Antibacterial Activity of Protease that Production, Purification and Characterization from Proteus Mirabilis against Gram Positive and Negative Pathogenic Bacteria

Baydaa A. Hassan[1], Maha Diekan Abbas[2], Suzan Nazar Rasool[3]
[1]College of Science, Kufa University, Iraq
[2]College of Biotechnology, Al Qasim Green University, Iraq
[3]Ministry of health, Iraq
Email: baidaa.aljanabi@uokufa.edu.iq

Abstract
This research was accompanied in the laboratories of Biology Section, Faculty of Science, that deal with production, purification and characterization of protease by Proteus mirabilis which carried out for better making of protease using 1% casein as the substrate of enzyme, the manufacture was carried out by flooded fermentation, the greatest surroundings were the isolated of protease in synthetic medium, it donated great titer of protease activity, cultivation period 48 h, cultivation temperature 30 °C and pH = 7, the protease was purified using precipitation by ammonium sulphate (60%) and dialysis, the developed protease had finest activity at pH = 7, the protease was stable with pH values ranging between (6 - 9) and in temperature 40 °C also protease was stable in (30- 50) °C,examines of the protease for molecular weight was approved out by SDS-PAGE electrophoresis which discovered 47 KDa, Among the 5 pathogenic bacteria tested only 2 isolates, Micrococcus sp. and S. aureus showed inhibition zone on protein pellete treated agar after 24 hour with inhibition zone 9 mm for Micrococcus sp. and 12 mm for S. aureus, but no antibacterial activity of protease showed against gram negative bacteria involved E.coli, Salmonella typhi,, and Pseudomonas aeruginosa
Keywords: Protease; Proteus mirabilis; characterization

1. Introduction

Proteases are a large class of enzymatic molecules that catalyze the cleavage of peptide bonds, they are present in all living organisms, in which they display many essential physiological functions ranging from generalized protein degradation to more specific regulatory activity, proteases can be both intracellular and extracellular in nature, the extracellular proteases are less selective in their substrate recognition and can cleave both self and non-self-molecules with equal efficiency, it is therefore essential that these enzymes be expressed as zymogens or in their inactive forms so as to prevent premature proteolytic activity which is injurious to producer cell itself [1]. Proteases account for about 65% of the total industrial enzyme market [2]. These proteases have wide-ranging applications in detergent, pharmaceutical, food, chemicals, degelatinization of photo films and leather industries [3].

Among different sources, such as plants, animals, and microbes, proteases are generally produced by microbial sources, among microbes, Bacillus sp, Pseudomonas sp, Proteus sp are extensively studied for protease production in a large scale, and they are exploited in various industries like leather, detergent, pharmaceuticals, and textile; some fungal species like Aspergillus sp. have been studied thoroughly for the production of protease [4, 5].

2. Materials and Methods

2.1. Proteus mirabilis isolates

A total number of 50 samples were collected from patients with urinary tract infection admitted to AL-Sader Teaching Hospital, AL-Hakim General Hospital and AL-Furat Alawsat Hospital in AL-Najaf Governorate, after the end of incubation period of bacteria isolates, identification of these isolates were based on morphological and biochemical tests which described by MacFaddin [6].

2.2. Proteus mirabilis inoculum preparation: It was organized according to (7).

(Table 1): Fermentation medium compositions for Proteus mirabilis growth.
S/N Ingredients Quantity(g/ml)
1 (NH4)2SO4 0.1
2 Peptone 2
3 soybean meal 2
4 KH2PO4 0.1
5 glucose 1.5
6 sodium carbonate 0.5
7 Distilled water Up to 100 ml mark

2.3. Protease separation

The designated strains of isolates were transmitted in 500 ml flasks, the flasks were incubated at 37°C with a constant shaking at 200 rpm for 2 days, the cell was separated by centrifugation at 6000 rpm and

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4°C for 10 min, the supernatants were collected and used to conclude the protease activity, the separation expressed the maximum activity designated for advance study [7, 8].

