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Full Length Research Paper

Purification and biochemical characterization of a serine alkaline protease TC4 from a new isolated *Bacillus alcalophilus* TCCC11004 in detergent formulations

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An extracellular alkaline protease producing strain was isolated from alkaline soil and identified as *Bacillus alcalophilus* TCCC11004 on the basis of 16S rDNA gene sequencing and biochemical properties. The most appropriate medium for the protease production was composed of (g/l): maltodextrin 110, yeast extract 17.5, cotton seed meal 29.3, K_2HPO_4 18, trisodium citrate 3.3 and $CaCl_2$ 2.6. The alkaline protease TC4 was purified from the culture supernatant by ammonium sulfate precipitation, Sephadex G-75 gel filtration and SP-Sepharose HP ion exchange chromatography, with a 6.8 fold increase in specific activity and 15.2% recovery. The molecular weight was estimated to be 26 kDa on SDS-PAGE. The protease was highly active from pH 9.0-12.0 with an optimal at pH 11.0. It was active at 30 - 60°C and exhibited maximal activity at 50°C. The thermostability of the protease was increased by the addition of $CaCl_2$. It retained 70 and 81% of its initial activity after heating for 2 h at 50°C, in the absence or presence of 2 mM $CaCl_2$, respectively. The enzyme was inactivated by diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride, suggesting that it is a serine protease. The protease was stable in 0.5% SDS and retained 70.3% of its initial activity after 1 h of incubation. It was active in the presence of 3% Triton X-100 with 100% activity and stable towards oxidizing agent with 69.2% activity in the presence of 1% H_2O_2. The enzyme showed excellent compatibility with commercial detergents such as TaiZi, BiLang, DiaoPai and TianQing, retaining more than 90% of its initial activity in the tested detergents after 1 h of preincubation at 40°C.

Key words: Serine alkaline protease, *Bacillus alcalophilus*, stability, detergent compatibility.

INTRODUCTION

Alkaline proteases have great applications in a variety of industrial fields such as in detergent, food, pharmaceutical production, leather and textile processing (Gupta et al., 2002; Kumar and Takagi, 1999). Today, they account for about 60% of the total enzyme sales in various industrial market sectors, and are expected to increase in future

Alkaline proteases play an important role in detergent industry. Their application history dates back to 1914 when Rohm added alkaline protease into detergent (Gupta et al., 2002). Alkaline proteases are the most appropriate as detergent additives, as the pH of washing environment is generally from 9.0 to 12.0. Alkaline proteases, when used as additives in detergents, enable the digestion of protein-based stains under mild conditions, generate less byproducts and waste, reduce wear and tear, and result in more ecofriendly operations compared to the purely chemical-based detergents. Incorporation of alkaline protease has been essential for modern, compact detergents (Maurer, 2004).

A great number of microbes belonging to bacteria, fungi and

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Abbreviations: SDS-PAGE, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; **PMSF,** phenylmethylsulfonyl fluoride; **DFP,** diisopropyl fluorophosphates; **EDTA,** ethylenediaminetetraacetic acid.

yeast are well known to produce alkaline protease (Donaghy and McKay, 1993; Gupta et al., 2002; Hajji et al., 2007; Kumar and Takagi, 1999). Of these, the bacteria, particularly the genus *Bacillus*, are used most often in commercial production. For one thing, they are non-pathogenic and well known with respect to fermentation technology; for another, they have a huge capacity for secreting proteins directly into the fermentation broth, which greatly simplifies the downstream processing (Kumar and Takagi, 1999; Maurer, 2004). However, there are many more parameters involved in the selection of a good detergent alkaline protease, such as activity and stability at washing pH and temperature, compatibility with detergent components like surfactants and oxidizing agents. The demand prompts us to explore new alkaline protease with enhanced properties. Many *Bacillus*-derived alkaline proteases have been well documented and characterized (Hadj-Ali et al., 2007; Haddar et al., 2009; Jacobs et al., 1985; Jaouadi et al., 2008; Oberoi et al., 2001; Wells et al., 1983). But there are few reports about *Bacillus alcalophilus* with enhanced properties. In this paper, a detergent-stable alkaline protease TC4 from a new isolated *B. alcalophilus* TCCC11004 was purified and examined for its characteristics in detergent formulations.

MATERIALS AND METHODS

Microorganism and taxonomic study

An alkaline protease producing bacterium TCCC11004 was isolated from alkaline soil in TangGu (Tianjin, China) by selective screening on skim milk agar plates containing (g/l): tryptone 10, yeast extract 5, NaCl 10 and skim milk 100 ml. A clear halo around the colony gave an indication of protease-producing microorganism. The isolate was examined according to the methods described in Bergey's Manual of Determinative Bacteriology and on the basis of the 16S rDNA sequence analysis.

The genomic DNA of *Bacillus* was prepared from overnight grown cultures by Bacterial Genomic DNA Extraction Kit (TaKaRa, China). 16S rDNA sequence was amplified from it with the upstream primer: 5'-GAGAGTTTGATCCTGGCTGGCTCAG-3' and the downstream primer: 5'-AAGGAGGTGATCCAGCCGCA-3', which generated a DNA fragment of approximately 1500 bp. Amplification of DNA was carried out under the following conditions: denaturation at 94℃ for 5 min followed by 30 cycles of 94℃ for 30 s, 55℃ for 30 s, 72℃ for 1.5 min and final extension at 72℃ for 10 min. PCR product was cloned using pUCm-T vector (BBI, Canada). Nucleotide sequencing of the cloned PCR product was performed following the application of automated DNA sequencing method based on dideoxynucleotide chain termination method (Sanger et al., 1977). The nucleotide sequence was analyzed with the GenBank database using BLAST program (NCBI).

Protease production

Inocula were routinely grown in seed culture medium containing (g/l): yeast extract 5, tryptone 5, glucose 10, and K_2HPO_4 18. The initial medium used for protease production was composed of (g/l): glucose 55, yeast extract 17.5, K_2HPO_4 18, trisodium citrate 3.3, and $CaCl_2$ 2.6. Media were autoclaved at 121℃ for 20 min.

Cultivations were conducted in 50 ml of production medium in a 250 ml flask maintained at 34℃. Incubation was carried out with agitation at 180 rpm/min for 50 h. The cultures were centrifuged at 8000 rpm/min, 4℃, and the supernatant was subjected to proteolytic activity determination.

