

INHIBITION ACTIVITY OF SOME PLANT SEEDS EXTRACTS AGAINST THE PROTEASE PRODUCED FROM LOCAL ISOLATE OF *CANDIDA ALBICANS* ISOLATE USING SOLID STATE FERMENTATION

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ABSTRACT

This study was aimed at inhibition of purified protease produced by *C. albicans* using plant seeds extract. One hundred two local *C. albicans* isolates that were isolated and identified by microscopic and biochemical tests were submitted to primary and secondary screening techniques in order to select the qualified *C. albicans* isolate for protease synthesis. Among these isolates, nineteen isolates with the highest hydrolysis zone on skim milk media (primary screening) were chosen for secondary screening. It has been found that *C. albicans*V56 had the highest productivity of the enzyme (41.8U/mg protein). The optimum conditions of protease production by selected isolate using solid state fermentation by using wheat bran as best substrate , temperature 28 °C and pH 7, after 5 days of incubation . The enzyme was purified by concentration with sucrose, then used gel filtration chromatography using Sephadex G-75. The results show two peaks. The first peak has a purification folds 6.5 time with an enzyme yield of 13.08 % , while peak 2 has a purification folds 3.3 time with an enzyme yield of 20%. The purified enzyme exhibited maximal activity at pH 6.0 for peak 1 and 3.0 for peak 2, whereas the maximum stability was 7.0 for peak 1 and 8 for peak 2. The optimal temperature for purified enzyme activity was 40 for peak 1 and 30 for peak 2, and it was stable until 45°C. Protease activity was inhibited with local plant seed extracts. *Lathyrus sativus* extract inhibits approximately 50 % of protease activity.

Keywords: *C. albicans* ,protease ,inhibitors, plant extract, seeds

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الفعالية التثبيطية لبعض المستخلصات النباتية ضد البروتياز المنتج من العزلة المحلية لخميرة *C. albicans* بواسطة تخمرات الحالة الصلبة

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باحث

المستخلص

هدفت الدراسة الحالية الى تثبيط انزيم البروتياز المنقى من العزلة المحلية لخميرة *C. albicans* بواسطة مستخلصات بعض بذور النباتات المحلية، حيث تم عزل مئة واثنى عشر عزلة من خميره *C. albicans*، شخضت هذه العزلات بواسطة الاختبارات المجهرية والكيموحيوية وخضعت لعملية الغرلة الاولى باستخدام الوسط الصلب لتحديد العزلة الاكفأ في انتاج الانزيم، اختيرت 19 عزلة كقوة اعتماداً على نسبة (قطر النمو/ قطر الهالة) على الوسط الصلب. ادخلت هذا العزلات الى الغرلة الثانوية باستخدام تخمرات الحالة الصلبة، وانتخبت العزلة *C. albicans* V56 كونها الاكفأ في انتاج الانزيم اذ بلغت الفعالية النوعية 41.8 وحدة/ملغم بروتين. حددت الظروف المثلى لإنتاج الانزيم حيث تم اختيار نخاله الطحين كوسط انتاج امثل بدرجة حراره 28م وبرقم هيدروجيني 7 بعد 5 ايام من الحضانه. نقي انزيم البروتياز باستخدام التركيز بواسطه السكروز ثم كروماتوغرافيا الترشيح الهلامي بواسطة هلام السيفادكس ج-75، حيث بينت نتائج التنقية ظهور قمتين متتاليتين امتلكت القمه الاولى عدد مرات تنقيه مقدارها 6.5 بحصيلة انزيم 13.08% بينما كانت عدد مرات تنقيه القمه الثانية 3.3 بحصيلة انزيمه 20%، اظهر الانزيم المنقى اعلى فعالية عند رقم هيدروجيني 6 للقمه الاولى و3 للقمه الثانية، كما امتلكت قمتي الانزيم ثبات عند الاس الهيدروجيني 7 و8 للقمه الاولى والثانية على التوالي. كما اظهر الانزيم المنقى اعلى فعالية عند درجه 40 للقمه الاولى و30 للقمه الثانية وكانت درجه الثبات الحراري للأنزيم عند 45 لكلا القمتين. ثبت الانزيم المنقى باستخدام مستخلصات بعض النباتات المحلية اذ بلغت اقصى نسبة تثبيط للأنزيم المنقى عند معاملته مع مستخلص نبات الهرطمان اذا بلغت ما يقارب 50 %

كلمات مفتاحية: *C. albicans*، protease، مشطبات، مستخلصات نباتية، بذور

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INTRODUCTION

Candida species are opportunistic eukaryotic pathogens. They're unicellular and dimorphic. Meaning they can grow as both yeast and mycelium (26, 32), *Candida* has a variety of virulence factors, involving the ability of adherence to epithelial and endothelial cells, the ability of exoenzymes synthesis (phospholipase and proteinase), and the ability to switch phenotypic, all of which aid its penetration of host cells (15,23). Proteases, also known as proteolytic enzymes, are enzymes that break down proteins by catalyze the hydrolysis of peptide bonds (31,34). *C. albicans* is the main reason for the infection of candidates, the infection occurs at the weakness of the immunity or disorder in the host environment (14). The protease enzymes are important virulence factors that contribute to pathogenicity. They make it easier to enter the host organism and attack its defense system. Mammalian, plant, bacterial, and fungal have all been identified and described as good sources of extracellular enzymes. Microorganisms are a suitable source for production of protease enzyme because of they grow quickly and take up a little space (17,31). Purification microbial protease is difficult because there are various forms and other proteases with similar physical and chemical characteristics. The purified form of protease is essential to research its biochemical properties, enzyme structure, and catalytic mechanism, Microbial protease was purified using a variety of methods (ammonium sulfate precipitation, ultra-filtration, immobilized metal affinity chromatography, gel filtration chromatography, ion exchange chromatography, etc.). Many chemical compounds, such as HgCl_2 , CaCl_2 and EDTA, modify the activity of enzymes since some of these substances work as activators of enzymes and others operate as inhibitors of enzymes (31). Protease inhibitors are proteins or peptides that reduce the catalytic activity of proteolytic enzymes. They are found in many plants, particularly grains, potatoes, and legumes (2, 24), Plants have a specific defense mechanism for attacking several organisms, as they begin to produce many secondary metabolic compounds that accumulate in many tissues of the plant during its stages of growth.

