Production of Partially Purified Collagenase from *Bacillus licheniformis* and It's Use to Tenderize Aged Buffalo Meat

Wasan K. A. Al-Temimi[1*], Salah N. Aziz[1] & Ali A. Khalaf[2]

[1] Department of Food Sciences, college of Agriculture, University of Basrah

[2] General State Company for Foodstuff Trading, Ministry of Trade, Basrah, Iraq

*Corresponding author email: wasan.abdul_razzaqi@uobasrah.edu.iq; S.N.A.: salah.aziz@uobasrah.edu.iq; A.A.K.: aliwtslhs73@gmail.com

Abstract: This study aimed to isolate and identify the *Bacillus licheniformis* bacteria that produces the enzyme collagenase. Due to the selective nature of this enzyme in breaking down and degrading collagen, it was used to tenderize aged buffalo meat. The bacterial isolates were screened using a selective medium and then grown on collagen-agar medium to identify enzyme-producing isolates. Thirteen enzyme-producing isolates were obtained based on the formation of a transparent corona around the colony. Biochemical assessments of the bacterial isolates were carried out using the ABIS online program. The isolates BL_1–BL_9, BL_{12} and BL_{13} were found to be related to *B. licheniformis* with a percentage match of 98%, while BL_{10} and BL_{11} were related to *Bacillus pumilus* and *Bacillus subtilis* with a percentage match of 95% and 95.3%, respectively. The enzyme activity of the isolates varied, with the highest activity reaching 200.71 units.ml^{-1} for isolate BL_{12}. The enzyme of this isolate was chosen to complete the other tests. The optimal time required to obtain the highest enzymatic activity was found to be 20 h, which amounted to 210.25 units.ml^{-1}, with a specific activity of 135.64 units.ml^{-1}. The enzyme was partially purified using ammonium sulphate at a saturation rate of 20–70% as it showed an enzymatic activity and specificity of 355.81 units.ml^{-1} and a specific activity of 423.58 units.ml^{-1}. Partially purified collagenase was used to tenderize aged buffalo meat. The results showed that treating aged buffalo meat with the enzyme solution for different immersion durations improved the pH, water-holding capacity (WHC) and cooking yield, with an increase in the concentration of hydroxyproline and a significant percentage of soluble collagen; the treated samples showed the best properties with a 60-min immersion duration.

Keywords: *Bacillus*, Biochemical test, Buffalo meat, Collagenase, Tenderization.

Introduction

Bacillus species are Gram-positive, motile, aerobic or facultatively anaerobic bacilli, some of which may be anaerobic. This genus includes >280 species (>390 synonyms) (Parte, 2018) and is of great importance in the field of biotechnology for its ability to grow in different culture media, especially the poor, as well as its production of many important metabolic products such as enzymes, proteins and polysaccharides. The United States Food and Drug Administration (FDA) classified *Bacillus subtilis* and *Bacillus licheniformis* as safe for use in medicines and nutritional supplements (Jin *et al*., 2016). Biotechnologists have focused their attention on *B. licheniformis* because it

possesses many features such as the ability to produce antibiotics and vitamins as well as proteins, which has been widely used to produce commercial enzymes (Schroeter *et al*., 2013). Several studies indicated the possibility of obtaining collagenase from bacterial sources, including *Achromobactin* spp. and strains of *Clostridium histolyticum*, *Clostridium perfringens* and *Vibrio alginolyticus*. Moreover, many researchers could extract enzymes from different *Bacillus* species (Quaglia & Gennaro, 2003; Watanabe, 2004; Bassetto *et al.,* 2016).

Buffalo meat is characterised by a lower fat mass and higher muscle mass compared to beef and pig meat. The Food and Agriculture Organization (FAO) stated that the buffalo is a unique animal and an important asset that holds great promise for meat production. It is also characterised by its rapid adaptation to tropical and subtropical environments, the flexibility of its diet due to its ability to convert and digest low-quality feed, its high metabolic efficiency and its resistance to diseases; in addition, its consumption is not prohibited by religion (Antonio, 2005; Wanapat *et al*., 2013). Several studies showed that buffalo meat is hard, dark, rough and tough, with a high collagen content. These traits impart poor sensory characteristics to the meat because most buffalo meat comes from dairy farms; when these animals become uneconomical, they are sold to slaughterhouses and used to produce meat (Kandeepan *et al*., 2009; Naveena *et al*., 2011; Singh *et al*., 2018).

Tenderness is one of the most important palatability characteristics for consumers, being the first characteristic that an individual feels when chewing meat. This characteristic is affected by several factors, including the age and type of the animal, the effectiveness of internal enzymes, and the amount and solubility of collagen. Many techniques have been used to speed up the tenderization process but enzymatic methods are the most common and effective in tenderizing solid meat to improve its quality, increase consumer demand and address the issue of toughness (Anandh, 2013).

Collagen is the main protein in connective tissues, especially in aged animals, due to its characteristic resistance to many proteolytic enzymes. Therefore, studies have focused on breaking down collagen using collagenase as it is a highly specialized enzyme that works on collagen by disengaging the α-triple helix and weakening the connective tissue network (Sorushanova *et al*., 2018; Al-Abadi & Al-Temimi, 2022).

Based on the above information, it would be valuable to take advantage of the ability of microorganisms to produce enzymes as well as the abundance of buffaloes and the health and nutritional advantages of their meat. Therefore, this study aimed to isolate and identify collagenase-producing *Bacillus licheniformis* and partially purify the enzyme and use it to tenderize the meat of aged buffaloes.

Materials & Methods

Culture medium

A selective culture medium (*Bacillus* medium) was prepared according to the components of Himedia Co., comprising 4 g of L-glutamic acid, 2 g of citric acid, 0.5 g of potassium hydrogen phosphate, 0.5 g of ferric ammonium citrate and 0.5 g of magnesium phosphate. A total of 7.5 g of this mixture was added to 20 g of glycerol and the volume was topped up to 1 L with distilled water, following which the pH was adjusted to 7.4 and the solution was sterilised at 121ºC for 15 min.

Collagen-agar plates

A medium consisting of 1% collagen and 2% agar was prepared according to the method described by Liu *et al*. (2010) to distinguish

collagenase-producing isolates based on the hydrolysis of collagen.

Culture and screening of isolates

Bacterial isolates were collected by taking swabs and parts of the skin, meat and bones of livestock in Basrah Governorate, Republic of Iraq. The samples were transferred to test tubes containing sterile *Bacillus* medium and incubated for 24h under anaerobic conditions. The process was repeated three times, with a predominance of *B. licheniformis*. After activating the bacterial isolates for 24 h, collagen-agar plates were inoculated using a loop by planning method and incubated at 37ºC for 24–48 h under anaerobic conditions. The process was repeated to obtain single isolates. Mercuric chloride precipitation reagent was prepared (15 g of $HgCl_2$ and 20 ml of concentrated HCl in a total volume of 100 ml made up with distilled water), and a drop was added to the samples.

