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RESEARCH ARTICLE

Purify and Characterize the Xylanase from the Local Isolation of Trichoderma Longibrachiatum

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Abstract

Fungi are well known for their ability to produce extracellular enzymes into their surrounding environment. Xylanases are an important class of hydrolytic enzymes involved in degradation of xylene a backbone of hemicellulose, in this study; we have isolated three isolates of xylene analytic fungi taken from five samples of sand gathered from different sites in the University of Mosul. Depending on the halo's diameter of analysis and by using the Congo red indicator, one isolate has got a high activity in producing the exocellular xylanase enzyme related to *Trichoderma longibrachiatum* on the base of morphological, microscopic and internal area (ITS) features. The xylanase has been purified from the raw extracted enzyme of the selected isolate throughout four subsequent steps included: sedimentation by Ammonium Sulphate, dialysis, gel filtration in column (Sephadex-G 100) and the Ionic exchanger (DEAE Cellulose). After this step, the number of filtrations is (49.22) with enzymatic outcome reaching to (33.93%). Studying the description of the filtered enzyme, PH the optimal for the enzyme activity is 0.5 and the optimal range of PH for enzyme stability is (4-6). Moreover, the optimal temperature degree for enzyme activity is (50 °C), whereas the optimal temperature degree for enzyme stability is (50-60 °C).

Keywords: *Mosul university, Xylanase, Trichoderma longibrachiatum.*

Introduction

Xylene belongs to inhomogeneous polysaccharides and is one of the most spreading polysaccharides, after cellulose, in nature. The xylene biomolecule is made up of D-xylose units which connect to each other by glycosidic linkage (B 1-4). As for its components and structure, xylene shows a huge comparison according to its various resources in nature[1, 2, 3].Because of the wide uses of enzymes, especially xylanase, dealing with its production has been commercially increased from different microorganisms during recent years; since it is used in improving the flour features, woven fabrics and cleaners manufacture in addition to bleaching the paper paste in order to get rid of lignin and to.

To add, it is used for producing some materials such as monocyte protein and xylitol and ethanol [4, 5].Types related to Trichoderma, especially *T. viride* & *T. reesei*, are ones of the most important filamentous fungi producing xylanase [6, 7].*Filamentous fungi* is characterized by its huge importance in the field of producing enzymes for several reasons including its growth easiness in nutritious cheap media taken from agricultural and industrial waste products to discharge enzymes in the growth medium, and that will decrease the cost of its extraction and to be produced in great amounts in comparing with bacteria and yeast [8, 9]. The kinds related to *Trichoderma* are the most important filamentous fungi which produce xylanase, especially the *T. viride* & *T. reesei* kinds [7]. The process of clarifying xylanase has been greatly dealt with and widely studied to know its biochemical features and to introduce the way of its catalysis for specific interaction without interference with other materials that may be found with the enzyme and may give different results [10].

However, several methods for splitting and clarifying enzymes have been focused by researchers at the recent decades, specifically the researchers and companies dealing with the food industry [11]. Accordingly, this study represents an attempt to get local fungi isolates that is able to produce xylanase enzyme and to clarify and study some of its features.

Materials and Methods

Isolates Origin: the isolates origins include soil samples collected from various sites in the University of Mosul. These samples have been taken from 20cm depth. To add, it has been used the sequenced dilute method in order to get the fungi isolates.

Culture Media

- Potato dextrose agar: it has been prepared according to the Oxoid Company instructions throughout adding 39gm to a limited amount of distilled water to get 1 litre as a final size. The media has been used to save and activate the isolates.

- Isolation Media: the nutritive media (Czapek-Dox) has been used in accordance with the method mentioned in [12], as follows: (g/l)

$NaNO_3$ -2, $MgSO_4$.7H2O-0.5, FeSO4.7H2O-0.01 K_2HPO_4-1, KCL- 0.5

With adding xylene as the only source of carbon.

- Screening Media (for fungi isolates): it detects the ability of gotten isolates to produce xylanase enzyme according to the method of [13]. In this process, it has been used the media prepared by the following materials (g/l):

Brichwoodzylan - 0.8% , $CaCl_2$,-0.05, $NaNo_3$ – 0.005, $FeSO_4.7H_2O$ –0.009, $ZnSO_4$- 0.002 ,$MnSO_4$ –0.012, KCL –0.23, KH_2PO_4 –0.23, Pepton - 2.0, Agar –20.

