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# Purification,characterization and application of alkaline protease enzyme produced by *Streptomyces rochei* NRC24

**Abdallah N.A[1]., Farid M.A[2]., Nayera A. M. Abdelwahed and Asmaa[2] I. El Shazly**

1- Microbiology Department, Faculty of Science, Ain-Shams University, Cairo, Egypt
2- Chemistry of Natural and Microbial Products Dept., National Research Center, 12311, Dokki, Cairo, Egypt.

## ABSTRACT

The crude extracellular alkaline protease from *Streptomyces rochei* NRC 24 was partially purified by fractional precipitation with ammonium sulphate yielded the highest specific activity of 365.5(U/mg of protein) at 50% saturation and reached 13.18 fold purification of the culture filtrate. Gel filtration was carried out with columns of sephadex G-100 revealed the presence of two protein peaks. The first protein component covered by fractions 7-13 comprised the majority of alkaline protease activity. The molecular weight of partial purified enzyme was about 38 KDa. The maximum activity was found at 60$^{o}$C, pH 8 after 10 min. incubation time and 10 mg/ml casein using enzyme concentration of 0.096 mg. The calculated $K_m$ value was found to be 2.43 mg/ml, while $V_{max}$ was 0.124 µg/ml/min. The enzyme was nearly stable for 60 min at temperature 40-50 $^{o}$C. Maximum activity was also recorded with $CaCl_2$, $MnCl_2.4H_2O$, $MgCl_2.6H_2O$, $ZnSO_4.7H_2O$, $BaCl_2.2H_2O$ and $H_2O_2$ that caused an increase in the enzyme activity with different ratios. The enzymatic activity was strongly inhibited by EDTA, $CuSO_4.5H_2O$, $HgCl_2$, $FeCl_3$, and KMnO4 and $(NH_4)_2S_2O_8$.The enzyme was able to hydrolyze different keratin-containing wastes with different ratios. X-ray film decomposition was investigated where gelatin layer was completely stripped within 60 min at pH 8 and 37$^{o}$C.

**Key words**: *Streptomyces rochei* NRC 24, alkaline protease, purification, characterization, applications

## INTRODUCTION

Proteases are enzymes that catalyze the splitting of proteins into smaller peptide fractions and amino acids by a process known as proteolysis (Rao *et al.*, 1998).Microorganisms represent an attractive source of proteases as they can be cultured in large quantities in a relatively short time. In general, microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer (Gupta *et al*., 2002a).

Enzymes are found in nature in complex mixtures, and in order to study a given enzyme properly, it must be purified. In most cases some of the other enzymes present will interfere, either by attacking the substrate, or by transforming the product into some other substances, or by attacking the coenzyme or even the enzyme itself (Dixon *et al*., 1958).There are no set rules for alkaline proteases as each enzyme has its own suitable purification method (Gupta *et al*., 2002a).After separating the culture from the fermentation broth by filtration or centrifugation, the culture supernatant is concentrated by means of ultrafiltration, salting out by solid ammonium sulfate or solvent extraction methods using acetone and ethanol (Kumar, 2002 and Thangam and Rajkumar, 2002).

The molecular weights of alkaline proteases produced by some actinomycetes ranged from 15 to 35 kDa (Hui *et al.,* 2004 and Dastager *et al.,* 2008). Alkaline proteases from *Bacillus* sp., *Streptomyces* sp. and *Thermus* sp. are quite stable at high temperatures, and the

addition of $Ca^{2+}$ further enhanced enzyme thermostability (Kumar and Takagi, 1999). Some of the major commercial uses of alkaline proteases required high temperatures, thus improving the thermal stability of the enzyme is distinctly advantageous. Thermostability can be enhanced by adding certain stabilizers to the reaction mixture. Metal ions like $Ca^{2+}$ and $Ba^{2+}$ were reported to enhance *Streptomyces* sp. protease stability in 1.24 and 1.37 times, respectively, during 1h at 55$^{o}$C (De Azeredo *et al*. 2004).The optimum pH range of alkaline proteases is generally between pH 8 and 12 (Mitsuiki *et al*., 2002 ,Ningthoujam *et al.*, 2009).

Alkaline proteases are robust enzymes with considerable industrial potential in detergents, leather processing, silver recovery, medical purposes, food processing, feeds, and chemical industries, as well as waste treatment. For environmental reasons, the biotreament of leather using an enzymatic approach is preferable (Andersen, 1998). De Azeredo *et al*. (2006a, b) used alkaline protease from *Streptomyces sp.* for the management of waste feathers from poultry slaughterhouses. Alkaline proteases also play a crucial role in the bioprocessing of used X-ray or photographic films for silver recovery (Gupta *et al*., 2002b).

This study was constructed to partially purify and characterize alkaline protease enzyme produced by *Streptomyces rochei* NRC 24 and studying some possible applications for the crude enzyme.

## MATERIALS AND METHODS

### Microorganism

*Streptomyces rochei* NRC 24 was isolated from an Egyptian soil using serial dilution method (Basavaraj *et al.*, 2010). The isolate was identified using 16S-rDNA with the help of Mubarak City for Scientific Research & Technology Applications, Alexandria, Egypt, and was used as producer of alkaline protease.

### Partial purification of alkaline protease

The crude culture supernatant obtained from seven days old cultures of *S. rochei* NRC 24 was subjected to the following purification steps according to Scopes (1994).

### Fractional precipitation with ethanol

The cold culture filtrate (crude enzyme) was subjected to a slow addition of cold ethanol in an ice bath during stirring till the desired concentration of ethanol was reached 20% (v/v). The precipitate was collected by cooling centrifugation at 2500 rpm for 30 min. The precipitated protein was dissolved in 50mM glycine-NaOH buffer of pH 8 and the process was repeated. Different fractions were thus obtained at ethanol concentrations of 40, 60 and 80% (v/v).

### Fractional precipitation with acetone

This was carried out as described for ethanol fractional precipitation. The precipitated enzyme fractions obtained at 20, 40, 60 and 80% (v/v) were assayed for alkaline protease activity and protein content.

### Fractional salting-out with ammonium sulphate

Solid ammonium sulphate was slowly added to the cold culture filtrate with constant stirring until the required saturation of ammonium sulphate was reached (ranging from 35-80%). The mixture was kept at 4$^{o}$C overnight and then centrifuged. The precipitate was removed and dissolved in 50mM glycine-NaOH buffer of pH 8. The enzyme was assayed for its activity and protein content. The active fraction (at 50% saturation) was kept for the study of the properties and kinetics of the partial purified alkaline protease enzyme.

**Purification,characterization and application of alkaline protease enzyme produced by *Streptomyces rochei* NRC24**

**Chromatographic purification of the alkaline protease on sephadex G-100 column (Gel filtration chromatography)**

This was achieved according to the method of Bhaskar *et al*. (2007) with some modifications. A column of sephadex G-100 equilibrated with 50mM glycine-NaOH buffer (pH 8) was used for chromatographic purification. The column was eluted with 50mM glycine-NaOH buffer (pH 8) at room temperature. 5 ml fractions were collected with a flow rate of 60 ml/h. The protein content and alkaline protease activity for collected fractions was determined.

