ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Production and Partial Purification of Protease from Chicken Feather using *Gonatorrhodiella parasitica* LS D40.

Sherien M M Atalla*.

Department of Chemistry of Natural and MicrobialProducts, National Research Centre, Giza, Egypt.

ABSTRACT

The local fungal strain *Gonatorrhodiella parasitica*LS D40 was used for protease production using chicken feather as substrate. The culture conditions for high protease production were optimized using 2.0g/l sodium nitrate ,0.5 g/l of KH_2PO_4, 1.5 g/l of KCl and 0.25 g/l of $MgSO_4$ after 6 days incubation period .Protease from *G. parasitica* LS D40 was partially purified using 80% acetone (v/v) with a yield 82.8 %. The enzyme was optimized at pH6.0 at 35 °Cafter15 min of the reaction using 2 % casein as substrate.

Keyword: protease, *Gonatorrhodiella parasitica*, chicken feather.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Proteases are the most important group of enzymes from biotechnological techniques. Protease occurs naturally in all microorganisms and is an essential constituent for all the existing live forms. Microorganisms such as bacteria and fungi and yeast are the main source of protease enzyme. Fungal protease are more promising for commercial application as these microorganism are more resistant to harsh climatic conditions and produces proteins/enzymes in their habitats (Srilakshmi, *et al*., 2015)Proteases are single class of enzyme, which occupy the pivotal position with respect to their physiological role and commercial application. They represent one of the largest groups of industrial enzymes widely used in detergents, leather, waste management and silver recovery (Babu. Proteases also have wide range of functions in nature like, in the regulation of biological metabolic processes such as spore formation, spore germination, protein maturation in viral assembly, activation of certain viruses in pathogenicity, various stages of mammalian fertilization process, blood coagulation, fibrinolysis, complement activation, phagocytosis and blood pressure control (Johnson, *et al*., 2006).

The use of agro-industrial residues as the basis for cultivation media is a matter of great interest, aiming to decrease the costs of enzyme production and meeting the increase in awareness on energy conservation and recycling (Singh,*et al.,* 2009).

The environmental conditions of the fermentation medium play a vital role in the growth and metabolic production of a microbial population. The most important among these are the medium, incubation, temperature and pH (Al-Shehri,*et al.,*2005).

The aim of this work was production and partial purification of protease obtained from fungi and some of their physiological factors.

MATERIALS AND METHODS

Organisms:

The fungal strain *Gonatorrhodiella parasitica* LS 40 used in this study was local strain obtained from soil and identified at the Department of Chemistry of Natural and Microbial Products at the National Research Centre, Dokki, Cairo, Egypt.

Protease fermentation media:

Substrates, like chicken feather, wheat bran, soy bean and corn steep liquor were screened for enzyme production using different fermentation media in Table (1).Two discs (6 mm in diameter) from 7 days old cultures were transferred to 250 ml Erlenmeyer conical flasks each containing 50 ml fermentation medium. The inoculated flasks were incubated on a rotary incubator shaker at 180 r.p.m for 7 days at (28-30° C). At the end of incubation period, cultures were centrifuged at 8000 r.p.m. The cell free supernatant was used as a crude enzyme for further determinations.

Table 1

Medium No.	Composition (g/l)	References
1	Substrate 5 ,glucose 2.5,yeast 0.5, K_2HPO_4 1, $MgSO_4.7H_2O$0.2	Hamidreza, *et al.* ,2007
2	Substrate10,$K_2HPO_4$1,$MgSO_4.7H_2$00.5,KNO_3 2,$ZnSO_4.7H_2O$0.4,$FeSO_4$ 1, $MnSO_4$ 0.2	Chinnasamy, *et al.,* 2011
3	Substrate 5, glucose 10, peptone 10, yeast 5,NaCl 10	Rahman , *et al.,* 2007
4	Substrate 10, $NaNO_3$ 2.5,K_2 HPO_4 1, $MgSO_4$.$7H_2O$ 0.5,KCl 0.5	Sangeeta and Rintu, 2006

Determination of protease activity:

