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Purification and Characterization of Cellulase enzyme from *Trichoderma longibrachiatum* isolated in Iraqi soil

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Abstract: *Cellulase produced by Trichoderma longibrachiatum was purified throughout four purification steps includes ammonium sulfate precipitation step with a saturation ratio of 80% then dialysis step followed by purification with ion exchange chromatography by using DEAE-cellulose, and then gel filtration step throughout sephadex-G200 which allows larger ability of separation with high degree of purification. Results of purification showed that the specific activity of the purified enzyme was 6680.8 U/mg protein with a purification fold and yield of 9.69 and 68.8% respectively. Purified cellulase from Trichoderma longibrachiatum was well characterized by studying some enzyme characteristics. Results showed that the molecular weight of cellulase was 17782 kilodalton; pH 4 was the optimum for enzyme activity and stability, while 40 ºC was also the optimum for enzyme activity and stability.*

Keywords*: Cellulase, Characterization ,Purification, Trichoderma longibrachiatum.*

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I. Introduction

Cellulases are glycoside hydrolases(GHs) that decompose cellulose a hydrophilic, water-insoluble polymer composed of repeated units of D-glucose interlinked by β-1,4- glycosidic bonds into shorter chain polysaccharides such as cellodextrin, cellobiose, and glucose (Garvey and Fischer, 2013). Cellulase are industrially important enzymes with a market share of 500 million dollars that is expected to rise to 1.5 billion dollars by 2018. Cellulases play a crucial role in generating sugar feedstock for lignocellulosic based biorefinery platform. In addition, their demand in textile, paper, feed and food industries is rising steadily. However, these industrial applications require thermostable, catalytically highly efficient cellulases for making the processes commercially viable (Sani and Krishnaraj, 2017). Cellulase has been produced from different organism, mainly fungi, bacteria, and protozoans, but the fungus *Trichoderma* spp was recorded as one of the most important commercial cellulase producers and has been widely used in a variety of industries. *Trichoderma* spp. are widely distributed all over the world and occur in nearly all soils and other natural habitats, especially in those containing organic and inorganic mattar (Montoya *et al* ., 2016). They are ubiquitous colonizers of cellulosic materials and can thus often be found wherever decaying plant material is available as well as in the rhizosphere of plants, where theycan induce systemic resistance against pathogens (Atanasova *et al*., 2013).

Solid state fermentations have been reported to have high enzyme productivities as compared to submerged fermentations (Prévot *et al*., 2013). The exact reasons for higher titres in SSF as compared to submerged fermentations are currently not wellknown. There are however suggestions that higher biomass and lower protein breakdown contribute to better production in SSF. SSF holds a tremendous potential for the production of enzymes especially where the crude fermented products maybe used directly as an enzyme source. SSF processes have lower energy requirements and produce less wastewater(Singhania *et al*.,2010). SSF offers numerous advantages lower cost and reduced need for asceptic techniques compared to submerged fermentations (SmF)(De la Cruz Quiroz R *et al*., 2015).

This study aimed to investigate the purification and characterization of cellulase enzyme from *Trichoderma longibrachiatum* isolated in Iraqi soil .

II. Materials and Methods

2.1. Fungal strain and production of cellulase

Trichoderma longibrachiatum strain AB1 previously isolated from Iraqi soil was identified based on 18S rDNA sequencing. The nucleotide sequence of the strain was deposited to NCBI (National Center for Biotechnology Information) GenBank with accession number KY750397.1. The newly isolated *Trichoderma longibrachiatum* strain AB1 is maintained on potato dextrose agar (PDA) enriched with carboxymethyl cellulose (CMC) at 4 °C. After that, this isolate was mutated according to Gadgil *et al* ,(1995) to produce higher

cellulase activity. Cellulase was produced by mutant isolate *Trichoderma longibrachiatum* in SSF using wheat bran as substrate. SSF was carried out in 250 mL Erlenmeyer flasks containing 10 g of substrate in Mineral Salt Solution (Mekala *et al*.,2008) containing (g/L) urea 2; NaCl 5; NH_4NO_3 5; KH_2PO_4 5; $CaCl_2$ 1; $MgSO_4.7H_2O$ 21; peptone 5; $FeSO_4.7H_2O$ 0.005; $MnSO_4.7H_2O$ 0.001; $ZnSO_4.7H_2O$ 0.001; $CoCl_2$ 0.0002; Tween-80 0.5 and pH 5 with 70 % moisture content. Sterilized flasks were inoculated with 1ml spore suspension and incubated at 28 °C under static condition for 7 days. The crude enzyme was extracted with 50 ml of D.W and shaked vigorously for 1-2 minutes, followed by filtration through muslin cloth. The filtrate was centrifuged at 8000 rpm for 5 min at 4 °C. The clear supernatant was used as crude enzyme for purification.