**Physicochemical properties of the partial purified alkaline protease**

**Molecular weight determination**

The molecular weight of the partial purified enzyme (by gel filtration, fraction 10) was determined by using SDS-gel electrophoresis. Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis technique (PAGE) was carried out according to Laemmli (1970) method. Reagents used were electrophoresis buffer (Tris, 0.030 g; glycine, 0.1441 g; pH, 8.3 and SDS, 0.1% w/v), separating gel buffer (Tris, 18.182 g; SDS, 0.4 g% and pH, 8.8), stacking gel buffer (Tris, 7.1 g; SDS, 0.5g and pH, 6.8 with final volume 125 ml, sample buffer (Tris, 0.6 g; glycerol, 5 ml; SDS, 2 g and pH, 6.8 then complete to 50 ml and add 20 mg bromophenol blue) , reducing sample buffer (2-Mercaptoethanol (8%), 80 µl in 1 ml sample buffer), acrylamide stock solution (Acrylamide, 30 g; bisacrylamide, 0.8 g and distilled water, 100 ml), ammonium persulphate solution 10% (Ammonium persulphate, 0.1 g in 1 ml of distilled water). The resolving gel was prepared for one gel of 12.5% SDS-PAGE, as follows: acylamide solution, 2.5 ml; resolving buffer, 1.5 ml; distilled water, 2 ml; ammonium persulphate (10% w/v), 50µl and TEMED, 7µl added last. Ingredients were mixed gently ensuring that no air bubbles were formed and then the mixture was poured into glass plate assembly using Pasteur pipette. Gel was overlayed with isopropanol or water carefully excluding air bubbles and ensuring a flat surface. After polymerization, isopropanol was carefully washed with deionized water. Stacking gels (4.0%), was prepared by mixing distilled water, 2.02 ml; acrylamide solution, 0.44 µl; stacking gel, 0.83 µl; ammonium persulphate (10%), 33 µl and TEMED, 3.3 µL added last.Ingredients were mixed as before and poured onto top of resolving gel and then the comb was inserted avoiding air bubbles. After polymerization of the stacking gel, the comb was removed and the top of the gel was filled with the electrophoresis buffer removing any unpolymerized acrylamide. Protein markers were broad range protein molecular weight ranging from 35-245 KDa (Gene Dire X) was included on the same gel. The protein molecular weight marker is a mixture of 8 purified proteins supplied in gel loading buffer (20 mM Tris-phosphate, pH 7.5 at $25^0$C, 2% SDS, 1 mM 2-mercaptoethanol, 3.6 M urea and 15% (v/v) glycerol). The logarithmic of molecular weight of the protein standard plotted against the rate of flow which represents the distance migrated by the protein divided by the total distance migrated by the dye and the curve was used to determine the molecular weights of the unknown proteins through using Gel Analysis program.

**Procedure**

Control, crude and partial purified enzyme samples were subjected to SDS-PAGE; through 4% stacking and 12.5% resolving gels in 0.75 mm thick vertical slab gels. SDS-PAGE consisted of a 12.5% polyacrylamide in 0.375 M Tris, pH 8.8 resolving gel and a 4.0% gel, in 0.125 M Tris, pH 6.8 as a stacking gel and a total of 20 µl of a sample was applied to each lane. Electrophoresis was run at 80 mV constant voltage and stopped after the bromophenol blue dye reached to the end of the plate. After electrophoresis, the gel was

coomassie blue stained. The gel plate was removed and placed in the staining solution(coomassie brilliant blue R-250 dye 0.25 g; methanol,50 ml; glacial acetic acid 10 ml and water 40 ml) for 1 h then de-stained several times till gained clear bands with destaining solution, methanol 50ml; glacial acetic acid 10ml and water 40 ml.

**Characterization of partial purified alkaline protease**

**Effect of pH reaction**

The optimum pH was determined with casein as a substrate dissolved in the following buffer systems (50mM), namely sodium-phosphate buffer (pH 6-8), glycine-NaOH buffer (pH 8-10) and sodium hydrogen orthophosphate-NaOH buffer (pH 11-12).

**Effect of incubation temperature**

Proteolytic activity of the partial purified enzyme on casein was analyzed at different temperatures from 30 to 70$^{o}$C under standard assay conditions.

**Effect of incubation time**

The partial purified enzyme was incubated with the substrate for different time intervals up to 60 minutes and then the reaction product was measured.

**Effect of substrate concentration**

Alkaline protease activity was determined with different casein concentrations (2.5-60 mg/ml) under standard conditions. Then the tyrosine as a reaction product was estimated to calculate the kinetics parameters ($K_m$ and $V_{max}$) according to Hanes (1932).

**Effect of enzyme concentration**

The reaction was carried out under standard assay conditions with varying the amount of the partial purified enzyme (0.048, 0.096, 0.144, 0.192, 0.288, 0.385, 0.481 and 0.577 mg protein) and then the enzyme activity was determined.

**Thermal stability**

Small aliquots of the partial purified enzyme were pre-incubated at different temperatures 40, 50, 60 and 70$^{0}$C and then the enzyme activity was determined.

**Effect of some metal salts**

The effect of different metal salts of concentrations (0.6, 1.2, 2.4 and 4.7 mM) on the activity of partial purified enzyme was studied. The metal salts which were used namely, $MgSO_4.7H_2O$, $BaCl_2.2H_2O$, NaCl, $ZnSO_4.7H_2O$, $CuSO_4.5\ H_2O$, $HgCl_2$, KCl, $MgCl_2.6H_2O$, $CaCl_2$, $FeSO_4.7H_2O$, $FeCl_3$, $K_2HPO_4$ were incubated with the reaction mixture at 60$^{o}$C for 10 minutes then the enzyme activity was determined. The control was assayed without any metal salts.

**Effect of some oxidizing and reducing agents**

This was carried out as described for metal ions but the concentrations of the oxidizing and reducing agent were different as they were 0.06, 0.12, 0.24 and 0.47 mM.

**Some applications of *S. rochei* NRC 24 alkaline protease.**

**Degradation of some keratinous wastes**

The experiment was conducted to investigate the keratinolytic activity of the produced alkaline protease on different keratin-containing substrates like waste chicken feathers, nails, raw wool and hair according to (Bockle *et al*. 1995; Andersen, 1998). with some

modifications. Additionally, untreated and the treated wastes with the enzyme were examined microscopically under scanning electron microscope (JEOL, JXA-840A) at different magnification powers.

**Recovery of silver from waste X-ray film by alkaline protease from *S. rochei* NRC 24**

This experiment aimed to study the ability of the crude alkaline protease produced by *S. rochei* NRC 24 to decompose the gelatin layer of waste X-ray film. This was done according to Shankar *et al.* (2010).Gelatin hydrolysis was monitored by measuring increasing of turbidity in the hydrolysates spectrophotometrically by measuring the absorbance at 660 nm. At the same time, the concentration of released amino acid was also determined every 15 min.

