Optimized Recombinant Expression and Characterization of Collagenase in *Bacillus subtilis* WB600

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Abstract: Background: The collagenase encoding gene *col* was cloned into a pP43NMK vector and amplified in *Escherichia coli* JM109 cells. The shuttle vector pP43NMK was used to sub-clone the *col* gene to obtain the vector pP43NMK-*col* for the expression of collagenase in *Bacillus subtilis* WB600. The enzyme was characterized and the composition of the expression medium and culture conditions were optimized. Methods: The expressed recombinant enzyme was purified by ammonium sulfate, ultrafiltration, and through a nickel column. The purified collagenase had an activity of 9405.54 U/mg. Results: The recombinant enzyme exhibited optimal activity at pH 9.0 and 50 °C. Catalytic efficiency of the recombinant collagenase was inhibited by Fe$^{3+}$ and Cu$^{2+}$, but stimulated by Co$^{2+}$, Ca$^{2+}$, Zn$^{2+}$, and Mg$^{2+}$. The optimal conditions for its growth were at pH 7.0 and 35 °C, using 15 g/L of fructose and 36 g/L of yeast powder and peptone mixture (2:1) at 260 rpm with 11% inoculation. The maximal extracellular activity of the recombinant collagenase reached 2746.7 U/mL after optimization of culture conditions, which was 2.4-fold higher than that before optimization. Conclusions: This study is a first attempt to recombinantly express collagenase in *B. subtilis* WB600 and optimize its expression conditions, its production conditions, and possible scale-up.

Keywords: collagenase; *Bacillus subtilis* WB600; protein purification; characterization; recombinant protein expression

1. Introduction

Collagenases can be classified as metallocollagenases and serine collagenases that specifically hydrolyze the three-dimensional spiral structure of natural collagen [1,2]. They are involved in the degradation of the extracellular matrices of animal cells [3]. Enzymes catalyzing collagen hydrolysis include matrix metalloproteinases (MMPs), which are zinc-containing enzymes [4]; they usually require calcium for their optimum activity and stability [5]. Collagenases are found in several bacterial species such as *Bacillus cereus* [6,7], *Clostridium histolyticum* [8], *Bacillus subtilis* [9], and *Actinomyces* [10]. However, activity efficiency of most bacterial collagenases does not meet the needs of industrial processes, which are often carried out under conditions not optimal for the stability and activity of collagenases. Nevertheless, collagenases have several industrial, biotechnological, pharmacological, and medicinal applications. They are used to treat cardiovascular diseases, neurodegenerative diseases, cancer, arthritis [10–14], and liver fibrosis [15].

Collagenase genes of different origins were heterologously expressed in *Escherichia coli* (E. coli) [6–8]. However, *E. coli* is not a suitable host for enzyme production in the food industry due to plasmid instability and the presence of endotoxins. *Bacillus subtilis* (*B. subtilis*) is a non-pathogenic and bacteriophage-resistant host, which is generally regarded as safe [16] compared to *E. coli*. Moreover, *B. subtilis* can use various signal peptides to
non-specifically secrete recombinant proteins for the production of industrial enzymes [17] and act as microbial cell factories [18]. *B. subtilis* WB600 lacks six protease genes; therefore, secreted proteins are not digested [19].

The present study describes the successful expression of collagenase in *B. subtilis* WB600 by using a modified shuttle vector pP43NMK. Furthermore, the culture conditions, including the key media components and the fermentation conditions, were optimized. The results obtained here may be useful for the industrial production of collagenase. The production of recombinant collagenase in *Bacillus subtilis* can simplify the purification process for its secretory expression, and the enzymes can be safely and widely used in the food industry due to their non-pathogenicity, which avoids the effects of harmful virulence factors [20].

2. Materials and Methods

2.1. Media Composition and Culture Conditions

The *Bacillus* strains were cultured in Luria Bertani (LB) medium containing peptone (10 g/L), yeast extract (5 g/L), and sodium chloride (10 g/L) at 37 °C and shaken at 200 rpm. Terrific Broth (TB), comprising peptone (12 g/L), yeast extract (24 g/L), glycerol (0.4% v/v), KH₂PO₄ (0.23 g/L), and K₂HPO₄ (1.25 g/L), was used as the fermentation medium for collagenase expression. Kanamycin (Kan; 50 µg/mL) was added to the LB and TB media. Collagenase was expressed in 100 mL of growth media in 300 mL conical flasks at 37 °C for 48 h.

