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Production and Characterization of Exopolygalacturonase for *Fusarium oxysporum* and *F. sacchari*

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Abstract: ***These experiments were conducted to study the production; purification, characterization, and molecular identification of Polygalacturonase enzyme from F. oxysporum and F. sacchari were investigated. The results showed that the optimum incubation period for Polygalacturonase in F. oxysporum and F. sacchari were occurred within two days. Polygalacturonase exhibited maximal activity at pH 6, 27˚C and 0.5% pectin concentration. The results revealed that the best ratio for exo Polygalacturonase precipitation for F. oxysporum and F. sacchari was 70 and 90% of ammonium sulphate respectively. Two peaks of PG were appeared in ion exchange chromatography purified from F.oxysporum. While in case of F. sacchari, only three peaks of protein and one peak of enzyme activity were shown The results showed that the molecular weight of Polygalacturonase from F. oxysporum (two peaks) using SDS-PAGE were approximately 53KD, and about 41 KD for PG purified from F.sacchari under denaturation conditions. For characterization of Polygalacturonase purified from F.oxysporum, the results reported the highest activity of PG (A) occurred at pH 5 while peak (B) was showed highest activity at pH 6. On other hand, the maximum enzyme activity of Polygalacturonase in F. sacchari was obtained at pH 5. Best growth (two peaks) achieved at 40˚C for F. oxysporum and 45˚ C for F. sacchari respectively. Finally, molecular identification of exo Polygalacturonase gene in F. oxysporum and F. sacchari was investigated using PCR technique. The PCR primers designed from the conserved region of known fungal produced* **1700***bp product from the genomic DNA of F.oxysporum and 1200 bp product from the genomic DNA of F.sacchari.*

Keywords: Purification, polygalacturonase enzyme, molecular identification of pgx1 gene.

1. Introduction

Fungi can produce wide extracellular enzymes, especially Fusarium species; these are used to break down complex polysaccharides into simple sugars to be important and used for growth and reproduction. These enzymes are very important in an industry of detergent, starch, drinks, food, textile, animal feed, chemicals and bio medical products. These enzymes include, protease, cellulase, chitinase, lipase, and pectinase are found [1,2]. Pectin is an important structural component of primary cell walls and middle lamella providing firmness and organization to plant tissue [3]. Pectinase is one of the most of important enzymes that contribute to the degradation of pectin by various mechanisms. The family of pectinase consists of protopectinase, polygalacturonase, lyase and methyl esterase. Polygalcturonase is used to cleavage of the polygalactyronic acid with the introduction of water across the oxygen bridge. Based on the mode of action, polygalacturonase are classified into two groups ,(i) endo polygalacturonase (E.C.3.2.1.15) that used to breakdown the polymer chain in a random pattern liberating saturated oligogalacturonides and galacturonic acid .(ii) exopolygalacturonase (E.C.3.2.1.67) split the polymer bonds releasing one saturated galacturonic acid residue from non-reducing end of homogalacturonan. Polygalacturonase are one of the most important pathogenicity factors for fungi such as *Aspergillus flavus*, *Alternaria* citri and *Fusarium oxysporum*, in addition to some genera of bacteria such as Agrobacterium tumefaciens [4]. The characterization of purified polygalacturonase is an important that used for research since it exhibited on being able to distinguish between the enzymatic complex components of the substrate degradation mechanisms, optimum conditions for enzymatic activity and the regulation of enzyme by inhibiters [5]

2. Material and Methods

Source of organisms:
The species of *Fusarium* are obtained from the Unit of Advanced Mycology / Department of Biology / College of Science/ University of Babylon / Autum 2013.

