

## **Activity *Aspergillus oryzae* to cellulase production**

### **فعالية *Aspergillus oryzae* في انتاج انزيم السليلز**

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#### **Abstract**

This study aimed to extraction cellulase enzymes and purified by using many methods The crude enzymes when using ammonium sulfate had total activity (14 , 90 , 2835 )U, total protein 10.57 mg and the specific activity ( 0.013 , 1.57, 2.68 ) U/mg protein The crude culture filtrate containing Filter paperase (FPase) , Carboxymethylcellulase (CMCase) and  $\beta$ -glucosidase were subjected to ammonium sulfate precipitation. Different concentrations (20, 40,60, 80 and 100%) of ammonium sulfate were used for that purpose. After conducting some preliminary trials, 40- 60 % concentration were selected for the enzyme precipitation.

On the other hand, the protein of crude enzyme extract was separated in (33 ) fractions of fungi from DEAE-cellulose column is ,It was found that only the fractions ( F-8) contained cellulase activity., while other fractions were eluted by the buffer containing different concentrations of Nacl .On the other hand, the component of the active fraction ( F-8) , obtained after DEAE cellulose chromatography was separated by gel filtration and gave only one peak of enzymes alone and the fraction also found to be contained cellulase activity. as given in the specific activity of cellulase were ( 15.67 , 7.94 , 1972 .16 ) U/mg for FPase ,CMCase and  $\beta$ -glucosidase respectably and characterized purified enzyme effect of various pH values the highest enzyme activity was found at 5.5 pH. When the pH of the enzyme medium was increased up to 6.0 , FPase enzyme giving activity ( 0.91 ) U , but CMCase and  $\beta$ -glucosidase giving highest activity at pH 6 were ( 0.34 , 65.12 ) U and the activity declining at pH 5 to ( 0.25 , 59.95 ) IU . and It found that the enzyme exhibited maximum activity at 25 C° in CMCase was ( 0.33 ) U , but 30 C° for  $\beta$ -glucosidase was ( 38.22 ) U . On the other hand, FPase was having activity at 25 and 30 C° were (0.14,0.15)U.

Finally, the crude enzyme preparation was subjected to SDS-PAGE to determine the molecular weight of the enzyme. While conducting the electrophoresis, one major band showing cellulolytic activity. The molecular weight of the protein was found to be about (38) kD, (52) kD and (49) kD for (FPase ,CMCase ,  $\beta$ -glucosidase ) respectively.

#### **الخلاصة**

تهدف هذه الدراسة إلى استخلاص أنزيمات التحلل السليلوزي وتنقيتها باستخدام عدد من الطرق حيث استعمل الانزيم الخام وتم ترسيبه بواسطة كبريتات الامونيوم حيث اعطى فعالية كلية للأنزيم (14، 90، 2835) وحدة دولية والبروتين الكلي 10.7 ملغم و الفعالية النوعية كانت ( 0.013 , 1.57 , 2.68 ) وحدة دولية ، فالزرع الخام والمرشح حاوي على أنزيمات Filter paperase (FPase) ، Carboxymethylcellulase (CMCase) و  $\beta$ -glucosidases المترسبة بواسطة كبريتات الامونيوم بمختلف التراكيز (20، 40، 60، 80 و 100) % و بعد الاستنتاج في المحاولات الاولى وجد ان تركيز (40-60) % استعمل لغرض ترسيب الانزيم. اما البروتين ( الانزيم الخام ) فقد تم فصله في (33) جزء حمل على عمود DEAE-cellulose حيث وجد فقط ان الجزء (F-8) الحاوي على الفعالية الانزيمية ، بينما الاجزاء الاخرى حملت على بفر حاوي على تراكيز متدرجة من كلوريد الصوديوم.

من جانب اخر المكونات الفعالة للجزء (F-8) الحاصل عليها من كروتوكرافيا التبادل الايوني فصلت بواسطة الترشيح الهلامي و اعطى قمة واحدة للأنزيم والجزء الحاوي على الفعالية الانزيمية اعطى فعالية نوعية كانت (7.94، 15.67، 1972.16) / وحدة دولية / ملغم للأنزيمات FPase ، CMCase و  $\beta$ -glucosidases على التوالي . بعد تنقية الانزيم تم توصيفه حيث وجد ان أعلى نشاط للأنزيم عند pH 5.5 ارتفع نشاط الانزيم الى 6 من جانب اخر اظهر الانزيمات فعالية عالية عند pH (4.5 - 6) فإن انزيم FPase اعطى فعالية 0.91 وحدة دولية ولكن CMCase و  $\beta$ -glucosidases اعطى

اعلى فعالية انزيمية عند PH 6 كانت ( 0.34 و 65.12)وحدة دولية وانخفضت عند pH 5 وكانت الفعالية (0.25,59.95) وحدة دولية. وجد ان فعالية الانزيم في درجة الحرارة ملائمة لنشاط CMCCase في 25<sup>0</sup>م وكانت 0.33 وحدة دولية ولكن في 30<sup>0</sup>م ارتفعت فيها فعالية انزيم  $\beta$ -glucosidases الى 38.22 وحدة دولية بينما امتلك فعالية عالية عند 25 و 30 م وصلت الى ( 0.14 , 0.15 ) وحدة دولية. واخيرا فان الانزيم الخام المنقى تم تحميله SDS-PAGE لتحديد الوزن الجزيئي للأنزيم. عند إجراء الترحيل الكهربائي حيث تم تحديد الوزن الجزيئي و استنتج من الترحيل ظهور ثلاث قطع تشير الى الانزيمات فالوزن الجزيئي كان (38، 52 و 49) كيلو دالتون للانزيمات FPase، CMCCase و  $\beta$ - glucosidases على التوالي.