2.4. Protease assay

Protease activity was assayed according to [9]. One unit of protease defined as the amount of enzyme required to produce an increase of 0.1 in optical density under optimal defined conditions.

2-5. protein Determination: Protein content was calculated according to [10]

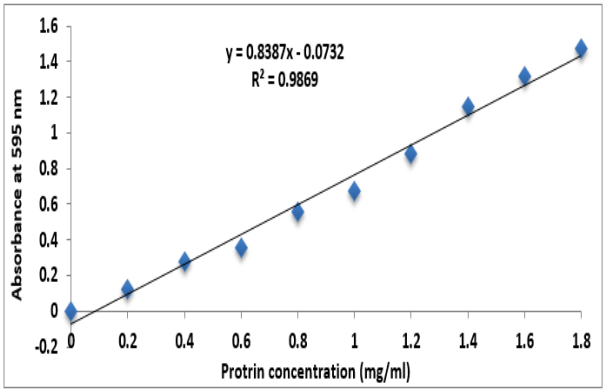

Figure (1): Standard curve for the various concentration of bovine serum albumin (BSA) at 595 nm.

2.6. Factors affecting production of protease: include pH regulation, medium composition, fermentation temperature and incubation period

2.7. Enzyme purification

2.7.1. Ammonium sulfate fractionation

For ammonium sulfate precipitation solid ammonium sulfate was added to supernatant at 20% saturation and left for 4 hours. The process of centrifugation was used to separate the precipitate. The process was performed again with 40% saturation. The precipitate was then again refined using 60% and 80% saturation in supernatant. The finalized precipitates were added to small amount de-ionized water. All the concentrated fractions were subjected to protein and enzyme activity assay to choose the fraction containing maximum activity [11].

2.7.2. Dialysis

The gained precipitation was introduced into dialysis bag against 0.05 M Tris buffer solution (pH 8.0) [12].

2.7.3. Gel filteration chromatography technique

It was achieved by using Sephadex G-100 was soaked in 100ml Tris buffer for 72 hours., the protein fractions was calculated at 280 nm [12].

2.7.4.Measurement of protease molecular weight

The molecular weight of purified protease was determined by SDS- PAGE according to [13].

2.8Characterization of protease

2.8.1. Optimal pH for enzyme activity

The 1% casein as the (substrate) was prepared with dissimilar pH ranges (4, 5, 6, 7, 8, 9, 10)

2.8.2. Optimal temperature for enzyme activity

The 1% casein as the (substrate) was prepared in tubes, the tubes were incubated in dissimilar temperatures (10, 20, 30, 40, 50, 60) °C.

2.9. Antibacterial activity of protease from Proteus mirabilis

Pathogenic bacteria that isolated from raw meat samples which used in this assay were E. coli, S. aureus, Micrococcus sp., Salmonella typhi, and Pseudomonas aeruginosa. Bacteria were diluted using McFarland standard and measured on spectrophotometer (600 nm). Bacteria were then diluted with 0.85% NaCl, each well was added with 50 mL sample of pellet protein (protease). Incubation was performed at 37C for ±24 hours, then the diameter of inhibition zone (mm) was measured in each well.

3. Results and Discussion

3.1. Identification of bacterial isolates

The Table (2) shown that the results of biochemical tests which used for the Proteus mirabilis isolates identification, this results found all the bacterial isolates are catalase,citrate, motility, methyl red and H2S production and urease test positive, As well as all the Proteus mirabilis isolates are oxidase, indole and Voges- Proskauer test negative [6].

Table (2): Biochemical tests for Proteus mirabilis isolates

	Differential tests								
Proteus mirabilis Isolates	Catalase	Voges-Proskauer	Indole Production	H2S Production	Methyl Red	Urease	Citrate	Motility	Oxidase
	+	-	-	+	+	+	+	+	-

3-2: Factors affecting protease production

3-2-1: best culture medium for protease production

The maximum protease production by Proteus mirabilis was occurred using the synthetic medium, it gave higher titer of lipase (0.975 U/ml) followed by Luria – Bertoni medium with casein (0.721 U/ml) while nutrient broth gave low titer of protease activity (0.166 U/ml), figure (2). The choice of the suitable fermentation medium is a critical factor for microbial development and enzyme making, the growth of an organism in culture medium is inclined by the nutrient composition of the medium and the accessibility of these nutrients [14].

