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Biodisintegration of human mucin protein by protease produced from Escherichia coli AJ55 isolated from Urinary Tract Infection of Iraqi patients

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ABSTRACT

Proteases have various applications in the food, pharmaceutical, medicine, pathogenicity of some pathogenic bacteria, and detergent sectors as well as meeting the needs of approximately 60% of the global enzyme industry, whereas they catalyze the breakdown of protein molecules into peptides and amino acids. Production and purification of protease enzyme by the isolate *Escherichia coli* AJ55 was scrutinized in the present study. Cultivation optimum conditions, were various complex medium, carbon source, nitrogen source, temperature, pH of the medium, and time of incubation were optimized to enhance the total protease production in shake flask culture of *E.coli* AJ55. The nutrient broth supplemented with 2% glucose and 2% yeast extract, with a pH of 7.0 and incubated at 37 °C for 24 hours, better conditioned for producing the maximum production of protease. *Escherichia coli* AJ55 proteolytic enzyme was separated and purified using ion-exchange chromatography on a DEAE-Cellulose column and Sephadex G-150 gel after being precipitated with 0-70% saturated ammonium sulfate. Protease that had been partially purified had a yield of 34%, a purification fold of 13.4, an activity of 12.16 U/ml, a protein concentration of 0.005 mg/ml, and a specific activity of 2432 U/mg. By using gel filtration chromatography on a Sephadex G-150 gel, partially purified protease was examined for its ability to cleave the mucin protein. The findings of mucin biodegradation showed that the five fractions of the small peptides were produced after treatment of mucin with partially purified protease.

Keywords: Optimum conditions, Purification, Mucin, Cleavage.

التحلل الحيوي لبروتين الميوسين باستخدام انزيم البروتيز المنتج من بكتيريا *Escherichia coli* AJ55 الممرضة المعزولة من اللتهابات المسالك البولية للمرضى العراقيين

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الخلاصة

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ان انزيمات البروتيزات لها تطبيقات مختلفة في قطاعات الأغذية والأدوية والطب والإمراضية لبعض البكتيريا المسببة للأمراض والمنظفات بالإضافة إلى تلبية احتياجات ما يقرب من 60٪ من صناعة الإنزيمات العالمية ، حيث أنها تحفز تكسير جزيئات البروتين إلى ببتيدات وأحماض أمينية . في هذه الدراسة تم فحص إنتاج وتنقية إنزيم البروتيز من بكتريا *Escherichia coli* AJ55. كما تم تحديد الظروف المثلى للزراعة ، من حيث الوسط المعقد للتنمية ، ومصدر الكاربون ، ومصدر النيتروجين ، ودرجة الحرارة ، و الاس الهايدروجيني للوسط ، ووقت الحضانة لتعزيز إجمالي إنتاج انزيم البروتيز من بكتريا *E.coli* AJ55 في حاضنة زرعية هزازة. حيث ان وسط المرق المغذي و المضاف إليه 2٪ جلوكوز و 2٪ مستخلص الخميرة ، مع اس هايدروجيني 7.0 ودرجة حرارة حضن في 37 درجة مئوية لمدة 24 ساعة ، كانت افضل ظروف لانتاج انزيم البروتيز. تم فصل انزيم البروتيز المنتج من بكتريا *E.coli* AJ55 وتنقيته باستخدام كروماتوجرافيا التبادل الأيوني على عمود DEAE-Cellulose و Sephadex G-150 gel بعد ترسيبه بنسبة 0-70٪ بكبريتات ألامونيوم المشبعة. حيث ان البروتيز الذي تم تنقيته جزئيًا كان له حاصل بحدود 34٪ ، وعدد مرات تنقية 13.4 ، وفعالية 12.16 وحدة / مل ، وتركيز بروتين 0.005 ملغم / مل ، مع فعالية نوعية حوالي 2432 وحدة / ملغم. ايضاً تم استخدام كروماتوغرافيا الترشيح الهلامي على هلام Sephadex G-150 ، لتحديد قدرة البروتيز المنقى جزئياً على تحطيم بروتين الميوسين. أظهرت نتائج التحلل الحيوي للميوسين ظهور خمسة اجزاء لببتيدات صغيرة تم إنتاجها بعد معالجة بروتين الميوسين بانزيم البروتيز المنقى جزئيًا.

Introduction

The predominant enzymes produced by microbial sources are proteases. The best microbial proteases for biotechnological processes are those with desired properties [1]. Protease is employed in a variety of industries, including the food, pharmaceutical, and detergent industries, and it meets the needs of over 60% of the global enzyme market [2]. It catalyzes the breakdown of protein molecules into peptides and amino acids. Because they can be grown in huge numbers quickly, produce abundance, and are easier to modify genetically than plants and animals, microorganisms are an appealing supply of protease enzymes [3, 4].

There are several publications in the literature on the secretion of proteins by fungi and Gram-positive bacteria, but there are relatively few researches on the secretion of proteins by Gram-negative bacteria, particularly *Escherichia coli*. Despite the fact that *E.coli* is frequently utilized as a model pathogen and for the generation of recombinant proteins in the biotechnology sector [5], this is the case. Short bacilli, non-spore-forming, facultatively anaerobic, gram-negative bacteria [6], belonging to the Enterobacteriaceae family, and *Escherichia coli* are all characteristics of this organism [7]. It is produced on ~~a~~ basic media. A significant portion of the typical gut flora in humans includes *E.coli*. Enteropathogenic *E.coli* (EPEC), enterotoxigenic *E.coli*, enterohemorrhagic *E.coli* (EHEC) [8], enteroinvasive *E.coli*, enteraggregative *E.coli*, and diffusely adherent *E.coli* are six groups of intestinal pathogens that have been well documented. Pilli, enterotoxins (LT, ST) [9], endotoxins (lipopolysaccharide), Shiga-like toxins, hemolysin, intimin, aerobactin, cytonecrotizing factor, and biofilm development are some of the virulence factors that contribute to the pathogenicity of *E.coli* [8, 10, 11].