Biochemical tests

Biochemical tests were carried out on the isolates that had the highest collagenase activity. The isolates were tested for Gram positivity, motility, haemolysis, growth at 45 ºC, growth at 65 ºC, growth at pH 5.7, growth in 7% NaCl medium, anaerobic growth, growth in 0.001% lysozyme, casein hydrolysis, gelatin hydrolysis, starch hydrolysis, β-galactosidase ONPG, catalase, oxidase, urase, arginine dihydrolase (ADH), indole production, citrate utilisation, nitrate reduction, Voges–Proskauer (VP), L-arabinose, cellobiose, fructose, gructose, inositol, lactose, D-mannitol, D-mannose, maltose, ribose, salcin, sorbitol, sucrose and D-xylose. After completion of the biochemical tests, the bacterial isolates were identified using the ABIS online program (Stoica & Sorescu, 2017).

Collagenase production

The production medium was prepared according to the method of Savita & Arachana (2015) with minor modifications. Briefly, 2 g of glucose, 1 g of collagen, 0.005 g of $CaCl_2$, 0.05 g of $NaH2PO_4$ and 0.05 g of K_2HPO_4 were mixed, and the volume was topped up to 100 ml with distilled water. The medium was inoculated with 3% of the selected bacteria and incubated at 37 ºC for 18 h in a shaker at 150 rpm. After completing the incubation at a speed of 4000 rpm at 4 ºC for 10 min, the supernatant was collected, representing the crude collagenase enzyme.

Estimation of enzymatic activity

The enzymatic activity was estimated according to the method described by Bergeyer (1974) with some modifications, wherein 0.05 ml of the crude enzymatic extract was mixed with 250 ml of substrate (5% bovine tendon collagen from Sigma, UK, dissolved in 0.02 M of sodium phosphate buffer at pH 7) and incubated at 37 ºC for 30 min. The mixture was then centrifuged at $4000 \times g$ for 10 min at 4 ºC. Thereafter, 0.2 ml of the filtrate was mixed with ninhydrin solution (4% ninhydrin solution in 200 mmol phosphate buffer) in a ratio of 1:1. The solution was heated at 100 ºC in a water bath for 20 min and then cooled to laboratory temperature with ice water, following which 5 ml of 1-propanol was added. An absorbance measurement was conducted at 570 nm. The activity was estimated based on the standard curve of the amino acid leucine. Enzymatic activity was defined as the amount of enzyme needed to convert a micromolar of the substrate into a product within 1 min under reaction conditions.

Protein concentration estimation

The Bradford (1976) method was used to estimate protein concentration.

Determining optimal enzymatic activity

The optimal enzymatic activity of collagenase was determined in the production medium through incubation for different durations that ranged from 0 to 24 h at 37 ºC, according to Savita & Arachana (2015). Enzymatic activity was estimated every 2 h.

Partial enzyme purification

The crude enzyme was precipitated by gradually adding solid ammonium sulphate

crystals to the crude extract with continuous stirring at a temperature of 4 ºC for 2 h at a saturation temperature of 20–70%. Centrifugation was carried out at 10000 × g for 20 min at 4 ºC. The precipitate was collected and re-dissolved using 0.02 M of sodium phosphate buffer at pH 7. The dialysis process was carried out against distilled water at a temperature of 4 ºC for 24 h, with water being replaced every 6 h (Adinarayana *et al.*, 2003).

Applications

The partially purified collagenase enzyme was evaluated and compared to a standard collagenase enzyme (for general use, Type I, 0.25-1 FALGPA units.ml^{-1} ≥ 125CDU.mg^{-1}, supplied by Sigma, UK) in the tendering process as a control.

Buffalo meat

Buffalo meat was obtained from a breeder in the Qarmat Ali area, Basrah Governorate, Republic of Iraq. Samples were taken from buffaloes aged 7–10 years immediately after slaughter, and the round muscle was used in the study.

Chemical content of buffalo meat

The chemical content of buffalo meat (moisture, protein, fat and ash) was estimated as previously described (AOAC, 2016).

Collagenase (EC.3.4.24.3)

Collagenase was prepared at a concentration of 0.1% by dissolving 0.1 g of the enzyme in distilled water and topping up the volume to 100 ml with distilled water.

Preparation of meat samples

After washing and removing the fatty parts, the meat samples were cut into slices of 1.5 cm thickness and approximately 100 g weight. The slices were treated by the immersion method with enzymatic solutions of both partially purified and standard collagenase enzyme for immersion durations of 0, 15, 30, 45 and 60 min at 45 ºC, following which qualitative tests were performed on the samples.

Physical and chemical properties of meat

pH

The pH was measured according to the method described by Feng *et al.* (2020). Briefly, 10 g of ground meat was mixed with 100 ml of distilled water for 10 s using an electric mixer, and the pH was measured using a pH meter (Sartorius, Germany).

Water-holding capacity (WHC)

The method of Zhao *et al.* (2012) was implemented with some minor modifications. Briefly, 4 gm of the meat samples was immersed in the enzymatic solutions at the concentrations and immersion durations mentioned above. After treatment, the samples were taken out, the surface moisture was scanned, and the samples were re-weighed. The WHC was expressed as a percentage according to the equation:

$$\% \text{ Water Loss} = 100 \times \left(\frac{\text{M untreated} - \text{M treated}}{\text{M untreated}} \right)$$

M=Meat

Cooking loss

Cooking loss was measured according to Habtu *et al.* (2020). Meat samples were weighed before and after cooking for immersion times of 0, 15, 30, 45 and 60 min, and cooking loss was expressed as a percentage.

Determination of hydroxyproline

he concentration of hydroxyproline was estimated according to the method described by Moon (2018) with minor modifications. Two grams of meat was hydrolysed using 40 ml of 6 N HCl for 18 h. After the decomposition process was completed, the solution was filtered, and 50 ml of distilled water was added to it. The absorbance of the solution was measured at 540 nm and the concentration of hydroxyproline was calculated based on the standard curve of hydroxyproline. Total collagen was calculated

by multiplying the concentration of hydroxyproline by 7.14, expressed as mg.g^{-1} of meat.