Preparation of *Fusarium oxysporum* and *Fusarium sacchari* Inoculum
Pure culture of newly isolated *F. oxysporum* and *F. sacchari* was maintained on PDA slants at 4 ˚C, synthetic medium was autoclaved for 15 min, cooled then the flasks(250 ml size), were inoculated by placing 10mm agar medium plugs containing active mycelium (5 days old) from each of *F.oxysporum* and *F.sacchari* growing in slant culture. Flasks were incubated at 28˚C on a rotary shaker at 150rpm for 5 days.

Isolation of polygalacturonase enzyme
At the end of fermentation, the mycelium was separated from the culture broth by filtering through filter paper (Whatman No.1), the filtrate was centrifuged at 10,000 rpm for 20 min at 4 ˚C to remove the suspended particles, and the supernatant was carefully collected and stored under refrigerated conditions for further purification steps after enzyme assay.

Measuring the activity of polygalacturonase enzyme
The reaction mixture contained 0.1 ml of the enzyme, 0.8 ml of citrus pectin substrate; 0.1ml of acetate buffer (pH

5.6).The mixture was incubated for 20 minutes at 40˚C. After incubation period, 1 ml of DNS was added and the mixture heated at boiling for 5 minutes in water bath to develop the color. After cooling in room temperature, the optical density was measured in triplicate at 560 nm using spectrophotometer. Absorbance was used to calculate the activity of enzyme using the formula:-

$$\text{Enzyme activity U/ml} = \frac{\text{O.D}}{\text{0.01*Time* volume (crude enzyme)}}$$

Purification of polygalacturonase

Ammonium sulphate precipitation

The crude polygalacturonase solution was precipitated by different concentrations of (NH4)2SO4 (20, 30, 40, 50, 60, 70, 80 and 90%) under cooled conditions, the precipitates were separated by centrifugation at 8000 rpm for 30 minutes and dissolved in small amount of acetate buffer solution prepared in (3.8.1.4) The final volume of solution, the activity of enzyme and protein concentration was measured and specific activity was calculated. The protein concentration was determined by Bradford method (1976).

Separation of enzyme through DEAE – cellulose

Enzyme solutions (3 ml) that produced after precipitation step was loaded in ion exchange column .The column was washed with 0.1 M of acetate buffer at flow rate 30 ml/ hours and eluted with gradient (1M- 0.1 M) of NaCl solution. Fractions of 3 ml/ tube were collected and the optical density at 280 nm was measured, enzyme activity was measured at the peak fractions with high polygalacturonase activity were collected, volume enzyme activity and protein concentration was determined.

Gel filtration chromatography technique

Enzyme separation through sephadex G-200

The enzyme solution separated from ion exchange chromatography was added gently on the surface of gel, the elution was achieved by using acetate buffer at flow rate 30 ml/ hour (3 ml for each fraction), the protein fractions was measured at 280 nm, the enzymatic activity was measured of fractions, then collected the activation parts and measured the activity, volume and protein concentration, divided in vials and stored in freeze to the following experiments.

SDS−Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE was performed to check the homogeneity of the enzyme on a 10%, 15% gel (Laemmli, 1970) and molecular weight determined by standard protein marker. The protein bands on gel were visualized by staining it with Coomassie brilliant blue R- 250.

Characterization of polygalacturonase enzyme

Optimum pH of enzyme activity

The substrate pectin for polygalacturonase was prepared with different pH ranges by using acetate buffer 0.1 M (pH= 4.5, 5, 5.5, and 6), phosphate buffer 0.1 M (pH= 6.5, 7, 7.5) and Tris Hcl buffer 0.1 M (pH= 8, 8.5, 9, 9.5 and 10), the purified enzyme was added and reaction solution was incubated at 40 ˚C for 20 minutes, after then reaction was stopped by adding 1 ml of DNS reagent in boiling water bath. The enzyme activity was estimated and the reaction was drawn between enzyme activity and pH ranges to determine optimal pH for enzyme activity.

