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Contributed Paper

Isolation of A Novel Collagenase-producing Strain from Animal Bone Wastes and Optimization of Its Enzyme Production

Lili Liu*, Yuanyuan Meng, Xiaoning Dai and Ke Chen
Henan University of Science and Technology, 263, Kaiyuan Road, Luoyang, Henan 471003, PR China.
*Author for correspondence; e-mail: yangliuyilang@126.com

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ABSTRACT

A collagenase-producing bacterium was isolated and identified from animal bone wastes. Based on its morphological, physiological and biochemical characteristics, and 16S rRNA gene phylogenetic tree analysis, the strain was identified belonging to *Bacillus cereus* and named as MBL13. The conditions of culture medium and fermentation were optimized. The results indicated that sucrose, bone gelatin and Ca^{2+} were optimal carbon, nitrogen sources and metal ion for *B. cereus* MBL13. By response surface methodology (RSM), the optimum fermentation conditions were made of temperature 33.8 °C, fermentation time (X_2) of 49.5 h, inoculum concentration (X_3) of 45.2 mg/L, medium volume (X_4) of 27.3 mL, and initial pH (X_5) of 6.8 with the maximum collagenase activity of 50.03 U/mL. From the culture supernatant, a novel collagenase was purified and its molecular weight was estimated by SDS-PAGE to be approximately 52.0 kD. Scanning electron microscopy (SEM) analysis showed that the purified collagenase could effectively degrade bovine bone collagen. This study suggested the collagenase produced by *B. cereus* MBL13 has a great potential as a novel protease in hydrolyzing animal bone wastes.

Keywords: animal bone wastes, collagenase, isolation, *Bacillus cereus* MBL13, optimization

1. INTRODUCTION

As the main structural protein of various connective tissues in animals, collagen is one of the most abundant proteins in mammals, making up about 25% of whole-body protein content. It is commonly found in skin, bones and connective tissue within body, providing structural support, strength and a degree of elasticity (in combination with elastin). Isolated from natural sources, it has been widely used in various branches of industries such as foods and pharmaceuticals [1-3]. It is also a very attractive substrate for many fragrance and cosmetic applications. It was reported that injections of collagen can improve the contours of skin and fill out depressions by removing various lines and wrinkles from the face, as well as scarring (including acne).

Collagen is primarily found in skin, tendons and cartilage as well as organic

components of animal bones, teeth and corneas. Bone, as a composite tissue, is basically made up of two phases of an organic and an inorganic one [4]. Mammalian bone is composed of a well organized extracellular matrix that contains embedded crystals of hydroxyapatite (HA). The major part, 90% of organic matrix, is collagen (mainly type I collagen), and remaining 10% consists of over 200 non-collagenous proteins [5]. Collagens and their peptide fragments are produced in large quantities as by-products in livestock and poultry industries [6]. Collagens consist of three polypeptide chains containing repeating triplet sequence Gly-X-Y. It is a unique protein that contains a right-handed triple superhelical rod consisting of three polypeptide chains, which forms insoluble fibers with a high tensile strength and mechanical stability [7-8]. Therefore, it is resistant to common proteolytic enzymes degradation, such as pepsin, trypsin, and papain. It is becoming a part of solid waste due to its rigid structure. Hence, there exists an urgent demand for developing biotechnological alternatives to such wastes recycling.

Annually, more than 55 percent of total animal bone wastes (over 200 million tons per year) are discarded as inedible byproducts. As animal bone contains bioactive and nutraceutical molecules, many studies were performed to utilize large amounts of proteins, oil and mineral originating from these bone [9]. There have been many interests in recycling collagen from animal bone wastes, mainly focusing on enzyme hydroxylation [10-12]. However, only a few proteases with unique characteristics can trigger bone collagen degradation [13]. Collagenase belongs to a typical enzyme capable of degrading native triple helix of collagen [14-15]. It was reported that many collagenases originates from microorganisms, such as *Nocardiopsis dassonvillei*, *Bacillus licheniformis* F11.4, *Pseudomonas sp.* SUK, *Bacillus stearothermophillus*, *Alkalimonas collagenimarina* AC40^{T}, *Penicillium aurantiogriseum* URM 4622 [16-21]. Publications dealing with bacterial collagenases and collagen-degrading enzymes mainly focused on potential role of those enzymes in human diseases [22]. Although some strains have been isolated for collagenases production [23-25], few from animal bone wastes were studied.

Accordingly, this paper is aimed to isolate and identify collagenase-producing strain from animal bone wastes. Moreover, the culture conditions of the isolated strain and its enzyme specificity were optimized and determined separately as well. It is expected to be useful in exploiting its potential applications in animal bone industry.

2. MATERIALS AND METHODS

2.1 Materials

Bone gelatin used in this work was purchased from Wulong gelatin Co. LTD., China. Animal bones wastes were collected from animal bone process plants in China. All other reagents used were of the highest grade commercially available.

2.2 Isolation and Selection of the Strains

Microorganisms isolated from animal bone wastes (Changsha, Hunan province, China) were cultivated in medium agar plates (Diameter: 60 mm, Lianman industry Co. Ltd, Shanghai, China) and overlaid with bone collagen medium (containing 10 g/L bone collagen, 20 g/L agar, pH 7.2-7.5). These plates were incubated at 37 °C for 48 h. Colonies that grew well under such conditions were isolated and retained for subsequent screening. The individual colony was injected by dropping mercuric chloride precipitation (consisting of 15 g

$HgCl_2$, 20 mL concentrated hydrochloric acid and 65 mL distilled water). Thirty-two bacteria with larger transparent circles were obtained followed by inoculating in medium culture (consisting of 0.5 g/L $NaH_2PO_4 \cdot 2H_2O$, 2.5 g/L $K_2HPO_4 \cdot 3H_2O$, 0.05 g/L $CaCl_2$, 20 g/L glucose and 10 g/L tryptone, pH 7.0-7.2) with inoculum concentration of 40 mg/L in baffled shake flasks (100 mL liquid medium in 250 mL flasks). They were shaken on an orbital shaker at 180 rpm (37 °C). After incubation for 48 h, the culture broth was centrifuged (4 °C and 4000 × *g* for 10 min) and the supernatants were collected for measurement of enzymatic activity. The strain is maintained on nutrient agar and used throughout the study.