Percent soluble collagen

The soluble collagen content was determined according to Abdeldaim & Ali (2013). Five grams of the enzyme-treated meat was placed in a closed 250 ml beaker and transferred to a water bath. The temperature was raised to boiling point for 30 min. When the cooking was completed, the meat samples were cut into small pieces and 50 ml of cold distilled was added, mixed for 2 min and centrifuged at 2000 rpm. The solution was collected, and acid hydrolysis was carried out for 18 h. The concentration of hydroxyproline in the precipitate and the filtrate was measured throughout the process. Collagen solubility was calculated as:

% Soluble Collagen
= 7.14 x % Hydroxyproline Concentration

Statistical analysis

The experiment was designed using a completely randomised design (CRD), and the averages were compared according to LSD at a significance level of $P \leq 0.05$, with three replicates per treatment. Values or diagrams with the same superscripts indicate that there is no significant difference at $P \leq 0.05$.

Results & Discussion

**Isolation and screening of microorganisms**

Table (1) shows the bacterial isolates obtained from different isolate sources through growth on selective Bacillus medium under anaerobic conditions to target *Bacillus licheniformis*, as the repetition of the activation process on selective medium and the dominantly anaerobic conditions ensured the predominance of the target bacteria. The bacterial isolates were screened using the collagen-agar medium. The presence of a transparent circle around the bacterial colony indicated the ability of the bacterial isolate to produce the collagenase enzyme as it is

formed as a result of the decomposition of collagen by collagenase. Thirteen isolates showed this ability to degrade collagen in the culture medium. Biochemical tests were conducted on the selected isolates as shown in table (1). The results showed that 11 isolates were positive for Gram staining, mobility, growth at 45ºC, growth at pH 5.7, growth in NaCl medium, growth under anaerobic conditions, and growth at 0.001% lysozyme, as well as for the cationic hydrolysis of casein, gelatine, starch, ONPG, catalase, ADH, citrate consumption, nitrate reductase, and PV. Moreover, different growth capacities appeared in the fermentation medium to which L-arabinose, cellobiose, fructose, glucose, glycerol, lactose, mannitol, mannose, ribose, sucrose, and xylose were added. The isolates were negative for haemolysis, growth at 65 ºC, oxidase, urase, and inositol. Isolate BL_{10} was positive for haemolysis, did not grow under anaerobic conditions, could not hydrolyse starch, and was negative for VP. Isolate BL_{11} was positive for haemolysis, negative for growth under anaerobic conditions, positive for starch hydrolysis, negative for ADH, and positive for inositol. All the data obtained from the biochemical tests and presented in table (1) were entered into the ABIS online program. The results showed that isolate BL_1–BL_9, BL_{12} and BL_{13} were related to *B. licheniformis* with a match percentage of 98%, while isolate BL_{10} was related to *B. pumilus* with a percentage of 95% and isolate BL_{11} was related to *B. subtilis* with a percentage of 95.3%. Several researchers have used the ABIS online program to identify bacterial strains. Saeed (2020) identified *Bacillus* bacteria and observed a match of 92.2% with *B. subtilis*, 92.5% with *Bacillus megaterium* and 87% with *B. licheniformis*. Dumitru *et al.* (2021) used the ABIS online program to identify *B. megaterium* with a concordance rate of 91.8%. Banoon & Ali (2018) identified some strains of *B. licheniformis* using VITEK-2.

Enzymatic activity

The enzymatic activity of collagenase of the selected 13 isolates was examined using colorimetric methods and is shown in table (1). The estimation was based on the ninhydrin method through its interaction with amino acids or peptides formed by the decomposition of collagen by the enzyme. The highest enzymatic activity belonged to isolate BL_{12} (200.71 units.ml^{-1}), while the isolate BL_{10} showed an activity of 14.37 units.ml^{-1}. The variation in the enzymatic activity of the isolates may be attributed to different bacterial strains, the time and conditions of fermentation, and the nature of the production medium. Collagenase extracted from isolate BL_{12} was used in the subsequent tests. The optimum time for the highest enzymatic activity of collagenase was determined during the incubation duration of 0–24 h in the production medium as shown in fig. (1). The enzymatic activity rose after 10 h of incubation to 60.8 units.ml^{-1} and reached the maximum activity at an incubation duration of 20 h, with an efficiency of 210.25 units.ml^{-1} and a specific efficiency of 135.65

units.ml^{-1}. The enzymatic activity gradually decreased as the incubation duration increased further. This increase in enzymatic activity may be attributed to the fact that the enzyme was produced in the stationary growth phase with the availability of the substrate, which led to an increase in the effectiveness. As for the decrease in enzymatic activity with the progression of the incubation, it may be attributed to the depletion of the substrate or the saturation of the active sites of the enzyme. Savita & Arachana (2015) found that the highest activity of collagenase enzyme extracted from *Bacillus* KM369985 was 600 units.ml^{-1} at 72 h of incubation. Tran & Nagano (2002) observed that the best incubation time for collagenase production from *B. subtilis* CN2 was 14 h. Sartika *et al*. (2019) showed that the highest activity of collagenase extracted from *Bacillus* sp. 6-2 reached 1.7 units.ml^{-1} in 30 h. The difference in enzymatic activity in previous studies may be attributed to the bacterial strain, the medium and conditions of fermentation used, and the size of the inoculum.
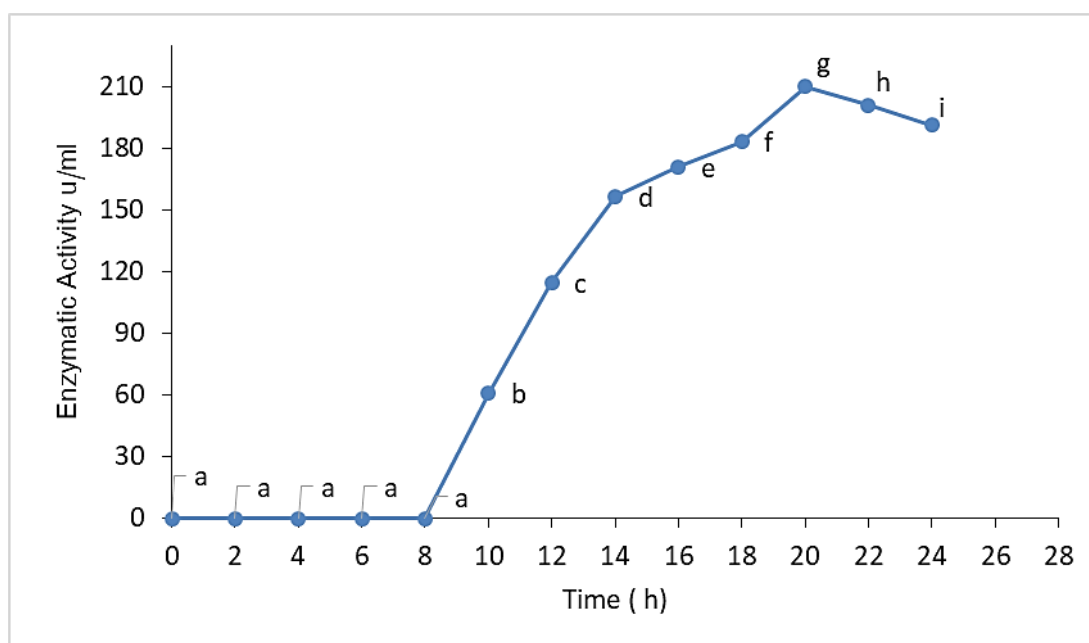
Fig. (1): Optimal collagenase enzyme activity for the isolate (BL12) at varied incubation times (0–24 h). The different letters indicate that there are significant differences at p≤0.05.

