Production and Partial Purification Of Protease By *Fusarium spp.* By Solid State Fermentation.

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Abstract

In this paper, A strain of *Fusarium spp.*, is grown on potato dextrose agar and on wheat bran Czapek Dox medium. It was found to produce neutral protease. Fructose, when added to the culture medium was found to give rise to the neutral protease produced in the presence of fructose and was also found to enhance the production of neutral protease. production was carried out by solid state fermentation The fungi were grown on czepek- Dox with wheat bran culture medium. The optimum conditions were determined as follows, dilution ratio 1:30, temperature 25° C, pH 7.0, incubation time 9 days, inoculums size 1×10^6 (spore /ml), carbon source and nitrogen source were fructose ,ammonium nitrate respectively. The protease was isolated from the culture filtrates and was DEAE-(diethylaminoethyl) DEAE-cellose. The specific activity of pure enzyme was 30.211 u/mg, with No. of fold 8.945 and yield 0.188%, gradient buffer treatment,and characterization of pure enzyme optimum pH ,pH stability 6- 11 and 11 respectivly. Optimum temperature and stability temperature 40° C and 20° C and had a Vmax of 2 of protein and Km value of 1.5mg/ml towards casein and 3.9 km ,3 vmax of BSA.

إنتاج إنزيم البروتييز المنقى جزئيا من عفن *Fusarium spp.* بتخمرات المزارع الصلبة.

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الخلاصة

استخدمت في هذا البحث عزلة *Fusarium spp.* المعزولة من التربة والمنماة على وسط potato dextrose agar ومن ثم نميت على وسط نخالة الحنطة– جابك دوكس لغرض انتاج انزيم البروتييز المتعادل، وجد أن اضافة الفركتوز الى وسط النمو رفع من انتاجية انزيم البروتييز ووجد كمدعم لانتاج الانزيم ايضا، وحصلت انتاجية الانزيم بتخمرات المزارع شبه الصلبة بعد تنمية العفن على وسط النخالة المرطب بوسط جابك– دوكس، قدرت الظروف المثلى لانتاج الانزيم كالاتي: كانت نسبة الترطيب 1:30، درجة الحرارة المثلى 25 م، الرقم الهيدروجيني الامثل 7.0، مدة الحضن 9 أيام بحجم اللقاح 10^6 1× خليه\مل، والمصدر الكاربوني والنيتروجيني الامثل الفركتوز والامونيوم نايتريت على التوالي، أستخلص البروتييز من راشح المزرعة الفطرية وأجريت خطوات التنقية بالترسيب باملاح الامونيوم وكانت نسبة التشبع المثلى 60% ومن ثم تمت ديلزة الانزيم ضد دارئا، وأخيرا التنقية بعمود المبادل الايوني السالب (DEAE-cellose) وكانت الفعالية النوعية للانزيم المنقى(u/mg) 30.211 وعدد مرات تنقية 8.945 وحصيلة 0.188%، اتصف الانزيم البروتييز المنقى بالفعالية المثلى والثبات على رقم هيدروجيني 11 –6·0 و11.0 على التوالي، درجة الحرارة المثلى للفعالية والثبات 40م 20م لمدة 20 دقيقة وقيم السرع القصوى 2 و 3.9 تجاه الكازئيين والبومين المصل البقري على التوالي وقيم الثوابت الحركية 1.5 mg/ml و 3 على التوالي تجاه الكازئيين والبومين المصل البقري.

Introduction

A strain of *Fusarium spp.* was isolated from soil. Proteolytic enzymes are by far the most important group of enzymes produced commercially and are used in many areas of application, such as in detergents, brewing, photographic, leather and dairy industries (27). For these reasons also proteolytic enzymes are the object of this study. The price of commercially available enzymes which are produced mostly by submerged fermentation is usually too high for agro-biotechnological applications (22; 24). An alternative technique for enzyme production is solid state fermentation (6; 10) .Solid state fermentation has some advantages compared to liquid state cultures LSC: higher product yield; better product quality; cheaper product recovery and cheaper technology (5).

In addition, the hydrolytic enzymes which are excreted in this case are found more concentrated in the culture filtrate than in the case of LSC (13). It was also reported by the authors (20) that supplementation of normal wheat bran Czapek Dox medium with fructose led to a greater production of proteases in the culture medium. It was also seen that fructose does not affect the secretary properties of the membrane (21).

This indicated that fructose enhances the production of these enzymes. The effect of fructose could be either due to an increase in the production of one or more of these enzymes and/or due to the production of a new protease. Another possibility was that fructose might alter some of the properties of proteases which are reflected in greater than normal activities of these enzymes. To elucidate these points, proteases produced in normal medium and in the presence of fructose were purified and compared. The present paper describes apartial purification of enzyme from *Fusarium spp.* by solid state fermentation.