- Production Media: it has been prepared depending on what has been stated by [14]including the following materials (g/l): Brich wood xylan –0.5, Yeast extract –0.2, NaCl –0.25, KH_2PO_4 –1.5, NaH_2PO_4 –3.0, NH_4CL –0.5, $MgSO_4.7H_2O$ –0.025 As for pH, it has been accurate at (7). The production media has been prepared in conical flasks (250 ml.). This media has been distributed as (50 ml.) for each flask, as twice as for each process. Flasks have been tightly sealed by using cotton stopples and aluminium flakes which is sterilized by the autoclave with pressure (1) km/cm^2 and the temperature are at (121°C) for (15) minutes. After sterilization completed, flasks have been left to be cool, then they are vaccinated by shaker incubator prepared recently (7 days ago) with 40% rate. After that, they are put in Eppendorf tubes at about (28±2°C), and with a shaking rate reach to (150) lap for each minute for five day period.

Extracting the DNA:

After activating the fungi isolate on the (PDA) media, it has been gathered the biological mass aged five days throughout Vacuum. Parts of the fungi lines have been taken in Eppendorf tubes under sterilized circumstances. The process of extraction has been made by using the extracting Kits for DNA, supplied by Bioneer Company, including a group of liquids in addition to DNA filter columns and collection tubes. Depending on instructions of the supplier company and according to the method of [15], it has been founded the concentration and purity of DNA extracted by using Biodrop Spectrophotometer.

DNA Amplification

Using the Kit of Accu Power ® PCR Pro Mix supplied by Bioneer Company and by tackling two initiative specifications (Forward ITS5) having the sequence: (GGAAGTAAAGTCGTAACAAGG) and (Reverse ITS4) having the sequence: (TCCTCCGCTTATTGATATGC), it has been enlarged the area of ITS in the DNA according to the method followed by [16] . The reaction mixture has been prepared and mixed well in the Eppendorf Tubes depending on the attached bulletin of Master Mix. Then, these tubes have been moved to thermo cycler and the reaction has been achieved according to the following protocol: The initial denaturation for 5 minutes in 90°C, denaturation for 1 minute in 94°C, annealing for 2 minutes in 56°C, extension for 3 minutes in 72°C, and the final extension for 5 minutes in 72°C. The number of cycles is 35.

Gel Electrophoresis

The gel electrophoresis has been made by agarose gel prepared with the rate 1.2% according to the method followed by [17] 60 volt for 3 hours. It has been revealed the results of PCR split by the electrophoresis while the loading dye reaches to the end of gel compared with the size guide (DNA ladder 100 bp) supplied by Bioneer Company by using the UV-Transilluminator.

DNA Sequencer

After enlarging the DNA, the result has been transmitted into the Korean Macrogen Company to get the DNA sequence for the (ITS) area; it has also been used te AB DNA Sequencing System. The isolate has been determined by adding a serial number into the database of National Center of Biotechnology Information (NCBI).

Xylanase Activity Assessment

It has been assessed the xylanase activity depending on the method followed by [18] by using the detector of 3.5-Dinitro Salicylic Acid (DNS). A solution of reaction has been prepared by mixing 1 %(w/v) of xylene by using the regulator solution (Sodium acetate 0.05 M) at PH - 5. The enzyme unit can be introduced as the amount of enzyme which releases 1 micromole of glucose during one minute.

Determination of Protein

It has been determined the amount conducting the method followed by [19] by using spectrophotometer at 280nm wavelength. Bovine serum albumin (BSA) has been used as a regular solution to prepare the protein curve.

Isolate Determination at the genus and Type level

It has been determined the isolate which proved its ability to produce xylanase enzyme throughout the initial screening by following the classifying keys stated by[20, 21].

Purification of Xylanase

The raw extraction of xylanase has been purified depending on [22]Ammonium Sulphate with satisfaction rates reaching 80% for enzyme sedimentation. The process of central expulsion of sediment with 10000 rpm for 30 minutes. The sediment has been taken and performed a dialysis operation vs. the regulator solution (sodium acetate 0.05M) when the PH is 5 for 24 hours, taking into consideration substituting the regulator solution every 6 hours.

The next step is gel filtration by using Sephadex G-100 prepared in accordance with the instructions of the supplier company (Sigma-Aldrich) with (1.5 × 60 cm.) dimensions. As for balance and recovery, it has been made by the same buffer solution with 3ml. per part.