Optimum temperature of enzyme activity

The substrate pectin for polygalacturonase enzyme was prepared at PH 5.8 for *F.oxysporum* and pH 5 for *F.sacchari* in tubes, the tubes were incubated at different temperatures (30, 35, 40, 45, 50, 55, 60, and 65) ˚C for 40 at 10 min for mixing, after then purified enzyme was added and incubated for 20 min to start the reaction, the activity was estimated and the reaction was drawn between enzyme activity and different temperature.

Molecular characteristic of *Fusarium* species

DNA extraction kit solution

These methods were determined according to SolGent Company**.**

3. Results

Purification of polygalacturonase enzyme

The ammonium sulphate used in different saturation ratios (20, 30, 40, 50, 60, 70, 80, and 90%), then the 70% ratio and 90 % for *F.oxysporum* and *F.sacchari* was selected as best ratio for precipitate the crude extract of enzyme in the first step respectively, followed by Ion exchange chromatography two peaks of protein and enzyme activity appeared in the eluted fractions from *F.oxysporum* that shown in figure (1) The first peak of protein with specific activity 3.4U/mg with purification fold 5.1 and yield 44.3%, while the second peak of protein has specific activity 26.4U/mg with purification fold 39.4 and yield37.8% that showed in table 1. In the state of *F.sacchari*, the results in figure (2) showed appearance of three beak of protein with enzyme activity .It was obtained one peak of protein after concentrated with specific activity 0.3U/mg with purification fold 2.1 and yield 36.8% that showed in table 2. The enzyme solution obtained from ion exchange chromatography was passed through sephadex G-200 column (1.5*85) cm that equilibrated with acetate buffer (0.1M, pH 5.6), the fraction were collected and measured at 280nm absorbency. One peak of protein with one peak of enzyme activity was appeared, as shown in figure (3). This peak of protein has specific activity 0.7 U/mg with purification fold 4.2 and yield 34.3%, shown in table (2). SDS PAGE electrophoresis of purified enzyme revealed five samples, first sample was crude enzyme extract while other sample produced from ion exchange (peak A and B) these samples belong to *F,oxysporum* has molecular weight 53 KD (figure 4). In the state of *F.sacchari* revealed four samples, first sample was crude extract enzyme, second sample was precipitation of ammonium sulphate, third sample produced from ion exchange and the four sample produced from gel filtration (figure 5). The optimum pH for PG enzyme activity (2 peaks) purified from *F.oxysporum* showed in (figure 6), peak A has enzyme activity 18.9U/ ml at pH 5 while peak B has optimum activity 19.6U/ml at pH 6. *F.sacchari* showed in (figure 7) has maximum enzyme activity at pH 5 20.4U/ml. The optimum temperature for PG enzyme activity (peak A and B) purified from *F.oxysporum* showed in (figure 8), peak A has enzyme activity 18U/ml at

temperature 40˚C while peak B has optimum activity 17.5U/ml at temperature 40˚C.. *F.sacchari* showed in (figure 9) has maximum enzyme activity 23.4 U/ml at temperature 45˚C

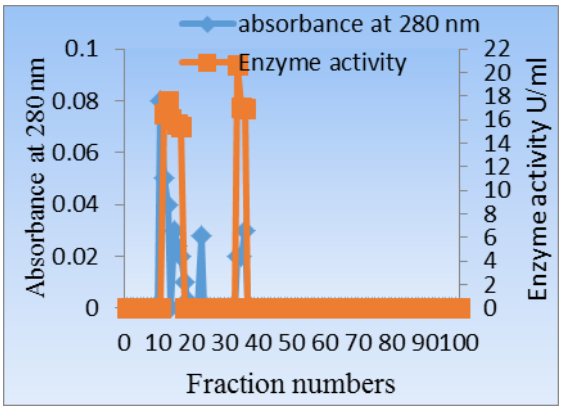


Figure 1: Ion exchange chromatography using DEAE-cellulose column (2.8×18) cm with acetate buffer (0.1M, pH 5.6), for purification from *F.oxysporum* polygalacturonase enzyme from *F.oxysporum*

