Iraqi Journal of Biotechnology, 2022, Vol. 21, No. 2, 488-498

Optimum Conditions of Production and Purification of Gastroenteritis *E. coli* Protease Isolated from Iraqi Patients

Ataa R. Khayoon , Ali J. AL-Sa'ady

Department of Biotechnology, College of Science, University of Baghdad

Received: 1/6/2022 Accepted: 10/10/2022 Published: December 20, 2022

Abstract: This study was performing on isolates of *Escherichia coli* bacteria, which isolated from stool of people with gastroenteritis and diarrhea. These isolates were examined on skim milk agar medium in order to screening if isolates produce protease enzyme, as well as determination some optimal conditions for protease production like carbon source, nitrogen source pH, temperature, and duration of incubation. The protease was purified by ion exchange chromatography and gel filtration after its precipitated by 0-80% ammonium sulphate. The results showed that the highest production of protease from local *E. coli* 29 isolate in the medium containing of brain heart infusion broth supported with fructose, sodium nitrate, pH 6.0, and incubated at 37 °C, for 24 hours. The results of protease purification by ion exchange and gel filtration chromatography, showed that the specific activity was 19250 U/mg, yield 49 and purification fold 4.94.

Keywords: Optimum conditions, Ion exchange chromatography, gel filtration chromatography.

Corresponding author: (Email: ataa.raghad1206a@sc.uobaghdad.edu.iq).

Introduction

Proteases are the most common enzymes produced by microbial sources. For biotechnological processes, Microbial proteases with attractive features are optimal enzymes for these purposes (1). Proteases are enzymes that catalyze the breakdown of protein molecules into peptides and amino acids. It has been applied in a variety of industries including pharmaceutical, food, and detergent industries, also provide the requirements of 60% of the market for enzymes worldwide, since they required a short period of time to be cultured in large quantities, as a result, the production is abundant and genetic modification is easier than in plants and animals (2,3, 4). According to their optimum pH, they are divided into acidic, neutral, and alkaline proteases, and alkaline proteases account for around 89% of the overall sales of proteases (5). Based on the functional group in their binding sites, proteases are categorized into six types. They are metallo, serine, threonine, aspartic, cysteine, glutamic, and cysteine proteases (6). Different factors such as nitrogen and carbon source, pH, media, temperature, and incubation time can all be optimized to increase the output of industrially important protease (7,8). Proteases are important virulence factors in diseases because they increase organism's invasiveness by destroying host tissue and interference with the

host's antibacterial immune system (9). *Escherichia coli* is an adult-lifelong colonizer of the gastrointestinal tract of human and it is a Gram-negative bacterium, belong to Enterobacteriaceae family, and its facultative anaerobic. This species interacts with the host in the mucosal layer as an unharmful commensal. However, some strains of *E. coli* have pathogenic characteristics and can cause infections, and in some situation, nonpathogenic intestinal *E. coli* may ultimately cause or lead to diseases in the hosts (10). When *E. coli* reaches unnatural places, it can cause a wide range of infectious diseases, including septicaemia, meningitis, wound infections, urinary tract infections, and other infections of soft tissues (11). The objective of this study was to find the best possible conditions for the maximum production of protease enzyme from *E. coli* bacteria that was isolated from the stool of patients suffering from diarrhea and gastroenteritis, also the study involved purification of this enzyme by ion exchange chromatography and gel filtration.

Materials and method

Materials

Skim milk agar, brain heart infusion broth, brain heart infusion agar (Hi-media, India). Coomassie brilliant blue G-250 from BDH-England, trichloroacetic acid (TCA) from Alpha-chemika-India.

Samples collection

147 Bacterial isolates on MacConkey agar were obtained from many hospitals in Maysan city, which isolated from stool of patients suffering from diarrhea and gastroenteritis, then cultured on EMB media and diagnosed with Vitek device. Then the isolates were culture in Brain Heart infusion Broth and incubated for 24 hours at 37°C until used.

Primary screening for protease production

The bacterial suspensions for 147 isolates were prepared by culturing each bacteria colony taken by loop in 25 ml Brain Heart Infusion broth medium and incubated at 37 °C for 24 hours. Then the skim milk agar (51grams in 1000 ml) was prepared, sterilized by Autoclave, warmed and poured into sterilized Petri dishes, then holes (5mm) were prepared on the skim milk agar by using sterile tips under sterilizing conditions. this media was used to determine bacteria's ability to produce protease enzymes. A volume of 20 µl of bacteria suspensions was added to the prepared holes on skim milk agar plates and incubated at 37 °C for 24 hours. The clearance zone area in diameters (mm) that appear around the holes (which indicated for protease activity), were measured. Based on which isolates had given the largest clearing zones ratio, the isolates were chosen (12). 5 isolates were selected due to they showed the largest clearance zone which indicate for protease activity.

