ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 11, Issue, 08, pp.6306-6310, August, 2019

DOI: https://doi.org/10.24941/ijcr.36118.08.2019

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

PRODUCTION, PURIFICATION AND BIOCHARACTERIZATION OF XYLANASE FROM TRICHODERMA VIRENS

[1, 2, *]Yaaser Q. Almulaiky

[1]Chemistry Department, Faculty of Sciences and Arts, University of Jeddah, Khulais, P.O. Box 355, Khulais, 21921, Saudi Arabia
[2]Chemistry Department, Faculty of Applied Science, Taiz University, Taiz, Yemen

ARTICLE INFO

Article History:
Received 16th May, 2019
Received in revised form
14th June, 2019
Accepted 10th July, 2019
Published online 31st August, 2019

Key Words:
Xylanase, Trichoderma Virens,
Solid-State Fermentation, Purification.

**Corresponding author:*
Yaaser Q. Almulaiky

ABSTRACT

The current study describes the purify and characterize xylanase from *Trichoderma virens*. Xylanase (Xy1a) was purified to homogeneity 47.6-fold via sephacryl S-200. The molecular mass of Xy1a estimated by gel filtration was once 20 kDa with 24.7% recovery. Purity was proven by means of SDS-PAGE and a single band was observed. The highest activity of the Xy1a was observed at pH 5.5 and at temperature 50°C. The Xy1a enzyme was very stable up to 50°C. The purified xy1a showed higher affinity for Birchwood xylan with Km and Vmax of 5.83 mg/ml and 0.575µmol min^{-1} mg^{-1}, respectively. Metallic cations, such as Ca^{2+} and Ni^{2+} were found to enhance the enzymatic activity of the purified Xy1a, while Cu^{2+}, Co^{2+}, $Pb^{2+}$and Zn^{2+} ions were found to be partially inhibitory. Metallic cations, such as Hg^{2+} and Cd^{2+} were found to be strongly inhibited the enzyme. Based on the results, it could be confirmed that the purified enzyme has potential role in some application such as the food, feed, pharmaceutical and paper industries.

***Citation: Yaaser Q. Almulaiky,* 2019.** "Production, purification and biocharacterization of xylanase from Trichoderma virens", *International Journal of Current Research*, 11, (08), .6306-6310.

INTRODUCTION

Plant biomass should be investigated as a noteworthy wellspring of segments that can be changed over into items utilized in mechanical preparing. It is the most plenteous inexhaustible wellspring of vitality and for the most part comprises of lignocellulose, the muddled heterogeneous complex made up of hemicellulose, lignin and cellulose (Tuck *et al.,* 2012). As of late, analysts have concentrated on creating distinctive imaginative advancements to change over lignocellulosic materials into valuable mechanical items (Thanaporn *et al.,* 2015). The lignocellulosic materials and their proficient biodegradation are among the best zones of enthusiasm for scientists because of their potential application in mechanical exercises (Govumoni *et al.,* 2013). Among them, the real part of hemicellulose, xylan has a high capacity for change to pertinent items. Xylanase is the class of catalysts produced by microorganisms which break down hemicellulose. Hemicellulose comprises of polymer glucose known as xylan and after cellulose, xylan was second most copious sustainable biomass present on earth after. Xylanases (3.2.1.8) catalyze the breakdown of xylan into xylooligosaccharides and D-xylose. Xylan is a heterogeneous polysaccharide and comprises of a direct backbone of β-1, 4-D-xylopyranoside buildups and quick side-chain branches (Thmoas *et al.,* 2015). Xylanases application in one-of-a-kind sectors has improved markedly in the past decade. Due to its hydrolysis and different properties, Xylanases are broadly used in many industrials such as –pulp and paper, food, textiles, bio-fuel, animal feed and drinks (Bajpai, 1999). Recently Awalgonkar *et al.* mentioned that xylanase additionally aid pleasant of papads, an Indian usual food based on black gram (Awalgaonkar *et al.,* 2016). Xylanases for industrial use are produced mostly from filamentous fungi (Sunna *et al.,* 2007) and bacteria (Bajaj *et al.,* 2010) in solid state fermentation (SSF) or submerged fermentation (SmF). The current finds out about describes the production, purification and biochemical characterization of xylanase with the aid of *Trichoderma virens* using solid-state fermentation.