## Introduction

Several fungi and bacteria are capable of producing multiple groups of enzymes, collectively known as cellulases, acting synergistically to hydrolyze  $\beta$ -1,4-glycosidic bonds within the cellulose molecules. Filamentous fungi, particularly *Aspergillus* species have been reported as efficient producers of cellulases. The members of *Aspergillus* species are major agents of decomposition and thus possess the ability to produce a range of enzymes like cellulase [1]. Considering the importance of CMCCase in oil industry, [2] produced a mixture containing polygalacturonase, cellulase, xylanase and protease enzymes using *Aspergillus niger* 3T5B8 on different industrial residues and applied in vegetable oil extraction. The use of sugar cane bagasse as substrate, pH 4.2 and 24 hr of fermentation time were the optimum conditions for production of enzymes cellulase 1.75 U/ml , xylanase 30.62 U/ml , protease 5.27 U/ml. The objective for increase the yields of cellulase and xylanase , [3] used different reactor to grow *Aspergillus niger* on rice-straw and compared the activities, yields and productivities of both enzymes. In general, better yield and productivity was observed in bubble column and external loop air-lift reactors. The highest yield and productivity of  $\beta$ -glucosidase were 84 FPA IU/g and 9.7 FPA IU/l/h, obtained by fed-batch mode in the bubble-column and in the stirred-tank reactor, respectively. In case of  $\beta$ -glucosidase the highest yield and productivity were 370 U/g and 26 U/l/h in an external loop air-lift reactor. During enzyme production process (in addition to the target enzyme) growth medium may have some undesirable metabolites of the micro-organisms. The purified enzymes exhibit higher activity, lesser risk of harmful substances and thus better affectivity for the specific product. Recently, in order to evaluate the role and mechanism of action of cellulose components during the solubilization of cellulose. [4] Purified a thermostable CMCCase from culture supernatants of *Daldinia eschscholzii* (Ehrenb. Fr.) Rehm grown on 1.0% (w/v) CMC using ammonium sulfate precipitation, ion-exchange, hydrophobic interaction and gel filtration chromatography. The molecular weight of the enzyme was 46.4 kDa. The isoelectric point of the enzyme was pH 4.9 while the optimum temperature was 70°C. The enzyme retained 85% of its maximum activity after 150 min at 50°C, but was rapidly inactivated at 70°C .

This study aimed to produce cellulase enzymes from fungus *Aspergillus oryzae* and purified by using ammonium sulfate, Ion exchange, Gel filtration, Electrophoresis.

## Material and methods

### Isolation *Aspergillus oryzae*

For the isolation of fungi. One gram a soil was transferred to aliquots of 9ml sterile distilled water in test tube. It was shaking vigorously at constant speed for 15min. suspension was then subjected to serial dilutions from the appropriate plate in duplicate with 15 ml of medium The plates were contain 0.25 mg / l of chloromphencoil to reduce contamination with fast growth bacteria , that incubated for 7 days at 25C<sup>0</sup>. The well-grown spreaded single colonies were picked up and sub cultured on potato dextrose agar slants (Burnset al ., 2010) and its identification [5] , [6] .

### Enzymes Purification

#### Extraction and concentration of crude enzyme.

*Aspergillus oryzae* was cultivated in 100 ml of Mandel s broth medium .The stock culture was sub cultured and organism was inoculated in the medium and allowed to grow at 30C<sup>o</sup> with manual

shaking for 4 days. When the organism was grown profusely, the culture medium was filtered and the filtrate was centrifuged at 10,000 rpm for 15 minutes. The supernatant was used as the crude enzyme solution for the experimental purposes [7].

### **Enzymes Assay**

#### **Total Cellulase activity or Filter paper activity (FPase)**

Filter paper activity (FPase) for total cellulase activity in the culture filtrate was determined according to the standard method [8] and the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method according [9].

#### **Endoglucanase activity (CMCase)**

Endoglucanase activity (CMCase) was measured according [8].

#### **$\beta$ -glucosidase activity**

$\beta$ -glucosidase activity was assayed by the method of [10].

#### **Protein assay**

To determine the concentration of protein using Bradford assay. The absorbance at 595nm [11].

#### **Ammonium sulfate precipitation**

The method Ammonium sulfate precipitation assayed by [7].

#### **DEAE-Cellulose Chromatography**

The crude enzyme solution after dialysis against distilled water and 0.2M Sodium acetate buffer pH 5.2, for 24 hour was applied to DEAE-cellulose column which was previously equilibrated with the same buffer at 4 C<sup>0</sup>. The separation of protein from the column was performed by stepwise elution with the buffer containing increasing concentrations of NaCl (0.05M, 0.1M, 0.2M, 0.5M and 1M). About 3.0 ml fraction was collected in different test tubes. The absorbency at 280 nm and enzymatic activity of each fraction was estimated. The fraction (F-8) containing enzyme activity obtained from DEAE cellulose column was purified further by gel filtration.

