Studies on Alkaline Serine Protease Produced by Bacillus clausii GMBE 22

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Abstract: An alkali tolerant Bacillus strain having extracellular serine alkaline protease activity was newly isolated from compost and identified as Bacillus clausii GMBE 22. An alkaline protease (AP22) was 4.66-fold purified in 51.5% yield from Bacillus clausii GMBE 22 by ethanol precipitation and DEAE-cellulose anion exchange chromatography. The purified enzyme was identified as serine protease by LC-ESI-MS analysis. Its complete inhibition by phenylmethanesulfonylfluoride (PMSF) also justified that it is a serine alkaline protease. The molecular weight of the enzyme is 25.4 kDa. Optimal temperature and pH values are 60°C and 12.0, respectively. The enzyme showed highest specificity to N-Suc-Ala-Ala-Pro-Phe-pNA. The K_m and k_cat values for hydrolysis of this substrate are 0.347 mM and 1141 min^{-1} respectively. The enzyme was affected
by surface active agents to varying extents. The enzyme is stable for 2 h at 30°C and pH 10.5. AP22 is also stable for 5 days over the pH range 9.0–11.0 at room temperature. AP22 has good pH stability compared with the alkaline proteases belonging to other strains of *Bacillus clausii* reported in the literature.

**Keywords:** Alkaline protease, *Bacillus clausii*, Enzyme purification and characterisation, Serine protease

### INTRODUCTION

The strains of the *Bacillus* genus are of considerable importance for biotechnology due to their capability to produce some industrial enzymes having high pH optima, such as proteases, amylases, or xylanases.[1] Microbial alkaline proteases are among the most important groups of industrial enzymes produced commercially and are used in various industrial sectors, such as detergents, leather, and silver recovery from X-ray films.[2–4] Extensive studies have been performed on alkaline proteases from other alkaliphilic *Bacillus* species; however, few such studies on the different types of *B. clausii* strains have appeared in the literature.[5–8] It is very important to find microorganisms producing enzymes with better properties suitable for commercial exploitation. The activity and stability of the alkaline protease over a broader range of pH would definitely make this enzyme an important candidate for various industrial applications. In this paper, we are reporting the *B. clausii* GMBE 22 having some different phenotypic and genotypic properties from the other *B. clausii* strains and the purification and characterization of the alkaline protease (AP22) having a good pH stability.

### EXPERIMENTAL

**Chemicals**

Chemicals used in the cultivation of the microorganism were supplied by Oxoid Ltd (Hampshire, England) and Merck AG (Darmstadt, Germany). All other chemicals used were of analytical grade and obtained either from Merck AG (Darmstadt, Germany) or Sigma Chem. Co., Ltd., (St Louis, USA).

**Identification of the Strain GMBE 22**

The strain GMBE 22 was isolated from compost according to procedures described by Denizci et al.[5] Reference strain *B. clausii* DSM 8716^T^ was
obtained from DSMZ (Germany). The phenotypic characters of the strain GMBE 22 were determined according to the methods described by Sneath et al.\[9\] and Fritze et al.\[10\] API 50 CHB gallery (Biomerieux, Marcy L’Etoile, France) was used for carbohydrate utilization tests. The cellular fatty acids were determined by the Sherlock-MIDI Automated Microbial Identification System (MIDI Inc., Newark, DE, USA) according to the procedures described in the MIDI user manual. The 16S rDNA gene of strain GMBE 22 was amplified by polymerase chain reaction (PCR) using the primers f27 (5'-AGAGTTTGATCMTGGCT CAG-3') and U1492r (5’-ACCTTGTTACGACTT-3'); afterwards, sequence analysis was performed with an ABI 310 DNA sequencer. Homology search (GenBank = EMBL = DDBJ) was carried out by using the Basic Local Alignment Search Tools (BLAST) program\[11\] and 16S rDNA sequences were aligned by the Clustal multiple alignment program (Clustal W 1.8).\[12\] A phylogenetic tree was displayed by the neighbor-joining method.\[13\] The 16S rRNA sequence of the strain GMBE 22 reported in this article has been submitted to the NCBI database under Genbank accession number DQ131908.