Protease activity in the culture supernatant was determined according to the method of (Tsuchida , *et al.,* 1986)using casein as a substrate. A mixture of 500 µl of 1% (w/v) of casein in phosphate buffer (pH 7) and 200 µl crude enzyme extract were incubated in a water bath at 40°C for 20 minutes. After 20 minutes, the enzyme reaction was terminated by the addition of 1 ml of 10% (w/v) Trichloro acetic acid (TCA) and was kept at room temperature for 15 minutes. Then, the reaction mixture was centrifuged to separate the un reacted casein at 10,000rpm for 5 minutes. The supernatant was mixed with 2.5 ml of 0.4MNa_2CO_3 and 1 ml Follin Ciocalteus phenol reagent, was added. The resulting solution was incubated at room temperature in the dark for 30 minutes and the absorbance of the blue colour developed was measured at 660 nm against a reagent blank using a tyrosine standard. One unit of protease was defined as the amount of enzyme that releases 1 µg of tyrosine per ml per minute under the standard conditions of supernatant solution. The protein content of the enzyme solution was carried out according to **(**Lowry, 1951) [11].

Optimization of culture condition:

Several fermentation process parameters have been studied to improve the yield of protease production by *G.parasitica*LS D40. The influence of different substrate concentrations using different fermentation media, different nitrogen sources $NaNO_3$,$(NH_4)_2SO_4$,in comparison with media free from nitrogen source. Optimization of mineral media components by using different concentrations of KH_2PO_4, KCl, $MgSO_4$ ranging from (0.25 - 2.5 g/l). Different incubation periods(2-14 days) were tested for their enzyme production. Each media was incubated at 28-30 °C for 7 days at 200 r.p.m

.

Fractional precipitation of protease with acetone:

A Known volume of cold acetone was added slowly to the cold enzyme solution until the required concentration of acetone was reached. After removing the precipitate fraction in refrigerator centrifuge, acetone was added to the supernatant and the process was repeated until the final concentration of acetone was reached. The enzyme fractions obtained ranged from 20-80 %. Determination of protease activity and protein content were carried out.

Factors affecting protease activity:

Optimization of partial purified enzyme was carried out by incubating the reaction mixture at different pH values ranging from (4.0 to 8.5) using 0.2 M phosphate buffer, different temperature (20 to 50) °C ,different reaction time from (5-40) min and different substrate concentrations.

RESULTS AND DISCUSSION

The results obtained in this work revealed the ability of *Gonatorrhodiella parasitica*LS D40 for protease production.

Different fermentation media:

Four different fermentation media varied in their substrate were inoculated with the fungal strain and incubated for 7 days at 28 °C. Results in Table (2) showed that enzyme production can be affected while using different substrates. Chicken feather produce maximum protease activity using medium 4 (0.64 U/ml) followed by wheat bran and corn steep liquor (0.57,0.56 U/ml respectively) then soy bean produce(0.50 U/ml) , media1 produce low protease activity with all substrate while medium 2 and 3 produce medium to low activity. Medium should contain likely inducers of the product and be devoid of constituents that may repress enzyme synthesis. It has been reported that *B. licheni form is* produces very narrow zones of hydrolysis on casein-agar despite being very good protease producers in submerged cultures (Mao ,*et al .,*1992)[12]. Chicken feathers are composed of beta keratin which is an insoluble protein rich in amino acid and has a stable rigid structure because of several cross linking disulfide bonds involving cysteine (lakshmi , *et al* .,2014).

ISSN: 0975-8585

Table (2): Effect of different fermentation media

Different substrates	Protease activity (U/ml)			
	Media 4	Media 3	Media 2	Media1
Chickenfeather	0.64	0.29	0.22	0.066
Soy bean	0.50	0.24	0.13	0.061
Wheat bran	0.57	0.25	0.17	0.052
Corn steep liquor	0.56	0.17	0.24	0.042

Effect of different substrate concentrations in presence of$NaNO_3$, $(NH_4)_2SO_4$ as additional nitrogen source:

This experiment was done to determine the suitable substrate concentrations using media containing 2.5 g/l $NaNO_3$, 1.2g/l$(NH_4)_2SO_4$as nitrogen source and media free of nitrogen sources. Results in Fig (1) showed that 1 g of feather using sodium nitrate as nitrogen sources produced maximum protease activity, increase in substrate concentrations led to decrease in protease production. Media free of nitrogen source and containing $(NH_4)_2SO_4$showed moderate to low activity. This results was the same as (Johnvesly and Naik,2007)noticed that 1% (w/v) of sodium nitrate seems to be good nitrogen source for protease production. These results in contrast with (Mostafa,*et al*., 2006) who found that the lowest protease yield was recorded using potassium nitrate in the production medium