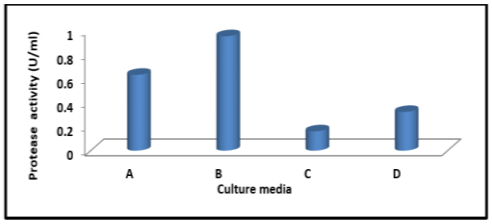

Figure (2): Effect culture media A: Luria – Bertoni medium with casein, B: synthetic medium, C: nutrient broth, D: Brain-Heart Infusion broth on the protease production from Proteus mirabilis.

3.2.2. Greatest incubation period for protease production

The figure (3) shown the increasing of protease activity with increasing the incubation period until reach to highest activity (0.842 U/ml) in second day (48 h) using the casein as a substrate of enzyme, then it began to decreased (0.453, 0.212 U/ml) in 72, 96 h respectively. Increase in cultivation period caused in decline in the creation of protease by Proteus mirabilis, this may be due to the certainty that after protease maximum creation, there was manufacture of other by products and a lessening of nutrients these results inhibited the organisms growing and later, enzyme formation (14). This result agree with other research [15] when they found the best protease production from Bacillus sp. accrued in the same incubation period, while other research [16] when they proved the best protease production from Bacillus sp. accrued in 72 h.

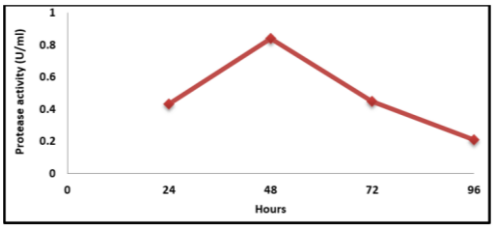

Figure (3): Effect of incubation period on creation of protease from Proteus mirabilis

3.2.3. Maximum temperature for protease production

The effect of temperature on enzyme production exhibited that the activity of protease increased gradually with rise in temperature from 10 °C attainment a full in 30°C (0.772 U/ml) above this temperature there was a lessening in the protease activity (0.345, 0.163 U/ml) in 40, 50 °C respectively (Figure 4). Growing heat is a very chief factor which varies from organism to organism and minor changes in growth temperature may change enzyme formation [17, 18]. previous research [19] they showed that highest protease production from Bacillus cereus occurred in 35 °C.

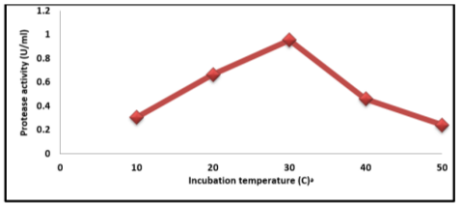

Figure (4): Effect of incubation temperature on the production of protease from Proteus mirabilis

3.2.4. Maximum pH for enzyme production

The figure (5) shown the rising of protease activity with rising the pH until reach to maximum activity (0.881 U/ml) in pH = 7 using the 1% casein as a substrate of enzyme, then it began to decline in greater pH values (0.591, 0.238 U/ml) in pH= 8, 9 respectively. Similar research [20] when they demonstrated the best protease production from Bacillus sp. accrued in the same pH.

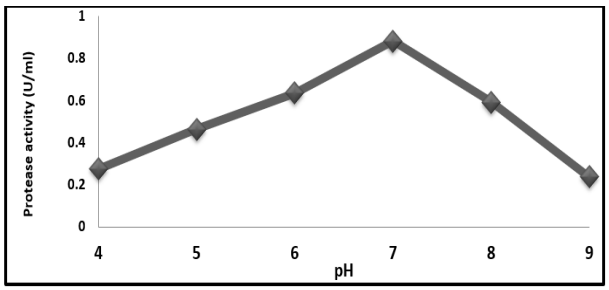

Figure (5): Effect of pH on the protease production from Proteus mirabilis.