Microorganism and Cultivation

*B. clausii* GMBE 22 was used for production of alkaline protease (AP22). Enzyme production was carried out in a medium (100 mL) containing (w/v) 1.0% starch, 0.5% yeast extract, 0.1% K₂HPO₄ and 0.02% MgSO₄·7H₂O in 500 mL Erlenmeyer flasks. Flasks were incubated at 37°C by shaking at 160 rpm for 72 hours after inoculation with 18 h old inoculum culture at 1% (v/v) ratio. The medium pH was adjusted to 10.0 by aseptic addition of 10% Na₂CO₃ solution after sterilization.

Purification of Alkaline Protease

After 48-hour cultivation, *B. clausii* cells were removed from the culture medium by centrifugation (6,000 rpm, 30 min, 0–4°C) and the culture filtrate was fractionated by the addition of ethanol. Only the proteins precipitated between 40 and 80% of volume fraction of ethanol were collected by centrifugation (12,000 rpm, 30 min, 0–4°C) and the remaining ethanol in the precipitate was removed. The precipitate was dissolved in 50 mM glycine-NaOH buffer, pH 10.5 and applied to a DEAE-cellulose column (25 × 2.5 cm diameter). Elution was carried out with 50 mM glycine-NaOH buffer, pH 10.5 at a 15 mL min⁻¹ flow rate. The alkaline protease was eluted as the first major protein peak. Those fractions were collected, pooled, and used for characterization studies.
Determination of Alkaline Protease Activity

The method described by Takami et al.\cite{14} was used for determination of alkaline protease activity. One unit of alkaline protease activity was defined as the amount of enzyme able to produce one µg tyrosine per minute under the assay conditions. Activity measurements were repeated five times, and the results were calculated as mean values. The standard errors of the mean values were lower than 4%.

Protein Measurement

Protein was measured by the Coomassie Blue G-250 binding method using bovine serum albumin as the standard.\cite{15,16}

Electrophoretic Analysis of Enzyme Purity

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli\cite{17} using 5% (w/v) stacking gel and 12% (w/v) resolving gel. Electrophoresis was performed at a constant 100 V for 120 min. Enzyme samples were denatured by boiling for 3 min in the presence of 5 mM PMSF before loading onto the gel. After electrophoresis, proteins in the separating gel were visualized by silver staining.\cite{18} Fermentas Protein Molecular Weight Marker SM0431 containing seven proteins within the 14.4–116 kDa range was used in order to determine the molecular weight of the enzyme with an Image Analyzer System (UVI, BTS-20.M).

Peptide Mass Mapping

Peptide mass mapping was performed by LC/MS (Waters/Micromass). The purified alkaline protease visualized on SDS-PAGE gel by Coomassie blue staining as a major protein band was cut out of the gel, destained from the Coomassie blue, and digested with trypsin. Peptide masses obtained were searched against a comprehensive non-redundant protein sequence database (NCBI-nr) using the Mascot program for protein identification.

Estimation of the Temperature and pH Activity Profiles of Alkaline Protease

In the case of temperature-activity profile estimation of the enzyme AP22, 0.5 mL of enzyme solution (235 U/mg specific activity,
0.118 mg/mL protein) was mixed with 2.5 mL 0.6% Hammersten casein solution prepared in 50 mM glycine-NaOH buffer, pH 10.5 and incubated 20 min at various temperatures between 30 to 80°C in the presence and absence of Ca^{2+} ions. The pH-activity profile of the enzyme was determined at pH values in the range of 7.0 to 13.0. 0.5 mL of enzyme solution was mixed with 2.5 mL 0.6% Hammersten casein solution prepared in 50 mM sodium phosphate (pH 7.0–8.5), 50 mM glycine-NaOH (pH 9.0–12.0) and 200 mM glycine-NaOH (pH 12.5–13) buffers and incubated for 20 min at 30°C.