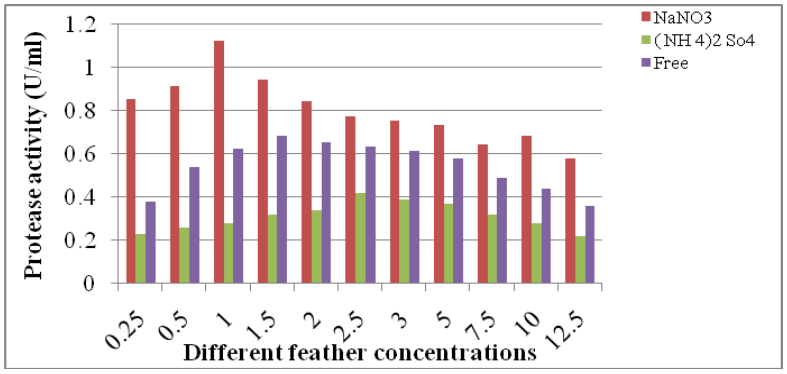

Fig (1): Effect of different substrate concentrations on protease production by *G. parasitica* LS D40.

Effect of different sodium nitrate concentrations:

Different concentrations of sodium nitrate affect on protease production. Results in Fig (2) showed that increase in sodium nitrate concentration led to increase in protease production. Maximum protease production was obtained at 2.0g/l which produce 1.88 U/ml then activity begin to decrease. This result was in agreement to (Banerjee and Bhattacharyya, 1992) who found that 0.25%sodium nitrate was the optimum for alkaline protease production from *Rhizopusoryzae*.

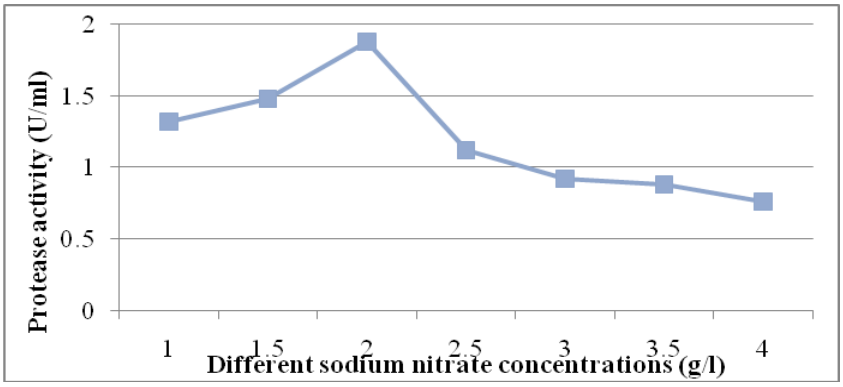

Fig (2): Different sodium nitrate concentrationson protease production by *G. parasitica* LS D40.

ISSN: 0975-8585

Optimization of mineral media components:

Mineral media component of KH_2PO_4, KCl, $MgSO_4$ were optimized for better protease production. Results in Fig.(3) showed that 0.5 g/l of KH_2PO_4, produced maximum activity of 1.89 U/ml . 1.5 g/l of KCl produce maximum activity 2.0 U/ml while 0.25 g/l of $MgSO_4$ was the optimum for protease production produce 2.03 U/ml. This results was not coincided with (Latshaw, *et al.,* 1994) who found that the optimum concentration of minerals was at 0.2 g/l for bothKH_2PO_4, $MgSO_4$ and 1.0 g/l for NaCl.