2.3 Identification of Strain MBL13

The isolated strain was identified according to the method described in "The Shorter Bergey's Manual of Determinative Bacteriology" and also via 16S rDNA sequence [26]. The entire length of the 16S rDNA gene of strain MBL13 was almost amplified and sequenced [27]. A neighbor-joining phylogenetic tree was constructed based on comparison of 16S rDNA sequence of this strain and those of *Bacillus* species.

The colony morphology of the isolated strain was observed on agar plates after 48 h culture at 37 °C. The cell morphology of the isolated strain was examined after being cultured in liquid for 24 h, 48 h, 72 h, and 96 h using light microscopy (× 1500 magnification). Moreover, a JEM-120 transmission electron microscope (JEOL, Japan) was employed as well to achieve a better view of strain cell that was cultured for 72 h. The physiological and biochemical characteristics of collagenase-producing organism were examined using standard procedures.

Sequences analysis of 16S rDNA was performed by amplifying 16S rDNA of the isolated strain with PCR [28]. The aligned 16S rDNA sequences of related species were retrieved from the NCBI nucleotide database. The accession number of the 16S rDNA in GenBank is DQ148914.

2.4 Collagenase Assay

The activity of collagenolytic protease was determined as described by Lima *et al* [29] with minor modifications. Specifically, 0.01 mL collagenolytic protease solution was added into 0.3 mL of 1 g/L acid-soluble type I bovine collagen (the typical collagen in animal bone) in 20 mM Tris-HCl buffer (pH=7.5) followed by incubating at 37 °C for 30 min. The reaction was terminated by the addition of 0.6 mL 10 % (by mass per volume) trichloroacetic acid (TCA). The hydrolysate was centrifuged at 10000 × *g* for 10 min (Avanti J-E, Beckman Coulter Inc, Brea, CA, USA). 0.2 mL of the supernatant was mixed with 0.5 mL of ninhydrin solution followed by heating them at 100 °C for 15 min and cooling in ice water for 5 min. The mixture was diluted with 2.5 mL of 60% (by volume) 1-propanol and was centrifuged again at 10000 × g for 10 min for the measurement of absorbance. The sample was analyzed spectrophotometrically at a wavelength of 570 nm using a spectrophotometer (UV-1800 spectrophotometer Shimadzu Co., Kyoto, Japan). One unit (U) of collagenase activity was expressed as one μmol of glycin equivalents released per mL per minute. The amount of glycin was determined based on the glycin standard curve. All the experiments were performed in triplicate and the results were expressed as the means ± SD.

2.5 Growth Curve and Collagenase Production

The growth and protease production were investigated in fermentation medium incubated in 1 L flask. The bacterium was cultured at 37 °C for 52 h on a rotary shaker at 180 rpm with a inoculum size of 40 mg/L. Samples were withdrawn at 2 h intervals for its growth and collagenase activity determinations. The growth was monitored by OD_{600nm} value (UV-1800 spectrophotometer Shimadzu Co., Japan).

2.6 Optimization of Medium for Collagenase Production

To test effect of different carbon sources, nitrogen sources and metal ions on collagenase production, glucose in basal medium was employed with 20 g/L sucrose, glucose, starch, xylose, fructose, lactose, mannose, galactose, dextrin and fiber (Sigma Chemical Co., St. Louis, MO, USA). All carbon sources were filter sterilized with 0.22 μm filter membrance. Nitrogen source (tryptone) in basal medium was substituted with various nitrogen sources at 10 g/L: ammonium sulfate, ammonium dihydrogen phosphate, urea, tryptone, yeast extract, casein and bone gelatin. Calcium chloride in basal medium was substituted with 0.05 g/L Mn^{2+}, Ca^{2+}, Ba^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, Li^{+}. The pH values and enzyme activities were monitored after 48 h growth with an initial pH 7.2 at 37 °C. The experiments were conducted in triplicate.

2.7 Optimization of Fermentation Conditions for Collagenase Production

At first, the effect of changing a single factor on the collagenase activity was employed to determine the preliminary range of the fermentation variables: fermentation time (X_2), inoculum level (X_3), medium volume (X_4), initial pH level (X_5). Single factor experiments were undertaken for determining optimum range of each factor. Initially, 50 mL fluid medium was added in 250 mL Erlenmeyer flasks and adjusted to pH 7.0, mixed thoroughly and autoclaved at 121 °C for 30 min. The flasks were cooled to room temperature and then inoculated (with inoculums concentration 40 mg/L) of 24 h grown bacterial culture (O.D. at 600 nm between 0.49 and 0.51) under sterile conditions and incubated at various temperatures (27 °C, 32 °C, 35 °C, 37 °C, 40 °C, 42 °C, 54 °C) for 36 h. To investigate the influence of other culture parameters on collagenase activity, growth time (12 h, 24 h, 36 h, 48 h, 60 h, 72 h, 84 h), inoculum concentration (10 mg/L, 20 mg/L, 30 mg/L, 40 mg/L, 50 mg/L, 60 mg/L, 70 mg/L), medium volumes (20 mL, 30 mL, 40 mL, 50 mL, 60 mL, 80 mL, 100 mL) in the 250 mL flask and initial pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0) were studied. Collagenase activity was expressed as mean and standard deviations based on the results obtained with triplicate flasks. Then, the fermentation parameters were optimized using RSM. To determine the combined effect of the independent variables on the response, a central composite design (CCD) with five independent variables at five levels was performed. For each factor, a conventional level was set to zero as a coded level. Among these five factors, each one with five coded levels consisting of 36 experimental runs, were used [30-31].