**Temperature and pH Stability Profiles of Alkaline Protease**

In order to determine the thermal stability profile of the enzyme AP22, 0.5 mL of enzyme solution (235 U/mg specific activity, 0.118 mg/mL protein) in 50 mM glycine-NaOH buffer, pH 10.5 was incubated at a temperature range between 30–70°C for 2 h and then residual activity at each temperature was measured. The thermal stability was expressed as % residual activity by taking the initial activity of the enzyme as 100% at each temperature studied. In the case of pH stability profile estimation of the enzyme, 0.05 mL of the enzyme solution in 50 mM glycine-NaOH buffer, pH 10.5 was mixed with 0.450 mL of buffers at pH ranging from 7.0–13.0. The mixtures were incubated for 5 days at room temperature and then residual activity at each pH value was measured. The pH stability was expressed as % residual activity by taking the initial activity of enzyme as 100% at each pH value studied.

**Effect of Active Site Inhibitors on Enzyme Activity**

The effects of active site inhibitors on protease activity were studied using diethyl-pyrocarbonate (DEPC), N-p-tosyl-L-lysine chloromethylketone (TLCK), and Np-tosyl-L-phenylalaninechloro-methyl ketone (TPCK) for the modification of histidine residues, phenethylmethylsulfonylfluoride (PMSF), ethylacetimidate, phenylglyoxal, N-bromosuccinimide, and N-ethyl-5-phenyl-iso-oxasolium-3-sulfonate for the modification of serine, lysine, arginine, tryptophan, and aspartate residues, respectively. Iodoacetic acid, iodoacetimidate, and N-ethylmaleimide were used for modification of cysteine residues. PMSF, N-bromosuccinimide, and TPCK were freshly prepared in absolute ethanol, acetone, and methanol, respectively as stock solutions, and were diluted to appropriate concentrations with 50 mM NaOH–glycine–NaCl pH 10.5. Other active site inhibitors were dissolved in 50 mM NaOH–glycine–NaCl buffer at pH 10.5. Enzyme solution (235 U/mg specific activity, 0.118 mg/mL protein)
was preincubated with 1 and 10 mM concentration of each inhibitor for 2 h at 30°C. The % residual activity of enzyme solution was measured by using casein as substrate.

Substrate Specificity

p-Nitroanilide (pNA)-conjugated synthetic peptide substrates, such as N-Suc-Ala-Ala-Pro-Phe-pNA, Nα-benzoyl-L-Arg-pNA (L-BAPNA), N-Suc-Ala-Ala-Ala-pNA, and L-Leu-pNA were used to determine the substrate specificity of the enzyme. Dimethylsulfoxide (DMSO) was used to prepare a stock solution (5 mM) of each substrate. Each substrate stock solution (25 μL) was mixed with 465 μL 50 mM glycine–NaOH buffer, pH 10.5 and pre-incubated at 37°C for 5 min. Each reaction mixture was incubated for 10 min, after addition of 10 μL enzyme solution (646 U mg⁻¹ specific activity and 0.0557 mg mL⁻¹ protein concentration). 100 μL 2% (v/v) acetic acid solution was added to the reaction mixture in order to stop the reaction, and the absorbance of the mixture was measured at 410 nm. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 μmol pNA per minute at 37°C and pH 10.5.

Effect of Metal Ions on Alkaline Protease Activity

The effects of metal ions on alkaline protease (646 U mg⁻¹ specific activity, 0.0557 mg mL⁻¹ protein) were investigated by adding Mn²⁺, Ca²⁺, Fe²⁺, Co²⁺, Mg²⁺, and Cu²⁺ ions to the reaction mixture at a concentration of 5 mM. Relative enzyme activities were measured at 30°C.