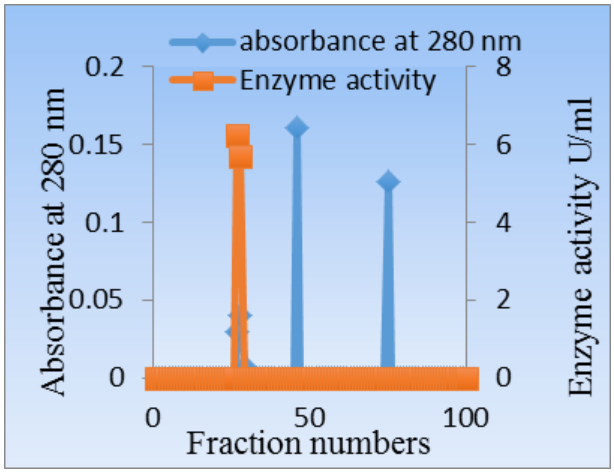


Figure 2: Ion exchange chromatography using Deae-Cellulose column (2.8x18) cm with acetate buffer (0.1M, pH 5.6), for purfication from *F. sacchar* polygalacturonase enzyme

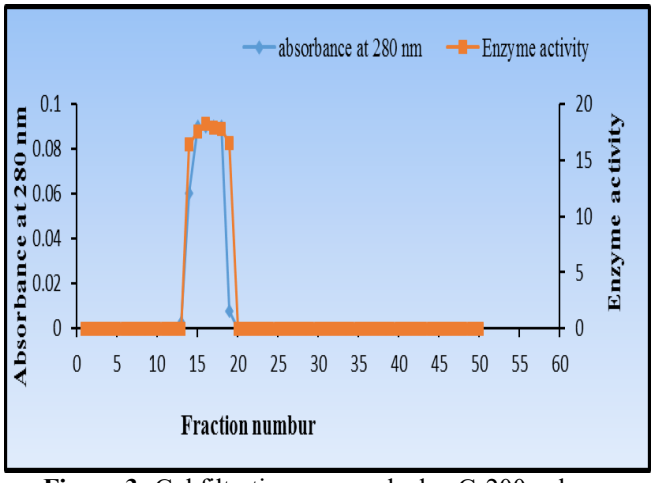


Figure 3: Gel filtration uses sephadex G-200 column (1.5×85 cm) cm with acetate buffer (0.1M, pH 5.6), for purification polygalacturonase enzyme from *F.sacchari*

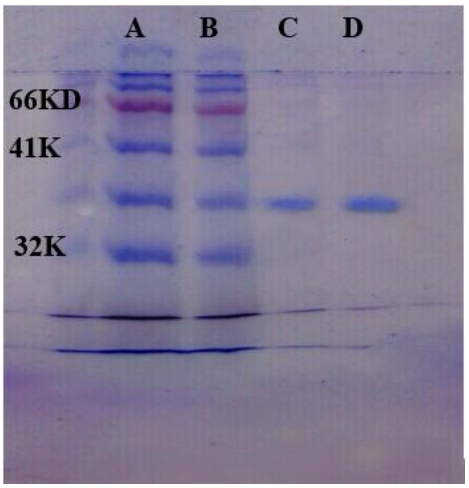


Figure 4: Polyacrylamide gel electrophoresis of the Polygalacturonase from *F.oxysporum* since A=molecular weight marker, B=crude extract enzyme, D=represents the purified enzyme produced from Ion exchange chromatography