Among these compounds are enzyme inhibitors (22).

MATERIALS AND METHODS

Sample collection, isolation and identification of protease-producing isolates: One hundred thirty-three samples which were previously collected from Baghdad medical city and 20 ready isolates from biotechnology department in Baghdad University, during periods (September to December 2020). The provenience of these samples from skin, vaginal, mouth, urine, ear. These samples were cultivated on Sabroid dextrose broth then Sabroid dextrose agar for identification. Some biochemical tests, microscopic examination and API test were done. The identified *C. albicans* isolates were prepared for screening experiment.

Primary (qualitative screening) of *C. albicans* isolates for protease production

One hundred two *C. albicans* isolates were screened using skim milk medium to find the best ones that produced protease, A single colony of yeast isolate that had previously been activated in sabroid dextrose broth was spotted to the middle of skim milk agar plate and then incubated at 37°C for 48 hrs. Clear zone hydrolysis around the colony was an indication of protease secretion.

Secondary screening (Quantitative screening) of *C. albicans* for protease production :

Nineteen isolates with maximum productivity based on primary screening were selected and cultivated on skim milk media. A 250 ml flask containing 10g of wheat bran moisted with 10 ml sodium acetate 0.05M with pH 5.0 and inoculated with 1.0 ml of yeast inoculum with concentration of 1×10^6 cell/ml. The flasks were incubated at 37°C in the incubator for 5 days.

Enzyme extraction: After the desired incubation, a known quantity (10 g) of the fermented material was mixed with 40 ml of distilled water and homogenized by shaking for 30 min and filtered through cheese cloth. Cell free supernatant was obtained by centrifuging the extract at 10,000 x g for 30 min. The supernatant which contained the crude enzyme was used for the protease assay.

Protease activity: Protease activity determination method described by Supuran *et al.* (28), using bovine serum albumin as a

substrate, the hydrolysis of BSA was detected by measuring the absorbance at 280 nm using spectrophotometer. Protein concentration measured according to the method described by Bradford *et al.* (3).

Optimum conditions for protease production: Effect of fermentation media:

Three different types of media were used to determine the optimal medium for protease production. Including: wheat bran, rice bran and millet bran. Erlenmeyer flasks (250) ml containing 10 g of each tested medium moisted with 10 ml of 0.05M, PH 5.0 sodium acetate buffer in duplicate were autoclaved, then inoculated with 1.0 ml of yeast inoculum consisting of 1×10^6 cell/ml of selected isolate and incubated at 37°C for 5 days. After incubation, the enzyme activity, protein concentration, and specific activity were determined in the supernatant from each flask.

Effect of pH value

To determine the effect of initial pH value of the culture medium on enzyme production, after selected medium type that gives the maximum productivity of protease A 250 ml Erlenmeyer flasks containing 10 ml of the selected medium were adjusted to different pH values (3, 4, 5, 6, 7, 8, and 9), then the culture medium was inoculated with yeast inoculum at a concentration of 1×10^6 cell/ml and incubated at 37°C for 5 days. After incubation, the enzyme activity, protein concentration, and specific activity were determined in the supernatant from each flask.

Optimum incubation period

Different incubation times were examined to determine the best incubation period for protease production. The selected medium at optimum pH, inoculated with yeast inoculum (1×10^6 cell/ml) and incubated at 37°C for different incubation time; 2, 3, 4, 5, 6, 7 and 8 days. Enzyme activity, protein concentration, specific activity were measured for each time.

Optimum temperature

Optimum temperature was determined for best protease production. The selected medium with optimum pH and incubation period, inoculated with 1ml of yeast inoculum 1×10^6 cell/ml and incubated at different temperature included 28, 35, 37, 40, 45, and 50°C for 5 days. After enzyme extraction with centrifugation, the enzyme activity, protein

concentration and specific activity were measured for protease productivity.

Optimum moisture content

The best moisture content for protease production was determine by experimenting with different moisture content. Selected medium in different moisture content include: 1:0.5, 1:0.75, 1:1, 1:1.5, 1:1.75, 1:2 (w\v) for production was inoculated with an inoculum of 1×10^6 cell/ml and incubated at 37 C. After incubation, the enzyme activity, protein concentration, and specific activity were determined in the superannuate from each flask.

Purification of protease

The protease was purified from *C. albicans* isolate (V56) by concentrated with sucrose followed by gel filtration chromatography using sephadex G-75

Enzyme concentration by sucrose

Dialysis tube with Mw CO 10000 KD used for concentration of the protease and the enzyme activity, protein concentration, and specific activity were all measured.

Separation of enzyme through sephadex G-75 column:

The column was attended and packed according to the manufacturing company's directions (Pharmacia-Sweden). The crude enzyme was passed over a sephadex G-75 column (25×2.5), and the elution step was performed with sodium phosphate buffer solution 0.2 M, pH 7.0, flow rate 20 ml/h, 3 ml for each fraction. The enzyme activity of these fractions was determined after the protein fractions were assessed at 280 nm in each fraction. The volume of effective fractions was measured, and subsequently activity and protein concentration were estimated.

Characterization of partial purified protease: Effect of pH on protease activity:

The effect of pH on activity of the partial purified protease was determined by preparing BSA as a substrate in different buffer solutions with ionic power 0.2M include citric acid buffer (pH 3 and 3.5), acetate buffer (pH 4, 5 and 6), phosphate buffer (pH 7 and 8) and Tris-HCL buffer (pH 9). The enzyme activity was estimated, and the relationship between enzyme activity and pH values was plotted to find the optimal pH for protease activity.

