ejpmr, 2016,3(2), 113-118

SJIF Impact Factor 2.026

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 3294-3211

EJPMR

OPTIMIZATION OF EXTRACELLULAR PROTEASE EXTRACTED FROM *ESCHERICHIA COLI*

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Article Received on 09/12/2015 **Article Revised on 30/12/2015** **Article Accepted on 21/01/2016**

ABSTRACT

Protease production from *Escherichia coli* isolated from patient suffering Urinary Tract Infection (UTI) was studied. The ability of this bacteria to produce protease enzyme was screening on skim milk agar , as well as the culture conditions like culture medium , inoculum concentration , pH , temperature , incubation period were optimized. The high level of protease production of isolated bacteria (*E.coli* 1 , *E.coli* 2) were found in Tryptic soy broth culture medium with inoculum concentration 1.5×10^{8} CFU/ml at 40 °C after 24 hrs at pH = 6 with continuous agitation (150 rpm).

KEYWORDS: Protease, *E.coli*, UTI, Optimization.

INTRODUCTION

Proteases are the main enzymes produced form Microbial Sources. Microbial protease with desirable characteristics are most suitable for biotechnological processes.[1]

Protease catalyse the degradation of protein molecules to peptides and amino acid and has been used in various industries such as food, pharmaceutical, detergent industries and cater to the requirement of nearly 60% of the world enzyme market.[2]

Microorganisms offer an attractive source of protease enzyme because they can be cultured in large quantities in a short period time they produce an abundant and they can be genetically manipulated easily than plants and animals.[3-4]

There are many reports in the literature regarding protein secretion form fungi and Gram – positive bacteria, the studies on protein secretion from Gram – negative bacteria especially from *Escherichia coli*, are quite limited. This is in spite of the fact that *E.coli* is widely used in the biotechnology industry for expression of recombinant proteins and as a model pathogen.[5-6]

Proteases are well known virulence factors that promote survival pathogen and immune evasion of many pathogen,[7] *E.coli* are generally classified as nonsecretors of protein. However, in pathogenic *E.coli*, the secretory mechanism of a number of virulence factors has been studied in some detail,[8, 9, 5, 6] in contrast, nonpathogenic *E.coli* are typically believed no to secrete protein, however, reports have shown that cryptic genes coding for secretion and pilation are also present in *E.coli* K_{17} laboratory strains.[10, 11]

These gens can be expressed during environmental variation and lead to the release of extracellular protease.[12]

Protease are considered important virulence factors in the infection were contributed to invasiveness of organisms which damage host tissue and interfere with host antibacterial defense mechanisms.[13]

The present study was aimed to optimize the conditions for maximum production of extracellular protease form *E.coli*, which isolated from UTI patients.

MATERIALS AND METHODS

Samples collection and isolation of bacteria

Hundred and sixty samples of urine were collected from patients suffering Urinary Tract Infection (UTI) from many hospitals in Baghdad, during the period from September 2014 to – May 2015. The urine specimens were inoculated on both MacConkey agar and blood agar plates by direct streaking methods.[14]

Bacteria were isolated as a pure colonies on MacConkey agar and Eosin methylene blue agar.[15]

Identification of bacteria

Bacterial isolates were examined and identified by cultural characteristic, biochemical test and using vitek2 system.

Enzyme production medium

Prepared according to Benson (2002) by dissolving 4 g of skim milk in 50 ml distilled water, heated at 50°C and sterilized in autoclave for 5 min and then dissolved 2 g agar in 50 ml distilled water adjusted the pH = 7.5 sterilized in autoclave, cooled to 50°C, Mixing well and poured into sterile petri dish This medium used to detected the protease producing ability in bacteria.

Protease production test

Isolated bacteria were screened for protease production ability on skim milk agar and Casein agar. The zone formation around the bacterial colony indicated the protease positive result which may be due to the hydrolysis of Casein.[16]

Assay of protease

Qualitative assay of protease

Wells were made with a sterile Pastour pipette in skim milk agar and Casein agar and 0.2 ml of culture supernatant were dispensed into each well and incubated at 37°C, after 24hrs enzyme activity was visualized as clear zone around the well and proteolytic zone was measured.[17, 18, 19]

Quantitative assay of protease

Transferred one colony of bacterial isolate from sub culture on brain heart infusion agar plates to 5 ml of Tryptic soy broth and incubated in shaking incubator (150 rpm) at 37°C for 24hrs and transferred 1% ml of this culture to 100 ml of Tryptic soy broth incubated at 37°C for 24hrs in shaking incubator (150 rpm), then centrifuged it at (5000 rpm) for 20 min in 4°C. Collected culture supernatant (crude enzyme) to assay protease activity and protein concentration.