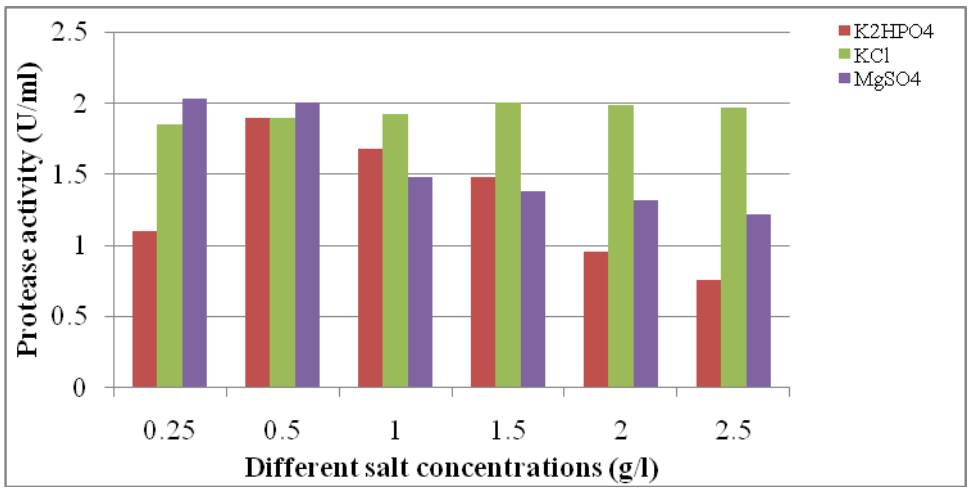

Fig (3): Effect of different concentrations of KH_2PO_4, KCl and $MgSO_4$ on protease production by by*G. parasitica* LS D40.

Effect of different incubation periods:

Protease production was carried out over 14 days incubation periods. Results in Fig.(4) showed that protease reached its maximum activity 2.32 U/ml after 6 days incubation period , after 8 days protease activity begin to decline. Shahina, *et al.,*2009 found that *Aspergillus tamarii* showed maximum protease activity after 6 days incubation period while *Aspergillus funiculosus* produce maximum activity after 5 days of incubation. Rashed, 2013found that, enzyme production was observed maximum on the fourth day of fermentation. Chinnasamy, *et al.,* 2011 found that highest enzyme activity of *Aspergillus flavus* was obtained on 7th day of incubation.

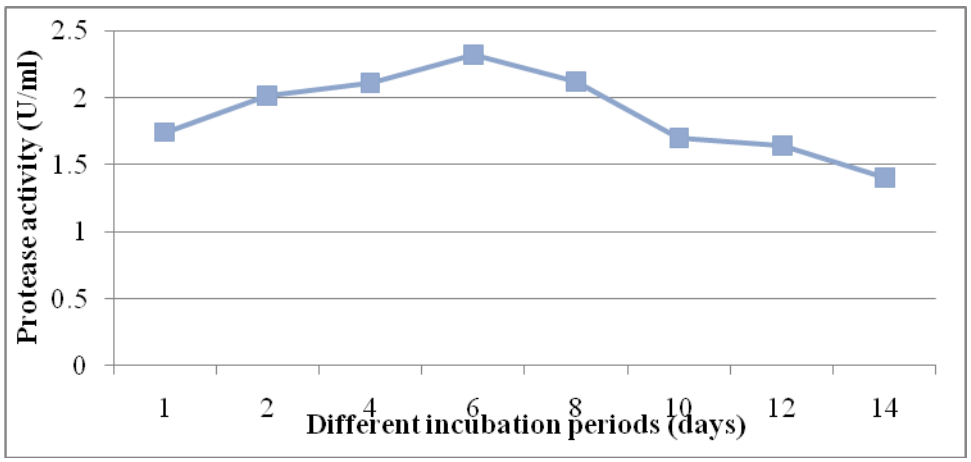

Fig (4): Effect of different incubation periods on the production of protease by*G. parasitica* LS D40.

Partial purification of protease:

Protease was partially purified using different concentrations of acetone. Results in Table (3) illustrated that the acetone fractions recovered 84.9% of the culture filtrate proteins and yielded 82.8%

recovered enzyme activity. Moreover, the acetone fraction(80%) was the richest in recovered activity (24.0%) and possessed (34.1%) of recovered protein. This fraction gave specific activity of (1.50) which reached about 0.7 fold purification. The purification of alkaline protease using acetone at 80% saturation was also reported by Thangam and Rajkumar,2002 and Omran , 2005) .

