Annals of R.S.C.B., ISSN: 1583-6258, Vol. 25, Issue 1, 2021, Pages. 6812 - 6830
Received 15 December 2020; Accepted 05 January 2021.

Determination of Optimal Conditions for the Activity and Stability of the Protease Produced from Bacillus Subtilis Isolated from Meat

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Summary:
26 different samples of red and white meat were collected from different areas in Erbil city for the period from 12-3-2019 until 15-6-2019, and the contaminated bacteria types were isolated and diagnosed with the meat samples using the Vitek-2 device, as well as depending on the phenotypic characteristics and biochemical tests, which appeared to be prepared automatically. Types of E.coli, Klebsiella, Micrococcus luteus, Staphylococcus aureus, Staph epidermidis, Enterococcus faecalis, Streptococcus pneamoniae, Micrococcus luteus, Pseudomonus aeruginosa, Bacillus cereus and B. Subtilis The isolate, which is believed to belong to B.subtilis, was selected, the phenotypic and microscopic characteristics of this bacteria were studied, in addition to biochemical tests, where it was confirmed belong to B. subtilis bacteria, and used to produce the protease enzyme. Ionic and gel filtration chromatography. The results showed that the optimum conditions for the enzyme activity were at o30°c, with enzymatic efficacy 2955 units / ml, pH 9.0, and enzymatic efficacy 544.5 units / ml. As for the stability of the enzyme, the best temperature for the stability of the enzyme was at 50°c, and the enzyme efficiency was 328.5 units / ml for a full 15 minutes. The pH is 8.0, with an enzymatic activity of 583.5 units / ml as the enzyme has retained 100% of its activity. The effect of Nano carbon dot on pure protease enzyme was also studied with different pH numbers and the absorbance was obtained by Spectro photo meter as it was the highest absorbance at pH 5.0 as the protease activity was increased by 3 times of the free protease.

Key words: Protease, B.subtillus, enzyme activity, Enzyme stability, meats.

Introduction:

Protease enzymes have a great role and importance due to their entry into many industries, such as the dairy industries, meat softening, detergents and others, as they represent more than 60% of the total global enzyme sales. (Ningthoujam et al., 2009) Protease enzymes break down the peptide bonds of proteins and can be obtained from plants, animals, and micro-organisms and are classified into acidic, neutral or alkaline proteases based on the optimum pH (Mohammed et al., 2015) The best microbiology relied upon in the production of enzymes, especially the protease enzyme, in the industry are Bacillus subtilis, Aspergillus niger and A.oryzea as these species produce health-safe enzymes in addition to their high production (Ningthoujam et al., 2009). B. subtilis is a bacterial species Gram positive, which belongs to the Bacllaceae family, which was called Vibro subtilis from the year 1835 and was renamed in the year 1872 to B. subtilis, and these bacteria are found naturally in meat, soil and vegetables and grow at medium temperatures and that the optimum temperature growth ranges between 25-35°c (Al-Ezzi, 2015).

And to talk about these bacteria in the field of biotechnology, they have tremendous benefits in many diverse fields, for years, their biochemical properties have been studied and it has been concluded that they are not harmful to human , because it does not cause any harm through contact, but some harmful effects have been described to it, despite this, the many proven benefits of this bacteria , whether in agriculture,

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medicine or industry, have made it one of the best options when it comes to the positive effect of some types of bacteria on humanity (Miyazaki and Sarti 2013).

Materials and Methods:

1- Sampling: 26 samples of red, white, fresh and frozen meat were collected from different places and at different time periods. It varied between being cooked, semi-cooked and fresh, as the samples were immersed in normal saline and left for two hours until the meat ingredients mixed with it.

2- Isolation of bacteria types from meat samples: For the purpose of isolating the types of contaminated bacteria from the meat samples, the decimal alleviation method was used for the meat samples. As for the cultivation, it was done by using MacConkey agar, Manitol Salt agar, Nutrient agar, Blood agar.

3- Isolation of B. subtillus bacteria: For the purpose of isolating this bacteria, the decimal fear method was used for the meat samples, as each diluted sample was added to the surface of the medium.

4- Diagnosis of isolated bacteria types: - The diagnostic process was carried out depending on the phenotypic characteristics, microscopic examination and biochemical tests, which included examination of catalase, oxidase, urease, casein, gelatin , Methyl red and Triple Sugar (TSI).

5- Diagnosis using the Vitek2 compact system, which is supplied by Bio Merieux (USA), using the usual biochemical tests referred to above.

6- Production of protease from B. subtilis bacteria:

A- Semi-quantitative detection of the protease enzyme production: The bacterial isolates were grown on a Nutrient broth medium in test tubes at a Loop full for each tube and incubated at 37^{o}c for 24 hours. 40 µl were taken from each tube and placed in pits located in the center of Skim milk agar with several pits per plate and the plates were incubated at 37^{o}c for 48 hours. 72 hours.

B- Quantitative detection of protease enzyme production: In it, the isolates were activated on the medium of the nutrient broth, then incubated at a temperature of 37 ^{o}c for 24 hours, after which the isolate producing the enzyme that belonged to B. subtilis bacteria isolated from meat was inoculated with a loop full in the flask containing the medium Nutrient broth and the flask was placed in the shaker incubator at the speed 120 rpm at of 37°c for 24 hours. After that, it was centrifuged in a centrifuge at a speed of 6000 rpm at a temperature of 4 °c for 20 minutes, and then the supernatant was taken which contain crude enzyme.

7- Proteolytic activity test A plate containing skim milk agar was prepared, then we made a hole in the plate with a size of 1cm with 6 holes, and 40 µl of raw enzyme were added to each hole and incubated at 37°c for 24 hours.The presence of a different color around the edge of the hole in the form of a gel indicative of the activity of the crude enzyme extracted (Ponnuswarmy and Vincent 2013).

8-Assay of enzyme activity: of the enzyme According to Bradford's method, (1997) using bovine serum albumin (BSA) the protein concentration was determined by making a standard curve for bovine serum albumin using different concentrations of stocked BSA and the absorbance was measured at a wavelength of 595 nm.

9- Determination of the optimum conditions for the crude protease enzyme: The Muyan et al. (2006) method has been used as follows: A- The pH, in which 2 ml of 0.5% casein was prepared with 100 µl of EDTA (0.04 ml / liter) with 400 µl of phosphate buffer (Tris-Hcl, Ph = 8) with 200 µl of crude enzyme with 800 µl water It was distilled, mixed and treated with different pH numbers (8,9,10) at a temperature of 40°c for 15 minutes, which was the optimum degree of enzyme activity, and the reaction was stopped by adding 1 ml of TCA (30%) and then centrifuged at 4000rpm for 15 minutes and then 1 ml of the filtrate was withdrawn and added to a test tube and 5 ml of Na_2Co_3 (0.55 ml / liter) was added to it with 1 ml of Fulen's

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reagent, mixed and left for 15 minutes,and the absorbance was measured with a Spectrophotometer at a wavelength of 680nm.

B- Temperature: 2 ml casein (0.5%) was mixed with 100 µl EDTA (0.04 mol / liter) with 400 µl of crude enzyme with 800 µl of distilled water and mixed and treated at different temperatures (30,40,50,60,70,80 ^{o}c) and a pH of 9.0, which is optimal for enzyme activity for 15 minutes, after which the reaction was stopped by adding 1 ml of TCA (30%) and centrifuged at 4000rpm for 20 minutes. Then 1 ml of the filtrate was withdrawn and placed in a test tube, and 5 ml of Na_2Co_3 (0.55 mol / liter) was added to it with 1 ml of Follen's reagent, mixed and left for 15 minutes, and the absorbance was measured with a Spectrophotometer at a wavelength of 680nm.