Then, the ionic exchanger (DEAE Cellulose) has been prepared with (1.5 × 60 cm.) dimensions. As for balance and recovery, it has been also made by the same buffer solution with acceleration amount as 30ml/hr. and 3ml. per part. The split parts, that have enzymatic activity, have been gathered and lyophilized to study their biochemical features.

Enzyme Characterization

The experiments of enzyme characterization include the study of the optimal PH for enzyme activity and stability in addition to the effect of temperature on the enzyme activity and its temporal stability.

Results and Discussion

Isolation and Screening

From stand, it has been gotten three fungi isolates characterized by speed growth and their intensity after enriching the hard media of isolate with xylanase to determine the isolates that have the ability to discharge the enzyme which is responsible for xylene solvent throughout observing the xylene solvation in the areas surrounding the growth of fungi and its colonies.

The isolates have been grown in the above-mentioned media for 5 days in 28°C temperature. It has been followed their ability to produce xylanase enzyme depending on the halo diameter of solvation after submerging the growth plates with the Congo red indicator. The results have revealed that there is a great difference in the ability of the gotten isolates to produce xylanase.

Figure (1) shows the distinction of isolate labelled (T2) to which its solvation rate for xylene reaches to (9.5) comparing with the other two isolates that are later disregarded for their weak production of the enzyme. The difference of their ability to solve xylene is attributed to genetic contrast existed among them. Browsing other studies, it has been also observed such a contrast in the componence of isolates, genealogies, or types in solving xylene.

Among 53 isolates of fungi belonging to various types, seven ones have been distinguished with solvation rate higher than the others [23].In another study, it has been found 5 isolates related to the type

(*Cladosporium*) which are distinguished by giving a solvation rate higher than the others after growing them in the media of modified Czapek-Dox [24].

Figure 1: the componence of Isolate (T2) for solving xylene

Isolate Characterization

It has been characterized the isolate selected from the process of initial isolation and screening after growing it in the (PDA) media depending on phenotypic and microscopic tests on the genus and type levels and according to classifying keys stated in the scientific references [20,21]. In this regard, it has been observed that the isolate forms colonies having a speed growth and a dark green to olivaceous colour with an intensive growth rate at 30°C.

Their spores are characterized with: green coloured, mediate sized, 4-9 micron length, oval shaped and transparent. As for the main conidiophore, it is easy and carries short sideward branches having shorter cylindrical ones. These ramifications are carried on the branch singularly on wide or irregular distances.

Partial Characterization

To confirm the isolation diagnosis, it has been used a PCR for DNA and primers concerning the internal area (ITS). Such ITS have characterized as highly differentiation between geniuses and types and can be used in distinguishing various fungus types because it provides specific sequences for each type. At the time of analysis the result of electrophoresis on agarose gel (Figure 2), the enlargement results show that the extracted isolate belongs to *Trichoderma longibrachiatum* in terms of existing an obvious alone pack of DNA with a partial weight of 700 pg. of basic pairs in comparing with the volumetric guide (Marker ladder). This asserts the truth of used primers sequences and its connection to the ITS area. Figure (3) shows sequences of nitrogen bases of isolates which had been submitted to Blast Program within the National center of biotechnical information.

Throughout the comparison among the nucleotide sequences of isolate, it has been founded that those sequences provide a case of perfect matching 100%with the ones of nitrogen bases, i.e. with the deposit sequence *Trichoderma longibrachiatum* in the genes bank under the serial no. (Ky789475.1) and also with *Trichoderma longibrachiatum* enrolled under the serial no. (Kt278853.1).

As for the concluded results, they are identical with what has been got [25]while isolating several isolates related to *Trichoderma* and characterizing them partially by using specialized Primers in purifying PCR. In a trial to characterizing an isolate for *Trichoderma longibrachiatum* by using specialized primers [26] has concluded that the results of enlargement, which has been got by sequenced PCR as a result of electrophoresis on agarose gel, led to arising on isolate pack with partial weight of 700 pg. of basic pairs. Accordingly, it could be asserted that the isolate used in this study is *Trichoderma longibrachiatum*.