2.2. Enzyme assay and protein determination

Cellulase was hydrolyzed CMC to produce free carboxymethyl glucose units. Cellulase activity was estimated using CMC as substrate under standard method described by Otajevwo and Aluyi,(2011). The reducing sugars released from the reaction were determined according to Fu *et al*.,(2012) by dinitrosalicylic acid (DNS) method. In the above assay, one unit (U) of enzyme was defined as the amount of enzyme that released 1 lmol of the glucose per minute under standard assay conditions (30 min incubation at 50 °C with 0.5% CMC in 50 mM acetate buffer pH 5.0). The concentrations of soluble proteins were estimated according to Bradford ,(1976) using bovine serum albumin (BSA) as the standard.

2.3.Cellulase purification

More than one-step (using different purification techniques) were used to purify cellulase which produced by the mutant isolate of *Trichoderma longibrachiatum* under optimum conditions(Yin *et al*., 2010).

2.3.1. Ammonium sulfate precipitation

The first step of cellulase purification was achieved by precipitation with ammonium sulfate. Culture medium was centrifuged at 10000 rpm for 15min at 4ºC , then ammonium sulfate was added to the supernatant (crude enzyme) with gradual saturation ratios ranging between 40% and 80% and kept for 4 - 6 hours at 4°C . The mixture was then mixed gently on a magnatic stirrer at 4ºC for 20min. The resulting precipitate was collected by centrifugation at 10,000 rpm for 15 min at 4ºC. After centrifugation , supernatant was decanted and the pellet was dissolved in 5 ml of 0.02 M Tris-HCl buffer solution (pH 8). Enzyme activity and protein concentration were estimated before and after ammonium sulfate precipitation.

2.3.2. Dialysis of crude enzyme

Enzyme solution obtained after ammonium sulfate precipitation at suitable saturation ratio was kept a dialysis bag at 4 ºC and dialyzed against distilled water with three increments of substitutions every six hours.Total proteins and activity of dialysed cellulase were determined before and after dialysis, and dialysed enzyme was lyophilized for further purification steps and molecular weight determination respectively.

2.3.3. Purification by ion exchange chromatography

Cellulase obtained after dialysis step was further purified by ion exchange chromatography using DEAE-Cellulose column , A DEAE-Cellulose column was prepared according to Whitaker and Bernard, (1972) by dissolving 20 g of resin in 1L of D.W. beads were then left to settle down,and washed several times with D.W until getting clear appearance. The suspension was filtered throughout Whattman No. 1 using Buchner funnel,then resin was resuspended in 0.25 M NaCl and 0.25 M NaOH solutions. Gel suspension was filtered again as mentioned above and washed several times with 0.25 M HCl solution and next by D.W. before it was equilibrated with 0.02 M Tris-HCl buffer solution pH 8. After preparation, dialyzed cellulase was then applied to ion exchange chromatography column(1.5×31 cm) packed with DEAE Cellulose equilibrated previously with 0.02 M Tris-HCl buffer pH8,then column was washed with an equal volume of the same buffer, while attached proteins were stepwise eluted with gradual concentrations of NaCl (0.1, 0.2, 0.3, and 0.4 M respectively). Flow rate throughout the column was 3ml/fraction and the absorbance of each fraction was measured at 280 nm using UV-VIS spectrophotometer. Cellulase activity was determined in each fraction according to assay method described in above mention. Fractions representing cellulase activity were pooled together and concentrated for the last step of purification by gel filtration chromatography.

2.3.4. Purification by gel filtration chromatography

Gel filtration chromatography was used as a last step for purification of cellulase produced by the mutant isolate of *Trichoderma longibrachiatum*. SephadexG-200 was prepared as recommended by Pharmacia Fine Chemicals Company. A quantity of Sephadex G-200 was suspended in 0.2 M Tris-HCl buffer solution pH 8 for 24 hr, degassed, and packed in a glass column (1.7 × 28.5 cm), and equilibrated with 0.02 M Tris-HCl

buffer solution(pH 8) . 3 ml Aliquot of cellulase obtained after the ion exchange purification step was applied on to SephadexG-200 column equilibrated previously with 0.02 M Tris-HCl buffer solution pH8. Elution was achieved at a flow rate of 3 ml/fraction using the same buffer for equilibration. Absorbance of each fraction was measured at 280 nm. Cellulase activity was also determined in each fraction.

2.4.Cellulase Characterization

2.4.1. Determination of Molecular weight

Molecular weight of pure cellulase was determined by gel filtration chromatography by using the same column that used for purification (Sephadex G-200). Pure cellulase was left to flow through the glass column ,and eluted in a flow rate of 3ml / fraction. Standard proteins (Lysozyme, urease , Pronase, pepsin and Bovin Serum Albumin) at a concentration of 3 mg/ml were also applied separately through the same column and were eluted in the same manner of cellulase elution. Absorbance at 280 nm was measured for each fraction, while blue dextran was applied and eluted to determine the void volume, then the linear relationship between ve /v◦ against log molecular weight for each standard protein was plotted.

2.4.2. Determination of optimal pH for cellulase Activity

Purified enzyme was incubated at different pH values ranging from 4,5,6,7,8, and 9 in water bath for 30 min . Then cellulase activity was assayed.