Materials and Methods

Microorganisms and culture medium:

the microorganism used throughout this work was isolated from soil of university. The culture was grown on potato dextrose agar medium at 25◦C for 5 days before being used for enzyme production. In order to study enzyme production, supplementation of normal wheat bran Czapek Dox medium with fructose led to a greater production of protease in the culture medium.

Preparation of culture filtrate:

Wheat bran under the conditions of solid state fermentation, Erlenmeyer conical flasks of 250 ml capacity were used containing 20 ml of liquid media without organic sources and 10g of dry wheat bran medium and sterilized at 121°C / 15 min/ 15 pis . Fructose solution was adjusted to pH 7.0 and mixed with normal medium at 3% level after sterilization. Inoculation of normal and fructose supplemented medium was carried out at 5% level with spore suspension of organism prepared in sterile distilled water and adjusted to (1×10^6) spores/ml. Incubation was carried out at 26 ± 2°C under static conditions. After 7 days the contents of the flasks were filtered through Whatman No. 3 filter paper discs. Culture filtrates were centrifuged at 5000 x g for 10 min /4°C and the supernatant was used as the crude enzyme.

The contents of each flask were gathered thoroughly mixed with cooled distilled water 65 mland rapidly filtered through a Buchners funnel. The filtrate was then subjected to enzyme activity assay for the determination of protease production .

Assay of protease activity:

Protease activities of crude and purified enzymes were measured as described by (19), using casein as substrate and measuring the release of peptide fragments. Two ml reaction mixture consisting of 0·5 ml of 1% casein, 0·5 ml of 0.2 M sodium phosphate buffer of respective optimum pH for neutral protease activity and 1 ml of suitably diluted enzyme were incubated for 20 min at 37°C. Reaction was stopped by the addition of 3 ml of 5 % TCA. The contents of the tubes were centrifuged at 5000 x g for 10 min / 4°C and then the tubes were assayed for TCA soluble peptide fragmentsat 280 nm. protein determination was calculated by the method of (11).

Appropriate zero time controls were also run simultaneously. One unit of protease activity was defined as that amount of enzyme which liberates 1 mg equivalent of peptide fragments under the assay conditions using bovine serum albumin as Standard. Specific activity is expressed as units per mg protein.

Optimization of the enzymes:

Moisturization:

The influence of moisturizing ratio for the production of neutral protease was studied by incubating media at 1: 20, 1: 30, 1: 40, 1: 50, 1: 60 g/ml.

pH:

The pH optimum of the neutral protease enzyme production was determined by using different pH 5.0, 6.0, 7.0, 8.0, and 9.0.

Incubation period:

The incubation period of the neutral protease enzyme production was determined by using different 7, 9, 11, 13, 15 days.

Temperature:

The influence of temperature of the production of neutral protease was studied by incubating media at different temperatures 25, 30, 35, 40, and 45°C.

Carbon source:

The optimum carbon source of the neutral protease enzyme production was determined by using different carbon source (maltose, glucose, fructose, sucrose, lactose).

Nitrogen source:

The optimum nitrogen source of the neutral protease enzyme production was determined by using different nitrogen source (peptone ,casein ,ammonium nitrate, yeast extract ,ammonium sulphate).

Inoculum size:

The influence of inoculation represented (1×10^6) of the production of neutral protease was studied by inoculating media at different volumes 1×10^6, 2×10^6, 3×10^6, 4×10^6, 5×10^6 spore/ml.

Purification of proteases produced in the presence of fructose:

Purification steps included ammonium sulphate precipitate obtained at 60–90 % saturation was dissolved in a small amount of 0·02 M sodium phosphate buffer, pH 6.8 and was dialyzed three times overnight against the same buffer. Any sediment formed was removed by centrifugation and the supernatant was loaded on DEAE-cellulose column 2× 30 cm previously equilibrated with 0·02M sodium

phosphate buffer, pH 7 Column elution was performed by the same buffer with an increase in molarity from 0·02–0·5 M by inclusion of NaCl. Protease activity was assayed at pH 6.8 and the peaks obtained were in turn tested for optimum pH. Fractions showing maximum activity in the respective peak areas were pooled.

Characterization of the enzyme:

Optimum and stability temperature:

The influence of optimum temperature on the activity of protease was studied by incubating the reaction mixture at different temperatures (20 ,30, 40, 50, or 60◦C). The enzyme was incubated at its optimum stability temperature for different time intervals 20, 30, 40, 50 or 60°C at 10 and 60 min then coold in ice bath and the reaction mixture was incubated at the same temperatures to study the stability of the enzyme.

