Isolation and identification of a novel fibrinolytic *Bacillus tequilensis* CWD-67 from dumping soils enriched with poultry wastes

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A newly isolated strain, CWD-67, which exhibited high fibrinolytic activity, was screened from dumping soils enriched with poultry wastes. The strain was identified as *Bacillus tequilensis* (KF897935) by 16Sr RNA gene sequence analysis and biochemical characterization. A fibrinolytic enzyme was purified to homogeneity from the culture supernatant using ammonium sulfate precipitation, membrane concentration, dialysis, ion-exchange, and gel filtration chromatography. SDS-PAGE analysis showed that the purified enzyme was a monomeric protein with an apparent molecular weight of 22 kDa, which is the lowest among *Bacillus fibrinolytic* enzymes reported to date. The purified enzyme was confirmed to have fibrinolytic activity by a fibrin zymogram. The optimal pH and temperature values of the enzyme were 8.0 and 45°C, respectively. The enzyme was completely inhibited by PMSF and significantly inhibited by EDTA, TPCK, Co2+, Zn2+, and Cu2+, suggesting a chymotrypsin-like serine metalloprotease. *In vitro* assays revealed that the purified enzyme could catalyze fibrin lysis effectively, indicating that this enzyme could be a useful fibrinolytic agent.

**Key Words:** *Bacillus tequilensis*; fibrinolytic bacteria; serine metalloprotease; zymogram

**Introduction**

There has recently been increased attention to life-threatening cardiovascular diseases, such as coronary heart disease, blood clotting, peripheral vascular disease, and cerebrovascular disease (Lim, 2013). Among these diseases, blood clotting is an emerging problem that seriously threatens the health of human beings. There are more than twenty enzymes in the body that assist in the clotting of blood, while there is only one capable of breaking clots down. Fibrin is an important protein constituent of blood clots that is normally formed from fibrinogen via the action of thrombin (EC. 3.4.21.5) following injury (Wolberg, 2007). Therefore, the accumulation of fibrin in blood vessels usually results in thrombosis, leading to myocardial infarction and other cardiovascular diseases (Collen and Lijnen, 1991). Owing to the increased incidence of thrombosis, the search for novel thrombolytic agents is of the utmost importance.

A variety of thrombolytic agents, including tissue plasminogen activator (t-PA) urokinase (EC 3.4.21.73), streptokinase (EC 3.4.99.0), and staphylokinase (EC 3.4.99.22), have been investigated (He et al., 2008). Fibrinolytic enzymes have attracted attention as thrombolytic agents owing to their widespread applications. Several studies have demonstrated that fibrinolytic enzymes can be obtained from different sources, including animals (Geng et al., 2014), plants (Siripatetawee et al., 2012), foods (Montriwong et al., 2012), and microbes (Huang et al., 2013). Soils have been extensively investigated for the presence of microorganisms that produce useful biologically-active molecules. However, soil microorganisms have only recently been investigated as potential biofactories for the synthesis of various enzymes. Currently, use of bacteria in this field is rapidly gaining importance owing to a growing success, ease of handling and possibilities for genetic modification. Enzymes produced by soil bacteria can provide numerous advantages over traditional
enzymes owing to the wide range of environments from which they are recovered (Mander et al., 2011).

The first fibrinolytic enzyme isolated from Bacillus natto, nattokinase (EC 3.4.21.62), has been widely applied as a thrombolytic agent (Cao et al., 2009). Additionally, Pseudomonas sp. TKU015 (Wang et al., 2009), B. subtilis (Chang et al., 2012), and Streptomyces sp. CS624 (Mander et al., 2011) have recently been reported to synthesize fibrinolytic enzymes. Enzyme synthesis methods can be categorized into intracellular and extracellular synthesis according to the place at which the enzymes are formed. Investigations of extracellular enzyme synthesis are currently being conducted to elucidate the mechanisms of synthesis to enable easy downstream processing and rapid scale-up processing. For these reasons, bacterial systems have the potential for use in the extracellular synthesis of such enzymes. Here, we report a newly isolated Bacillus tequilensis CWD-64 from dumping soils that possess fibrinolytic activity. Further, extraction, purification, and characterization of chymotrypsin-like serine metalloprotease from the culture supernatant of B. tequilensis CWD-64 are reported, and their fibrinolytic activity is described.