10- Purification of enzyme The enzyme was partially purified according to Hao et al. (2013) by performing the following steps:

A- Precipitation with ammonium sulfate and different saturation ratios until the best saturation percentage was obtained and it was 50%, where 50 gm of aluminum sulfate was added to 100 ml of raw enzyme gradually in an ice bath with continuous stirring for 30 minutes and centrifuged at a speed of 5000rpm at 4^{o}c. for 15 minutes, the precipitate was dissolved in a small amount of (Tris-Hcl, Ph = 8). The activity and concentration of the protein were estimated and applied in the bucket solution itself for 24 hours.

B- Dialysis membrane screening of the ammonium sulfate precipitate using dialysis bags in distilled water for 48 hours, with the addition of $CaCl_2$ (0.002 mL) to distilled water in a 500 ml container. The water was changed every three hours at a temperature of 4^{o}c with continuous stirring for 48 hours and centerifuged. At a speed of 7000rpm at 4 oC for one hour, then dissolve the precipitate in 10 mL of a solution of glycin and a NaOH buffer with pH 8 (Muhsin and Aubaid, 2000 and Al-Janabi, 2005). C- Ion exchange chromatography, DEAE-cellulose: In it, 5 ml of concentrated enzyme extract were added to the ion exchange column and the column was washed with a Tris buffer (Tris-Hcl, Ph = 8), where 2 ml of the concentrated solution was added, followed by 2 ml of phosphate buffer solution for washing, and the enzyme was recovered at a flow rate of 1 ml / minute and follow-up. The enzymatic content by reading the absorption intensity at the wavelength of 650nm and by using a UV-visible spectrophotometer and the specific activity of the enzyme was estimated.

E- Electrophoresis in an acryl amide gel in the presence of SDS (SDS Poly acryl amide gel electrophoresis) This method was used to estimate the molecular weight of the protease enzyme and to know its homogeneity, as the necessary solutions were prepared and the electrophoresis process was carried out in the presence of SDS (Hames, 1985).

11-Characterization of the enzyme:

A- Determination of the optimum temperature for enzyme activity Prepare 2 mL of casein (0.5%) with 100 µl EDTA (0.04 mol / L) with 400 µl of phosphate buffer with 200 µl of enzyme purified extract with 800 µl distilled water, mixed and incubated at different temperatures (37-40-45-50- 60-70^{o}c for 15 minutes at pH 9 and the reaction was stopped by adding 1 ml of TCA (30%) and discarded using centrifugation at 4000rpm for 15 minutes, where the filtrate was withdrawn and placed in a test tube and 5 ml Na_2Co_3 (0.55 mol/lit) was added to it. / L) with 1 ml of Follen's reagent, mixed and left for 15 minutes, the absorbance and enzymatic activity were calculated using a Spectrophotometer at a wavelength of 680nm (Wing and Pan, 1997).

B- Determination of the thermal stability of the enzyme: Incubate 200 µl of the enzyme at 100 ° C for 10 minutes without substrate with 100 µl EDTA (0.04 mol / L) with 400 µl of buffer (Tris-Hcl, Ph = 9.0) with 800 µl distilled water at different temperatures (30-40-50-60^{o} c)for 15 minutes and the reaction was stopped by adding 1 mL of TCA (5%) and centerifuged at a 4000rpm centrifuge for 15-20 minutes, 1 mL of filtrate

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was withdrawn and 5 mL Na_2Co_3 (0.55 mol / L) was added to it. and 1 ml of Follen's reagent, mixed it and left for 15 minutes, after which the effectiveness was estimated using a Spectrophotometer with a wavelength of 680nm.

C- Determination of the optimum pH for the activity: Prepare 2 ml of (0.5%) casein with 100 µl of 0.04 mol / liter EDTA with 400 µl of phosphate buffer Tris_Hcl with 200 µl of purified extract enzyme with 800 µl of distilled water and mixed and treated with different pH (4,5, 6,7,8,9) at a temperature of 40°C for 15 minutes and the reaction was stopped by adding 1 ml TCA (30%) and centrifuged at 4000 rpm / min for 15 min. Withdraw 1 ml of the supernatant liquid, add 5 ml of Na_2Co_3 (0.55 mol / liter) to it, add 1 ml of the folien reagent to it, mixed and leave for 15 minutes and then the effectiveness was estimated using a Spectrophotometer with a wavelength of 680 nm (Muyan, 2006).

D- Determination of the optimum pH for the stability of the enzyme: 200 µl of the enzyme incubated at 100 °c for 10 minutes without substrate with 100 µl EDTA (0.04 mol / L)with 400 µl of buffer(Tris Hcl pH = 9.0) at 0.05 mol concentration with 800 µl distilled water at different pH numbers (6,7, 8,9,10) at a temperature of 40°c for 15 minutes. Then the reaction was stopped with the addition of 1 ml TCA (5%) and centrifuge at 4000 rpm for 15-20 minutes and 1 ml of the purified enzyme supernatant was withdrawn and 5 ml Na_2Co_3 (0.55 mol / L) was added to it. Then 1 ml of Folin's reagent was added to it, mixed and left for a period 15 minutes after which the efficiency was estimated using a spectrophotometer of 680nm wavelength.

12- The interaction of the protease enzyme with NANO Carbon dot, in order to find out its effect on the pure protease enzyme, and by using two methods to measure the activity affecting the enzyme:

A- Measurement of absorbance with the presence of the enzyme. 100 µl of pure protease enzyme was used with 100 µl H_2O_2 and 200 µl Tmb with 100 µl of nanoparticles. 2 ml were obtained by adding acted buffer and absorbance was measured at a wavelength of 650nm.

B- Absorbance measurement without the presence of an enzyme, where 100 µl H_2O_2, 200 µl Tmb and 100 µl of nanoparticles were used. 2 ml were obtained by adding aceted buffer and absorbance measured at a wavelength of 650nm.

C- Measurement of optimal enzymatic activity in different hydrogen bases: To measure the effectiveness of the enzyme with carbonel NANO partecal and by using different pH numbers from the enzyme (5-7-9-10), 100 µl $H_2O_2$with 200 µl Tmb was used with 100 µl of nanoparticles and 2 ml was obtained by adding distilled water and measuring the absorbance in a spectra device. photo meter. D- Measurement of absorbance and enzymatic activity in different volumes: Different volumes of purified protease enzyme were used (10-50-100-150-200-500) µl, 100 µl H2O2, 200 µl Tmb and 100 µl nanoparticles were added. 2 ml were obtained by adding distilled water and measuring absorbance by spectrophotometer.

Results and discussion: -

1- Types of bacteria isolated from meat samples: The results of isolation and diagnostics showed that samples of red and white meat were contained, using MacConkey agar, Manitol Salt agar, Nutrent agar, Blood agar. It belongs to the following bacterial species: E.coli, Klebsiella, Micrococcus luteus, Staphylococcus aureus, Staph epidermidis, Entero coccus faecalis, Streptococcus pneamoniae, Micrococcus luteus, Pseudomonus aeruginosa, Bacillus cereus and B. Subtilis This is based on morphological properties, microscopy and biochemical tests, as well as using the Vitek2 device.

2- Isolation and diagnosis of B. subtilis bacteria: The isolation of the bacteria believed to belong to the B. subtilis type was carried out using the Bacillus differentiation agar .which was used to produce the protease enzyme used in the study the morphological features for colonis and Microscopic examination using gram

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stain and spore stain using Malachite green in addition to biochemical tests , the diagnosis confirmed by Vitek_2 device showed it is g^+,spore forming (subterminal),table-1 showed the results of biochemical. The following table shows the results of the biochemical tests for this bacteria.

Table (1): Biochemical tests for Bacillus Subtilis

Result	Name of the test	No
+	**Catelas**	**1**
+	**Oxsies**	**2**
_	**Ureas**	**3**
+	**Dissolve gelaten**	**4**
+	**Hadrolases casein**	**5**
_	Triple polysaccharide fermentation test	**6**
+	Reagent mythel red	**7**

3- Semi-quantitative detection of protease enzyme production: The semi-quantitative screening of the protease enzyme production was accomplished by local isolation from white and red meat samples through its ability to grow on the medium in Skim milk agar, as shown in Figure 1, depending on the formation of a region of degradation around the colony for each of the bacterial isolates. B. subtilis isolate showed efficiency in the production of the protease enzyme depending on the capacity of the hydrolysis zone around the colony in the medium of Skim milk agar, which confirms the ability of this isolate to produce the protease enzyme by converting protein materials into amino acids and peptides.