Secondary screening for protease production

The secondary screening for protease included culturing $8*10^6$ cells\ml from each 5 bacterial isolates (that gave the largest clearance zones on skim milk agar), in 25 ml Brain Heart infusion Broth and incubated at 37 °C for 24 hours. Then 10 ml of the suspension was filtered by filter paper and centrifuged at 5000 rpm for 20 min. Protease activity and protein concentration were determined using the supernatant (13).

Protease activity assay methods

Protease activity was done depend on the method described by (14), as following: whereas add 1.8 ml of casein solution (1%) into test tubes and incubated in water bath at 30 °C for 5 minute. Then add 0.2 ml of enzymatic solution to casein solution and placed in the water bath at 37 ^{0} C for 30 min. The reaction was ended by adding (3 ml) of the (5%) TCA, and the solution was centrifuged at 6000 rpm for 15 minutes. The control test was prepared by adding (3 ml) of 5 % TCA to (1.8 ml) of the 1 % casein solution and then added (0.2 ml) enzymatic solution. The absorbency for the supernatant was measured at 280 nm of UV-VIS-spectrophotometer. Enzymatic activity (unit/ml) was determined depended on the degradation of casein protein into small peptides and soluble amino acids.

Protein concentration assay

The concentration of protein was determined according to Bradford assay(15).

Optimization for protease production

Carbon sources

Several carbon sources were used to find out which carbon source is able to increase protease production by *E. coli* bacteria, where used 6 flasks each one containing 25 ml of sterilized(by autoclave) Brain Heart Infusion broth media with 0.25 mg % of ml of carbon sources (glucose, fructose, maltose, sucrose, cellulose, and starch) which added to each flask separately, then all flasks(with pH 7) was inoculated with 8* 10^{6} cells\ml of the bacterial suspension and incubated in the incubator at 37°C for 24 hours. After that the protein concentration and protease activity was estimated(15).

Nitrogen sources

Different nitrogen sources were used to check which nitrogen source is able to increase protease production by *Escherichia coli* bacteria, where used 6 flasks each one containing 25 ml of sterilized(by autoclave) Brain Heart Infusion broth and 0.25 mg % fructose with 0.25 mg % of ml of nitrogen sources (yeast extract, trepton, ammonium sulphate, Sodium nitrate, urea, and Calcium nitrate) which added to each flask separately, then all flasks (with pH 7) was inoculated with 8*10^{6} cells\ml of the bacterial suspension and incubated at 37°C for 24 hours. After that the protein concentration and protease activity was estimated (16).

pH

To examine their impact on the production of proteases, optimized production medium was changed with various pH values (3, 4, 5, 6, 7, 8, and 9), then incubated at 37°C for 24 hours and measured the protein concentration and protease activity (17).

Temperature

This experiment involved incubated the optimum medium (that consist of brain heart infusion broth medium, fructose, Sodium nitrite, pH 6 and inoculated with 8 * 10^{6} cells\ml of bacterial suspention) at different temperatures (25, 30, 35, 37, 40, and 45) for 24 hours to find out which is the optimum temperature for production the highest value of protease by *Escherichia coli*. Then the protein concentration and enzyme activity were measured (18).

Incubation period

Incubation time consider as important factor for enzyme production. The brain heart infusion broth with the fructose, Calcium nitrate, pH 6,

inoculated with 8×10^6 cells\ml of bacterial suspension and incubated in different hours (12, 24, 36, 48). The protein concentration and enzyme activity were estimated to each period (20).

Purification of protease by Ion exchange chromatography

The enzyme was precipitated using an ammonium sulfate technique with 0-80% as saturated ratio. The concentrated enzyme was applied in a circular motion using a clean pasteur pipette to an exchange chromatography column that was packed with CM-Cellulose (28×1.7 cm). Tris-HCl (0.005 M, pH 8.0) was used to equilibrated the column and the column eluted by NaCl gradient (0.1-1 M) with a flow rate of 30 ml/hr and 3 ml for each fraction. The absorbance at wavelength 280 nm by spectrophotometer for all fractions, were measured. Then the enzyme activity and protein concentration were measured for the activation fractions.