2.4.3. Determination of optimal pH for cellulase stability

Purified enzyme was incubated at different pH values ranging from 4,5,6,7,8, and 9 in water bath for 1 hr. The remaining activity was then measured after assaying enzyme activity.

2.4.4. Determination of optimal temperature for cellulase activity

Cellulase activity was determined after incubation of the purified enzyme with subtrate(CMC)at different temperature (20, 25, 30, 35, 40, and 45 ºC) for 30 min. Then cellulase activity was assayed after each incubation temperature.

2.4.5. Determination of optimal temperature for cellulase stability

A purified enzyme was incubated at different temperatures (20, 25, 30, 35, 40, and 45 ºC) for 1 hr, then the subtrate(CMC) was immediately added, The enzyme activity was assayed for each treatment, and The remaining activity (%) for cellulase was plotted against the temperature.

Results and Discussion

3.1.Purification of cellulase

To purify cellulase produced by mutant isolated *T.longibrachiatium* under the optimum conditions, four purification steps were used, they included the following:

3.1.1. Ammonium sulfate precipitation

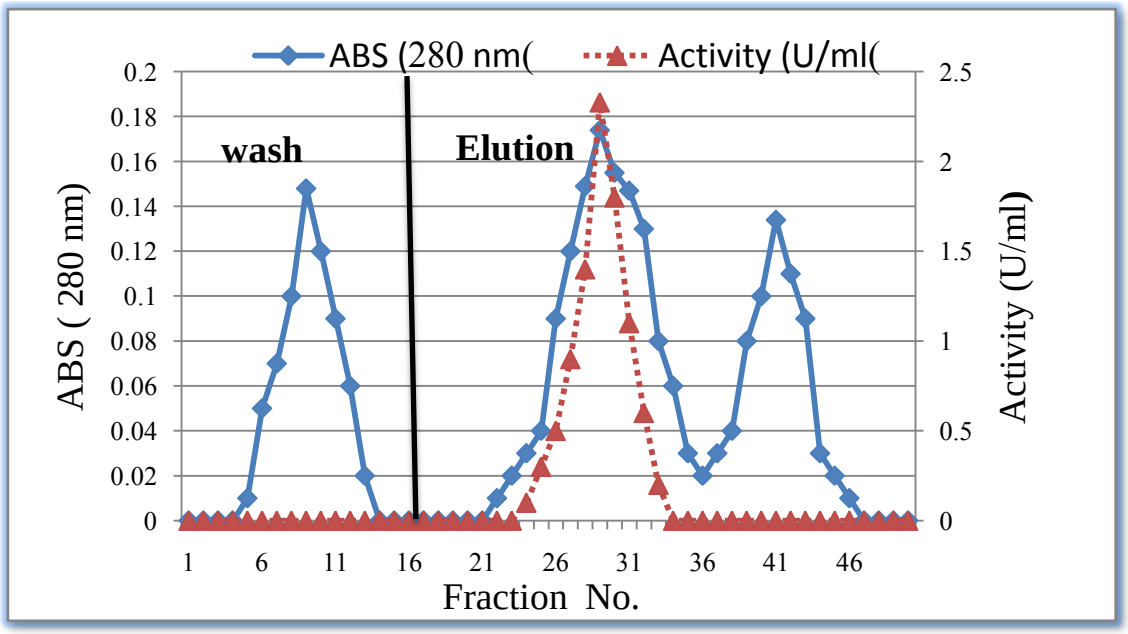
In this experiment, ammonium sulfate precipitation method was saturation for enzyme solution was done at 40-80% for precipitation of the crude enzyme (cell free culture filtrate). Results show that 80% of saturation with ammonium sulfate precipitated the enzyme, under the optimum conditions, and the specific activity of cellulase was 764.8 U/mg with a purification fold of 1.11 as shown in table(1). Ammonium sulfate is a common salt used in protein precipitation due to its high solubility, stabilized protein structure and cheap. Therefore, it was used in precipitating cellulase and different enzymes (Mawadza, 2000). Pachauri *et al*.,(2017) found that 1.351 fold of purification and 72.4% of cellulase yield were obtained from *T.longibrachiatium* after ammonium sulfate precipitation with a saturation percent reached 40-80%. Another study of Sahin *et al*.,(2016) indicated that 9.57 fold of purification and 2.65% of cellulase yield were obtained from *T.atroviride* after ammonium sulfate precipitation with a saturation percent reached 60-80%.

After ammonium sulfate precipitation, the precipitate was redissolved as the first step in cellulase purification. The ammonium sulfate in small amount of 0.02 M Tris-HCl buffer pH 8 and dialyzed against the same buffer. Results showed that after dialysis, cellulase specific activity and the unit activity increased to1904.4 U/mg, 1047.45 U/ml respectively. Elakkiya *et al*.,(2014) observed that the recovery of enzyme yield in *Trichoderma viride* for dialyzed step was 1525.34 U/gm while the specific activity was 144 U/mg .