Measurement of protease activity

Protease activity was determined according to the method originally described by[20, 21] and modified by[22] as following:

Added 0.2 ml of culture supernatant to 1.8 ml Casein solution 2% the mixture was incubated in water bath at 37°C for 10 min, then 3 ml of TCA 5% was added to this mixture and placed at 4°C for 10 min to form precipitate, then it was centrifuged at (5000 rpm) for 15 min also control was made from 3 ml of TCA 5% added to 1.8 ml Casein solution before 0.2 ml of culture supernatant addition. Absorbance was measured at 280nm and the amino acids produced by the degradation of Casein. The protease activity was determined by using the following equation.

$$\text{Protease Activity Unit/ml} = \frac{Absorbance\ \ at\ \ 280\ nm}{0.01 \times 10min \times 0.2ml}$$

Unite of enzyme activity was defined as the amount of enzyme which gives 0.01 increase of absorbance 280nm/min under assay condition.

Determination of protein concentration

The protein concentration was estimated using absolute method as described by[23] as following:

- Prepared standard curve of Bovin Serum Albumin (BSA) by preparing a series of protein standard diluted with (0, 20, 40, 60, 80, 100) mg/ml.
- Added 0.1 ml of each above diluted to separate test tube.
- Added 0.4 ml of Tris-HCl buffer solution (0.1 M, pH 8).
- Added 2.5 ml of coomassie blue to each tube and mixed by vortex.
- Adjusted the spectrophotometer to wavelength 595nm, and blank using the tube which contains no protein.
- To estimated unknown protein the same steps were used, (0.1 ml sample, 0.4 ml Tris-HCl, 2.5 ml of coomassie blue) will addition.
- Waited 5 min and read each of the standards and each of the samples at 595nm wavelength.
- Plotted the absorbance of the standard of their concentration. Computed the correlation coefficient and calculated the concentrations of unknown samples.

Also estimated specific activity of enzyme using the following equation:

$$\text{Specific activity unit/mg protein} = \frac{\text{enzyme activity unit/ml}}{\text{protein concentration mg/ml}}$$

Optimization process condition for protease production

Determination of optimized culture medium for protease production

Tested five culture medium to determined optimized production medium include:

- Nutrient broth.
- Brain heart infusion broth.
- Tryptic soy broth.
- Tryptic soy broth with peptone.
- Luria – Bertoni medium.

Inoculated all culture media by 1% ml of bacterial overnight culture in 37°C at 24 hrs and incubated with shaking incubator at 37°C for 24 hrs with controlled agitation at (150 rpm) at the end period the broth culture was centrifuged at (5000 rpm) for 30 min the supernatant was collected to assay protease activity and protein concentration.

Determination of optimized inoculum concentration of protease production

Different concentrations of isolates were used depending on Mcfarland standard No.(0.5). The standard inoculum obtained from this number by adjust the turbidity of bacterial suspension of colonies selected from 18 – 24 hrs agar plate to obtain the desired standard (10^6, 10^7, 10^8, 10^9) CFU/mL, added 1% ml of each inoculum

concentration to the production medium and incubated in shaking incubator at (150 rpm) for 24 hrs . Centrifuged at (5000 rpm) for 30 min then assay protease activity and protein concentration.

Determination of optimized initial medium pH for protease production

Optimized production medium was adjusted with different initial pH values (5, 6, 7, 8, 9) to study their effect on protease production the cultures were inoculated with 1% ml of optimized inoculum concentration and incubated in shaking incubator for 24 hrs. Centrifuged and assay protease activity and protein concentration.

Determination of optimized incubation temperature for protease production

Production medium was inoculated by the addition of 1% ml of optimized inoculum and incubated at different temperature (25, 37, 40, 45)°C for 24 hrs. Then centrifuged and assay protease activity and protein concentration.