3.3. Protease purification

3.3.1. Precipitation with ammonium sulfate

The ammonium sulfate used in different saturation ratios (20,40,60, and 80)%, then the 60% ratio was taken as finest ratio for precipitate the crude extract of enzyme, when the specific activity extended to (2.766U/mg), with a purification fold (1.084) and yield (0.422) as shown in table (3), while the additional saturation ratios (20,40,80) gave low down titer of specific activity (1.793, 2.056, 2.370 U/mg) respectively. Several studies [7] they showed the purified protease from newly isolated bacterial strain T3 used ammonium sulfate with 70 % saturation ratio.

3.3.2. Dialysis against Tris Hcl buffer

The acquired ammonium sulfate precipitate was introduced into dialysis bag over night against (50mm) Tris Hcl buffer, then the specific activity reached to (3.133 U/mg), with a purification fold (1.228) and yield (0.291) as shown in table (3).The additional results of Nassar et al. [21] they purified protease from Bacillus amyloliquefaciens used the same buffer.

3.3.3. Gel filteration chromatography technique

The enzyme solution produced from dialysis was passed during gel filtration using (Sephadex G -100) column (2× 40 cm) that equilibrated with (0.05M) Tris buffer at (pH= 8.0), the fractions were collected from column and calculated at 280 nm absorbency. In the first step of gel filtration the specific activity was the protein peak of (6.046 U/mg) with purification fold (2.370), while in the second step of gel filtration the specific activity reached (12.744 U/mg) with purification fold (4.995) shown in table (3). The other results recorded by Adesegun et al. [12] when they get specific activity (345.0µmol/min/mg protein) with purification fold (4.49) when they purified protease from Proteus vulgaris by using Sephadex G -100 column.

Purification steps	Volume (ml)	Activity (U/ml)	Total activity(U)	Protein con.(mg/ml)	Specific activity(U/mg)	Fold	Yield %
Crude enzyme	20	2.171	43.42	0. 851	2.551	1	100
Ammonium sulfate precipitation (60)%	10	1.834	18.34	0.663	2.766	1.084	0.422
Dialysis against phosphate buffer	10	1.266	12.66	0.404	3.133	1.228	0.291
Gel filtration (First step)	5	0.913	4.565	0.151	6.046	2.370	0.105
Gel filtration (Second step)	5	0.548	2.74	0.043	12.744	4.995	0.063

3.4. Protease characterization

3.4.1. The optimum pH for protease activity

The figure (6) shown the rising the activity of protease purified from Proteus mirabilis with rising the pH until reach to highest activity (0.976 U/ml) in pH = 7 then it began to decreased in higher pH values (0.737, 0.512 U/ml) in pH= 8, 9 respectively. Added results like [22] they establish the maximum activity of protease purified from Pseudomonas aeruginosa ATCC 27853 happened at the same pH,, also [23] found the optimum activity of protease purified from P. aeruginosa ME4 occurred at pH =7

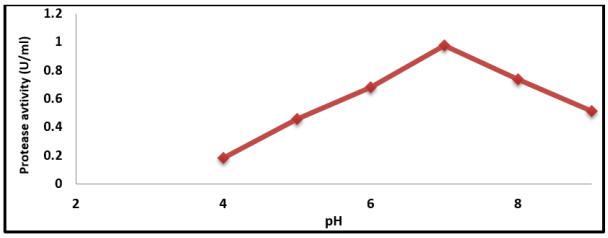

Figure (6): Effect of several pH on the activity of purified protease from Proteus mirabilis

3.4.2. The greatest pH for protease stability

To study the effect of optimum pH for protease stability the protease solution was incubated with varied buffers pH values ranging between (4-10), for 30 minutes at room temperature, then calculated the remaining activity. The figure (7) demonstrates the best pH for protease stability ranging between (6- 9) and the stability was decreased in great alkaline and acidic pH. the enzyme was kept 60% of activity in pH= 5 while the activity was decline in pH= 4 and pH =10 to 37%, and 45 % respectively. Various researches pointed to the finest pH of protease stability similar [21] they presented that the optimum pH of protease stability purified from Bacillus amyloliquefaciens 35s ranging between (7–10).