Assay of protease activity

Protease activity was measured using casein as a substrate with minor modifications (Keay et al., 1970). 1.0 ml of diluted enzyme preparation was preincubated at 40℃ for 2 min. The reaction was started by addition of 1.0 ml casein (1% (w/v), pH 11.0). The reaction mixture was then incubated at 40℃ for 10 min and stopped by addition of 2.0 ml of 0.4 M trichloroacetic acid. The mixture was allowed to stand at room temperature for 10 min and then centrifuged at 8000 rpm/min to remove the precipitate. 1.0 ml of supernatant was mixed with 5.0 ml of 0.4 M Na_2CO_3 and 1.0 ml Folin reagent. The mixture was incubated at 40℃ for 20 min and was estimated spectrophotometrically at 680 nm. A standard curve was generated using solutions of 0 - 6 mg/l tyrosine.

One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg tyrosine per millilitre in 1 min under the experiment conditions used.

Protease activities represent the means of at least two determinations carried out in duplicate. The difference between values did not exceed 5%.

Protease purification

Ammonium sulfate precipitation

For ammonium sulfate precipitation, 47.2 g of ammonium sulfate was slowly added to 100 ml of culture supernatant at 25℃, to achieve 70% saturation. After 5 h, the precipitate was recovered by centrifugation (8000 rpm/min, 4℃), dissolved in a minimal volume of phosphate buffer (pH 7.6).

Sephadex G-75 gel filtration

The ammonium sulfate fraction was subjected to gel filtration on a Sephadex G-75 column (1.6 cm × 60 cm) which had been equilibrated previously with phosphate buffer (pH 7.6). Fractions of 5 ml were collected at a flow rate of 1 ml/min with the same buffer. Protein contents (Absorbance at 280 nm) and protease activity were determined. Fractions showing protease activities were pooled.

SP-Sepharose HP separation

The active fractions from Sephadex G-75 gel filtration were applied to a SP-Sepharose HP column (2.6 cm × 20 cm) equilibrated with phosphate buffer (pH 7.6). After being washed with the same buffer, bound proteins were eluted with a linear gradient of NaCl in the range of 0-0.6 M in the equilibrating buffer. Fractions showing activities were collected at a flow rate of 1.5 ml/min and monitored by the absorbance at 280 nm.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out for the determination of molecular weight of the enzyme as described by Laemmli (1970), using a 5% (w/v) stacking and a 12% (w/v) separating gels. Samples were heated at 100℃ for 5 min before electrophoresis. The gel was stained with

Table 1. Partial morphological and biochemical characteristics of strain TCCC11004.

Characteristic	Result
Shape	Rod
Gram stain	Positive
Spore forming	+
Aerobiosis	+
Catalase activity	+
Reduction of nitrate	-
Hydrolysis of starch	+
Hydrolysis of casein	+
Growth temperature	20 - 55℃
Acid from	
Xylose	+
Arabinose	+
Mannitol	+
Maltose	+
Fructose	+
Galactose	+

0.1% (w/v) Coomassie Brilliant Blue R 250 and destained by conventional procedures.

Protein concentration

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard. During the course of enzyme purification, concentrations of protein in the fractions were monitored by measuring the absorbance at 280 nm.

Effect of pH on activity and stability

The optimum pH of the purified enzyme was studied in the range of 5.0 - 13.0 with 1% casein (w/v) as a substrate. The pH stability of the alkaline protease was determined by incubating enzyme preparation in buffers of different pH in the range of 9.0 - 12.0 for 24 h at room temperature. Aliquots were withdrawn and proteolytic activity was determined under standard assay conditions.

The following buffer systems were used: Na_2HPO_4-citric acid buffer for pH 5.0 - 7.0, Tris-HCl buffer for pH 8.0 - 9.0, Glycine-NaOH buffer for pH 10.0 - 11.0, and KCl - NaOH buffer for pH 12.0 - 13.0.

Effect of temperature on activity and stability

The effect of temperature on the enzyme activity was examined at various temperatures with 1% casein (w/v) at pH 11.0. Thermal stability was determined by incubating the enzyme preparation in the presence or absence of Ca^{2+}. Aliquots were withdrawn at intervals to test the residual activity at pH 11.0.

Effect of various metal ions, enzyme inhibitors and surfactants on protease stability

The influence of various metal ions (Ca^{2+}, Mg^{2+}, Cu^{2+}, Hg^{2+}, Na^{+}, K^{+}, Zn^{2+}, and Mn^{2+}, 5 mM) on protease stability was studied by incubating the enzyme in the presence of different ion for 1 h at 40℃. The residual activity was determined with 1% casein (w/v) at pH 11.0 and 50℃.

The effects of enzyme inhibitors were studied using phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP) and ethylenediaminetetraacetic acid (EDTA). Enzyme was preincubated with inhibitors at 40℃ for 30 min and then the residual activity was measured using casein as a substrate at pH 11.0 and 50℃.

The effect of some surfactants (Tween 60, Tween 80, Triton X-100 and SDS) and oxidizing agents (H_2O_2) on enzyme stability was studied by preincubating the enzyme with these additives at 40℃ for 1 h. The residual activity was measured at pH 11.0 and 50℃. The activity of the enzyme (without any additives) was taken as 100%.

Commercially available detergents like TaiZi (China), BiLang (China), DiaoPai (China) and TianQing (China) were used to test the compatibility of the enzyme. The detergents were diluted in tap water to give a final concentration of 10 mg/ml to simulate washing conditions. Endogenous protease present in these detergents was inactivated by incubating the detergents at 70℃ for 1 h, prior to the addition of the enzyme. The enzyme preparation was incubated with the diluted detergents at 40℃ and the residual activity was determined at pH 11.0 and 50℃. The enzyme activity of a control (without detergent) incubated under the same conditions was taken as 100%.

Nucleotide sequence accession number

The 1543 bp 16S rDNA gene sequence of TCCC11004 strain has been submitted to GenBank database and assigned accession number EU231621.