Effect of Hydrogen Peroxide and Surface-Active Agents on Alkaline Protease Activity

The effect of hydrogen peroxide (H₂O₂) at 5%, 10%, and 15% concentrations (w/v) on alkaline protease activity (646 U mg⁻¹ specific activity, 0.0557 mg mL⁻¹ protein) was investigated by incubating the reaction mixture for different time intervals at 30°C and measuring the percent residual activity of enzyme at pH 10.5 and 30°C.

The effects of 0.2% (w/v) SDS, 1.0% (w/v) sodium perborate (SPB), Tween-20, Tween-40, Tween-60, and Tween-80 on alkaline protease activity (646 U mg⁻¹ specific activity, 0.0557 mg mL⁻¹ protein) were investigated by incubating the reaction mixtures for 1 h at 30°C and measuring the % residual activity of enzyme at pH 10.5 and 30°C. The activity of the enzyme having no surfactant was considered as 100%.
Determination of the Kinetic Parameters of Enzyme

The initial reaction rates for N-Suc-Ala-Ala-Pro-PhepNA were measured in the 0.2–0.5 μM concentration range of this substrate at 37°C and pH 10.5. The initial reaction rates for Hammerstein casein were measured at the 0.25–2.00 mg mL⁻¹ concentration range of this substrate at 30°C and pH 10.5. The $K_m$ and $V_{max}$ values of the purified enzyme for both substrates were determined according to Michaelis-Menten kinetics using a Lineweaver-Burk plot and were used to calculate $k_{cat}$ and $k_{cat}/K_m$ values. The $k_{cat}$ values were calculated from the equation $V_{max} = k_{cat} \times [E]_t$, where $[E]_t$ is the total enzyme concentration in reaction mixtures.

RESULTS AND DISCUSSION

The Identification of Strain GMBE 22

Strain GMBE 22 was identified as the member of *Bacillus* species by considering its morphological and physiological characteristics. It has white colonies on agar plates with 1–3 mm diameters. The strain is Gram-positive, strictly aerobic, motile, sporulating, and rod-shaped (2.6 × 0.7 μm) bacterium. Its spores are ellipsoidal and subterminally located in swollen sporangium. The phenotypes of the strain GMBE 22 and the reference strain *B. clausii* DSM 8716ᵀ are found to be nearly identical, according to 38 characteristics tested (Table 1). The carbohydrate utilization patterns of both strains showed similarity in 37 of the 49 tests (Table 2). The overall biochemical and physiological traits suggest that this strain can be placed into the phylogenetic group 6 among the members of the *Bacillus* species.¹⁹,²⁰ The major fatty acid components in the cellular fatty acid composition of strain GMBE 22 were found to be iso-C 15:0 (46,04 %) and ante-iso-C 15:0 (12,36 %). (Table 3). These fatty acids were also found as major components of strain *B. clausii* DSM 8716ᵀ. The strain GMBE 22 is in the same cluster of phylogenetic tree (Figure 1) with different strains of alkalophilic *B. clausii* such as *B. clausii* LMG 19610, *B. clausii* LMG 19634, *B. clausii* DSM 8716ᵀ and *B. clausii* GMBAE 42.⁵ The results related to 16S rRNA sequence and cellular fatty acid composition analyses indicates that it is a different strain of *B. clausii*.

Purification and Identification of the Alkaline Protease Strain GMBE 22

The maximal alkaline protease (AP22) production by *B. clausii* GMBE 22 was obtained within 48 h of cultivation (Figure 2). Table 4 shows
Table 1. Phenotypic properties of the strain GMBE 22 and reference strain *B. clausii* DSM 8716<sup>T</sup>