The well-known virulence factor known as proteases helps many pathogens evade the immune system and survive. In general, *Escherichia coli* is categorized as a non-secretor of protein. However, a variety of virulence factors' secretory mechanisms in pathogenic *E.coli* have been extensively researched. Contrarily, it is generally accepted that nonpathogenic *E.coli* do not produce protein; nonetheless, reports have revealed that *E.coli* laboratory strains also include cryptic genes that code for secretion and pilation . These genes can be expressed in response to changes in the environment, which causes extracellular protease to be released [8].

Infections with proteases are thought to have major virulence factors that increase the invasiveness of organisms that cause harm to host tissue and interfere with the host's antibacterial defense mechanisms [9, 12].

High-molecular-weight proteins known as salivary glycoproteins, or mucins, are often produced and secreted in a variety of organs. Two distinct forms of mucins are secreted by human salivary glands: oligomeric mucin (MG1), which has a molecular weight exceeding 1000 k Da, and monomeric mucin (MG2), which has a molecular weight between 200 and 250 k Da. Mucin-coated bacteria may be unable to connect to the surface, which plays a function in oral bacterial adhesion [14]. The current study aims to the optimizing the conditions and purification of extracellular protease, which was produced from *E.coli* AJ55 isolated from UTI patients, and mucin protein cleavage by this protease enzyme.

MATERIALS AND METHODS

Nutrient agar and broth from Himedia, India. Sodium acetate (CH_3COONa), Sodium hydroxide (NaOH), Ethanol 95%, and other materials from BDH, England.

Microorganism and inoculum preparation

Collection of one hundred and seventy-three isolates (In a previous study) from different patients with urinary tract infections (UTI) (were taken from Biotechnology Department, College of Science\Baghdad University), and on skim milk agar was screening for protease production. The isolate of *E.coli AJ55* was best isolate for protease production, then the isolate of *E.coli AJ55* was subcultured and incubated at 37 ℃ on a nutrient agar slant, and at 4℃ the tubes were stored. The bacterial growth on nutrient agar medium was transferred to the culture broth to prepare the inoculum. The inoculum was cultivated in a 250 mL flask with 50 mL of brain heart infusion broth for 24 hours at room temperature and 120 rpm on a rotary shaker.

Determination of protease activity and protein concentration

The Abdallah method [1] with some modifications was used to examine the proteolytic activity of the protease against casein. A 200 µl of a protease enzyme and 1.8 ml of 1% (w/v) casein were combined and incubated at 45 C in a water bath for 30 minutes. The reaction was halted by adding 3 ml of 15% trichloroacetic acid (TCA). The mixture was centrifuged at 6000 rpm for 15 minutes. After combining 3 ml of TCA with 1.8 ml of 1% casein to create a blank, 200 µl of extracted enzyme was added. The identical procedures as with the samples under investigation were applied to the blank. A cuvette for the spectrophotometer was filled with 3 ml of the supernatant. To determine the proteolysis activity of the protease, the absorbance at 280 nm was measured. To calculate and ascertain the protein concentration in samples, the Bradford method was used [14].

Optimization of cultural conditions of protease production

Optimization for protease production employing the selected isolate was performed using submerged fermentation (SmF). These parameters comprise fermentation media, Carbone sources, nitrogen sources, incubation temperature, the pH, and incubation period.

Efficient medium

To select efficient medium for enzyme production from *E.coli* AJ55 isolate, six flasks were used in this experiment, the first flask containing nutrient gelatin broth, the second flask containing casein + nutrient broth medium (1:1) (w:w), the third flask containing casein medium, the fourth flask contain soya bean + nutrient broth medium (1:1) (w:w), fifth flask con-

tain soya bean and the sixth flask containing nutrient broth, then inoculation with inoculum size 8 $\times10^6$ cells/ml and incubated at 37 °C for 24 hours; enzymatic activity and protein concentration were estimated [15].

Carbon sources

Seven types of carbon sources were used for the determination of optimal carbon source for protease production from *E.coli* AJ55 isolate. These carbon sources including (20 g/l): lactose, starch, fructose, sucrose, cellulose, maltose, and glucose, were added separately in different flasks containing nutrient broth. Then inoculation with 8 $\times10^6$ cells/ml and incubated at 37 °C for 24 hours; enzyme activity and protein concentration were estimated [16].

Nitrogen sources

Six types of nitrogen sources were used for the determination of optimal nitrogen source for protease production from *E.coli* AJ55 isolate. These nitrogen sources including (5 gm/l): Treptone, Yeast extract, Urea, Ca(No3)2, NaNo2, and (NH3)2So4, were added separately in different flasks containing nutrient broth plus glucose. Then inoculation with inoculum size 8 $\times10^6$ cells/ml and incubated at 37 °C for 24 hours; enzyme activity and protein concentration were estimated [15].

Temperature

To select optimum temperature for enzyme production from *E.coli* AJ55 isolate, flasks containing medium that was selected as an efficient medium for protease production were inoculated with inoculum size 8 $\times10^6$ cells/ml and incubated at different temperatures 25 , 30 , 35, 37 , 40, and 45 °C for 24 hours, the enzymatic activity and protein concentration was estimated [1].

Determination pH

The selective broth medium was prepared at different pH 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, and 8, then flasks were inoculated with 1 % (8×10^6 cells/ml) of the *E.coli* AJ55 isolate and incubated at 37 C° for 24 hours, the enzymatic activity and protein concentration was estimated [16].