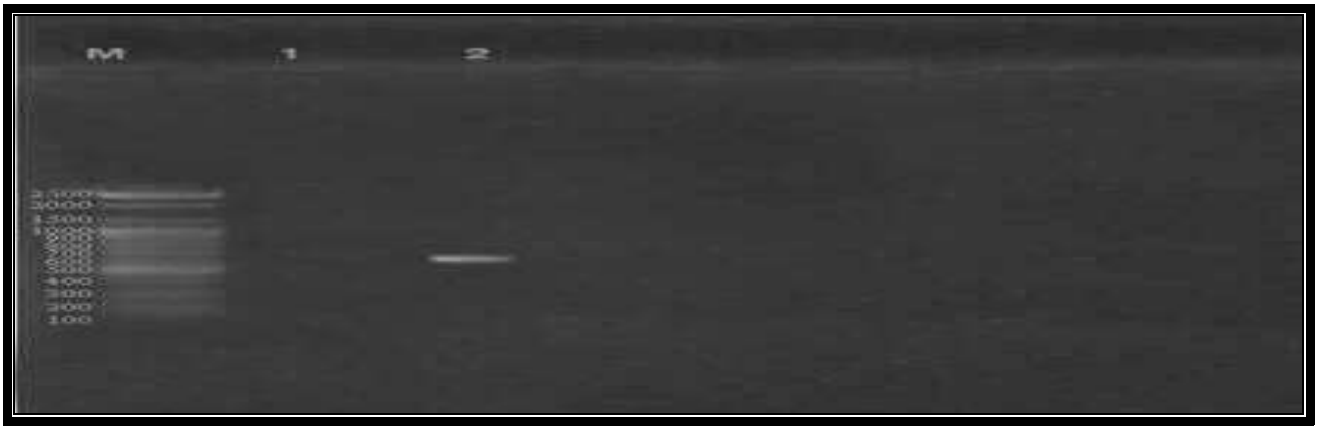

Figure 2: the Result of DNA Enlargement for *Trichoderma longibrachiatum* Isolate on Agarose Gel

1	GAGGCATCAT	TACCGAGTTT	ACAACTCCCA	AACCCCAATG	TGAACGTTAC	CAATCTGTTG
61	GAGGGATCAT	TACCGAGTTT	ACAACTCCCA	AACCCCAATG	TGAACGTTAC	CAATCTGTTG
181	CCTCGGCGGG	ATTCTCTTGC	CCCGGGCGCG	TCGCAGCCCC	GGATCCCATG	GCGCCCGCCG
241	CCTCGGCGGG	ATTCTCTTGC	CCCGGGCGCG	TCGCAGCCCC	GGATCCCATG	GCGCCCGCCG
361	GAGGACCAAC	TCCAAACTCT	TTTTTCTCTC	CGTCGCGGCT	CCCGTCGCGG	CTCTGTTTTA
421	GAGGACCAAC	TCCAAACTCT	TTTTTCTCTC	CGTCGCGGCT	CCCGTCGCGG	CTCTGTTTTA
481	TTTTTGCTCT	GAGCCTTTCT	CGGCGACCCT	AGCGGGCGTC	TCGAAAATGA	ATCAAAACTT

Figure 3: the Sequences of Nitrogen Bases of *Trichoderma longibrachiatum* isolate

Purifying Xylanase Enzyme

After getting the raw extracted enzyme from the fungi, it has been submitted to sedimentation by Ammonium Sulphate. Consequently, it has been founded that the best rate of satisfaction was 70% in which it has been got the quality effectiveness reaching to (28.88 unit/ml.) with (2.14) times of purification as shown in the table (1). Many researchers refer to the efficiency of Ammonium Sulphate in the concentration of xylanase enzyme produced by the microorganisms.

Then, the process of dialysis has been done by using a membrane after sedimentation. In this step, it has been noted that the enzymatic activity and the quality effectiveness are (123.40 & 44.54 unit/ml.) in addition to the decreasing of protein concentration by half because proteins with low molecular weights were exited so that times of enzyme purification were increased.

As shown in Figure (4), the results of gel filtration step submit an increasing of purification times to 7.51 with enzymatic outcome reaching to (49.14%) by using the chromatography column of ionic exchange to complete the enzymatic purification. To add, it has been observed four vertexes of protein arising in the recovered parts of the gel. While assessing the enzymatic effectiveness by using xylene, as a substrate, it has been found that the activity is concentrated at the third vertex which expresses the enzyme; whereas the other ones are completely inactive. In this step, the purification times are 49.22 with the enzymatic outcome of 33.93%. The Results obtained point out that the enzyme sedimentation process leads actively to the concentration of enzyme by Ammonium Sulphate; since it works on equaling the existing charges on the surface of protein which consequently affects on the quality effectiveness of enzyme and also increases the purification times.

