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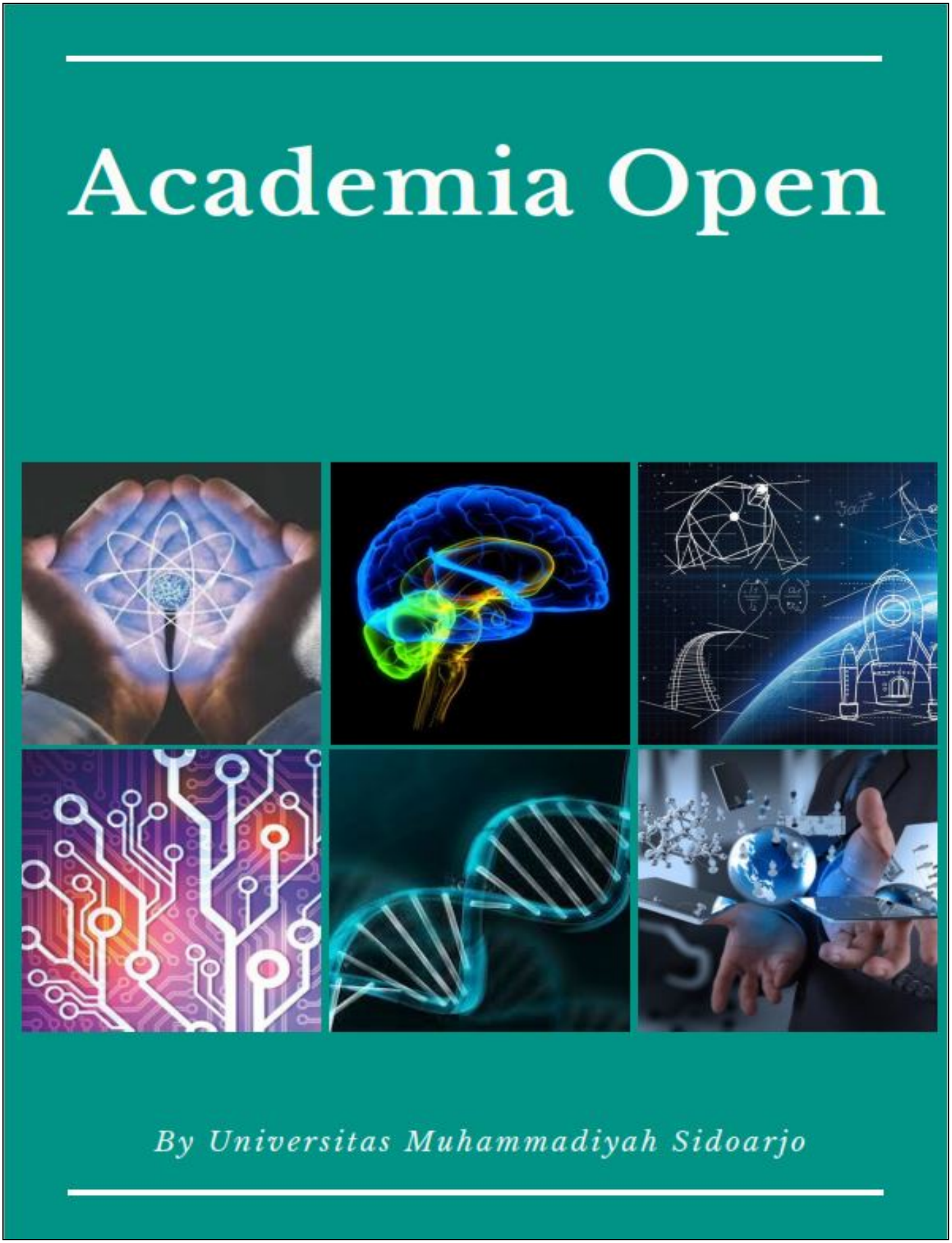

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Enhanced Protease Production from Diabetic Ulcer-Derived E. coli