Incubation period

The selective broth medium was inoculated with 8 $\times10^6$ cells/ml of the *E.coli* AJ55 isolate and incubated at 37 °C for different incubation periods 4, 8, 12,16, 20, 24, 28, 32 36,40, 44, and 48 hours, the enzymatic activity and protein concentration was estimated [1].

Isolation and purification of protease

Separation of protease

The broth culture was centrifuged at 12000 rpm for 15 minutes, at 4^0C. Protein content and protease activity were estimated.

Purification of protease by ion exchange chromatography

Precipitation step was done by adding solid ammonium sulfate to the crude extract with (0-70) saturation rates. The exchanger DEAE-cellulose was prepared as described by Whitaker [17]. The DEAE-cellulose was packed into the column (28×1.6 cm), and then the column was equilibrated with the Tris-HCl (0.005 M, pH 8) buffer overnight. Dialyzed protease in a volume of 13 ml was carefully pipetted through a DEAE-cellulose column, washed with 0.005 M Tris-HCl buffer pH 8.0, and then associated proteins were eluted with Tris-HCl buffer pH 8.0 and NaCl gradient 0.1-1 M. The flow rate through the exchanger was 30 ml/hour. Protein

fractions (3 ml) were collected. Protein contents were traced in accordance with absorbency at 280 nm and the activity were estimated for each fraction, then the fractions showed activity were collected and concentrated with sucrose, the peaks were estimated by plotting the absorbance at 280 nm versus fraction number.

Purification of protease and mucin elution by gel filtration chromatography

According to the manufacturer's instructions, a Sephadex G-150 column (65×1.5 cm) was manufactured and packaged (Pharmacia- Sweden). A 0.02 M Tris-HCl buffer with a pH of 8.0 was used to equilibrate the column overnight at a flow rate of 30 ml/hour. A 15 ml dialyzed protease that had undergone ion-exchange chromatography for purification was then run through a gel filtration column together with 225 ml of Tris-HCl buffer at pH 8.0. Protein fractions totaling three ml were collected. In order to evaluate the enzyme activity, protein contents were tracked in accordance with absorbency at 280 nm. The active portions were gathered and sucrose was used to concentrate them. The same steps of gel filtration procedure above were performed with standard Mucin protein (0.11 mg/ml) [1, 17].

Cleavage of mucin by protease enzyme from *E.coli* AJ55 Treated mucin with protease enzyme

A 0.2 ml partially purified enzyme with a protein concentration of 0.005 mg/ml, and ~~an~~ a specific activity of 2432 U/mg was mixed with 2 ml of standard mucin (0.11 mg/ml), then the mixture was incubated for 16 hours at 37 ^{O}C [1, 11].

Determination of mucin cleavage

Using the gel filtration method, the cleavage of mucin was evaluated on a Sephadex G-150 column (65×1.5 cm). The column was equilibrated overnight with 0.02 M Tris-HCl buffer pH 8.0, at flow rate of 30 ml/hour. A 2.2 ml solution of partially purified protease and standard mucin was carefully pipetted through into the column and afterward washed with Tris-HCl buffer 0.02 M and fractions accumulated, each three ml, and the peaks were determined by charting the absorbency at 280 nm versus fraction number, and the enzyme activity was estimated [1, 11].

RESULTS AND DISCUSSIONS

Efficient medium

For optimization of protease production from *E.coli* AJ55 isolate, we used different media. Results shown in Figure (1) indicate that nutrient broth medium was selected as it gave 76.2 U/mg of protease specific activity which is considered the highest value compared with other used media.

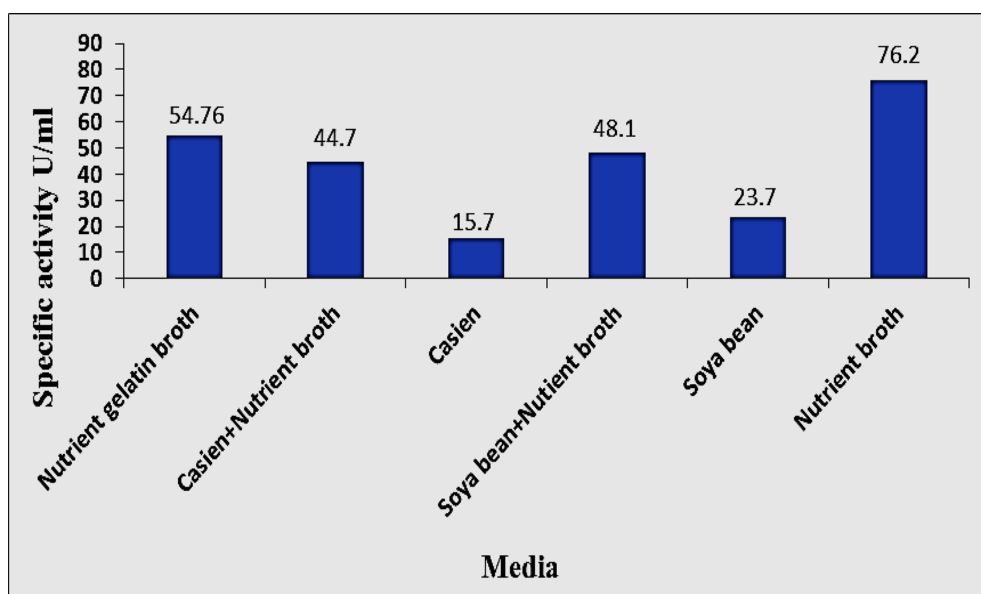

Figure 1: Effect of complex media on protease production from E.coli AJ55 isolate using SmF, pH 7, incubation at 37 °C for 24 h.