<table>
<thead>
<tr>
<th>Property</th>
<th>GMBE 22</th>
<th><em>B. clausii</em> DSM 8716&lt;sup&gt;T&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Form</td>
<td>Rod</td>
<td>Rod</td>
</tr>
<tr>
<td>Mobility</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Spore</td>
<td>+ (subterminal)</td>
<td>+ (subterminal to paracentral)</td>
</tr>
<tr>
<td>Gram stain</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>NO&lt;sub&gt;3&lt;/sub&gt; to NO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td><strong>Growth at</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5&lt;sup&gt;°&lt;/sup&gt;C</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>30&lt;sup&gt;°&lt;/sup&gt;C</td>
<td>+</td>
<td>+</td>
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<tr>
<td>40&lt;sup&gt;°&lt;/sup&gt;C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>50&lt;sup&gt;°&lt;/sup&gt;C</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>55&lt;sup&gt;°&lt;/sup&gt;C</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>pH 5.7</td>
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<td>pH 6</td>
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<td>pH 7</td>
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<td>pH 8</td>
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<tr>
<td>pH 9</td>
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<td>+</td>
</tr>
<tr>
<td>pH 10</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Growth in</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% 2 NaCl</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>% 5 NaCl</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>% 7 NaCl</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>% 10 NaCl</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VP test</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>pH in VP Broth &lt;6</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>pH in VP Broth &gt;7</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Hydrolysis of</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cellulose</td>
<td>−</td>
<td>ND</td>
</tr>
<tr>
<td>Production of</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>dihydroxyacetone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Deamination of</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>phenylalanin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Utilization of citrate</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Indol production</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Degradation of tyrosine</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Growth with lysozyme</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*Results of Denizci et al.,<sup>[5]</sup> ND; No data available.*
Table 2. Carbohydrate utilization patterns of the strain GMBAE 22 and reference strain *B. clausii* DSM 8716\(^T\)

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>GMBAE 22</th>
<th><em>B. clausii</em> DSM 8716(^T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Erythritol</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>D-Arabinose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Arabinose</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Ribose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Xylose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adonitol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Methyl-D-Xyloside</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Glucose</td>
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<td>+</td>
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<tr>
<td>Fructose</td>
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<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sorbose</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Rhamnose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inositol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α-Methyl-D-Mannoside</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>α-Methyl-D-Glucoside</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N-Acetyl Glucosamine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Amygdalin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arbutin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Esculin</td>
<td>+</td>
<td>+</td>
</tr>
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<td>Salicin</td>
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<td>+</td>
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<td>Cellobiose</td>
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</tr>
<tr>
<td>Maltose</td>
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<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td></td>
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</tr>
<tr>
<td>Melibiose</td>
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<td>+</td>
</tr>
<tr>
<td>Saccharose</td>
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<tr>
<td>Trehalose</td>
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<td>+</td>
</tr>
<tr>
<td>Inulin</td>
<td></td>
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</tr>
<tr>
<td>Melezitose</td>
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<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
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<tr>
<td>Starch</td>
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<td>+</td>
</tr>
<tr>
<td>Glycogen</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Xylitol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gentiobiose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Turanose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-Lyxose</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>D-Tagatose</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(Continued)
the purification profile of the enzyme. AP22 was eluted in the unbound fractions of the anion exchanger DEAE-cellulose column (Figure 3a), since the alkaline proteases do not bind to the anion-exchange column materials due to their positive surface charges. A 4.66-fold purification of enzyme was obtained with 51.5% yield and 2,800 U mg$^{-1}$ specific activity. The SDS-PAGE analysis of the DEAE-cellulose column eluate of enzyme AP22 showed that the molecular weight of the enzyme is 25.4 kDa (Figure 3b).

Peptide mass mapping was used for the identification of alkaline protease. The matching of peptide masses was achieved to subtilisin savinase.