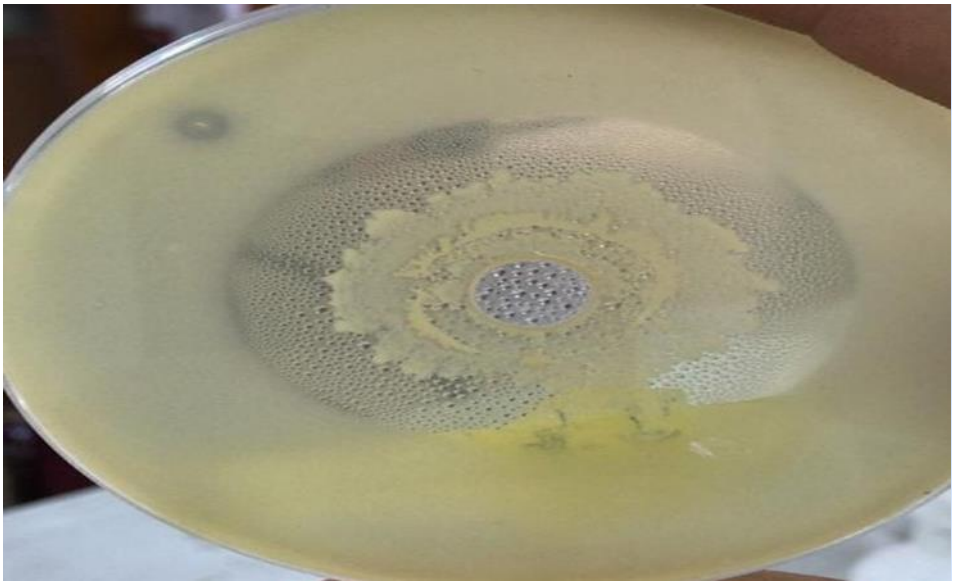

Figure 1: Semi-quantitative detection of the protease secreted by B. Subtilis

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4- Quantitative detection of protease enzyme production:

Quantitative screening of local isolates to produce protease enzyme was accomplished by determining the enzymatic activity in the culture medium to produce the enzyme at a temperature of 37ºC for a period of 72 hours, as it gave the enzymatic activity of the crude enzyme 600 units /ml with a specific efficacy of 260 units / mg and a protein concentration of 0.433 under the conditions of the experiment. The results are consistent. With what Mahdi and Ali (2009) pointed out that the highest activity of this enzyme is due to the strains of B. Subtilis.

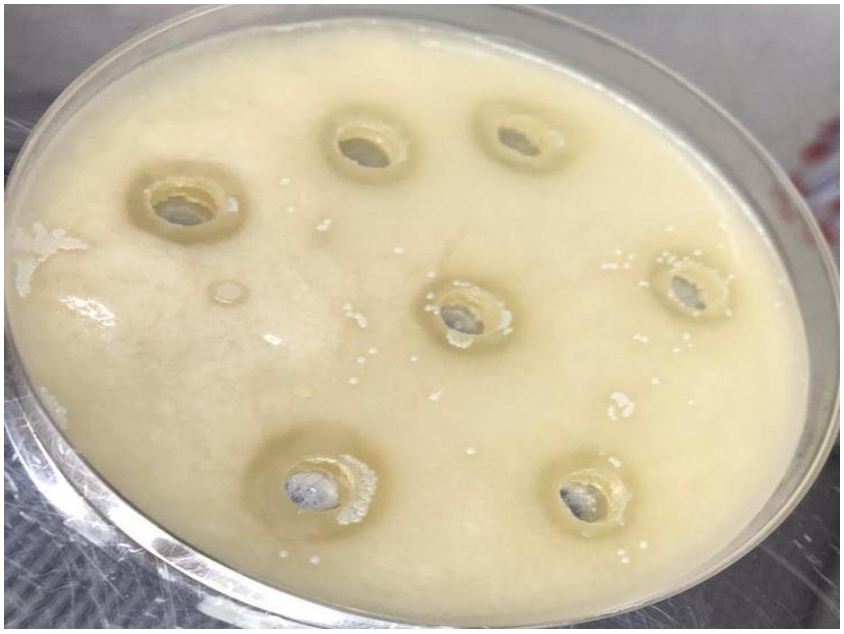

Figure 2: Decomposition of the media in the presence of B. subtilis, which produces the protease enzyme

5- The optimum conditions for the production of the enzyme from B.subtilis on the medium, Skim milk agar. When studying the effect of the incubation period, it was noticed that the largest diameter of the decomposing zone was obtained when incubating for 24 hours, as the diameter of the decomposition zone reached 40 mm, which represents the best production and this result is consistent with what It was referred to Al-Khafaji and others (2009) who found the best enzyme yields obtained at a 24-hour incubation period, but differed with Muller_ Alouf et al. (1997) in that their protease-producing isolates gave diameters ranged between 15.2-15.5 mm when using Str.pyogens during a period of time. 48 hour incubation.

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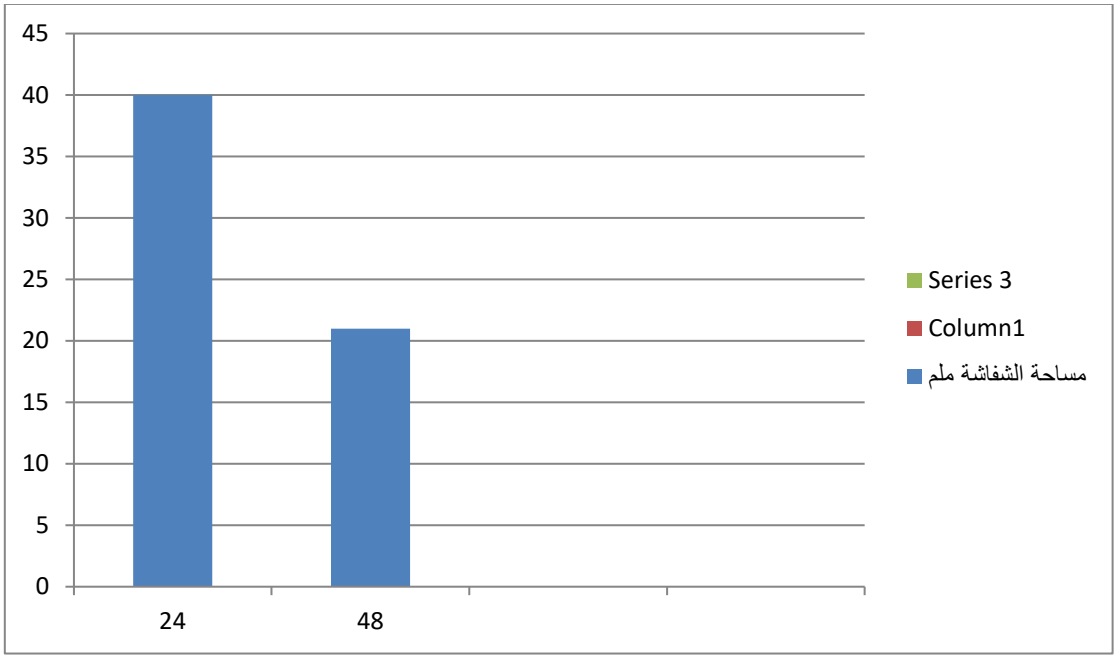

Figure 3: Shows the enzyme production from B. subtilis after a 24-hour incubation period