METHOD AND MATERIALS

Chemicals: Birchwood xylan, oat spelts xylan, xylose and dinitrosalicylic acid were acquired from Sigma Chemicals. Other chemicals for column chromatography and electrophoresis were obtained from Amersham Pharmacia Biotech.

Microorganisms: *Trichoderma virens* was provided from Plant Pathology Unit, National Research Centre, Cairo, Egypt.

Solid-state fermentation: Ten gm of citrus orange strip was utilized to develop *Trichoderma virens* for 7 days at 28 °C. Two ml of spore suspension was utilized to inoculate

Erlenmeyer flasks (25 ml). The flask was aseptically vaccinated with 2 ml of spore suspension of *T. virens*. On revolving shaker, the cultures were incubating at 28°C at 150 rpm for 7 days.

Purification of *T. virens* xylanase: Ion exchange chromatography was performed using DEAE-Sepharose column at a 0.5 ml/min flow rate with 20 mM Tris-HCl buffer, pH 7.2. the elution enzyme was done with a 0.0–3 M NaCl stepwise gradient in the same buffer. The eluted fractions were assayed for enzyme activity and protein concentration. The eluted fractions exhibiting high xylanase activity were pooled. Xylanase (Xy1) was concentrated through dialysis against solid sucrose and was loaded into a Sephacryl S-200 column with 20 mM Tris-HCl buffer, pH 7.2 at 30 ml/h flow rate. The active fractions were pooled and used for the characterization experiments.

Xylanase assay: Xylanase activity was estimated by deciding the liberated reducing end products using xylose as standards (Miller, 1959). The reaction mixture containing 0.5ml: 125 µl Birchwood xylan (1%), 250 µl of 200mM acetate buffer (pH 5.5) and an amount of crude extract. The assay was completed at 37 °C for 1 h. At that point 0.5 ml dinitrosalicylic acid reagent was included and warmed in a bubbling water bath for 10 min. The absorbance was estimated at 560 nm. One unit of xylanase activity was determined as the measure of compound which freed 1 µmol of xylose for every min under standard examine conditions.

Protein determination: Bradford method was used to determine the protein (Bradford, 1976), bovine serum albumin (BSA) was used as a standard.

Molecular weight determination: To determine the molecular weight, gel filtration technique using a Sephacryl S-200 and SDS-PAGE were employed as described by Laemmli (Laemmli, 1970).

Characterization of xylanase

pH optimum: The activity of xylanase was determined using different pH buffers as follow: sodium acetate (pH 4.0 - 6.0) and Tris-HCl (6.5 - 9) at 50 mM. The maximum activity was taken as 100% and % relative activity was plotted against different pH values.

Temperature optimum: At temperature range of 30-80 °C, the activity of xylanase was investigated. 100% is the maximum activity which was being taken.

Thermal stability: The enzyme was incubated at a temperature range of 30- 80°C for 15 min prior to Birchwood xylan addition. The % relative activity was plotted against different temperatures.

Kinetic constant (Km): The Km values were determined from Lineweaver-Burk plots by using Birchwood xylan and oat spelts xylan concentrations.

Effect of metal ions: The enzyme before the addition of the Birchwood xylan substrate was incubated with 2 and 5 mM solution of different metal ions for 15 min. 100% has been taken as an enzyme activity without metal ion. The relative enzyme activity in the presence of each metal ion was determined.