The increasing of purification times and decreasing the enzymatic outcome refer to the important evidence of enzyme pureness [27].In a study submitted by [28], they could purify two types of xylanase enzyme produced by *Trichoderm alongibrachiatum* after passing by the purification steps including sedimentation by ethanol, gel filtration, and ionic exchanger. In this regard, the purification times have been increased to reach to 5.0 with an enzymatic outcome amounted (43%) for enzyme Xylanase A; whereas the contrary has been done for Xylanase B to reach to (0.5%) and purification times are amounted (1.3%).

On the other hand, [29]have found that the quality effectiveness of xylanase enzyme produced by the fungi isolates *Trichoderma longibrachiatum*is (6.630 unit/mg.) and the

purification times of enzyme is (55.8). To add, the enzymatic outcome reaches to (5.1%), after purification times including extra filtration, sedimentation of ammonium sulphate, in addition to the ionic exchanger and gel filtration as a final step. As for the differences of purification times for xylanase enzyme concluded from various sources, it is attributed to different technology used in the extraction process and enzymatic purification in addition to the circumstances, extracting duration, type of buffer solution, and the enzyme source.

Table 1: Xylanase purification steps from Trichoderma longibrachiatum

Purification steps	size	Activity mg/ml	Protein mg/ml	Quality activity mg/ml	Total activity	Purification times	Enzyme yielded %
Enzyme	200	67.35	5.09	13.23	13470.0	1	100
AmmoniumSulphate precipitation	88	106.25	3.75	28.33	9350.0	2.14	69.41
Dialysis	56	123.40	2.77	44.54	6910.4	3.36	51.30
Gel filtration	43	147.11	1.48	99.39	6619.95	7.51	49.14
Ionicexchanger	26	175.83	0.27	651.2	4571.58	49	33.93

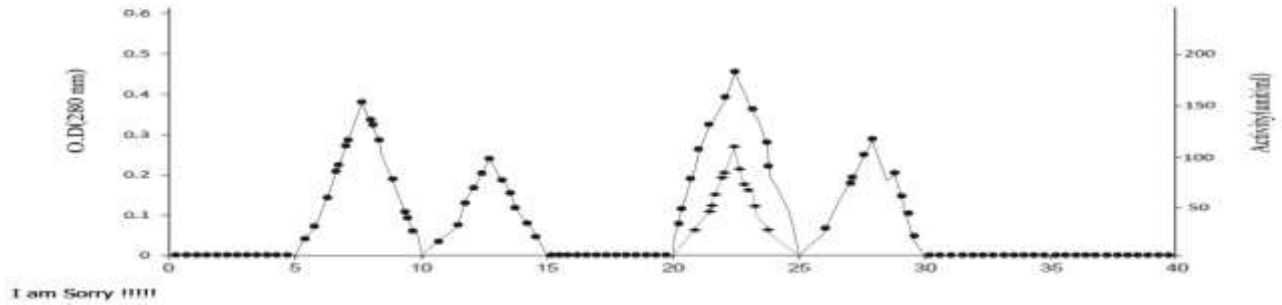

Figure 4: The xylanase enzyme purification produced by *Trichoderma longibrachiatum* throughout the ionic exchanger process

Enzyme Characterization:

The Typical Activity of pH:

The results have been shown in figure (5) which represents the optimal pH carve of activity of the purified enzyme. The highest rate of enzyme activity at (5) pH is (67.28 unit/ml.). it is also noted the gradual decreasing of activity at the two sides of optimal pH; since the enzyme activity is (36.88 unit/ml.) at (3) pH and (25.79 unit/ml.) at (8) pH. This indefinitely denotes that the increasing or decreasing of pH towards the optimal values has negative effects on the activity of purified enzyme form fungs which has been found that it works at the pH values nearby the equalization. The reason behind decreasing the enzyme activity is attributed to the change of ionic group's structure existing in the active site of the enzyme or at the basic material, or in both of them. Accordingly, the ionic state of such groups has been changed and reflected negatively on the ability to connect the enzyme with the basic material [30].This result has been matched with the one submitted by [29]where the pH (5) gives the highest activity of xylanase enzyme produced by *Trichoderma longibrachiatum*. On the other hand, [31]state that the proper pH of xylanase enzyme activity is (5.6) for the isolate of *T.ressei.*