Materials and Methods

Reagents and media. Culture media was purchased from HiMedia (HiMedia Ltd., Mumbai, India). Fibrinogen, thrombin, and plasmin were procured from Sigma (Sigma Aldrich, CA, USA). Sephadex G-75 FF was purchased from GE Healthcare (GE Healthcare BioSciences AB, Uppsala, Sweden). DEAE-Cellulose FF was acquired from GE (GE Healthcare Life Sciences, UK). Phenyl methyl sulfonyl fluoride (PMSF), ethylene diamine tetraacetic acid (EDTA), and N-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were obtained from Merck (Merck, Mumbai, India). Other reagents and chemicals used were of analytical grade.

Isolation and screening of bacteria. Samples were collected from dumping soils enriched with broiler wastes containing muscles, blood, and skin, from different sites in Potheri, Chennai, India. The soils were serially diluted with sterile water until a dilution containing 10^6 colonies forming units (CFU) g⁻¹ of soil was obtained, after which they were inoculated onto skim milk agar plates and incubated at 30°C for 24 h. Strains exhibiting a clear zone (degradation of casein) around the colony were selected and sub-cultured several times on nutrient agar medium until purity, after which they were stored until further analysis. Fibrin plate assay. Fibrinolytic activity was investigated by a fibrin plate assay as previously described (Astrup and Mullertz, 1952), with minor modifications. The fibrin agarose gel (5-mm thick) contained 2.0% agarose, 0.12% (w/v) fibrinogen, 0.5 U/ml thrombin and 0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl. The clot was allowed to set for 30 min at room temperature, after which cell-free bacterial culture supernatant of the selected proteolytic strain was carefully loaded onto the fibrin agar plate. Un-inoculated broth medium was used as a negative control. The loaded plates were incubated at 37°C for 24 h, after which the clear zones around the paper discs indicating fibrinolytic activity were measured.

Bacterial identification. To identify the bacterium, a polymerase chain reaction (PCR) was conducted to amplify the 16S rRNA gene from the genomic DNA of selected fibrinolytic strains as previously described (Weisburg et al., 1991) using the following primers: forward 5′-TGGGCTCAAGAACGCTGCGGC-3′, reverse 5′-CCCACTGCTCCCGTAAAGGAT-3′. The temperature cycle for the reaction consisted of 30 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min 30 s followed by final extension for 5 min at 72°C. The PCR product was cloned into pGEM-T easy vector (Promega, Madison, WI, USA), after which the nucleotide sequence of the 16S rRNA gene was sequenced using an ABI Prism 377 DNA sequencer (PE Applied Biosystems, Foster City, CA, USA). The obtained sequence was then subjected to a BLAST search of the GenBank database (NCBI, Bethesda, MD, USA) to identify the bacterial isolates. Identification of the strain was confirmed by biochemical tests according to Bergey’s Manual of Systemic Bacteriology.

Purification of extracellular enzyme. The selected bacterial strain was cultured in nutrient broth medium (peptone, 5 g/L; beef extract, 1.5 g/L; yeast extract, 1.5 g/L; sodium chloride, 5 g/L) and incubated at 30°C in a shaker (180 rpm) for 96 h under aerobic conditions. Cell-free supernatants were then collected by centrifugation at 8,000 rpm for 20 min, after which ammonium sulfate was added to the supernatant at 50% saturation and the mixture was incubated overnight. The precipitate was then centrifuged at 8,000 rpm for 20 min, after which the pellet was resuspended in 50 mM Tris-HCl buffer (pH 8.0) and dialyzed against the same buffer overnight. Next, the dialysate was loaded onto a DEAE-Cellulose column (2.4 × 45 cm) that had previously been equilibrated with Tris-HCl at pH 8.0 by fast protein liquid chromatography (FPLC Amersham Bioscience). Elution with a linear gradient of 0.0 to 1.0 M NaCl resulted in a single peak of fibrinolytic activity. Peaks that exhibited higher fibrinolytic activity were pooled, concentrated and then polished using a Sephadex G-75 FF column (1.5 × 25 cm) that had previously been equilibrated with Tris-HCl at pH 8.0 by FPLC. All fractions were collected separately and the absorbance at 280 nm was measured with a spectrophotometer. All purification steps were conducted at 4°C.