3.1.2. Ion exchange chromatography

Ion exchange chromatography technique was used to purify cellulose produced by fungal isolate *T.longibrachiatium* after ammonium sulfate precipitation and dialysis step. In this technique, the dialyzed

cellulase was applied onto DEAE-cellulose column, then the column was equilibrated and washed with an equal volume of 0.02 M Tris-HCl buffer solution at pH 8 to wash uncharged and positively charged proteins in protein mixture of crude cellulase. The negatively charged bound proteins were then eluted using gradient concentration of sodium chloride ranged between 0.1 and 0.4 M. Cellulase activity and absorbance at 280 nm were measured at each fraction. Results in figure (1) showed the appearance of one protein peak in the washing fractions, while two protein peaks appeared after elution by gradient concentrations of sodium chloride. Cellulase activity was measured in the fractions of these three protein peaks. Data indicated that cellulase activity occurred in the fraction of the eluting step and confined between fraction No.23 and fraction No.33. The highest cellulase activity was in the fraction No.28. Accordingly, fractions represent cellulase activity were pooled and concentrated to 5ml, then protein concentration, cellulase activity and specific activity were measured. It was concluded from the separation step by using DEAE-cellulose, that cellulase has a positive net charge, since it was not bound with the same charge of the ionic matrix (anionic ion exchanger) of DEAE-cellulose. The proposed data in table (1) also show that protein concentration, cellulase activity and specific activity in this step were 0.35 mg/ml, 1894.5U/ml, 5412.8 U/mg protein respectively, with purification fold of 7.85 and enzyme yield of 78.06%. These results were in agreement with Zeng *et al*. (2016) who reported an increase in cellulase activity after purification by using anionic ion exchange of DEAEcellulose. Sahin *et al*.,(2016) also purified cellulase using the same method, and the enzyme was eluted from the column in the elution fractions ion exchanger enzyme was washed when they purified the cellulase from *T.atroviride*. After purification by ion exchange chromatography step, cellulase was further purified using the gel filtration chromatography technique to purify the enzyme for further enzyme characterization studies.



Figure(1): Ion exchanger chromatography of cellulase produced by the mutant isolated *T.longibrachiatium* using DEAE-Cellulose column(1.5x31cm) with a flow rate of 30 ml/hour.

3.1.3. Gel filtration chromatography

The gel filtration chromatography technique was the final step in the purification of cellulase produced by the mutant isolated *T.longibrachiatium*. After purification by the ion exchange purification step, fractions representing cellulase activity were collected, pooled and concentrated to be applied in sephadex-G200 previously equilibrated with 0.02 M Tris- HCl buffer pH 8. Sephadex-G200 has separation limits ranging between (5000-600,000 dalton) allowing larger capacity of separation with a high purification degree. A aliquot of 3 ml of cellulase concentrate was injected into the column and eluted with the same buffer. Results displayed in figure (2) show one protein peaks appeared after elution with buffer solution, and this peak represents cellulase activity manifested after elution with Tris-HCl buffer at fraction number 18. Protein concentration, activity and specific activity were measured in 7 ml aliquot of concentrated enzyme. Table (1) confirms an increase in the specific activity of the purified enzyme (6680.8 U/mg) recording purification fold reached 9.69 producing 68.8%cellulase. In another study, cellulase produced by was purified by *T.longibrachiatium* gel filtration using sephadex-200 resulting in an enzyme with specific activity of 30 U/mg and 14.82%

yield(Pachauri *et al*., 2017). Zeng *et al*. (2016) purified the enzyme by *T.virens* gel filtration achieving specific activity of 31.5 U/mg and a purification up to 35.8 folds. Similarity, Sahin *et al*. (2013) purified the enzyme by gel filtration achieving specific activity of 6.85 U/mg and a purification up to 7.2 folds.