Table (1): The biochemical characteristics and enzymatic activity of bacterial isolates.

Abbreviation (BL: Bacterial Isolates).

No.	Biochemical examinations	Isolates												
		BL1	BL2	BL3	BL4	BL5	BL6	BL7	BL8	BL9	BP10	BS11	BL12	BL13
1	Gram Stain	+	+	+	+	+	+	+	+	+	+	+	+	+
2	Motility	+	+	+	+	+	+	+	+	+	+	+	+	+
3	Hemolysis	-	-	-	-	-	-	-	-	-	+	+	-	-
4	Growth at 45ºC	+	+	+	+	+	+	+	+	+	+	+	+	+
5	Growth at 65ºC	-	-	-	-	-	-	-	-	-	-	-	-	-
6	Growth at pH 5.7	+	+	+	+	+	+	+	+	+	+	+	+	+
7	Growth at 7% NaCl Medium	+	+	+	+	+	+	+	+	+	+	+	+	+
8	Anaerobic Growth	+	+	+	+	+	+	+	+	+	-	-	+	+
9	Growth in Lysozyme 0.001%	+	+	+	+	+	+	+	+	+	+	+	+	+
10	Casein hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+
11	Gelatin Hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+
12	Starch hydrolysis	+	+	+	+	+	+	+	+	+	-	+	+	+
13	ONPG	+	+	+	+	+	+	+	+	+	+	+	+	+
14	Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+
15	Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-
16	Urase	-	-	-	-	-	-	-	-	-	-	-	-	-
17	Arginine dihydrolase ADH	+	+	+	+	+	+	+	+	+	+	-	+	+
18	Indole production	-	-	-	-	-	-	-	-	-	-	-	-	-
19	Citrate Utilization	+	+	+	+	+	+	+	+	+	+	+	+	+
20	Nitrates reduction	+	+	+	+	+	+	+	+	+	+	+	+	+
21	VP	+	+	+	+	+	+	+	+	+	-	+	+	+
22	L-Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+
23	Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	+
24	Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
25	Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
26	Glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+
27	Insitol	-	-	-	-	-	-	-	-	-	-	+	-	-
28	Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+
29	D-mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+
30	D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+
31	Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+
32	Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+
33	Salcin	+	+	+	+	+	+	+	+	+	+	+	+	+
34	Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+
35	Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+
36	D-Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+
37	Enzymatic Activity	96.44[f]	38.76[c]	78.33[e]	139.66[h]	151.11[i]	95.45[f]	23.77[b]	55.89[d]	110.12[g]	14.73[a]	25.97[b]	200.71[j]	80.55[d]
	Type of Isolate	*B. licheniformis*									*B. pumilus*	*B. subtilis*	*B. licheniformis*	

The enzyme was partially purified from isolate BL_{12} using ammonium sulphate at 20–70% saturation. A rise in enzymatic activity was observed, and the specificity reached 355.81 units.ml^{-1} and 423.58 units.ml^{-1}. The increase in enzymatic activity upon partial purification may be attributed to the precipitation process that resulted in the removal of contaminating proteins, polysaccharides and compounds that impede the binding of the substrate with the active site of the enzyme. Another reason might be the competition of pollutants with the substrate for binding to the active site. Liu *et al*. (2010) noted that the activity of collagenase partially purified with ammonium sulphate from *Bacillus cereus* MBL13 reached 51,563 unit.ml^{-1}. The enzyme was partially purified from isolate BL_{12} using ammonium sulphate at 20–70% saturation. A rise in enzymatic activity was observed, and the specificity reached 355.81 units.ml^{-1} and 423.58 units.ml^{-1}. The increase in enzymatic activity upon partial purification may be attributed to the precipitation process that resulted in the removal of contaminating proteins, polysaccharides and compounds that impede the binding of the substrate with the active site of the enzyme. Another reason might be the competition of pollutants with

the substrate for binding to the active site. Liu *et al*. (2010) noted that the activity of collagenase partially purified with ammonium sulphate from *Bacillus cereus* MBL13 reached 51,563 unit.ml^{-1}.

Chemical content of buffalo meat

Table (2) shows the chemical composition of buffalo meat (percentage of moisture, protein, fat and ash). The moisture percentage was 77.85%, protein amounted to 18.75%, and the percentage of fat and ash was 1.89% and 0.97%, respectively. These results were similar to those of several researchers who studied the chemical composition of buffalo meat. Ziauddin *et al*. (1994) found that the chemical composition of aged buffalo meat included 79.69%, 17.81%, 0.44%, and 0.98% moisture, protein, fat and ash, respectively. Naveena *et al*. (2004) reported that the moisture content and protein in buffalo meat was 76.51% and 20.08%, respectively. Gecgel *et al*. (2019) assessed the chemical content of buffalo meat and reported values of 48.6–59.73% moisture, 15.12–17.65% protein, 18.90–30.02% fat and 2.48–3.56% ash.

Table (2): Chemical content of aged buffalo meat.

Treatment	Chemical content				
Buffalo Meat	Moist.%	Protein%	Fat%	Carbohydrate%	Ash%
	77.85±0.1	18.75±0.3	1.89±0.1	0.54± 0.02	0.97± 0.01

pH

Table (3) shows the change in the pH of buffalo meat treated with partially purified collagenase compared with 0.1% standard collagenase at different immersion durations. A gradual increase in pH values was observed, reaching a maximum of 6.07 and 6.14, respectively, at 60 min of immersion. The gradual rise in pH values may be attributed to the ability of collagenase to degrade collagen in the connective tissues of meat, causing an increase in the WHC as a

result of the association of water with the active hydrophilic groups present in the protein and leading to an increase in pH values. These results agreed with a number of studies. Naveena *et al*. (2004) indicated that there were significant differences in the pH values of buffalo meat treated with enzyme extracts compared to untreated samples. Naveena *et al*. (2011) also noted an increase in pH values when treating buffalo meat with concentrations of 0.1–1% ammonium hydroxide. Detected an increase in the pH

values of beef with increasing immersion durations in 0.1% of collagenase standard and collagenase purified from bighead fish intestines, reaching 5.9 and 5.8, respectively, at an immersion duration of 120 min, as well as an increase in the protein's ability to retain water, causing the sample to retain its quality and tenderness (Al-Temimi, 2014).