#### **Gel Filtrations**

The enzyme containing fraction (F-8) was dialyzed against distil water and 0. 2 M sodium acetate buffer pH 5.2 for 24 hours at 4C<sup>o</sup>. After centrifugation, the clear sample was loaded onto the gel bed Sephadex G- 75 column (1.6 cm×60 cm), pre-equilibrated with 0. 2 M sodium acetate buffer pH5.2 at a flow rate of 1.0 ml/min. Fractions of 5.0 ml were collected examined at 280 nm and assayed for enzymes activity. The active fractions containing Enzymes activities from the column were pooled and dialyzed against the buffer or further analysis [12]. After diffusion the sample, about 1 ml of elute buffer was poured on the top of gel bed and was allowed to diffuse. An additional amount of buffer was then added, so that the space about 3-4 cm above the gel bed was filled with elute. The buffer was allowed to flow continuously through the column and 3 ml fraction of elute was collected by an automatic fraction collector and monitored for enzyme activity as well as for protein concentration at 280 nm.

#### **SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

SDS-PAGE was conducted in 10% gel at 28C<sup>o</sup>, pH 7.0 according to the method of [13]

#### **Characterization**

Characterization of the purified enzyme was carried out according to the procedure of [1]

#### **Optimum temperature**

For estimation of the optimum temperature of the enzymes, the activity was determined by carrying out the assay at several temperatures between (10 -90) C<sup>o</sup>.

**Optimum pH**

The pH profile of the enzymes was evaluated by incubating the enzymes for 10 min at 50°C in appropriate buffers: 50 mM sodium acetate (pH 3–4.5), 50 mM sodium citrate (pH 5–5.5) and 50 mM sodium phosphate buffer (pH 6–9).

**Molecular weight**

Molecular weight was determined by SDS-PAGE. The molecular mass of enzyme was determined by loading the standard markers along with the prepared enzyme. Molecular weight of purified cellulase was determined by following the procedure as described by [13]. RNAase 13.5KD , Lysozyme 14 KD , Trypsin 23 KD , Egg albumin 45 KD, Bovine serum albumin 67KD , Phosphorylase 97 KD , *B*-glactosidase 116 KD , Alkaline phosphatase 140 KD were used as marker proteins.

**Results and Discussion**

**Enzyme purification**

**Concentration crude enzymes**

The Tables (1),(2) and (3), gives the summary of results when FPase ,CMCase and  $\beta$ -glucosidase were subjected to purification process. The crude enzymes had total enzyme activity (14 , 90 , 28.35 ) U, total protein 10.57 mg for all enzymes and the specific activity ( 0.24 , 1.57, 2.68 ) U/mg protein , The crude culture filtrate containing FPase ,CMCase and  $\beta$ -glucosidase were subjected to ammonium sulfate precipitation. Different concentrations (20, 40,60, 80 and 100%) of the ammonium sulfate were used for the purpose. After conducting some preliminary trials, 40- 60 % concentration was selected for the enzyme precipitation. After standing overnight, the precipitate formed was collected by centrifugation. Later, the enzyme was loaded on the column packed with Sephadex G-75 and fractions of 5 ml were collected and analyzed for enzyme activity. Each of the purification step resulted in enhanced specific activity; the ammonium sulfate precipitation resulted in (38.69, 1.25, 5767.5) IU of the enzyme with 0.012 mg protein.

Each step of the process increased the purification fold (26.28,60.50,93.93) fold purification to FPase, CMCase and  $\beta$ -glucosidase were calculated after ammonium sulfate precipitation.

Tables (1) FPase purification process Satisfy the sedimentation rate of ammonium sulfate

Concentration ammonium sulfate (%)	Volume (ml)	Activity Unit \ ml	Protein concentration	Specific Activity Unit/mg	Total Activity	purification (fold )	Yield %
Crude extract	100	0.14	10.57	0.013	14	1	100
0- 20	20	0.093	0.017	5.47	1.86	22.79	13
20-40	10	0.16	0.029	5.51	1.6	22.95	11
40-60	15	0.87	0.020	43.5	13.5	181.25	93
60-80	15	0.26	0.86	0.30	3.9	1.25	27
80-100	15	0.08	0.023	3.47	1.2	14.45	86
40-60	15	0.89	0.023	38.69	13.3	26.28	95

Tables (2) CMCase purification process Satisfy the sedimentation rate of ammonium sulfate

Concentration ammonium sulfate (%)	Volume (ml)	Activity Unit \ ml	Protein concentration	Specific Activity Unit/mg	Total Activity	purification (fold )	Yield %
Crude extract	100	0.90	10.57	1.57	90	1	100
0- 20	20	0.17	0.017	10	3.4	6.36	3.77
20-40	10	0.19	0.029	6.55	1.9	4.17	2.11
40-60	15	0.46	0.86	0.53	6.9	0.33	7.66
60-80	15	0.25	0.020	1.25	3.75	0.12	4.1
80-100	15	0.24	0.023	1.04	3.6	0.66	4
40-60	15	1.14	0.012	1.25	17.1	60.50	60.5

Tables (3)  $\beta$ -glucosidase purification process Satisfy the sedimentation rate of ammonium sulfate

Concentration ammonium sulfate (%)	Volume (ml)	Activity Unit \ ml	Protein concentration	Specific Activity Unit/mg	Total Activity	purification (fold )	Yield %
Crude extract	100	28.35	10.57	2.68	2835	1	100
0- 20	20	40.21	0.017	2365.29	804.2	47.56	28.36
20-40	10	49.48	0.029	1706.20	494.8	34.30	17.45
40-60	15	69.38	0.86	80.67	1040.7	1.62	36.70
60-80	15	69.25	0.020	3462.5	1038.75	69.62	36.64
80-100	15	67.34	0.023	2927.82	1010.1	58.87	35.62
40-60	15	69.21	0.012	5767.5	1038.15	93.93	36.60

The basis for the precipitation of enzyme and concentration by ammonium sulfate depends on process called salting out , salts can change the structure of the solvent , which can to lead large changes in protein conformation by altering the electrostatic interaction between charged groups on the protein surface , the salts also competes with the protein for solvent molecules and thereby lowers its solvation , the large scale enzyme precipitation use of many other neutral salts is preferred over by ammonium sulfate which corrosive and releases NH<sub>3</sub> at higher temperature.