RESULT AND DISSECTION

The various enzymes production and secondary metabolites can be made by fermentation on a solid substrate (SSF). The benefits of the procedure are plant material usage with at the same time high production of the ideal item or gathering of items. In our investigation, we utilized citrus orange strips as a development medium. Expansive amounts of citrus strips stay from the generation of juices, purees and sticks. As indicated by Marin (Marin *et al.,* 2007), the strips of ready orange natural products principally are involved in 37.08% of cellulose, 23.02% gelatin, 11.04% hemicellulose, 9.75% free sugars and 9.06% protein, in view of dry weight. The purification scheme is summarized in Table 1. DEAE-Sepharose chromatography of xylanases from *T. virens* separated five isoenzymes (Xy1, Xy2, Xy3, Xy4 and Xy5 with specific activities 843.8, 122.7, 56.7, 38.6 and 20 units /mg protein, respectively) (Figure 1). The Xy1 isoenzyme showing maximum activity was concentrated by sucrose, loaded on a Sephacryl S-200 gel filtration column, pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.2). The final Xy1a obtained (Figure 2a) showed a single peak with the highest specific activity of 2633 units /mg protein, 46.5-fold purification and 24.7% recovery (Table 1). Many research of xylanase purification with specific activities had been also separated from special organisms, *Aspergillus tamarii Kita* (specific activities 1215 units /mg protein with 7.43 fold) (Heinen *et al.,* 2018), *Aspergillus niger* SCTCC 400264 (xynB with specific activities 1201.7 units/mg protein) (Yi *et al.,* 2010) and *A. niger* GS1 (specific activities 522 units/mg protein) (Amaro-Reyes *et al.,* 2011). The purified xylanase (Xy1a) confirmed a single band of protein using SDS-PAGE, in addition, the molecular weight was decided to be 20 kDa the usage of Sephacryl S-200 column (Figure 2a and b). A similar result was bought for the xylanase from *Aspergillus tamarii Kita* (19.5 kDa) (Heinen *et al.,* 2018), *Aspergillus terreus* NRRL1960 (19.0 kDa) (Kocabas *et al.,* 2011) and *Scytalidium thermophilum* ATCC No. 16454 (21 kDa) (Kocabas *et al.,* 2015). The outcomes have proven in Fig. 3a current that the purified Xy1a enzyme has maximum activity at pH 5.5. quite a few research stated that many xylanases from microorganism had an optimum pH ranging from 5 and 8. A similar result was once said for xylanase produced with the aid of *Aspergillus tamarii Kita* and *Aspergillus kawachii*, which showed optimal activity at pH 5.5 (Heinen *et al.,* 2018; Ito *et al.,* 1992).

The optimal activity was once detected at 50 °C for purified enzyme (Xy 1a) (Fig. 3b), This result is agreement with these from many different xylanases from *Trichoderma* lines that are found in temperatures from 45 to 60°C (Silva *et al.,* 2015; Silveira *et al.,* 1999). In relation to thermal stability, the purified enzyme (Xy1a) was secure to 50°C but also keep 80% of its activity when incubated at 60°C (Fig. 3C). Earlier, thermal stability at 50°C used to be detected for xylanase produced with the aid of *Trichoderma sp.* K9301 (Chen *et al.,* 2009) and *Talaromyces thermophiles* (Maalej *et al.,* 2008). The kinetic parameters of the purified xylanase (Xy 1a) relative to Birchwood xylan and oat spelts xylan are shown in Fig. 3d. The Km values for the enzyme had been 11.05 and 5.83 mg/ml for oat spelts xylan and Birchwood xylan, respectively.

Table 1. Purification scheme for *T. virens* xylanase

Steps	T. units*	T. Protein mg	S.A Unit/mg protein	Fold purification	Recovery 100%
Crude extract	640	11.3	56.6	1	100
Chromatography DEAE-sepharose					
0.0 M NaCl (Xy1)	270	0.32	843.8	14.9	42.2
0.05M NaCl (Xy 2)	92	0.75	122.7	2.17	14.4
0.1M NaCl (Xy 3)	68	1.2	56.7	1.00	10.6
0.2M NaCl (Xy 4)	54	1.4	38.6	0.68	8.4
0.3M NaCl (Xy 5)	38	1.9	20	0.35	5.9
Gel filtration on sephacryl S-200					
Xy 1a	158	0.06	2633	46.5	24.7

* One unit of enzyme activity was defined as the amount of enzyme which liberated 1 µmol of xylose per minute under standard assay conditions.