The ability of meat to retain moisture is one of the important requirements of meat producers due to its association with flavour, appearance and juiciness. Fig. (2) shows the ability of aged buffalo meat to retain water when treated with 0.1% purified collagenase

compared to standard collagenase for different immersion durations.

The results showed an increase in WHC starting from 12.63% at an immersion duration of 0 min and reaching 14.29% and 14.46% at immersion durations of 60 min for meat treated with both purified and standard collagenase, respectively. These results agreed with a study by Naveena *et al*. (2004), which indicated that the WHC was directly affected by the pH as a decrease in pH negatively affected the active groups present in the protein, which hindered its ability to bind water.

**Table (3): Change in pH values of aged buffalo meat when treated with enzymatic solutions of collagenase**.

Treatments	Immersing Time (min.)				
	0	15	30	45	60
Partial Purified Collagenase	$5.67^a \pm 0.2$	$5.71^a \pm 0.1$	$5.83^a \pm 0.2$	$5.95^a \pm 0.3$	$6.07^a \pm 0.1$
Standard Collagenase	$5.67^a \pm 0.2$	$5.76^a \pm 0.3$	$5.89^a \pm 0.1$	$6.04^a \pm 0.2$	$6.14^a \pm 0.1$

The different letters indicate that there are significant differences at $p \leq 0.05$

Zhao *et al*. (2012) found that the water loss capacity of meat between the control sample and the sample treated with collagenase amounted to 1%, while the loss percentage increased to 5.8% and 8% for samples treated with bromelain and papain, respectively, indicating that collagenase improves WHC. It was also shown that meat treated with collagenase retained a fresher appearance and colour compared to samples treated with bromelain and papain. Irurueta *et al*. (2008) indicated that the WHC of buffalo meat cuts or minced meat ranged from 23.73% to 39.76%, while Naveena *et al*. (2011) reported that the WHC of buffalo meat cuts ranged

from 25.3% to 40.20% when treated with different concentrations of ammonium hydroxide and sodium chloride.

In this study, the increase in the WHC can be attributed to the ability of water to penetrate into the meat tissues as a result of the decomposition of the triple helical structure of collagen, causing an increase in the binding of water with the hydrophilic active groups in the protein and leading to a significant improvement in WHC. Badr (2008) indicated that hydrolysed collagen derived from connective tissues has an excellent ability to bind water, which improves the tenderness of cooked meat.
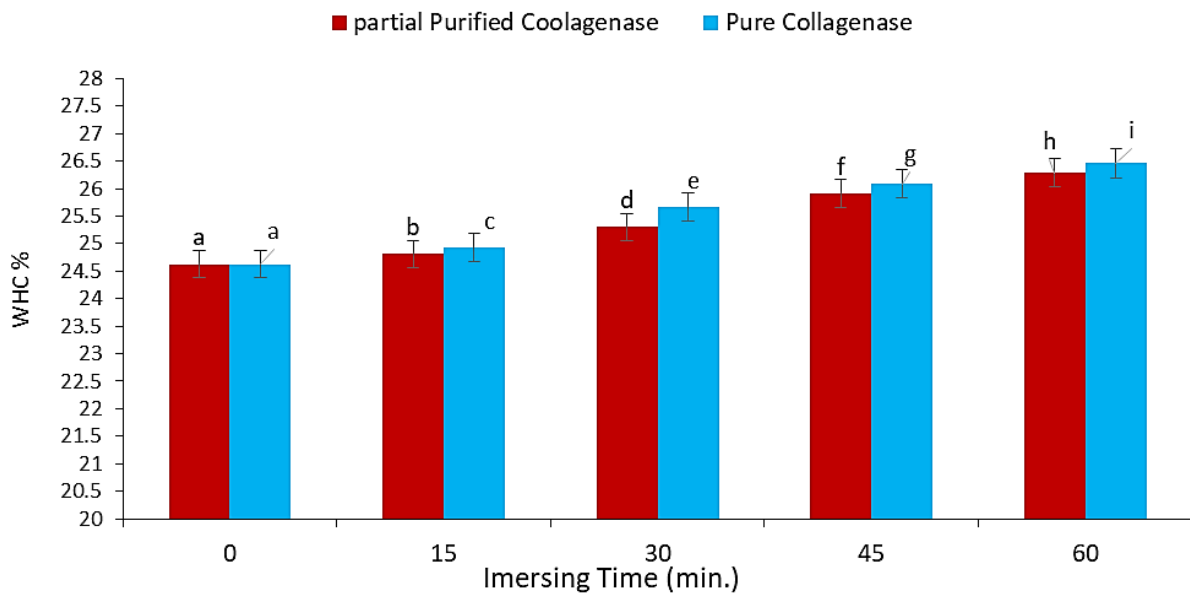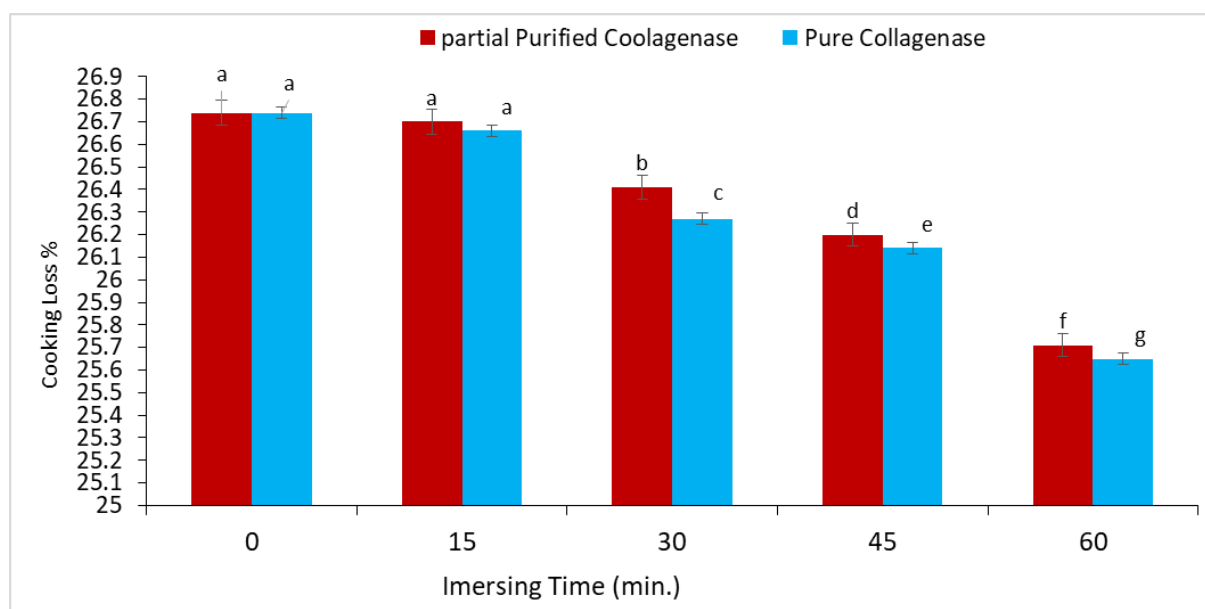
Fig. (2): WHC of aged buffalo meat treated with enzymatic solutions at a concentration of 0.1% at different immersion durations. The different letters indicate that there are significant differences at p≤0.05.