Table 3: Partial purification of protease from *G. parasitica*LS D40

Acetone concentration (%)	Protein content (mg/ml)	Protease activity (U/ml)	Specific activity (U/mg protein)	Recovered protein %	Recovered activity %	Fold of purification
C.F.	1.133	2.4	2.12			
20	0.176	0.45	2.56	15.5	19.1	1.23
40	0.195	0.47	2.41	17.2	19.7	1.15
60	0.205	0.48	2.34	18.1	20.0	1.10
80	0.387	0.58	1.50	34.1	24.0	0.70
Total	0.963	1.98		84.9	82.8	0.98

Characterization of Partially Purified Enzyme:

Different pH values of the reaction:

The effect of different pH values of the reaction mixture was determined by incubating the reaction mixture at different pH values ranging from 4.0-8.5.Results in Fig.(5) illustrated that protease activity increased with increasing pH values till reached its maximum activity (2.86 U/ml) at pH 6.0 then the activity begin to decrease. This results was not coincided with (Towhid, *et al.,* 2006) who found that the maximum protease activity of the fungal strain *As pergillus flavus* was at pH 8.0of the reaction . Abirami , *et al.,*2011found that the optimum pH of protease from *Penicillium janthinellum* was estimated to be 6.5 and from *Neurosporacrassa was* 6.5 ,proteases from *Mucor*sp. described by (Maheshwari, *et al.,* 2000)also exhibited low optimum pH The enzyme appeared to be slightly acid, which was expected for proteases produced by fungi (Reed and Nagodawithana, 1993).

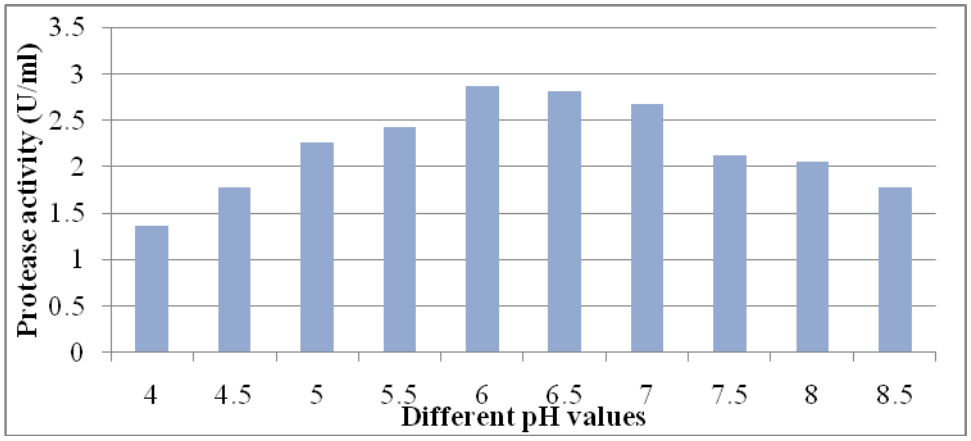

Fig (5): Effect of different pH values of the reaction on the activity of protease by *G. parasitica* LS D40.

Temperature of the reaction:

Results in Fig.(6) showed that the optimum temperature of protease from *G. parasitica* NRC D40 was at 35 °C followed by temperature 40°C, above and below this temperature moderate to low activity was determined. Proteases from the*Aspergillus*sp.(Coral, *et al*., 2003) and from *Penicillium*sp.Tunga, *et al.,* 2003 showed optimum activities at temperatures 40°C and 45°C respectively.

ISSN: 0975-8585

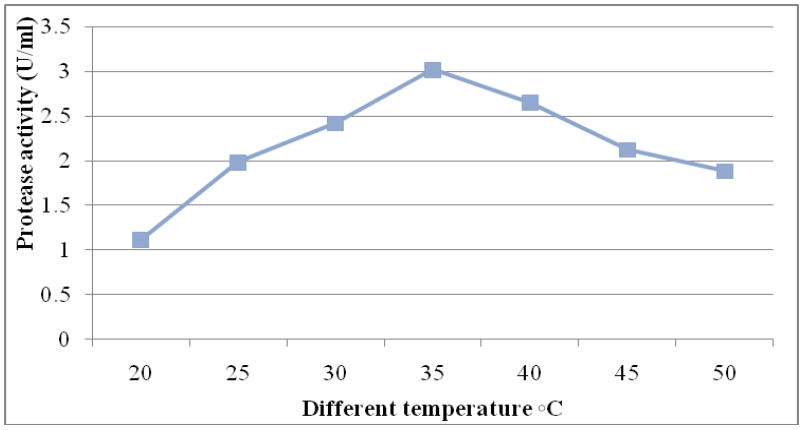

Fig (6): Effect of different temperature of reaction mixture on the activity of protease by *G. parasitica* LS D40.