Determination of optimized incubation period for protease production

The optimized protease production medium was inoculated with 1% of optimized inoculum concentration and incubated at different time period (18, 24, 30, 48) hrs, centrifuged at (5000 rpm) for 30 min, the culture solution obtained was used to assay protease activity and protein concentration.

RESULT AND DISCUSSION

Selected two isolated bacteria of *E.coli* (*E.coli* 1, *E.coli* 2) were identified based on morphological, biochemical characteristics and the Vitek2 system which showed the maximum protease production on skim milk agar by zone of hydrolysis around colonies were found (20, 16) mm respectively and which showed higher protease activity by Qualitative and Quantitative assay of protease, these isolated used for protease optimization studies.

Optimization of culture condition for protease production

Effect of culture media on protease production

The effect of culture media on protease production was shows in (figure 1), maximum protease production was observed in Tryptic Soy Broth for (*E.coli* 1, *E.coli* 2) after 24 hrs of incubation with specific activity (3.09, 2.71) unit/mg respectively, this result similar to the study reported by,[24] which found that the highest protease production in Tryptic Soy Broth.

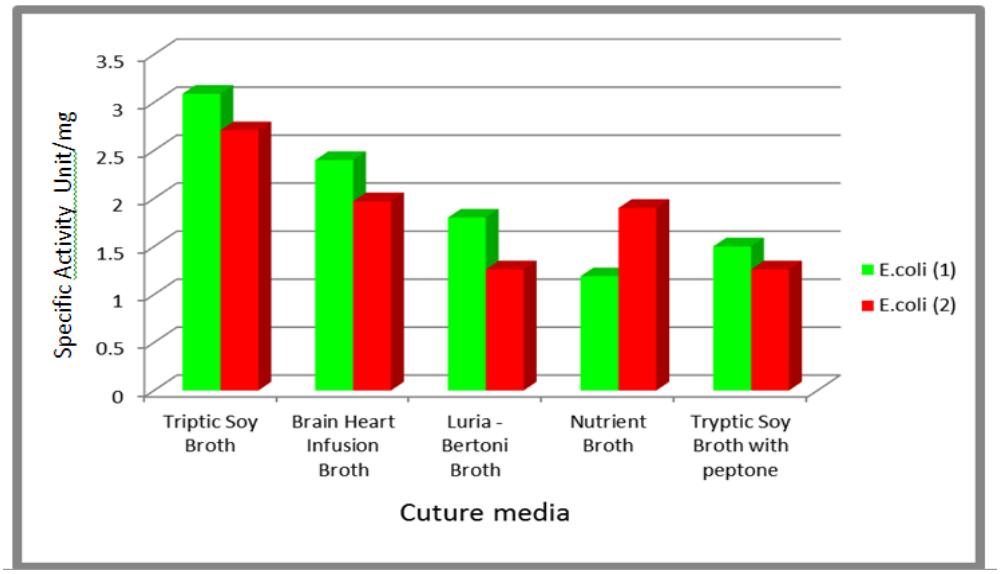

Figure 1: The optimized culture medium for protease produced from *E.coli*.

Effect inoculum concentration on protease production

The study shows that optimum inoculum concentration of bacteria isolate for protease production was 1.5×10^8 CFU/ml (3.3, 3.0) unit/mg, (figure 2). The less protease production (1.5, 1.3) unit/mg in small inoculum concentration 1.5×10^6 CFU/ml may be due to insufficient number of bacteria which lead to reduce the amount of secreted protease and also the enzyme activity was decreased even in higher inoculum concentration and that due to reduced dissolved Oxygen and increased competition towards nutrient,[25] optimum inoculum concentration was 1.5×10^8 CFU/ml form (*E.coli* 1, *E.coli* 2) for maximum protease production.

