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Characterization and overexpression of a novel keratinase from *Bacillus polyfermenticus* B4 in recombinant *Bacillus subtilis*

Yu-Ze Dong[1], Wen-Shous Chang[1,2] and Po Ting Chen[1*]

Abstract

Background: Keratins, insoluble proteins with a robust structure, are a major component of epidermal tissue and appendages such as hair, feathers, nails, and walls. Keratinous waste mainly emanates from poultry and leather industries, thereby severely contaminating the environment. Keratinase can lyse proteins with robust cross-linked structures, such as keratin, and can hence be used in animal feed, fertilizer, detergent, leather, pharmaceutical, and cosmetic industries. *Bacillus polyfermenticus* B4, isolated from feather compost, secretes keratinase to metabolize feathers. Hence, this study aimed to investigate the enzymatic characteristics and recombinant production of keratinase from *B. polyfermenticus* B4.

Methods: A novel keratinase KerP was isolated from *B. polyfermenticus* B4 and overexpressed in *B. subtilis* PT5, via the T7 promoter.

Results: The highest keratinolytic activity of recombinant KerP was observed at pH 9.0 and 60 °C. Enzyme activity was enhanced with Fe^{2+}, Mn^{2+}, and SDS, and inhibited by Zn^{2+}, Ni^{2+}, EDTA, PMSF, and β-mercaptoethanol. KerP production was the highest at 473 ± 20 U/mL with *B. subtilis aprE* signal peptide using LB broth.Conclusions: The novel keratinase KerP has potential industrial applications, particularly in the treatment of keratinous waste.

Keywords: Keratinase, *Bacillus polyfermenticus*, Keratinous waste, Characterization, Overexpression, *Bacillus subtilis*

Background

Keratin is an essential protein constituent of the animal epidermis and associated integuments such as feathers, hair, nails, scales, horns, and hooves. It is an insoluble protein and can be classified into two types, based on the content of disulfide bonds: soft and hard (Coulombe and Omary 2002). Soft keratin has low disulfide bond content and a soft and flexible texture, and is present in skin and healing tissue, whereas hard keratin has high disulfide bond content and a hard and less flexible texture, and is present in feathers, nails, and horns (Coulombe and Omary 2002). Keratins in the hair or wool primarily contain α-helices, whereas those in feathers or nails primarily contain β-sheets (Onifade et al. 1998; Schrooyen et al. 2001; Wang and Parsons 1997). Cross-linking in keratin fibers occurs through disulfide bonds, hydrogen bonds, and hydrophobic interactions. General proteases such as trypsin and pepsin cannot degrade keratin (Gupta and Ramnani 2006; Kreplak et al. 2004; Olajuyigbe and Falade 2014).

Currently, keratinous wastes from poultry, fur, leather, and slaughter industries are gradually causing serious environmental problems (Korniłłowicz-Kowalska and Bohacz 2011; Onifade et al. 1998). Many attempts have been made to convert keratinous waste to smaller debris or protein hydrolysates through physical and chemical treatments (Gupta et al. 2013), which can then be used as nutrient additives in animal feed, agriculture, and cosmetic industries (Bhange et al. 2016). However, the conventional treatment methods could deteriorate the quality and stability of the hydrolyzed products, adding

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to environmental concerns such as strong acidification or alkalinization of water through the generated waste.

Keratinases are proteases that can particularly lyse recalcitrant proteins such as keratin (Onifade et al. 1998). Keratinases have been reported in many microorganisms such as bacteria, actinomycetes, and fungi (Bhange et al. 2016). Moreover, KerA, isolated from *Bacillus licheniformis* PWD-1, is the most well-characterized keratinase (Lin et al. 1995; Onifade et al. 1998). Keratinases bind to and cleave keratins at their hydrophobic sites. Based on their catalytic mechanism, most keratinases are either metalloproteases or serine-metalloproteases (Brandelli 2008; Bressollier et al. 1999).

Microorganisms isolated from keratin-rich environments such as poultry feather wastes, slaughterhouse wastes, and leather wastes, have high keratinolytic activity (Brandelli et al. 2010; Fakhfakh-Zouari et al. 2010; Khardenavis et al. 2009). Most of the keratinases from these strains are extracellular enzymes, with only a minute fraction being intracellular or associated with the cell wall. Because of their unique characteristics, keratinases have potential applications in industrial fields such as stock farming, poultry feather treatment, leather depilation, laundry detergents, and cosmetics. However, large-scale production of keratinase is essential for its application at the industrial level.