These results were similar to the results of [1], who proved that selection of efficient media for protease production by *E.coli* depends on changing the media components. While Abed, *et al*., [16] discovered that the highest levels of protease synthesis for *E.coli* 1 and 2 were seen in Tryptic Soy Broth after 24 hours of incubation with specific activities of 3.09 and 2.71 U/mg, respectively.

Effect of carbon source

We looked at how carbon sources affected bacterial development and protease synthesis. *Escherichia coli* AJ55 were cultivated for 24 hours in nutrient broth media that had 20 g/L additions of a variety of carbon sources. As can be shown in Figure 2, among the 7 types of carbon sources investigated, the results suggested that *E.coli* may have employed a variety of carbon sources to produce proteases. Protease is infrequently produced in other carbon sources, with glucose medium having the highest production rates (146.75 U/mg). Although glucose is often a good source of energy for growth, it interferes with the formation of numerous secondary and primary metabolites [18]. The findings ~~of~~ illustrate the impact of alternative carbon sources on bacterial development and protease production (Figure 2).

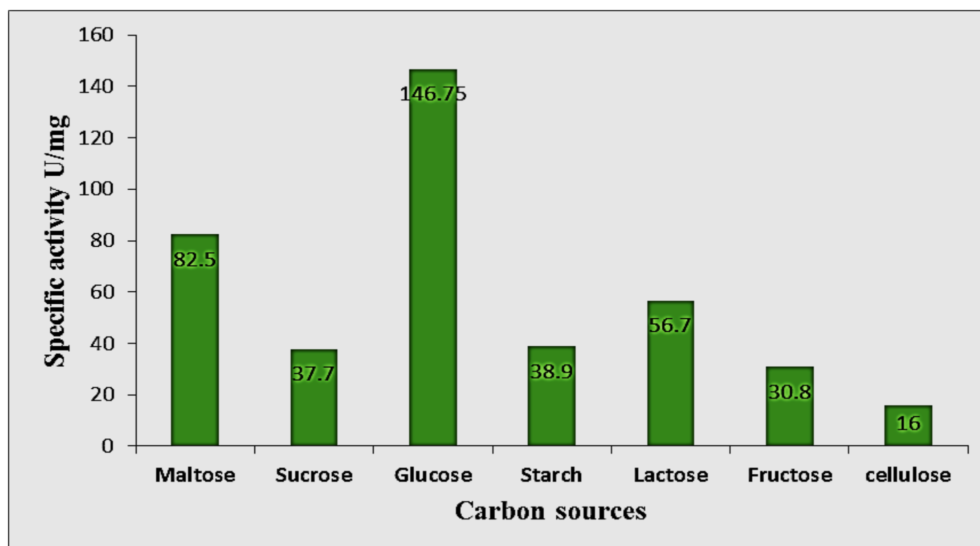

Figure 2: Effect of carbon source on protease production from *E.coli* AJ55 isolate using SmF, pH 7 incubation at 37 °C for 24 h.

These results were similar to the results of [1], who mention that the best carbon source for maximum protease production from *streptococcus mutans* 67 was glucose. Other studies that employed various sugars such starch, sorbitol, lactose, maltose, and sucrose also found increased protease production, suggesting that various bacteria have varying preferences for the optimum carbon source for enzyme production [15, 18].

Effect of nitrogen source

For the protease synthesis from *E.coli* AJ55, various nitrogen sources including treptone, urea, sodium nitrate (NaNO3), yeast extract, (NH4)2SO4, and Ca(NO3) were independently added. It is well understood that the use of various nitrogen sources in fermentation had an impact on the growth of microorganisms and the production of proteases. The findings demonstrated that the optimal nitrogen source for the formation of protease was yeast extract. Yeast extract, when used as a nitrogen source, produced high yield of protease than the other nitrogen sources, as seen in Figure (3). It has been claimed that a variety of amino acids found in organic nitrogen sources are crucial for the formation of primary metabolites. In actuality, *E.coli* AJ55 may manufacture the protease enzyme in the presence of several amino acids.

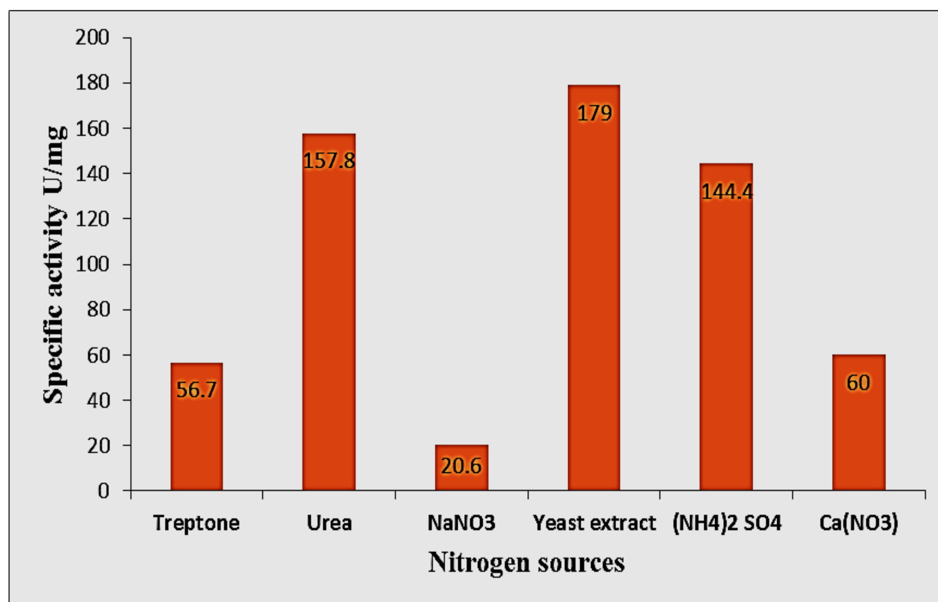

Figure 3: Effect of nitrogen source on protease production by *E.coli* AJ55 using SmF, pH 7, incubation at 37 °C for 24 h.

of studies demonstrate that the amino acid and peptides were much better nitrogen sources for protease production from bacteria than ammonium compounds. Therefore, it is believed that the existence of zinc-containing salts and nitrogen in their two forms (NO3 and NH4 +) is necessary for the synthesis of protease [1, 15].