Effect of different pH on stability of partial purified protease: Equal volume from

partially purified enzyme was mixed with the buffers at different range of pH (3.5-9) at ratio of (1:1) the mixture was incubated at 37°C for 15 min., The enzyme activity was assayed and estimate the remaining enzymatic activity (%), then the relation between remaining activity % toward pH values was plotted to determine the optimum pH of protease stability.

Effect of temperature on protease activity

Partial purified protease activity was estimated at different range of temperature (25-70) °C, and the relation between enzyme activity and temperature was evaluated to determine the optimal temperature of enzyme activity.

Effect of temperature on protease stability

Partially purified protease was incubated at different temperature (25-70) °C for 15 min then transferred to ice bath, the remaining activity (%) was estimated then the relation between remaining activity % toward temperature was plotted to determine the optimum temperature of protease stability.

Effect of some chemical compounds on protease activity:

The effect of chemical compounds include (CaCl₂, HgCl₂, CuCl₂, AgCl and EDTA) on purified protease activity was studied. Solution for each one was prepared at concentration 0.1mM and 0.05mM by dissolving in distilled water. Enzyme solution was incubated with metal ions solution at a ratio of 1:1 (v/v) for 15 min at 37°C, then the enzyme activity was assayed and compared with control that represents the untreated enzyme, thereafter remaining activity % was calculated.

Effect some plants sources on protease activity:

Plant seeds: Plant seeds, which are locally available in market, include (*Lathyrus sativus*, *vicia faba* and *Phoenix dactylifera*) were used as the source of material to screen for protease inhibitor activity. The seeds dried at room temperature, then crushed using a clean mortar and pestle, and the powdered plant was sieved to obtain the fine powder

Preparation of plant extract

1. aqueous extract: The plant extracts were prepared by mixing 3 g of plant seed powder separately with 30 ml of sterile distilled water in a 250 Erlenmeyer flask and left the suspension with stirring in a shaking incubator over night at 37 C. Then the extracts were filtered using several layers of gauze, then

centrifugation at 4000 rpm for 30 min obtain crude extract.

2. alcohol extract: Prepare the alcoholic extract in the same way as the aqueous extract, replacing the sterile distilled water with ethyl alcohol 80 %, then put the filtrate in clean petri dishes to dry and the powder obtained from drying the alcoholic extract was dissolved in distilled water for enzyme inhibition experiments Partial purified enzyme was incubated with plant extract in ratio 1:1(v/v) at 37C for 15 min; the residual activity and inhibitory activity % were calculated after enzyme activity determination.

Inhibitory effect of plant extracts against *C. albicans* growth:

Five holes of equal diameters were made in the middle of the PDA media with diameter 6 mm by means of a cork borer, 0.1 ml of the extract was added in a hole preceded by spreading 0.1 ml of inoculum containing 10^5 cell/ml on the medium, then the plates were left for a period of time for the diffusion of the plant solutions into the culture medium, then incubated at 37C for 24 hours, after which the diameter of the inhibition zone was measured using a ruler (23). the drug sensitivity of candida also tested, 0.1 ml from yeast suspension spread on SDA medium by (L-spreader), the dishes were left after inoculation for 30 minutes, after which 0.1 ml of antifungal (nystatin) was added . The samples were incubated for 24 hours, and then the diameter of the inhibition zone was measured using a numbered ruler.

RESULTS AND DISCUSSION

Isolation and identification of *Candida albicans*

Eighty two local isolates of *Candida albicans* diagnosed from 133 Specimens in addition to 20 ready isolate which were collected from various sources (vaginal, urine, mouth, skin), all of these sample subject to microscopic and biochemical examination as following, when cultured the specimen on SDA noted that the colonies of Candida Circular or convex oval, creamy white in color also The colonies were examined microscopically after staining with crystal violet ,the cells appeared spherical in shape to oval or elongated, single and budding. it is appeared in a light green color on chrome agar. *Candida* was distinguished from other

species by the ability to formation of germ tubes and growth in temperature 45 °C.

Primary screening (qualitative screening) of *C. albicans* isolate for protease production

One hundred and two isolates were subjected for qualitative screening method using skim milk agar medium and clear hydrolysis zone assay was done. By calculating the ratio between the colorless zone diameter around the yeast culture (Z) and the colony growing diameter (G), the efficiency of yeast isolates was compared. Among all *C. albicans*.

isolates, nineteen isolates were protease producer whenever a clear zone of hydrolysis was screened around colony of isolate in the skim milk agar media (Fig. 1). These isolates were chosen for further secondary screening (quantitative screening) using solid state fermentation. Mohanasrinivasan *et al.* (18) Used skim milk media for screening the production of protease from four different fungus species and protease production was confirmed by the formation of a proteolysis zone surrounding colonies (Table 1).

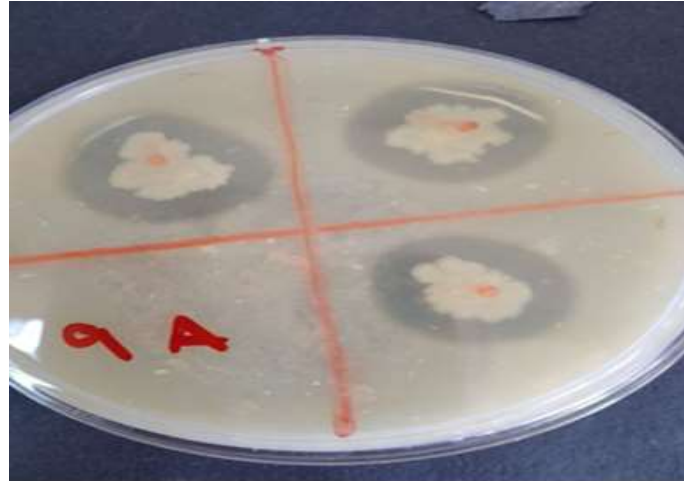


Figure1. The growth of *C. albicans* on skim milk media

Table 1. The clear zone ratios of selected *C. albicans* isolates on the skim milk plate medium

