercuribenzoate, which suggests that disulfide bonds are essential to maintain the active conformation of the xylanase from *B. amyloliquefaciens* as mentioned by Taibi et al. [39] for xylanase from *Actinomadura* sp. It can be seemed that metal ions are not necessary for enzyme activity.

Table 3: Effect of addition of various salts on the activity of pure *B. amyloliquifaciens* xylanase

Salts and chemicals added	Relative activity (%)	
Control (none)	100.00	100.00
NaCl	76.40	59.14
KCl	68.29	57.14
$CaCl_2$	64.83	68.50
$MgCl_2$	2.80	Nil
$CoCl_2$	81.15	55.99
$HgCl_2$	1.99	Nil
$MnSO_4$	70.34	57.60
$CdSO_4$	62.30	29.40
$CuSO_4$	53.97	41.42
$ZnSO_4$	54.97	37.48
$FeSO_4$	78.39	63.67
$Pb(CH3COO)_2$	63.72	14.36
EDTA	77.9	69.90
P-Chloromercuribenzoate	58.44	2.76

Xylanase activity without added metal ions was taken as 100% activity. The enzyme was preincubated with each salt at 30˚C for 30 min. and residual activity was assayed. Data are means of triplicate tests.

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Amino acid composition for *B. amyloliquifaciens* xylanase was estimated (Figure 5).The results revealed the presence of 17 amino acids. The enzyme was consistently rich in glutamic acid (31.54%) followed by tyrosine (12.83%), alanine (9.54%), glycine (9.36%), serine (5.85%), phenylalanine (5.06%) and threonine (4.27%). Leucine, lysine, cystine, histidine and aspartic acid represented (2.45-3.80%). While, it contained low levels of isoleucine, methionine, arginine, valine and proline. These results resemble to some extent those for different microbial origin xylanases [31, 45, 46]. Amino acid compositions of xylanases reported from various sources indicate the presence of predominantly aspartic acid, glutamic acid, glycine, serine and threonine. There was no remarkable homology between amino acid composition of the xylanase and those of reported xylanases from the closely related fungi and other microorganisms [47].

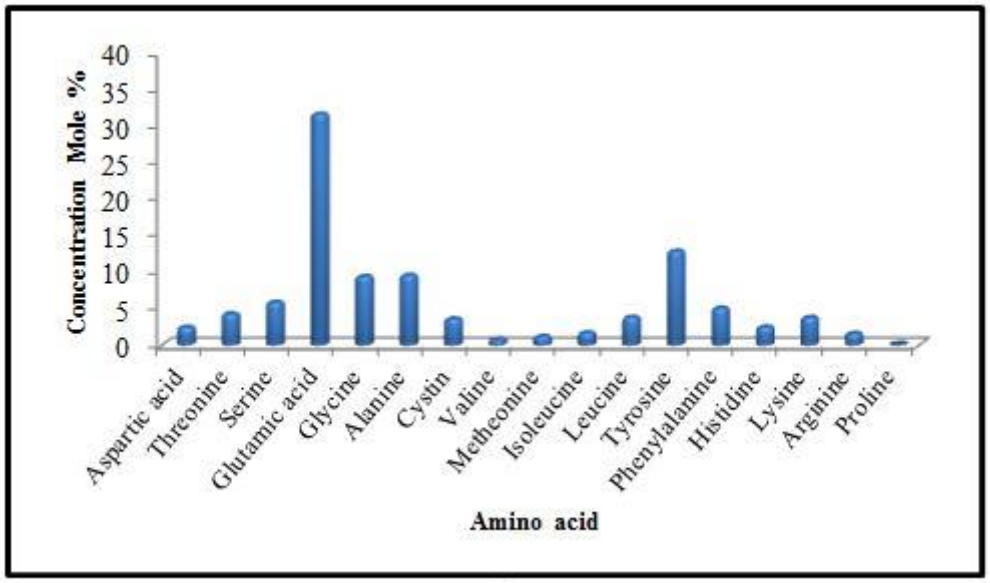

Figure 5: Amino acid composition of B. *amyloliquefaciens* pure xylanase enzyme

The presence of carbohydrates in purified *B. amyloliquifaciens* xylanase was detected and found to be 20%.This enzyme being glycoproteins or containing carbohydrate bound non covalently [48]. This interpretation was confirmed as *B. amyloliquifaciens* xylanase gave broad diffused on SDS-PAGE (Figure 2). This result was similar to xylanases from *P. ostreatus* [31] and *P. thermophila* xylanase [46]. This carbohydrate content is higher than that of xylanases from *P. occitanis* (10.83%) as reported by Driss et al. [49], but lower than that of xylanases from *Talaromyces byssochlamydoides* (37%) [50]. Wong et al. [51] stated that carbohydrate groups play an important role in stabilizing the enzymes protein structure as well as in their activities, thermostabilities and the multiplicity of these enzymes. Poorna and Prema [52] stated that glycosylation improves stability in more extreme pH and temperature conditions.

Such pure *B. amyloliquefaciens* NRRL B-14393 xylanase, which is free from cellulase activity, will be attractive and useful in various industrial applications such as the food, fruit juice, coffee and tea industries as well as large scale production of xylooligosaccharides. The most promising area will be the paper and pulp industries, where these enzymes can be used in biopulping processes to remove the xylan remaining in the pulp and so improve paper quality.

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