## RESULTS

**Partial purification of *S. rochei* NRC 24 alkaline protease.**

It was achieved by fractional precipitation using ethanol, acetone and ammonium sulphate. Ethanol fractionation of the culture filtrate afforded 4 fractions (Table 1). The fraction precipitated at 40- 60% ethanol possessed the highest activity (7594 U/mg fraction), specific activity (213.28 U/mg proteins) and purification fold (1.37) as compared with other fractions. Also acetone fractionation afforded 4 fractions where the fraction precipitated at 40-60% possessed the highest specific activity (360.73 U/mg protein) and possessed 38.34% recovered activity with 2.31 fold purification. On the other hand, the precipitation with ammonium sulphate afforded 5 fractions. The enzyme fraction salted out at 50% ammonium sulphate saturation was the most active fraction showing specific activity (365.0 U/mg proteins) and possessed a recovered activity of 48.53% and exhibited 13.18 fold purification.

**Table (1): Partial purification of *S. rochei* NRC 24 alkaline protease**

| Precipitant % | Total Protein (mg) | Recovered Protein % | Total activity (U/mg) | Recovered activity (%) | Specific activity (U/mg) | Purification fold |
|---|---|---|---|---|---|---|
| Crud enzyme | 260.1 | 100 | 4057.3 | 100 | 155.89 | 1 |
| Ethanol | | | | | | |
| 0-20 | 3.06 | 1.18 | 298.61 | 0.74 | 97.58 | 0.63 |
| 20-40 | 23.33 | 8.97 | 2265.5 | 5.59 | 97.13 | 0.62 |
| 40-60 | 35.61 | 13.69 | 7594.83 | 18.73 | 213.28 | 1.37 |
| 60-80 | 48.51 | 18.65 | 6551.67 | 16.16 | 135.07 | 0.87 |
| Acetone | | | | | | |
| 0-20 | 6.99 | 2.69 | 266.36 | 1.41 | 38.11 | 0.24 |
| 20-40 | 31.93 | 12.28 | 2038.71 | 5.03 | 63.86 | 0.41 |
| 40-60 | 43.1 | 16.57 | 15547.66 | 38.34 | 360.73 | 2.31 |
| 60-80 | 71.11 | 27.34 | 208.08 | 0.51 | 2.93 | 0.02 |
| Ammonium Sulphate | | | | | | |
| 35 | 6.99 | 1.39 | 236.12 | 1.69 | 33.8 | 1.22 |
| 50 | 18.53 | 3.68 | 6763.53 | 48.53 | 365.0 | 13.18 |
| 60 | 14.6 | 2.9 | 3312.52 | 23.77 | 226.88 | 8.19 |
| 70 | 24.55 | 4.88 | 342.7 | 2.46 | 13.96 | 0.50 |
| 80 | 16.08 | 3.19 | 0.0 | 0.0 | 0.0 | 0.0 |

**Purification of partially purified *S. rochei* NRC 24 alkaline protease by column chromatography on Sephadex G-100.**

As it was shown ammonium sulphate at 50% saturation was the most appropriate agent for precipitating active fraction of the extracellular alkaline protease. For further purification, the aforementioned enzyme fraction was subjected to purification by column chromatography on sephadex G-100.

The results (Fig. 1) indicated that the first 5 fractions were devoid of any protein or activity. The column affected the recovery of 12% protein and 52.1% activity of the applied sample. The column also affected the resolution of 2 protein components. A major component covered by fractions 7-13 and comprised 3.35 mg protein which constituted 0.67% of the protein content of the culture filtrate. The total recovered activity of this protein component represented about 52.1% of the applied sample. From this component, fraction 10 was the most active, showed 17.76 fold purification and comprised 17% of the alkaline protease activity of the applied sample.

On the other hand, the second protein component was not well defined, it was covered by fractions 14-22 and not possess any alkaline protease activity and comprised 4.24% of the applied protein.

From the above results, it is obvious that the first protein component (fractions 7-13) comprised most of the alkaline protease activity.

Furthermore, the molecular weight of the first peak (fraction 10) was estimated to be about 38 KDa by comparison of its mobility on SDS- PAGE with those of standard proteins (Photo 1).

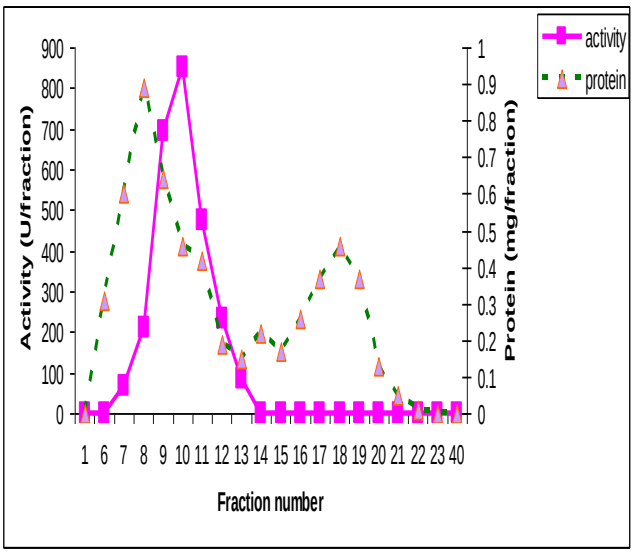


**Fig.(1): Purification of partially purified *S. rochei* NRC24 alkaline protease by gel filtration.**

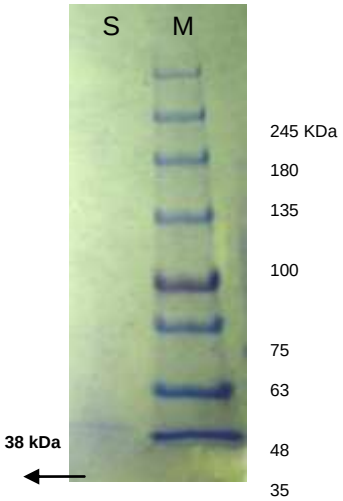


**Photo.(1): SDS-gel electrophoresis of the partial purified enzyme (fraction 10) (S), and protein markers (M).**

**Properties of the partial purified *S. rochei* NRC 24 alkaline protease.**

The partial purified enzyme showed maximum activity at pH 8(100%).Lowering or raising the pH than that value led to decrease in enzyme activity (Table 2). Concerning the temperature effect, the enzyme activity gradually increased with the increase of reaction temperature up to $60^0$C where it reached its maximum activity (11134.83 U/ml). Further

increase in the reaction temperature above 60°C resulted in a gradual loss in the enzyme activity.