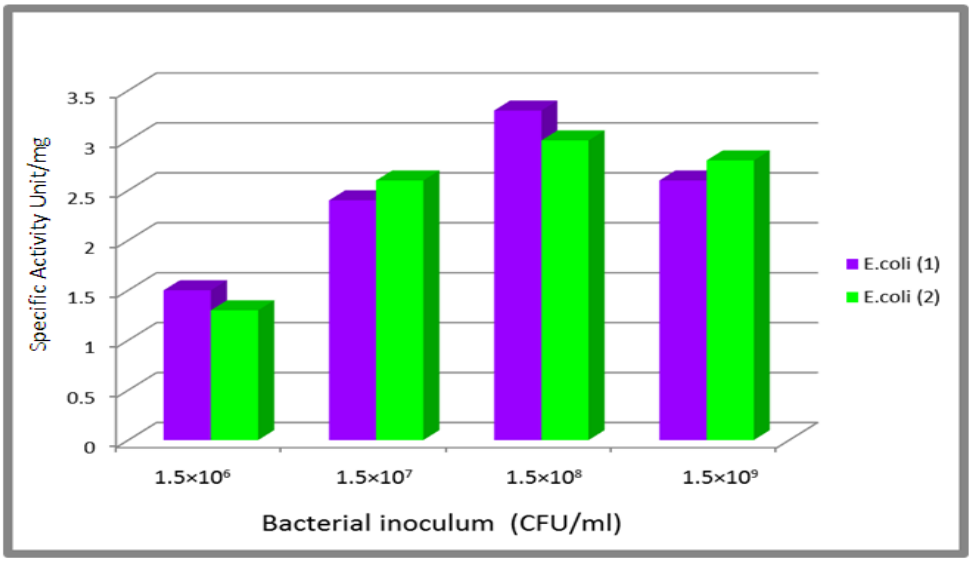

Figure 2: The optimized inoculum concentration of protease produced from *E.coli.*

Effect of pH on protease production

Different pH values (5 , 6 , 7, 8 , 9) were used to study their effect on protease production the optimum pH for protease production was found to be 6 with specific activity (2.98, 2.94) unit/mg (figure 3), this result was similar to acidic protease produced by *E.coli* and Enterobacter 5.4 in similar study reported by.[2]

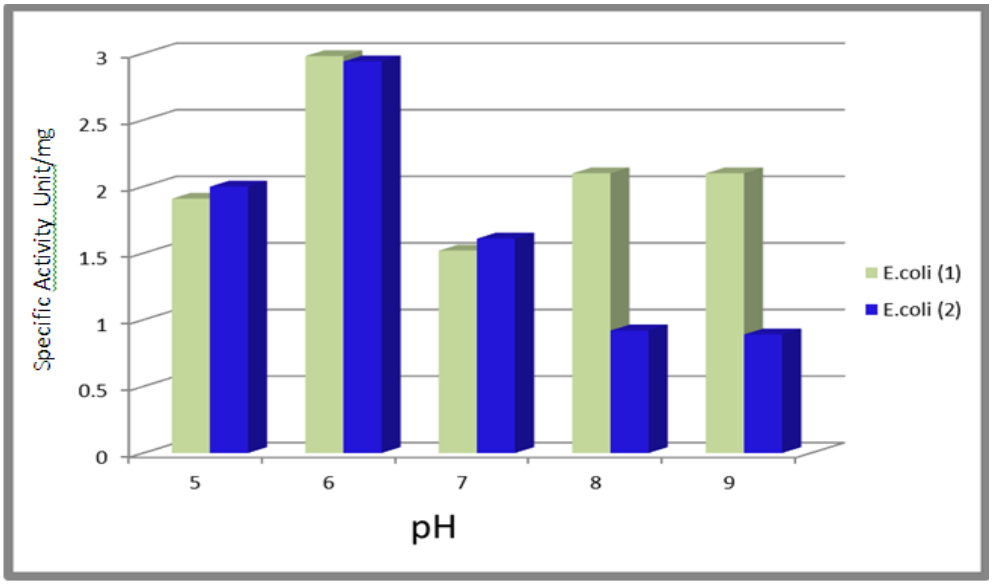

Figure 3: The optimized pH for protease produced from *E.coli.*

Effect of incubation temperature on protease production

Different temperature ranges (25, 37, 40, 45)°C were used to study the effect of temperature on protease production, the maximum protease production was observed at 40°C when the specific activity recorded (2.9, 2.5) unit/mg of protease (figure 4), this result agreed with the study reported by,[26] who found that the optimum protease activity from *Bacillus frimus* was observed at 40°C.