Optimum and stability pH:

The optimum pH and stability pH of the protease enzyme was determined by using buffer solutions of different pH (citrate buffer, phosphate buffer, glycin buffer 4.0, 5.0, 6.0, 7.0 , 8.0, 9.0, 10.0 and 11.0 at 10 and 30 min respectivily).

Determination of kinetic parameters:

Enzyme was incubated with various concentrations of casein and bovine serum albumin (0.1- 1%) in Na-phosphate buffer pH 7.0 at 37°C. Kinetic parameters Km and Vmax were calculated by linear regression from (9) .

Resultes and discussion

Influence of mouisturized ratio

That mouisturized ratio 1 : 30 was found the most effective ratio for protease production 4.02 U /ml (Fig. 1) Initial moisture content usually varies depending on the type of substrate, and here, any increase in decrease of the moisture was not favourable for enzyme production.

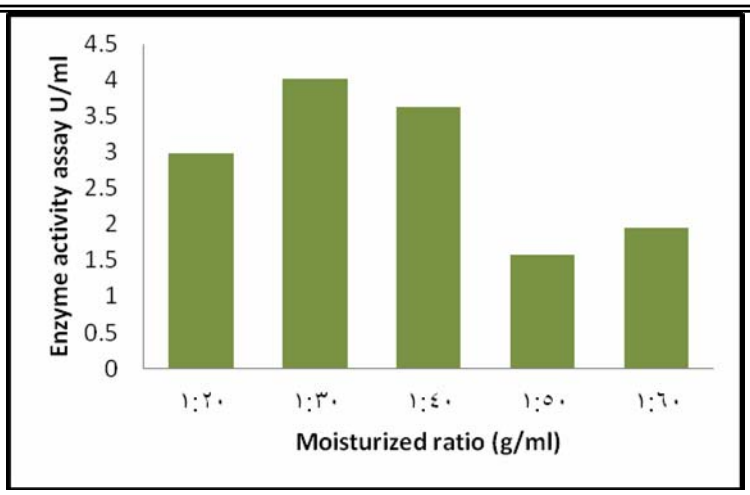


Fig.(1): Effect of moisturized ratio on the production of protease by *Fusarium spp.*

Influence of pH:

Maximum protease production 4.74 U/ml was found at pH 7.0 (Fig.2). The results clearly indicated neutrophilic nature of the fungus. The medium Protease production by *Aspergillus niger* was observed in the range pH 7 - 9. Growth and protease production ceased at pH 10. Optimum pH 7.0 has been reported for neutral protease of Conidiobolus coranalis. Likewise pH 7.0 has been reported to be optimum for *Aspergillus flavus* (25).

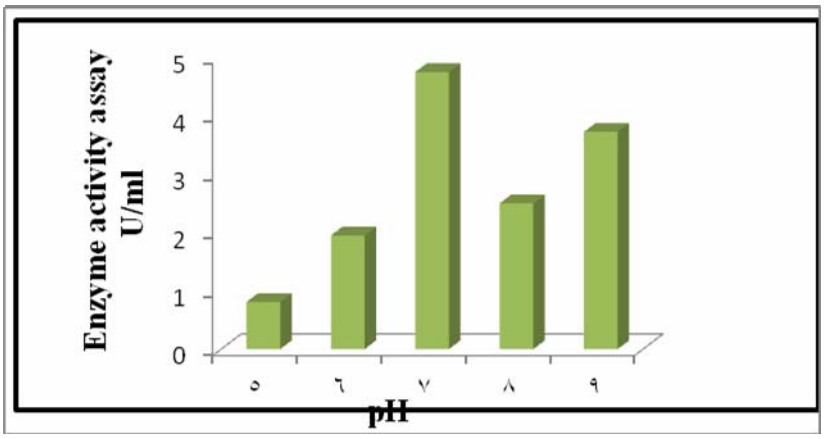


Fig. (2): Effect of pH on the production of protease by *Fusarium spp*

Influence of incubation period

The mximum activitiy of enzyme was observed after 9 days of fermentation process (Fig.3) when the fungi growth passed the lag phase and the enzyme production started . (16) stated that *A. flavus* optimally produces protease after 10 days of incubation.