Previous studies have used various bacterial expression systems for keratinase production (Lin et al. 1997; Liu et al. 2014; Radha and Gunasekaran 2008). *Bacillus subtilis* can efficiently secrete native or heterologous proteins into the culture broth, which renders it an attractive strain for enzyme production at an industrial level. Moreover, *B. subtilis* is a generally recognized as safe (GRAS) bacterium, with promising application in protein requirement in food and in the clinical setting (de Boer Sietske and Diderichsen 1991; Harwood 1992; Pohl and Harwood 2010; Schallmey et al. 2004; Westers et al. 2004). Signal peptides play a very important role in protein secretion in *B. subtilis* (Brockmeier et al. 2006; Tjalsma et al. 2004); a suitable signal peptide could significantly enhance target protein secretion (Brockmeier et al. 2006; Li et al. 2004).

In this study, a keratinolytic bacterium *Bacillus polyfermenticus* B4 was isolated from soil feather composts in a chicken farm in Tainan and a novel keratinase KerP was isolated and cloned into *B. subtilis* PT5 for heterologous production. Furthermore, the biochemical characteristics and catalytic mechanism of KerP were investigated. Moreover, enzyme production in *B. subtilis* was evaluated by fusing the mature KerP polypeptide with different signal peptides.

Methods

Isolation and screening of keratinolytic microorganisms from feather composts

To isolate keratinolytic microorganisms, soil samples from feather composts were extracted from six areas (sample A to F) of a poultry farm in Tainan, Taiwan. Each 5-g sample was suspended in 100 mL feather defined medium (defined medium containing 10 g of feathers) in a 500-mL shake flask and incubated at 37 °C at 150 rpm. After 2 days of incubation, the feathers were disrupted in the culture medium containing sample B, and after 12 days of culturing, the feathers were decomposed to small debris, except for some broken rachis. Thereafter, 0.1 mL of diluted culture broth (10^{-6}) containing sample B was spread on a plate containing defined medium and 1% skimmed milk. The halo-forming colonies thus formed were considered potential candidates for keratinase production, and inoculated into feather defined medium at 37 °C at 150 rpm. Furthermore, 16S rDNA sequencing was performed with primers 27F and 1497R (Kim et al. 2000) to identify the microorganisms.

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. Gene cloning was performed in *E. coli* DH5α and *B. subtilis* strain PT5 containing the T7 RNA polymerase for gene expression driven by the T7 promoter (Chen et al. 2010). For DNA manipulation, *E. coli* were grown in 10 mL of Luria–Bertani (LB) medium in a 125-mL shake flask, and cell densities were determined through the optical density at 600 nm (OD_{600}). For keratinase production, recombinant *B. subtilis* strains were cultured overnight at 37 °C and inoculated into a 250-mL shake flask containing fresh LB medium (30 mL) with cell density of $OD_{600} = 0.1$, and then incubated in an orbital shaker at 200 rpm and 37 °C for 24 h. For the selection of *E. coli* harboring plasmids or *B. subtilis* integrants, the medium was supplanted with antibiotics ampicillin (50 μg/L for *E. coli*) and erythromycin (10 μg/L for *B. subtilis*).

Construction of expression vectors for keratinase secretion

The vectors and primers used in this study are listed in Tables 1 and 2, respectively. The full-length keratinase gene *kerP* was amplified from *B. polyfermenticus* B4 through polymerase chain reaction (PCR) with primers KP01 and KP02. The PCR product of *kerP* (0.802 kb) was digested with *Nru*I/*Bam*HI and ligated with the integration vector pDMT to generate pDMT-KerP.

The PCR product of DMT-mKerP0102 (5.033 kb) was amplified from vector pDMT-KerP designed to eliminate the *kerP* signal peptide sequence, which was amplified