Gel filtration chromatography

Gel Filtration Chromatography technique was using for more purification of protease. Proteins and peptides can be separated by Gel Filtration Chromatography depend on their size. Firstly, prepared and packed of Sephacryl S-300(100×1.5 cm) column (Pharmacia-Sweden). The fractions that provided the most activity of protease enzyme, which was 6 ml of enzyme solution, was add to the Sephacryl S-300 column and with flow rate of 30 ml/hour, Tris-HCl (0.005 M, pH 8.0) for equilibrated and mucin (2ml) was add to the column, eluted with Tris-HCl (0.005 M, pH 8.0). 5 ml for each fraction was collected, then the absorbance at 280nm was measured for each fraction by spectrophotometer.

Results and discussion

The result of screening for protease production from *E. coli* were showed that the *E. coli* A29 was given the highest yield of protease enzyme on skim milk agar by formation of hydrolysis zone around colonies 65 mm (Figure 1), and in secondary screening which involved assay of protease activity and enzyme concentration, the specific activity of *E. coli* A29 was (3441) unit/mg as in figure 2.

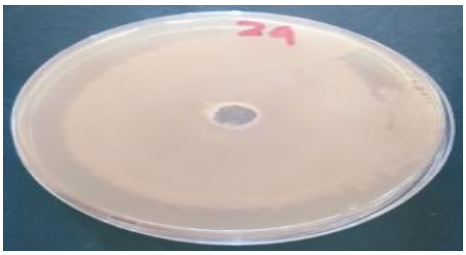

Figure (1): Formation of hydrolysis zone by *E. coli* A29.

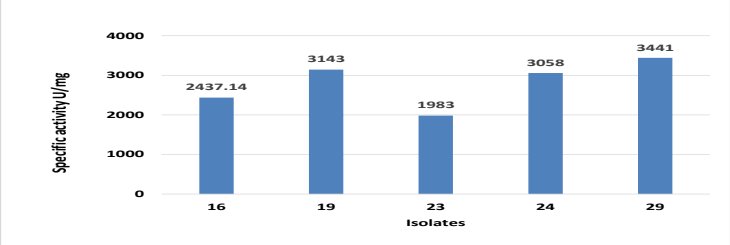

Figure (2): The optimized of isolate *E.coli* A29 for protease production. Optimization of culture condition for protease production.

Carbon source

Fructose was the most carbon source that provided the highest level of protease production with specific activity (2293.75 U/mg), as shown in figure (3). In contrast to another research on *Enterococcus faecium* by Ahmed *et al*. (21), sucrose exhibited to be the optimal carbon source that gave the highest level of protease production, while in other study on *Bacillus subtilis* by Qureshi *et al*. (22), the most optimal carbon source was glucose.

\

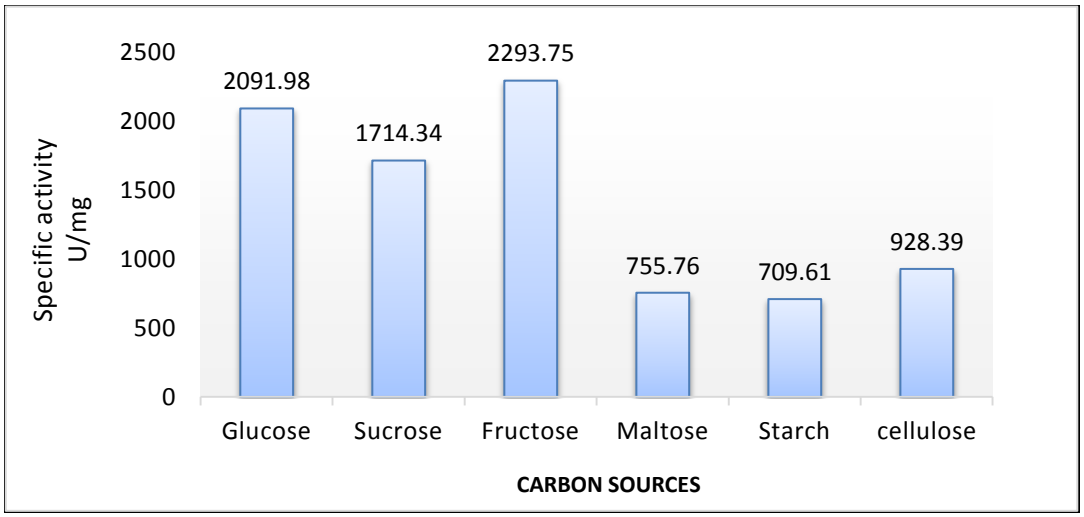

Figure (3): The effect of carbon sources on protease production from E.coli A29 using bran heart infusion broth media, pH 7, incubation at 37 for 24 hours.

Nitrogen source

Figure (4) display that the Sodium nitrite, between others nitrogen sources, showed the highest level of protease production with specific activity (2329.75 U/mg). In contract with other study on *Enrerococcus faecium* bacteria by Ahmed *et al*. (21), the yeast extract act as the best nitrogen sources that increase protease activity.