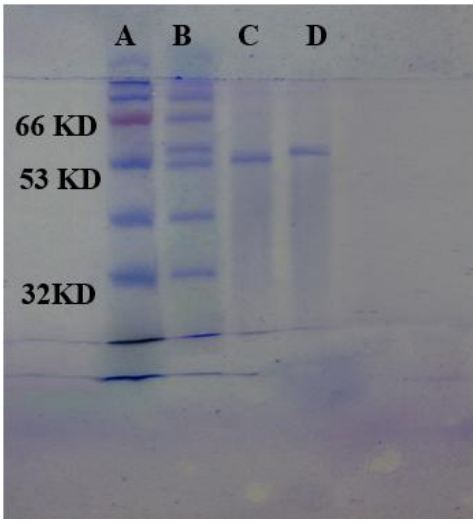
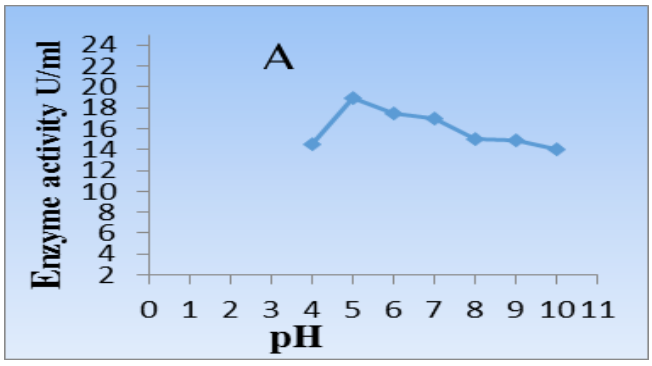


Figure 5: Polyacrylamide gel electrophoresis of the Polygalacturonase from *F.sacchari* since A=molecular weight marker, B=crude extract enzyme, C, =represents the purified enzyme produced from Ion exchange chromatography. and D= Purified enzyme produced from Gel filtration.



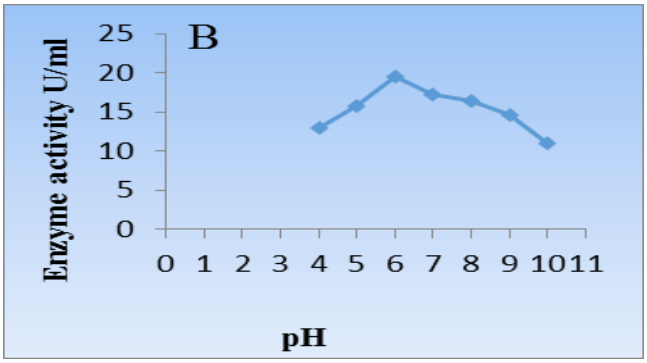


Figure 6: Effect of pH value on the polygalacturonase activity purified from *F.oxysporum*. A= peak 1and B= peak 2.

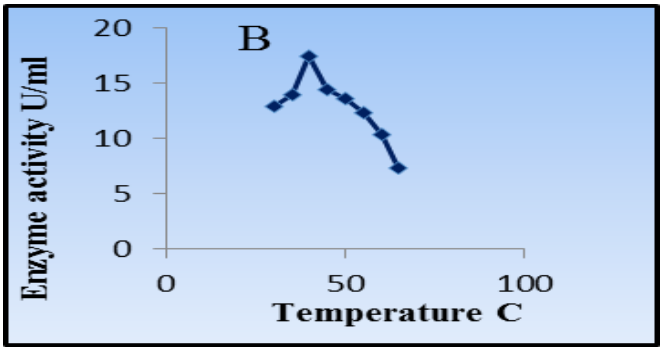


Figure 8: Optimum temperature of polygalacturonase activity purified from *F. oxysporum.* A= peak 1and B= peak 2

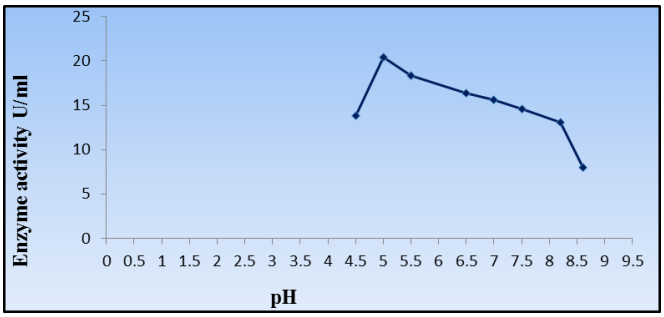


Figure 7: Effect of pH value on the polygalacturonase activity purified from *F. sacchari.*