The salting out which includes the equation of charge on the surface of protein molecules by ions of ammonium sulfate, and breach of a layer of water surrounding the protein , leading decrease solubility and precipitation, and affects the size of the protein and its shape and the presence of other vehicles, it's in speed solubility as depends on the salt concentration of the protein shipments in number and distribution , as well as non-ionic aggregates and the distribution of hydrophobic aggregates and number in addition to the higher solubility and not deterring enzyme [14].

[4] Observed 45.31 U/mg protein after ammonium sulfate precipitation during the purification of endoglucanase from crude culture of wood-decaying fungus *Daldinia eschscholzii* (Ehrenb.:Fr.) Rehm. The difference in the results as compared with present study may be due to the difference in the concentration of the ammonium sulfate used for precipitation of enzymic protein. The results are also supported by those of [15] when they observed specific activity 24.61 mol/min/mg protein, 4.51 purification fold and 13.46% enzyme recovery during the process of cellulase purification.

Likewise, [16] during five step purification of cellulase from *Chaetomium thermophilum* CT2 observed specific activity 38.7 IU/mg, 8.8 % enzyme recovery and 20.4 purification fold. The higher values observed than the present study were due to the extent of purification i.e. more steps

were involved in the purification process resulting in the increased purification of enzyme protein. The results of the study are also agreed by [17] as they found 6.5 IU/mg specific activity, 2.4% enzyme of *B*-glucosidase recovery and 13 fold enzyme purification during their research work. On the other hand, using ammonium sulfate to purify CMCase enzyme, the crude enzyme had total enzyme activity 70,000 IU and 7502.68 mg total protein the specific activity 9.33 IU/mg protein and using concentrations (30, 60 and 80%) of the ammonium sulfate used for the purpose. After conducting some preliminary trials, 80% concentration was selected for the enzyme precipitation [18].

### **Ion exchange chromatography**

The elution profile of crude enzyme solution of *Aspergillus oryzae* from DEAE-cellulose column is shown in (Figure 1) it was revealed that, the protein of crude enzyme extract was separated in (33) fractions. It was found that only the fractions (F-8) contained cellulase activity, this fraction was pooled and subjected to gel filtration for further purification, On the other hand, i.e. (F-30) was eluted from the column by buffer only, while other fractions were eluted by the buffer containing different concentrations of NaCl.

Table (4) refer to total activity when Ion exchange chromatography (6.1, 4.1, 729.7) U for FPase, CMCase and  $\beta$ -glucosidase respectively, and the specific activity (8.47, 5.69, 1013.47) U/mg protein to same enzymes, this step of process increased the purification to (0.605, 0.406, 72.39) fold respectively.

While the above, the Figure were appear the second peak at fraction NO. (20) to FPase, CMCase and  $\beta$ -glucosidase respectively. When using gradually concentration of NaCl (0.05M, 0.1M, 0.2M, 0.5M and 1M) M in elution phase.

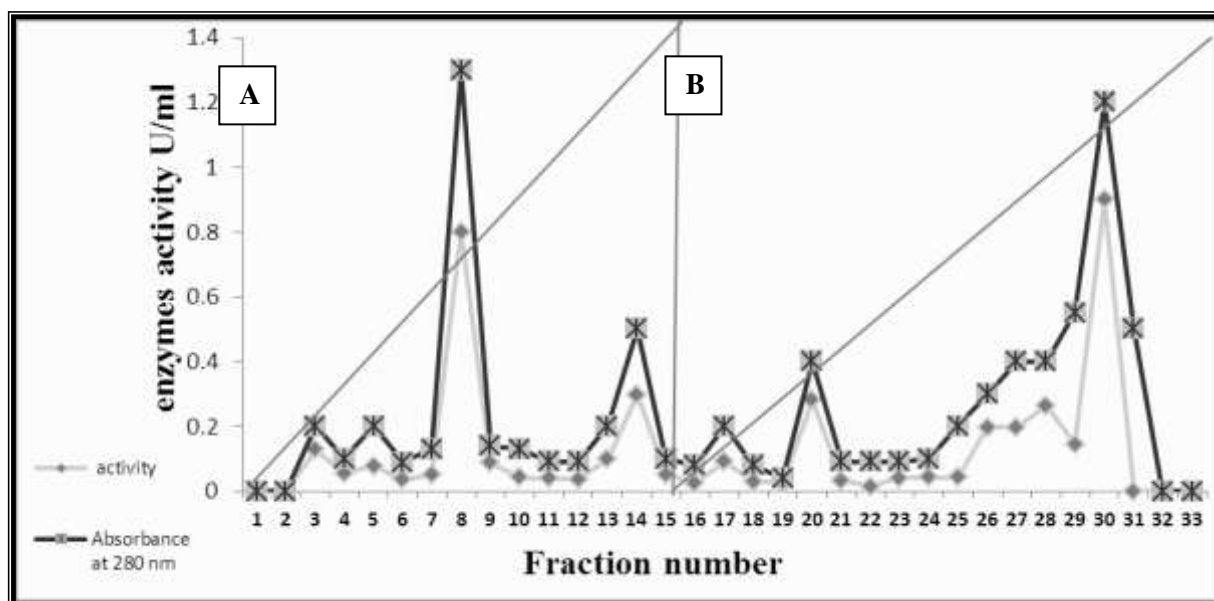


Figure (1) Ion exchange chromatography A-fractions contain mixed enzymes B- gradually concentration of NaCl