Assay of enzyme activity. Enzyme activity was estimated by measurement of the acid-soluble material released from azocasein (Huang et al., 2013) using the following buffer systems: 0.1 M glycine-HCl (pH 3.0), 0.1 M disodium hydrogen phosphate-sodium citrate (pH 4.0–7.0), 0.1 M Tris-HCl (pH 8.0–9.0), 0.1 M glycine-NaOH (pH 10.0), 0.1 M disodium hydrogen phosphate-NaOH (pH 11.0), and 0.1 M KCl-NaOH (pH 12.0–13.0). The reaction mixture, which was composed of 0.6 mL of 1% (w/v) azocasein solution and a 20-μL enzyme sample, was incubated at 37°C for 20 min, after which it was supplemented with 1.2 mL of 10% (w/v) trichloroacetic acid. Next, the sample was placed on ice for 10 min, then centrifuged at 4°C and 12,000 rpm for 10 min. The concentration of acid-soluble material in the supernatant was measured based on the absorbance at 360 nm. One unit of protease activ-
Fibrinolytic enzyme from Bacillus tequilensis

Results and Discussion

Isolation and identification of fibrinolytic bacterium

A total of 23 soil samples were collected from broiler waste dumping sites. Pilot scale screening revealed 137 strains that exhibited proteolytic activity on skim milk agar media (Fig. 1a). Among these, 12 were able to degrade fibrin in a fibrin agarose medium after 24 h of incubation using culture supernatant. Strain CWD-67, which exhibited the maximum halo zone (4.3 cm in diameter) around the colony, was selected from among the 12 positive strains (Fig. 1b). Biochemical characterization revealed that strain CWD-67 was Gram-positive, endospore-forming, motile, and round in shape. In addition, the bacterium was catalase and oxidase positive and capable of growth at 20°C to 40°C, with optimum growth occurring at 30°C. The genomic DNA of strain CWD-67 was amplified using universal primers, and the 16S rRNA gene sequence was analyzed. Alignment of the sequence (819 bp) of strain CWD-67 with similar 16S rRNA gene sequences in the GenBank database revealed a high similarity (99%) to Bacillus tequilensis. Based on these results, strain CWD-67 was identified as a member of Bacillus tequilensis, and designated as B. tequilensis CWD-67 (GenBank accession number KF897935). Other Bacillus species have been shown to produce fibrinolytic enzymes. For example, Bacillus natto is a well-known fibrinolytic bacteria isolated from natto, a Japanese fermented soybean. Natto has been extensively studied and shown to produce a strong fibrinolytic enzyme known as nattokinase, which is currently used as a natural alternative blood thinner and blood clot dissolver (Cao et al., 2009; He et al., 2008). Many other studies have reported strains of bacilli that produce fibrinolytic activity, including Bacillus subtilis HQS-3 and Bacillus amyloliquefaciens DC-4 isolated from douchi, a traditional Chinese soybean (Huang et al., 2013; Peng et al., 2003), Bacillus sp. KA38 isolated from the Korean salty fermented fish, jeot-gal (Kim et al., 1997), and Bacillus sp. CK11-4 from the Korean fermented-soybean sauce, Chungkook-Jang (Kim et al., 1996). However, to the best...
of our knowledge, this is the first report of the isolation and identification of *B. tequilensis* with fibrinolytic activity from broiler waste soils.

**Purification of extracellular fibrinolytic enzyme**

Fibrinolytic enzyme was purified from a culture supernatant of *B. tequilensis* CWD-67 using ammonium sulfate precipitation followed by DEAE-Cellulose and Sephadex G-75 FF chromatography. Following ammonium sulfate precipitation, the dialyzed sample exhibited fibrinolytic enzyme activity. DEAE-Cellulose chromatography yielded several protein peaks that could be detected at 280 nm. The active fraction of fibrinolytic peak was collected and applied to a Sephadex G-75 FF column that had been equilibrated with 50 mM Tris-HCl buffer (pH 8.0) at a flow rate of 0.5 ml/min. Only one protein peak with fibrinolytic activity could be purified (Fig. 2). Based on the purification scheme, the fibrinolytic enzyme produced by *B. tequilensis* CWD-67 was purified 34.7-fold with an overall yield and specific activity of 8.2% and 58.5 units/mg, respectively (Table 1).