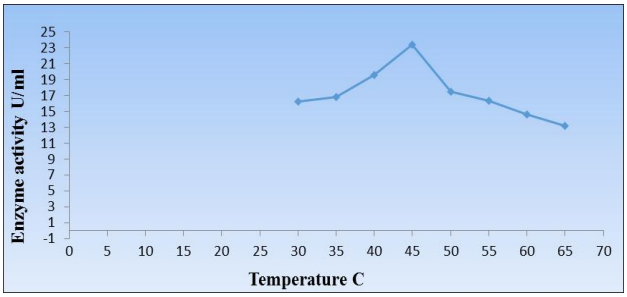


Figure 9: Optimum temperature of polygalacturonase activity purified from *F. sacchari.*

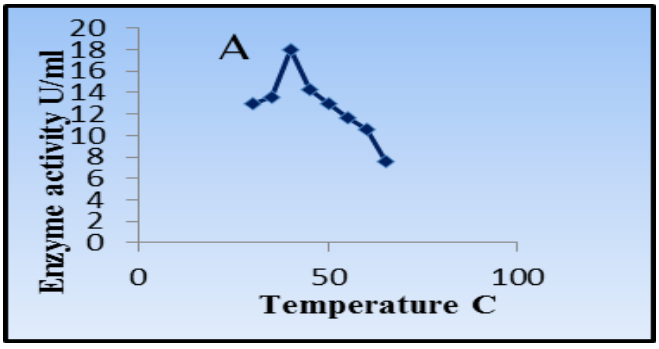


Table 1: Purification steps of polygalacturonase enzyme purified from F.oxysporum.

Purification steps	Volume (Ml)	Total activity (U)	Enzyme activity /ml)	Protein concentration (mg/ml)	Specific activity /mg	yield	Purification fold
Crude extract	8	139.5	17.4375	26	0.670673	100	1
Ammonium sulfate precipitation 70%	6.5	132.977	20.458	18.666	1.096003	95.324	1.6341843
Ion exchange chromatography Peak1	3	61.8	20.6	6	3.43333	44.3011	5.11923
Ion exchange chromatography Peak2	3	52.8	17.6	0.666	26.4	37.8	39.4

Table 2: Purification steps of polygalacturonase enzyme purified from *F.sacchari*.

Purification steps	Volume (Ml)	Total activity (U)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity U/mg	yield	Purification fold
Crude extract	8	159.16	19.895	122	0.1630738	100	1
Ammonium sulfate precipitation 70%	7.5	151.7175	20.229	78.666	0.2571505	95.323	1.576896
Ion exchange chromatography	4.5	58.59	13.02	38	0.3426316	36.812	2.10108331
Gel filtration	3	54.687	18.229	26	0.701115	34.35	4.29937556

Diagnosis by polymerase chain reaction technique (PCR)

The genomic DNA of *Fusarium* species (*F.oxysporum, F.sacchari, F.globosum, F.proliferatum,* and *F.solani)* was isolated and gene coding for (*pgx1*) was amplified by PCR. This result showed that the gene *pgx1* encoded for polygalacturonase enzyme is present. The PCR primers designed from the conserved region of known fungal produced 1700 pb product from the genomic DNA of *F.oxysporum* and 1400 bp product from the genomic DNA of *F.sacchari*, as shown in figure 10.