RESULTS AND DISCUSSION

Isolation and identification of the microorganism

Several strains producing extracellular proteases were isolated from alkaline soil. Of these, isolate TCCC11004 exhibiting a large zone of hydrolysis on skim milk agar plates was selected. It was Gram-positive, aerobic, sporeforming, rod-shaped bacterium (Table 1). In order to identify TCCC11004 strain, 16S rDNA gene was amplified, cloned in pUCm-T vector and sequenced. The nucleotide sequence was analyzed with the GenBank database using BLAST program. Strain TCCC11004 was identified as *B. alcalophilus* TCCC11004 combining the analysis of morphological and biochemical characteristics with the alignment of 16S rDNA gene sequence. It was deposited in Tianjin University of Science and Technology of centre of culture collection (TCCC; deposition No: TCCC11004).

Production of TC4 protease

In commercial production, it is important to optimize the production medium for maximum protease production as well as an economy. Firstly, different carbon sources were tested for the production of alkaline protease. Glucose in the initial medium was substituted with starch,

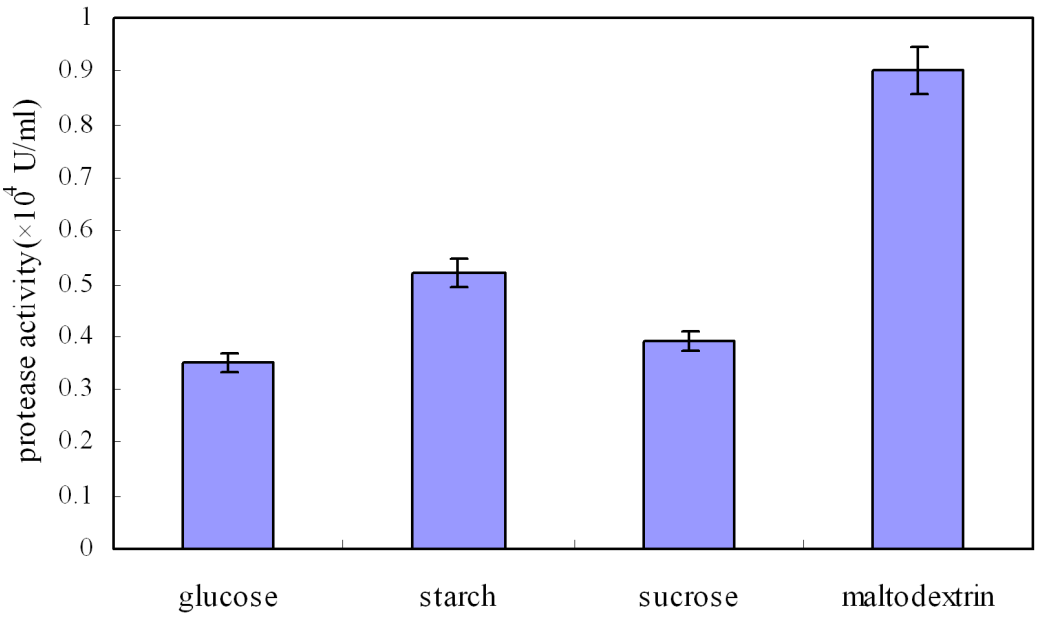

Figure 1. Effect of different carbon sources on alkaline protease production by *B. alcalophilus* TCCC11004. Cultivations were performed for 50 h at 34℃ in initial medium containing (g/l): carbon source 55, yeast extract 17.5, K_2HPO_4 18, trisodium citrate 3.3, $CaCl_2$ 2.6.

sucrose, and maltodextrin at a concentration of 55 g/l. As shown in Figure 1, the addition of 55 g/l starch resulted in about 1.5 fold increase of the protease activity (0.52 × 10^4 U/ml). The production of protease by TCCC11004 was greatly enhanced by the addition of maltodextrin into the medium. Strain TCCC11004 exhibited highest productivity of alkaline protease in medium containing maltodextrin as carbon source (0.9 × 10^4 U/ml) compared with that of glucose.

To select the optimum nitrogen source for alkaline protease production, yeast extract was replaced with cotton seed meal, peptone, ammonium sulphate, or a combination of yeast extract and cotton seed meal (Figure 2). These nitrogen sources were added into the medium at a concentration of 17.5 g/l. Only the combination of yeast extract and cotton seed meal strongly increased protease activity (1.5 × 10^4 U/ml). Enzyme production in the absence of yeast extract and cotton seed meal was significantly low (about 0.12 × 10^4 U/ml), indicating the requirement of nitrogen source and some growth factors for the protease production.

Since maltodextrin was the best carbon source for protease production by TCCC11004, the effect of its concentration on the enzyme production was studied. As shown in Figure 3, protease activity reached a maximum value (2.0 × 10^4 U/ml) at the concentration of 110 g/l.

It had been demonstrated that a combination of yeast extract and cotton seed meal was the best candidate for nitrogen sources, so the optimum concentrations of yeast extract and cotton seed meal were tested. As shown in Figure 4, addition of yeast extract (17.5 g/l) and cotton seed meal (29.3 g/l) to the medium was found to increase the protease activity to 2.5 × 10^4 U/ml.

In order to investigate the effect of some mineral ions on protease production, different mineral ions were added to the initial medium. Of the tested additives, K_2HPO_4, trisodium citrate and $CaCl_2$ stimulated enzyme production. K_2HPO_4 has been used as a source of phosphate in most studies, with their buffering capacity in the medium being an added advantage (Mao et al., 1992; Moon and Parulekar, 1991). Trisodium citrate and divalent metal ions such as Ca^{2+} are also required in the production medium for maximum protease activity. The protease activity was increased when the medium was supplemented with 2.6 g/l $CaCl_2$ and improved to 2.75 × 10^4 U/ml when 3.3 g/l trisodium citrate and 18 g/l K_2HPO_4 were added (data not shown).