**SDS-fibrin zymogram**

SDS-PAGE and fibrin-zymography of the purified enzyme were conducted to verify the enzyme purity and estimate its molecular mass. The molecular weight of the purified enzyme was estimated to be 22 by SDS-PAGE using a standard marker (Genei, India). Fibrin-zymography indicated that the protein purified from the culture supernatant of *B. tequilensis* CWD-67 showed fibrinolytic enzyme activity (Fig. 3). Taken together, these results indicated that the enzyme is a monomeric protein that differs from other fibrinolytic enzymes produced by the *Bacillus* species. The molecular mass of nattokinase from *B. natto*, which is known to be a direct fibrinolytic enzyme, is 28 kDa (Cao et al., 2009). However, fibrinolytic enzymes produced by *Bacillus* species have molecular masses varying from 20 to 41 kDa (Chang et al., 2012; Huang et al., 2013), while those produced by *Pseudomonas* sp. and *Streptomyces* sp. are 21 kDa, and 18 kDa, respectively (Mander et al., 2011; Wang et al., 2009). In contrast, the fibrinolytic enzyme isolated in this study was much less than that from *Bacillus subtilis* ICTF-1 (28 kDa) (Mahajan et al., 2012), while it was greater than that from *A. mellea* (20 kDa) (Lee et al., 2005) and (14 kDa) from *P. eryngii* (Cha et al., 2010).

**Effects of pH and temperature on enzyme activity and stability**

The effects of pH and temperature of the purified enzyme from *B. tequilensis* CWD-67 are presented in Fig. 4. The results showed that the fibrinolytic enzyme was active at pH 6.0–8.0, with the optimum activity occurring at pH 8.0 (Fig. 4a). The activity decreased greatly in the acidic and alkaline range, indicating that it is a neutral enzyme. The pH optimum of *B. tequilensis* CWD-67 coincides with that of subtilisin-like protease from *Bacillus subtilis* (Kim et al., 2006) and chymotrypsin-like serine metalloprotease from *Cordyceps militaris* (Choi et al.,...

### Table 1. Purification of fibrinolytic enzyme from *B. tequilensis* CWD-64.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Purification fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>329.6</td>
<td>1582.1</td>
<td>1.8</td>
<td>1.0</td>
<td>100.0</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation</td>
<td>106.2</td>
<td>930.9</td>
<td>5.0</td>
<td>5.8</td>
<td>77.2</td>
</tr>
<tr>
<td>DEAE-Cellulose</td>
<td>37.1</td>
<td>744.7</td>
<td>22.0</td>
<td>19.1</td>
<td>35.3</td>
</tr>
<tr>
<td>Sephadex G-75 FF</td>
<td>9.7</td>
<td>363.8</td>
<td>58.5</td>
<td>34.7</td>
<td>8.2</td>
</tr>
</tbody>
</table>

*Enzyme activity was measured using the synthetic substrate azocasein (see Section “Materials and Methods”).*
Fibrinolytic enzyme from *Bacillus tequilensis* 245

...2011), while its pH stability was wider than of *B. subtilis* (Kim et al., 2006). As shown in Fig. 4b, the enzyme was active at 35–50°C, exhibiting maximum activity at 45°C and showing a rapid decrease in activity above 50°C, mainly due to thermal denaturation (Choi et al., 2011). The enzyme was stable and retained more than 80% of its initial activity between 45°C and 50°C, but rapidly lost activity when the temperature increased above 55°C. The optimal temperature for the purified enzyme coincides with that of serine proteases from *B. subtilis* A26 (Agrebi et al., 2009). The thermal stability range for the reported enzyme was comparatively wider than that of chymotrypsin-like serine protease from *Paenibacillus polymyxa* EJS-3 (Lu et al., 2010) and chymotrypsin-like serine metalloprotease from *C. militaris* (Choi et al., 2011).

**Effects of inhibitors and metal ions on enzyme activity**

The effects of various inhibitors on enzyme activity were investigated using PMSF, Bestatin A, Pepstatin, EDTA, and TPCK. The purified enzyme was completely inactivated by PMSF, which is considered a serine protease inhibitor. Moreover, the purified enzymes were significantly inhibited by metalloprotease (EDTA) and chymotrypsin-like proteinase (TPCK) inhibitors, whereas acid protease inhibitor (pepstatin) and amidopeptidase inhibitor (Bestatin) did not exert an inhibitory effect (Table 2). Metal ions also exerted varied effects on the purified enzyme. Specifically, the activity was significantly inhibited by Co²⁺, Zn²⁺, and Cu²⁺, while it was slightly enhanced by Ca²⁺, Mg²⁺, and Fe²⁺. These findings suggest that the purified enzyme might belong to the chymotrypsin-like serine metalloprotease family, which is known to be involved in the treatment of thrombosis (Choi et al., 2011). The enzyme was similar to the fibrinolytic enzyme from *Fusarium* sp. CPCC 480097 (Wu et al., 2009) and *A. mellea* metalloprotease (AMMP), a fibrinolytic enzyme from cultured mycelia of *A. mellea* (Wu et al., 2009), which are known to be a chymotrypsin-like serine metalloprotease and chymotrypsin-like metalloprotease, respectively.