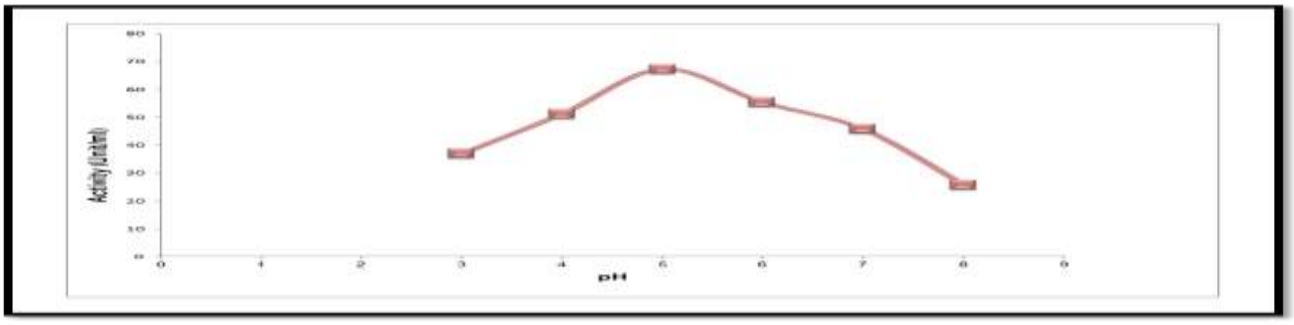

Figure 5: The Optimal pH for Producing Xylanase Enzyme from the Isolate of *Trichoderma longibrachiatum*

The Optimal pH to Prove Enzyme Activity:

The results show that xylanase enzyme purified from the fungi isolate of Trichodermalongibrachiatum has stability at the pH values ranged between (40-60) in which enzyme keeps all its activity as shown in Figure (6). But, the activity started to be decreased gradually at 7pH and deteriorated considerably at 8 pH. This case of increasing and decreasing the enzyme activity may be attributed to changes in the enzyme

structure in addition to the change of ionic state of the active site of the enzyme and also the change of chemical nature of buffer solution. All these factors affect the optimal pH to characterize the enzyme. On the other side, [32] have found that xylanase enzyme produced by the isolate of *T.reesei* with solid state fermentation has stability at the pH values ranged between (4-5), [33] also point out that the limited value of pH is between (4.6-6.5) used for the stability of enzyme effectiveness produced by *T.inhamatum*.