Plasmid extraction kit: Tiangen Biotech (Beijing) Co., Ltd., Beijing, China. DNA Marker: Takara Bio Co., Ltd., Beijing, China. Ultra-micro spectrophotometer: NP80 Mobile, Implen International Trading Co., Ltd., Beijing, China. Shaking incubator: ZQTY-70V, Shanghai Zhichu Instrument Co., Ltd., Shanghai, China. Gel imaging system: 4600SF, Shanghai Tianneng Technology Co., Ltd., Shanghai, China. Autoclave pot: D-I autoclave, Beijing Faen Science and Trade Co., Ltd., Beijing, China. Vertical electrophoresis instrument: Mini-protean, Bio-Rad Laboratories, Inc., Shanghai, China. Agarose level electrophoresis instrument: DYCP-31D, Beijing Liuyi Biotechnology Co., Ltd., Beijing, China. UV/VIS spectrophotometer: TU-1810, Beijing General Instrument Co., Ltd., Beijing, China. High-speed refrigerated centrifuge: ALLegra, Beckman Coulter, Inc., California, USA. Digital display constant temperature water bath pot: HH-4A, Guohua Instrument Manufacturing Co., Ltd., Changzhou, China. Thermal cycler: T100, Bio-Rad Laboratories, Inc., Shanghai, China.

2.2. Gene Optimization Methodology

Codon usage bias of the optimization sequence was adjusted against a proprietary reference codon bias established by multi-omics data analysis to fit the profile of highly expressed genes in the target host and optimized by Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China, with their homemade software (http://192.168.19.50:8080/cool/, accessed on 6 May 2022). Various parameters were meticulously evaluated, including codon usage bias, codon context, GC profile, negative CpG islands, splicing sites, negative cis-acting elements, hidden stop codon, mRNA secondary structure, RNA instability, repeat sequences, restriction sites, and any undesired motif. Artificial gene synthesis was carried out by PCR, digestion, ligation, cell transformation, and other technologies.

2.3. Plasmid Construction and Transformation for Collagenase Expression

*B. amyloliquefaciens* has been proven able to synthesize collagenase and secrete it extracellularly, and the dermal collagen could be degraded by its fermentation broth, which could be applied to leather production [21]. The application of collagenase in the food industry has attracted much attention, and it is necessary to find a collagenase gene with stable enzymatic properties, accompanied by the potential for mass production in the microbiology industry. According to the GenBank database, the sequence identity of the collagenase encoding gene from *B. amyloliquefaciens* and *Bacillus velezensis* is 100%;
therefore, the gene synthesis was carried out according to the sequence published by *Bacillus velezensis* in this study, whose GenBank code is CP011686.1 with the locus tag AB13_2557. The collagenase gene sequence was extracted from GenBank (*col*, GenBank ID CP011686.1, locus tag: AB13_2557) and restriction enzyme sites’ sequences for *Pst* I and *Hind* III were synthesized and ligated into the plasmid pP43NMK purchased from Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China. The ligated plasmid was used to transform *E. coli* JM109 competent cells. Colonies carrying the pP43NMK-*col* plasmid were confirmed by colony PCR and double digestion by the restriction enzymes *Pst* I and *Hind* III. Following amplification in *E. coli* JM109, the expression plasmids were extracted and were used to transform *B. subtilis* WB600 [1].

2.4. Recombinant Expression and Purification of Collagenase

The *B. subtilis* cells were grown for 14–16 h in 50 mL LB media at 37 °C with stirring at 220 rpm. An aliquot of the overnight culture (2% *v/v*) was inoculated into 100 mL of fermentation medium in a 300 mL conical flask and incubated for 48 h and harvested by centrifugation at 10,000 rpm at 4 °C for 20 min.