Isolate No.	Ratio (Z/G)	Isolate No.	Ratio (Z/G)	Isolate No.	Ratio (Z/g)
V3	3.1	V18	1.9	V34	2
V4	1.6	V20	1.4	V45	0.8
V9	1.3	V24	1.3	V49	1
V10	2.2	V34	2.2	V51	1.1
V11	2.3	V37	2.6	V53	1.3
V12	1.2	V41	1.1	V56	2.5

Secondary screening (quantitative screening) of *C. albicans* isolate for protease production: The eighteen isolates of *C. albicans* with the highest zone of hydrolysis in primary screening were screened again for enzymatic activity using the solid-state fermentation method for better detection and selection. Among the nineteen isolates six isolates has highest specific activity and *C. albicans* V56 had the highest specific activity,

with 41.8 U/mg protein protease specific activity in crude supernatant,(Table.2). For further research, the *C. albicans* V56 isolate with the highest specific activity was chosen. Castro *et al.* (6) found that the maximal lipase and protease production from *C. utilis* in SSF utilizing supplemented olive cake was 14.31 U/mg, the variability between isolate in production due to the characteristics of the isolate and its genetic origin.

Table 2. The specific activity of protease produced from local isolate of *C. albicans* by solid state fermentation

Isolate Number	Specific activity (U/mg protein)
V3	8.5
V10	4.3
M11	9.3
M34	8.16
Sk37	3.08
V56	41.8

Optimum conditions for protease production: Optimum production medium

Three different types of solid substrate were examined for their efficiency in protease production, these medium included: Wheat bran, rice bran and millet bran, among the three media tested, wheat bran proved to be the most effective for protease production with specific enzyme activity 43.3 U/mg, while rice bran and hulk bran had 4.42 and 16.6 U/mg, respectively (Fig.2), as a result, this medium was selected to enhance the activity of the

others fermentation parameters. The coarser variant of bran is thought to be a better substrate since it does not accumulate and allows for better air circulation, heat dissipation, and mycelia penetration, It's also a better economically option because it's less expensive than the finer form of bran. This coarse wheat bran can be employed in a form that makes the nutrients in it freely available to the organism because of the extended sterilizing (25).

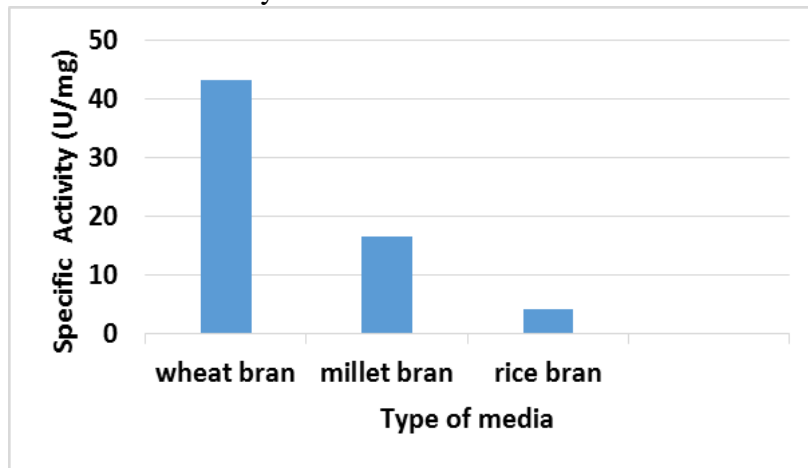


Figure 2. Effect of different types of the medium on protease production from *C. albicans*V56 isolate after incubation at 37°C for 5 days

Optimum PH

To evaluate the effect of the initial pH on protease production, *C. albicans* V56 was cultivated in the production medium with various pH values (3, 4, 5, 6, 7, 8 and 9). As indicated in Fig. (3), the maximal protease specific activity (40.7) U/mg was obtained at

pH 7.0; however, raising or lowering the pH value above or below 7.0 resulted in a decrease in enzyme activity. The influence of pH on the solubility of medium ingredients, substrate ionization and availability for the microbe, and enzyme stability are all important factors in enzyme synthesis (5).

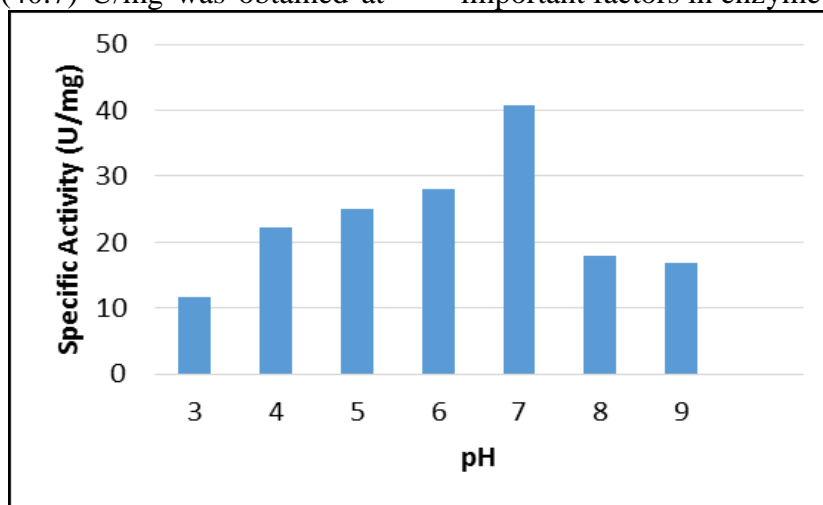


Figure 3.Effect of pH on protease production from *C. albicans*V56 isolate using Solid state fermentation after incubation at 37°C for 5 days

Optimum moisture content

Candida albicans V56 was grown on a production medium with varying moisture

content to investigate the effect of moisture content on protease production (1:0.5, 1:0.75, 1:1,1:1.25, 1:1.5, 1:1.75 and 1:2) (w:v),The

Maximum Protease specific activity was 35.56 U/ml protein, achieved at 1:1 moisture content, while the lowest specific activity was at moisture content 1:1.5 as shown in (Fig.4), It's thought that increased SSF moisture content

reduces solid particle porosity, limiting oxygen transport. While a decrease in SSF moisture content causes decreased substrate solubility and minimal swelling (9).