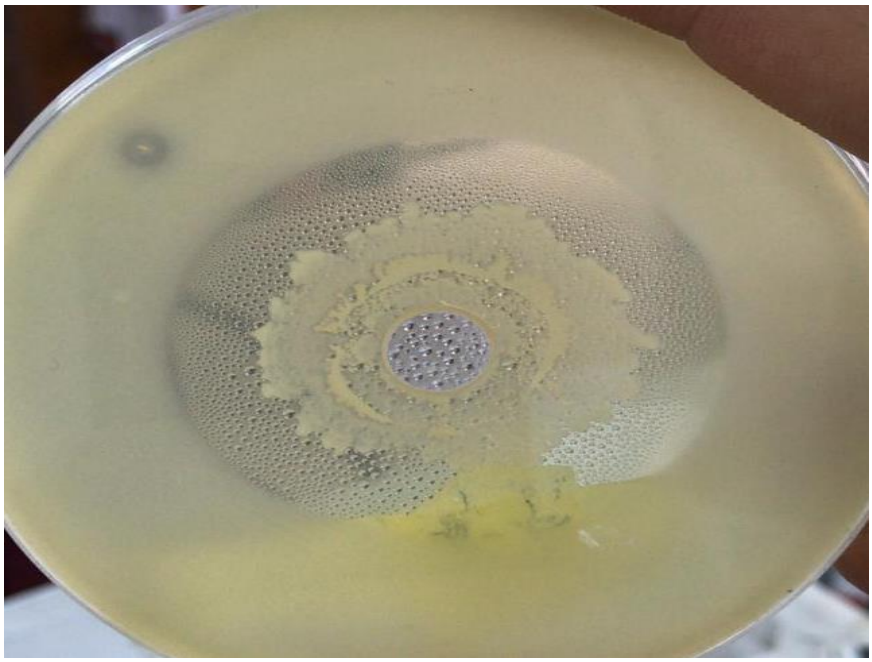

Figure 4- shows the area of the transparent region analyzed by the enzyme during the incubation period

As for the temperature, different temperatures were used 25,30,37,45, 55,60 ^{o}c and the results indicated that there was a difference in the diameter of the transparent zone about growth with different temperatures, as this isolation gave the highest effectiveness at a temperature of 45°c and reached The diameter of the transparent area is 35mm, and the results are similar to Behzad Otroshi etal, (2014) who obtained the best activity with a degree of 40°c. As for Pant etal, (2015) who obtained the best decomposition diameter of 2.2 cm at an incubation temperature of 35°c Basu et al, (2007) recorded that the optimum temperature for enzyme production was 35°c. The results agreed with A.sujatha and subash (2018) who indicated that A temperature of 40°c is the optimum temperature for production, as shown in Table 2: Table-

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2:Diameter of the degradation zone of the protease enzyme produced from B.subtilis at different temperatures.

Decomposition zone diameter/ mm	**temperature**
27	**25**
10.5	**30**
21	**37**
35	**45**
6	**55**
3	**60**

Regarding the pH, the pH of the production medium was amended to (4-6-7-9-10) to study the effect of the optimal pH, as the results showed that the best production of this enzyme was at a pH of 7, where the diameter of the decomposition zone reached 2 mm and the effect of the number The hydrogen peroxide in the production of the enzyme is due to its effect on the properties of the medium, such as the solubility of nutrients and the concentration of bicarbonate resulting from the dissolution of Co_2, which affects the buffering capacity of the medium, which is reflected in the growth of bacteria and their production of enzymes (Bull and Bushnell, 1976). On the other hand, the pH affects the stability of the enzyme, the best production of most of the exocrine enzymes is in the optimal pH numbers for growth, except that some of them deviate from that, and it is not necessary for the optimal pH of production to match the optimal pH for the enzymatic activity (Volesky and Luong, 1985) as shown in Fig. 5

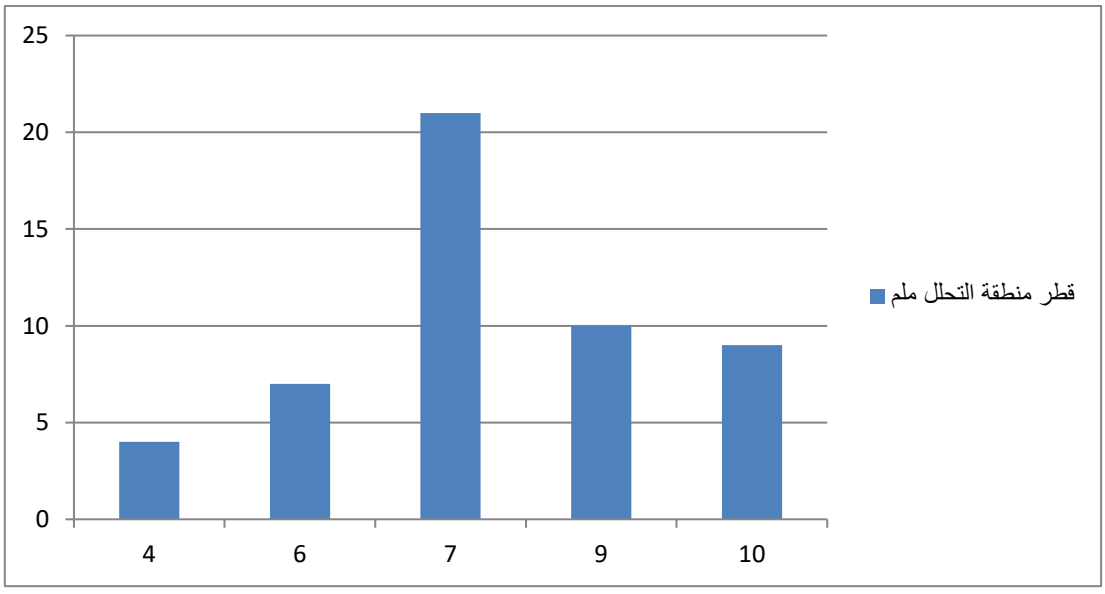

Figure 5 -The optimal pH number for the production of the butase enzyme from B. subtilis

These results were consistent with those obtained by Al-Rubaie and others (2012) who obtained the best dissolution diameter of 2.1 cm when using the protease enzyme at a pH of 7 for the enzyme produced from

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the bacterium P. aeruginosa. Also, it agreed with the results of Al-Juni (2017) who obtained the best degradation area of 4.5 cm at a pH of 7 for the enzyme produced from the same bacteria above.

6- Effectiveness of raw enzyme under different conditions:

A- pH:different pH numbers were used, and the results showed that the optimum pH with enzymatic efficacy was1378.5 units / ml. This result was identical with Al-Shehri et al. (2004) and it was similar to the liping wing (2015), where the optimum pH was 10 as for Anil Prakash. et al. (2018) showed that the best pH for the production of the protease enzyme from B. subtilis is 7.4 and the enzymatic activity is 143.73 units / ml. Whereas Qureshi et al. (2017) indicated that the optimum pH is 8.5 at a temperature of 50°c, as shown in Figure -6.