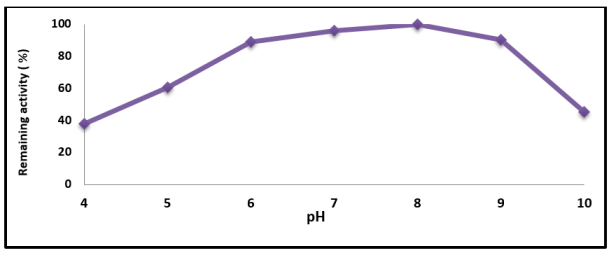

Figure (7): Effect of pH on the stability of purified protease from Proteus mirabilis.

3.4.3. Finest temperature for protease activity

To establish the best temperature of protease activity purified from Proteus mirabilis, the enzyme reaction was done in dissimilar range of temperature (10 - 60) °C, and the results shown in figure (8) rising the activity of enzyme with increasing the temperature until reached to highest activity of lipase (0.787U/ml) in 40°C then it began to decreased in elevated temperature values (0.332, 0.164U/ml) in 50, 60 °C respectively. Further readings similar [20] they found the maximum activity of protease purified from Bacillus cereus was in 50 °C

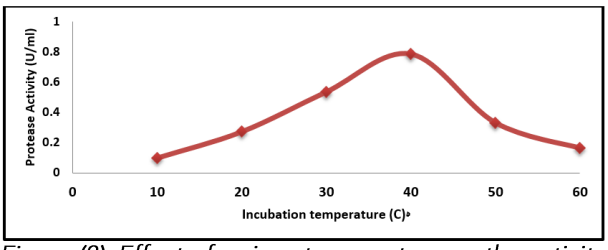

Figure (8): Effect of various temperature on the activity of purified

protease from Proteus mirabilis

3.4.4. The optimum temperature of protease stability

The figure (9) showing the results of incubation of enzyme with varied temperature ranging between (10 -60) °C for 30 minutes, the enzyme was kept the activity when it incubated into (30 -50) °C. while keep 71% of its activity in temperature 20 °C, while its keep only 56 % in 60, °C, the variances of thermo stability values and reserve period of enzyme depend on the kind of substrate, buffer ionic strength, enzyme molecular weight and the enzyme cause.

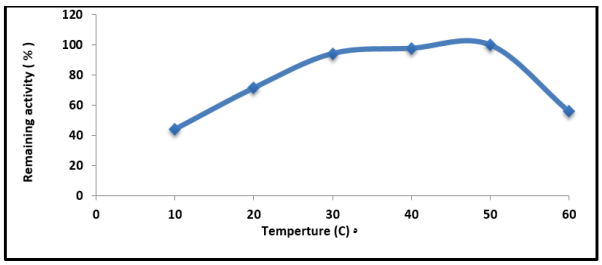

Figure (9): Effect of different incubation temperature on the stability of

purified protease from Proteus mirabilis

3.4.5. Molecular weight determination of protease by polyacrylamide gel electrophoresis technique

In order to examine the purity of the protease, which was purified from Proteus mirabilis, polyacrylamide gel electrophoresis under denaturing and with concentration12.5 %, the electrophoresis involve three samples, the first sample was crude enzyme extract, the second sample was the first step of gel filtration, the third sample was the second step of gel filtration, when the gel is engrossed in coomassie

brilliant blue G- 250, numerous protein bands seemed with diverse molecular weight along the gel in crude extract sample, while one band appeared in the second and third sample. The appearance of many protein bands along the gel is imputed to that crude extract contains large number from different proteins with diverse molecular weights, the third sample was gave one band, this means that one protein with one molecular weight of approximately 47 kDa is found, other results like they found the molecular weight of protease isolated from P. mirabilis approximate 50 kDa, also [22] they originate the molecular weight of the protease isolated from Pseudomonas aeruginosa ATCC 27853 was 15 kDa when its mobility relative to those of standard proteins on SDS-PAGE.