Saturated ammonium sulfate solution was carefully added to the supernatant at different concentrations (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%) and incubated for 14–16 h at 4 °C. The precipitates were dissolved in 10 mM Tris-HCl buffer (pH 7.5) and dialyzed against the same buffer to completely remove the ammonium sulfate. The enzyme solution was concentrated using a 10 kDa Amicon filter (Merck, Munich, Germany) and purified using a His-Tag Purification Resin (Beyotime Biotechnology, Shanghai, China). The purified recombinant collagenase was then analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out to determine the successful purification of the recombinant protein and its molecular mass. Coomassie Brilliant Blue R-250 was used for staining the gel, and the molecular mass of collagenase was estimated on a 12% polyacrylamide gel based on protein standards [22].

2.6. Protein Quantification and Enzyme Assay of Collagenase

The protein concentration was estimated using the Bradford method; the absorbance was measured at 595 nm and bovine serum albumin was used as the protein standard [23].

Collagenase activity was determined by the colorimetric method. Briefly, 1 mL of 1 mg/mL gelatin solution was mixed with 500 µL of Tris-HCl (pH 7.5) and 100 µL of the enzyme solution and incubated for 40 min at 37 °C. Subsequently, the reaction was stopped by adding 500 µL of trichloroacetic acid (10%, *m/v*), 900 µL of acetic acid buffer (pH 5.4), and 1 mL of indigohydrone to the mixture, followed by incubation in boiling water for 10 min, then immediately cooling down in ice water. Finally, 4 mL 60% ethanol was added to the mixture, and the absorbance was read at 570 nm.

One unit (U) of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1.0 µg glycine per minute.

2.7. Effects of pH, Temperature, and Metal Ions on Collagenase Activity and Stability

Collagenase activity was assayed under standard conditions at various temperatures (30–80 °C) to determine the optimum temperature of the recombinant enzyme. Thermal stability of the enzyme was measured for any residual activity at different temperatures for 2 h. All experiments were performed in triplicate and repeated three times. The residual activity was expressed as a percentage of the control sample activity, based on the assumption that the activity of control sample (in the absence of any additives) was 100%.

For the pH dependence assay, the collagenase enzymatic activity was measured using buffers at different pH levels, including sodium citrate buffer (pH 5.0), phosphate buffer (pH 6.0–8.0), and glycine–NaOH buffer (pH 9.0–11.0). To determine the pH stability of the
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recombinant collagenase, the enzyme was incubated in the above buffers for 2 h, followed by the measurement of the residual enzymatic activity under standard assay conditions.

To analyze the effect of metal ions on the enzyme activity, the enzyme solution was incubated in different metal ion solutions, including Fe$^{3+}$, Zn$^{2+}$, Co$^{2+}$, Ca$^{2+}$, Mg$^{2+}$, and Cu$^{2+}$ (5 mM) for 2 h. The residual activity of the enzyme in each solution was measured individually and compared.

2.8. Optimization of the Fermentation Conditions for the Recombinant Expression of Collagenase

The effects of media composition and culture conditions on the recombinant expression of collagenase in B. subtilis WB600 were examined.

The carbon sources (glucose, mannitol, maltose, fructose, glycerin, and sucrose) and nitrogen sources (beef paste, ammonium sulfate, urea, gelatin, peptone, and yeast extract) were varied with an initial total nitrogen concentration of 10.0 g/L. The initial concentrations of carbon source and nitrogen source analyzed were 5.0, 10.0, 15.0, 20.0, 25.0, and 30.0 g/L and 6.0, 12.0, 18.0, 24.0, 30.0, 36.0, and 42.0 g/L, respectively. The effect of metal ions (Zn$^{2+}$, Mg$^{2+}$, Na$^{+}$, and Ca$^{2+}$) on collagenase production was also investigated.

The optimized medium was adjusted at different initial pH levels (5.0, 6.0, 7.0, 8.0, 9.0), stirring speeds (200, 220, 240, 260, 280 rpm), temperatures (25, 30, 35, 40, 45 °C), and inoculation concentrations (5%, 7%, 9%, 11%, 13%, 15%). All the values of enzyme activities were averaged from three replicates with standard deviations.