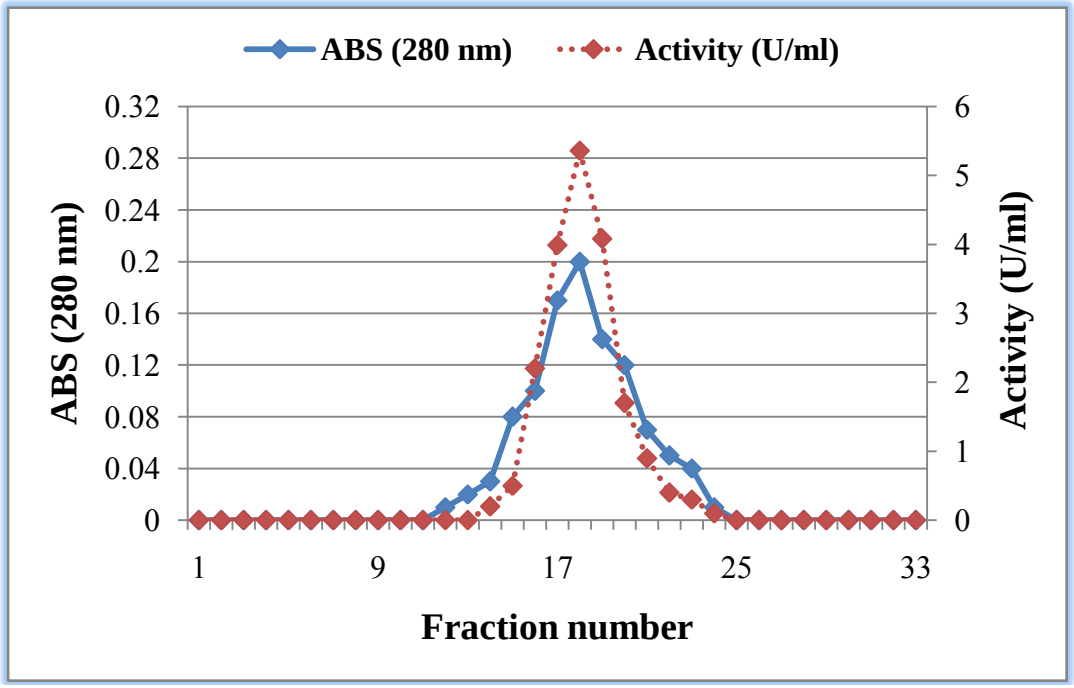


Figure (2): Gel filtration chromatography of cellulase produced by the fungal isolated *T.longibrachiatium* using sephadex-G200 column(1.7 cm x 28.5 cm) equilibrated with Tris-HCl buffer solution pH, fraction volume was 3 ml at flow rate of 30 ml/hours.

Table(1): Purification steps for cellulase produced by mutant isolated *T.longibrachiatium*.

Purification Steps	Vol. (ml)	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Total Activity (U)	purification (Fold)	Yield (%)
Crude enzyme	15	808.95	1.174	689.05	12134.2	1	100
$(NH_4)_2SO_4$ 40-80%	10	1162.5	1.52	764.8	11625.0	1.11	95.8
Dialysis	10	1047.45	0.55	1904.4	10474.5	2.76	86.3
DEAE-cellulose	5	1894.5	0.35	5412.8	9472.5	7.85	78.06
Sephadex-G200	5	1670.2	0.25	6680.8	8351.0	9.69	68.8

3.2.Characterization of purified cellulase

3.2.1. Molecular weight of cellulase

Molecular weight of the purified cellulase produced by *T.longibrachiatium* was determined by gel filtration using sephadex-G200 in the presence of five standard proteins (Lysozyme, pepsin, Pronase, urease and BSA). Cellulase and standard proteins were applied and eluted individually under the same conditions. Figure (3) denotes that cellulase has a molecular weight of 17782 Kilo dalton. The difference in the molecular weight of cellulase may due to carbohydrate contents in the protein which increase the molecular weight of protein(Abdul-Hadi *et al*.,2016) or could be due to the biological aspect of the fungal strains, these aspects include intrinsic genomic traits, identity of the gene encoding enzyme and the proteomic level(Soliman *et al*.,2013). Azimova *et al*.,(2016) indicates the molecular weight of EGII and EGIII from *Trichoderma harzianum* are nearly equal to 135 and 75 KDa. Additionally, Pachauri *et al*.,(2017) found that molecular weight of cellulase was to be 67 KDa from *T.longibrachiatium*. Similarity, Makhdum,(2013) indicated that Endoglucanase of 23.5 KDa with purified from *Trichoderma harzianum*. Additionally, Onsori *et al*.,(2005) who observed that molecular weight of CMCase from *Aspergillus* sp. were 18KDa, 23KDa, 28KDa.

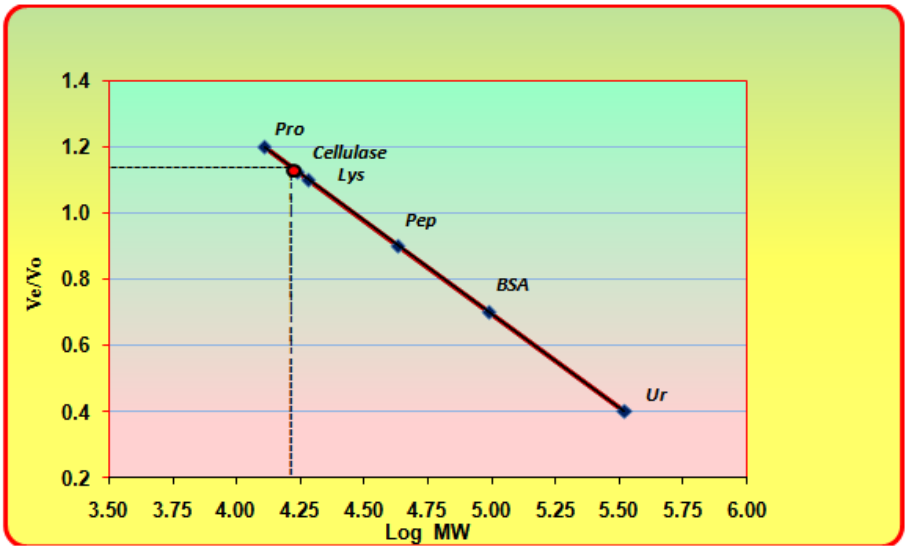


Figure (3): The relation ship between the v_e/v_o and log MW for molecular weight determination of purified cellulase produced by the mutant isolated *T.longibrachiatium* by gel fitration using sephadexG200(28.5x1.7cm).