The design of experiments, analysis of the results and prediction of the responses were carried out using Design-Expert software (Version 8.0.6.1). Comparisons of means were performed by one-way ANOVA (analysis of variance) followed by Tukey's test (p-value < 0.05).

2.8 Purification of Collagenase

All purification steps were carried out at 4 °C. After cultivation, the cells were removed by centrifugation at 12000 × g for 25 min. Solid $(NH_4)_2SO_4$ was slowly added into the culture medium supernatant (4 L) until the concentration reached saturation of 30%, which was calculated using 3.9 mol/L at 0 °C. The volume of the resultant mixture increased and was kept at 4 °C overnight. A volume of 4.1 L of the supernatant was collected by centrifugation at 12000 × g for 30 min. Solid $(NH_4)_2SO_4$ was continuously added into the supernatant (30 %) until the concentration reached saturation of 75%, stirred for 1 h and left overnight at 4 °C. The precipitate was harvested by centrifugation at 12000 × g for 30 min, dissolved in Tris-HCl (pH=7.5) and the dialyzed againstsame buffer overnight (4 °C), and then concentrated in a freeze dryer (Labconco Corp, Kansas City, MO, USA) [20]. The concentrate was dissolved in 15 mmol/L Tris-HCl buffer (pH=7.5) and the insoluble components were removed by centrifugation at 10000 × g for 30 min. The supernatant was dialyzed against the same buffer for 24 h. The resultant dialysate was concentrated by ultrafiltration using an Amicon PM10 membrane (Mr=10000 Da, Millipore, Billerica, MA, USA). The concentrate was applied to a DEAE-Sepharose Fast Flow column (1.6 × 40.0 cm, Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same buffer, and eluted with a linear gradient of 0-2.0 mol/L NaCl in 20 mmol/L Tris-HCl buffer, pH=7.5, at a flow rate of 2.0 mL/min. The active fractions were pooled, dialyzed and concentrated. The concentrate was reloaded onto a Sephadex G-100 column (1.6 × 60 cm, GE Healthcare, Uppsala, Sweden) equilibrated with the same Tris-HCl buffer. The active fractions were pooled and concentrated. Afterwards, the collagenolytic protease solution was concentrated and stored at -20 °C until use. Fractions (1 mL) of purification steps were collected and tested for collagenase activity and analyzed by SDS-PAGE to confirm purity. All chromatographic purification steps were carried out using an AKTA TM purifier (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

2.9 Specificity of Purified Collagenase Degrading Type I Collagen

The purified collagenase were investigated by using the concentration of 3 mmol/L type I collagen as a substrate. Reaction was performed for 30 min at pH and temperature of 7.5 and 37 °C. The proteolytic specificity of the purified collagenase was determined by analyzing peptides. The hydrolysis products were analyzed by SDS-PAGE. Experiments were tested by regarding the same enzyme preparation purchased from the United States of type-I collagenase standard as controlled trials.

2.10 SEM Analysis of the Purified Collagenase Degrading Bovine Bone Crude Collagen

After being treated (removing muscle, cartilage, sponge periosteum) and degreased (121 °C, 45 min), the bovine bone was demineralized (using different HCl concentrations and soaking times) and the pH value was adjusted (0.05% NaOH) to neutral state. Then, the bovine bone was dried (with moisture content of < 6%) and finely ground to bone crude collagen powder (<150 μm). The powder was hydrolyzed by the purified collagenase under the experimental conditions (pH 8.0, 45 °C, enzyme/substrate: 3/100, substrate concentration: 2% (w/v) at different times (2 h, 4 h and 6 h). After incubation at 95 °C

for 10 min to inactivate the enzyme, The inactivated reaction mixture was centrifuged at 5000 × g for 10 min. The separated bone collagen powder was dried by vacuum freeze-drying. Using scanning electron microscopy (JSM-6390, JEOL Ltd, Tokyo, Japan), the surface of crude bone collgen was analyzed after the treatment with purified collagenase.

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of Collagenase-producing Strains

To date, different *Bacillus* species that produce protease, such as *B. licheniformis, B. amyloliquefaciens* and *B. cereus* have been reported [17, 32-33]. However, no report is available on isolation of *B. cereus* producing collagenase from animal bone wastes. So, in this study, collagenase-producing bacteria were isolated from animal bone wastes by selective media. Among 32 isolated strains, MBL13 showed the highest enzyme activity in supernatant. It was incubated onto agar plates containing 20 g/L bone gelatin for 24 h at 37 °C. Colonies with a single morphtype were obtained and tested. The single colony of MBL13 was a round, elevated, white, opaque, large wax-like and drip-like. The morphological characteristic of MBL13 was a rod-shaped Gram-positive bacterium with spore (Figure 1a). The SEM images showed that the cells were 0.56 μm in width and 2 μm in length (Figure 1b). Table 1 summarized the parameters of physiological and biochemical characteristic of MBL13 strain. These characteristics are listed according to standard layout in recommended identification scheme of Bergey's manual of determinative bacteriology [27]. The results showed a typical characteristic of the *Bacillus cereus*. 16S rDNA sequences comparison study supported a strong relationship between strain MBL13 and members of genus *Bacillus*, particularly revealed the highest homology (98 percent) with *Bacillus cereus*. Phylogeny relationship of closely related microorganisms was shown in (Figure 1c).