The protease production was carried out in the optimized medium at 34℃, 180 rpm/min. At the initial 20 h incubation period, there was no protease activity in the culture. The production of protease TC4 began after 20 h and increased exponentially up to 50 h in the complex medium. As shown in Figure 5, the total protease activity was up to 2.8 × 10^4 U/ml. At present, many alkaline proteases which originated from *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, and *Bacillus lentus* are used in detergents worldwide (Gupta et al., 2002). However, *B. alcalophilus* TCCC11004 in this study was also seemed to be a good candidate. The strain plays an important role in the industrial alkaline protease production. The alkaline protease TC4 from *B. alcalophilus* TCCC11004 was highly active compared with some earlier reported proteases (Hadj-Ali et al., 2007; Abidi et al., 2008). The production medium used in the industrial fermentations

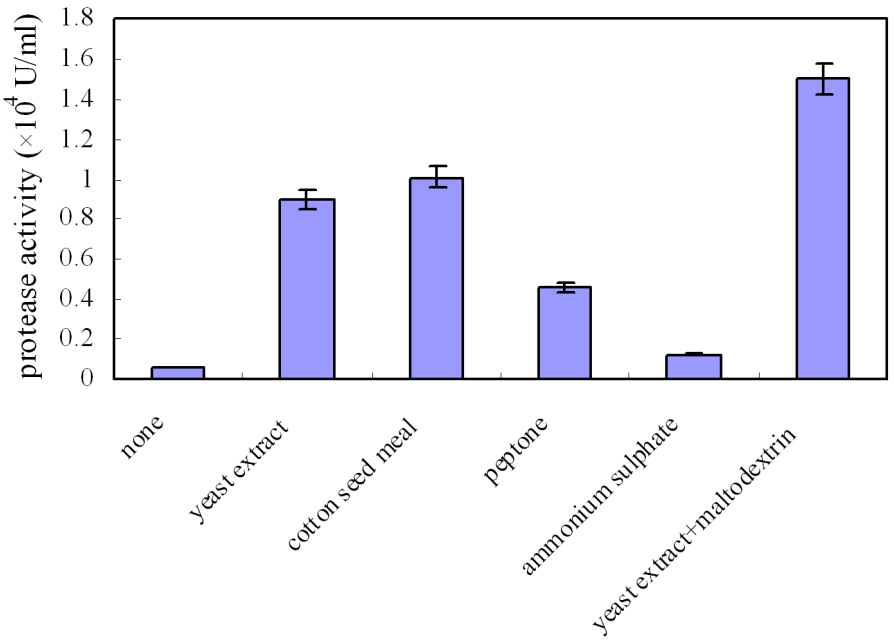

Figure 2. Effect of different nitrogen sources on alkaline protease production by *B. alcalophilus* TCCC11004. Cultivations were performed for 50 h at 34℃ in initial medium containing (g/l): maltodextrin 55, nitrogen source 17.5, K_2HPO_4 18, trisodium citrate 3.3, and $CaCl_2$ 2.6.

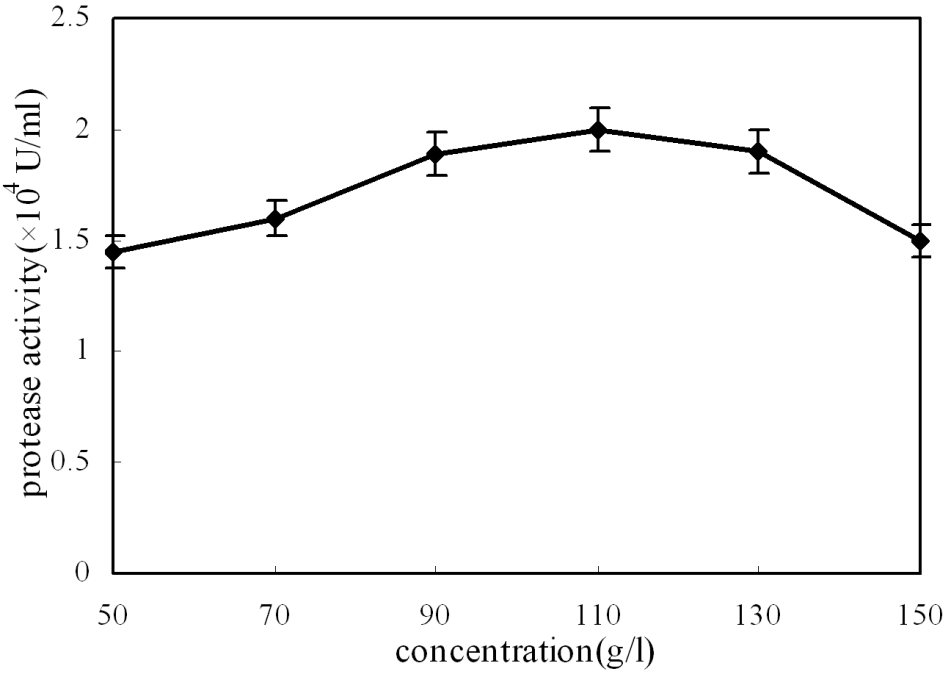

Figure 3. Effect of maltodextrin concentration on protease production by *B. alcalophilus* TCCC11004. Cultivations were performed for 50 h at 34℃ in medium containing (g/l): yeast extract 17.5, cotton seed meal 17.5, K_2HPO_4 18, trisodium citrate 3.3, and $CaCl_2$ 2.6.

also has to fulfill economic requirements. As a result, these media are often based on the complex and cheap carbon and nitrogen sources. Both the maltodextrin and cotton seed meal in the production medium are economic