The effect of reaction time indicated that the enzyme reached its maximal activity when the reaction lasted for 10 min. (1609.43 U/mg proteins and dropped significantly by performing the reaction for 30 min (744.07 U/mg proteins) reached 464.83 U/mg proteins after 60 min. Similar results revealed that substrate concentration of 10 mg/ml was sufficient for the production of maximal enzyme activity (1883.87 U/mg proteins). Further increase in substrate concentration did not increase enzyme activity.

The effect of enzyme concentration upon the reaction rate indicated that enzyme concentration of 0.096 mg protein was sufficient for achieving maximal enzyme activity (18026.46 µg/ml).Higher enzyme concentration did not increase enzyme activity.

$K_m$ and $V_{max}$ values were determined for the partial purified alkaline protease using casein as a substrate and calculated from Hanes-Woolf Plot. Casein concentration ranged from 2.5 to 10 mg/ml in 50 mM glycine-NaOH buffer and the reaction was conducted at 60°C for 10 min. Figure (2) showing the $K_m$ value (2.43 mg/ml) and $V_{max}$ value (0.124 µg/ml/min).

The thermostability of alkaline protease produced by *S. rochei* NRC 24 was examined at different temperatures (Fig.3) between 40 and 50°C. The enzyme was nearly stable for 60 min. and did not lose much of its activity. On the other hand, preheating the enzyme at 60°C and higher led to adverse effect on enzyme activity, the extent of these effects depended on the temperature and time of treatment. It should be noted that the enzyme lost about 90.6% and 97.2% of its activity by heating for 20 min at 60 and 70°C, respectively. The enzyme was completely inactivated at 70°C after 30 min.

It was observed in Figure (4) that most of the tested metal salts had an inductive effect on the enzyme activity. The maximum protease activity was recorded with the presence of $CaCl_2$ at 1.2mM. Additionally, $MnCl_2.4H_2O$ and $MgCl_2.6H_2O$ at 1.2mM caused a marked increase in the enzyme activity. Similarly, $ZnSO_4.7H_2O$ and $BaCl_2.2H_2O$ caused increasing in the enzyme activity but with fewer ratios. On the other hand, activity was strongly inhibited by EDTA at all concentrations used. Also $CuSO_4.5\ H_2O$, $HgCl_2$ and $FeCl_3$ at 0.6 mM concentration caused 24.48%, 75.38% and 84.32% inhibition of the enzyme activity, respectively (Fig.4).

The effect of different oxidizing and reducing agents on partial purified alkaline protease showed that among the oxidizing agents used, the enzymatic activity was strongly inhibited by $KMnO_4$ and $(NH_4)_2S_2O_8$, whereas $H_2O_2$ caused a slight increase in the enzyme activity. However, reducing agents such as ascorbic acid and cysteine had no significant effect on the enzyme activity (Fig.5).

**Table (2):- Properties of the partial purified *S. rochei* NRC 24 alkaline protease.**

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| pH value | 6 | 7 | 8* | 8.5 | 9 | 9.5 | 10 | 11 | 12 | | |
| R.A. (%) | 28.44 | 70.03 | 100 | 93.12 | 78.63 | 65.87 | 37.77 | 11.24 | 0 | | |
| Temp. C° | 30 | 37* | 40 | 50 | 55 | 60 | 65 | 70 | | | |
| R.A. (%) | 43.52 | 100 | 156.9 | 213.82 | 221.97 | 255.57 | 193.68 | 25.22 | | | |
| Time (min.) | 1 | 2 | 3 | 4 | 5 | 10 | 15 | 20* | 25 | 30 | 35 |
| R.A. (%) | 8.16 | 87.77 | 119.59 | 145.29 | 148.29 | 155.77 | 121.77 | 100 | 83.35 | 71.99 | 65.36 |
| Substrate Conc.mg/ml | 2.5 | 5 | 10* | 20 | 25 | 40 | 60 | | | | |
| R.A. (%) | 43.57 | 75.12 | 100 | 92.74 | 81.18 | 47.66 | 23.45 | | | | |
| Enzyme Conc.mg/ml | 0.048 | | 0.096 | 0.144 | | 0.192 | 0.288 | 0.385 | 0.481 | | 0.577 |
| Tyrosine Conc. (µg/ml) | 17215.51 | | 18026.46 | 13507.96 | | 11031.52 | 7867.58 | 6029.78 | 4805.68 | | 4307.19 |