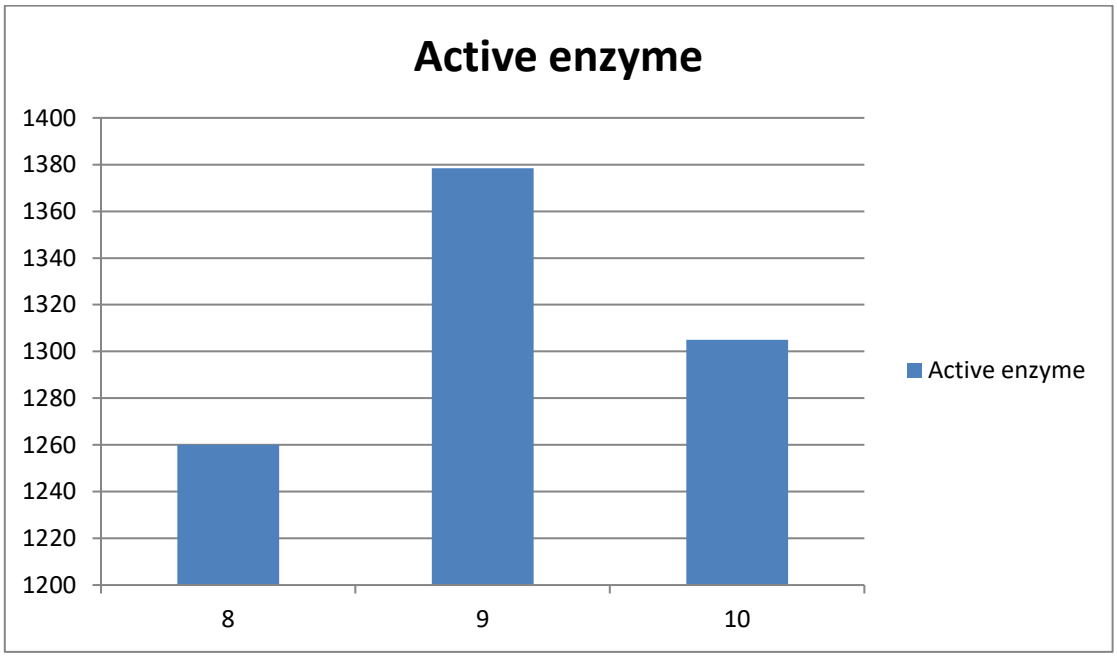

Figure 6-Raw enzyme activity at different pH levels

B- **Temperature**: Figure-7 shows the enzymatic and absorption activity of the crude protease at different temperatures, where an optimum temperature for the crude protease enzyme was obtained at a temperature of 30^{o}c with an enzymatic activity of 1065 units / mol and the results are consistent with what was reported by Anil Prakash et al. (2018) that The optimum temperature for the crude protease enzyme extracted from B. subtilis is 30^{o}c, but it differs with Qureshi et al. (2017) that the optimum temperature for the protease enzyme is 50^{o}c and with Ashwini (2014) who indicated that the optimum temperature for the production of this enzyme from B.subtilis subtilis are 40^{o}c.

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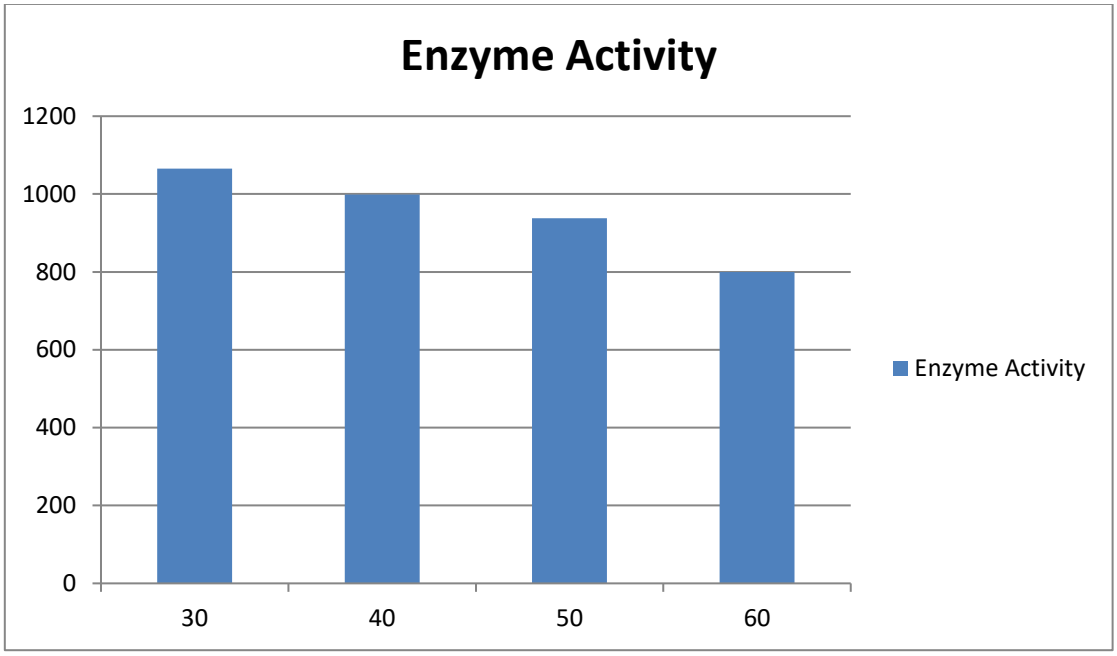

Figure-7: The enzymatic and absorbance activity of crude protease at different temperatures

7- Purification of the protease enzyme from B. subtilis The protease enzyme was purified in several steps that included precipitation with ammonium sulfate at a saturation rate of 40-60%, followed by the use of ion exchange chromatography with DEAE-Cellulose, and then followed by the first gel filtration using Sephadex-6B. First, ammonium sulfate was used with a saturation ratio of 40-60% and an enzymatic activity of 490 units / ml was obtained a protein concentration of 0.428 units/mg a high quality of 260 units / mg and an enzymatic yield of 49%, as shown in Figure -8, where in this step the charges on the The protein surface due to the action of salt and disruption of the nature of the water surrounding the protein molecules, which leads to a decrease in protein solubility and results in its precipitation (Scopes, 1987). The use of ammonium sulfate is due to its positive advantages, which are its high solubility, availability, appropriate cost and no harm to enzymes (Volesky and Luong, 1985). The results agreed with the findings of Sujatha and Subash (2017) that the optimum proportion of ammonium sulfate is 40-60%, but it differed with Ranganathan Kabilan (2016) where the optimum percentage was 70% with a pH of 7 and it converged with Asker and others (2013) who found that the ratio The optimum precipitation percentage for the protease enzyme produced from B. Subtilis is 60%.

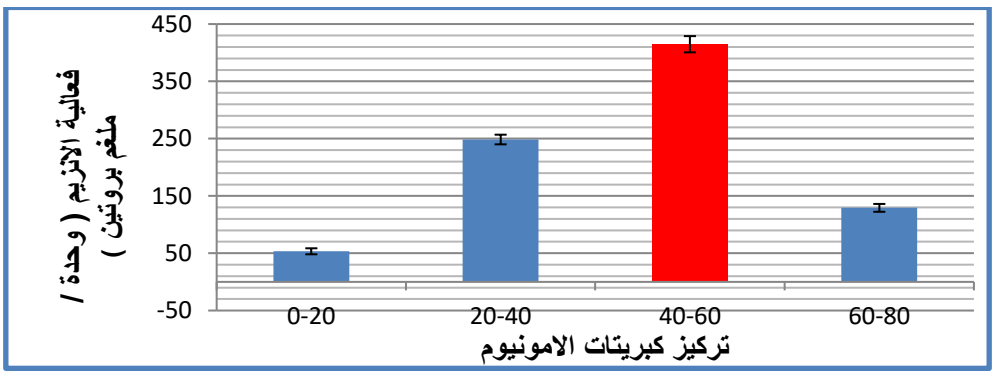

Figure (8) shows the specific activity of the protease enzyme after its concentration using different saturation ratios of ammonium sulfate.

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Then followed by the use of ion exchange chromatography technique using the DEAE-Cellulose ion exchanger after performing the dialysis process to concentrate the enzyme and get rid of the excess water by placing it inside the dialysate bags for 42 hours and submerged in the buffer used in the purification process (Pohl and others, 1995), and after that the enzyme solution was added. To an ion exchanger column with dimensions of 1.5 x 24 cm, the protein was recovered using a gradient of concentrations of 0.1 NaCl in molarity as shown in Figure -9.