Table 1 Strains and plasmids used in this study

	Relevant characteristics	Source
Strain		
E. coli		
DH5α	*deoR endA1 gyrA96 hsdR17 supE44 thi1 recA1 lacZM15*	Lab. collection
B. subtilis		
PT5	DB428, Δ*wpr::T7gene 1*	
PT5(DMT-KerP)	PT5, Δ*mpr*:: P_{T7}/*kerP*-erm	This study
PT5(DMT1-mKerP)	PT5, Δ*mpr*:: P_{T7}/*aprE* SP-m*kerp*-erm	This study
PT5(DMT7-mKerP)	PT5, Δ*mpr*:: P_{T7}/*xynD* SP-m*kerp*-erm	This study
PT5(DMT8-mKerP)	PT5, Δ*mpr*:: P_{T7}/*bglS* SP-m*kerp*-erm	This study
PT5(DMT12-mKerP)	PT5, Δ*mpr*:: P_{T7}/*mpr* SP-m*kerp*-erm	This study
PT5(DMT16-mKerP)	PT5, Δ*mpr*:: P_{T7}/*xynA* SP-m*kerp*-erm	This study
PT5(DMT21-mKerP)	PT5, Δ*mpr*:: P_{T7}/*abnA* SP-m*kerp*-erm	This study
Plasmid		
pDMT	Integration vector contains by P_{T7} and *mpr′*	
pDMT-KerP	pDMT with full-length *kerP* gene	This study
pDMT1-mKerP	*aprE* SP instead of *kerP* SP in pDMT-KerP	This study
pDMT7-mKerP	*xynD* SP instead of *kerP* SP in pDMT-KerP	This study
pDMT8-mKerP	*bglS* SP instead of *kerP* SP in pDMT-KerP	This study
pDMT12-mKerP	*mpr* SP instead of *kerP* SP in pDMT-KerP	This study
pDMT16-mKerP	*xynA* SP instead of *kerP* SP in pDMT-KerP	This study
pDMT21-mKerP	*abnA* SP instead of *kerP* SP in pDMT-KerP	This study

erm, selection marker of erythromycin resistant; *kerp*, keratinase KerP full-length gene; m*kerP*, keratinase KerP mature sequence; P_{T7}, T7 promoter; SP, signal peptide

by PCR with primers DMKP01 and 02 and plasmid pDMT-KerP as a template. Six signal sequences were amplified from the *B. subtilis* chromosome, using specific primer pairs. The PCR products of signal sequences were digested with *Xho*I/*Bam*HI and cloned into the PCR product DMT-mKerP0102 to generate the six integration vectors (Table 1).

Polymerase chain reaction was carried out using Q5 DNA polymerase (New England BioLabs) per the following protocol: pre-denaturation at 98 °C for 30 s, 25–30 cycles of DNA amplification during amplification 98 °C for 10 s, annealing at 60 °C for 10 s, elongation at 2 kb/min at 72 °C; and a final extension step at 72 °C for 5 min. The generated vectors were integrated into *B. subtilis* PT5 chromosome through double homologous recombination to generate KerP-producing strains.

Analytical methods

The keratinolytic activity assay protocol developed previously (Vermelho et al. 2010) was performed in this study, with minor modifications. The 0.1-mL cell-free culture broth was added to a 0.4-mL reaction mixture containing 0.05 M Glycine–NaOH buffer (pH 9.0) and 10-mg feather powder (purchased from an agricultural materials store in Tainan, Taiwan). The reaction was carried out at 60 °C for 1 h and terminated by adding 20% trichloroacetic acid (0.5 mL). After centrifugation for 10 min at 4 °C, 14,000 rpm, the optical density of the supernatant was determined at 280 nm. One unit of keratinolytic activity (one unit) was defined as the amount of enzyme required to increase the A_{280} by 0.01.

The cell-free culture broth was harvested through centrifugation for 10 min at 4 °C, 14,000 rpm for evaluating keratinase activity or production via SDS-PAGE on a 10% (w/v) polyacrylamide gel, as described previously (Chao et al. 2002).

Temperature and pH dependence

The optimal pH and temperature for KerP activity were determined using buffers of pH 5–12 at 30–90 °C. Different buffers were selected to cover a range of pH value by using Mcilvaine (pH 5–6), Tris–HCl (pH 7–8), and Glycine–NaOH (pH 9–12) buffers. Enzyme activity was analyzed as described above. To estimate the pH stability of KerP, the enzyme was incubated for different durations at 4 °C. The thermal stability was determined by measuring the residual activity of the enzyme solution incubated at 30–90 °C for 10–60 min.

Batch fermentation

A 5-L fermenter was used for batch fermentation of recombinant *B. subtilis*. After overnight fermentation, the