3. Results and Discussion

3.1. Gene Optimization

Gene expression is regulated and influenced by various factors such as codon usage bias, ribosome binding, and mRNA structure. The gene optimization process takes into consideration as many factors as possible, resulting in gene sequences that can optimally express proteins. There are numerous possibilities of mRNA sequences coding for the same protein. However, an advanced algorithm was used to screen and generate an optimal sequence from tens of thousands of candidate sequences.

The 936 bp collagenase sequence was optimized for optimal protein expression in B. subtilis, as shown in Figures 1 and 2. Codon usage bias was adjusted to fit the highest expression profile of the target host; CAI (Codon Adaptation Index) was upgraded from 0.81 to 0.96 (a CAI of 0.8–1.0 is regarded as good for high expression). Average GC content was adjusted from 47.1% to 40.3% and unfavorable peaks were removed. Repeated sequences in the original sequence were removed to avoid the formation of stem-loop structures in the mRNA and to facilitate the protein synthesis process. Undesired motifs, including restriction enzyme sites for use in sub-cloning and negative cis-acting sites, were modified. The whole sequence was fine-tuned to increase the translation efficiency and prolong the half-life of mRNA.

3.2. Expression of Recombinant Collagenase

DNA fragments around 936 bp were obtained, encoding for a protein comprising 312 amino acids. The appropriate recombinant plasmid was confirmed by double digestion with the restriction endonucleases Pst I and Hind III (Figure 3a) and was subsequently transformed into competent B. subtilis cells.
Figure 1. (a) Original sequence. (b) Optimized sequence. Relative codon frequency distribution. Color of codons indicates the frequency of that codon with respect to the host. Rarely used codons are shown in cyan and frequently used codons are shown in red. A redder codon indicates higher frequency. Redder codons compared with the other codons mean that they are better suited for the host codon bias, resulting in high expression. (c) Original sequence. (d) Optimized sequence. Codon relative frequency radar plot. The relative codon frequency distribution shows the frequency of each individual codon and this radar plot shows the suitability of the codon usage profile between the optimized sequence (shown in red) and host (shown in blue). A better curve match means it is more adequate.
Figure 2. (a) Original sequence. (b) Optimized sequence. Frequency of optimal codons. Shows the percentage distribution of codons; codons with a higher percentage are more frequently used. (c) Original Sequence (47.1%). (d) Optimized sequence (40.3%). GC content adjustment. The ideal percentage range of GC content is between 30% and 70%, ideally 40–60%.
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The molecular mass of the recombinant protein was predicted to be 35.4 kDa using the ExPASy program (http://web.expasy.org/protparam/ accessed on 6 May 2022). Meanwhile, recombinant collagenase from other bacteria such as Actinomyces [10], Pseudalteromonas agarivorans [24], Bacillus cereus [7], Vibrio alginolyticus [8], and Lucilia Sericata [25] were reported as 150 kDa, 52.5 kDa, 55 kDa, 90 kDa, and 52 kDa, respectively.

B. subtilis WB600/pP43NMK-col was cultured in the fermentation medium at 220 rpm and 37 °C for the expression of collagenase. Under such expression conditions, the extracellular activity of collagenase was 1145.16 U/mL. SDS-PAGE analyses of the supernatant of the culture broth indicated a distinctive protein band at around 35.4 kDa, confirming the expression of collagenase. This band was not present in the negative control samples (Figure 3b), consistent with the enzyme activity assay results. In summary, recombinant collagenase was successfully expressed in B. subtilis WB600.

3.3. Purification of the Recombinantly Expressed Collagenase

The supernatant of the bulk-cultured recombinant collagenase was concentrated with 70% ammonium sulfate, and the precipitate was dissolved in Tris-HCl (pH 7.5) buffer. The solution was then ultrafiltered against the Tris-HCl (pH 7.5) buffer. Subsequently, the ultrafiltrate was passed through a nickel column. The fractions containing the collagenolytic activity were pooled, concentrated, and stored at −20 °C. The results of the collagenase