Peningkatan Produksi Protease dari E. coli yang Berasal dari Ulkus Diabetes

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Abstract

This study explored a local Escherichia coli isolate from diabetic foot ulcer patients for protease enzyme production. Qualitative screening on skim milk agar showed a 6 mm hydrolysis halo, while quantitative screening with casein revealed a specific activity of 144.55 U/mg protein. Purification via ion exchange chromatography on DEAE-cellulose increased the specific activity to 767.5 U/mg, with a 5.3-fold enhancement and a 29.5% yield. The findings suggest E. coli as a viable source for protease production and demonstrate the efficiency of ion-exchange chromatography for enzyme purification, with potential significant industrial applications.

Highlight:

1. E. coli sourced from diabetic ulcers shows promise for protease production.
2. Skim milk agar and casein used for qualitative and quantitative screening.
3. Ion exchange chromatography significantly enhances enzyme specific activity.

Keywoard: Escherichia coli, protease production, diabetic foot ulcer, enzyme purification, ion exchange chromatography

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Introduction

The biological processes can now successfully rival traditional chemical processing thanks to recent developments in the field of biocatalysts. Utilizing the most desirable characteristics of biocatalysts, or enzymes, such as high selectivity with fewer side effects and increased yields, mild response circumstances, and frequently low environmental consequences, combinations of chemical and biocatalytic systems are being created there. [1] Two thirds of all the enzymes used in different businesses were proteases [2]. It is used in the waste treatment, food, detergent, and pharmaceutical industries, among others [3]. While proteases used in medicine are manufactured in tiny amounts but need to be thoroughly purified, those prepared in large quantities and utilized as raw materials in the food and detergent industries. formulations.established and characterized extracellular proteases showed proteolytic activity when cultured on skim milk plates [4]. identified and characterized extracellular proteases [5].when cultivated on skim milk plates, shown proteolytic activity [6]. Gram-negative E. coli is a facultative anaerobic bacteria that can convert to fermentation or anaerobic respiration in the absence of oxygen. If oxygen When present, aerobic respiration is used to make ATP.The size of cells are typically 2.0 µm in length, 0.25–1.0 µm in width, and 0.6–0.7 µm3 in volume. Typically, cells appear as rods.[7][8]. Escherichia coli, or just E. coli, is a type of bacteria that can be found in food, the environment, and the intestines of both people and animals. The E. Coli bacteria are a diverse group of bacteria. Some forms of E. Coli can cause illness, but most types are not hazardous.[9][10].

Method

Equipment and apparatus

Apparatus	Company/origin Apparatus
Autoclave	*Express/Germany*
Balance (sensitive)	*Ohaus/ Germany*
Centrifuge (Portable)	*Selectap/Spain*
Incubator	*Gallenkamp/UK*
Laminar air flow	*Memmert/ Germany*
pH meter	*Metter-Tolledo/UK*
Vortex	*Buchi/ Switezerland*
UV-Visible spectrophotometer	*Shemadzu /Japan*
Water Distillator	*GFL*

Figure 1. *The instruments and tools utilized in this investigation were as follows: Materials Both Biological and Chemical*

Material	Company/origin
Tris- HCl, K_2HPO4, KH_2PO4	BDH/ England
TCA	Sigma/USA
DEAE-Cellulose	Whattman/ England

Figure 2. *The research used the following materials.*

Media

Medium	Company/Origin
Nutrient agar	Difco/USA
Nutrient broth	Oxoid

Figure 3. *Ready-to-use cultural media were all organized following the guidelines provided by industrialized businesses and autoclaved at 121°C, (15 pound/In2) For 900 seconds.*

Laboratory prepared media

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Skim milk agar medium [11], buffers and solutions, Potassium phosphate buffer solution (pH 7) and It was prepared according to [12]

Protease assay solution

1. 0.1M Tris-HCl buffer with a pH of 8It was made by dissolving 500 milliliters of distilled water with 6 grams of basic HCI.