Table 2 Primers used in this study

	Relevant characteristics	Source
Primer		
27F	AGAGTTTGATCCTGGCTCAG	
1492R	AAGTCGTAACAAGGTAACC	
BP01	GTGAGAGGCAAAAAGGTATG	
BP02	TTACTGAGCTGCCGCCTG	
KP01	ATCGTATCGCGAGTGAGAGGCAAAAAGGTATG (*Nru*I)	
KP02	CTATGAGGATCCTTACTGAGCTGCCGCCTG (*Bam*HI)	
DMKP01	TGACTATCGCGATTACCCTCTCCTTTTAAAAAATC (*Nru*I)	
DMKP02	TATGCTCTCGAGGGCAGGGAAATCAAACGGGGA (*Xho*I)	
aprE01	AGAGGGTCGCGAGTGAGAAGCAAAAAATTGTGG (*Nru*I)	*aprE* SP
aprE02	GATCGACTCGAGGCAGCCTGCGCAGACATGTTG (*Xho*I)	*aprE* SP
xynD01	ATGCATTCGCGAATGAGGAAAAAGTGTAGCGT (*Nru*I)	*xynD* SP
xynD02	TAGCTACTCGAGGCAGCATACGCAGACTTCCCAG (*Xho*I)	*xynD* SP
bglS01	ATGCATTCGCGAATGCCTTATCTGAAACGAGTG (*Nru*I)	*bglS* SP
bglS02	TAGCTACTCGAGGCAGCTGAGGCAGTAGCAGTGA (*Xho*I)	*bglS* SP
mpr01	GGAAGTTCGCGAATGAAATTAGTTCCAAGATTCAG (*Nru*I)	*mpr* SP
mpr02	TAGCTACTCGAGGCCGCTTTTGCCGGTACGCCA (*Xho*I)	*mpr* SP
xynA01	ATGCATTCGCGAATGTTTAAGTTTAAAAAGAATTTC (*Nru*I)	*xynA* SP
xynA02	TAGCTACTCGAGGCTGCAGAGGCGGTTGCCGAA (*Xho*I)	*xynA* SP
abnA01	GATGACTCGCGAATGAAAAAGAAAAAAACATGG (*Nru*I)	*abnA* SP
abnA02	TAGCTACTCGAGGCTGCCTCTGCGGGAGCAG (*Xho*I)	*abnA* SP

cell culture was inoculated into three 500-mL shake flasks containing 200 mL fresh LB medium, with the initial cell density adjusted to 0.1 (OD_{600}). After 2 h of culturing, cultures from the three shake flasks were transferred into the fermenter. The final volume in the fermenter was adjusted to 3 L and the initial cell density was adjusted at 0.1 (OD_{600}). The working condition was set at 37 °C; initial pH, 7.0; air flow, 1.5 vvm; and dissolved oxygen concentration (maintained through agitation), 20%.

Results and discussion

Isolation and screening of keratinolytic microorganisms from feather composts

After 12 days of culturing in the feather meal medium, complete decomposition of feathers was observed only in flask B4. The results indicated that only the B4 strain could produce keratinase, while the other strains produced general proteases. Furthermore, 16S rDNA sequencing (Kim et al. 2000) performed using strain B4, followed by sequence analysis by National Center for Biotechnology Information (NCBI) indicated that B4 strain showed 99.8% sequence similarity to *B. polyfermenticus*. Hence, the B4 strain was identified as *B. polyfermenticus* B4.

Keratinase isolation from *B. polyfermenticus* B4 and production of recombinant keratinase

The protein sequence of the commercially available keratinase KerA from *B. licheniformis* PWD-1 (Lin et al. 1995) was compared with that of *B. polyfermenticus* through a protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). KerA showed 65% similarity to alkaline protease precursor (GeneBank: ADP23919.1), using the protein database of *B. polyfermenticus*. This suggested that the alkaline protease precursor of *B. polyfermenticus* maybe attributed to keratinase and also existed in *B. polyfermenticus* B4. Two primers, BP01 and BP02, were designed for *B. polyfermenticus* SCN11 alkaline protease precursor (GeneBank: HQ436407.1). The single DNA fragment BP0102 was amplified through PCR with primers BP01–BP02 and *B. polyfermenticus* B4 chromosome as a template. The PCR product BP0102 was cloned into the plasmid pBluescript II SK(−) digested with *Eco*RV to generate pBlue-BP0102, which was then subjected to DNA sequencing (Tri-I Biotech, Inc., New Taipei City) with T7 and T3 primers. The DNA fragment was translated to the corresponding polypeptide in silico and a BLASTX was performed to compare the translated sequence with alkaline protease precursor protein. The result was showed in Additional file 1: Figure S1 revealed a 99% similarity with alkaline protease precursor (GeneBank: ADP23919.1). Therefore, this gene fragment was putatively referred to as KerP.

The KerP gene was amplified through PCR with primers KP01 and KP02 and then cloned into the integrant plasmid pDMT to generate pDMT-KerP, using *Nru*I and *Bam*HI. The plasmid pDMT-KerP was integrated into the *B. subtilis* PT5 chromosome, resulting in the new KerP-expressing strain PT5(DMT-KerP). This strain formed a clear halo in the LB plate containing skim milk. Moreover, this recombinant strain could digest the feathers completely in feather defined medium after 7 days of incubation. This proved that the *kerP* gene encodes a keratinase.