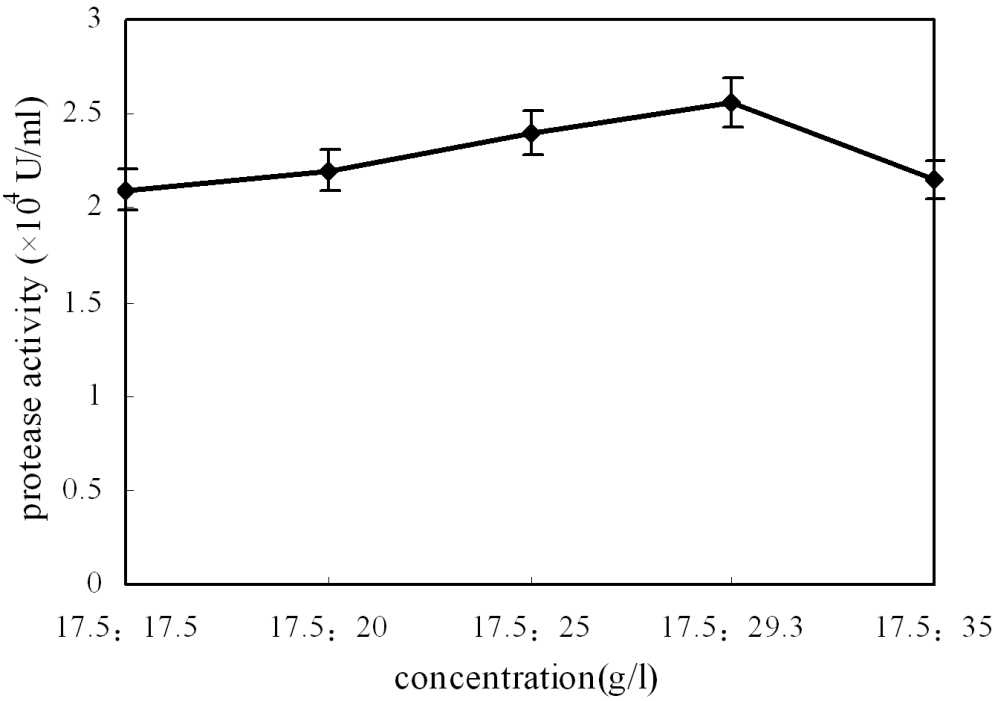

Figure 4. Effect of yeast extract and cotton seed meals concentrations on protease production by *B. alcalophilus* TCCC11004. Cultivations were performed for 50 h at 34℃ in medium containing (g/l): maltodextrin 110, K_2HPO_4 18, trisodium citrate 3.3, and $CaCl_2$ 2.6.

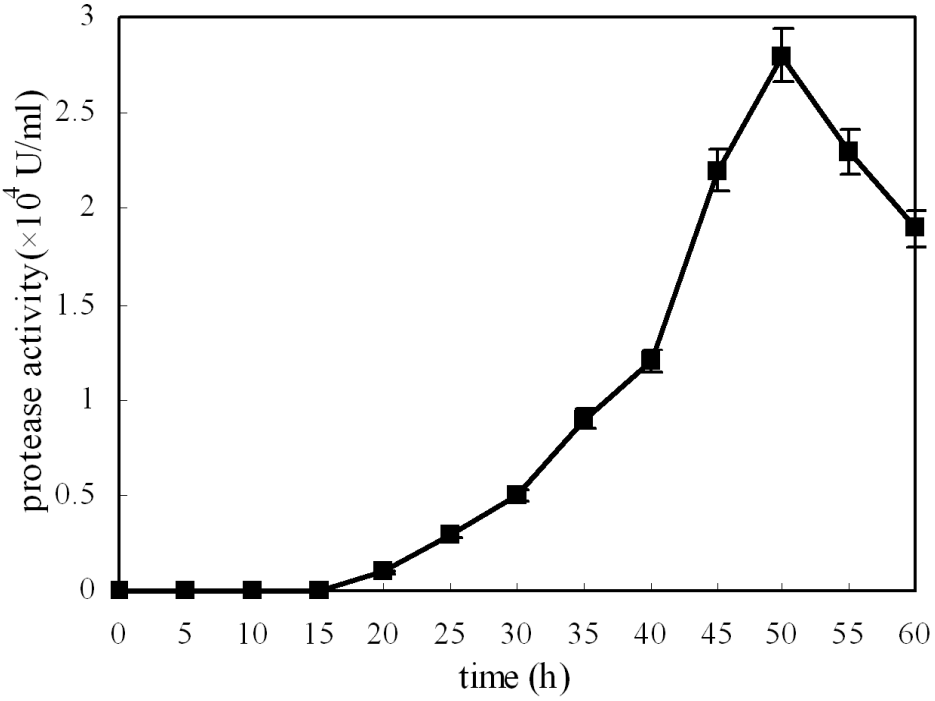

Figure 5. Time profile of protease production at 34℃ under shaking (180 rpm/min).

nutritional sources for the strain's growth and synthesis of alkaline protease. The highly productive strain as well as the low-cost production medium suggested the application potential at the industrial scale.

Table 2. Summary of the purification of TC4 protease from *B. alcalophilus* TCCC11004.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Recovery (%)	Purification fold
Crude extract	7.2×10^5	202.23	3560.3	100	1
Ammonium sulfate	4.3×10^5	46.45	9257.3	59.7	2.6
Sephadex G-75	2.3×10^5	13.46	17087.7	31.9	4.8
SP-Sepharose HP	1.1×10^5	4.53	24282.6	15.2	6.8

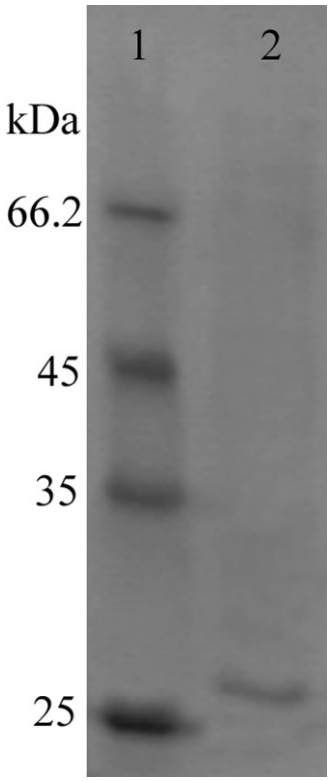

Figure 6. SDS-PAGE of the purified protease from *B. alcalophilus* TCCC11004. **Lane 1:** protein molecular mass markers; **Lane 2:** purified TC4 protease.

Purification of TC4 protease

Purification of the TC4 protease from *B. alcalophilus* TCCC11004 was carried out by the three-step procedure described above. In the first step, ammonium sulfate precipitation resulted in a 2.6 fold increase of specific activity. The 70% ammonium sulfate fraction was then subjected to gel filtration on a Sephadex G-75 column and to ion exchange chromatography on SP-Sepharose HP column. After the final purification step, the protease was purified 6.8 fold with a recovery of 15.2% and a specific activity of 24282.6U/mg protein. The results of the purification procedure were summarized in Table 2. As shown in Figure 6, the molecular weight of purified protease was estimated to be 26 kDa on SDS-PAGE.