2. Solution of casein (1%): One gram of casein was dissolved in one hundred milliliters of 0.1M Tris-HCl buffer solution to create it.

3. 5% Acid trichloroacetic: It was made by dissolving 5g of TCA in an amount of Distilled water ., and then adding Distilled water. to bring the volume up to 100 ml.

Laboratory prepared media

Skim milk is used to make agar medium [13]. Five grams of skim milk were dissolved in fifty milliliters of distilled water, and the mixture was autoclaved for five minutes to create this medium. Next, 50 milliliters of distilled water were used to dissolve 2 grams of agar, and the mixture was autoclaved for 15 minutes. Before being distributed, the medium was well mixed after it had cooled to 45°C. into sterilized Petridis plates, which are then stored at 4°C until needed.

Solutions and buffers

Solution of potassium phosphate buffer, pH 7 [12]. In accordance with [12], it was prepared.

Solution for protease assay

1. 0.1M Tris-HCl buffer with a pH of 8It was made by dissolving 500 milliliters of distilled water with 6 grams of basic HCI.

2. Solution of casein (1%): To prepare it, 1g of casein was dissolved in 100 ml of 0.1M Buffer solution Tris-HCl.

3. 5% Acid trichloroacetic: This was created by adding D.W. Protein Assay Solution to bring the volume up to 100 ml after dissolving 5g of TCA in a quantity of distilled water.

4. Protein assay solution [14]

5. Bright blue G-250 Coombassie solution.

6. 50 cc of 95% ethanol was used to dissolve 0.1 g of Coombassie Brilliant Blue in order to prepare it.

7. A pH 8.0 Tris-HCl buffer (0.1M).

Methods

Scheme (1) provided a summary of the primary steps in the research strategy, starting with the samples., protease production.

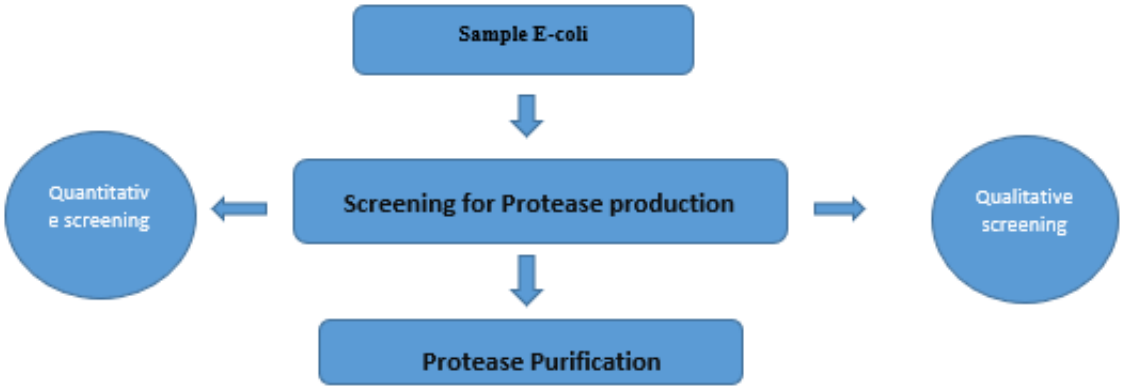

Figure 4.

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Scheme(1):mainstep of the research plan

Sterilization Methods [15]

Autoclaving: Moist Heat Sterilization

Autoclaving was used to sterilize the media and solutions for 15 minutes at 121°C (15Ib/in2).

1. Sterilization via Heat Using an electric oven, glassware was disinfected for three to five hours at 160 to 180°C.

2. Membrane Filtration for Sterilization Using (0.22) µm millipore filters, heat-sensitive liquids were sterilised by membrane filtration.