Effect of pH and temperature on KerP activity

With feather powder as the substrate, the highest KerP activity was observed at pH 9.0, (Fig. 1a) and the enzyme retained complete activity at pH 7–8 after 48 h of storage

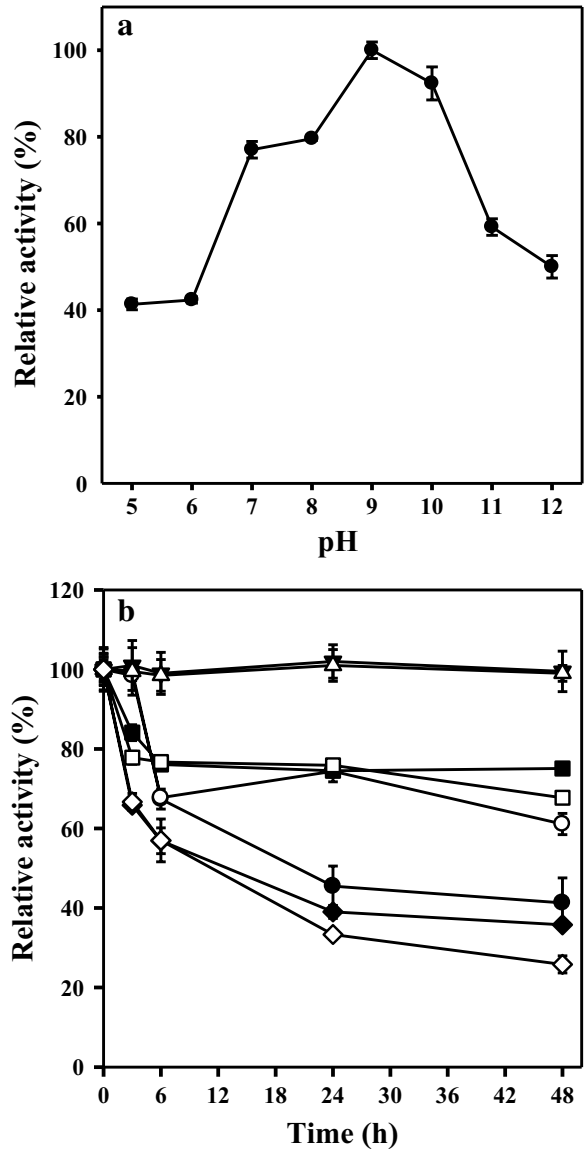

Fig. 1 **a** Effect of pH on KerP activity. **b** pH stability of KerP. Enzyme activity was preserved at various pH levels between 0 and 48 h before assaying enzyme activity. pH 5.0 (filled circle), pH 6.0 (open circle), pH 7.0 (filled inverted triangle), pH 8.0 (open triangle), pH 9.0 (filled square), pH 10.0 (open square), pH 11.0 (filled diamond), pH 12.0 (open diamond). The enzyme activity was assayed with feather powders as the substrate at 60 °C for 1 h