Different reaction time:

Protease reach its maximum activity after 15 min of the reaction time then activity begin to decrease as shown in Fig.(7). Towhid, *et al.,* 2006found that maximum protease activity of *Aspergillus flavus* was after 60 min

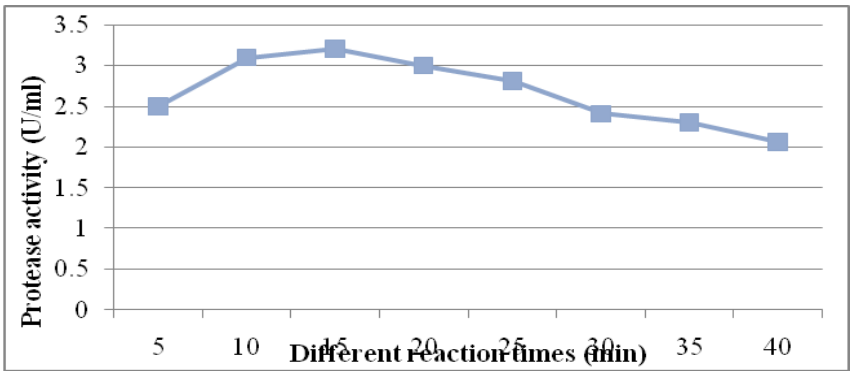

Fig (7): Effect of different reaction timeson the activity of protease by *G. parasitica* LS D40.

Different substrate concentrations:

Results in Fig.(8) showed that maximum protease activity was detected with 2% casein concentration as substrate. The activities of the crude protease from *Aspergillusflavus* at various substrate concentrations (0.5 to 5.0% of casein solution) were observed and maximum activity was obtained with 3% casein as substrate (Towhid, *et al.,* 2006).

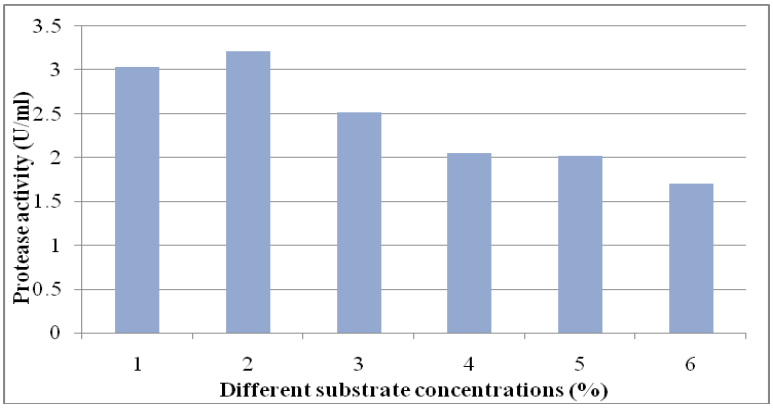

Fig (8): Effect of different substrate concentrations on protease activity by *G. parasitica* LS D40.

ISSN: 0975-8585

Thermal stability:

Results in Fig (9) showed that relative activity was stable at temperature 20 &25 ˚C for 60 min then activity begin to decrease till it reached 35 &40 ˚C enzyme loss its activity. The thermal stability of protease from *Penicillium janthinellum* and *Neurosporacrassa* was determined to be 30 - 40 ˚C and 30-40˚Crespectively. *Aspergillusparasiticus* which maintained 100% of activity at only 40˚C for 1 hour (Tunga, *et al.,* 2003). The second possible explanation may be related to the rigidity of the conformation of the enzyme molecules resulting from binding to the carrier (Afaq and J. Iqbal, 2001)