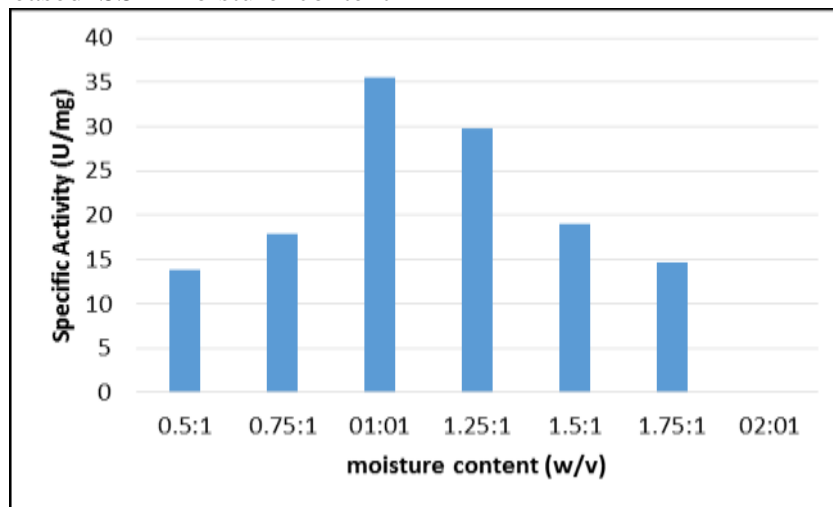


Figure 4. Effect of different moisture content on the production of Protease from *C. albicans* V56 isolate

Optimum temperature

To study the influence of temperature on protease production *C. albicans* V56 was grown on production medium with temperature ranging from 28 to 50 C as shown in (Fig.5), The highest Protease specific activity (63.6 U/mg protein) was obtained at 28°C, which is the optimum temperature,

while lower yields (31.6 U/mg protein) were obtained at higher temperatures (50°C). Temperature plays a vital role in the growth and metabolism of any microbe, (21) reported optimum temperature for protease production by *C. coronatus* to be 28°C while growth and enzyme production were adversely affected at 30°C and above.

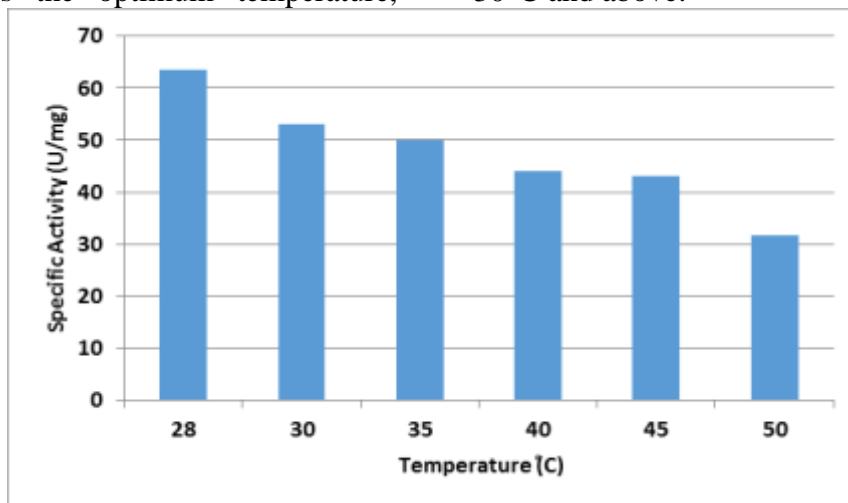


Figure 5. Effect of temperature on protease production from *C. albicans* V56 isolate for 5 days at pH 7

Optimum incubation period

To investigate the effect of incubation time on protease production. *C. albicans* V56 was grown on production medium for different incubation periods (2, 3, 4, 5, 6, 7 and 8) days, (Fig.6) shown the maximum protease activity was on the fifth day, with specific activity of 61.90 U/mg protein, when the incubation

period was lengthened, the specific activity decreased. This is due to changes in culture conditions over time, such as a reduction in oxygen, nutrients, and toxic compounds that inhibited yeast growth. (19) grown *C. guilliermondii* on the medium containing 1 % dry okra and achieved highest enzyme activity after 3 days (155 U/mg).

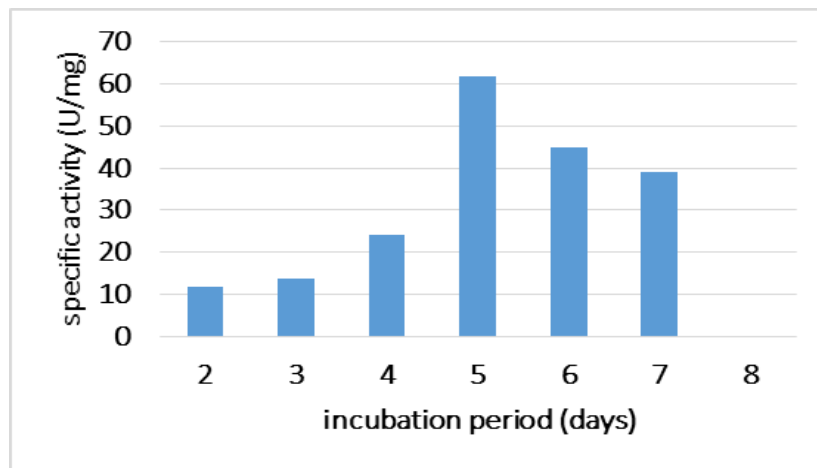


Figure 6. Effect of incubation periods from on protease production from a local isolate of *C. albicans*

Purification of protease

C. albicans V56 was grown under optimum conditions for the production of Protease. The crude protease was purified from unwanted proteins and other components by sucrose concentration followed by (Sephadex – G75) gel filtration as follows:

Concentration by sucrose

The crude enzyme was concentrated with sucrose, and the results showed that 64.51 % of protease enzyme was obtained with a purification fold of 1.93, as shown in (Table 1). Sucrose was once widely used for protein concentration, almost as an inexpensive way of precipitating and concentrating a protein extract.