Table (4) activity Ion exchange chromatography and Gel Filtration

Purification Step	Volume (ml)	Activity Unit/ml	Protein concentration	Specific Activity	Total activity	purification (fold )	Yield %
Crude extract FPase	100	0.14	10.57	0.013	14	1	100
Crude extract CMCCase	100	0.90	10.57	1.57	90	1	100
Crude extract $\beta$ -glucosidase	100	28.35	10.57	2.68	2835	1	100
40-60% $(\text{NH}_4)_2\text{SO}_4$ FPase	15	0.89	0.023	38.69	13.3	26.28	95
40-60% $(\text{NH}_4)_2\text{SO}_4$ CMCCase	15	1.14	0.012	1.25	17.1	60.50	60.5
40-60% $(\text{NH}_4)_2\text{SO}_4$ $\beta$ -glucosidase	15	69.21	0.012	5767.5	1038.15	93.93	36.60
Ion exchange FPase	10	0.61	0.072	8.47	6.1	65.1	61
Ion exchange CMCCase	10	0.41	0.072	5.69	4.1	66.94	58
Ion exchange $\beta$ -glucosidase	10	72.97	0.072	1013.47	729.7	72.39	52.75
Gel filtration FPase	5	0.58	0.037	15.67	2.9	1.11	20.7
Gel filtration CMCCase	5	0.29	0.037	7.94	1.47	0.567	10.5
Gel filtration $\beta$ -glucosidase	5	72.97	0.037	1972.16	364.85	140.86	73.5

This decrease in yield might be due to denaturation of enzyme during the purification steps or other reasons, using DEAE-cellulose that recognized first high ability to separation the protein (high resolving power). Second, the large capacity to protein binding according the charge [19]. I've been use of ion exchanger systems chromatography commonly used among these features, the ability to high separate and high capacity to absorb the associated proteins and clarity and simplicity of the principle of separation which depends the basis of the difference in the charge capacity used ion exchangers natural, which is a derivative of cellulose because of their suitability for the separation of proteins with molecular weights of different as well as the possibilities few of the metamorphosis of the important things that must be noted is that the summit, which did not Colum linked to the positive ion and appeared in part washing back to the enzymes that carry a positive charge prevented the outcome of the link colum positively charged ion them like the charge while the enzymes which carry on their surface charge in negative of charge exchanger increases its ability to adsorption on the surface. Exchanger offending her link to the charge may be very difficult to be separated so that, therefore, increasing the concentration of salt and recovery in the buffer helps in the process of separating such proteins [20].

### Gel Filtration

The component of the active fraction (F-8), obtained after DEAE cellulose chromatography was separated by gel filtration that gave only three peaks of enzymes alone and the fraction also found to be contained cellulase activity.

A brief summary of the purification procedure was presented in Table (4) As given in the table, the specific activity of cellulase were ( 15.67 , 7.94 , 1972.16 ) U/mg for FPase ,CMCase and  $\beta$ -glucosidase respectably, Although the yield were only about ( 20.7 , 10.5 , 73.5 ) % of the extracted protein was removed during the purification steps and the enzyme was purified with an increase in purification fold more than ( 1.11 , 0.567 , 140.86 ). That might be due to denaturation of enzyme during the purification steps or it may be other reasons. (Figure 2).