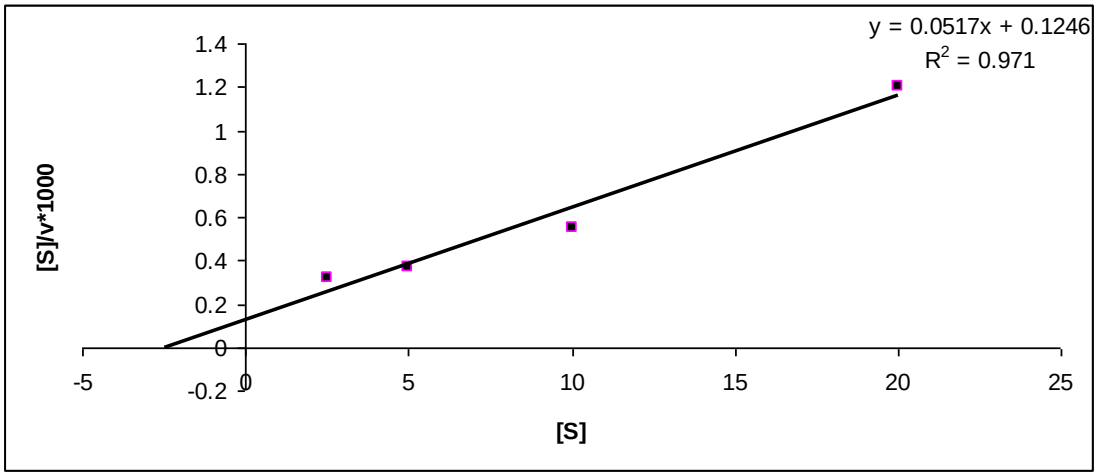


**Fig.(2): Hanes-Woolf Plot of partial purified *S. rochei* NRC 24 alkaline protease.**

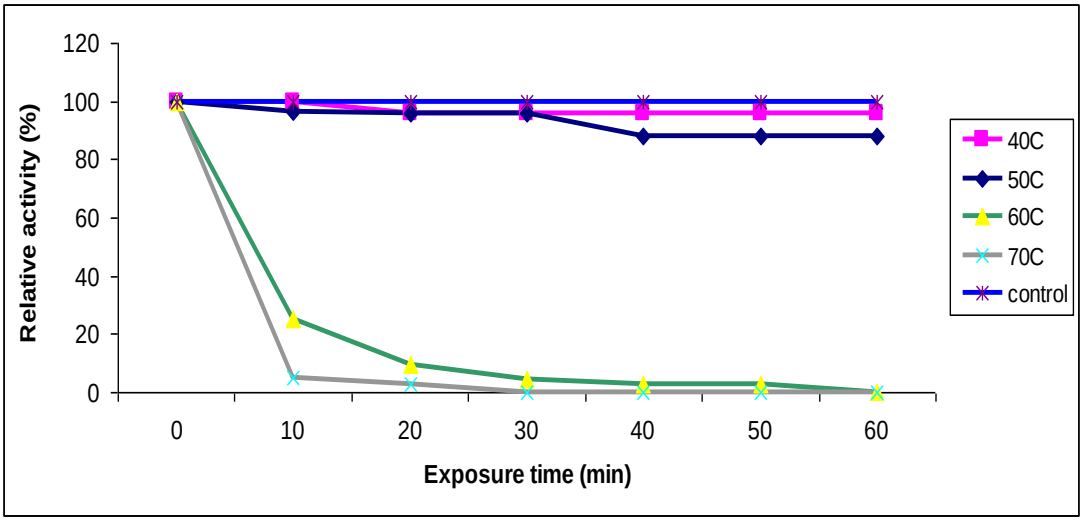


**Fig. (3): Thermal stability of partial purified *S. rochei* NRC 24 alkaline protease.**

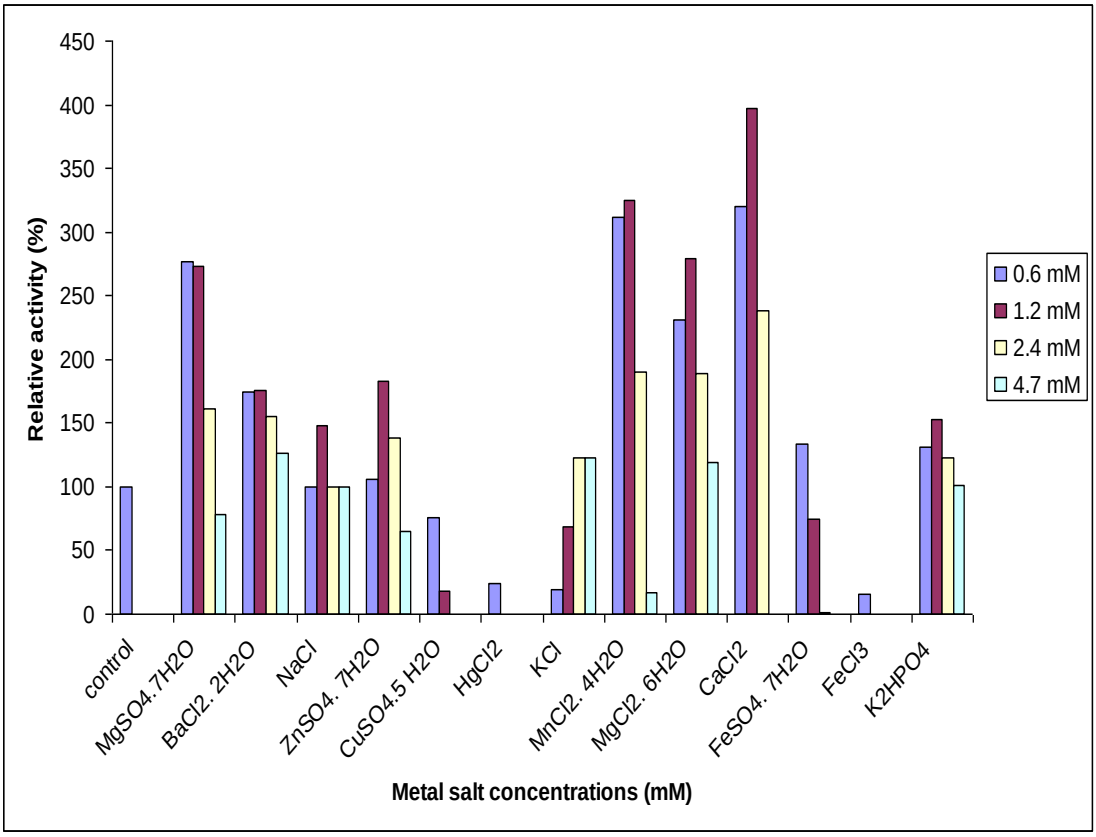


**Fig. (4): Effect of some metal salts addition on the activity of the partial purified *S. rochei* NRC 24 alkaline protease.**

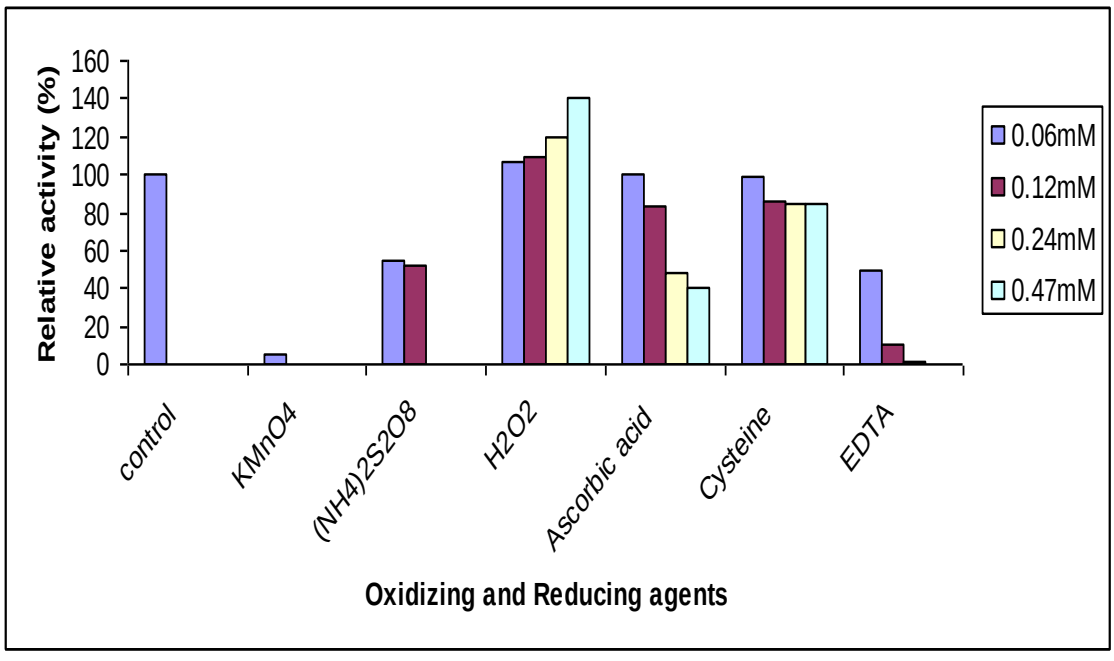


**Fig. (5): Effect of some oxidizing reducing and chelating agents on the activity of the partial purified *S. rochei* NRC 24 alkaline protease.**

**Some applications on extracellular alkaline protease produced by *S. rochei* NRC 24**

**A. Degradation of some keratinous substrates waste by alkaline protease from *S. rochei* NRC 24.**

It was obvious from the results that the extracellular alkaline protease produced by *S. rochei* NRC 24 was able to hydrolyze different keratin-containing substrates with different ratio (Table 3, photos 2,3 and 4). It hydrolyzed waste feathers more efficiently (796.31 µg/ml) than did with hair and raw wool (158.32 and 131.11 µg/ml, respectively). However, it was found that the enzyme had no hydrolyzing effect on nails.