Effect of temperature

The impact of temperature on bacterial proliferation is obvious. However, temperature has an impact on protease synthesis, with a rise in yield at 37°C. At 37°C, the maximum yield (180.6 U/ml) of protease was found (Figure 4). This is comparable to how marine bacteria produce proteases [15].

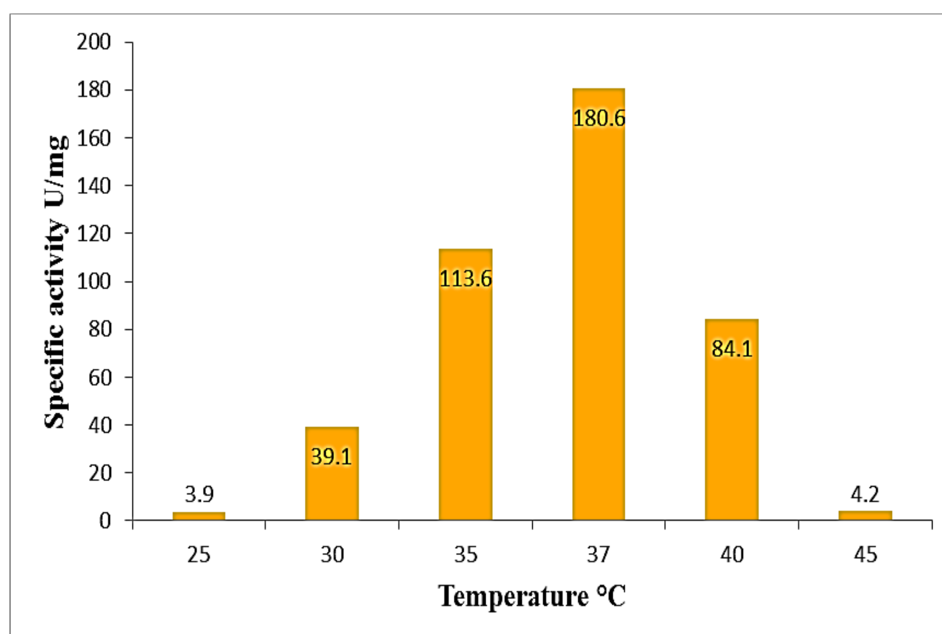

Figure 4: Effect of temperature on protease production by *E.coli* AJ55 using SmF, pH 7, incubation for 24 h.

These bacteria produced protease best at a temperature of 37 °C. These are consistent with other microbes' synthesis of primary metabolites. One of the crucial factors that impact the effectiveness of the SmF system is temperature. Some of the data showed a close correlation between the synthesis of the enzyme and bacterial growth, and *E.coli* AJ55 produces protease at a temperature that is also optimal for bacterial development. This finding was in agreement with that obtained by Abed *et al*., in their study [16], which showed that the maximum protease levels were produced at temperatures best for the growth of the bacteria during submerged fermentation.

Influence of pH

The isolate of *E.coli* AJ55 was grown on a nutrient medium with 5 g/L of yeast extract and 20 g/L of glucose throughout a pH range of 3-8 for 24 hours in order to examine the impact of the pH values on bacteria growth and protease production. At pH 7.0, the maximum protease synthesis (181 U/mg) was discovered. Figure (5) illustrates how pH impacts the production of proteases and the growth of bacteria. For *E.coli* AJ55, a correlation between starting pH and protease synthesis between pH 3 and 8 was noted. This finding indicated that pH may play a role in protease production. When the pH of the culture medium was set to 7.0, the best pH for the production of 181 U/mg of protease was noticed. In a similar study, researchers discovered a correlation between the production of protease during bacterial growth and an appropriate concentration of hydrogen ions in the medium (pH 7). On the other hand, when the pH of the culture media elevated or fell by roughly pH 7.0, bacterial growth was suppressed.