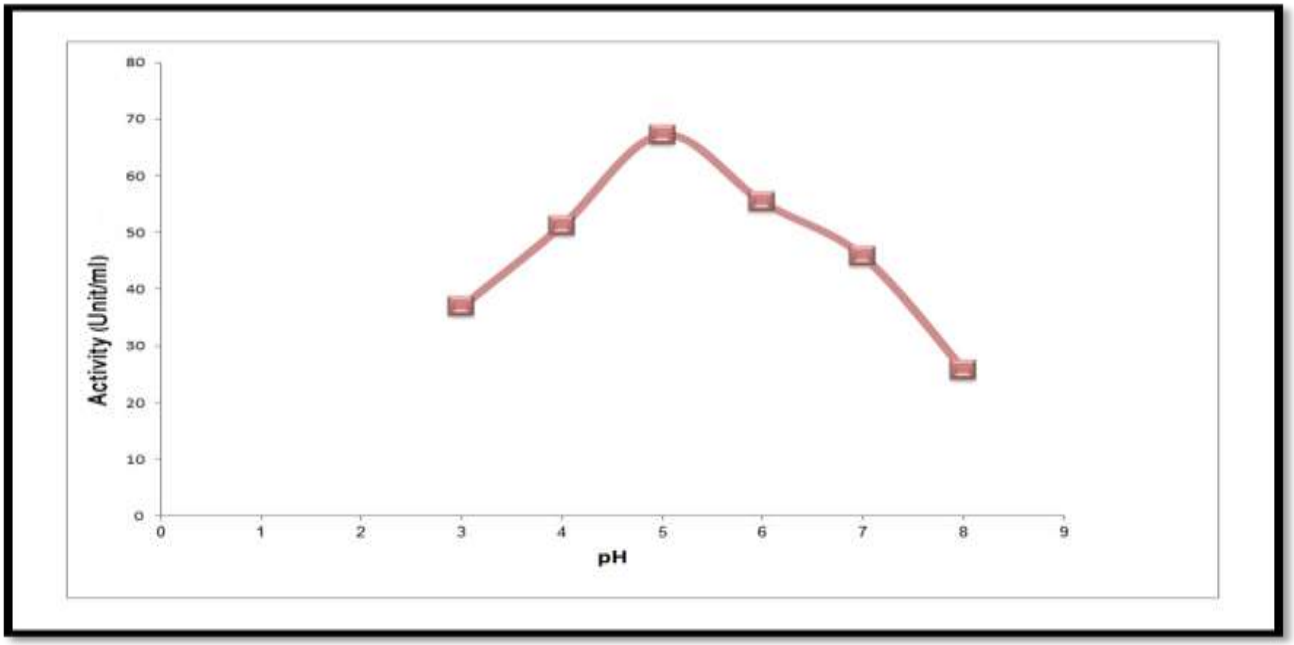

Figure 6: The Optimal pH for Stability of Xylanase Enzyme Effectiveness Produced by the Isolate of *Trichoderma longibrachiatum*

The Optimal Temperature for Enzyme Effectiveness:

The stated resulted in Figure (7), which expressed the carve optimal temperature for purified xylanase enzyme, shows the increased effectiveness of enzyme with increasing the optimal temperature degree reaching to 50°C while the effectiveness is (68.31), then followed by decreased effectiveness due to increased temperature degree considerably to be (23.9 unit/ml.) at 80°C. this case of increased effectiveness may be attributed to the speed of enzymatic interaction with increasing temperature degree to a specific measurement because of the increasing of dynamic energy for the molecules of interacted materials and then the increasing of collision chances among molecules of the basic material and the active site of the enzyme [27]. As for the reason behind the decreased enzymatic effectiveness, it is due to occur a case of metamorphosis in the effective site of enzyme which leads to incompatibility between the enzyme and the basic material in a correct aspect, in addition to a change existing in the enzyme structure [34]. The optimal temperature degree for xylanase enzyme effectiveness differs according to the difference of the enzyme source.

In this regard [29] point out that the optimal temperature for xylanase enzyme effectiveness extracted from *Trichodermalongibrachiatum* is (45°C). The concluded result matches with what [35]refer to; since the optimal temperature degree for enzymatic effectiveness extracted from the fungi isolate of *T. ressei* is (50°C). nevertheless, this result matches what have been founded by [36] since the highest rate of

xylanase enzyme effectiveness produced by *Humicolainsolens* Y1 is (50°C).

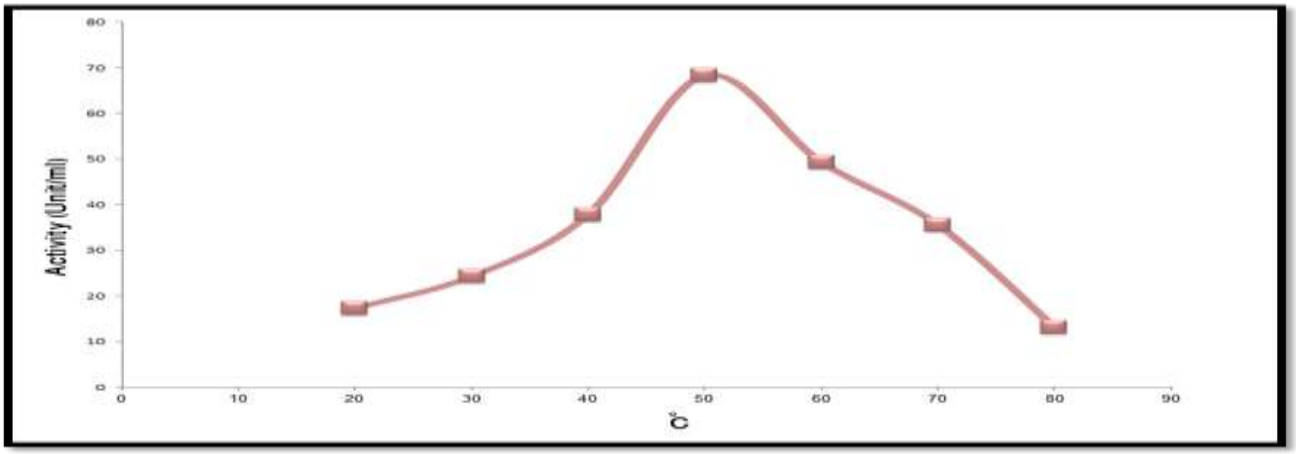

Figure 7: The Optimal Temperature Degree of Producing Xylanase Enzyme from the Fungi Isolate of *Trichoderma longibrachiatum*