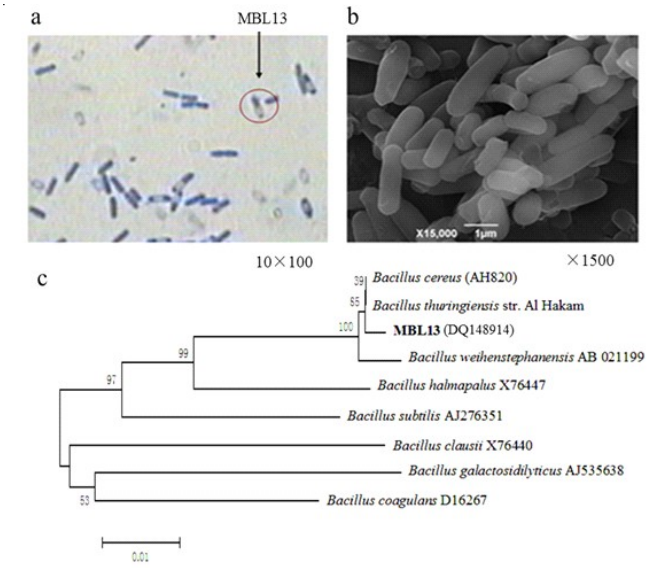

Figure 1. Identification of MBL 13 strain. (a) Gram staining of MBL13 under microscope; (b) SEM photograph of MBL13; (c) The phylogenetic tree based on 16S rDNA sequence analysis.

Table 1. Physiological and biochemical characteristics of the isolate MBL13.

Characters		Results
Growth temperature and pH	25 °C	+
	37 °C	+
	45 °C	+
	pH 5.7	+
Tolerance of NaCl	2%	+
	5%	+
	7%	+
Biochemical characteristics	Catalase	+
	V.P.	+
	Citric acid salt	+
	Urease	-
	Nitrate reduction	+
	Anti-lysozyme	+
Hydrolysis	Gelatin	+
	Starch	+
	Triolein	+
	Casein	-
Carbon sources	D-Glucose	+
	Maltose	+
	Lactose	+
	Sucrose	+
	D-Xylose	-
	D-Mannitol	-
	Arabinose	-

+, positive results; -, negtive results.

3.2 MBL13 Strain Growth and Enzyme Activity

A time course study was performed to determine the growth and collagenase production of *B. cereus* MBL13 strain with respect to time. (Figure 2a) shows the different parameters determined at 4 h intervals. Maximum growth and optical density of *B. cereus* MBL13 was achieved after 32 h incubation. In basal medium, the logarithmic phase commenced at 8 h and continued for another 20 h. Protease production was detected in the late logarithmic phase (after 16 h) and it increased until its optimal production after 44-h incubation. The production of enzyme normally occurs in the late logarithmic phase of growth, when the cell density is high. This production occurs through a phenomenon called quorum sensing. It involves the activation of specific genes at high cell densities in response to chemical signals released by *B. cereus* MBL13. Swift reported that once the cell densities and auto-inducers have reached certain threshold level, generally in the late logarithmic phase, the expression of genes encoding exoproteins and secretion systems is induced [34]. The maximum enzyme activity is 36.80 U/mL at 44 h and prolongation of the fermentation was

accompanied with a gradual decrease of enzyme activity. Secretion of collagenase has been linked to the bacterial growth either associated with the growth or with stationary phase [33, 36].

3.3 Effect of Carbohydrate Source, Nitrogen Source and Metal Ions on Enzyme Production

The ability of *B. cereus* MBL13 to utilize various carbon sources for collagenase production is shown in (Figure 2b). Enzyme production varied with the replacement of 20 g/L glucose in basal medium by various carbon sources. As shown, carbon sources had different effects on fermentation broth pH and enzyme activity. Maximum collagenase production was obtained after 48 h incubation. Media containing glucose, fructose and lactose showed enhancement of enzyme activity by as much as 35.6%, 37.2% and 38.7%, respectively. Meanwhile, the pH values of those medium were different. On the other hand, starch, dextrin and cellulose reduced the enzyme productivity. Their pH values were 7.5, 7.6 and 7.4 respectively. In this study, the best carbon source was found to be sucrose, showing 44.7 percent increase in collagenase production compared to the basal medium. Readily metabolized or utilized carbon sources in the media could decrease or inhibit enzyme synthesis. This result is different from those reported by Li [35] who found that glucose is the best carbon source for enzyme production by *Bacillus cereus*. Aljohani [36] found that starch were the best carbon sources for enzyme production by *Bacillus subtilis*, whereas glucose were ineffective.

In most microorganisms, nitrogen sources are metabolized to produce amino acids, nucleic acids, proteins and cell wall components [37]. In this study, the nature of organic nitrogen sources greatly influenced collagenase production. The ability of *B. cereus* MBL13 to produce collagenase in liquid media was examined in various nitrogen sources. The result (Figure 2c) indicated that organic nitrogen of tryptone, bone gelatin (final medium pH 8.3), and inorganic nitrogen of $(NH_4)_2SO_4$ and $NH_4H_2PO_4$ extract enhanced collagenase productivity. On other hand, enzyme activity decreased dramatically when using casein. Bone gelatin, the special nitrogen source, promoted the collagenase production of *B. cereus* MBL13 more than 45.1% compared to the control (Broth medium). However, this result is different from several strains of *Bacillus cereus*, which were Abfalter et al reported [34].