Cooking loss percentage

Fig. (3) shows the cooking loss percentages of aged buffalo meat treated with enzymes at a concentration of 0.1% for immersion durations of 0, 15, 30, 45 and 60 min. The results showed a gradual decrease in weight loss percentage for both enzymes across cooking durations, with the values for the last immersion duration reaching 25.71% and 25.65%, respectively. The decrease in the rate of loss may be attributed to two important factors. The first is the role of the collagenase enzyme, which caused breakage of the triple helical structure of collagen in connective tissues, leading to the penetration of water between the tissues in association with active

hydrophilic groups. Second, the tendering process with enzymes led to a rise in the pH of the meat, which led to an increase in the WHC, thereby reducing the volume of the extracted liquid. Naveena *et al*. (2004) indicated that the decrease in pH during the tendering process leads to the reduction of the active hydrophilic groups in the protein, causing an increase in the rate of loss during cooking. These results agreed with Al-Temimi (2014), who noticed a decrease in the cooking yield of aged beef treated with collagenase extracted from the inner viscera of a bighead fish and standard collagenase.

Fig. (3): The percentage of cooking loss of aged buffalo meat treated with enzymatic solutions at a concentration of 0.1% at different immersion durations.

The different letters indicate that there are significant differences at $p \leq 0.05$

Determination of hydroxyproline and soluble collagen

Table (4) shows the concentration of hydroxyproline in aged buffalo meat treated with collagenase at a concentration of 0.1% for immersion durations of 0–60 min. The results showed a gradual increase in hydroxyproline concentrations with the progression of immersion periods in the enzymatic solutions, reaching a maximum concentration at the highest immersion period of 60 min, in which standard collagenase showed clear superiority compared to partially purified collagenase.

Geles *et al*. (2003) reported that collagen constitutes 80% of the connective tissues in meat, making its targeting and estimation important factors in tenderizing. The results of the current study showed a significant increase in the percentage of soluble collagen with the progression of immersion durations for both enzymes. The high percentage of soluble collagen may be attributed to the high specificity and selectivity of the collagenase enzyme in the degradation of collagen, breaking of bonds and dismantling the α-helical structure of collagen.

Table (4): Hydroxyproline concentration for aged buffalo meat treated with enzymatic solutions at a concentration of 0.1% at different immersion duratios.

	Enzymes	Immersing Time (min.)				
		0	15	30	45	60
Hydroxyprline	Purified Collagenase	0.0531[a]±0.02	0.0561[a]±0.01	0.0595[a]±0.02	0.0609[a]±0.03	0.0618[a]±0.01
	Standard Collagenase	0.0531[a]±0.01	0.0571[a]±0.02	0.0611[a]±0.02	0.0619[a]±0.02	0.0630[a]±0.01
% soluble collagen	Purified Collagenase	35.43[a]± 0.01	37.5[a]±0.01	39.7[a]±0.03	40.63[a]±0.03	41.23[a]±0.03
	Standard Collagenase	35.43[a]±0.03	38.1[b]±0.03	40.77[b]±0.01	41.3[b]±0.01	42.03[b]±0.01

The different letters indicate that there are significant differences at p≤0.05

These results are in agreement with Zhao *et al.* (2012) that collagenase is one of the enzymes specialized and selective in its work, and it is highly effective in degrading collagen, especially when tenderizing meat compared to other enzymes. Abdeldaiem & Ali (2013) showed that the increase in collagen hydrolysis in the connective tissue leads to an increase in the water holding capacity and an increase in the tenderness of meat during cooking. These results are in agreement with Al-Temimi (2014) who observed an increase in the concentration of soluble collagen for aged beef treated with collagenase enzyme purified from the intestines of standard Bighead fish compared to papain enzyme and distilled water.

Conclusions

Thirteen bacterial isolates were obtained and identified as *B. licheniformis*, *B. pumilus* and *B. subtilis*. All isolates showed the ability to produce collagenase with different levels of enzymatic activity. The collagenase from isolate BL12 was selected for subsequent experiments. The enzyme was partially purified using ammonium sulphate and used to tenderize aged buffalo meat. Treatment with partially purified collagenase showed promising results in tenderizing aged meat by improving its physicochemical properties. The results regarding pH, WHC, cooking yield and hydroxyproline acid concentration were not significantly different for both enzymes. However, the standard collagenase was significantly superior with regard to the percentage of soluble collagen.

Contributions of authors

W.K.A.A.: Constructed the idea and hypothesis for research; planned the methodology, analyzed data and conducted enzyme detection experiments, Provided financial support

S.N.A.: Collection of samples, Analysed bacterial isolates and performed isolates diagnostic experiments, Provided financial support.

ORCID

W. K. A. Al-Temimi: https://orcid.org/0000-0003-2428-790X
S. N. Aziz: https://orcid.org/0000-0002-6942-7331
A.A. Khalaf: https://orcid.org/0000-0001-8706-846X

Conflict interest

The authors declare that they have no conflict of interest.

A.A.K.: Analysed bacterial isolates and performed isolates diagnostic experiments, wrote the manuscript. Provided financial support.