Table 2. Effect of metallic cations and EDTA on the activity of purified Xy1a

Effect of metals	2 mM	5 mM
	Relative activity (%)	
Control	100	100
Ca^{2+}	98	111
Ni^{2+}	102	123
Pb^{2+}	75	63
Co^{2+}	81	73
Hg^{2+}	45	17
Cu^{2+}	96	80
Zn^{2+}	87	59
Cd^{2+}	51	32
EDTA	93	89

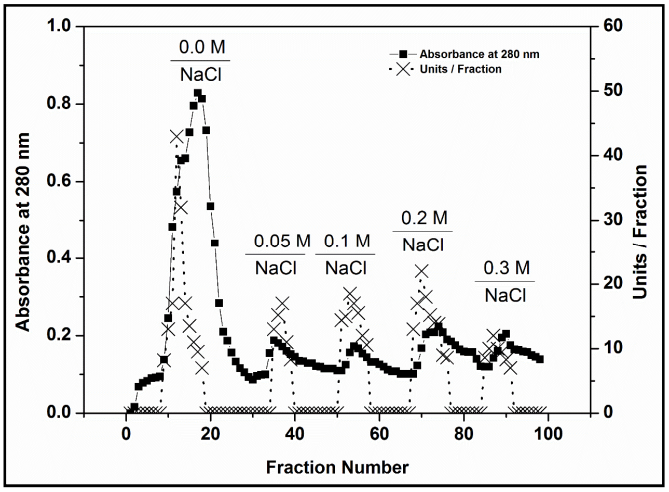

Figure 1. A typical elution profile for the chromatography of xylanase using a DEAE-Sepharose column