Gel filtration chromatography

A Sephadex – G75 gel filtration column with diameters of (25×2.5) cm was used to purify the concentrated enzyme solution. The results showed the presence of two protein peaks and two peaks for enzyme activity. the first peak of protease activity in fractions 6-12 and the second peak in fractions 12-20, as shown in. (Fig.7) and (Table 2), in this step the specific activity reached to 298 U/mg protein with a purification fold 6.5 and yield reached to 13.08% in peak 1 while 153.3U/mg specific activity, and 3.3 purification fold and 20 % yield in peak 2 as shown in Table 3. Sutar *et al.* (29) achieved 12 fold purification of *C. coronatus* protease with 3.8% yield in 6 steps for enzyme purification.

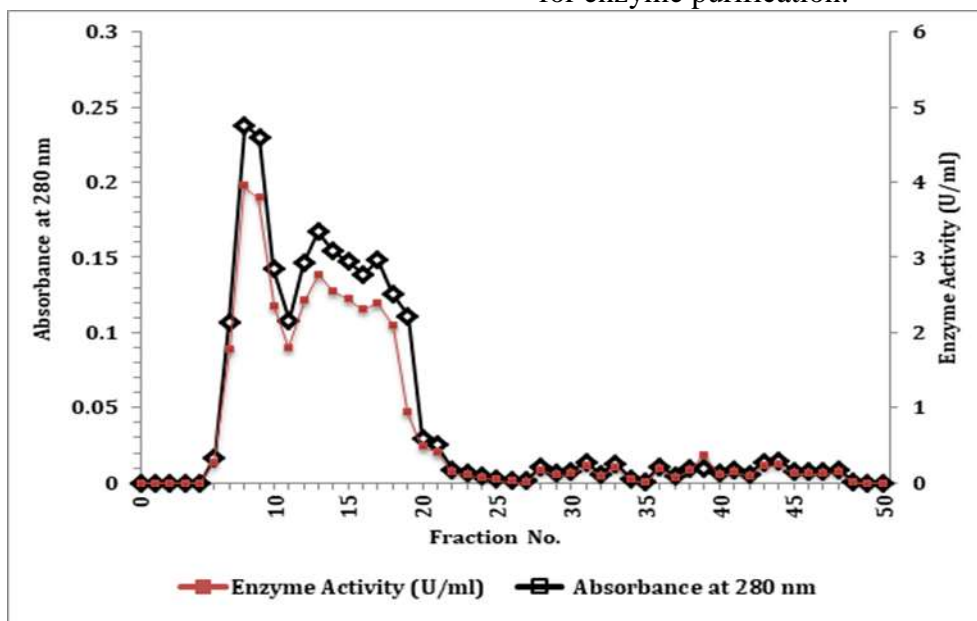


Figure 7. Gel filtration chromatography for protease purification from *C. albicans* V56 by using Sephadex G75 column (2.5 x 25) cm equilibrated and eluted with phosphate buffer (0.2 M, pH 7), in flow rate 20ml/hr. 3ml for each Fraction

Table 3. The purification steps of protease from *C. albicans* V56

Purification Steps	Volume (ml)	Enzyme Activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/mg)	Total Activity (U)	Purification Fold %	Yield
Crude enzyme	50	8.2	0.18	45.5	410	1	100
Concentration by sucrose using dialysis	10	26.45	0.3	88.1	264.5	1.93	64.5
Gel Filtration by sephadex G-75 (Peak 1)	18	2.98	0.010	298	53.64	6.5	13.0
Gel Filtration by sephadex G-75 (Peak 2)	27	3.051	0.019	153.3	82.3	3.3	20

Characterization of partial purified protease: Effect of pH on protease activity

The effect of pH on partial purified protease from *C. albicans* V56 was evaluated in a pH range of 3.0-9.0. (Fig. 8) indicate that protease in Peak 1 has the maximum activity in pH 6 with enzyme activity 1.066 U/ml, while the maximum activity in Peak 2 was in PH 3 with enzyme activity 1.23 U/ml, These findings led

to the conclusion that protease activity was higher at acidic or neutral pH values than at nearly alkaline pH values, and that this difference was due to pH's influence on enzyme activity in a variety of ways, including affecting on the ionization of groups in the enzyme active site, influencing the ionization of groups in the substrate (10).

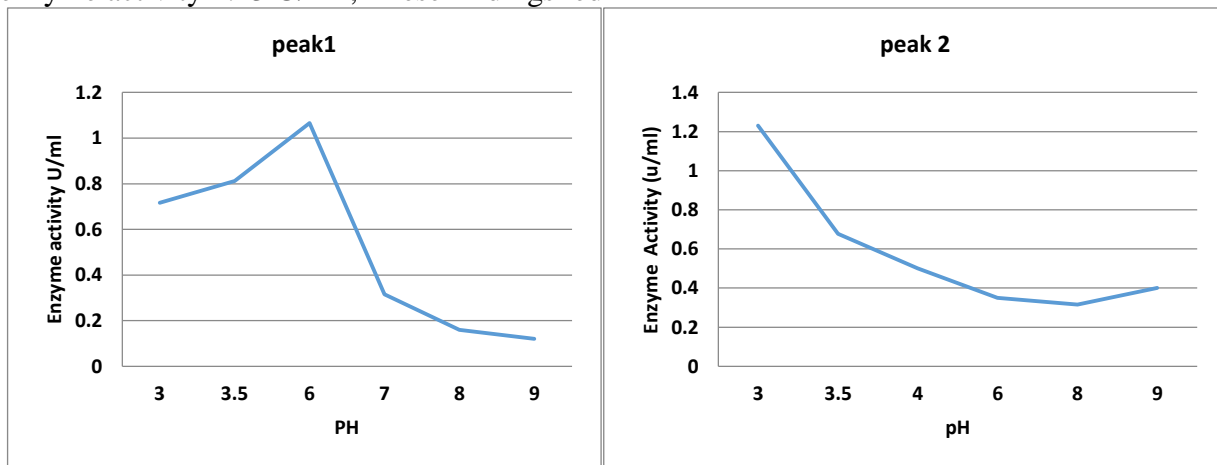


Figure 8. Effect of different pH values (3.0-9.0) on partially purified Protease activity from local isolate *C. albicans* V56