**B. Hydrolysis of gelatin layer of waste X-ray film by alkaline protease from *S. rochei* NRC 24.**

The enzyme had the ability to decompose the gelatin layer of the X-ray film completely (Table 4, Photo 5) after 60 min releasing silver and polyester base to be reused.

**Table (3): Degradation of some waste Keratinous substrate by *S. rochei* NRC24. alkaline protease**

| Keratinous Released Substrate | Conc. Of amino acid(tyrosine) (µg/ml) | Total released amino acids (µg/ml) |
|---|---|---|
| Feathers | 796.31 | 1592.62 |
| Hair | 158.32 | 316.64 |
| Raw wool | 131.11 | 262.22 |
| Nails | 0.0 | 0.0 |

**Table (4): Hydrolysis of gelatin layer of X-ray film by *S. rochei* NRC24. alkaline protease.**

| Incubation time(min.) | O.D. of turbidity (nm) | Conc.of released amino acids (µg/ml) |
|---|---|---|
| 15 | 0.141 | 801.87 |
| 30 | 0.652 | 149.27 |
| 45 | 0.667 | 1739.21 |
| 60 | 2.5 | 1829.92 |

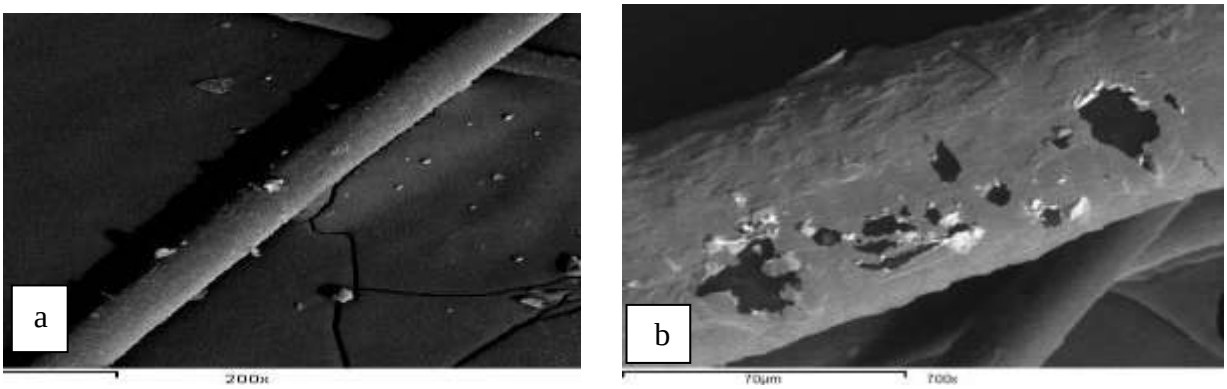


**Photo (2): Scanning electron micrograph of sections of raw wool.**
**(a):wool without enzyme treatment. (b):wool treated with alkaline protease from *S. rochei* NRC 24 for 24 h.**

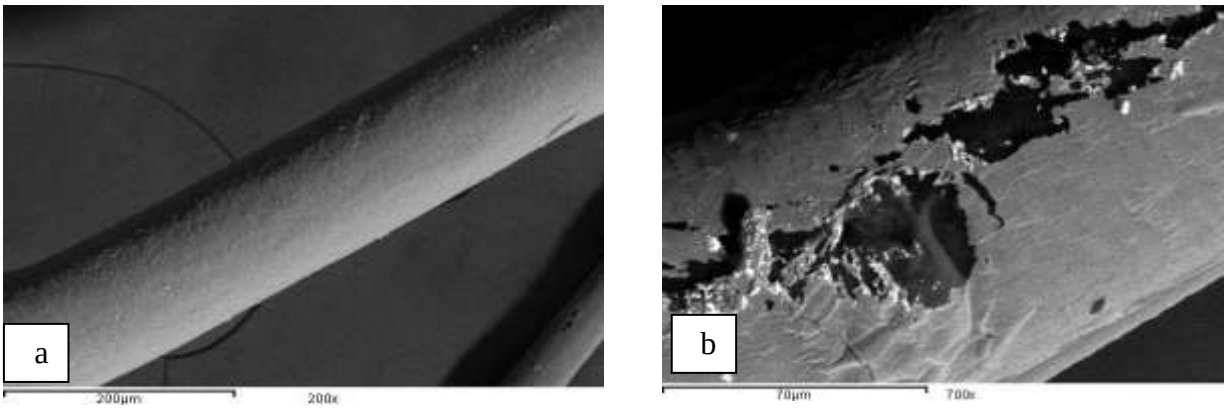


**Photo (3): Scanning electron micrograph of sections of raw hair.**
**(a): hair without enzyme treatment. (b): hair treated with alkaline protease from *S. rochei* NRC 24 for 24 h.**

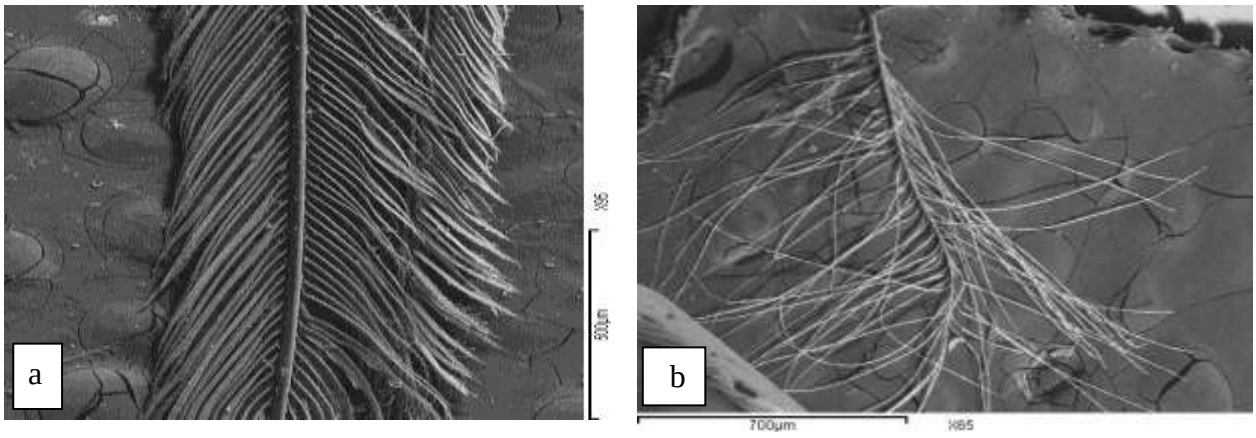


**Photo (4): Scanning electron micrograph of sections of native feathers. (a): feathers without enzyme treatment.(b):feathers treated with alkaline protease from *S. rochei* NRC 24 for 24 h.**