**Table 2.** Continued

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>GMBAE 22</th>
<th>*B. clausii DSM 8716$^{T}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Fucose</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>L-Fucose</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D-Arabitol</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>L-Arabitol</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Gluconate</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2-Keto-Gluconate</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5-Keto-Gluconate</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Results from Denizci et al.[5]*

**Table 3.** Cellular fatty acid compositions of the strain GMBE 22 and *B. clausii DSM 8716$^{T}$

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Strain GMBE 22</th>
<th>*B. clausii DSM 8716$^{T}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10:0</td>
<td>0.51</td>
<td>0.29</td>
</tr>
<tr>
<td>11:0 iso 3OH</td>
<td>2.22</td>
<td>–</td>
</tr>
<tr>
<td>iso-C 14:0</td>
<td>1.14</td>
<td>1.70</td>
</tr>
<tr>
<td>14:0</td>
<td>1.61</td>
<td>0.60</td>
</tr>
<tr>
<td>iso-C 15:0</td>
<td>46.04</td>
<td>38.81</td>
</tr>
<tr>
<td>anteiso-C 15:0</td>
<td>12.36</td>
<td>17.76</td>
</tr>
<tr>
<td>16:1 $\omega$7c alcohol</td>
<td>7.19</td>
<td>3.32</td>
</tr>
<tr>
<td>iso-C 16:0</td>
<td>3.21</td>
<td>2.24</td>
</tr>
<tr>
<td>16:1 $\omega$1 Ic</td>
<td>0.87</td>
<td>2.72</td>
</tr>
<tr>
<td>C 16:0</td>
<td>5.09</td>
<td>2.06</td>
</tr>
<tr>
<td>iso 17:1 $\omega$10c</td>
<td>2.23</td>
<td>1.94</td>
</tr>
<tr>
<td>iso-C 17:0</td>
<td>6.67</td>
<td>16.35</td>
</tr>
<tr>
<td>anteiso-C 17:0</td>
<td>2.09</td>
<td>9.95</td>
</tr>
</tbody>
</table>

*Results from Denizci et al.[5]*
Alkaline Serine Protease Produced by *Bacillus clausii* GMBE 22

(alkaline protease), denoted as gil267048 with a protein score of 91. In view of the high sequence coverage, the enzyme AP22 was identified as serine alkaline protease (Table 5). Totally 19 mass values were searched, where matching mass values were found to be 17. The sequence coverage was 34% and the matched peptides were AQSVPWGISRVQAPAAHNR (1–19), GVLVVAA (144–180), QKNPSWSNVQIR (230–241) and NTATSLGSTNLYGSGLVNAEAATR (246–270).

**Figure 1.** Phylogenetic tree of *B. clausii* GMBE 22 associated with the other members of the *Bacillus* genus. The 16S rRNA sequence of *Geobacillus thermoleovorans* was chosen arbitrarily as the out-group sequence.
There are a few reports available in the literature for purification and characterisation of alkaline proteases from *B. clausii* strains. Joo et al.\(^{[21]}\) reported the partial purification of oxidant and SDS-stable alkaline protease from a *B. clausii* strain. Kazan et al.\(^{[22]}\) also reported the 16-fold purification of an alkaline protease from another *B. clausii* strain with 58% yield.

**Figure 2.** Time course of the growth and alkaline protease production of *B. clausii* GMBE 22.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (cm(^3))</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U mg(^{-1}))</th>
<th>Yield (%)</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Supernatant of culture fluid</td>
<td>200</td>
<td>12.63</td>
<td>7606</td>
<td>601</td>
<td>100.0</td>
<td>1.00</td>
</tr>
<tr>
<td>2. Fractional precipitation by ethanol at 40–80% saturation</td>
<td>14</td>
<td>4.32</td>
<td>6245</td>
<td>1444</td>
<td>82.0</td>
<td>2.40</td>
</tr>
<tr>
<td>3. Pooled fractions of DEAE-cellulose column chromatography</td>
<td>13</td>
<td>1.40</td>
<td>3920</td>
<td>2800</td>
<td>51.5</td>
<td>4.66</td>
</tr>
</tbody>
</table>
Temperature and pH Optimums and Stability Profiles of the Alkaline Protease AP22