In general, the molecular weights of bacterial alkaline proteases range from 15 to 35 kDa (Gupta et al., 2002). The molecular weight of the TC4 protease was higher than that of alkaline protease from *Bacillus mojavensis* A21 (20 kDa) (Haddar et al., 2009) and lower than that of Subtilisin Carlsberg (27.3 kDa) (Jacobs et al., 1985), as well as that of subtilisin-like alkaline protease BPP-A from *Bacillus pumilus* MS-1 (33 kDa) (Miyaji et al., 2006).

Effect of pH on enzyme activity and stability

Protease TC4 was highly active from 9.0 to 12.0, with an optimum pH at 11.0 (Figure 7). The relative activities at pH 7.0 and 9.0 were about 35 and 81%, of that at pH 11.0. However, protease activity decreased greatly above pH 12.0 and was less than 40% of the maximum activity at pH 13.0.

Furthermore, the pH stability was determined by incubating the enzyme in buffers of different pH in the range of 9.0 - 12.0 for 24 h at room temperature, followed by the activity determination at pH 11.0. As shown in Figure 8, TC4 was stable between 9.0 - 12.0 and showed 100% of its activity after 24 h of incubation at pH 11.0. When TC4 protease was incubated at pH 9.0 and 10.0 for 24 h, it retained 87 and 92% of its initial activity, respectively.

The detergent alkaline protease should be active and stable under the washing pH. Many alkaline proteases produced by *Bacillus* have a high optimum pH. The optimum pH of the TC4 enzyme was comparable with protease isolated from *B. licheniformis* NH1, which showed an optimum pH of 10.0 - 11.0 (Hadj-Ali et al., 2007), whereas protease A21 isolated from *B. mojavensis* A21 had an optimal pH of 8.5 (Haddar et al., 2009). The pH stability of TC4 was also found greater than that of earlier reported proteases from *Bacillus* sp.RGR-14 (Oberoi et al., 2001) and *B. licheniformis* NH1 (Hadj-Ali et al., 2007). The stability of the TC4 enzyme in alkaline suggested its usefulness in detergent.

Effect of temperature on enzyme activity and stability

The effect of temperature on the protease activity using casein as a substrate was examined at various tempe-

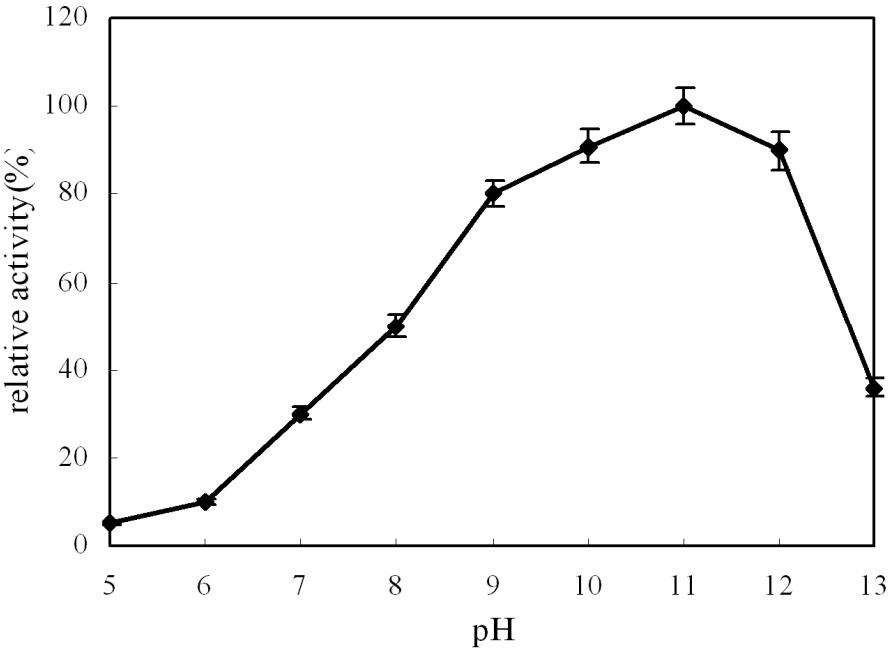

Figure 7. Effect of pH on the activity of protease TC4. The protease activity was determined in the pH range of 5.0 - 13.0 using buffers of different pH values. The activity of the enzyme at pH 11.0 was taken as 100%.

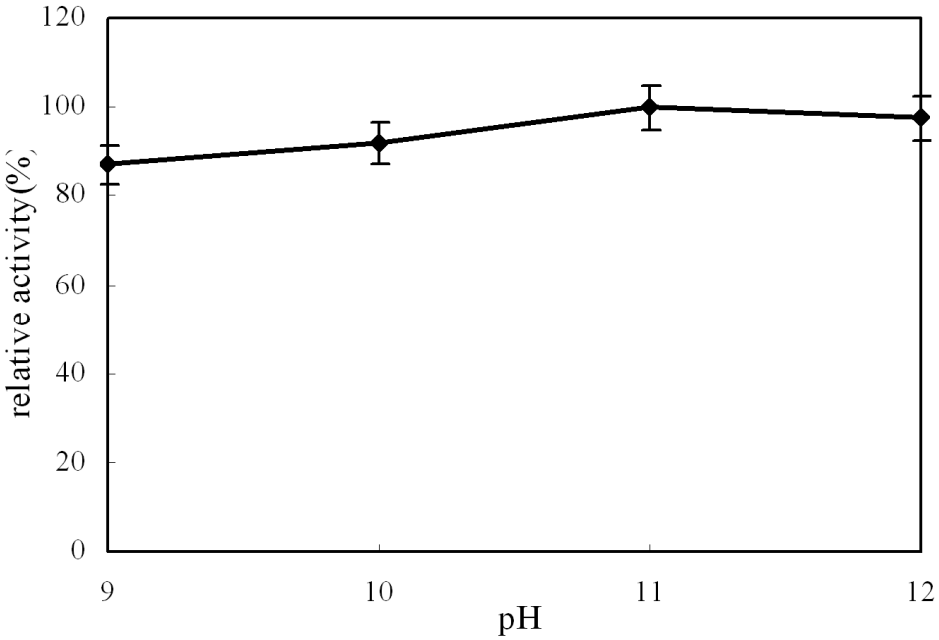

Figure 8. Effect of pH on the stability of protease TC4. The pH stability of the protease was determined by incubating the enzyme in different buffers for 24 h at room temperature and the residual activity was measured at pH 11.0 and 50℃. The activity of the enzyme before incubation was taken as 100%.