Quantitative screening [16]

A fresh culture of bacterial isolate containing 100 µl was added to a conical flask containing nutritious broth, and the mixture was allowed to culture for 24 hours at 30°C. in a shaker incubator spinning at 150 rpm. Following incubation, the pellets were disposed of, the culture was centrifuged, and the supernatants were removed and subjected to a protease activity analysis. To quantify TCA soluble peptide release, protease activity was assessed in triplicate using 1% (w/v) solution of casein. 0.8 milliliters of casein solution and 0.2 milliliters of enzyme solution made up the test combination. It was incubated for thirty minutes at 37°C. . 1 milliliter of TCA was added to halt the reaction, and it was then cooled in an ice bath for ten to fifteen minutes before being centrifuged for twenty minutes at maximum speed. The control was set up with the help of the identical procedures, with the exception that 0.2 milliliters After adding the enzyme solution to the casein solution, the absorbance at 280 nm was determined According to the following equation, enzyme activity is the quantity of enzyme needed in an experimental setting to raise absorbance at 280 nm by 0.01 in a minute.

$$\text{Enzyme activity (U/ml)} = \frac{\text{Absorbance at 280 nm}}{0.01 \times 30 \times 0.2}$$

0.01: Constant

30: Reaction time (min)

0.2: enzyme volume (ml)

$$\text{Specific activity (U/mg)} = \frac{\text{Activity (U/ml)}}{\text{Protein concentration (mg/ml)}}$$

Figure 5. *Protease purification by ion exchange chromatography*

The following procedures for protease purification were followed, as suggested [17][18] According to [21], g of resin were dissolved in 1 liter of distilled water to create a DEAE-Cellulose column. The beads were first given time to settle before being extensively cleaned with D.W. . until they took on a clear look. Using a Buchner funnel, the suspension was filtered through Whattman No. 1 before being discharged. The resin was again suspended in a solution of sodium hydroxide and chloride (0.25 M). The suspension underwent the previously described filtering process once more, followed by multiple washes in a 0.25 M hydrochloric acid solution and D.W, before being adjusted to a pH of 8 using 0.05 M Tris-HCl. Following preparation, the enzyme was added to an equilibrated ion exchange chromatography column. earlier using a pH 8 Tris-HCl buffer at 0.05M. Gradual amounts of sodium chloride (0.1–0.6 M) were used to elute the associated proteins from the column, which was subsequently cleaned using a volume equivalent of the same buffer. Protease was eluted at a flow rate of 30 ml/h (3 ml/fraction). A UV-VIS spectrophotometer set at 280 nm was used to calculate the absorbance of each fraction.

Result and Discusion

Isolation of E.coli E.coli was obtained from Furkan M.Kadhim m.phD student in AI-Nahran University /Bioseparation lab.

Semi- quantitative screening

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When the isolate of E. coli was grown on agar media with skim milk, forming a hydrolysis halo for the colony allowed for semi-quantitative screens for protease production (Figure 3-1).shown the isolate's ability to hydrolyze skim milk agar media and generate hydrolysis halos. Halos varied in diameter from 6 mm [19]

Quantitative screening

After cultivating in the production broth medium at 37°C for a full day. the local isolate of E. coli was quantitatively screened to assess its capacity to produce protease. Protease's particular activity in the crude filtrates was then measured after centrifugation. Findings indicated that the isolate produced proteases. According to [20], In culture filtrates, the specific activity of protease was 144.55 U/mg of protein. . Genetic variations in the genes involved for producing protease account for the variations in the isolates' capacities to produce protease [21].