Effect of pH values on protease stability

The results in Fig. (9) Show that pH ranged between 5.0-7.0 were the optimum pH for protease stability, the enzyme kept about 100% of its activity in pH 7.0 in peak 1 while kept about 100 % in pH 8.0 for peak 2. The enzyme activity was decreased away either side of the optimum pH values. The results

lead to conclusion that the Protease of *C. albicans* V56 is more stable in alkaline pH. This decrease in enzyme activity occurs when pH values are outside of the optimal range, because of the effect of pH on enzyme structure, which causes denaturation or a change in the ionic state of the enzyme active site (27).

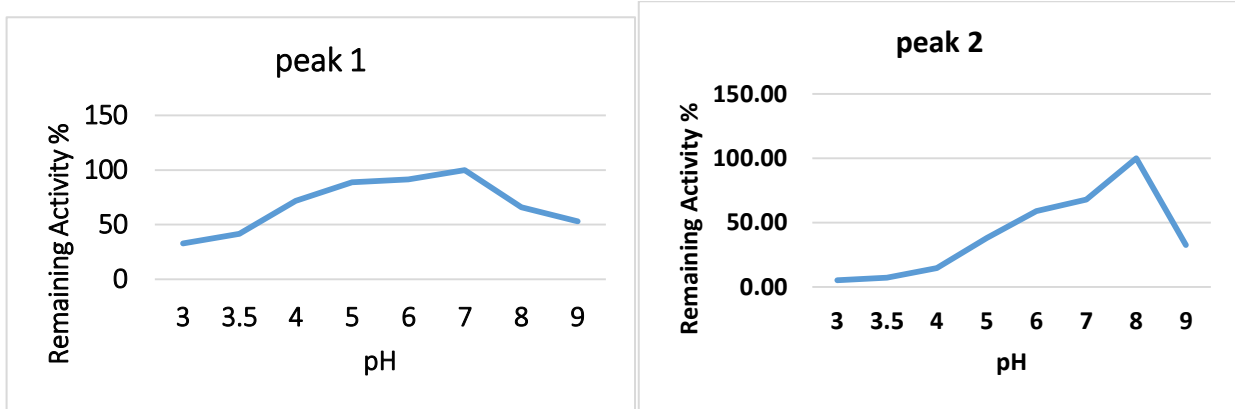


Figure 9. Effect of different pH values (3.0-9.0) on the stability of partially purified protease from a local isolate of *C. albicans* V56

Effect of temperature on protease activity

To study the effect of temperature on protease activity Incubation of the partially purified enzyme and substrate mixture at various temperatures ranging from 25°C to 70°C. Fig.10 The protease activity was increased when the temperature and it reached its

highest value at 40°C for peak 1 with maximum activity was 1.49U/ml, while peak 2 the maximum activity was 1.271 U/ml at 30°C; however, as the temperature increased, the activity fall until reached 70°C for both peaks

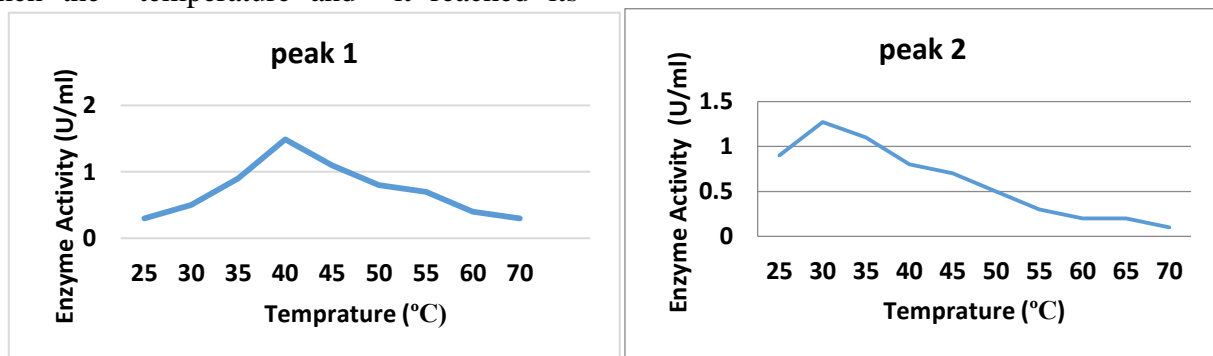


Figure 10. Effect of different range of temperatures (25-70) °C on partial purified protease activity from *C. albicans* V56

Effect of temperature on protease stability

The stability of protease was tested by incubating the purified enzyme for 15 minutes at temperatures ranging from 25°C to 70°C and then calculating the remaining activity percent. Protease activity was maintained in the current investigation at temperatures ranging from 25 to 45°C for both peak, then the activity began to decrease with increasing

temperature and although at 70°C more than 80% of the activity was lost. (Fig.11), the sensitivity of protease to high temperatures indicates the effect of high temperatures on the 3D structure of the protein by damaging R-groups of amino acids, resulting in denaturation and loss of action at temperatures above 55°C (8).