ratures and pH 11.0 (Figure 9). The TC4 protease was active between 30 and 60℃, with an optimum at 50℃. The activity at 40 and 60℃ were about 85 and 98% of the maximum activity, respectively. However, the inactivation was observed at 70℃. The optimum temperature of TC4 was different from some other reported proteases,

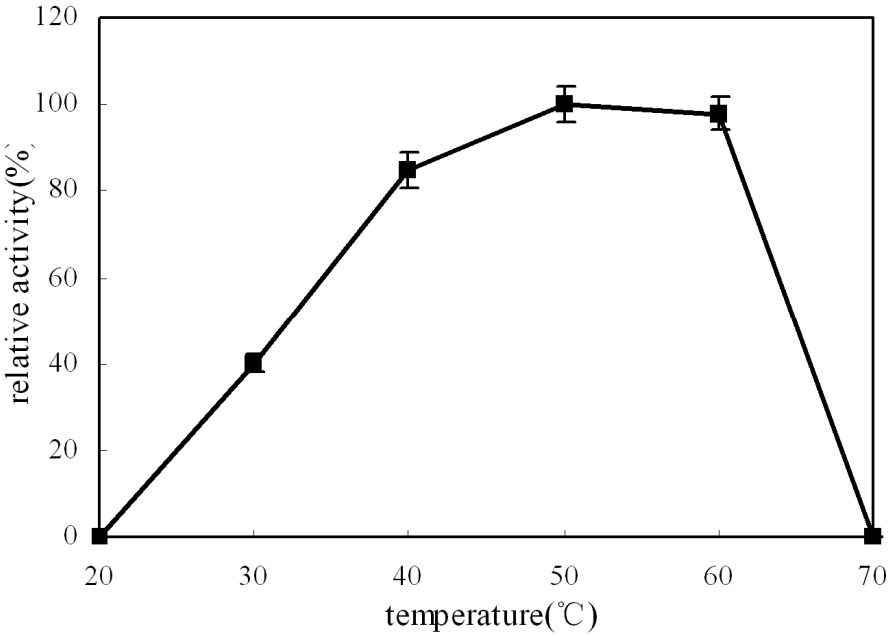

Figure 9. Effect of temperature on the activity of protease TC4. The temperature profiles were determined by assaying activity at different temperature varied from 20 to 70℃. The activity of the enzyme at 50℃ was taken as 100%.

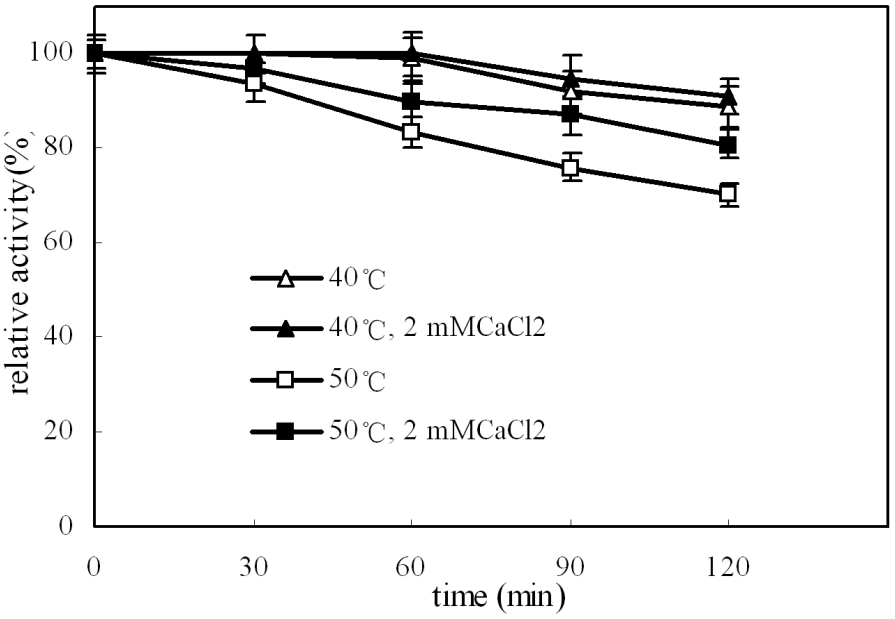

Figure 10. Effect of temperature on thermal stability of protease TC4. The enzyme was preincubated in the absence or presence of 2 mM $CaCl_2$. Remaining activity was determined at intervals under the optimal conditions. The original activity before preincubation was taken as 100%.

whose optimum temperatures were higher than that of TC4. This includes proteases from *Bacillus circulans* (Rao et al., 2009), *B. pumilus* CBS (Jaouadi et al., 2008) and *Bacillus* sp. GX6638 (Durham et al., 1987), which are optimally active at 65℃ or higher. The thermal stability of TC4 in the presence of 2 mM $CaCl_2$ was also examined (Figure 10). The stability of the TC4 was enhanced in the presence of $CaCl_2$. At 40℃, the TC4 retained 94% of its

Table 3. Effect of metal ions (mM), inhibitors (mM), surfactants (% v/v or w/v) and oxidizing agent (% v/v) on alkaline protease activity from TC4.

Metal ions	Concentration	Residual activity (%)
None	0	100 ± 5.0
Ca^{2+}	5 mM	105 ± 5.2
Mg^{2+}	5 mM	103 ± 4.9
Cu^{2+}	5 mM	75.3 ± 3.6
Hg^{2+}	5 mM	3 ± 0.15
Na^{+}	5 mM	98.6 ± 4.9
K^{+}	5 mM	94 ± 4.6
Zn^{2+}	5 mM	70.5 ± 3.3
Mn^{2+}	5 mM	91.4 ± 4.5
Inhibitors		
DFP	1 mM	2 ± 0.1
PMSF	1 mM	0
EDTA	1 mM	93.5 ± 4.5
Surfactants		
Tween 60	1% (v/v)	83.4 ± 4.0
Tween 80	1 %(v/v)	94.5 ± 4.6
TritonX-100	3% (v/v)	100 ± 5.0
SDS	0.5% (w/v)	70.3 ± 3.5
	1% (w/v)	48.2 ± 2.3
Oxidizing agent		
H_2O_2	0.5% (v/v)	72.3 ± 3.6
	1% (v/v)	69.2 ± 3.3