The effects of addition of some metal ions to culture medium on collagenase productivity were shown in (Figure 2d). Mn^{2+} and Fe^{3+} had adverse effects on enzyme production. Ca^{2+} and Cu^{2+} could enhance enzyme productivity; especially Ca^{2+} markedly affected protease activity and showed 43.08% increase in activity over the control (Broth). This might be attributed to the stabilizing effect of Ca^{2+} and Cu^{2+} on collagenase. Bhagwat et al. reported that Ca^{2+} had a stimulating effect on enzyme action [18].

3.4 Optimization of Fermentation Conditions for Collagenase Production

Based on the single factor test (data not shown), the minimum and maximum values for, temperature (X_1) were set at 27 and 47 °C, ferrmentation time (X_2) between 24 and 72 h, inoculum concentration (X_3) 20 and 60 mg/L, medium volume (X_4) 20 and 40 mL and initial pH (X_5) 4.0 and 8.0 (Table 2).

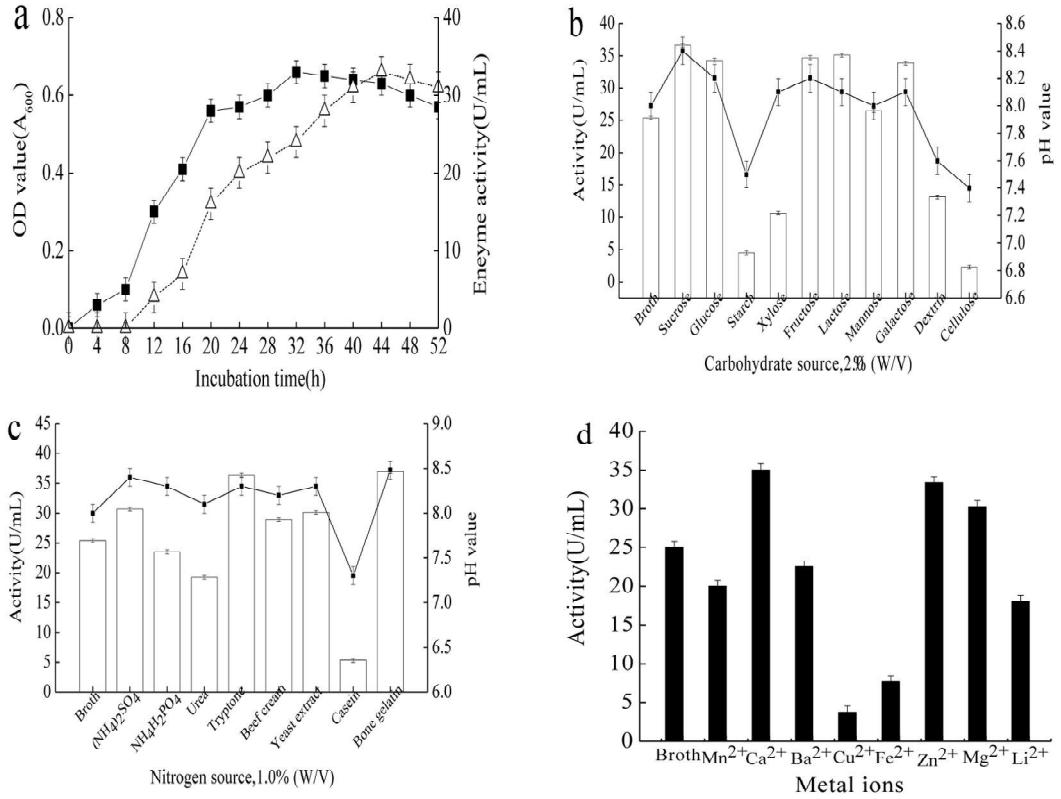

Figure 2. Change of the growth curve and enzyme activity of strain (a). The growth curve of the *B. cereus* MBL13 (■) was shown during different times (0-52 h). Enzyme activity (Δ) changed with times. Effects of carbohydrate sources (b), nitrogen sources (c) and on metal ions (d) on the enzyme production by *B. cereus* MBL13, pH value (-•-) and collagenase activity (▭) were quantified.

Table 2. Independent variables and their levels used in the response surface design.

Variables	Symbol	Coded levels				
		-2	-1	0	1	2
Temperature (°C)	X_1	27	32	37	42	47
Fermentation time (h)	X_2	24	36	48	60	72
Inoculum concentration (mg/L)	X_3	20	30	40	50	60
Medium volume (mL/250 mL)	X_4	20	25	30	35	40
Initial pH	X_5	4.0	5.0	6.0	7.0	8.0

The experimental design and responses are given in Table 3 and Table 4. The experimental results were fitted with a second-order polynomial equation to explain the dependence of collagenase activity (*Y*) on the different factors. In addition, the experiment results indicated that the collagenase activity ranged from 21.89 U/mL to 51.97 U/mL. The maximum collagenase activity 51.97 U/mL was found under the experimental conditions of temperature of 37 °C, time of 48 h, inoculum concentration

of 40 mg/L, medium volume of 30 mL, initial pH of 6.0. Through multiple regression analysis on the experimental data, the model for the predicted response could be expressed by the following quadratic polynomial equations (in the form of coded values):

$$Y=48.21-1.44X_1+2.91X_2+1.87X_3-0.20X_4+1.62X_5+0.49X_1X_2-0.93X_1X_3+1.50X_1X_4-3.97X_1X_5+0.38X_2X_3+1.71X_2X_4-1.64X_2X_5-3.09X_3X_4-0.48X_3X_5-2.01X_4X_5-4.60X_1^2-2.34X_2^2-2.23X_3^2-2.63X_4^2-2.95X_5^2 \quad (1)$$

Where X_1, X_2, X_3, X_4 and X_5 are temperature, fermentation time, inoculum, medium volume and initial pH, respectively.