Determining Temperature Degree of Enzyme Stability:

The investigation about xylanase enzyme with higher thermal stability is one of the important aims dealt with by several studies since such enzymes are often used in higher temperature circumstances in the practical applications. The results of enzyme raising point out that in various temperature degrees the rate of optimal pH for enzyme stability ranged between (20-70) for one hour as in the Figure (8) which represents the carve of thermal stability. The enzyme shows a stable state with temperature degrees ranged between (50-60°C). At 50°C, it keeps all its activity which reaches to (70.35 unit/ml.). On the other hand, we note at 20°C, the activity is greatly decreased; it is estimated as (30.66 unit/ml.). The same cases with 70°C & 80°C, the enzymatic effectiveness started to decrease severely to be estimated as (45.33 & 38.20 unit/ml.) respectively. The reason behind losing part of enzyme's activity during the period of raising is due to exposing enzyme to a metamorphosis which leads to changes in its structure, and that will be negatively reflected the enzyme interaction with the basic material. The concluded result matches with what [37] conclusion; both of them conclude that the thermal stability of purified xylanase enzyme *Saccharopolyspora pathumthaniensis* S582 is between (60-70°C). to add, this result is nearly similar to what has been concluded [38]in which xylanase enzyme produced by *Trichodermaressei* has thermal stability at 50°C after 30 minutes of raising.

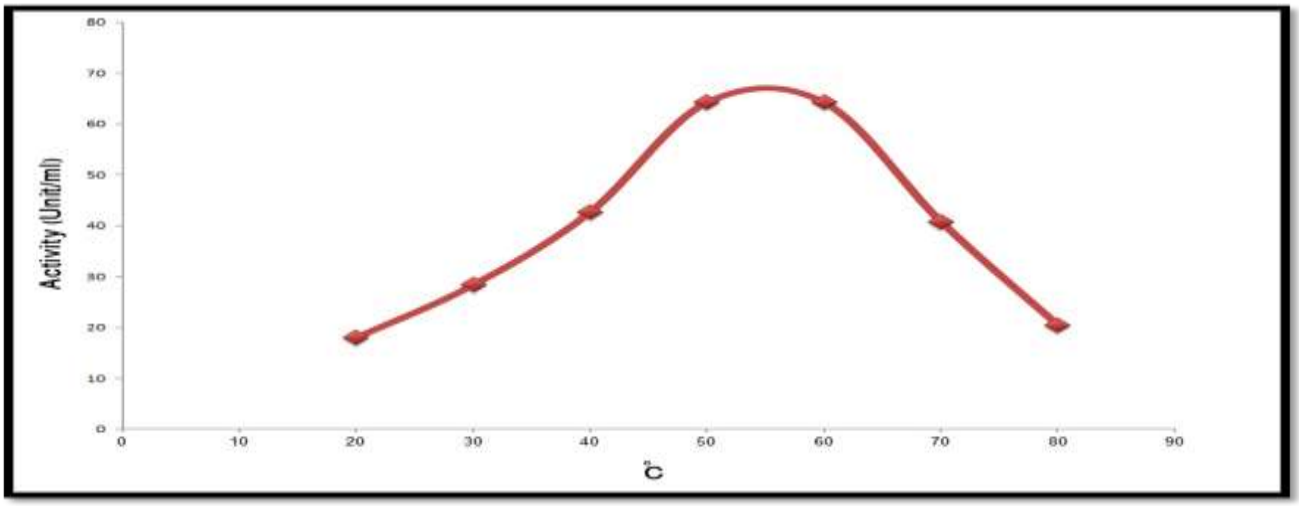

Figure 8: The Temperature Degree on Stability Effectiveness of Xylanase Enzyme from the Fungi Isolate of *Trichoderma longibrachiatum*

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