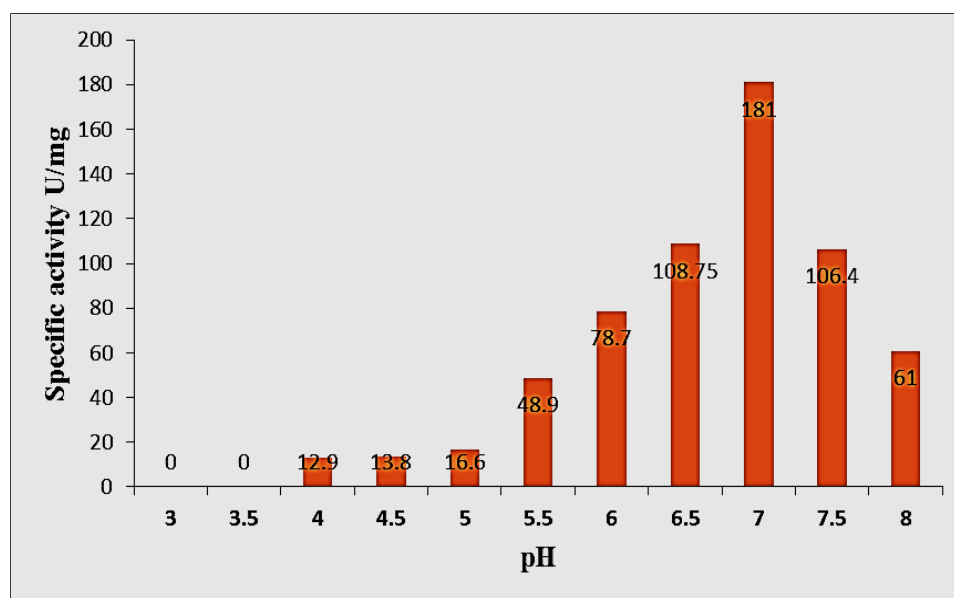

Figure 5: Effect of pH on protease production by *E.coli* AJ55 using SmF, incubation at 37 °C for 24 h.

The essential aspects in the increase of biomass and the synthesis of proteases were the varied morphology of bacteria with various initial pH values. The ionic state of substrates [19], the solubility of salts, the uptake of different nutrients, and product biosynthesis are all impacted by the pH of the medium. In general, cells can only expand inside a limited pH range, and pH frequently affects metabolite synthesis [2].

Effect of incubation time

By incubating the chosen isolate for various amounts of time, the protease production parameter was first optimized (4, 6, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 hours). Conical flasks were incubated for various amounts of time spaced (4 hours apart) to find the better incubation time for protease synthesis. After 24 hours of incubation, the highest amount of protease synthesis (181 U/mg) was achieved (Figure 6).

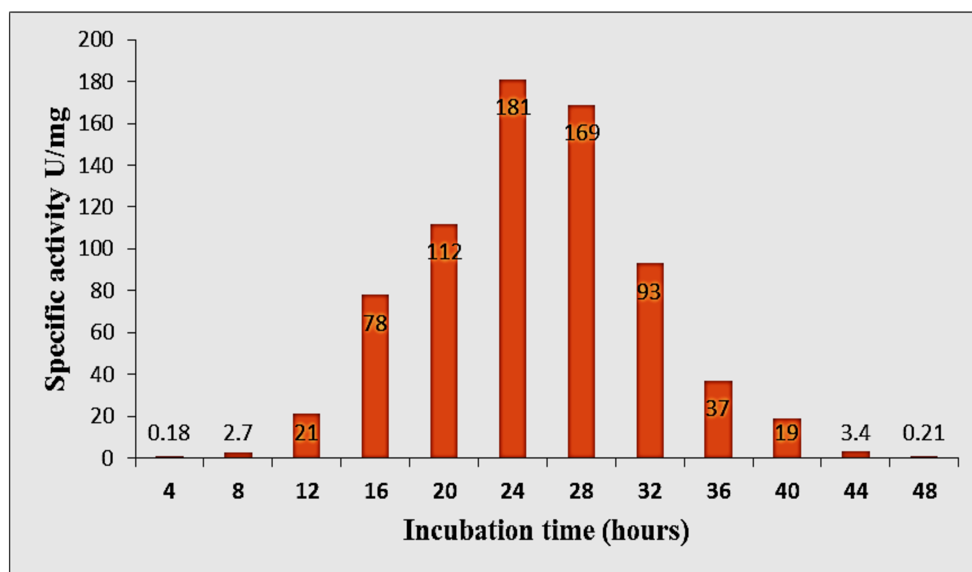

Figure 6: Effect of incubation time on protease production by *E.coli* AJ55 using SmF, pH 7.0, incubation at 37 °C.

The protease production was decreased after 28 hrs. This decrease in protease production occurred as a result of reduce in nutrients in the medium. Pant, *et.al*., [15], found that better incubation time for protease production by *Bacillus subtilis* was 36 hours.

Protease purification employs ion exchange chromatography

Purification of protease from *E.coli* AJ55 was done using ion exchange chromatography after protease precipitation by ammonium sulphate (0-70 % saturation). During the experiment, it was found that clearing with Tris-HCl (pH 8.0, 0.005 M), allows the manifestation of four peaks which are exemplified by fractions 18-20, 21-27, 28-30, and 31-38 as shown in Figure (7). Addition of 225 ml Tris-HCl with NaCl gradients (elution step) allows three peaks to be obtained and represented by fractions 66-87, 9397, and 98-99. Protease activity was assessed for each fraction. The fractions in steps 66–87 of the elution process displayed protease activity. The results also reveal that the protease produced by *E.coli* AJ55 carried negative charges that attracted to the positive charge of DEAE-cellulose, causing the protease to be eluted and proceed to the elution step. According to Table (1), the findings showed that the protease concentration was 0.012 mg/ml of protein, as well as of the other parameters; 19.22 U/ml of activity, 1600 U/g of specific activity, an 8.8 purification fold, and a yield of 62 % were obtained.