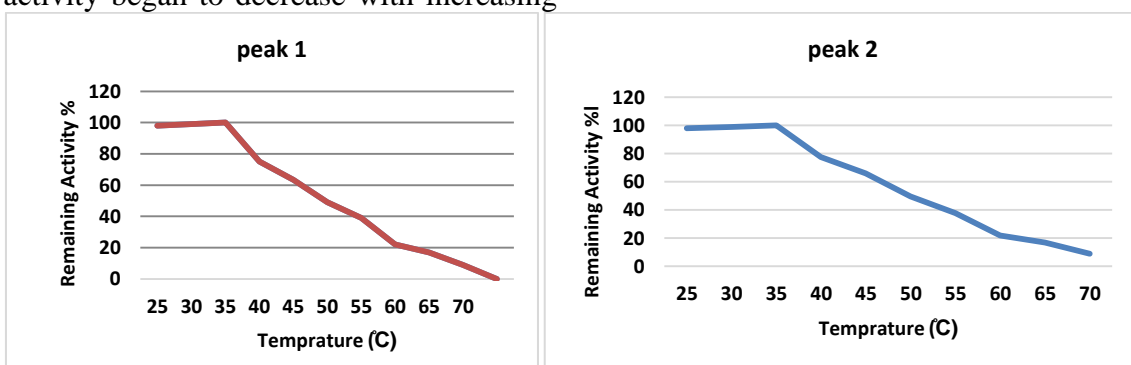


Figure 11. Effect of different ranges of temperature (25-70) °C in *C. albicans* V56 Protease stability

Effect some chemical compounds on protease activity: Protease purified from *C. albicans*V56 was treated with some of the chemical compound (Table 11) The Metal ions' influence on protease activity varies depending on the enzyme's origin, the presence of CaCl₂ were found to enhance enzyme activity to levels that were higher than the original activity (control value) at 0.05 and 0.1mM for both peak, Also the enzyme activity was decreased in the presence of CuCl₂, HgCl₂ and AgCl in the reaction media on which the remaining activity of CuCl₂ in peak 1 31.74 % and 22.35% while 33.4% and 29.9% for peak 2 in a concentration 0.05 and 0.1mM respectively while for HgCl₂ the remaining activity in peak 1 was 46.07% and 43.35% while peak 2 44.4% and 41.05%, while the remaining activity of AgCl for peak 1 37.03%, 32.9 % and 41.15 %, 35.6 % for peak 2 by using the aforementioned concentration. This may mean that protease of *C. albicans*V56 needs ions as cofactor certain cations, such as Ca⁺², improve the activity of

particular enzymes because of their involvement in protecting enzyme structure and thus providing a favorable reaction state (11), while CuCl₂, HgCl₂ and AgCl show marked inhibition in the enzyme activity, the inhibition of enzyme by HgCl₂ revealed the presence of SH groups in the enzyme active site, which oxidized by HgCl₂. Furthermore, HgCl₂ in the substrate processing solution may form a complex with the enzyme, preventing it from binding to the substrate and forming the product (17). The effect of inhibitors on protease activity was also examined by using EDTA. The remaining activity at concentration 0.05 and 0.1mM 62.20 % and 59.12% for peak 1 and 70% and 67% for peak 2. These results indicate that this enzyme was from metalloprotease on which the activities of enzyme was dependent on the ions such as Ca⁺² in the active site, so when it was added a chelating agent in a reaction media it form complexes with the ions in the active site which cause inhibition of enzyme activity (12).

Table 4. Effect of some chemical compounds on protease activity purified from *C. albicans* V56

Agent	Concentration (mM)	Remaining activity % (Peak 1)	Remaining activity % (Peak 1)
Control	–	100	100
CaCl ₂	0.1	107.7	105.4
	0.05	105.4	104.9
HgCl ₂	0.1	43.4	41.1
	0.05	46.1	44.4
AgCl	0.1	32.9	35.6
	0.05	37	41.2
CuCl	0.1	22.3	29.9
	0.05	31.7	33.4
EDTA	0.1	59.1	67.1
	0.05	62.2	70.9

Effect some plants sources on protease activity: This study was used some local plants seeds as a source of inhibition material for purified protease from *C. albicans*V56, the result in Table 5 show that the *Lathyrus sativus* has maximum protease inhibitor for both aqueous and alcohol extract in both peaks, the inhibitory activity in peak 1 of aqueous and alcohol extract 46.75% and 47.1% respectively, while peak 2, 47.50% and 48.41%, the result show that the *Lathyrus*

sativus have maximum percent of inhibition followed by *Phoenix dactylifera* L seed then *Vicia faba* seed. Plant is one of the most important groups for protease inhibition, plant considered as an important for healthy human (7). Plants contain a wide variety of compounds including polyphenols such as flavonoids, tocopherols, phenolic acids and tannins which have been found to provide inhibitory compounds or a platform on which to synthesize active molecules (1).

Table 5. The inhibitory ratio of some plant seeds on protease activity

Plant extract	Inhibitory ratio % of peak 1		Inhibitory ratio of peak 2	
	Aqueous extract	Alcohol extract	Aqueous extract	Alcohol Extract
<i>Lathyrus sativus</i>	46.75	47.1	47.50	48.41
<i>Phoenix dactylifera</i>	42.05	44.35	44.19	45.92
<i>Vicia faba</i>	39.35	36.18	37.89	42.31

Effect of the alcoholic extracts of the plant *Lathyrus sativus* on the growth of *Candida albicans*: The effect of alcoholic extract of *Lathyrus sativus* plant on candida isolated was studied using four extract dilutions (1:5, 1:10, 1:15, and 1:20). The results in (fig.12) showed that the concentration 1:20 had the largest inhibition diameter. In comparison to the other concentrations, the average inhibition diameter was 12.3mm, whereas the other concentration had no inhibitory action against yeast. The results show that the inhibition occurs when the extract concentration is high. Protease inhibitor plant possess antimicrobial action

against bacteria, fungi, and even viruses (27). Plant inhibitors affect fungi in different ways due to the natural of fungi itself include the differences of the structure in cellular membranes and its thickness, the size of fungal cells and the duration of the growth (1). The drug sensitivity of candida was also tested using anti-fungal Nystatin, as the result showed a region of inhibition with diameter 16.5 mm, this is due to the nystatin inhibition the manufacture of Ergosterol which is important in the manufacturing the cellular membrane of *Candida*.



Figure. 12. Antimicrobial activity of *Lathyrus sativus* seeds against *C. albicans* (A: compared with antifungal (nystatin), B: compared with other concentration).

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