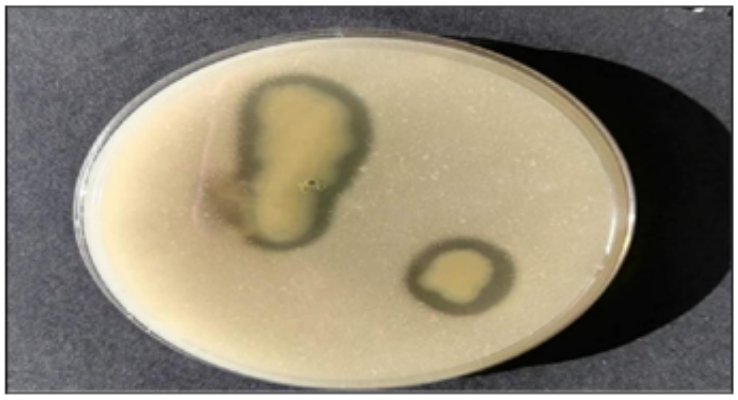

Figure 6. *E. coli's proteolytic activity on 10% skim milk agar following a 24-hour incubation period at 37°C*

Purification of protease by Ion exchange chromatography

Negatively charged protease generated by E. coli was purified using the ion exchange chromatography method.This method involved applying crude protease to To eliminate all uncharged and positively charged proteins, wash the protease sample in equal volumes with a 0.05 M Tris-HCl buffer solution (pH 8) after equilibrating the column using a DEAE-cellulose column.Next, with gradient sodium chloride concentrations between 0.1 and 0.6 M, the bounded proteins (negatively charged) were eluted.

Figure 2's results demonstrated that whereas three protein peaks emerged following elution by the varying sodium chloride concentrations, only one protein peak was visible throughout the washing step. By calculating each eluted fraction's absorbance at 280 nm, the four protein peaks were found.

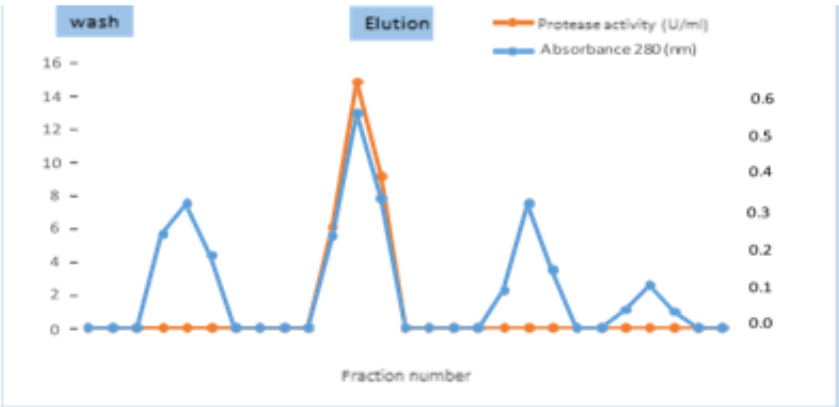

Figure 7. *A DEAE-Cellulose column (45 x 2 cm) is used in ion exchange chromatography to purify the protease produced by Escherichia coli.*

Protease activity was measured using the three protein peaks that were discovered during the elution process. The majority of the eluted proteins (fractions 46 to 66) had protease activity, which reached 15.35 U/ml, according to the results. Protein concentration, protease activity, and specific activity were assessed after the fractions that

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indicate protease activity were combined and concentrated. Given that protease associated with the anionic ion exchange (DEAE-cellulose), these findings support the notion that protease has a negative net charge. According to table (3-1) results, this concentrate had a protein concentration of 0.02 mg/ml, a specific activity of 767.5 U/mg and a protease activity of 15.35 U/ml. Additionally, it possessed a 29.5% enzyme yield and a 5.3 purification fold [25].

Purification step	Volume (ml)	Enzyme activity (U/ml)	Protein con.(mg/ml)	Specific activity (U/mg)	Total activite(U)	Purification (folds)	Yield(%)
Crude enzyme	100	13.01	0.09	144.55	1300	1	100
DEAE cellulose	25	15.35	0.02	767.5	383.75	5.3	29.5

Figure 8. *Purification steps for protease produced by the E.coli*

Conclusion

1-E.coli can be used as a good source for the fabrication of protease enzyme.

2- The ion-exchange chromatography method of purifying protease on DEAE-cellulose resulted in an enzyme with increased specific activity and purification fold.

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