3.2.2. Optimum pH for enzyme activity

Optimum pH for cellulase activity was determined by incubation of the purified cellulase with its substrate(CMC) at different pH values ranging from pH 3 to pH9. Results in (figure 4) showed that cellulase was active over a wide range of pH (3-9) with an optimum activity of 42.6 U/ml at pH 4, where as any further increase in pH from optimum value(pH 4) cellulase showed decreasing trends in its activity. The reason for this declining attributable efficiency at basal pH values returns to influence the groups of amino acids in active center or enzyme molecule on the ionic state of substrate (Englard and Sifter,1990),Also, changes in pH may also alter the three dimensional shape of the enzyme. Variations in pH of the medium results in the ionic form of active site of the enzyme and change the activity of the enzyme and hence the reaction rate(Rajesh *et al*.,2012). Another reason of little variations in pH optima may be to the genetic variability among different species (Iqbal *et al*.,2011).These observations were similar to the findings of Pachauri *et al*.,(2017) who reported maximum activity at pH 4.8 for the cellulase from *Trichoderma longibrachiatum* while examinations of Azimova *et al*.,(2016) revealed that optimum pH value of EGI and EGII was 4.5 from *Trichoderma harzianum*. Another study by Dobrev *et al*.,(2011) which enzyme produced from *Trichoderma longibrachiatum* showed maximum activity at pH 4.

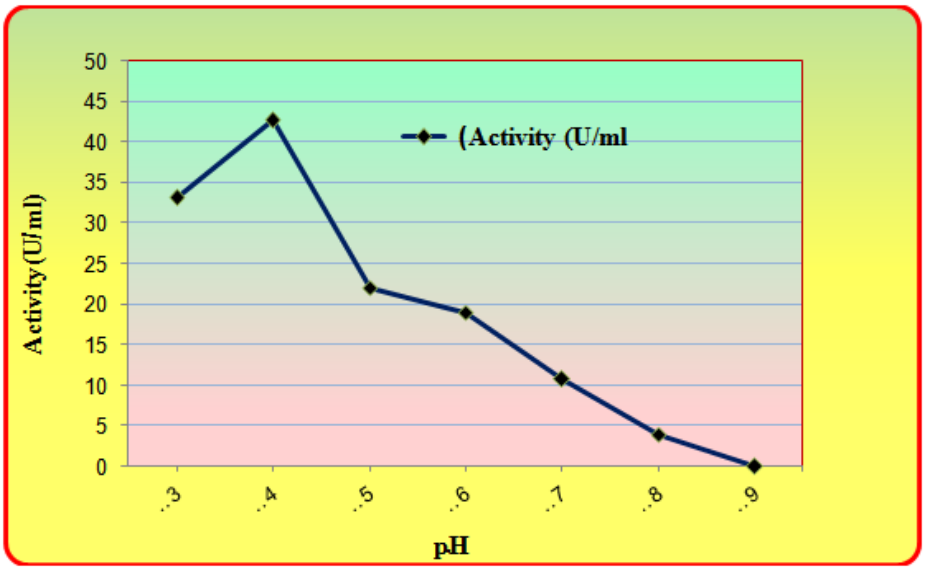


Figure (4): Effect of different pH values on activity of purified cellulase produced by mutant isolated *T.longibrachiatium*.

3.2.3. Optimum pH for enzyme stability

To determine the optimum pH for cellulase stability, purified cellulase was incubated at different pH values. The remaining activity was then determined after assaying enzyme activity. Results in figure(5) showed that cellulase was more stable at pH 4 since at this pH, enzyme gains maximum remaining activity (100%). While The remaining activity decreased when the enzyme was incubated at pH values less or more than 4. The instability of these enzymes at very low or very high pH values is due to the fact that they are proteins which are generally denatured at extreme pH values (Steiner *et al*.,1994). In general, the pH-stability curves of the enzymes are much broader than the pH-activity curves (Tong *et al*.,1980). Fungal cellulases, in general, are stable at over the pH range 3 –8 and usually active over the pH range 3.5 to 7, in citrate, phosphate or acetate buffers (Bhikhabhai *et al*.,1984).This corroborates the results of Soliman *et al*.,(2013) that CMCase II showed relatively stable in pH range(3-6), while Rahnama *et al*.,(2016) showed cellulase more stable at pH4.5 and remaining activity 100%.Another study by Leghlimi *et al*.,(2013) showed cellulase more stable at pH4 in *Trichoderma longibrachiatum* and remaining activity 100%.