Figure 3. Cloning and expression of collagenase. (a) Electrophoresis gel of the double-digested plasmid from B. subtilis WB600 to show the presence of the col gene. M, DNA ladder; lane 1, recombinant plasmid digestion; lane 2, recombinant plasmid PCR. (b) The recombinant collagenase was successfully expressed. M, molecular weight marker; lane 1, B. subtilis WB600/pP43NMK-col fermentation supernatant, the arrow points to the target protein; lane 2, B. subtilis WB600/pP43NMK fermentation supernatant.
purification using ammonium sulfate precipitation are summarized in Table 1. The optimal concentration of ammonium sulfate for purification was 70%, resulting in a specific activity of 6156.67 U/mg. Using a three-step procedure, the specific activity of the enzyme was increased from 1998.73 U/mg (crude enzyme) to 9405.54 U/mg (purified enzyme), and the enzyme was purified 4.71-fold with a yield of 2.14% from the crude extract (Table 2). The native bacteria *B. subtilis* DB104 expressing *col*H and its specific activity is estimated to be 1210 U/mg [26]. Cloned from *Grimontia (Vibrio) hollisae* 1706B and expressed by the *Brevibacillus* system, the purified recombinant enzyme had a specific activity of 5314 U/mg [27]. The strain SM1988T, which was a Gram-negative unipolar flagellar-shaped bacterium, expressed the collagenase enzyme with a specific activity of 384.14 U/mg [28]. Compared with them, the purified enzyme was higher, which may be due to the different measurement methods of enzyme activity, the different adaptation substrates, and the adequate purification effect of this study, which maintains the vitality of collagenase to a certain extent.

Table 1. Specific activity of the enzyme at different concentrations of ammonium sulfate precipitation.

| Ammonium Sulfate Concentration (%) | Specific Activity (U/mg) |
|-----------------------------------|--------------------------|
| 20                                | 4498.19                  |
| 30                                | 2925.32                  |
| 40                                | 2165.25                  |
| 50                                | 2177.78                  |
| 60                                | 4921.74                  |
| 70                                | 6156.67                  |
| 80                                | 5509.75                  |
| 90                                | 4000.21                  |

Table 2. Purification of collagenase from *B. subtilis* WB600/pP43NMK-col.

| Steps                        | Total Activity (U) | Total Protein (mg) | Specific Activity (U/mg) | Purification (Fold) | Yield (%) |
|------------------------------|--------------------|--------------------|--------------------------|---------------------|-----------|
| Cultivate supernatant        | 114,516.78         | 57.29              | 1998.73                  | 1                   | 100       |
| Ammonium sulfate precipitation| 58,034.55          | 9.42               | 6156.67                  | 3.08                | 50.67     |
| Ultrafiltration              | 8329.20            | 0.94               | 8860.85                  | 4.43                | 7.27      |
| Nickel column purification   | 2445.44            | 0.26               | 9405.54                  | 4.71                | 2.14      |

As observed from the SDS-PAGE, the recombinant collagenase was efficiently purified. Its relative molecular mass was estimated to be 35.4 kDa (Figure 4).

![Figure 4. SDS-PAGE of the purified collagenase expressed by *B. subtilis* WB600/pP43NMK-col. M, molecular weight markers; lane 1, purified collagenase.](image-url)
3.4. Effect of pH, Temperature, and Metal Ions on the Stability of Collagenase

As presented in Table 3, the maximum collagenase activity was observed at pH 9.0. The enzyme was relatively stable during the pH treatment for 60 min, with approximately 60% of its activity retained in the pH range of 8–10 for 90 min (Figure 5a). This result is consistent with the collagenase activity from *Streptomyces parvulus* [29].

Table 3. Effect of temperature, pH, and metal ions on recombination collagenase activity.

| Parameters | Enzyme Activity (U/mL) |
|------------|------------------------|
| **Temperature (°C)** |                      |
| 30         | 1449.39 ± 50           |
| 40         | 1600.96 ± 74           |
| 50         | 2055.65 ± 61           |
| 60         | 1202.49 ± 68           |
| 70         | 1035.44 ± 71           |
| 80         | 716.02 ± 53            |
| **pH**     |                       |
| 5          | 1644.96 ± 56           |
| 6          | 850.47 ± 82            |
| 7          | 877.36 ± 53            |
| 8          | 1419.24 ± 76           |
| 9          | 2005.94 ± 69           |
| 10         | 1173.15 ± 64           |
| 11         | 680.16 ± 78            |
| **Metal ions** |                    |
| Control    | 1599.33 ± 25           |
| Fe³⁺       | 1235.90 ± 34           |
| Zn²⁺       | 1816.08 ± 51           |
| Co²⁺       | 2343.30 ± 39           |
| Ca²⁺       | 2542.94 ± 43           |
| Mg²⁺       | 1908.97 ± 37           |
| Cu²⁺       | 174.95 ± 45            |

Figure 5. (a) Effect of pH on the stability of recombinant collagenase. (b) Effect of temperature on the stability of recombinant collagenase.