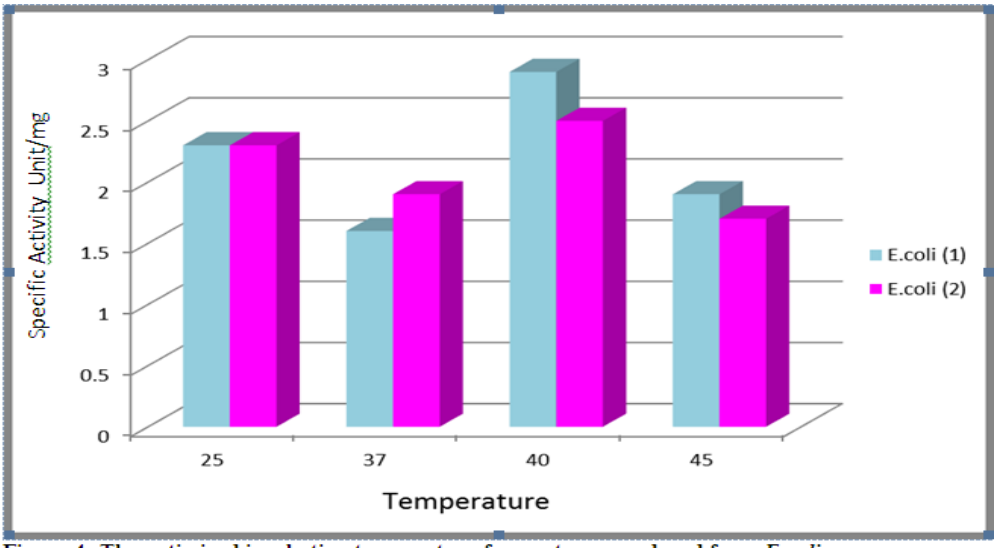

Figure 4: The optimized incubation temperature for protease produced from *E.coli.*

Effect of incubation period on protease production

Different time of incubation period was used (18, 24, 30, 48) hrs to determined optimum protease production, high level of protease was recorded at 24 hrs (2.7, 2.5) unit/mg (figure 5), the protease production decrease during longer incubation period to reached (2.1, 2.0) unit/mg during 48 hrs and this may be due to autolysis or may be due to change in culture condition, this result similar with the result obtained by.[27]

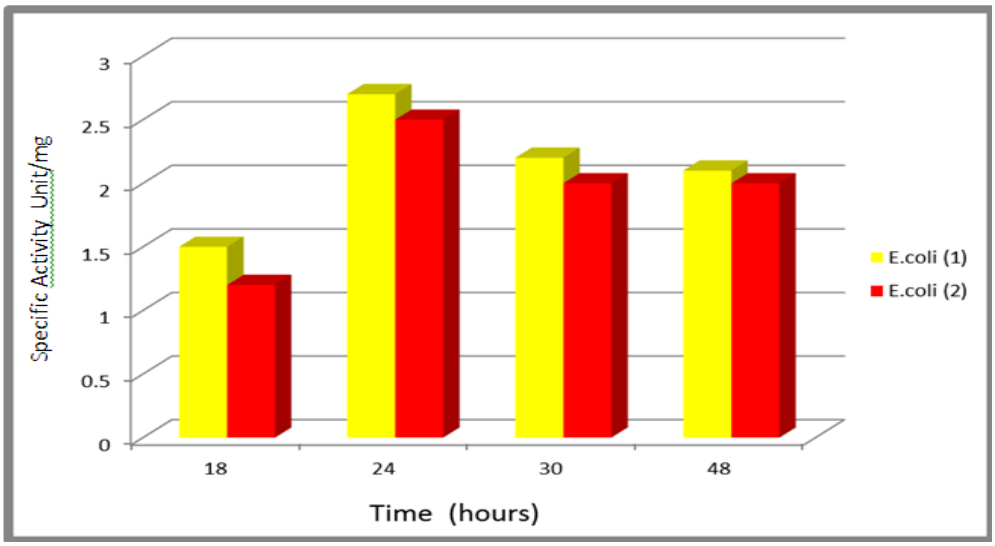

Figure 5: The optimized incubation period for protease enzyme produced from *E.coli.*

CONCLUSION

In the present study *E.coli* was isolated from UTI and investigated the optimum culture medium, optimum inoculum concentration, pH, Temperature, incubation period, for highest protease production were determined as (Tryptic Soy Broth, 1.5×10^{8} CFU/ml, 6, 40, 24) respectively. Extracellular acidic protease production by *E.coli* have application in meat tenderization in the production of fermented foods and also in acidic cleaning composition.

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