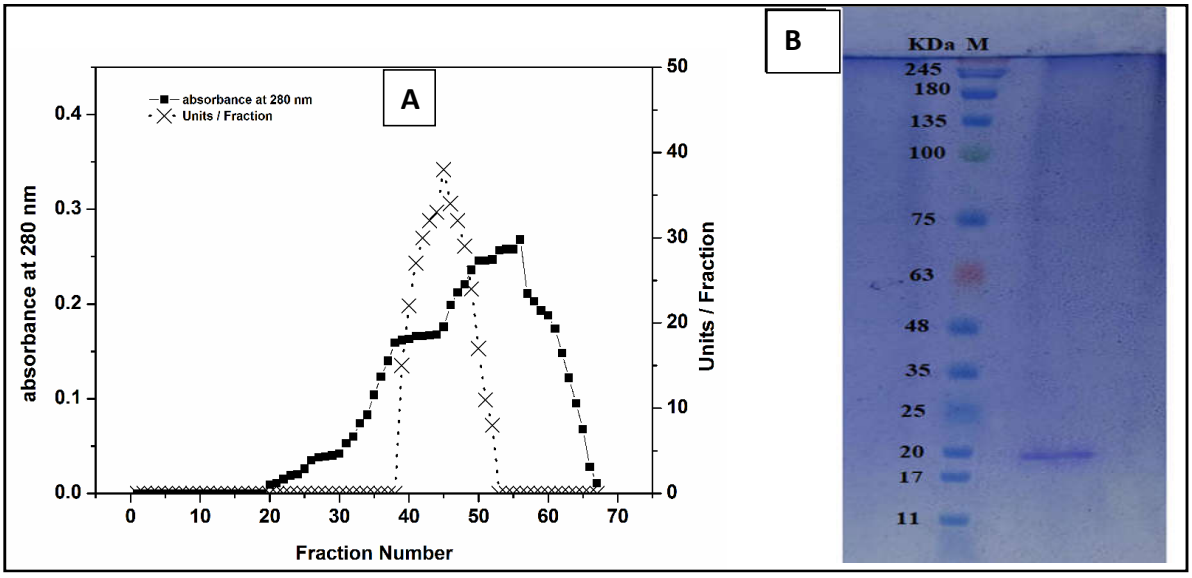

Figure 2. Gel filtration of Xy1a DEAE-Sepharose fractions using a Sephacryl S-200 column (A), SDS-PAGE for homogeneity and molecular weight determination of Xy1a (B)