Table 3 and Figure 5b illustrate the temperature effect on the collagenase activity. The optimum activity of the enzyme was observed at 50 °C, and about 75% of its activity was retained between 30 and 50 °C. The enzyme activity declined sharply when the temperature exceeded 50 °C. After 2 h of pre-incubation at different temperatures, collagenase activity decreased markedly after 40 min of incubation but showed stability in a temperature range of 30–50 °C, with more than 60% of its activity retained. However, pre-incubation above 60 °C substantially abrogated the enzyme activity, with less than 30% of its activity...
retained after incubation at 80 °C for 2 h. A similar result was observed with a thermophilic collagenase, which showed stability across a range of pH levels (7.0–8.5) and temperatures (40–60 °C), with an optimal pH of 8.0 and temperature of 60 °C [10]. The optimum collagenase activity of B. cereus was reported to be at a temperature of 45 °C and had a wide working ranges of pH values and temperatures (7.2–11.0 and 25–50 °C, respectively) [30]. Moreover, more than 50% of the collagenase activity remained after 10 min of incubation at 60 °C [31].

The effect of a few metal ions such as Fe\(^{3+}\), Zn\(^{2+}\), Co\(^{2+}\), Ca\(^{2+}\), Mg\(^{2+}\), and Cu\(^{2+}\) on the enzyme activity was evaluated at a final concentration of 5 mM (Table 3). The catalytic efficiency of the recombinant collagenase was inhibited by Fe\(^{3+}\) and Cu\(^{2+}\), but stimulated by Zn\(^{2+}\), Co\(^{2+}\), Mg\(^{2+}\), and Ca\(^{2+}\). The most notable inhibition was observed in the presence of Cu\(^{2+}\), while the biggest stimulation was observed in the presence of Ca\(^{2+}\). A similar result was observed in a Pseudoalleromonas agarivorans strain where the enzyme activity was strongly activated by Ca\(^{2+}\) [24], similar to that of a Bacillus pumilus Col-I [31]. This can be attributed to the collagenase structure (Figure 6) that shows high stability in the presence of Ca\(^{2+}\) owing to the formation of the collagen–Ca\(^{2+}\)–collagenase complex [24].

3.5. Optimization of Carbon Sources, Nitrogen Sources, Metal Ions for the Production of Collagenase by Recombinant B. subtilis WB600

Ten percent of different carbon sources were added to the fermentation medium, while the other components were not changed. After incubation by stirring for 48 h in the fermentation culture, the enzyme activity and mass were determined. As presented in Figure 7a, the best carbon source for collagenase production by B. subtilis was fructose, with a collagenase activity at 2237.78 U/mL. The recombinant B. subtilis grew better with the addition of glucose [32], showing that it could absorb glucose better and consume nutrients quickly, but with less enzyme production.

![Figure 6](https://swissmodel.expasy.org/) accessed on 6 May 2022. The gray ball represents zinc ion. Prediction and interpretation the zinc ion and acetate ion were through QM/MM optimization. Both the polarization by the catalytic site Zn301 and the interaction with the acetate moiety of Glu227 promote the elongation of the O-H bond of the Zn-ligated nucleophilic water [33]. (b) Spacefill representation of the collagenase (c) SWISS-MODEL matched template (SMRT ID: 5d88.1 The Structure of the U32 Peptidase Mk0906), U32 collagenase family. The interaction between zinc ions and residues is shown. According to the results of the experiment, it is also proved that Ca\(^{2+}\) and Zn\(^{2+}\) bind, which promotes the occurrence of enzymatic lysis and the improvement of enzymatic activity [24,34,35].