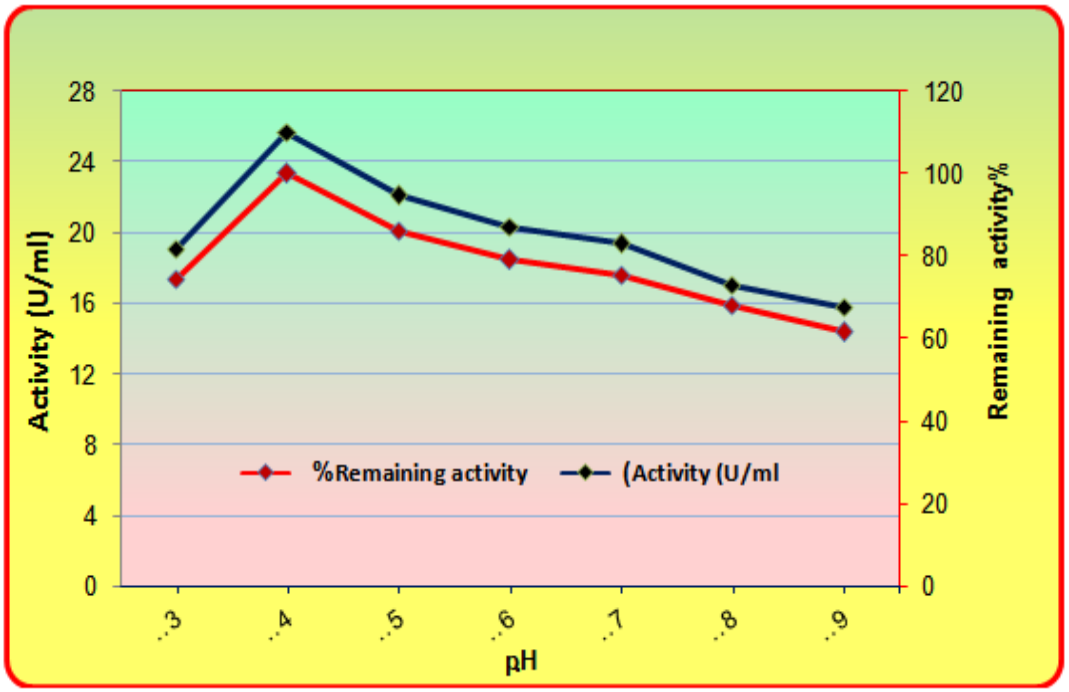


Figure (5): Effect of pH stability on activity of purified cellulase produced by mutant isolated *T.longibrachiatium* after 1 hr of incubation at 28 ºC.

3.2.4. Optimum temperature for enzyme activity

Optimum temperature for cellulase activity was examined at different temperatures ranging between 20 - 45 °C. Results showed that 40 °C was the optimum temperature for cellulase activity (figure 6). Activity decreased after enzyme incubation at values less or more than 40°C. Above a certain temperature enzyme activity decreases with increase in temperature because of enzyme denaturation (Rajesh *et al*.,2012). The temperature could speed up the reaction, but the activity of cellulase would fade along with the increasing temperature. The space structure of the enzyme would be destroyed when over an acidic or or basic environment, causing the change of conformation, and the loss of enzyme activity(Zeng *et al*.,2016).Similar results with Gorems,(2011) found the optimal temperature of cellulase to be 40ºC for isolate *Trichoderma* AUT1.

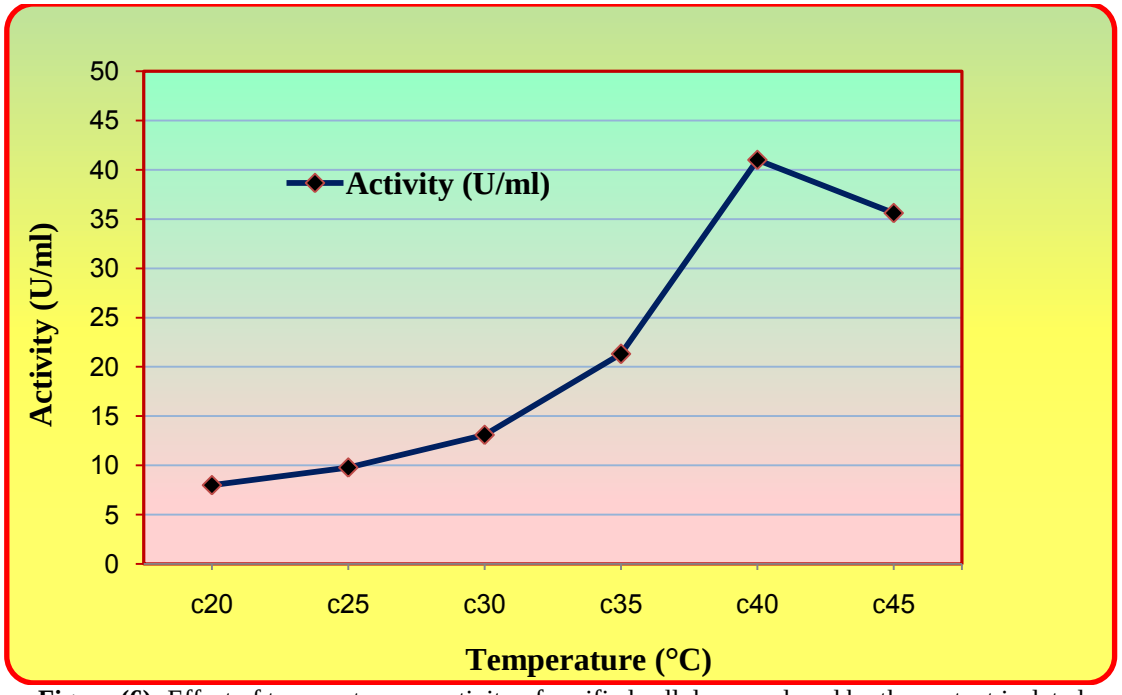


Figure (6): Effect of temperature on activity of purified cellulase produced by the mutant isolated *T.longibrachiatium*.