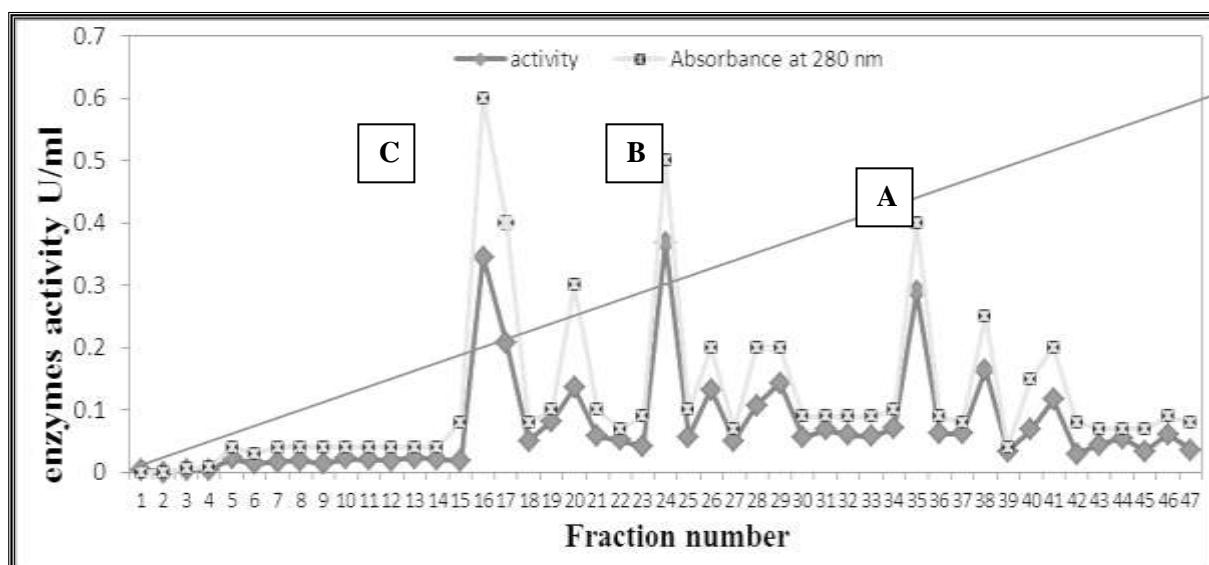


Figure (2 ) Gel filtration A- FPase B-β-glucosidase C- CMCCase

[18] Was using gel filtration to purification CMCCase enzyme from *A. niger* the maximum purification was observed after gel filtration (3.05 fold). Concomitantly, there was a decrease in the yield of enzyme after every step carried out during the process. The enzyme yield reduced to 55.67% after ammonium sulfate precipitation that reached to its minimum level i.e. 21.03%, after gel chromatography through Sephadex G-75, and total activity 25000 IU with Specific activity 28.46 IU/mg protein and 3.05 fold. The results explicated that every step in the enzyme purification resulted in the removal of undesirable fractions of protein therefore, decline in enzyme yield was found after the process of purification.

### Characterization

After purification, the enzyme was characterized to find the conditions at which showed best performance. The produced FPase ,CMCase and β-glucosidase were characterized for pH, temperature, heat stability and molecular mass. The detail of parameters is given below:

### pH

The effect of various pH values on the FPase , CMCase and β-glucosidase activity is depicted in (Figures 3,4 and 5) .

The graphic illustration elaborates that the enzyme activity was calculated at different pH values, the highest enzyme activity was found at 5.5 pH. When the pH of the enzyme medium was increased up to 6.0 , FPase enzyme that giving activity ( 0.91 ) U , but CMCase and β-glucosidase giving highest activityat pH 6 were ( 0.34 , 65.12 ) U and declining at pH 5 to ( 0.25 , 59.95 ) U . Likewise, when the activity of all enzymes was assayed at pH values higher the pH 4.5 - 6.



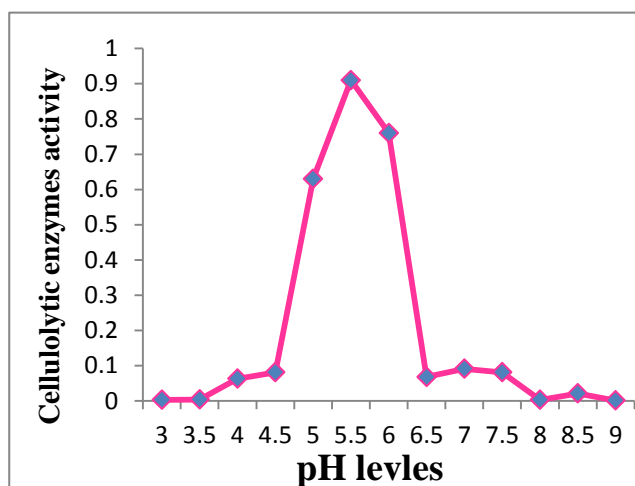
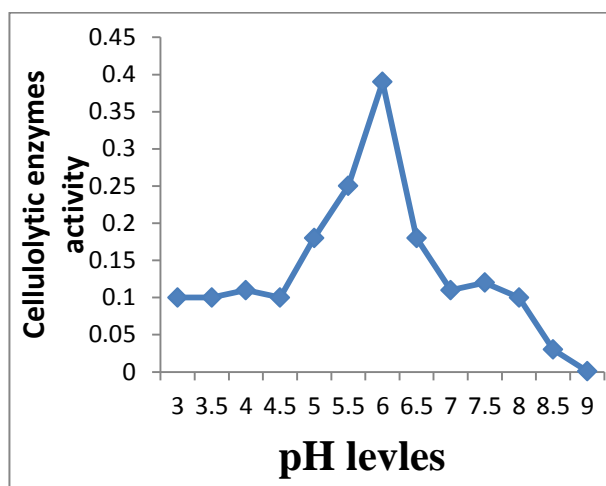
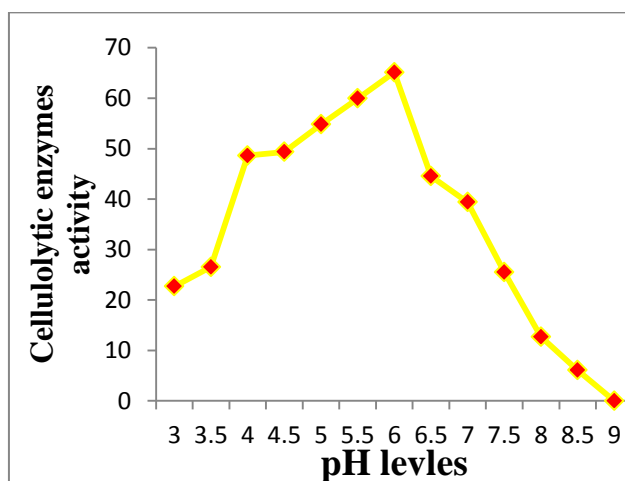