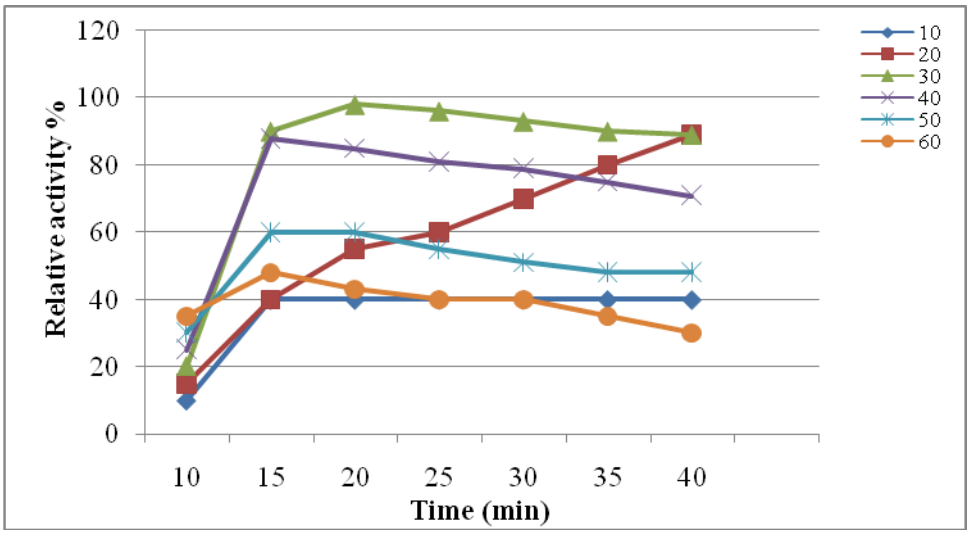

Fig (9): Effect of thermal stability on protease activity by *G. parasitica* LS D40.

CONCLUSION

The use of chicken feather as substrate was a cheap material for production and partial purification of protease from local isolate strain *G. Parasitica* LS D40 under optimum conditions.

ACKNOWLEDGMENTS

This work was supported by Prof. Dr. Mohamed Abdel Fattah Farid, Professor of Microbiology in Department of Chemistry of Natural and Microbial Products, National Research Centre, Dokki, Cairo, Egypt. The author express her sincere gratitude and gratefully acknowledge.

REFERENCES

[1] Abirami, V., Meenakshi, S. A., Kanthymathy, K., Bharathidasan, R., Mahalingam, R. and Panneerselvam, A.(2011). Partial purification and characterization of an extracellular protease from *Penicilliumjanthinellum* and *Neurosporacrassa*.European Journal of Experimental Biology, 1 (3):114-123

[2] Afaq, S. and J. Iqbal, (2001). Immobilization and stabilization of papain on chelating sepharose: a metal chelate regenertable carrier. Journal Biotechnology, 4(3): 120-124.

[3] Al-Shehri MA. (2004). Production and some properties of protease produced by *Bacillus licheniformis*isolated from TihametAseer, Saudi Arabic. Pakistan J. Biol. Sci., 7: 1631-1635.

[4] Babu N. K. S and Lakshmi K. D.(2005).Production and characterization of protease enzyme from *Bacillus laterosporus* .African Journal of Biotechnol., 4(7|) :724-726.

[5] Banerjee R., Bhattacharyya BC (1992). Optimisation of multiple inducers effect on protease biosynthesis by *Rhizopusoryzae.* Bioprocess Eng ;7:225–28.

[6] Coral, G., Arikan, B., unaldi, M.N., and Guvenmez, H. (2003), *Annals of microbiology*, 4:491 - 498.

[7] El Sayed E.M., Mostafa M.S., Hassan M.A., Mostafa, H.S. and Helemy M.H.(2012). Optimization conditions of extracellular proteases production from a newly isolated *Streptomyces pseudogrisiolus*NRC-15. CodenEcjhao E-Journal of Chemistry, 9(2): 4945-4973.

[8] Chinnasamy M., Duraisamy G., Dugganaboyana G. K., Ganesan R., Manokaran K. and Chandrasekar U.(2011). Production, purification and characterization of protease by *Aspergillusflavus*under solid state fermentation . Jordan Journal of Biological Sciences 4,(3):137-148.

[9] Germano, S., pandey, A., Osaku, C.A., Rocha, S.N., and Soccol, C.R. (2003), Enzyme and microbial technology,32: 246 -251.