3.2.5. Optimum temperature for enzyme stability

Different temperature regimes were used to determine the optimum temperature for cellulase stability (figure 7).Enzyme was incubated at a range of temperatures and the remaining activity was determined. The enzyme was stable at a temperature ranged between (20 – 45) ºC, while maximum remaining activity (100%) was gained at 40 ºC. The remaining activity decreased slowly when the enzyme was incubated at temperature values exceeds or less than the optimum temperature for stability(40°C). It is proposed that fungi obtained endoglucanase has mesophilic properties (Tao *et al*.,2010). It has been established for some time that the tertiary structure of proteins is stable. The conformational stability of a protein is the sum of a large number of weak, non-covalent, interactions, including hydrogenbonds, van der Waal interactions and others. All of these forces are affected by environmental conditions, including, for example, solvent and temperature (Roy *et al*.,1996). Segal, (1976) stated that crude and partially purified enzymes are more stable than purified enzymes due to the existence of carbohydrates and other proteins protecting them.

Similar results with Haung *et al*.,(2013) who proved that maximum activity obtained at 45 ºC and remaining activity 100% was reported. Another study of Azimova *et al*.,(2016) showed EGIII maximum remaining activity at 40 ºC increased by more than three times comparing to the initial of the enzyme that reported 300% i.e thermostability of EGIII among tested enzymes significantly varied.

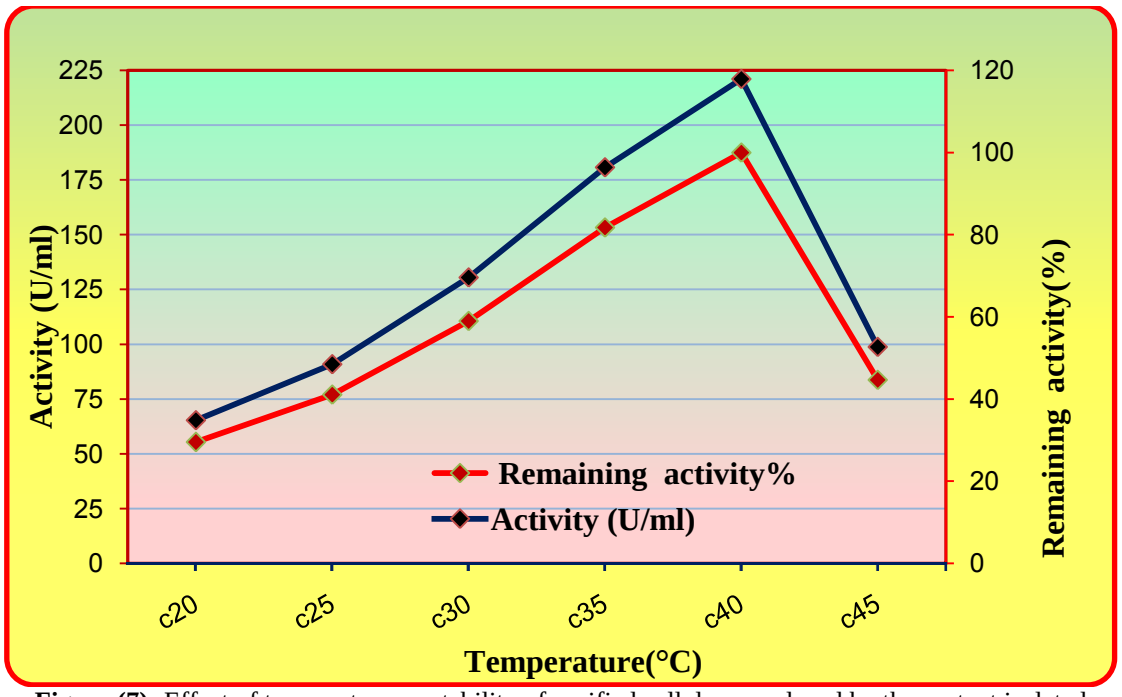


Figure (7): Effect of temperature on stability of purified cellulase produced by the mutant isolated *T.longibrachiatium*.

III. Conclusion

Purification of mutant isolate *Trichoderma longibrachiatum* gave higher cellulase specific activity with higher fold of purification. Purified cellulase enzyme was characterized on stable at acidic pH 4, this is an indication of the presence of carboxylic groups in the active site of the enzyme and stability temperature of 40°C

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