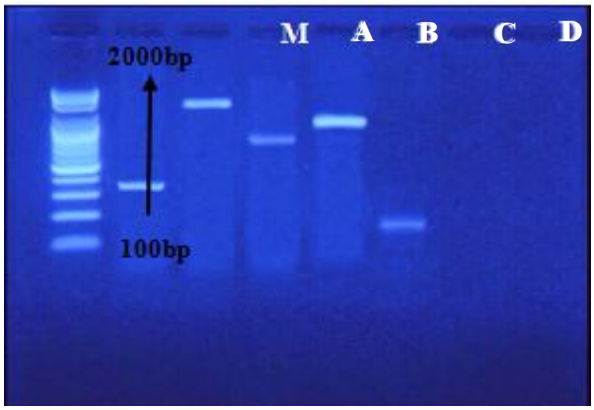


Figure 10: Agarose gel electrophoresis of PCR amplified products of *F.oxysporum* and *F.sacchari*, M= DNA ladder with molecular weight (100-2000bp),A= PCR products *pgx1*gene from *F.solani* B= PCR products *pgx1* gene from *F.oxysporum*, C= products *pgx1*gene from *F.proliferatum* D= PCR products of *pgx1* gene from *F.sacchari* and E= products *pgx1*gene from *F.globosum.*

4. Discussion

Purification of polygalacturonase enzyme
Precipitation with ammonium sulphate

Ammonium sulphate is a common salt using in protein concentration in view of higher solubility, cheap, and stabilizes protein structure. Crude polygalacturonase of *F.oxysporum* was concentrated with 70% ammonium sulphate. This result is in agreement with [6] who reported that the polygalacturonase was precipitated at 40-80% ammonium sulphate saturation with the highest activity obtained at 70% saturation from *Aspergillus niger (*SA6) and other studies by the same research have shown that polygalacturonase can be precipitated between 0-90% of ammonium sulphate depending on the source of enzyme.

In the case of *F.sacchari*, the maximum specific activity was obtained at 90%, these results are consistent with those obtained from [7] who found around 1.5 fold purification levels with 85% of enzyme recovery when they used 90% ammonium sulphate saturation to precipitate polygalacturonase from *Aspergillus niger*. This step was performed as a purification step to remove un wanted substances and protein as well as concentration step by resuspending the pellet in a less amount of buffer. Ammonium sulphate precipitation (salting out) is useful for concentrating dilute solutions of protein. It is also useful for fractionating a mixture of proteins since large proteins tend to precipitate first while smaller ones will stay in solution. After a protein has been precipitated and taken back up in buffer the solution contained a lot of residual ammonium sulphate which was bound to the protein. [8].

Ion exchange for polygalacturonase purified from *F.oxysporum* and *F.sacchari*

Ion exchange chromatography profile of Polygalacturonase on DEAE-cellulose showed that the enzyme eluted in the flow through. This step was used to purify protein, that indicated decrease in total protein and total activity, whereas specific activity increased .In this study, the results showed isoforms of PGase. These results in agreement with those results obtained by [9] that reported most PGs so far have several isozymes. Isoenzymes can be defined as the several forms of an enzyme that all catalyze the same reaction but may differ in reaction rate, pH, temperature, electrophoretic mobility and immunologic properties [10].

Most fungal organisms produce multiple forms enzyme [11] suggesting either that several genes are involved, or that post –translational modification results in different physical properties of the enzymes. In addition, these studies also compatible with other results reported by[12], which refer to various numbers of PGases with different specific activity, were reported from *Aspergillus niger*. According to [13] variation in the isoforms of extracellular enzymes obtained by solid state fermentation can be attributed to alteration of water potential that results in changes in the permeability of fungal membranes, limitation of sugar transport and presence or absence of inducer.DEAE-cellulose chromatography has many advantage, including high resolution power, high capacity, easy handling, good separation, ability of reactivation for using many times besides the simplicity of separation principle which depending on charge differences[14].