[10] Hamidreza F., Mahmoud J.,Naser B., Nadia M.,Kianoush K. D.(2007).Production and purification of a protease from analkalophilic*Bacillus*sp.2-5 strain isolated from soil. Iranian Journal of Biotechnology.5(2):110-113.

[11] Johnson, S., and Pellecchia, M. (2006). Structure- and fragment based approaches to protease inhibition. *Curl' Top Med Chem*6, 317-329.

[12] Johnvesly, B. and G.R. Naik, (2001). Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic*Bacillus* sp. JB-99 in a chemically defined medium. Process Biochem*.,* 37: 139-144.

[13] Kabli SA. (2007). Purification and characterization of protopectinase produced by *Kluyveromycesmarxianus*. JKAU Sci, 19: 139-153.

[14] Latshaw, J.D. Musharaf, N. Retrum, R. (1994). Animal Feed Sci. Technol., , 47, 179-188.

[15] lakshmi, J. Sri., Madhavi, J. and Ammani. K .(2014) .Protease production by *Humicolagrisea*through ssf . Global Journal Of Bio-Science and Biotechnology,3 (3):230-235.

[16] Mao W., Pan R., Freedman D. (1992). High production of alkaline protease by *Bacillus licheniformis*in a fed-batch fermentation using a synthetic medium. J IndMicrobiol 11:1-6.

[17] Maheshwari, R., Bharadwaj, G., and Bhat, M.K (2000).Microbiology and molecular biology reviews*,* 3: 461- 488.

[18] Mostafa, E.E., M.H. Awad, M.M. Saad, M.H. Selim and H.M. Sangeeta N. and Rintu B.(2006).Optimization of amylase and protease production from *Aspergillusawamori*in single bioreactor through EVOP factorial design technique.Food Technol. Biotechnol. 44 (2) 257-261.

[19] Omran, R. (2005).Charracterization of alkaline protease from *Thermoactinomyces*sp. National J. of Chem., 19: 446-456

[20] Shahina, Z., Hossain , M. T. andHakim, M.A.(2009). Characterization of protease producing fungi *A. funiculosus*and *A tamarii*and their proteases.The Chittagong Univ. J. B. Sci*.,. 4(1 &2):91-98 .*

[21] Shaukat A.; Qazi A. H; Khan M.R and Pakistan (2003). Protease activity in seeds commonly used as herbal medicine Pakistan J. Med. Res.,42 (2).

[22] Singh A, Singh N. and Bishnoi NR. (2009). Production of cellulases by *Aspergillusheteromorphus*from wheat straw under submerged fermentation. Inter. J of Civil and Environ Eng1: 23-26.

[23] Rahman, M.U.R., Shereen G. and Zahoor, M. (2007). Reduction of Chromium (VI) by locally isolated *Pseudomonas* sp.C-171. Turk. J. Biol. 31:161-166

[24] Reed, G., and Nagodawithana, T. (1993), Enzymes in food processing, New York academic press.

[25] Prasad, D. S. R. and Raju , K. Jaya (2013) . Studies on the production of Neutral Protease by *Rhizopusoligosporus*NCIM 1215 using *Lablab purpureus*seed powder under solid state fermentation.Journal of Chemical, Biological and Physical Sciences, 3(4):2772-2782.

[26] Thangam, E.B. and Rajkumar, G.S.,|(2002).Purification and characterization of alkaline protease from *Alcaligenesfaecalis.* Biotechnol. Appl. Biochem*.,* 35: 149-154.

[27] Towhid, H. MD., Flora, D., Marzan, L.W., Shafiqur, R. MD. and Anmwar, M.N. (2006). Some properties of protease of the fungal strain *Aspergillusflavus.* International Journal of Agriculture & Biology,2:162-164.

[28] Tunga, R., Shrivastava, B., and Banerjee, R. (2003).Purification and characterization of a protease from solid state cultures of Aspergillusparasiticus. Process biochemistry, 38: 1553 - 1558.

[29] Tsuchida O, Yamagota Y, Ishizuka J, Arai J, Yamada J, TakeuchiM, and Ichishima E (1986). An alkaline protease of an alkalophilic*Bacillus* sp. Curr. Microbiol. 14:7-12.