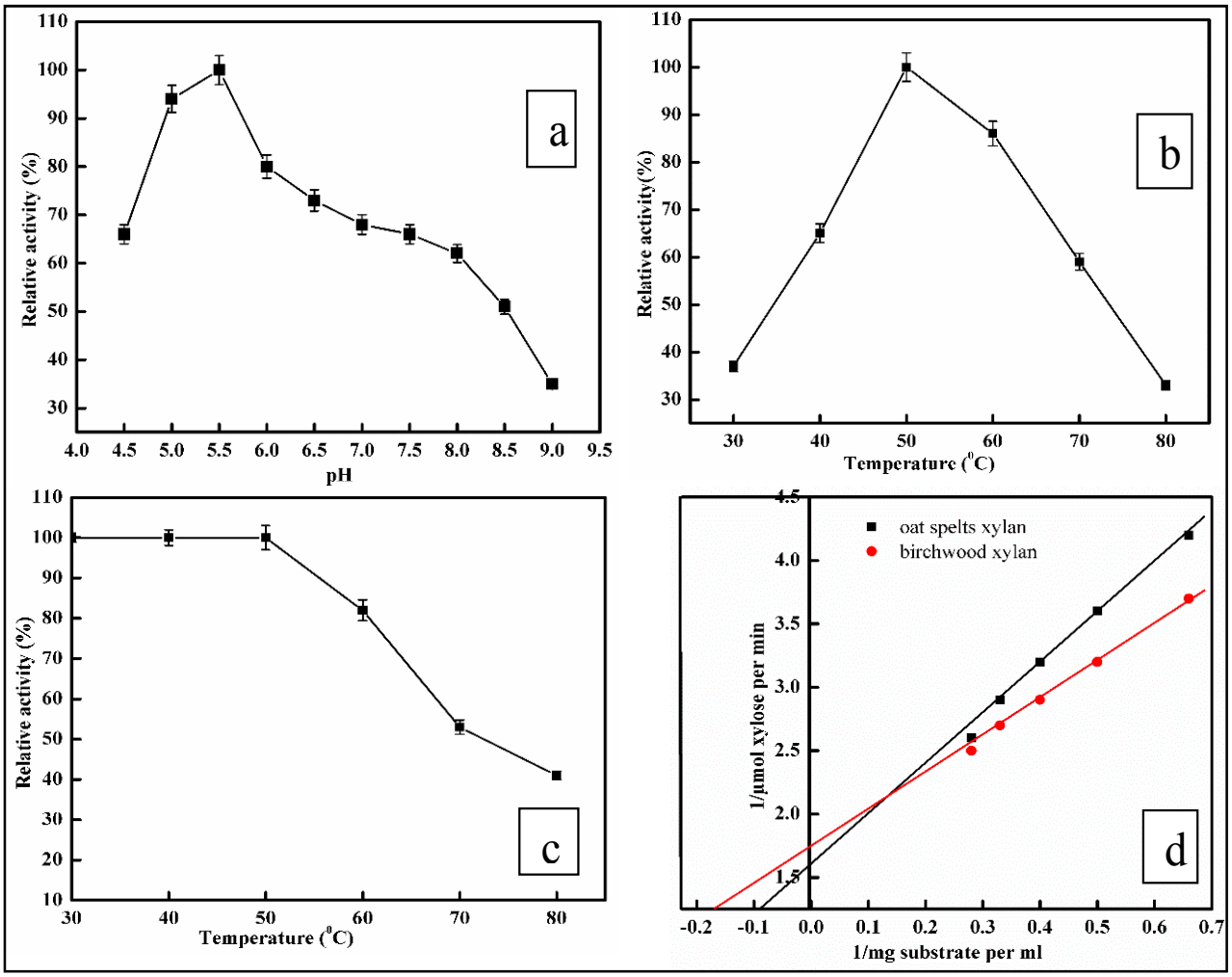

Figure 3. Optimum pH (a), effect of temperature on activity (b), the thermal stability (c), Kinetics parameters (Km) (d) of purified Xy1a

The Vmax values for the hydrolysis of these substrates found 0.627 and 0.575µmol min^{-1} mg^{-1}, respectively. The Km values indicated that the purified enzyme had greater affinity for birchwood than for oat spelts xylan. Silva *et al.* said that the two xylanase enzyme of *Trichoderma inhamatum* expressed the Km values of 1.6 and 14.5 mg/ml for xylanase I the usage of birchwood xylan and oat spelt xylan, respectively, whereas the Km values of xylanase II for these substrates had been 4 and 10.7 mg/ml (Silva *et al.,* 2015). Another study revealed that the Km values of *Penicillium sclerotiorum* xylanase using oat spelt xylan and birchwood xylan had been 2.6 and 6.5 mg/ml, respectively (Knob and Carmona, 2010). The impact of unique metal cations at 2 and 5 mM on the activity of the purified xylanase Xy1a from *T. virens* is introduced in Table 2. These metal cations confirmed activation and/or partial/strong inhibition outcomes on the xylanase Xy1a activity. At the attention of 5 mM, the metal cations (Cu^{2+}, Co^{2+}, $Pb^{2+}$and Zn^{2+}) presented a partial inhibitory effect on xylanase, while Hg^{2+} and Cd^{2+} were a sturdy inhibitor of the xylanase even at 2mM. The inhibition with the aid of Hg^{2+} appears to be a common feature of xylanases, showing the presence of cysteine thiol groups near or in the active site of the enzyme (Lappalainen *et al.,* 2000). Activation impact was found via Ni^{2+} and Ca^{2+} at 5 mM with improvement in activity by way of 23 and 11%, respectively. EDTA at both concentrations shown slightly lowered the activity of purified enzyme. The impact of EDTA on enzyme recommends that they may require divalent ion for catalysis. Similarly, EDTA had no impact on the xylanase activity from *A. niger* (Naganagouda *et al.,* 2009). In addition, metallic cations, such as (Co^{2+}, Cu^{2+}, Hg^{2+}, $Pb^{2+}$and Zn^{2+}) have been stated to have inhibitory effect on xylanase from *Aspergillus tamarii* Kita, which was once activated by Ca^{2+} and Ni^{2+}. A *Talaromyces thermophilus* Xylanase was once activated by using Co^{2+}, and Cu^{2+}, and inhibited by using Hg^{2+}, Ba^{2+}, and Mn^{2+} (Maalej *et al.,* 2008).

Conclusion

This is the first file about two-step chromatography purification of *Trichoderma virens* xylanase consisting of anion exchange and gel filtration. This is the first report about the usage of solid state fermentation to produced xylanase by *T. virens*. *T. virens* xylanase produced via SSF showed that the highest activity reached 2633 unit/mg protein. Biochemical characteristics of the purified Xy1a defined that it works at an excessive optimum temperature and prefers acidic conditions, and thermo stability at multiplied temperature are fabulous for industrial application. Moreover, the low molecular weight of the enzyme presents an extra benefit of its handy penetration into the lignocellulosic shape and efficient degradation of xylan.

Acknowledgements

Not Applicable.

Competing interests

The author declares that he has no competing interests.

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