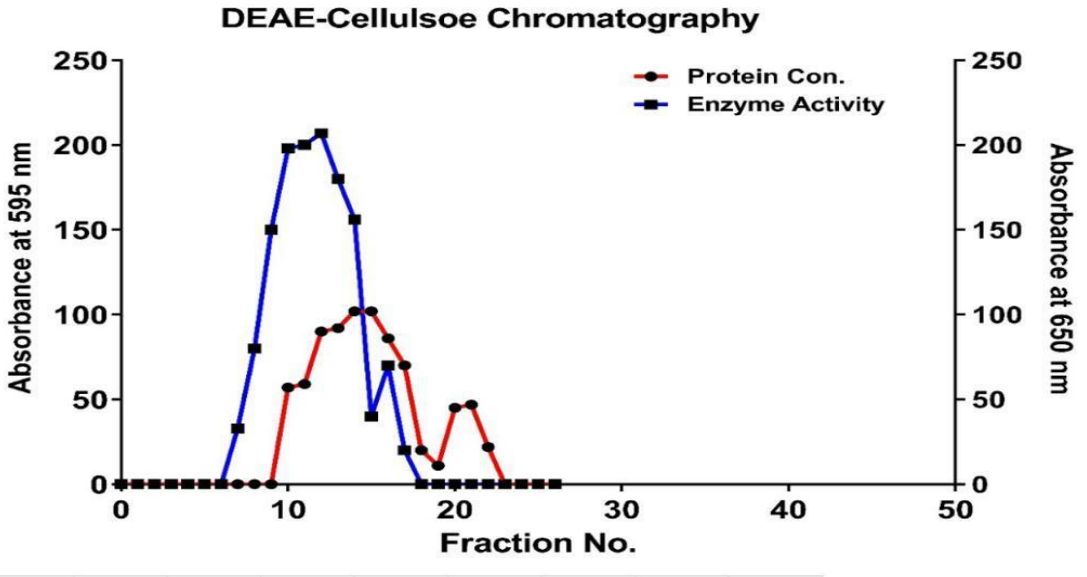

Fig. (9) Ion exchange chromatography of the protease enzyme produced from Bacillus subtilis 121 isolate using a DEAE Cellulose column(1.5x 24 cm) at a flow rate of (60 milliliters / hour).

The enzymatic activity in this step reached 200 units / mol, with specific efficacy of 200 units / mg, protein concentration 1 mg / ml, and an enzymatic yield of 20%. This result was consistent with the findings of Yin et al. (2010), where the enzymatic yield was 20.6%, and it was close to that of Vijayarahavan and Vincent, (2013)where the enzymatic yield was 16.7% using the DEAE-cellulose column with dimensions of 24 * 1.5 cm. The gel filtration step using a G-100 sephadex gel column followed the ion exchange step to complete the enzyme purification process at a flow rate of 1 mL / min with a 1: 1 volume gradient from 0.1 to 0.6 M NaCl in the same buffer. 5 ml of the fractions were collected, and passed on a Sephadex G-100 column with dimensions of 1.2 x 15 cm to complete the enzyme purification and to obtain high enzyme purity. This purification step of the recovered form resulted in a single peak of activity, and an enzyme activity of 120 units / mol was obtained. , With a protein concentration of 1.5 mg / ml, specific efficacy of 188 mg / ml, and an enzyme yield of 12%, as shown in Figure -10.

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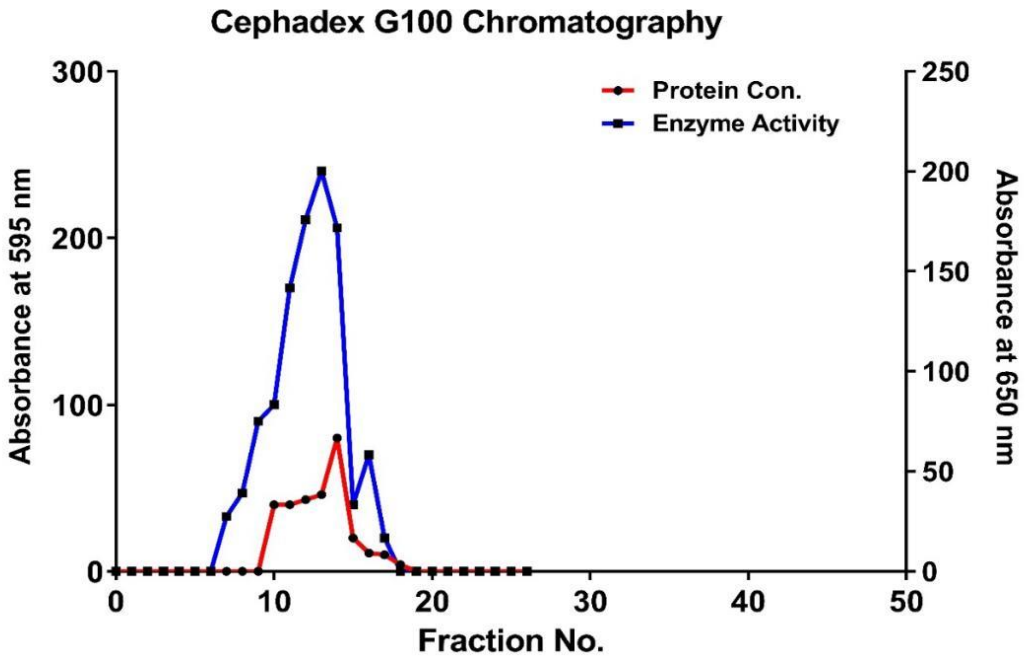

Figure(10)Gel-filtration step for the protease produced from B. subtilis 121 using a sephadex G-100 column equilibrated with a 100 mM solution of a base gear buffer PH=8 at a flow rate of (60)mL/ hr portion size:5 mL

at comparing the results obtained from other studies, the results of a study (Al-Rabei, 2001) showed that the protease produced from Proteus mirabilis bacteria as a second step by using Sephadex G-100 gel with a number of purification times 18.8 times and an enzyme yield of 27% as well as purifying the enzyme produced from Streptococcus bacteria. pyogenes as a third stage, using sephadex-G100 gel, 27.1 times purification, with a yield of 11.6% (Isa, 2000). As for the determination of the molecular weight of the protease enzyme, it was done by the electrophoresis method in the polyacrylamide gel in the presence of the teratogen (SDS - PAGE) in the calculation of the molecular weight of the purified enzyme. The molecular weight of that protein was calculated and it was 55,000 Dalton, while the standard curve for standard proteins in the acrylic amide gel in the presence of SDS was used to calculate the molecular weight of the purified protease enzyme, and it was 48,000 Dalton, as in Figure -11 In addition to determining the molecular weight of the protease enzyme, in this way, the homogeneity of the enzyme product was also confirmed by the appearance of a single protein package. When comparing the results obtained in this study with the results of other studies, K Saeki-M okudo Ito et al. (2002) estimated the molecular weight of the protease enzyme produced from B. Subtilis by SDS-PAGE, as the molecular weight was 45000 Dalton, while it was recorded. Bassem Jaouadi et al. (2008) The molecular weight of the purified protease enzyme from B. Punilas is 34,598 Dalton, while Hatem Rekik and his group (2018) estimated the molecular weight of the purified protease from B. Safensis to be 28,000 Dalton. S. Khairiar et al. (2020) estimated the molecular weight of the purified protease enzyme produced from B. Aryaphattai to be 26,000 Dalton.

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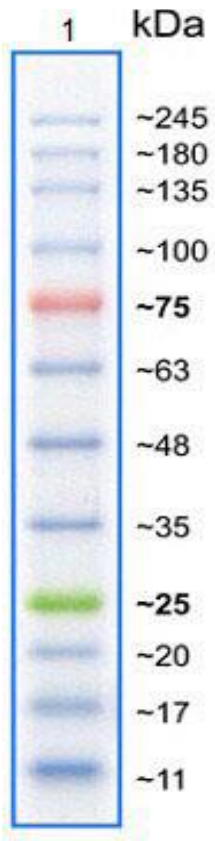

Figure_11 Pattern of electrophoresis of the (purified) protease enzyme produced from local isolate B. Subtilis in polyacrylamide gel in the presence of the SDS

8- **Characterization of the enzyme:**

A- The effect of temperature on the activity of pure protease enzyme:

For the purpose of studying the thermal effect on the activity of the protease enzyme purified from B. subtilis, the enzymatic reaction was carried out at different temperature ranges between 30-60°c, and the results shown in Figure -12 showed that the maximum activity of the pure enzyme was at a temperature of 30°c and then decreased after Incubation of the enzyme at a temperature of more than 30°c. The decrease in enzymatic activity when the temperature is higher than the optimum temperature of production may be attributed to an increase in the rate of vital activities and thus increased production of the enzyme (Al-Khafaji, 2008). This result did not agree with what Achrya et al. (2012) found. The maximum enzymatic activity of B. subtilis was at 50 ° C and pH 7.2.