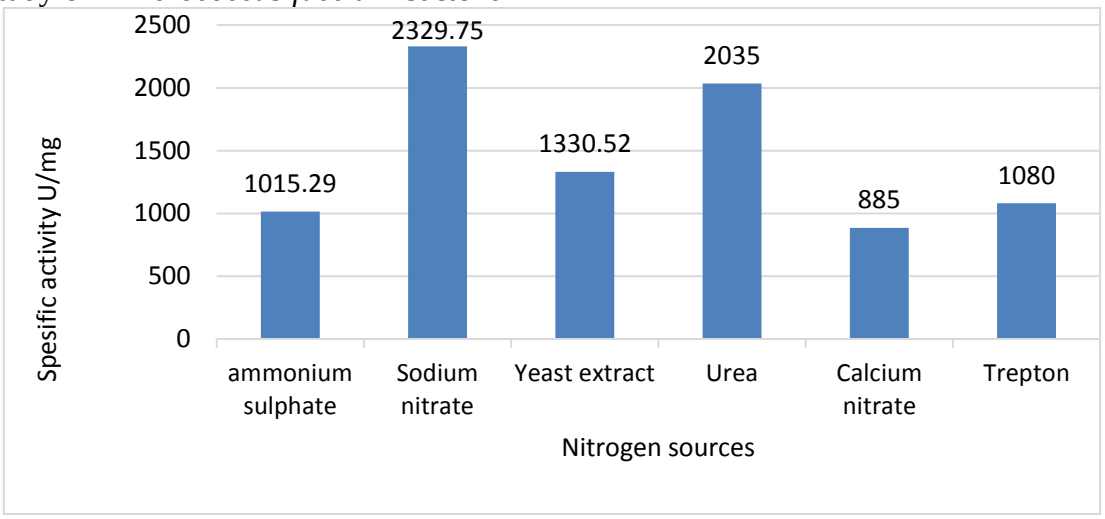

Figure (4): The effect of nitrogen sources on protease production from *E.coli* A29 using bran heart infusion broth media,fructose, pH 7, incubation at 37 for 24 hours.

Effect of pH on protease production

The effect of pH on protease production by *E.coli* A29 was studied in the pH range of (3 - 9). The pH of the medium was adjusted to the desired level by adding 0.1 N HCl or 0.1 N NaOH. Protease production increased with increasing initial pH of medium and reached a maximum at pH 6 at specific activity (3895.71 U/mg), however yield was low above and below this level of pH. The resulting pH was less than pH 9 of other study reported by Prakasham *et al*. (23) on *E.coli*. Figure (5) show the result.

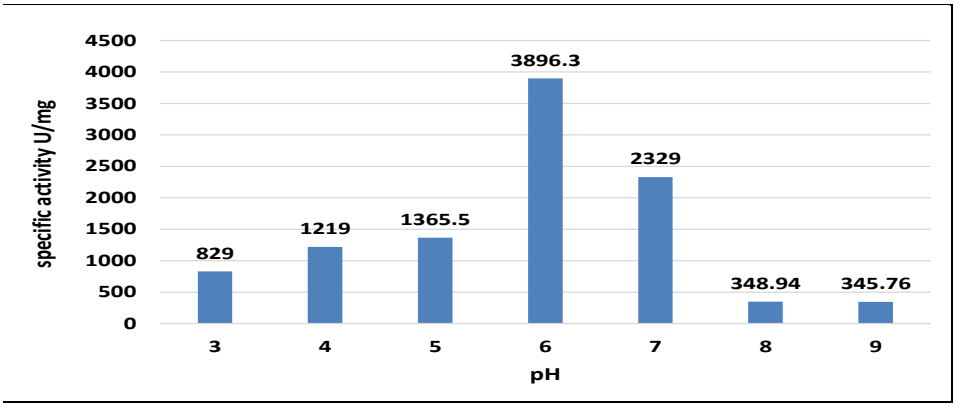

Figure (5): The effect of different pH on protease production from *E. coli* A29 using bran heart infusion broth media, fructose, Sodium nitrite, incubation at 37 for 24 hours.

Effect of temperature on protease production

The effect of temperature on protease production from *E.coli* A29 was performed the results in figure (6) showed that the highest specific activity was at 37 °C to give 3895.71 U\ml, compare with other temperature in other study by Baehaki *el al*. (24), in which the *E.coli* provided higher protease production at 40 °C while in other study by Nadeem *et al*. (25), the optimal temperature was 60°C for *Bacillus licheniformis*.