Figure ( 3) Effect of pH on the FPase activity



Figurer (4) Effect of pH on the CMCCase Activity



Figurer ( 5) Effect of pH on the  $\beta$ -glucosidase activity

Limited to the effect of pH on the case ion of the enzyme , and the extent of ionization of chains of amino acids side which are necessary to maintain the tertiary structure enzyme , and could lead to the changed situation ion which is reflected on the effectiveness of the enzyme , as the increased pH or decreased for certain limits can lead to deform the enzyme and loss of effectiveness , on the other side results, were noted that the enzymes are more active in moderate acetic reign than alkaline region. The decrease in activities at the extreme acidic pH or at alkaline pH values might be due to destruction of active site as well as changes in secondary or tertiary structure of cellulase . the decreased when hydrogen pH to severe effect of pH in the synthesis of the enzyme protein molecule may happen to irreversible and denaturated of an enzyme in solution highly acidic or basic change which leads to change active site and the effective loss of the effectiveness of the enzyme [21] .Fungal cellulase with pH values of (4.5 - 6.0) are common and have been obtained from *Trichodrma viridie* ,*Aspergillus niger* . *A. terreus* , *Neurospora crassa* , *Aspergillus aureolus* , *Aspergillus clavatus* , *Rhizopus oryzae* ,*Volvariella diplasia* and *Trichoderma reesei* QM 9414 [22] .

### Temperature

The effect of various temperatures on the relative activity of purified FPase , CMCCase and  $\beta$ -glucosidase were presented in (Figures 6 , 7 and 8 ) . It found that the enzyme exhibited

maximum activity at 25 C° in CMCase was ( 0.33 )U but 30 C° for  $\beta$ -glucosidase was ( 38.22 )U . While, FPase was having activity at 25 and 30 C°, activity were (0.14, 0.15)U.

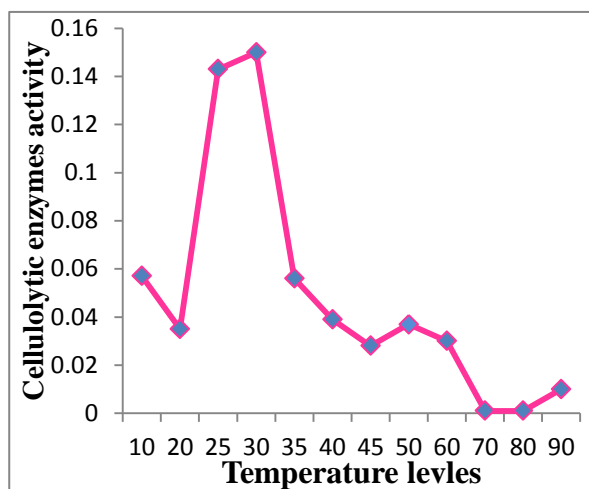


Figure (6)Effect temperature on the FPase activity

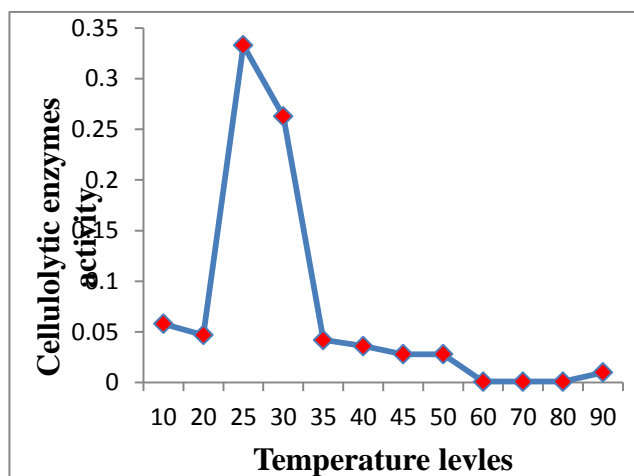


Figure (7) CMCase activity

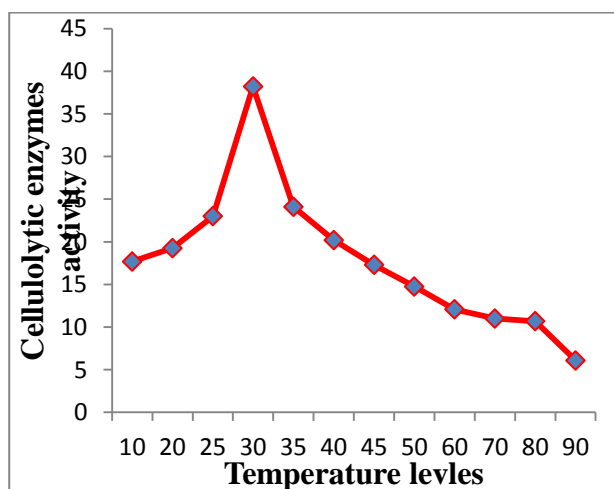


Figure ( 8) Effect temperature on the  $\beta$ -glucosidase activity

Enzyme was affected negatively and gradually reduced. In the same way, when the enzyme activity was determined below 40°C, a gradual decline in the three activity enzymes was observed. The the enzymic protein occurs at elevated temperatures therefore, after certain level of temperature increase (above 50C°), the enzyme activity decreased rapidly. In nutshell, the enzyme was most efficient at 50C° as compared with other tested temperatures. The speed of the enzymatic reaction increases with increasing temperature within a certain range because to the increased kinetic energy of the molecules to be up to the degree to which an then the speed of interaction maximum value increased temperatures from the border optimal leads to deform secondary and tertiary and quaternary structure enzyme and then reduced its effectiveness [21] The present results are in harmony with the recent findings of [17],[15] which were reported the same temperature (30C°) as optimum for cellulase activity. The results are in close agreement with the findings of [23] who reported that(25 - 30) C° as an optimum temperate during the characterization of Fpase and B-glucosidase produced from *Cryptococcus*. The results are also supported by [24]was found 30C° as a best temperature at which the enzyme was most active and stable. [25] Also reported the same temperature i.e. 30°C as optimum for cellulase activity.