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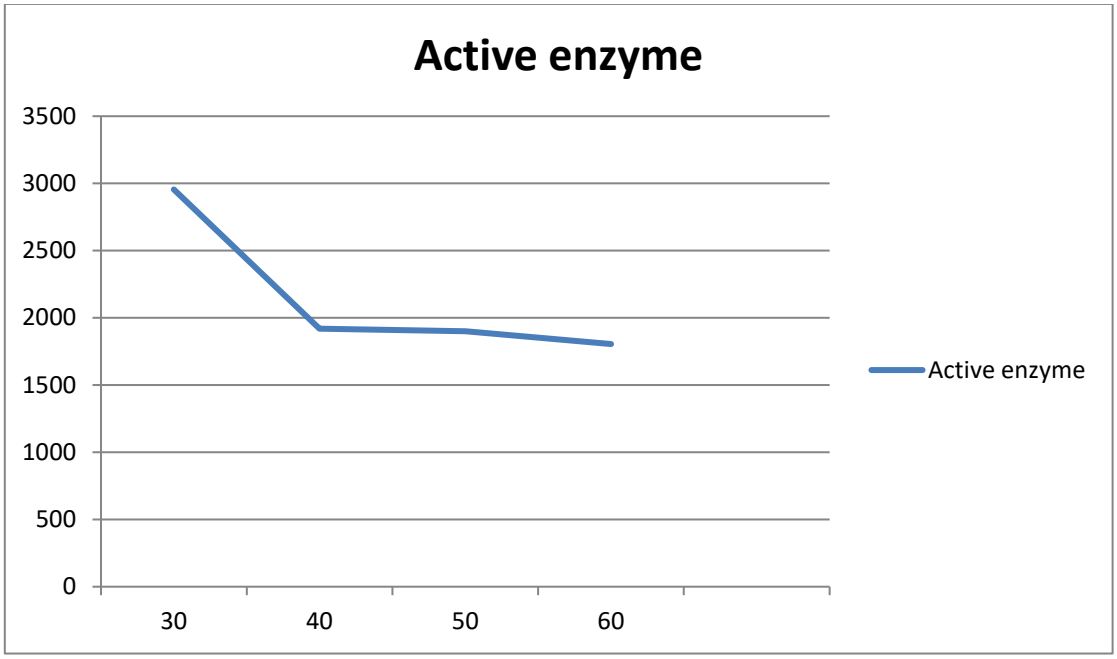

Figure (12) Effect of temperature on the activity of the purified protease enzyme produced from Bacillus subtilis

B- Thermal stability of pure protease at different temperatures:

Figure -13 shows the effect of temperature on the stability of the purified protease enzyme produced from B. subtilis within the temperature ranges of 30-40-50-60°c. It is evident from the results that the enzyme was stable at the temperature ranges of 50-60°c, while the maximum residual activity (100%) was at the temperature of 50°c, and the enzyme efficiency was 328.5 units/ml for a full 15 minutes. The results were identical to what was indicated by Raval et al. (2014) about the stability of the protease enzyme produced from halo alkaliphilic bacteria at 50°c. Then that activity began to decrease with the rise in temperature, where the remaining activity of the enzyme was 60% when incubated at 60°c for an hour perfect. The increase in temperature may lead to denaturation of the enzyme, loss of three-dimensional structures in the protein and the formation of random peptide chains, and this leads to a change in the active site that may lead to a loss of enzyme activity at high temperatures.

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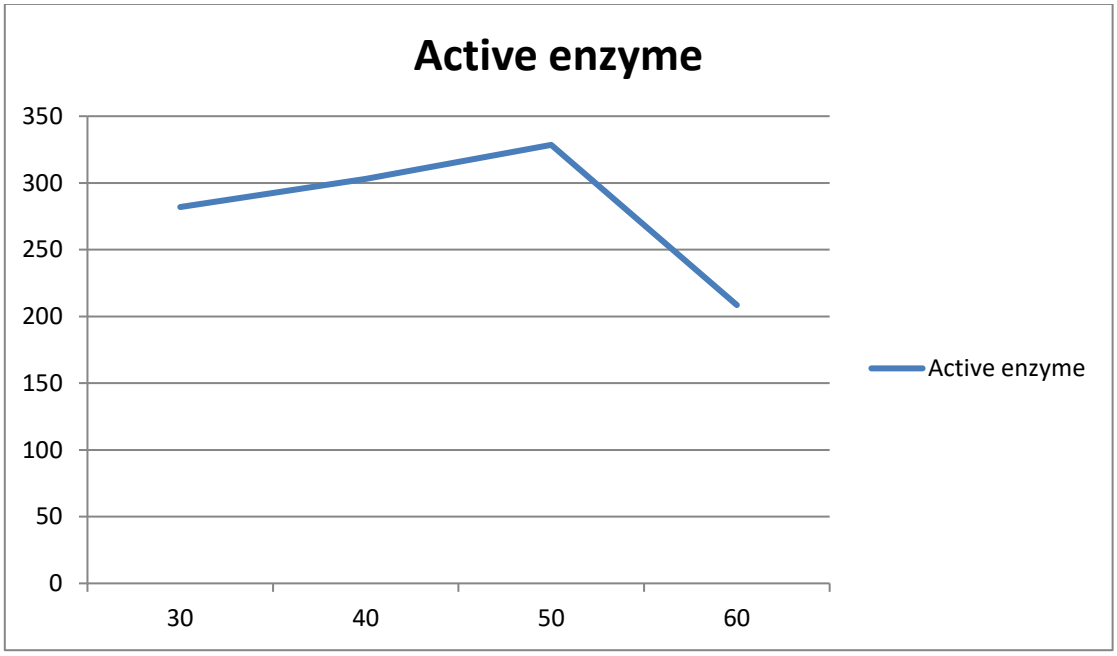

Fig. 13 Thermal stability of Bacillus subtilis purified protease

C- Determination of the optimal pH for the activity of the purified protease enzyme The effect of the optimal pH on the efficacy of the protease enzyme purified from B. subtilis at pH values (4,5,6,7,8,9) was studied. The results of Al-Hakim et al. (2018) and also with Al-Dulaimi (2010) agreed with 544.5 enzymatic units / ml. Extreme (4.0), in which the efficacy was significantly reduced.

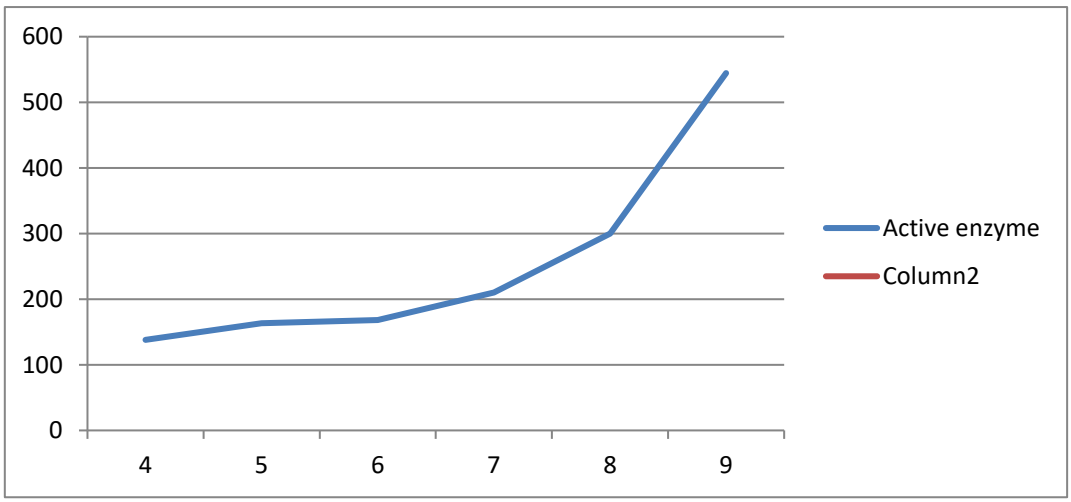

Figure (14) Optimal pH of activity of the purified protease produced from Bacillus subtilis