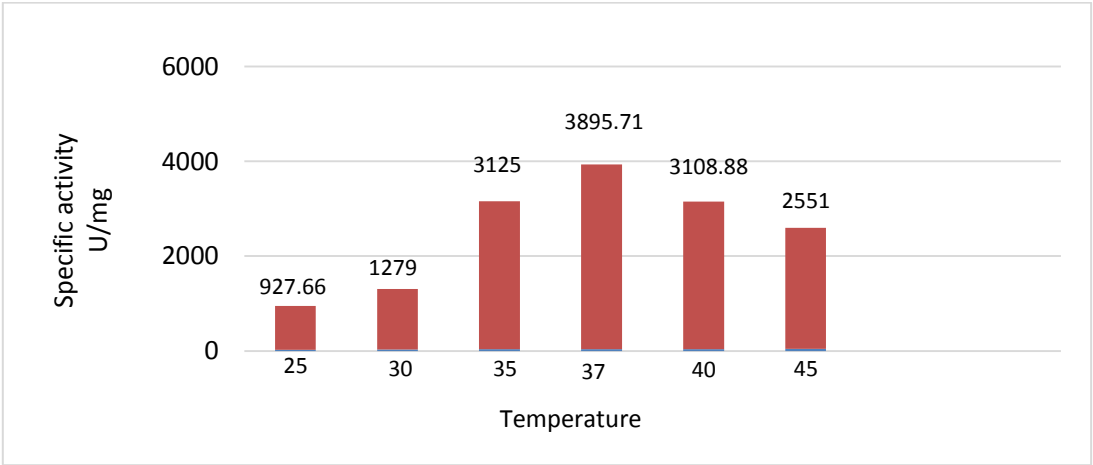

Figure (6): The effect of different temperatures on protease production from *E.coli* A29 using bran heart infusion broth media, fructose, Sodium nitrite, pH 6, for 24 hours.

Effect of incubation time on protease production

Different incubation times (18, 24, 36, 48) hrs. were utilized to identify the maximum protease production by *E.coli* A29, a high level of enzyme was observed after 24 hours (specific activity 3897.8 unit/mg), figure (7) show the result . This was similar to *E.coli* in previous study by Abed *et al*. (2) while in other study by Palsaniya *et al*. (26) , the optimal incubation period was 48 hours.

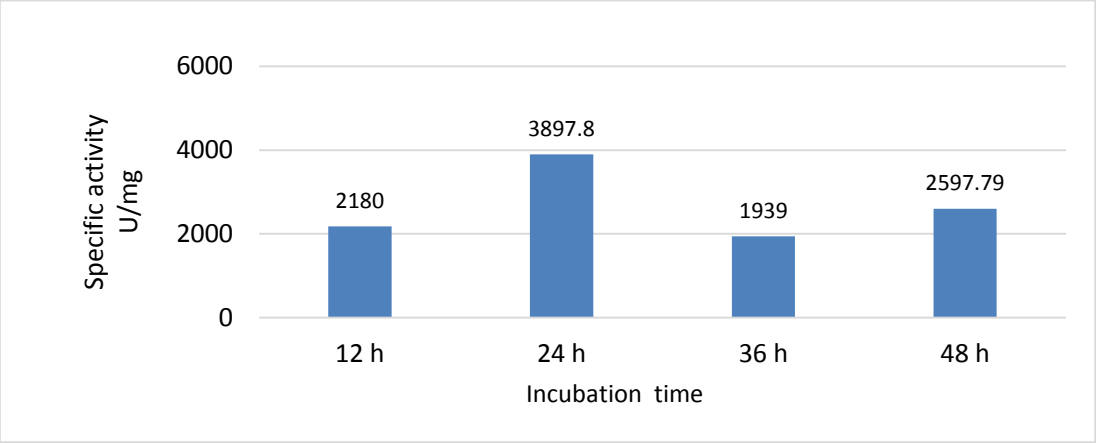

Figure (7): The effect of different incubation time on protease production from *E.coli* A29 using brain heart infusion broth media, fructose, sodium nitrite, pH 6, for 24 hours.

Purification of protease by Ion exchange chromatography

Ion exchange chromatography is a technique for separating molecules based on charge, shape, size, and solubility (27). In this procedure, there are mobile and stationary phases, CM-Cellulose is a type of weak cation exchanger, bind to the reverse charge of the proteins (28). The results as in figure 8, one peak appeared in washing step with 0.005 M Tris-HCl (pH 8), which represented between the fractions 14-41, and also three peaks in elution step which represented by fraction 61-71, 70-77, and 79-85. Enzyme activity and protein concentration for each fractions were determined. Protease activity was appeared at 13-29 fractions in washing step. As found in table (1), the protein concentration was 0.013 mg/ml, the activity was 90.56U/ml, and the specific activity was 18112 U/mg, the purification fold of 4.6 and 60 yield were obtained. Other study by Chung and Goldberg (29,32) was used CM-cellulose exchanger for protease purification from *E. coli*, showed that the protein concentration was 3.8, specific activity 75.1 U/ml, purification factor 363, recovery 121%. Additional research by Sharma and De included purification of protease enzyme produced from *Aspergillus tamari*, in which the protein concentration was 0.4mg/ml, the purification fold was 26, yield 50% and specific activity of 11078 U/mg and (30).