The fit statistics for collagenase activity (Y) for the selected quadratic predictive model are shown in Table 4. The determination coefficient (R^2=0.9651) by analysis of variance (ANOVA) of the quadratic regression model indicated that only 3.49% of the total variations were not explained by the model. However, a large value of R^2 does not always imply that the regression model is a good one. The value of the adjusted determination coefficient (Adj R^2=0.9185) also confirmed that the model was highly significant, which indicated good agreement between the experimental and predicted values of collagenase activity. The results of analysis of error indicated that the lack of fit test (0.0533) was insignificant at the 95% confidence level, confirming the validity of the model. At the same time, a relatively low value of coefficient of the variation (CV=6.24) indicated a very high degree of precision and a highly reliable experimental values [30]. Thus the model is adequate for prediction in the range of experimental variables. Moreover, the model *P*-value was very low (< 0.0001), indicating that the model terms were significant. The experimental ratio of 14.679 indicated an adequate signal. Hence, all these statistical parameters showed the reliability of the model.

Table 4 shows the regression coefficient values of Eq. (1). For each terms in the models, a large *F*-value and a small *P*-value would imply a extremely significant effect on the respective response variable [31]. The table shows that the linear coefficients (X_1, X_2, X_3 and X_5), quadratic term coefficients (X_1^2, X_2^2 , X_3^2, X_4^2 and X_5^2) and interaction coefficients (X_1X_5 and X_4X_5) were extremely significant, with very small *P*-values ($P < 0.01$). The simplified second-order polynomial Eq.(2) for collagenase activity (Y) in terms of actual factors was expressed as follows:

$$Y=48.21+-1.44X_1+2.91X_2+1.87X_3+1.62X_5-3.97X_1X_5-2.01X_4X_5-4.60X_1^2-2.34X_2^2-2.23X_3^2-2.63X_4^2-2.95\ X_5^2 \quad (2)$$

Response surface methodology was used to illustrate the effects of temperature, fermentation time, inoculum, medium volume and initial pH on the response. The regression model equation allowed the prediction of the effects of the five parameters on the collagenase activity. The type of interactions between the five tested variables and the relationship between responses and experiment levels of each variable was illustrated in 3D response-surface plots and 2D contour plots of the response surfaces. A circular contour plot indicated that the interactions between the corresponding variables are negligible, while an elliptical contour plot indicated otherwise [31].

Table 3. Observed values of response (collagenase activity in U/mL), Experimental values are average of five experiments.

Run	X_1	X_2	X_3	X_4	X_5	Collagenase activity (U/mL)
1	1	1	1	1	1	33.32±1.09
2	1	1	1	-1	-1	38.32±1.13
3	1	1	-1	1	-1	44.56±1.54
4	1	1	-1	-1	1	30.70±1.06
5	1	-1	1	1	-1	31.80±1.11
6	1	-1	1	-1	1	30.95±0.98
7	1	-1	-1	1	1	25.76±0.96
8	1	-1	-1	-1	-1	27.56±1.05
9	-1	1	1	1	-1	39.70±1.18
10	-1	1	1	-1	1	47.34±1.52
11	-1	1	-1	1	1	37.76±1.23
12	-1	1	-1	-1	-1	29.36±1.07
13	-1	-1	1	1	1	35.30±1.17
14	-1	-1	1	-1	-1	30.68±1.20
15	-1	-1	-1	1	-1	21.89±0.89
16	-1	-1	-1	-1	1	43.29±1.54
17	2	0	0	0	0	25.16±0.90
18	-2	0	0	0	0	31.28±1.01
19	0	2	0	0	0	41.23±1.55
20	0	-2	0	0	0	33.26±1.02
21	0	0	2	0	0	42.27±1.48
22	0	0	-2	0	0	33.13±1.16
23	0	0	0	2	0	36.90±1.28
24	0	0	0	-2	0	35.26±1.32
25	0	0	0	0	2	39.39±1.27
26	0	0	0	0	-2	30.23±1.19
27	0	0	0	0	0	47.09±1.53
28	0	0	0	0	0	48.48±1.52
29	0	0	0	0	0	49.01±1.50
30	0	0	0	0	0	47.11±1.47
31	0	0	0	0	0	47.08±1.49
32	0	0	0	0	0	50.14±1.51
33	0	0	0	0	0	47.01±1.50
34	0	0	0	0	0	47.32±1.52
35	0	0	0	0	0	50.05±1.48
36	0	0	0	0	0	51.97±1.50

Table 4. Analysis of variance ANOVA of the regression model of quadratic response.

Source of variation	Sum of squares (SS)	Degree of freedom (df)	Mean squares (MS)	*F*-value	*P*-value	Significance
X_1	49.85	1	49.85	8.69	0.0100	
X_2	202.83	1	202.83	35.35	< 0.0001	
X_3	83.66	1	83.66	14.58	0.0017	
X_4	0.97	1	0.97	0.17	0.6864	
X_5	62.95	1	62.95	10.97	0.0047	
X_1X_2	3.83	1	3.83	0.67	0.4266	
X_1X_3	13.89	1	13.89	2.42	0.1405	
X_1X_4	35.79	1	35.79	6.24	0.0246	
X_1X_5	252.57	1	252.57	44.02	< 0.0001	
X_2X_3	2.30	1	2.30	0.40	0.5359	
X_2X_4	46.75	1	46.75	8.15	0.0121	
X_2X_5	42.87	1	42.87	7.47	0.0154	
X_3X_4	2.43	1	2.43	0.42	0.5254	
X_3X_5	3.73	1	3.73	0.65	0.4324	
X_4X_5	64.68	1	64.68	11.27	0.0043	
X_1^2	675.80	1	675.80	117.80	< 0.0001	
X_2^2	175.11	1	175.11	30.52	< 0.0001	
X_3^2	158.49	1	158.49	27.63	< 0.0001	
X_4^2	221.43	1	221.43	38.60	< 0.0001	
X_5^2	278.11	1	278.11	48.48	< 0.0001	significant
Regression	2378.06	20	118.90	20.73	< 0.0001	
Residual	86.06	15	5.74			not significant
Lack of Fit	59.12	6	9.85	3.29	0.0533	
Pure Error	26.94	9	2.99			
Total	2464.12	35				
R^2	0.9651					
Adj-R^2	0.9185					
CV	6.24					
Std. Deviation	2.40					
Adeq precision	14.679					