D- The optimum pH for the stability of the purified protease

The effect of the optimum pH on the stability of the purified protease enzyme produced from B.subtilis was studied in a range of pH values, which ranged from 6-7-8-9-10 which is one of the important characteristics in determining the appropriate conditions for purification and storage of the enzyme. The results shown in Figure-15 That optimum pH for the stability of the protease enzyme ranged between 8.0-9.0, as the enzyme retained 100% activity at pH 8.0 and enzymatic activity 583.5 units/ml. Whereas, the enzyme retained 80% of its activity at pH 6.0 and the enzymatic stability decreased significantly in the extreme pH numbers due to the change in the secondary and tertiary structure of the enzyme molecule and a change in the active site of the enzyme molecule, as denaturation of the enzyme occurs in acidic and basic solutions (Whitaker, 2018). This result was in agreement with Rao et al. (1998), and based on these studies, it can be concluded

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that the ideal pH for the stability of the purified protease enzyme has certain limits of pH values for stability and outside these ranges a change in protein composition occurs and ultimately leads to denaturation (Zubay, 1993).

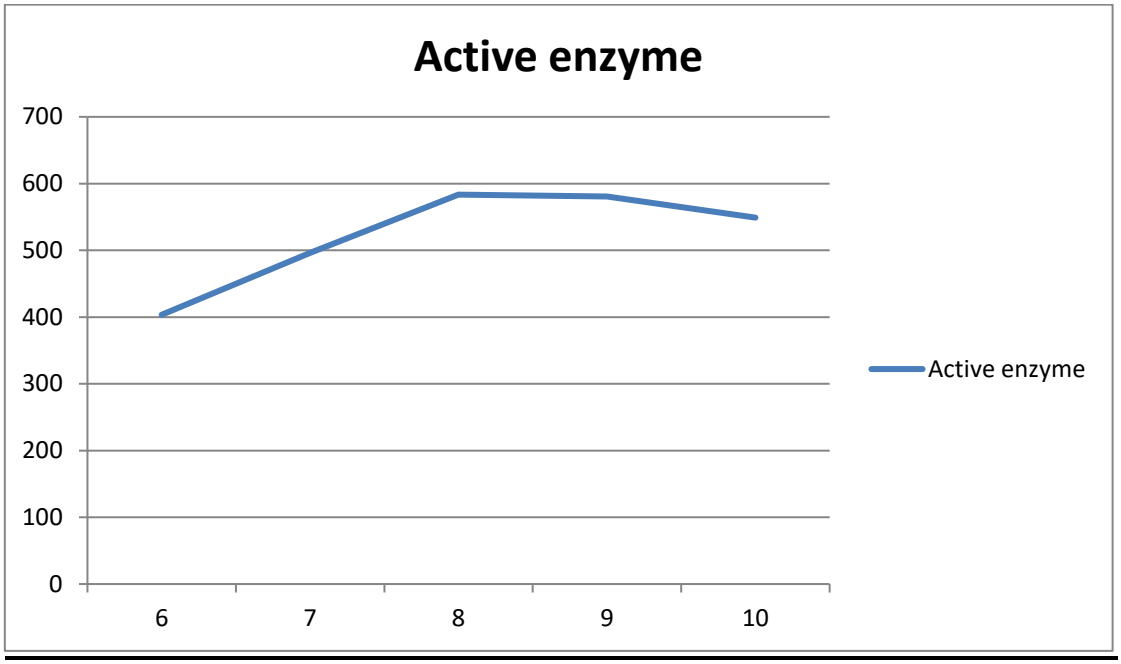

Figure 15: Optimal pH of the purified protease persistence

9- The effect of Nano carbom dot on the protease enzyme:

The results of the pure protease enzyme interaction with nanotechnology were shown, where the absorbance results were 0.710 On the wavelength of 650nm in the presence of pure Alazem, either when the enzyme was not added, the absorbance became 0.925 at the same wavelength. This type of nanoparticles was also studied on the pure protease enzyme with different pH numbers and the absorbance was obtained by a spectro photo meter, as shown in Figure-16, which shows The absorbance with different pH numbers when the pure enzyme interacted with nano carbon dot, where the results showed an increase in the absorbance up to several times at pH 5.0, which indicates the effect of nanoparticles on increasing the absorbance. The results agreed with Masi et al. (2018) where the inactivated protease activity was increased by 3 Two times the free protease PA-protease and twice the free protease of EH-protease at pH 5 (acidic) and the optimum pH of the pure protease enzyme was 9, Pathak et al. (2020) indicated that the pH is the optimal for the protease enzyme isolated from B.aryabhattai P1 With nano-ceramic technology it was 5.58 times more at pH 10 and 30 ^{o}c.

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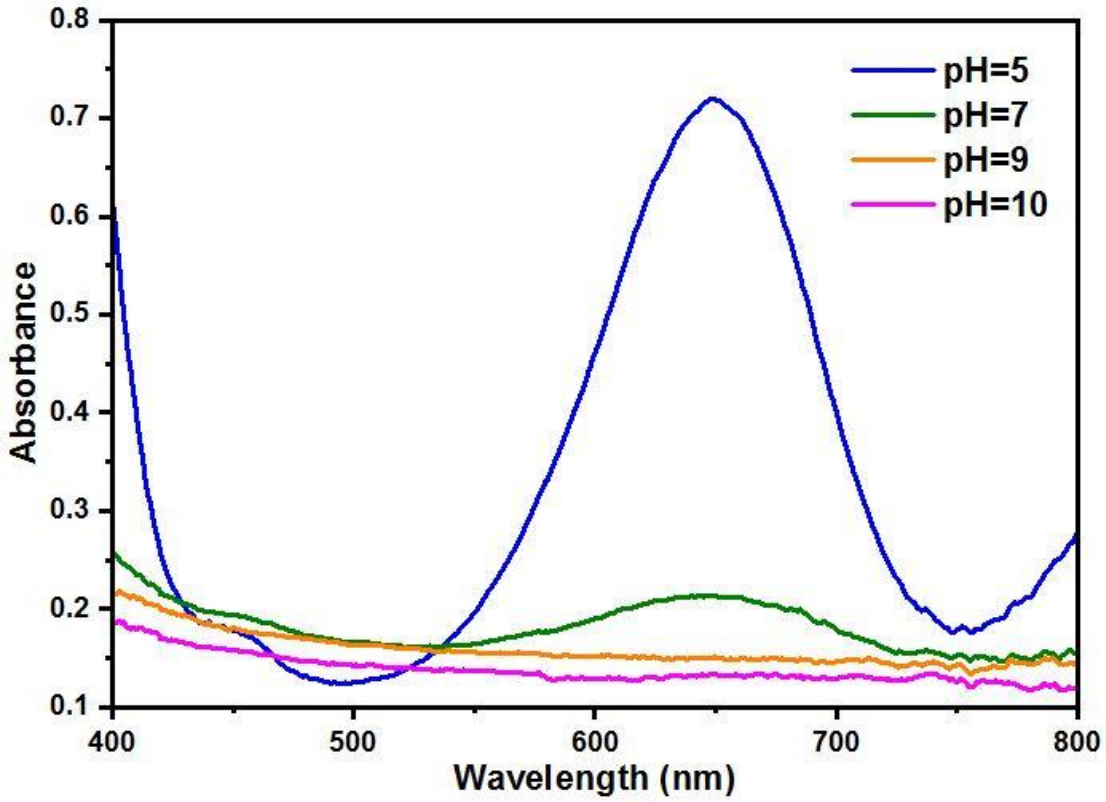

Figure (16) shows the absorbance in different pH of the effect of Nano Caron dot on the pure protease enzyme.

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