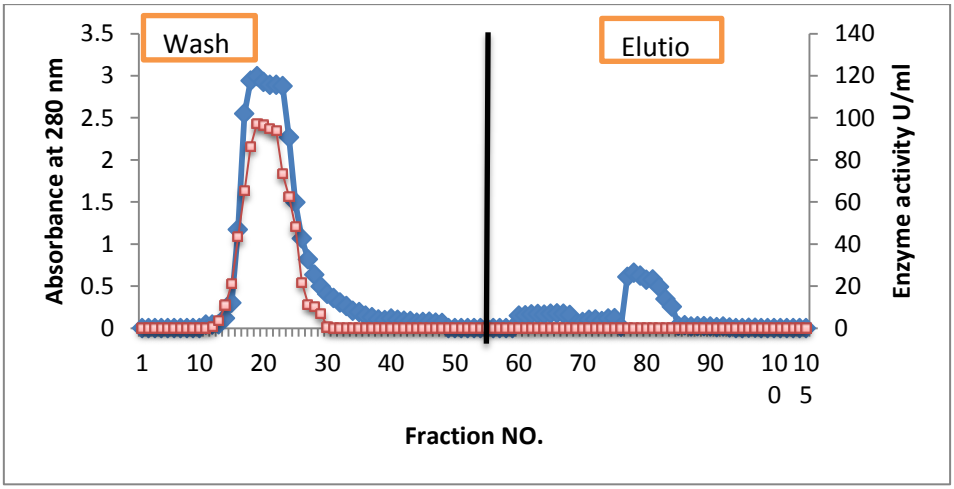

Figure (8): Ion exchange chromatography for purification of protease from *E.coli* by using CMC-column (28-1.7 cm) equilibrated with Tris-HCl (0.005 M, pH 8.0), eluted with Tris-HCl with NaCl gradient (0.1-1 M) in flow rate 30 ml/ hr , 3 ml for each fraction.

Gel Filtration Chromatography

Gel Filtration Chromatography technique was used as an additional step for protease enzyme purification. "Sephacryl S-300" (100×1.5 cm) column was prepared and utilized. The results that seen in figure (9) involved occurrence of two peaks was appeared which represented by fractions 29-41 and 63-67, one of them (29-41) referred to protease enzyme, one of them (29-41) referred to protease enzyme with specific activity was 19250 U/mg, yield 49 and purification fold 4.94 and, in contract with other research for purification of protease isolated from *Bacillus sp*, the specific activity of 916.76 U/mg, yield 1.68 and purification fold 4.31 (31).