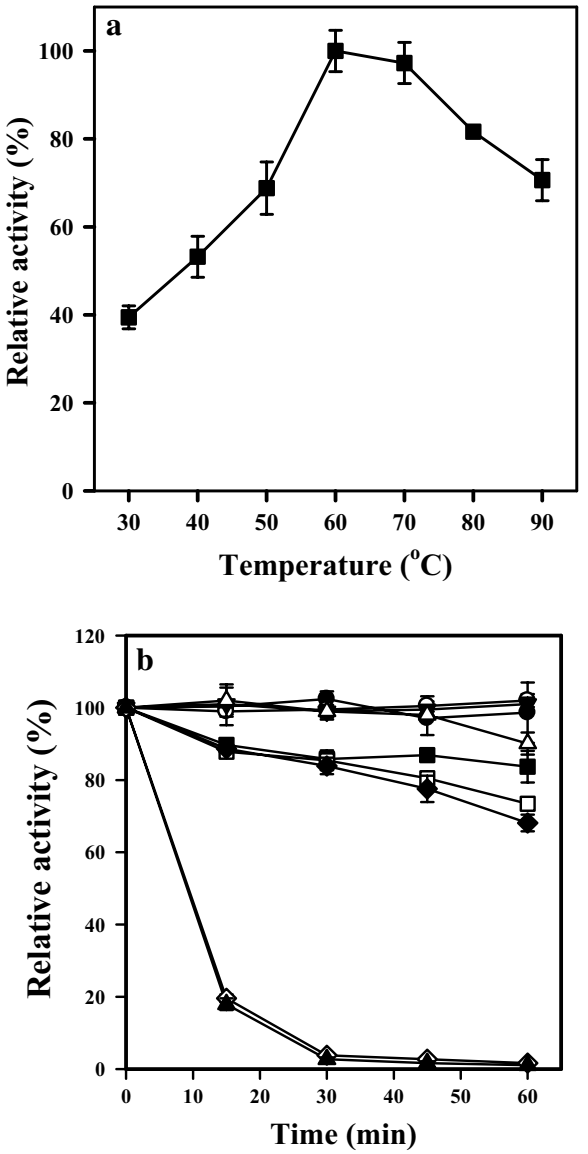

Fig. 2 **a** Effect of temperature on KerP activity. **b** Temperature stability of KerP. Enzyme activity was preserved at various temperatures between 0 and 60 min before assaying enzyme activity. 30 °C (filled circle), 40 °C (open circle), 50 °C (filled inverted triangle), 60 °C (open triangle), and 70 °C (filled square) for 60 min. The enzyme activity was assayed with feather powders as the substrate at pH 9.0 for 1 h

at 4 °C. However, enzyme activity was less than 50% after 48 h at pH 5, 11, 12 (Fig. 1b). The results showed that the optimal pH is 9.0 but the enzyme is stable at pH 7–8.

On incubation at pH 9.0, KerP exhibited the maximum activity at 60 °C (Fig. 2a). Enzyme activity was relatively stable below 60 °C after 60 min of pretreatment and declined rapidly beyond 80 °C (Fig. 2b). The optimal pH and temperature of the commercial keratinase KerA from *B. licheniformis* PWD-1 were pH 7.5 and 50 °C, respectively. Keratinase KerP is similar to other bacterial keratinases in optimal conditions (Sangali and Brandelli 2000; Sookkheo et al. 2000; Zhang et al. 2016). Moreover, the stability of KerP suggested potential industrial applications.

Effects of chemical compounds on KerP activity

The effects of metal ions, inhibitors, and surfactants on KerP activity were investigated (Table 3). Enzyme activity

Table 3 Effect of chemicals on KerP activity

Chemicals	Concentrations	Relative activity (%)
Control	–	100 ± 2
$CaCO_3$	10 mM	125 ± 2
$MgSO_4$	10 mM	151 ± 1
$MnSO_4$	10 mM	158 ± 2
$FeSO_4$	10 mM	308 ± 2
$ZnSO_4$	10 mM	60 ± 1
$NiSO_4$	10 mM	15 ± 1
EDTA	1 mM	47 ± 1
PMSF	1 mM	8 ± 1
SDS	1%	279 ± 2
β-mercaptoethanol	1%	31 ± 1
Triton X-100	1%	109 ± 5
Tween-80	1%	100 ± 1

was enhanced by Fe^{2+} and SDS but significantly inhibited by Ni^{2+}, Zn^{2+}, EDTA, PMSF, and β-mercaptoethanol. Because KerP was strongly inhibited by EDTA (relative activity 47%) and PMSF (relative activity 8%), it may be a serine protease or a serine-metalloproteinase (Brandelli 2008). Moreover, the detergent SDS enhanced the enzyme activity by 2.79 folds, thereby suggesting that KerP could resist the detergent and that the detergent could increase the surface contact between the feathers and the enzyme. Compared to other keratinases (Su et al. 2017a; Zhang et al. 2016), KerP has higher potential for application in commercial cleaning or bath products that contain SDS.

Enhancement of the KerP secretion in *B. subtilis*

Various bacterial expression systems were utilized for keratinase production, and *B. subtilis* was a better and more efficient host for keratinase production (Haddar et al. 2009; Lin et al. 1997; Liu et al. 2014; Zaghloul et al. 2011). All of them used the expression vectors to heterologously produce keratinase in *B. subtilis*. However, the expression plasmid used in *B. subtilis* would lead to problems of plasmid instability, which could reduce enzyme production, thereby interfering with enzyme production at the industrial level. The segregational and structural instability of *B. subtilis* plasmids are the two major, clearly described problems (Chen et al. 2007). To overcome these problems, researchers used theta mode plasmids that provide complete structural stability to express the enzyme. However, this type of plasmid still has the problem of segregational instability. The segregational instability of *B. subtilis* plasmids can be stabilized through the addition of selection marker (antibiotics or the deficiency components of amino acids) in the culture medium; however, this would increase the cost of fermentation and affect the environment in the industrial fermentation process. For industrial enzyme production, strain stability is critical for mass production of proteins of interest. Moreover, the enhancement of secretion of the recombinant protein is also a pivotal point in the *B. subtilis* expression system. For secretion, proteins need to be guided by the signal peptide. The appropriate signal peptide could enhance the secretion efficiency even though the original signal peptide may not be the best signal peptide (Chen et al. 2015). Therefore, screening of the signal peptide must be considered during the construction of the expression vector for the host.