References

Abdeldaim, M. H., & Ali, H. G. M. (2013). Tenderization of camel meat by using fresh ginger (*Zingiber officinale*) extract. *Food Science and Quality Management, 21,* 12-25. https://iiste.org/Journals/index.php/FSQM/article/view/10470

Adinarayana, K., Ellaiah, P., & Prasad, D. S. (2003). Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. *An Official Journal of the American Association of Pharmaceutical Scientists*, *4*(4), E56. https://link.springer.com/article/10.1208/pt040456

Al-Abadi, H. H. M., & Al-Temimi, W. K. A. (2022). Production of hydrolyzed collagen from common carp *Cyprinus carpio* L. scales by synergistic enzyme systems. *Basrah Journal of Agricultural Sciences, 35*(1), 94-105. https://doi.org/10.37077/25200860.2022.35.1.08

Al-Temimi, W. K. A. (2014). *Purification and Characterization of Collagenase Extracted from Fish Stomach by Products and Study it's Tenderizing Meat.* Ph. D. Thesis, Univ. Basrah: 151pp. (In Arabic, English abstract).

Anandh, M. A. (2013). Effect of Different Tenderizers on Tenderness and Quality of Buffalo Tripe. *Asian Journal. Dairy and Food Research*, *32*(2), 144-148. https://arccjournals.com/journal/asian-journal-of-dairy-and-food-research/ARCC347

Antonio, B. (2005). *Buffalo Production and Research*. FAO. 00100 Rome, 316pp.

AOAC. (2016). *Official methods of anaylsis*. Retrieved from http://www.eoma.aoac.org/methods/info.asp?ID=16264

Badr, H. M. (2008). Tenderness properties and microbial safety of spent hen meat treated by papain and gamma irradiation. *Journal of Radiation Research and Applied Sciences, 1*(2), 443-462. https://www.sciencedirect.com/journal/journal-of-radiation-research-and-applied-sciences

Banoon, S. R., & Ali, Z. M. (2018). Identification the local thermophilic strains of *Bacillus licheniformis* by VITEK-2 system and Gyrase B gene. *Al-Kufa University Journal for Biology*, *10*(2), 157-167. https://journal.uokufa.edu.iq/index.php/ajb/article/view/8125

Bassetto, F., Maschio, N., Abatangelo, G., Zavan, B., Scarpa, C., & Vindigni, V. (2016). Collagenase from *Vibrio alginolyticus* cultures: Experimental study and clinical perspectives. *Annals of Surgical Innovation and Research, 23*(6), 557-562. https://doi.org/10.1177/1553350616660630

Bergeyer, H. U. (1974). *Methods of Enzymatic Analysis*, Vol. 2, Second Edition, Academic Press, Elsevier Inc., 682pp. https://www.sciencedirect.com/book/9780120913022/methods-of-enzymatic-analysis

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72,* 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

Dumitru, M., Vodnar, D. C., Elemer, S., Ciurescu, G., Habeanu, M., Sorescu, I., Georgescu, S. E., & Dudu, A. (2021). Evaluation of non-encapsulated and microencapsulated lactic acid bacteria. *Applied Science, 11*(9867), 1-15. https://doi.org/10.3390/app11219867

Feng, Y. H., Zhang, S. S, Sun, B. Z., Wen, P. X. K. X., & Xu, C. C. (2020). Changes in physical meat traits, protein solubility, and the microstructure of different beef muscles during post-mortem aging. *Foods*, *806*(9), 1-10. https://doi.org/10.3390/foods9060806

Gecgel, U., Yilmaz, I., Soysal, M. I., Gurcan, E. K., & Kok, S. (2019). Investigating proximate composition and fatty acid profile of *Longissimus dorsi* from Anatolian water Buffaloes (*Bubalus bubalis*) raised in similar conditions. *Food Science and Technology, 39*(4), 830-836. https://doi.org/10.1590/fst.08918

Geles, K., Poschl, E., & Aiagner, T. (2003). Collagens-structure, function, and biosynthesis. *Advanced*

Drug Delivery Reviews, _28_, 1531-1546.
https://doi.org/10.1016/j.addr.2003.08.002

Habtu, E., Mekonnen, B., Kiros, H., Fesseha, H., & Getachew, B. (2020). Meat tenderization of efficiency of papain, bromelain and Zingiber officinale on old aged beef carcass of local zebu cattle. _Journal of Trends in Technical & Scientfic Research, 4_(1), 9-15.
https://doi.org/10.19080/TTSR.2020.04.555628

Irurueta, M., Cadoppi, A., Langman, L., Grigioni, G., & Carduza, F. (2008). Effect of aging on meat characteristics from water buffalo grown in delta del Paraná in Argentina. _Meat Science_, _79_(3), 529-533.
https://doi.org/10.1016/j.meatsci.2007.12.010

Jin, P., Zhang, L., Yuan, P., Kang, Z., Du, G., & Chen, J. (2016). Efficient biosynthesis of polysaccharides chondroitin and heparosan by metabolically engineered _Bacillus sublitis_. _Carbohydrate Polymers_, _140_, 424-432.
https://doi.org/10.1016/j.carbpol.2015.12.065

Kandeepan, G., Anjaneyulu, A. S. R., Kondaiah, N., Mendiratta, S. K., & Lakshmanan, V. (2009). Effect of age and gender on the processing characteristics of buffalo meat. _Meat Science_, _83_, 10-14.
https://doi.org/10.1016/j.meatsci.2009.03.003

Liu, L., Ma, M., Cai, Z., Yang, X., & Wang, W. (2010). Purification and properties of a collagenolytic protease produced by _Bacillus cereus_ MBL13 strain. _Food Technology and Biotechnology_, _48_(2), 151-160.
https://hrcak.srce.hr/53624

Moon, S. S. (2018). Effect of proteolytic enzymes and ginger extract on tenderization of _M. pectoralis profundus_ from Holstein steer. _Korean Journal for Food Science of Animal Resources, 38_(1), 143-151.
https://doi.org/10.5851/kosfa.2018.38.1.143

Naveena, B. M., Mendiratta, S. K., & Anjaneyulu, A. S. R. (2004). Tenderization of buffalo meat using plant proteases from _Cucumis trigonus roxb_ (Kachri) and _Zingiber officinale roscoe_ (_Ginger rhizome_). _Meat Science, 68_, 363-369.
https://doi.org/10.1016/j.meatsci.2004.04.004

Naveena, B. M., Kiran, M., Sudhakar, K. R., Ramkrishna, C., Vaithiyanathan, S., & Devetkal, S. K. (2011). Effect of ammonium hydroxide on ultrastructure and tenderness of buffalo meat. _Meat_

Science, _88_, 727-732.
https://doi.org/10.1016/j.meatsci.2011.03.005

Parte, A. C. (2018). LPSN-list of prokaryotic names with standing in nomenclature (bactrio.net), 20 years on. _International Journal of Systematic and Evolutionary Microbiology_, _68_(6), 1825-1829.
https://10.1099/ijsem.0.002786