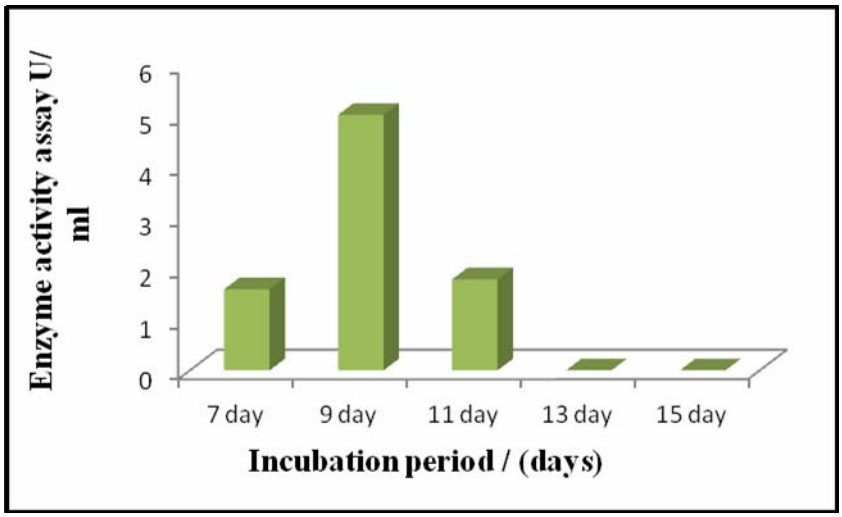
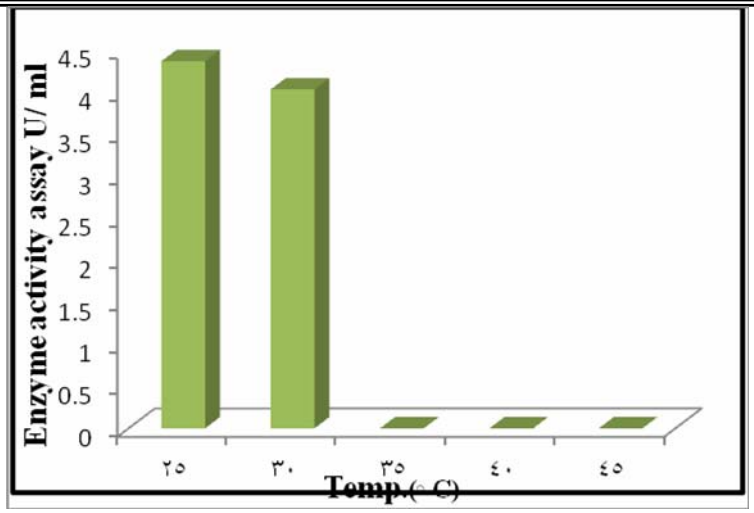


Fig.(3): Effect of incubation period on the production of protease by *Fusarium spp.*

Influnce of temperature

The protease production at different temperture reginswas examined after 9 days keeping the other fermentation conditions constant. Protease production increased at temperature of 25 °C. Maximum production of protease 4.41 U/ml was obtained at 25°C (Fig.4) , also (2 ; 3) found that *Phanerochaete chrysosporium* optimally produce protease at 25 ˚C when grown on a medium containing cotton linters and filter paper. Growth rates and protease production ceased at higher temperature 45°C .Similar observation were shown by (14) for *Aspergillus usami*. It was revealed that temperature does not only affects growth rates of organism but also exhibit marked influence on the levels of protease production.



Fig(4): Effect of temperture regines on the production of protease by *Fusarium spp.*

Influnce of carbon and nitrogen source

There are general reports showing that different carbon sources have different influences on extracellular enzyme production by different strains (26; 17) . Fructose and glucose showed the most effective carbon sources for protease production 6.35 , 5.61 U /ml respectivily and seemed to have a noticeable impact on enzyme production (Fig. 5).

Among the various nitrogen sources tested peptone and ammonium nitrate were found to be the most effective for protease Production 13.43 , 13.91 U /ml respectivily (Fig. 6). The mechanism that shows the formation of extracellular enzymes is influenced by the availability of precursors for protein synthesis. The effect of different nitrogen sources like peptone ,casein ,ammonium nitrate, yeast extract and ammonium sulphate has reported that nitrogen sources stimulate equal accumulation of protease in the culture medium of *Aspergillus terreus* (1) .