Determination of the optimum concentration of fructose was investigated by assaying various fructose concentrations ranging between 5 and 30 g/L at a pH of 7.5, temperature of 37 °C, and with shaking at 220 rpm (Figure 7b). The optimum fructose concentration was found to be 15 g/L, where collagenase activity was at a maximum (2158 U/mL). However, the cell growth was increased with an increasing starch concentration, reaching a maximum at 25 g/L.

In summary, in view to optimize both the collagenase activity and bacterial growth, the optimal concentration of fructose was selected at 15 g/L, and was used for subsequent studies.
Figure 7. Optimization of the growth media for recombinant expression of collagenase in B. subtilis WB600. The histogram represents the activity of recombinant collagenase and the line represents the bacterial growth. (a) Effect of carbon sources on the enzyme activity and cell growth. (b) Effect of concentration of fructose on the enzyme activity and cell growth. (c) Effect of nitrogen sources on the enzyme activity and cell growth. 1, beef paste; 2, ammonium sulphate; 3, yeast powder; 4, peptone; 5, urea; 6, gelatin; 7, mixture of peptone and yeast powder. (d) Effect of the concentration of nitrogen source on the enzyme activity and bacterial growth. (e) Effect of metal ions on the enzyme activity and cell growth. 1, Zn$^{2+}$; 2, Mg$^{2+}$; 3, Na$^{2+}$; 4, Ca$^{2+}$. (f) Effect of concentration of the Ca$^{2+}$ metal ion on enzyme activity and bacterial growth. The data are presented as mean ± standard deviation from three independent experiments.

To investigate the influence of nitrogen sources on the expression of recombinant collagenase by B. subtilis, the maximum collagenase activity (531.86 U/mL) was observed when peptone was used together with yeast powder; the bacterial growth increased slightly. As shown in Figure 7c, when yeast powder was added alone, the enzyme activity was relatively low, but the cell growth was high. This could have been due to the yeast powder being conducive to the growth of B. subtilis but less promotive of enzyme production.

The yeast powder and peptone were mixed in a ratio of 2:1, and Figure 7d shows the effects of different yeast and peptone concentrations (6–42 g/L) on the expression of collagenase by B. subtilis. Bacterial growth was increased at a concentration of 18 g/L. The best mixture concentration for optimal collagenase activity was 36 g/L.

Figure 7e shows that recombinant B. subtilis had the highest bacterial growth and collagenase enzyme activity (1926.9 U/mL) when Ca$^{2+}$ was added, which shows that
Ca²⁺ can promote the growth of bacteria and retain the optimal activity of collagenase. Different concentrations (1–30 g/L) of Ca²⁺ were added to the fermentation medium; the recombinant B. subtilis grew well and expressed collagenase at its highest when the concentration of Ca²⁺ was 10 g/L. This further confirms that Ca²⁺ affects the cell growth and expression potential of B. subtilis (Figure 7f).

3.6. Factors Affecting the Recombinant Production of Collagenase by B. subtilis WB600

The initial pH, rotational speed, temperature, and percentage of inoculation are process variables that affect the growth of bacteria and enzyme production. These variables were separately studied in order to evaluate their effects on collagenase production (Figure 8).

![Figure 8. Optimization of the expression conditions for producing collagenase in B. subtilis WB600. Histogram, the activity of recombinant collagenase. Line, bacterial cell growth. (a) Effect of initial pH on the collagenase activity and bacterial cell growth. (b) Effect of agitation speed. (c) Effect of expression temperature. (d) Effect of inoculation percentage. The data are presented as mean ± standard deviation from three independent experiments.](image)

3.6.1. Effect of Initial pH

The pH affects the growth of microbial cells and the protein expression yield [36]. The effect of the initial pH on the production of collagenase by B. subtilis was studied at 37 °C in the presence of 15.0 g/L of fructose and 36 g/L of a yeast and peptone mixture, and shaken at 220 rpm. Different initial pH levels ranging from 5.0 to 9.0 were studied. As indicated in Figure 8a, the cell growth was maximized at a pH of 9.0. However, the maximum enzyme activity (2391.4 U/mL) was obtained at pH 7.0. This is in agreement with the predicted pH for maximum collagenolytic activity at pH 7.21 [37]. In this context, pH 7.0 was selected for further experiments (Table 3).