Quaglia, G. B., & Gennaro, L. (2003). Enzymes: _Uses in Food Processing_. Pp., 2125-2139. In _Encyclopedia of Food Sciences and Nutrition_. Second Edition.
https://doi.org/10.1016/B0-12-227055-X/00420-X

Sartika, D., Natsir, H., Dali, S., & Leliani, L. (2019). Production and characterization of collagenase from _Bacillus_ sp. 6-2 isolated from fish liquid waste. _Jurnal Akta Kimia Indonesia, 2_(1), 58-66.
https://doi.org/10.20956/ica.v12i1.5924

Savita, K., & Arachana, P. (2015). Production of collagenase by _Bacillus_ KM369985 isolated from leather sample. _International Journal of Research in Biosciences, 4_(4), 81-87.
http://www.ijrbs.in/index.php/ijrbs/article/view/177

Schroeter, R., Hoffmann, T., Voigt, B., Meyer, H., Bleisteiner, M., Muntel, J., Jurgen, B., Albrecht, D., Becher, D., Lalk, M., Evers, S., Bongaerts, J., Maurer, K. H., Putzer, H., Hecker, M., Schweder, T., & Bremer, E. (2013). Stress responses of the industrial workhorse _Bacillus licheniformis_ to osmotic challenges. _PLOS One, 8_(11), 1-22.
https://doi.org/10.1371/journal.pone.0080956

Singh, P. K., Ahlawat, S. S., Sharma, D. P., & Pathera, A. (2018). Carcass characteristics of male buffalo calf & meat quality of its veal. _Buffalo Bulletin_, _37_(2), 129-144.
https://kuojs.lib.ku.ac.th/index.php/BufBu/article/view/134

Sorushanova, A., Delgado, L. M., Wu, Z., Shologu, N., Kshirsagar, A., Raghunath, R., Mullen, A. M., Bayon, Y., Pandit, A., Raghunath, M., & Zeugolis, D. I. (2018). The collagen suprafamily: From biosynthesis to advanced biomaterial development. _Advanced Materials, 31_(1), 1801651-1801690.
https://doi.org/10.1002/adma.201801651

Stoica, C., & Sorescu, I. (2017). _ABIS online – Advanced Bacterial Identification Software, an original tool for phenotypic bacterial identification_. Regnum Prokaryotae.

https://www.tgw1916.net/bacteria_Bacillus_input.php

Tran, L. H., & Nagano, H. (2002). Isolation and characteristics of *Bacillus subtilis* CN2 and its collagenase production. *Journal of Food Science, 67*(3), 1184-1187. https://doi.org/10.1111/j.1365-2621.2002.tb09474.x

Wanapat, M., Kang, S., & Phesatcha, K. (2013). Enhancing buffalo production efficiency through rumen manipulation and nutrition. *Buffalo Bulletin*, *32*(1), 258-275. https://kukrdb.lib.ku.ac.th/journal/BuffaloBulletin/search_detail/result/286523

Watanabe, K. (2004). Collagenolytic proteases from bacteria. *Applied Microbiology and Biotechnology*, *63*, 520-526. https://link.springer.com/article/10.1007/s00253-003-1442-0

Zhao, G.Y., Zhou, M. Y., Zhao, H. L., Chen, X. L., Xie, B. B., Zhang, X. Y., He, H. L., Zhou, B. C., & Zhang, Y. Z. (2012). Tenderization effect of cold-adapted collagenolytic protease MCP-01 on beef meat at low temperature and its mechanism. *Food Chemistry*, *134*, 1738-1744. https://doi.org/10.1016/j.foodchem.2012.03.118

Ziauddin, S. K., Mahendrakar, N. S., Rao, D. N., Ramesh, B. S., & Aml, A, B. L. (1994). Observations on some chemical and physical characteristics of buffalo meat. *Meat Science*, *37*, 103-113. https://doi.org/10.1016/0309-1740(94)90148-1

انتاج انزيم الكولاجينيز المنقى جزئيا من بكتريا *Bacillus licheniformis* واستعماله في تطرية لحم الجاموس المسن

اوسن كاظم عبدالرزاق التميمي[1]   و اصلاح ناجي عزيز[1]   و علي عبدالامير خلف[2]

[1]قسم علوم الاغذية، كلية الزراعة، جامعة البصرة

[2] الشركة العامة لتجارة المواد الغذائية، وزارة التجارة، البصرة ، العراق

المستخلص: استهدفت الدراسة الكولاجينيز من بكتريا *Bacillus licheniformis* وتنقيته جزئيا. أجريت عملية الغربلة للعزلات باستعمال وسط انتقائي ثم نميت على وسط Collagen Agar لتحديد العزلات المنتجة للانزيم. تم الحصول على 13 عزلة منتجة للانزيم من خلال تكون الهالة الشفافة التي تحيط المستعمرة، أجريت الاختبارات الكيموحيوية للعزلات البكتيرية وشخصت باستعمال برنامج ABIS online، اذ وجد ان عائدية العزلات BL_1-BL_9 و BL_{12} وBL_{13} كانت للبكتريا *B. licheniformis* بنسبة تطابق 98%، اما العزلات BL_{10} و BL_{11} فكانت عائديتها الى *B. pumilis* و *B. subtilis* بنسبة تطابق 95% و 95.3% على التوالي. تفاوتت الفعالية الانزيمية للعزلات، اذ بلغت اعلى فعالية 200.71 وحدة.مل$^{-1}$ كانت للعزلة BL_{12}، اختير انزيم هذه العزلة لاكمال الاختبارات الأخرى. وجد ان الوقت الأمثل للحصول على اعلى فعالية انزيمية هو 20 ساعة بلغت 210.25 وحدة.مل$^{-1}$ وبفعالية نوعية 135.64 وحدة.ملغم$^{-1}$. نقي الانزيم جزئيا باستعمال كبريتات الامونيوم بنسبة تشبع 20−70%، اذ اظهر فعالية انزيمية ونوعية بلغت 355.81 وحدة.مل$^{-1}$ وبفعالية نوعية 423.58 وحد.ملغم$^{-1}$. استعمل انزيم الكولاجينيز المنقى جزئيا في تطرية لحم الجاموس المسن، اذ بينت النتائج ان معاملة لحم الجاموس المسن بالمحلول الانزيمي لمدد غمر مختلفة، حسنت من الخواص pH و WHC و Cooking Loss مع زيادة في تركيز Hydroxyproline و% للكولاجين الذائب، اذ تفوقت فيها العينات المعاملة بمدة غمر 60 دقيقة.

كلمات مفتاحية: *Bacillus* sp ، الفحوصات الكيميوحيوية، انزيم الكولاجينيز، لحم الجاموس، تطرية اللحوم.