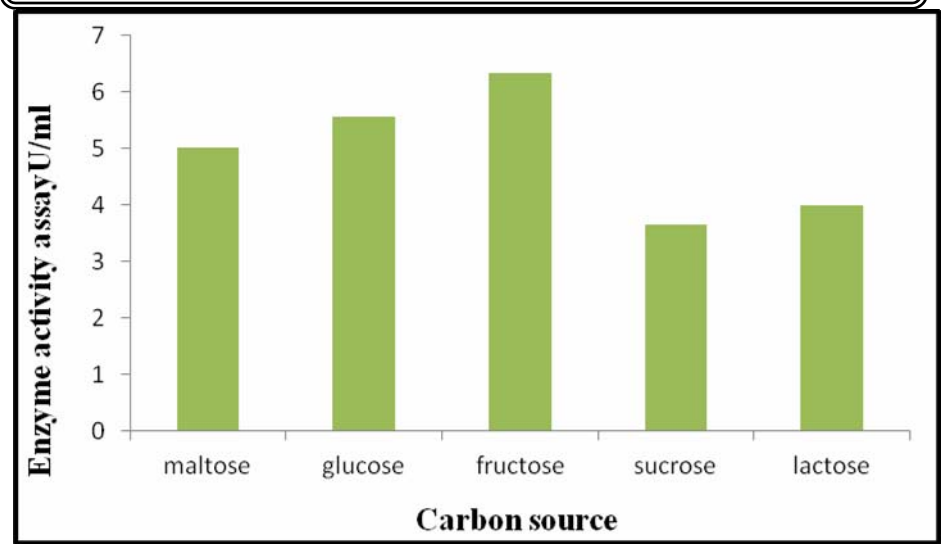


Fig. (5): Effect of carbon source on the production of protease by *Fusarium spp*

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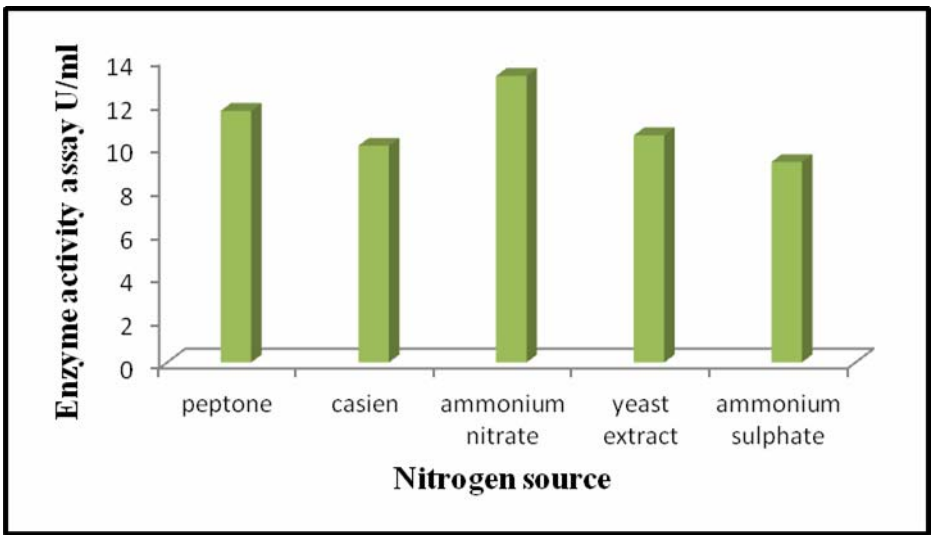

Fig. (6): Effect of nitrogen source on the production of protease by *Fusarium spp*

Influnce of Inoculoum size

To study the effect of inoculums size, fungus was grown at various inculoum size ($1x10^6$, $2x10^6$, $3x10^6$, $4x10^6$ and $5x10^6$) using production medium containing 1% casein as the main substrate. The highest activity was observed for protease Production (3.26 U /ml) in 1 x 10^6 sopre/ml inculoume size of spore suspension (Fig. 7). In order to verify the enzyme activity, the spore concentration must be high enough to colonize the substrate particles (23), many studies, however, have indicated that there can be a decline in this activity over a determined spore concentration.

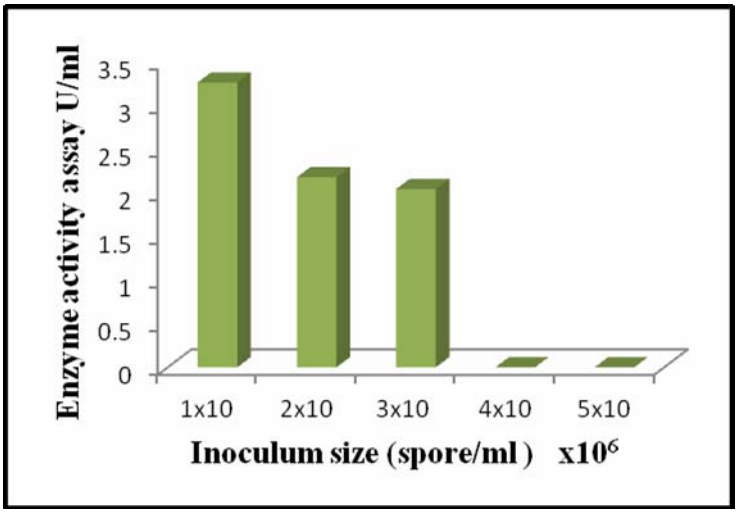

Fig.(7): Effect of inoculum size on the production of protease by *Fusarium spp*.