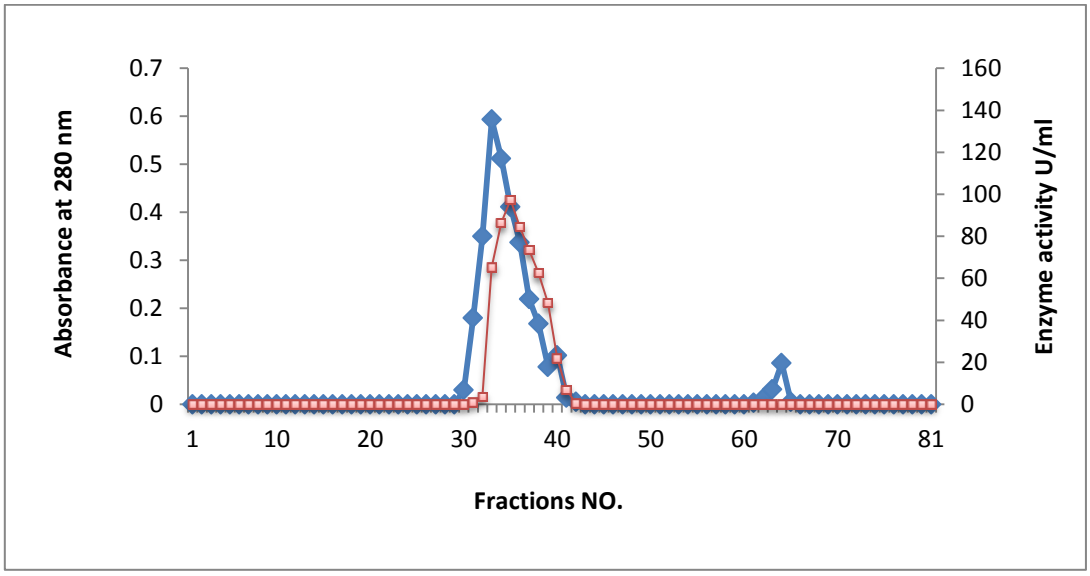

Figure (9): Purification of protease by using Sephacryl S-300 column (100×1.5 cm) with flow rate of 30 ml/hour, eluted with Tris-HCl (0.005 M, pH 8.0) and 5 ml for each fraction.

Table (1): Purfication steps of protease from *E.coli.*

Step	Volume ml	Activity U/ml	Protein concen. mg/ml	Specific activity U/mg	Total activity	Fold	Yield %
Crude enzyme	75	27.27	0.007	3896	2045	1	100
Precipitation by ammonium sulphate (0-80 % saturated)	15	98.88	0.013	7606	1483	1.95	73
Purification by Ion-exchange chromatography	14	90.56	0.005	18112	1267.9	4.6	62
Purification by Gel-filtrationchromatography	13	77	0.004	19250	1003	4.94	49

Conclusion

Escherichia coli bacteria isolated from stool of unhealthy human, is able to produce protease enzyme in some pathological conditions such as gastroenteritis. In this study, some optimum conditions were optimized for the production of protease enzyme in high quantities, such as carbon and nitrogen source, pH, temperature, and incubation period, respectively (Fructose, Sodium Nitrate, 6, 37°C, 24).

References

1. Naveed, M.; Nadeem, F.; Mehmood, T.; Bilal, M.; Anwar, Z. and Amjad, F. (2021). Protease-a versatile and ecofriendly biocatalyst with multi-industrial applications: an updated review. Catalysis Letters, 151(2): 307-323.
2. Abed, B.K.; Authman, S.H. and Yassein, K.H. (2016). Optimization of extracellular protease extracted from Escherichia coli. European Journal of Pharmaceutical and Medical Research, 3(2): 113-118.
3. Ahmad, M.S.; AbdEl-Salam, B.A.; Yaser, M.M. and Taha, S.S. (2018). Optimization and characterization of bacterial proteinase enzyme using whey as a fermentation medium. Journal Advance Pharmaceutical Education Research, 8(2): 63-76.
4. Chimbekujwo, K.I.; Ja'afaru, M.I. and Adeyemo, O.M. (2020). Purification, characterization and optimization conditions of protease produced by Aspergillus brasiliensis strain BCW2. Scientific African, 8: e00398.
5. Zhou, C.; Qin, H.; Chen, X., Zhang, Y.; Xue, Y. and Ma, Y. (2018). A novel alkaline protease from alkaliphilic Idiomarina sp. C9-1 with potential application for eco-friendly enzymatic dehairing in the leather industry. Scientific Reports, *8*(1): 1-18.
6. Lakshmi, B.K.M.; Sri, P.R.; Devi, K.A. and Hemalatha, K.P.J. (2014). Media optimization of protease production by Bacillus licheniformis and partial characterization of Alkaline protease. International Journal of Current Microbiology and Applied Sciences, 3(5): 650-659.
7. Sharma, S.A.V.I.T.R.I.; Bhat, G.K. and Shenoy, S. (2007). Virulence factors and drug resistance in Escherichia coli isolated from extraintestinal infections. Indian Journal of Medical Microbiology, 25(4): 369-373.
8. Maitig, A.M.A.; Alhoot, M.A. and Tiwari, K. (2018). Isolation and screening of extracellular protease enzyme from fungal isolates of soil. Journal of Pure and Applied Microbiology, 12(4): 2059-2067.
9. Shaheen, M.; Shah, A.A.; Hameed, A. and Hasan, F. (2008). Influence of culture conditions on production and activity of protease from Bacillus subtilis BS1. Pakistan Journal of Botany, 40(5): 2161-2169.
10. Palsaniya, P.; Mishra, R.; Beejawat, N.; Sethi, S. and Gupta, B.L. (2012). Optimization of alkaline protease production from bacteria isolated from soil. Journal of Microbiology and Biotechnology Research, 2(6): 858-865.
11. Nizet, V. and Klein, J. (2014). Bacterial sepsis and meningitis. Infectious diseases of the fetus and newborn infant, 8: 217-71.