3.6.2. Effect of Agitation Speed

Agitation speed is important for microorganism growth and the expression of collagenase during fermentation. Oxygen is required by bacteria to grow well and, therefore, to be able to express collagenase. Agitation of the medium ensures that the medium is well aerated. The effects of different agitation speeds (200–280 rpm) on B. subtilis growth and collagenase production were examined at 37 °C in 15.0 g/L of fructose and a 36 g/L of peptone and yeast mixture at a pH of 7.0. As depicted in Figure 8b, the highest collagenase
activity (2746.7 U/mL) was reached when the agitation speed was 260 rpm. However, the highest cell growth of recombinant *B. subtilis* was reached at 280 rpm. These results suggest that a high agitation speed promoted fast growth of the recombinant *B. subtilis*, but the higher shear force caused by the violent shaking and the over accumulation of metabolites could also negatively affect enzyme production [35,38]. The agitation speed for future experiments was taken at 260 rpm because maximum collagenase activity was achieved at that speed.

3.6.3. Effect of Temperature on the Cell Growth and Expression of Collagenase

Temperature is one of the most important factors to be considered because it affects the enzyme activity, cell growth, and expression of the enzyme [39]. Different temperatures (25–45 °C) were evaluated for their effects on collagenase activity and bacterial cell growth in 15.0 g/L of fructose and 36 g/L of a peptone and yeast mixture at pH 7.0, with shaking at 260 rpm. The maximum collagenase activity (2444.3 U/mL) and cell growth were reached at 35 °C (Figure 8c). Moreover, the bacterial growth increased significantly with the increase in temperature, particularly from 25 °C to 35 °C. However, with a further increase in temperature, the collagenase activity markedly decreased. A possible reason for the decrease in collagenase activity at a high temperature could be attributed to a decrease in the transport of intermediate metabolites to the cells [40]. Another reason could be that the enzyme was denatured at temperatures higher than 35 °C.

3.6.4. Effect of the Percentage of Inoculation

The percentage of inoculation has a significant effect on the yield of recombinant protein expression by bacteria. A small amount of inoculation will affect the cell growth of recombinant strains and will thus affect the enzyme production during the logarithmic growth period of the bacteria. A small percentage of inoculation will increase the consumption of nutrients, and it will be difficult to meet the needs of the growing recombinant strain in a short period of time.

To evaluate the effect of inoculation amount on the activity of collagenase, six different inoculation amounts ranging from 5% to 15% (v/v) were set up under the experimental situations of 35 °C, pH 7.0, and 260 rpm. As shown in Figure 8d, it can be concluded that different inoculation percentages have different effects on the recombinant expression of collagenase. The enzyme activity is the highest at 11% inoculation, but the cell growth is low, which may be due to the large number of bacteria but limited nutrients, affecting the cell growth but maximizing the use of the nutrients for collagenase production.

4. Conclusions

In this study, the collagenase encoding gene *col* was successfully expressed in *B. subtilis* WB600 under the pP43NMK vector. The collagenase sequence contains an open reading frame of 936 bp, which encodes a protein of 312 amino acid residues with a predicted molecular mass of 35.4 kDa. The recombinant enzyme was purified by ammonium sulfate, ultrafiltration, and nickel column with a collagenase activity of 9405.54 U/mg. The enzyme exhibited maximal activity at pH 9.0 and 50 °C. Catalytic efficiency of the recombinant collagenase was inhibited by Fe$^{3+}$ and Cu$^{2+}$, but stimulated by Co$^{2+}$, Ca$^{2+}$, Zn$^{2+}$, and Mg$^{2+}$. The optimal conditions for expression were at pH 7.0 and 35 °C, using 15 g/L of fructose and 36 g/L of yeast powder and peptone mixture (2:1) at 260 rpm with 11% inoculation. The maximal extracellular activity of collagenase reached 2746.7 U/mL after culturing in the optimization culture conditions, which was 2.4-fold higher than before optimization. This study is a first attempt to express recombinant collagenase and to optimize its production for possible scale-up.
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