Purification steps of protease produced by *F. spp.* was recorded in (Table. 1). The first step was carried out by precipitation of protein from the cellfree dialysate with ammonium sulfate at the saturation level of 60%. This resulted in 2.337 fold of purification of protease with yield of 9.787% of the original activity .

The precipitated enzyme was then purified by anion exchange chromatography on DEAE-Cellulose using linear sodium chloride gradient the wash step end until fraction no. 41 and then started elution step . The results showed that protease was purified 8.945 fold to a specific activity of 30.211 U mg-1 protein (Table. 1), (Fig. 8). This purification procedure was also used by (4) for *Phanerochaete chrysosporium* protease and resulted in 37-fold purifty.

Table (1): Purification of protease from *Fusarium spp.* by solid state fermentation.

sample	Volume (ml)	Activity (u/ml)	Protein (mg/m)	Specific activity (u/mg)	Total activity (u/ml)	Total protein (mg)	Yield %	fold
Crude extract	400	3.95	1.170	3.376	1580	468	100	1
Precipetate 60%	25	6.185	0.784	7.890	154.63	19.60	9.787	2.337
Dialysis	28	1.101	0.313	3.517	30.82	8.76	1.950	1.042
DEAE-cellulose	90	1.577	0.0522	30.211	141.93	4.68	0.188	8.945

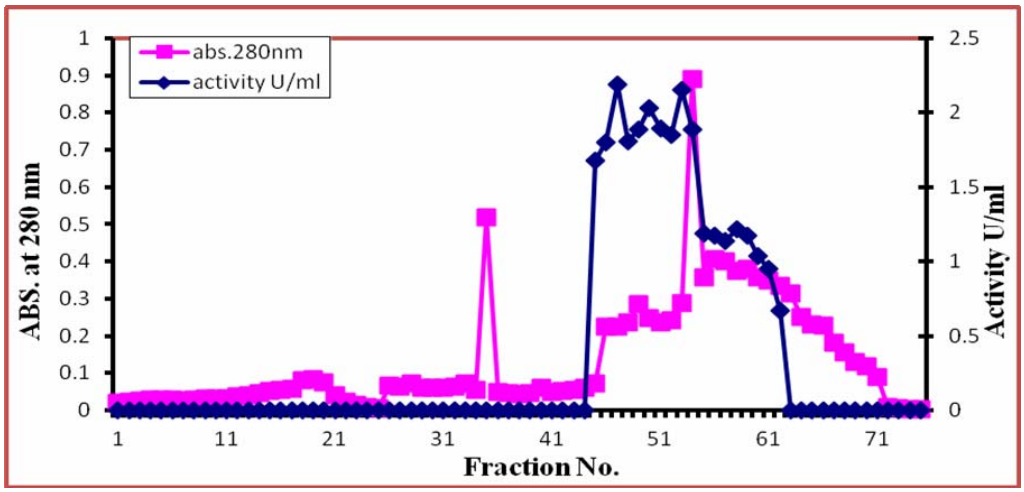

Fig.(8): Ion exchange chromotography of protease on DEAE-cellulose column (2x30 cm) elution was carried out with alinear grediant from (0.02 - 0.5M) NaCL in 2Mm Na- phosphate buffer pH(7.0). fraction 5 ml were collected and the flow rate was 40 ml/ 1h.

Optimum pH and stability:

Neutral protease was stable at pH 6 to 11.0 and 7·0– 11·0 when the enzymes were incubated for 1 h at 37°C. incubation in a pH range 4- 11 for either 20 or 60 min (Fig. 9). These results were in accordance with the results of (7), while protease was almost unaffected by incubation in the pH range 6.0- 7.0 for 20 min, and completely stable after 60 min of incubation at pH 6.5 (Fig. 10). These

results were in accordance with that of (23; 4). The enzyme exhibited maximum activity in the pH range of 7.0– 7.5 (Fig. 9). The activity of neutral proteases generally decreases towards the alkaline side. But in this case, a second increase in activity with increase in pH was observed, which was probably due to the presence of co-production of alkaline protease.