12. Shehada, W.; Uraz, G.; Demirel, R. and Hamamcı, H., 2021. Proteolytic Activity of Aspergillus niger Strains Isolated from Soil. International Journal Current Microbiological Applied Science, 10(02): 67-75.
13. Maitig, A.M.A.; Alhoot, M.A. and Tiwari, K. (2018). Isolation and screening of extracellular protease enzyme from fungal isolates of soil. Journal of Pure and Applied Microbiology, 12(4): 2059-2067.
14. Brock, F.M.; Forsberg, C.W. and Buchanan-Smith, J.G. (1982). Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. Applied and Environmental Microbiology, 44(3): 561-569.
15. Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72(1-2): 248-254.
16. Ikram-Ul-haq, H.M. and Umber, H. (2006). Production of protease by Penicillium chrysogenum through optimization of environmental conditions. Journal of Agriculture and Social Sciences, 2(1): 23-25.
17. Baehaki, A.; Nurhayati, T.; Palupi, N.S. and Suhartono, M.T. (2015). Production and characterization of protease from patogenic bacteria Escherichia coli. Journal of Chemical and Pharmaceutical Research, 7(10): 776-780.
18. Pushpam, P.L.; Rajesh, T. and Gunasekaran, P. (2011). Identification and characterization of alkaline serine protease from goat skin surface metagenome. AMB Express, 1(1): 1-10.
19. Bentubo, H.D.L. and Gompertz, O.F., (2014). Effects of temperature and incubation time on the in vitro expression of proteases, phospholipases, lipases and DNases by different species of Trichosporon. SpringerPlus, 3(1): 1-10.
20. Chimbekujwo, K.I.; Ja'afaru, M.I. and Adeyemo, O.M. (2020). Purification, characterization and optimization conditions of protease produced by *Aspergillus brasiliensis* strain BCW2. Scientific African, 8: e00398.
21. Ahmad, M.S.; AbdEl-Salam, B.A.; Yaser, M.M. and Taha, S.S. (2018). Optimization and characterization of bacterial proteinase enzyme using whey as a fermentation medium. Journal Advance Pharmaceutical Education Research, 8(2): 63-76.
22. Qureshi, A.S.; Bhutto, M.A.; Khushk, I. and Dahot, M.U. (2011). Optimization of cultural conditions for protease production by Bacillus subtilis EFRL 01. African Journal of Biotechnology, 10(26): 5173-5181.
23. Prakasham, R.S.; Rao, C.S. and Sarma, P.N. (2006). Green gram husk-an inexpensive substrate for alkaline protease production by Bacillus sp. in solid-state fermentation. Bioresource.
24. Baehaki, A.; Nurhayati, T.; Palupi, N.S. and Suhartono, M.T. (2015). Production and characterization of protease from patogenic bacteria Escherichia coli. Journal of Chemical and Pharmaceutical Research, 7(10): 776-780.
25. Nadeem, M.; Qazi, J.I.; Syed, Q. and Gulsher, M. (2013). Purification and characterization of an alkaline protease from Bacillus licheniformis UV-9 for detergent formulations. Songklanakarin Journal of Science and Technology, 35(2).
26. Palsaniya, P., Mishra, R., Beejawat, N., Sethi, S. and Gupta, B.L. (2012). Optimization of alkaline protease production from bacteria isolated from soil. Journal of Microbiology and Biotechnology Research, 2(6): 858-865.
27. Ebere, E.C.; Obinna, I.B. and Wirnkor, V.A. (2019). Applications of column, paper, thin layer and ion exchange chromatography in purifying samples: Mini review. SF Journal of Pharmaceutical and Analytical Chemistry.
28. Noori, H.A. and Aziz, G.M. (2020). Purification, Characterization, and Evaluation of Fibrinolytic Activity of Staphylokinase from Locally Isolated Staphylococcus aureus GH38. The Iraqi Journal of Agricultural Science, 51(4): 1195-1203.
29. Chung, C.H. and Goldberg, A.L. (1983). Purification and characterization of protease So, a cytoplasmic serine protease in *Escherichia coli*. Journal of Bacteriology, 154(1): 231-238.
30. Sharma, N. and De, K. (2011). Production, purification and crystallization of an alkaline protease from Aspergillus tamarii [EF661565. 1]. Agriculture and Biology Journal of North America, 2(7): 1135-1142.
31. Margino, S.; Jumi'ati, J. and Ngadiman, N., (2013). Purification and

Characterization of Protease From *Bacillus sp*. TBRSN-1. Indonesian Journal of Biotechnology, 18(2), 151-160.

32. AL-Shuwaikh, A. M., Ibrahim, I. A., & Al-Shwaikh, R. M. (2015). Detection of E. coli and rotavirus in diarrhea among children under five years old. Iraqi Journal of Biotechnology, 14(1).