**Hydrolysis of fibrinogen by the purified enzyme**

To investigate the degradation patterns of fibrinogen, purified enzyme was assayed using the fibrin plate method. After incubation at 37°C, the enzyme formed a larger clear zone around the disc than that generated by an equal amount of plasmin, as illustrated in the photograph taken at 12 h (Fig. 5). Since the area of the clear zone is directly

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**Table 2. Effect of metal ions and inhibitors on the activity of fibrinolytic enzyme.**

<table>
<thead>
<tr>
<th>Metal ions or inhibitors</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>PMSF</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>2</td>
<td>97 ± 1.5</td>
</tr>
<tr>
<td>Bestatin</td>
<td>2</td>
<td>94 ± 0.7</td>
</tr>
<tr>
<td>EDTA</td>
<td>2</td>
<td>9 ± 2.5</td>
</tr>
<tr>
<td>TPCK</td>
<td>2</td>
<td>4 ± 1.4</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>5</td>
<td>92 ± 5</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>5</td>
<td>34 ± 1.3</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>5</td>
<td>100 ± 0.6</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>5</td>
<td>39 ± 0.3</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>5</td>
<td>27 ± 0.4</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>5</td>
<td>99 ± 3.7</td>
</tr>
</tbody>
</table>

*Values are the mean ±SD of three experiments.*

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**Fig. 4.** (a) Effect of pH on the activity and stability of the fibrinolytic enzyme from *B. tequilensis* CWD-64. (b) Effect of temperature on the activity and stability of the fibrinolytic enzyme from *B. tequilensis* CWD-64.

**Fig. 5.** Analysis of fibrinolysis on fibrin-agarose plate: (1) Plasmin used as a standard, and (2) purified enzyme from *B. tequilensis* CWD-64.
proportional to the enzymatic activity, it can be assumed that the purified enzyme has a greater fibrinolytic activity than that of plasmin. Subsequently, the fibrinolytic activity of the enzyme was compared in the presence and absence of plasminogen. Additional plasminogen did not contribute to the enhancement of the clear zone, implying that the enzyme is a plasmin-like protease that directly degrades fibrin (data not shown). Fibrinogen is cross-linked by thrombin into fibrin, which has a structure that includes γ, α-, and β-chains, while a dimer γγ chain of fibrin is formed through Ca²⁺ cross-linking (Shen et al., 1975). The addition of the enzyme caused a clear hollow on the plasminogen-free plate, while the size of the clear hollow did not change obviously in the presence of plasminogen, suggesting that the purified enzyme was a plasmin-like, direct-acting, fibrinolytic protease that did not require endogenous fibrinolytic factors to lyse the thrombi. Hence, secondary effects, such as platelet activation related plasmin formation caused by plasminogen activators, could be avoided (Wu et al., 2009). The degradation pattern of fibrin by the purified enzyme showed that the enzyme preferentially hydrolyzed the α chain of fibrin, followed by the β chain, and finally the γγ chain, with a pattern similar to those of the enzymes from C. militaris (Choi et al., 2011) and Armillaria mellea (Lee et al., 2005).

Conclusion

In this study, a unique fibrinolytic enzyme was purified to homogeneity from Bacillus tequilensis CWD-67 via a three-step procedure with a 35-fold increase in specific activity and 8% recovery. The molecular weight of the purified enzyme was estimated to be 22 kDa by SDS-PAGE. The purified enzyme was homogenous on SDS-PAGE, forming a single band, suggesting that it was a monomer. The purified enzyme activity was completely inhibited by PMSF and significantly inhibited by EDTA, TPCK, CO²⁺, Zn²⁺, and Cu²⁺, indicating that the enzyme is a chymotrypsin-like serine metalloprotease. In addition, the enzyme can directly degrade fibrin with higher substrate specificity than the positive control (plasmin). Taking together, these findings indicate that the fibrinolytic enzyme from B. tequilensis CWD-67 is a potential candidate for the prevention and treatment of thrombosis. Microbes have the innate potential for synthesis of primary metabolites and can be regarded as potential biofactories for the synthesis of fibrinolytic enzymes. Further studies are needed to elucidate the effectiveness of thrombolysis by this enzyme in vivo.

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References