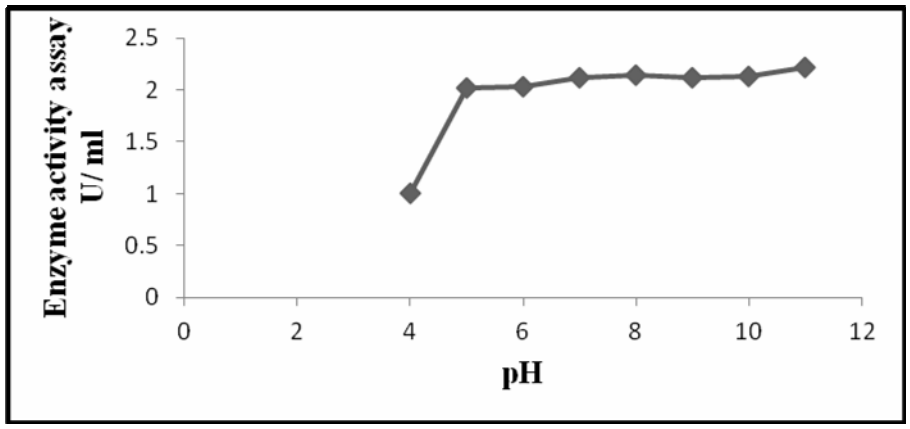

Fig.(9): The effect of optimum pH values on the activity of purified protease from *Fusarium spp.*

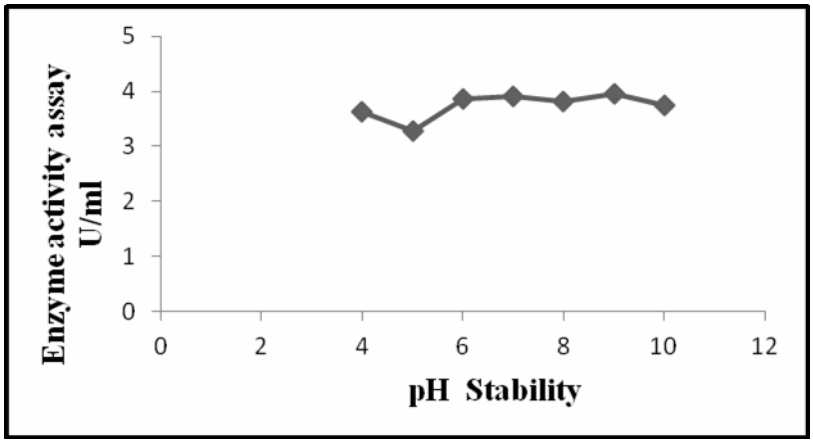

Fig.(10): The effect pH stability on the activity of purified protease from *Fusarium spp.*

Effect of temperature on activity and stability:

Maximum activity at was recorded 40°C which was stable at 20°C for 20 min.Concerning the effect of temperature on the enzyme activity, the results showed that protease achieved a high hydrolyzing effect at 55˚C (Fig. 11) .The optimal value of 65˚C reported for protease obtained from *Pleurotus ostreatus* (15). and that of 65˚C reported for protease obtained from *Pleurotus ostreatus* (18). Results of the experiments on the thermal stability of the enzymes shown in (Fig. 12) indicate that resdial enzyme activity 98.68% incubation for one hour at their optimal temperature was unable to completely retain itsr activity.

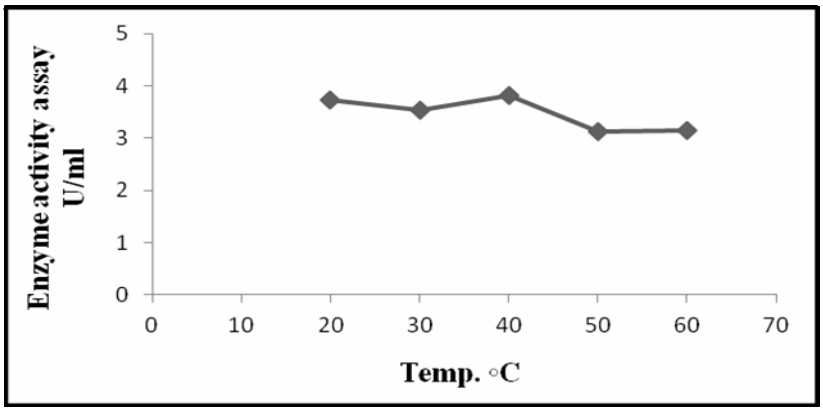

Fig. (11): The effect of Temp. on the activity of purified protease from *Fusarium spp*