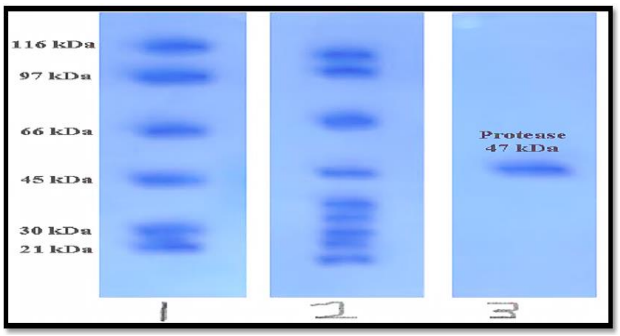

Figure (10): The polyacrylamide gel electrophoresis of the protease from Proteus mirabilisunder denaturing conditions. Lane (1) standard molecular weight markers, lane (2) crude extract enzyme, lane (3) purified protease produced from the second step of gel filtration

3.4.6: Protease antibacterial activity against gram positive and negative pathogenic bacteria

The antibacterial activity of protease was evaluated using 5 pathogenic bacteria that isolated from raw meat samples which used in this assay were E. coli, S. aureus, Micrococcus sp., Salmonella typhi, and Pseudomonas aeruginosa. Among the 5 pathogenic bacteria tested only 2 isolates, Micrococcus sp. and S. aureus showed inhibition zone on protein pellete treated agar after 24 hour with inhibition zone 9 mm for Micrococcus sp. and 12 mm for S. aureus, but no antibacterial activity of protease showed against gram negative bacteria, this result indicated that the protein pellet is specifically showing its antibacterial activity only to Gram-positive bacteria Figure (11).

Some bacterial proteases showed excellent anti-cancer, anti-microbial activities, scientists have found the broad use of proteases in medical field successfully, In medicine, different formulas, such as gauze, non-woven tissues, and ointment composition containing alkaline proteases produced by B. subtilis show promising therapeutic properties [24, 25]. It has been reported that fibrin degradation has been achieved by alkaline fibrinolytic proteases, the use of this fibrinolytic enzyme suggests its future application as an anticancer drug and in thrombolytic therapy [26]. This result similar to previous studies like [27] when they found the capacity of extracellular protease from Xylaria psidii KT30 against only gram positive bacteria included, B. subtilis and S. aureus with inhibition zone 8 ± 0.57 mm for B. subtilis and 7 ± 0.57 mm for S. aureus.

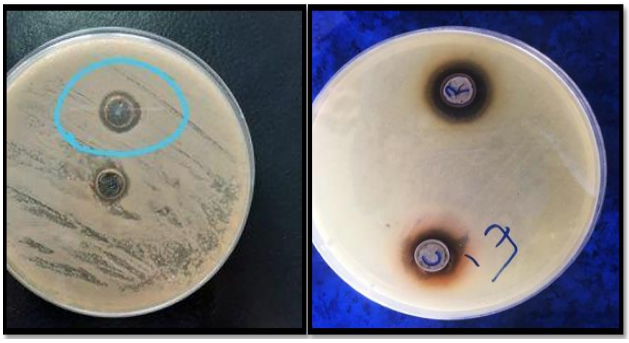

Figure (11): Antibacterial activity of protease against gram positive bacteria A. Micrococcus sp B. S. aureus

4. Conclusion

Lastly this research it can be finished that Proteus mirabilis isolates can be a good creation for the protease making being charity technologically. protease purified here was association to be stable in a pH extent of 6 to 9 and temperature extent of 30°C to 50°C. The ability of the protease developed here is like to the efficacy of the protease refined earlier by various investigators. The molecular mass was concluded by SDS- PAGE and a single band was identified later staining procedures giving signal of protease purity with 47 kDa. The prtotease is specifically showing its antibacterial activity only to Gram-positive bacteria.

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