Figure 3a showed the 3D graphic surface and contour plot of the combined effects of temperature and initial pH (X_1X_5) on the collagenase activity. These plots presented the response in function of two factors, keeping the other variable constant at its middle level (center value of the testing ranges). The tortuose surface and oval contour plot showed a strong interaction between these two factors. A higher activity of collagenase was obtained with a temperture between 34 and 37 °C and initial pH between 6.0 and 7.0. Collagenase activity decreased with the increase of temperature (37-47 °C) and initial pH (7.0-8.0). This demonstrated that the effect of temperature (X_1) and initial pH (X_5) on collagenase activity was significant, and was in good agreement with the results

in Table 4. The effects of medium volume (X_4) and initial pH (X_5) on collagenase activity are shown in Figure 3b. The maximum predicted value indicated by the surface was confined in the smallest ellipse in the contour diagram. The smallest ellipse in the contour plot indicated that there was a perfect interaction between the independent variables [30]. As indicated, the collagenase increased with increasing medium volume from 20 to 30 mL/250 mL (v/v) and decreased with volume from 30 to 40 mL/250 mL. The collagenase activity also increased with the increase of initial pH from 5.5 to 6.5, but decreased at values from 6.5 to 8.0. The optimized conditions were temperature (X_1) of 33.8 °C, fermentation time (X_2) of 49.5 h, inoculum concentration (X_3) of 45.2 mg/L, medium volume (X_4) of 27.3 mL, and initial pH (X_5) of 6.8. Under the optimal conditions, the model predicted yield is 50.04 U/mL. The results of analysis indicated that the experimental values (50.03 U/mL) were in good agreement with the predicted ones (not significant at the 5% confidence level) and consequently, indicated that the RSM model is satisfactory and accurate. Based on the result of the optimum media formulation and RSM, the optimum medium and fermentation conditions were tested for collagenase production and compared to the original conditions. Enzyme activity experiments were conducted in triplicate and the original conditions were set as blank controls. The enzyme activity of strain MBL13 was only 36.80 U/mL under initial culture and fermentation conditions, however, it increased by 35.95% up to 50.03 U/mL.

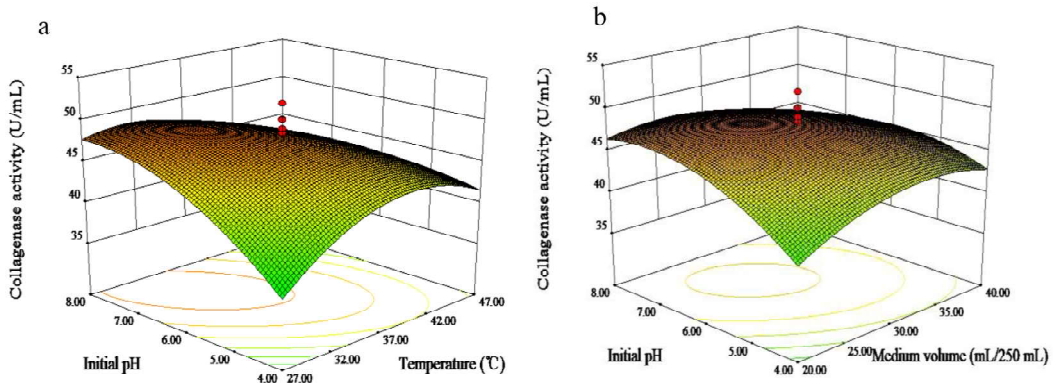

Figure 3. Response surface (3D) showing the interactive effect of temperature (X_1), medium volume (X_4) and initial pH (X_5) on the collagenase activity.

3.5 Purification of Collagenase

Under the optimal medium and fermentation conditions, *B. cereus* MBL13 secreted collagenase into the culture medium. As shown in Table 5, the enzyme was purified 42.85-fold with a 13.53% yield. The specific activity of the final enzyme preparation was estimated to be 5570 U/mg. Collagenase samples extracted in different purification steps were tested by SDS-PAGE (Figure 4). After Sephadex G-100 chromatography, collagenase activity was detected in a single peak. It showed the purified collagenase with a molecular mass of 52.0 kDa, which was different from those of other collagenases isolated from *Nocardiopsis dassonvillei, Bacillus cereus*, *Aspergillus tamarii* and *Pseudomonas sp* (molecular masses of 150.0 kDa, 100.0 kDa, 98.8 kDa and 123.0 kDa) [16, 34, 38, 39].

Table 5. The summary of the purification of *B. cereus* MBL13 collagenolytic protease.