The signal peptide sequence of KerP was compared with those in a *B. subtilis* database in our laboratory, and six different signal peptide candidates were amplified through PCR and replaced with the KerP original signal peptide on the vector pDMT-BpKer to increase keratinase secretion. The vectors were integrated into the *B. subtilis* PT5 chromosome to determine the keratinolytic activity of KerP. Results showed that the keratinase production was greater in three strains, PT5(DMT01-mKerP), PT5(DMT06-mKerP), and PT5(DMT12-mKerP), than in PT5(DMT-KerP) (Fig. 3). The highest production of KerP (473 ± 20 U/mL) was observed in PT5(DMT01-mKerP); its keratinase production was 1.55 times that of PT5(DMT-KerP), which used the original signal peptide of KerP, thereby indicating that the original signal peptide of KerP from *B. polyfermenticus* B4

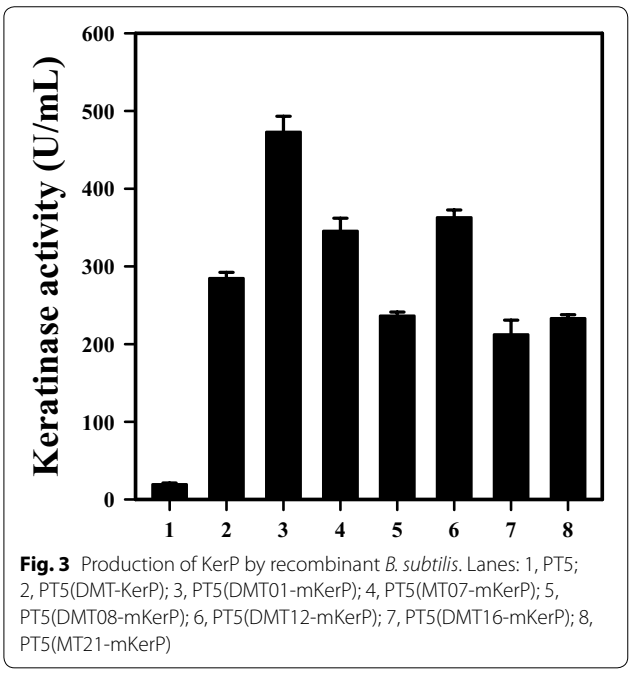

Fig. 3 Production of KerP by recombinant *B. subtilis*. Lanes: 1, PT5; 2, PT5(DMT-KerP); 3, PT5(DMT01-mKerP); 4, PT5(MT07-mKerP); 5, PT5(DMT08-mKerP); 6, PT5(DMT12-mKerP); 7, PT5(DMT16-mKerP); 8, PT5(MT21-mKerP)

could not result in the highest production of KerP in *B. subtilis*.

Batch fermentation of KerP-producing *B. subtilis*

Batch fermentation of PT5(DMT1-mKerP) was executed in a 5-L fermenter for producing KerP. Because of the characteristics of *B. subtilis* (Chen et al. 2007; Su et al. 2017b), the pH was not regulated during fermentation. KerP production was gradually increased with an increase in fermentation time. Keratinolytic activity was 467 ± 26 U/L (81.7 ± 5.6 mg/L) after 24 h of fermentation (Fig. 4a and b). The enzyme production through fermentation was similar to that through shake flask cultivation. Compared with that in previous studies (Chen et al. 2007, 2015; Su et al. 2017b), the yield of keratinase was the highest when using LB broth as the fermentation medium.

Conclusions

The keratinolytic strain *B. polyfermenticus* B4 was isolated from a poultry farm in Tainan, Taiwan, and its keratinase KerP was successfully cloned and expressed in *B. subtilis* PT5. Biochemical analysis revealed that the maximum keratinolytic activity of recombinant KerP was achieved at pH 9.0 and 60 °C. Enzyme activity could be enhanced with Fe^{2+}, Mn^{2+}, and SDS, and inhibited by Zn^{2+}, Ni^{2+}, EDTA, PMSF, and β-mercaptoethanol. The highest production of KerP was possible with the use of *B. subtilis aprE* signal peptide (Fig. 4a and b). KerP has potential applications in industry and in the treatment of