The protease was incubated with different metal ions, inhibitors, surfactants, oxidizing agent at 40℃ for 1h and the residual activity was determined at pH 11.0 and 50. The activity of the protease (without any additives) was taken as 100%.

initial activity after incubation for 1 h without $CaCl_2$. However, no activity was lost when incubated with $CaCl_2$. At 50℃, it retained about 90% of its initial activity in the presence of $CaCl_2$ for incubation of 1 h, while 83.5% of the initial activity was observed without $CaCl_2$. It also retained about 81% of its initial activity in the presence of $CaCl_2$ when incubated for 2 h, compared with the fact that it lost about 30% of its activity upon incubation in the absence of $CaCl_2$. The improvement in protease thermostability against thermal inactivation in the presence of Ca^{2+} could be ascribed to the binding of Ca^{2+} to autolysis sites as well as the strengthening of interactions inside the molecules (Lee and Jang, 2001).

Effect of metal ions on protease stability

The effect of some metal ions at the concentration of 5 mM on the stability of TC4 protease was studied at pH 11.0 and 40℃ (Table 3). A little enhancement in protease activity was observed in the presence of Ca^{2+} and Mg^{2+}. These cations (Ca^{2+}, Mg^{2+}) have also been reported to increase activity of A21 from *B. mojavensis* A21 (Haddar et al., 2009). It is believed that these cations protect the enzyme against thermal denaturation and play a role in maintaining the active conformation of the enzyme at higher temperatures (Donaghy and McKay, 1993; Steele et al., 1992). In contrast, the protease activity was strongly inhibited by 5 mM Hg^{2+}. Cu^{2+} and Zn^{2+} inhibited the protease activity by 24.7 and 30%, respectively. Mn^{2+}, Na^{+}, and K^{+} did not show much influence on protease activity.

Effect of inhibitors on protease activity

Inhibition studies shed light on the nature of the enzyme, so some inhibitors such as PMSF, DFP and EDTA were investigated (Table 3). This alkaline protease was sensitive to serine protease inhibitors, e.g PMSF, DFP, which sulfonated the essential serine residue in the active site and resulted in the complete loss of its activity. However, the enzyme was resistant to EDTA. The high activity of TC4 in the presence of EDTA was advantageous for application as detergent additive. EDTA is a kind of chelating agent used in most detergents. It functions as a

Table 4. Stability of the alkaline protease towards different commercial detergents.

Detergents	Remaining activity at different incubation time (%)		
	1 h	2 h	3 h
TaiZi	100 ± 5	97 ± 4.6	74 ± 3.2
BiLang	95 ± 4.2	91 ± 4.5	82 ± 4.0
DiaoPai	91 ± 4.5	85 ± 4.0	67 ± 3.0
TianQing	92 ± 4.0	86 ± 4.0	54.5 ± 2.6

The protease TC4 was mixed with the diluted commercial detergents (10 mg/ml) and incubated at 40°C. The residual activity was determined at pH 11.0 and 50. The protease activity of a control (without detergent) incubated under the same conditions was taken as 100%.

water softener and also plays a role in stain removal.

Stability in the presence of surfactants, oxidizing agent

The alkaline protease which was added to the detergent powder must be compatible and stable with all commonly used detergent components such as surfactants and oxidizing agents. The TC4 protease was stable in the presence of 1% Tween 80 and 3% Triton X-100, retaining 94.5 and 100% of its activity, respectively, after incubation for 1 h. Furthermore, the TC4 protease was stable towards SDS, retaining about 70.3 and 48.2% of its initial activity after incubation for 1 h at 40°C in the presence of 0.5 and 1% SDS. The stability of TC4 protease against SDS was greater than ES1 from *Aspergillus clavatus* ES1 (Hajji et al., 2007), which retained about 33% after incubation for 1 h with 0.5% SDS, but lower than proteases from *Bacillus* sp. RGR-14 (Oberoi et al., 2001), *B. licheniformis* RP1 (Kamoun et al., 2008), *B. pumilus* CBS (Jaouadi et al., 2008) and *B. licheniformis* NH1 (Hadj-Ali et al., 2007). Hydrogen peroxide is known to be a strong oxidizing agent; it inactivates proteins oxidatively (Joo et al., 2005). TC4 protease retained as much as 72.3 and 69.2% of its raw activity at a concentration of 0.5 and 1%, respectively. It was an important characteristic for its use in detergent formulations.

Compatibility studies with commercial detergents

The compatibility studies of the protease with commercial detergents showed that the enzyme was stable in the presence of these four detergents, retaining more than 90% of its initial activity after 1 h incubation (Table 4). The enzyme was stable in the presence of TaiZi and BiLang even after incubation for 3 h, retaining from 74% in TaiZi and 82% in BiLang. The performance of enzyme in the presence of DiaoPai and TianQing was found to be inferior to that of TaiZi and BiLang. However, it still retained more than 50% of its initial activity after incubation for 3 h.

Conclusion

This work described the purification and characteristics of a serine alkaline protease TC4 from a *B. alcalophilus* TCCC11004 isolated from alkaline soil. The most appropriate medium for the protease production was composed of (g/l): maltodextrin 110, yeast extract 17.5, cotton seed meal 29.3, K_2HPO_4 18, trisodium citrate 3.3 and $CaCl_2$ 2.6. The purification was achieved by ammonium sulfate precipitation, Sephadex G-75 gel filtration and SP-Sepharose HP ion exchange chromatography. After the final purification step, the protease was purified 6.8 fold with a specific activity of 24282.6U/mg protein and 15.2% recovery. Its molecular weight was 26 kDa on SDS-PAGE. The protease was active and stable at high pH and the optimal activity was at pH 11.0. It belonged to mesophilic enzyme, and it was optimally active at 50°C. One of the most important characteristics of the enzyme was its excellent stability towards detergents. The enzyme retained more than 90% of its original activity with four tested detergents even after 1 h incubation at 40°C.

The excellent performances of TC4 protease such as high activity and stability in high alkaline pH, stability in the presence of surfactants and oxidizing agent, like Tween 80, Triton X-100 and H_2O_2, as well as stability in the presence of commercial detergents suggested its feasibility for application in detergent.

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