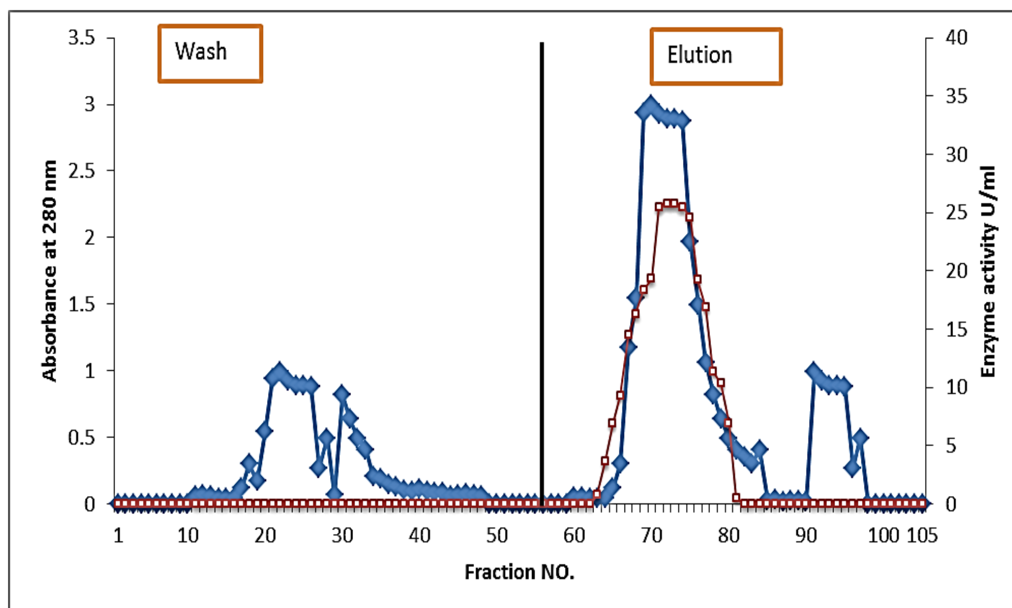

Figure 7: Ion exchange chromatography for purification of protease from *E.coli* AJ55 by using DEAE-Cellulose column (28-1.7 cm) equilibrated with Tris-HCl (0.005 M, pH 8.0) in flow rate 30 ml/ hr, eluted with Tris-HCl with NaCl gradient (0.1-1 M), and 3 ml for each fraction.

Gel filtration chromatography

Gel filtration step followed ion exchange, and this step was done using Sephadex G-150 gel. Figure (8) shows results of gel filtration where one big peak and three small peaks of protein are separated. After determining the amount of enzymatic activity, only one peak appears in the enzyme activity in fractions 30-42. These fractions are gathered and combined, and the volume is then concentrated by sucrose to 13 ml. According to the findings in Table (1), the gel filtration purification step produced an enzymatic yield of 34 %, a specific activity of 2432 Umg, and purification fold of 13.4.

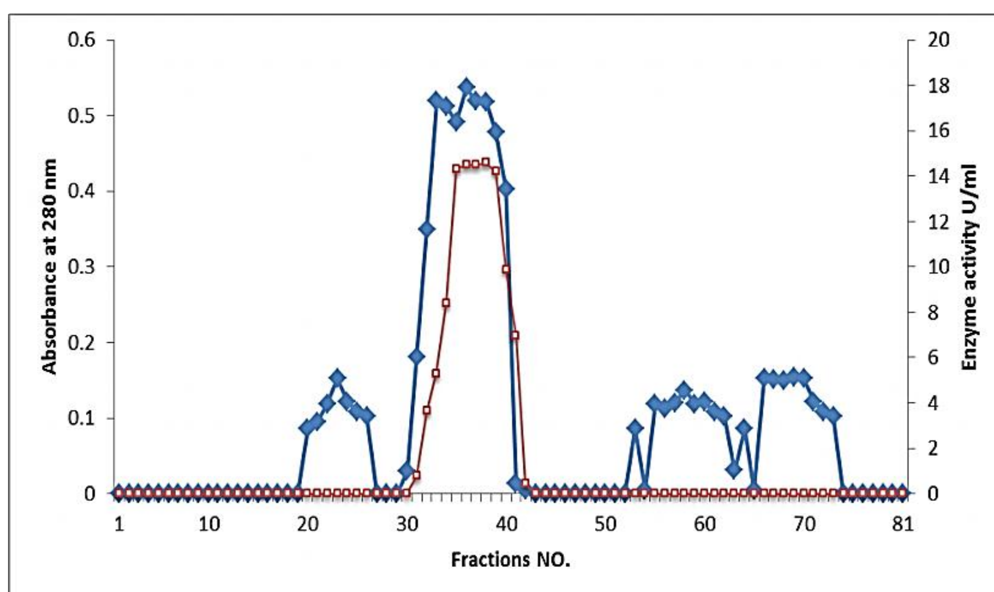

Figure 8: Purification of protease by using Sephadex G-150 column (65×1.5 cm) with flow rate of 30 ml/hour, 3 ml for each fraction, and eluted with Tris-HCl (0.02 M, pH 8.0).

Table 1: Purification steps of protease from *E.coli* AJ55.

Step	Volume ml	Activity U/ml	Protein concentration mg/ml	Specific activity U/mg	Total activity	Fold	Yield %
Crude enzyme	75	6.2	0.034	182	456	1	100
Precipitation by ammonium sulfate (0-70 % saturated) after concentration by sucrose	13	28.7	0.095	303	373	1.7	80
Purification by Ion-exchange chromatography (after concentration by sucrose)	15	19.22	0.012	1600	288.3	8.8	62
Purification by Gel-filtration chromatography (after concentration by sucrose)	13	12.16	0.005	2432	158	13.4	34

Elution of standard mucin employing gel filtration chromatography

This step was done using Sephadex G-150 gel. Figure (9) shows the results of gel filtration where appeared one big peak of standard mucin protein.