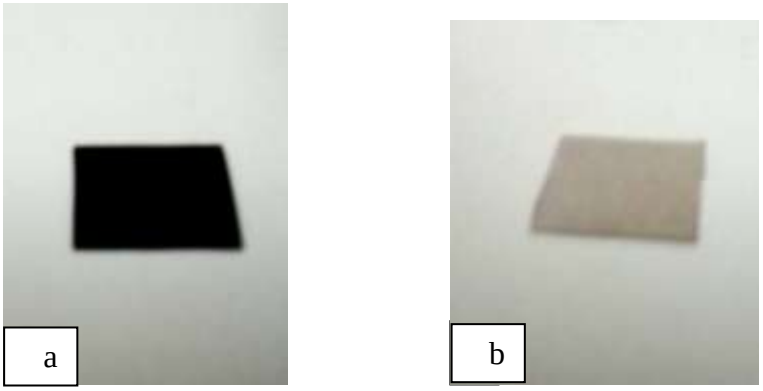


**Photo (5): Decomposition of gelatin layer of X-ray film by extracellular alkaline protease produced by *S. rochei* NRC 24.(a) Control and (b) treated with the enzyme.**

## DISCUSSION

Among different proteases, alkaline proteases produced by microorganisms are of main interest from a biotechnological perspective and are investigated not only in scientific fields of protein chemistry and protein engineering but also in applied fields (Jayasree *et al*., 2009).During this study partial purification of extracellular alkaline protease produced by *S. rochei* NRC 24 was achieved by fractional precipitation with acetone, ethanol and ammonium sulphate. The total respective recovered protein was 58.88%, 42.49% and 16.04%. The highest recovered activity was achieved with 50% saturation ammonium sulphate. In this respect, the result is in harmony with that obtained by ammonium sulphate precipitation of *Virgibacillus Pantothenticus* alkaline protease (4.62-fold purification) by Gupta *et al.* (2008). Salting out with ammonium sulphate was also reported by many investigators Hui *et al*. (2004) and Dastager *et al*. (2008). Fractional precipitation by organic solvents was reported by Ahmed *et al*. (2008).

The protein chromatographic eluted profile of the active fraction obtained from ammonium sulphate precipitation revealed the presence of two protein peaks. The first protein component covered by fractions 7-13 comprised almost of alkaline protease activity, whereas the second protein peak, covered by fractions 14-22, did not posses any proteolytic activity. Further purification of the partial purified *S. rochei* NRC 24 alkaline protease on sephadex G-100 afforded 52.1 fold purification. This result is in accordance with that found by Nilegaonkar *et al*. (2002) who reported 10.96-fold purification of alkaline protease on sephadex G-100 column.Furthermore, the molecular weight of the first peak (fraction 10) was estimated to be about 38 KDa by comparison of its mobility on SDS- PAGE with those of standard proteins. Alkaline proteases from different origins have various molecular weights. Nascimento *et al.* (2005) mentioned that *Streptomyces malaysiensis* AMT-3 produced alkaline protease with different molecular weights from 35 to 212 KDa according to SDS-PAGE. Dastager *et al.* (2008) also characterized alkaline protease from *Streptomyces gulbargensis* DAS 131 and reported its molecular mass was 19, 35 KDa on SDS-PAGE.

The general properties of the partial purified alkaline protease were studied. *S. rochei* NRC 24 alkaline protease was active over a pH range of 6 to 12 using sodium-phosphate, glycine-NaOH buffers with an optimum activity at pH 8. Similar result was reported by Ferracini- Santoes and Sato (2009).

The activity of the alkaline protease gradually increased with the increase of reaction temperature up to $60^0$C whereby the maximal activity of the enzyme was attained. Further increase in the reaction temperature above $60^o$C resulted in a gradual loss in the enzyme activity. This could be attributed to the fact that the rate of the chemical reaction generally doubles with every $10^o$C increase in reaction temperature. Enzymes, however like all proteins undergo thermal denaturation at elevated temperatures. Hence the enhancement of catalytic efficiency with increasing temperature is compromised by the competing effects of general protein denaturation at high temperatures. For this reason, the activity of a typical enzyme will increase with temperature over a definite temperature range and then diminish significantly above some critical temperature that is characteristic of the denaturation of the protein (Copeland, 2000). The result is similar to that reported by Hui *et al*. (2004) for alkaline protease from *Streptomyces* sp.

The partial purified enzyme showed highest activity after 10 minutes incubation. This result is similar to that reported by Takami *et al*. (1989) and Wang *et al*. (2005).

The enzyme activity increased with increasing casein concentration to reach a maximum activity at casein concentration of 10 mg/ml. At higher concentrations the enzyme activity remained more or less constant, probably due to the fact that there are so many substrate molecules competing for the active sites on the enzyme surfaces that they block the

sites and prevent any other substrate molecules from occupying them. Almost similar behavior has been reported by Ali and Dahot (2009) and Jameel (2011).

In the present study the $K_m$ and $V_{max}$ values were 2.43 mg/ml and 0.124 µg/ml/min, respectively. In this respect, many investigators found that $K_m$ values of purified alkaline protease of 0.25-3.92 mg/ml and $V_{max}$ values of 0.0286-17.53 U/mg protein (Thangam and Rajkumar, 2002 ; Gupta *et al*., 2005).

The proteolytic activity of the investigated enzyme increased exponentially upon increasing enzyme up to 0.096 mg/ml however, further increase in the enzyme concentration appeared to be unfavorable for the proteolysis process. This result could be attributed to that by adding more and more enzyme; the velocity can increase to the point at which significant amounts of the total substrate concentration are being depleted during the time window of our assay. When substrate depletion becomes significant, further increases in enzyme concentration will no longer demonstrate as steep a change in reaction velocity as a function of enzyme concentration (Copeland, 2000).

The data demonstrated that the metal salts had varied effect on partially purified alkaline protease activity. Most of the tested metal salts had an inductive effect on the enzyme activity. The maximum activity of protease was found with the presence of $CaCl_2$ and the presence of $MnCl_2.4H_2O$ and $MgCl_2.6H_2O$, $ZnSO_4.7H_2O$ and $BaCl_2.2H_2O$ caused increasing in the enzyme activity but with fewer ratios, while the enzymatic activity was strongly inhibited by EDTA, $CuSO_4.5H_2O$, $HgCl_2$ and $FeCl_3$. This is may be related to the toxic effect of some heavy metals are toxic to enzymes primarily because of their protein-binding capacity. The cationic metals are noncompetitive inhibitors, which bind irreversibly with sulfhydryl and carboxylate groups and with histidine, altering protein structure and the conformation and accessibility of the enzymes' active sites (Speir and Ross, 2002). The enzyme activity was inactivated by $Hg^{2+}$ indicating that the involvement of the thiol group in the conformation of the proteins or in the active site of the enzyme (Gomez De Segura and Fevre, 1993). The inactivation of protease by $Fe^{+2}$and $Cu^{+2}$ was reported by Hutadilok-Towatana *et al*. (1999). On the hand, many investigators found that proteases were activated by $Ca^{+2}$, $Na^{+}$, $Zn^{+2}$, $Mg^{+2}$, $Mn^{+2}$, $k^{+}$, $Fe^{3+}$, $Ba^{+2}$ while $Cu^{+2}$, $Na^{+}$, $Zn^{+2}$, $Mg^{+2}$, $Hg^{+2}$ and $Fe^{3+}$ inhibited them (Uchida *et al*., 2004).