Gel filtration chromatography of *F.sacchari*

Gel filtration chromatography by using sephadex G-200 Colum was the next step of PGase purification from *F.sacchari*. In this study, the results showed one peak of protein with high specific activity. These results considered with results presented [15] who reported PGase enzyme was purified from *Acrophialo nainiana* by using sephadex G-200 has one peak of protein and one peak of activity. The degree of purification of PGase based on specific activity from resistant and susceptible varieties, the fractionating of ammonium sulphate on sephadex G-200 tend to point to the pattern of synthesis of PGase. The position of enzyme on the chromatography suggested that only PGase was produced during growth [16].

Sephadex G-200 has many advantages included fast run , high recovery separation, simple preparation and its stability for long time permits reusing of the gel many times in protein separation [17].

Polyacrylamide gel electrophoresis technique

The SDS-PAGE revealed an apparent molecular weight of 53KD and 41KD for *F.oxysporum* and *F.sacchari* respectively. These results in this study agreement with [18] who reported that molecular weight of PGase enzyme at range 35-80KD. The majority of purified fungal PGase have molecular weight in range from 25-80KD [19].

The appearance of many protein bands along gel is imputed to that crude extract contain large number from different proteins with different molecular weight. Whearse the other samples gave only single band, these mean that there is no contamination from other proteins. Other studies suggested that molecular weight of PGases ranging from 35-496 Dalton's for different species of fungi [20] Of all the reasons adduced by [21] for the differences in molecular weight, nature and type of organisms used, substrates employed and analytical methods in addition to monomeric units of the polypeptides could justify the differences.

Characterization of polygalacturonase from *F.oxysporum* and *F.sacchari*

The optimum pH for enzyme activity

Effect of pH on purified PGase activity was determined; it was observed that PGase has the best activity in pH 5 and 6. It is not too sensitive to pH variation but it is more active at pH 5 and 6 than other values, this may give a conclusion that the fungus favored environmental conditions at pH 5 and 6 for degradation of lipids and utilization as carbon source or for their metabolic process.

Fungal PGases are known to operate mild acidic environment. pH optima for 23 PGases isolated from various species with a range of pH 3.8-6.5. These results were compatible with our results in this study. The optimum pH and possible amino acids in the active site of PGase is in agreement with those reported by previous workers. Other results showed similar observation of this study, which reported *Pencillium viridicatum* showed an optimum pH =6 as mentioned by [22], and pH 4.5-5 for Pencillium sp[23]. The effect of pH on structure and activity of PG from *A.niger* was demonstrated by [24]. They evidenced that the active conformation of PGase was favored pH between 3.5-6; alteration in the secondary and tertiary structures resulted in at pH=5-7.

The optimum temperature for enzyme activity

Temperature is a very important factor for microbial growth as well as microbial product formation and it varies from each of microorganism. In fact, slight changes in growth temperature may effect on enzyme activity [25]. In this study, the results showed optimum temperature of enzyme activity was 40, 45, and 50˚C. Regarding the influence of temperature was demonstrated, these results are agreement with [26] In addition, PGase produced by *A.flavus*, *A.fumigatus* exhibited maximum activity at 40 and 45˚C respectively [27] Optimal temperature of PGase showed a range from 40- 60˚C. These optimal temperatures were similar to those observed for PGase from *Streptomyces erumpens* [28].

Diagnosis by Polymerase chain reaction technique (PCR)

Exo polygalacturonase is important virulence factor in fungi and bacteria [29]. A single gene in many *Fusarium* species such as *F.oxysporum*, for this reason, encodes PGase the *pgx1* gene has been proposed as a molecular marker for taxonomic studies of *Fusarium* species [30,31]

In this study, the results showed that the gene encoding exo polygalacturonase of *F.oxysporum* and *F.sacchari* is present. These results are compatible with [32] who revealed that the gene *pgx1* is present in some *Fusarium* species with molecular weight 1400bp. Other studies revealed that the molecular weight of products that obtained from *F.oxysporum* was 1.793bp [33].

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