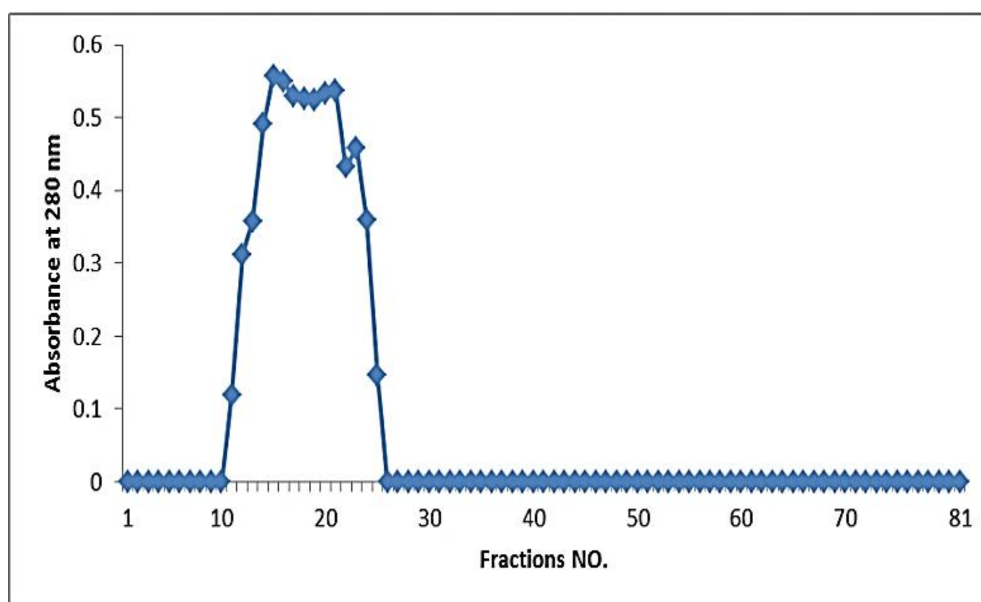

Figure 9: Elution of standard mucin protein using Sephadex G-150 column (65×1.5 cm) with flow rate of 30 ml/hour, eluted with Tris-HCl (0.02 M, pH 8.0) and 3 ml for each fraction.

Cleavage of mucin

A mixture solution of mucin and protease was prepared as mentioned by [1, 11, 17]. Cleavage of mucin protein was determined using gel filtration chromatography. Following the passage of the mixture sample through a Sephadex G-150 column (65×1.5 cm), the amount of elution proteins were calculated from the fractions that were collected by charting the absorbency of every fraction at 280 nm versus the number of fractions. In Figure (10), the outcomes show demonstrated that there are five peaks, with the initial peak being indicated by the fraction 32–46, the second peak being displayed by the fraction 52–59, the third peak being defined by the fraction 60–64, the fourth peak being indicated by via the fraction 68–73, and the fifth peak being indicated via the fraction 77–79. Our explanation for the presence of that first peak belongs to protease which eluted according to enzyme activity, while the mucin

represents the other peaks. However, the second, third, fourth, and fifth belongs to the fragment of mucin which was cleavaged by protease.

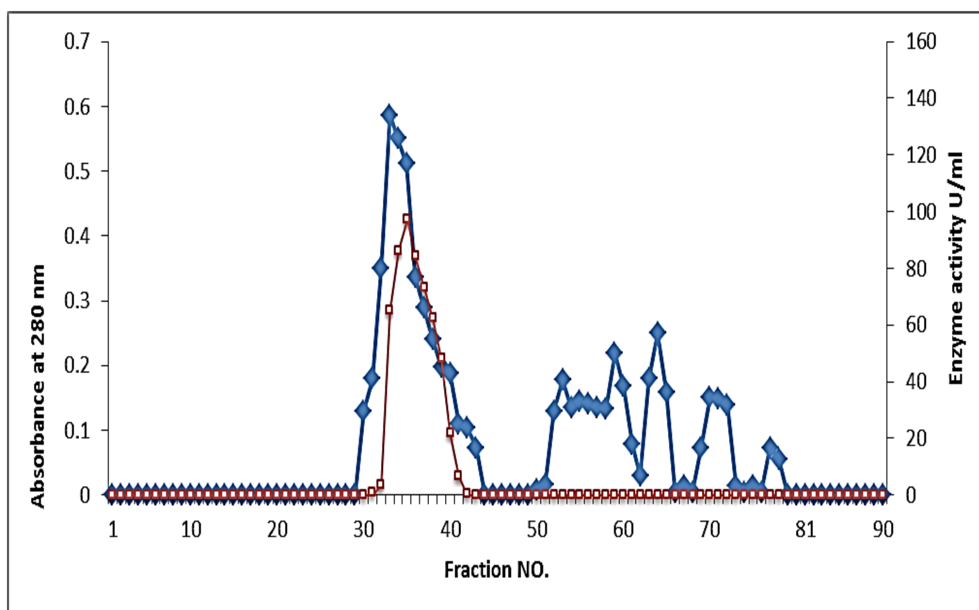

Figure 10: Gel filtration chromatography for mucin fractions treated with protease using Sephacryl S-300 column (100×1.5 cm), 3 ml for each fraction with flow rate of 30 ml/hour, and eluted with Tris-HCl (0.005 M, pH 8.0).

This result agrees with that reported by Wickstrom, *et al.* [20]. While Van der Hoeven and Camp [21], found that protease produced by *Streptococcus sangius* cleaves mucin to 4 peaks after passing the Sepharose-6B. In addition, Khayoon, and AL-Sa'ady [11] found that the protease produced from gastrointestinal *E.coli* A29 was cleavage of mucin into three peaks. In addition, Abdalah, *et.al*., [1], showed that treatment of mucin protein with partially purified protease from *Streptococcus mutans* gave three peaks after passing the sepharose-6B column. According to findings of Cordingley *et al.,* [3], the human rhinovirus protease was able to cleave the examined peptides at the breakage location with varying degrees of efficiency and could tell real substrate peptides apart from control peptides that included the dipeptide breakdown sequence pair Gln-Gly.

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