The present results indicated that the enzyme activity was strongly inhibited by $KMnO_4$ and $(NH_4)_2S_2O_8$, whereas $H_2O_2$ caused a slight increase in the enzyme activity. That activation indicates that the enzyme showed extreme activity towards oxidizing agent of immense commercial significance for detergent industry because peroxides are a common ingredient of modern bleach-based detergent formulation (Joo *et al*., 2003). This result was similar to that of Beg and Gupta (2003) and Nilegaonkar *et al.* (2007), where serine proteases were stimulated by $H_2O_2$ (1 and 5%). However, reducing agents such as ascorbic acid and cysteine had no significant effect on the enzyme activity.

Thermal stability is one of the most important factors in industrial applications (Cowan *et al*., 1985). Studying the thermal stability of the partial purified alkaline protease indicated that between 40 and $50^0$C the enzyme was nearly stable for 60 min and did not lose much of its activity. On the other hand, preheating the enzyme at $60^0$C and higher led to adverse effect on enzyme activity, the extent of these effects depended on the temperature and time of treatment. these results were inagreement with Oberoi *et al*. (2001) and Huang *et al*. (2003).

The present work provides new data on some keratin-rich wastes degradation by *S. rochei* NRC 24 alkaline protease. The data demonstrated that the extracellular alkaline protease produced by *S. rochei* NRC 24 was able to hydrolyze different keratin-containing substrates with different ratios. However, it was found that the enzyme had no hydrolyzing

effect on nails. In addition it was confirmed by SEM that *S. rochei* NRC 24 alkaline protease cracked and partially degraded the native feathers, raw wool and hair after 24 h.The ability of the enzyme to bind to the solid feather substrate makes it extremely attractive for detergent application (Egmond, 1997). In this respect, almost similar results were reported by Takami *et al*. (1992) and Bockle *et al*. (1995).

The present data demonstrated that alkaline protease from *S. rochei* NRC 24 was able to completely decompose the gelatin layer of the X-ray film after 60 min releasing silver and polyester base to be reused. This result is similar to Masui *et al*. (1999) and Karadzic *et al*. (2004) who reported that protease from *Bacillus* sp. and *Pseudomonas aeruginosa*, respectively, were able to decompose the gelatin layer on X-ray film.

In conclusion, the produced protease enzyme is active over a broad pH range, stable at high temperature and able to hydrolyze various solid substrates. Consequently, it has a considerable potential use in biotechnological processes particularly for degrading solid waste substrates like feathers, hair and X-ray film that cause environmental pollution.

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## تنقية وخواص وتطبيقات انزيم البروتينيز القلوى المنتج بواسطة جنس *Streptomyces rochei* NRC 24 جزئيا

**نجوي أحمد عبدالله[1] ،محمد عبد الفتاح فريد[2]، نيرة أحمد عبد الواحد[2]،أسماءإبراهيم الشاذلي[2]**
1- قسم الميكروبيولوجي- كلية العلوم- جامعة عين شمس-مصر
2- قسم كيمياء المنتجات الطبيعية والميكروبية- المركز القومي للبحوث

يهدف هذا البحث إلي تنقية انزيم البروتينيز المنتج بواسطة جنس *S. rochei* NRC 24 جزئيا ودراسة صفاتة والتطبيقات الممكنة للأنزيم الخام. وقداهتم البحث بتنقية الأنزيم الخام جزئيا بإستخدام الترسيب بواسطة كل من الاسيتون والكحول الاثيلى و كبريتات الامونيوم كل على حده و تم الحصول على أعلى عائد إنزيمى بإستخدام كبريتات الامونيوم عند درجة تشبع 50% حيث صاحب ذلك أعلى نشاط نوعى للانزيم (365 وحدة / ملجم بروتين) كما اعطى اعلى درجات تنقية (13.18 مرة) مقارنة بالرواسب الاخرى.وقد أسفرت نتائج التنقيةالأضافية للأنزيم عن فصل اثنين مكون بروتيني كن اولهم مكون من (الاجزاء 7-13) ذو نشاط أنزيمى يبلغ 52.1% من العينة المحملة على عامود فصل معبأ ب Sephadex G-100 و حيث أوضح نشاطها الانزيمى تنقية قدرها 59.29 مرة من العينة المحملة على العامود.هذا ويقدير الوزن الجزيئى للانزيم المنقى جزئيا باستخدام الفصل الكهربى على هلام بولى اكريلاميد أظهر انه 38 كليو دالتون.أظهرت الدراسة ان أعلى نشاط للانزيم المنقى جزئيا كان عند الاس الهيدروحينى 8عند درجة حرارة 60 درجة مئوية لمدة10 دقائق باستخدام 10 ملجم /مل من تركيز الكازين عندما كان تركيز الأنزيم0.096 ملجم بروتين .كما تم تعيين ثابت ميكائيل لانزيم البروتييز القلوى المنقى جزئيا وهو 2.43 ملجم /مل و السرعة القصوى لتحليل الكازين كانت 0.124 ميكرو مول / مل / دقيقة. وقد اظهر الأنزيم ثباتية لمدة 60 دقيقة عند درجة حرارة 40-50 درجة مئوية.وأدت إضافة كل من المواد الاتية على حدة $H_2O_2$, $CaCl_2$ $BaCl_2.2H_2O$ , $ZnSO_4.7H_2O$ , $MgCl_2.6H_2O$ ,$MnCl_2.4H_2O$ الى زيادة نشاط الانزيم بينما تسببت إضافة كل من $KMnO_4$ $(NH_4)_2S_2O_8$ ,EDTA, $CuSO_4.5H_2O$, $HgCl_2$ , $FeCl_3$ الى حدوث تثبيط جزئى للنشاط الانزيمى.وأثبتت النتائج قدرة الانزيم على تحليل بعض المخلفات الكيراتنية مثل ريش الدجاج , الصوف الخام والشعر. قد أظهرت النتائج قدرة الانزيم على تحليل تلك الطبقات الجيلاتينية بافلام اشعة اكس و ان النشاط الانزيمى يزداد بزيادة زمن التحضين الى ان يصل الى 60 دقيقة حيث تتم تحلل الطبقات الجيلاتنية كاملة (100%).