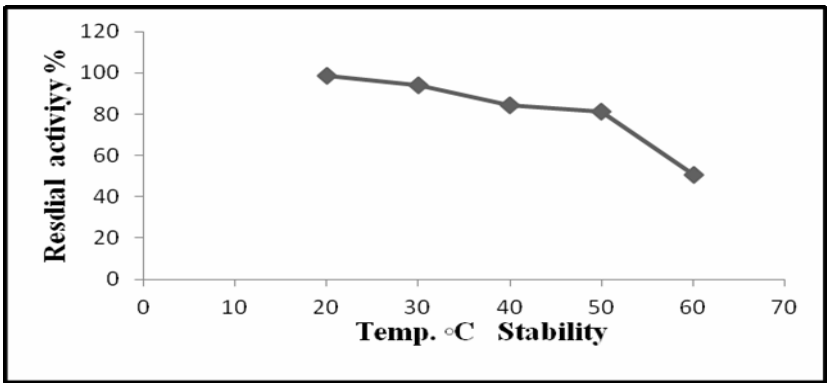

Fig.(12): Temp. stability on the activity of purified protease from *Fusarium spp.*

Optimum substrate concentration

Maximum enzyme activity was determined in terms of Vmax and Km using casein and BSA. Vmax and Km values were interpreted from Line Weaver. Optimum substrate concentration: Vmax and Km values for neutral protease enzyme from *Fusarium spp.* was determined from Line Weaver. Results revealed that neutral protease from *Fusarium spp.* had a Vmax of 2 of protein and Km value of 0.25mg/ml towards casein and 2.5km ,0.25vmax of BSA Fig. (13; 14). (12) has reported proteases with lower Km values with casein substrate from *Bacillus alkalophilus* and *Pseudomonas species*, which showed Km values of 0.4 and 2.5 mg/ml respectively. A slightly higher Km value of 3.7 mg/ml were reported for the enzyme from *Bacillus polymyxa* strain indicating higher affinity of the enzyme towards casein (8).

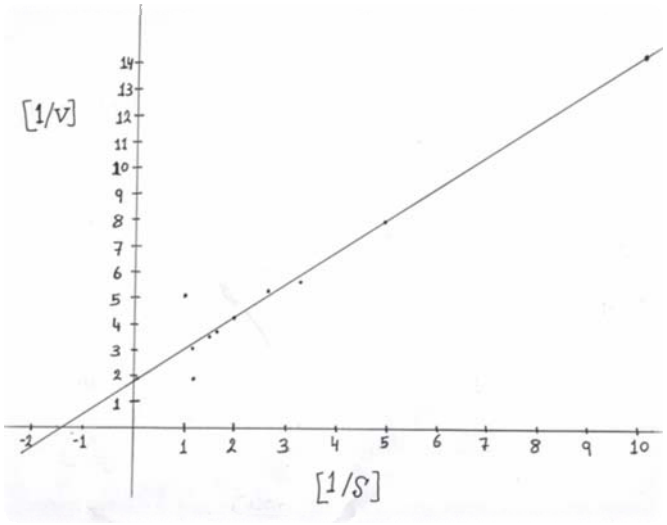

Fig. (13): Lineweaver-Burk plot for neutral protease under varying substrate (casein) concentrations (0.1-1%) indicating the Km and Vmax values.

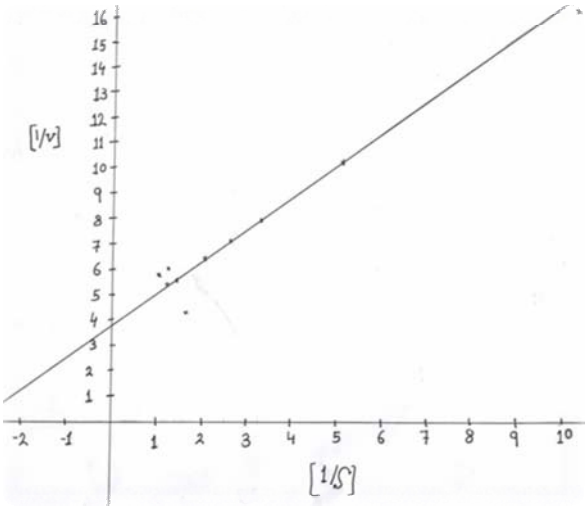

Fig. (14): Lineweaver-Burk plot for neutral protease under varying substrate (bovin serum albumin) concentrations (0.1-1%) indicating the Km and Vmax values.

Conclusions

Enzymes such as proteases have been traditionally produced by solid stare fermentation(SSF) of substrate such as wheat bran and determined the optimization and purification . Solid state fermentation is often viewed advantageously owing to its low operation cost. Enzyme production by SSF using agro by-products not only brings down the cost of production (both of fermentation and downstream processing), but it also provides an alternative path for the effective and productive utilisation of such nutrient-rich agro residues. SSF as the method of choice for the production of these proteolytic biocatalysts. The above study focused on the use of a fungus to convert agro-industrial by-products to a value-added product, an enzyme. From the results, it could be inferred that neutral protease produced through SSF,could possibly find useful application in food industrie.

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