### **Molecular weight**

The crude enzyme preparation was subjected to SDS-PAGE to determine the molecular weight of the enzyme. While conducting the electrophoresis, one major band showing cellulolytic activity was detected. The molecular weight of the protein was found to be about (38 ) kD, (52) kD and (49) kD for (FPase , -CMCase ,  $\beta$ -glucosidase ) respecyily (Figure 9) . The results regarding the molecular weight of the enzyme are close to the findings of [26] when purificatied of cellulolytic enzymes from *Aspergillus niger* appear three protein bands with the molecular weight of 33 and 24 , 34 kDa respectively .

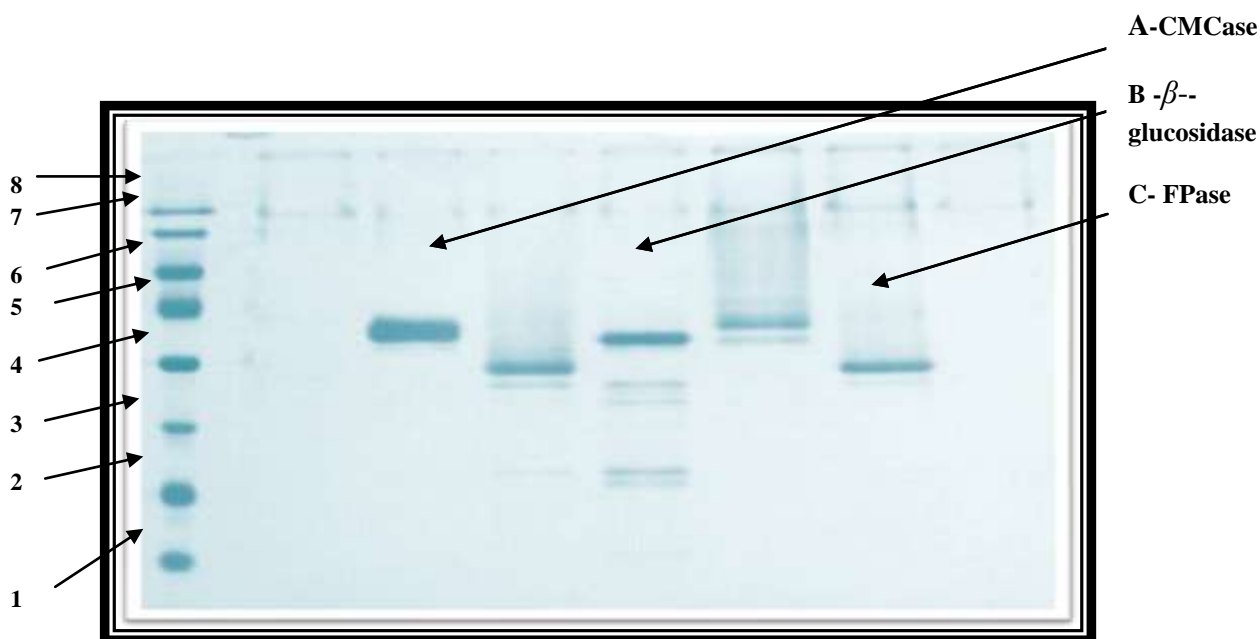


Figure (9) SDS-PAGE of purified cellulase from *A. oryzae* . Line 1, protein standard marker; line 2, purified enzymes

**1-RNAase 13.5KD 2- Lysozyme14 KD 3- Trypsin 23 KD 4- Egg albumin45 KD 5- Bovine serum albumin 67KD 6- Phosphorylase 97 KD 7-  $\beta$ -glactosidase116 KD 8- Alkaline phosphatase 140 KD**

[27]Show in their study the protein fractions have been assigned numbers in order of the increasing mobility towards the anode. *A. fumigatus* grown on coir waste, two protein bands were obtained each containing molecular weight of 32 and 21 kDa respectively. While, *A. niger* grown on the same substrate also showed two bands having molecular weight of 36 and 23 kDa respectively. In case of saw dust used as substrate, *A. niger* showed two bands with molecular weight of 22 kDa and 20 kDa respectively, while *A. fumigatus* showed two bands with molecular weight of 20 kDa and 17 kDa respectively. [28] Refer molecular weight of the partial purified enzyme was determined by SDS- PAGE (12%) as described previously in the materials and methods. Analysis of the enzyme by SDS- PAGE revealed the appearance of one band with molecular weight of 45.000 Daltons (calculated from the relation between the maker and the relative mobility of the calculated molecular weight) Similar results were mentioned, hence, a cellulolytic enzyme from *A. niger* was separated as a homogeneous entity in SDS- PAGE, but showed one major and two minor bands in disc, gel electrophoresis, these bands (proteins) may be isoenzymes or the different subunits of the same enzyme protein on electrophoresis gel [1] .According to research, two endoglucanase containing fractions were separated from *A. niger*. In comparison to the low-molecular-weight standards of Bio-Rad electrophoresis within SDS revealed a single band at a molecular weight of 90 KDa of thermophilic Actinomycetes [29].

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