Purification step	Total protein/mg	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	360.92	46920	130	1.00	100
Ammonium sulphate	99.21	28770	290	2.23	61.32
DEAE-Sepharose FF	5.07	12980	2560	19.69	27.67
Sephadex G-100	1.14	6350	5570	42.85	13.53

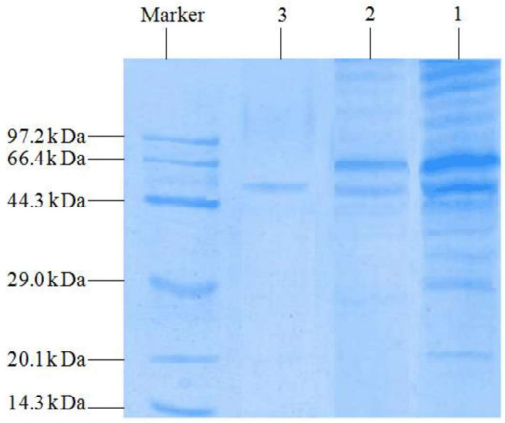

Figure 4. SDS-PAGE of the protein fractions obtained from various purification steps. Electrophoresis was performed on 10 % gels. Lane 1: fraction after the ammounium sulphate precipitation step, lane 2: fraction after the second DEAE-cellulose Fast Flow column chromatography, lane 3: purified collagenase.

3.6 Specificity of Purified Collagenase Degrading Type I Collagen

The specificity of the purified collagenolytic protease was tested using type I collagen as a substrate at 37 °C for 30 min. The hydrolysate was analyzed by SDS-PAGE (Figure 6). After enzymolysis, type I collagen became small uncontinuous molecular weight polypeptides. By comparing collagenase standard (lane 2), the purified collagenase from *B. cereus* MBL13 degraded type I collagen into hydrolysates of smaller molecular weight (lane 3), indicating that its degradation ability was superior to the collagenase standard (Sigma, USA). These results showed that the purified collagenase displayed a specificity in hydrolyzing type I collagen.

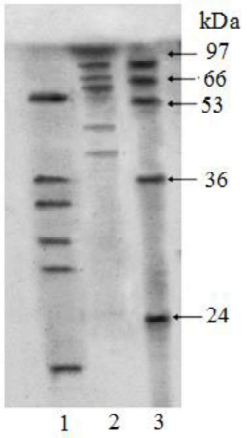

Figure 5. SDS-PAGE of type I collagen and its hydrolysis products obtained by the purified collagenase of *B. cereus* MBL13. Proteins were separated on 18% gel. Lane 1: Hydrolysates degrading by the purified collagenase, lane 2: Hydrolysates degrading by type I collagenase standard (Sigma, USA), lane 3: Protein markers (97, 66, 53, 36 and 24 kDa).

3.7 SEM Analysis of the Purified Collagenase Degrading Bovine Bone Crude Collagen

SEM showed the purified collagenase had a great influence on the surface structure of bovine bone crude collagen with the increase of degradation time in Figure 6. The surface of bone crude collagen (control) showed a long and tight and structural state in Figure 6(a). However, Figure (b) (c) (d) showed the surface structures of crude

collagens were gradually peeled off with the increase of degardation time, which made the tight and long shaped bovine bone crude collagen broken and more loose. This indicated the purified collagenase could obviously degrade type I collagen from bovine bone into small molecular polypeptides, which resulted in the destruction of the intact surface structure of bovine bone crude collagen.

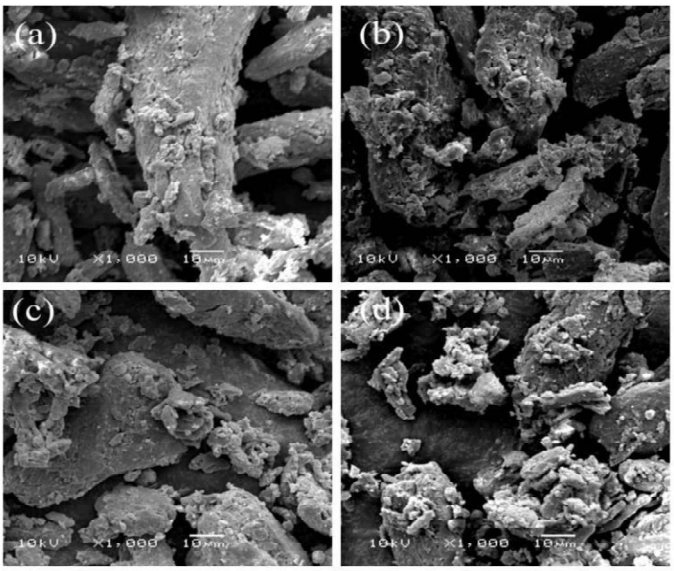

Figure 6. The SEM of bovine bone crude collagen at different degradation times. (a) Control, (b) 2 h, (c) 4 h, (d) 6 h.

4. CONCLUSION

In this work, a strain MBL13 capable of using bone gelatin as a carbon and nitrogen source was isolated and identified. According to the morphological characteristics, physiological and biochemical characteristics, combined with 16S rDNA sequence analysis, the strain was identified as *Bacillus cereus*, which shows the highest capability in producing collagenase. Culture and fermentation conditions were important for collagenase production. Optimization of collagenase production was carried out to enhance enzyme yield from *B. cereus* MBL13 strain. Investigation on the effects of nutritional and fermentation factors have enabled the formulation of media composition and fermentation conditions for optimal enzyme production. *B. cereus* MBL13 collagenase was purified to electrophoretic homogeneity using a combination of three purification steps. The molecular weight was approximately 52.0 kD, which was different from those of collagenases isolated from the other strains. With respect to substrate specificity, enzymatic hydrolysis was specific for type I collagen. The result of the action of the purified enzyme towards bovine bone crude collagen showed that the protease was a collagenase-type and was able to degrade bovine bone. Therefore, the presented results indicate that *B. cereus* MBL13 collagenase will most probably play an important role in the process of efficient waste animal bones hydrolysis.

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