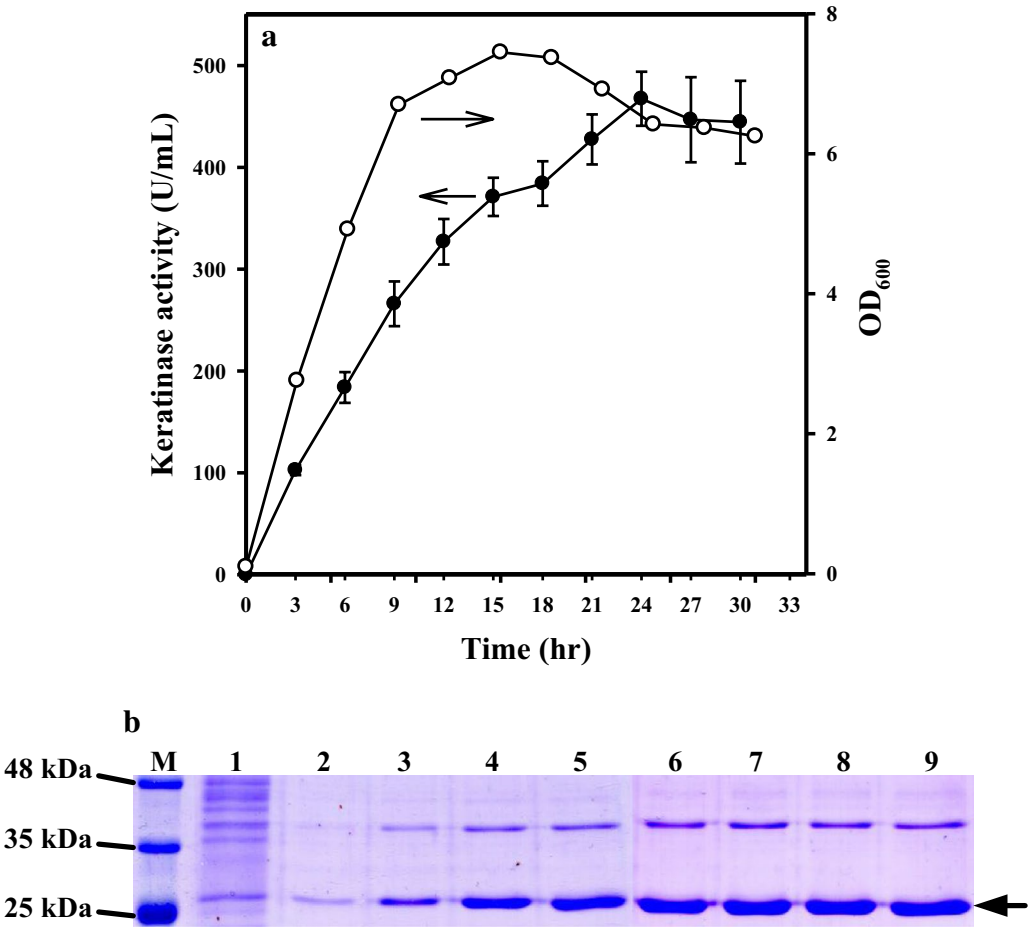

Fig. 4 **a** Batch fermentation of recombinant KerP-producing strain PT5(DMT01-mKerP) in a 5-L fermenter. Cell density was determined during fermentation (closed circles) at OD_{600}, and the production of KerP was assayed through measurement of keratinolytic activity (open circles). **b** SDS-PAGE analysis of KerP secreted by recombinant *B. subtilis* PT5(DMT01-mKerP). Fifteen microliters of culture broth from different fermentation periods was drawn out for PAGE. Lanes M, protein marker; lane 1, the culture broth of PT5 after 24-h fermentation; the culture broth of strain PT5(DMT01-mKerP) after fermentation for lane 2, 3 h; lane 3, 6 h; lane 4, 9 h; lane 5, 12 h; lane 6, 15 h; lane 7, 18 h; lane 8, 21 h; and lane 9, 24 h. The arrow indicates the position of KerP

keratinolytic waste. Future studies are required to standardize media composition and investigate scale-up fermentation strategies.

Additional file

Additional file 1: Figure S1. BLAST results of KerP and *Bacillus polyfermenticus* alkaline protease precursor (ADP23919.1). The data revealed 99% homology between KerP and ApBp. The amino acid residues at positions 1, 26, 68, and 103 are different (red words).

Abbreviations

erm: selection marker of erythromycin resistant; *kerp*: keratinase KerP full-length gene; m*kerP*: keratinase KerP mature sequence; P_{T7}: T7 promoter; SP: signal peptide.

Authors' contributions

PT designed the experiments, analyzed the data, and wrote the manuscript. YZ conducted most of the experiments, and WS provided the experimental materials and performed a few experiments. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in the main manuscript file.

Consent for publication

The authors approved the consent for publishing the manuscript.

Ethics approval and consent to participate

All authors have read and agreed the ethics for publishing the manuscript.

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