The optimum temperature of the enzyme AP22 was found to be 60°C. Addition of 5 mM Ca\(^{+2}\) ions caused a shift of temperature optimum from 60 to 70°C (Fig. 3a). Similar results were obtained for extracellular alkaline proteases obtained from different *B. clausii* strains,\(^{[21,22]}\) *B. mojavensis*,\(^{[23]}\) *B. brevi*,\(^{[24]}\) and *B. patagoniensis*.\(^{[25]}\) On the other hand, the pH optimum of enzyme was found to be 12.0 (Fig. 4b). Generally, the commercial alkaline proteases obtained from *Bacillus* species shows

**Figure 3.** (a) Elution profile of alkaline protease from *B. clausii* GMBE 22 from a DEAE-cellulose column equilibrated and eluted with 50 mM glycine-NaOH buffer, pH 10.5. (b) SDS-PAGE of alkaline protease (12% gel, 100 V for 120 min in Tris-glycine buffer, pH 8.3). Lane 1: Protein molecular weight markers, Lane 2: DEAE-cellulose column eluate treated with PMSF.

**Table 5.** Identification of the purified enzyme AP22 by LC-ESI-MS analysis

<table>
<thead>
<tr>
<th>Matched protein</th>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>gi</td>
<td>267048</td>
<td>91</td>
</tr>
<tr>
<td>gi</td>
<td>6137335</td>
<td>91</td>
</tr>
<tr>
<td>gi</td>
<td>14278487</td>
<td>91</td>
</tr>
<tr>
<td>gi</td>
<td>47168367</td>
<td>91</td>
</tr>
<tr>
<td>gi</td>
<td>3114348</td>
<td>91</td>
</tr>
</tbody>
</table>
optimal pH values within the range of 8.0–12.0\(^{[7,21–23,25]}\) The optimum pH of AP22 was higher than that of alkaline proteases obtained from other \(B.\ clausii\) strains\(^{[21,22]}\) and also commercial alkaline protease (Savinase).\(^{[26]}\)

The enzyme AP22 was found to be stable for 2 h at 30°C and pH 10.5. Only 6.35% and 9.96% activity losses were observed at 40°C and 50°C, respectively. However, the stability was considerably decreased at elevated temperatures and activity completely disappeared at 70°C (Fig. 4a). Enzyme was also stable within the pH range 9.0–11.0 for five days at 30°C; however, 20.14 and 96.70% activity losses were observed at pH values 12.0 and 12.5, respectively (Fig. 4b). On the other hand, the majority of alkaline proteases were found to be stable for 10–60 min in the pH range 6.0–12.0. Joo et al.\(^{[21]}\) and Kazan et al.\(^{[22]}\) reported the only 3 days stability for alkaline proteases from the different strains of \(B.\ clausii\) at room temperature and in the same pH range. Considering its higher pH stability, AP22 can be proposed as a better additive for commercial detergents.

**Effect of Divalent Cations on Alkaline Protease Activity**

The influence of divalent cations on AP22 are given in Table 6. Although the strong inhibitory effects of Cu\(^{2+}\) ions on the alkaline proteases of some \(Bacillus\) sp. have been reported,\(^{[23,24,27–29]}\) Cu\(^{2+}\) acted as a strong enhancer of enzyme AP22 activity. Ca\(^{2+}\) and Mg\(^{2+}\) ions slightly inhibited the enzyme AP22; however, Fe\(^{2+}\) and Co\(^{2+}\) ions caused 50% and 40% inhibition of enzyme, respectively. Nevertheless the stimulatory effect...
of Ca$^{+2}$ and Mg$^{+2}$ ions on the alkaline proteases of various *Bacillus* sp were reported in the literature.[23,30,31]

### Effect of Surface Active Agents and H$_2$O$_2$ on Alkaline Protease Activity

The enzyme AP22 was stable for almost 1 hr in the presence of 1% (w/v) SPB but 0.2% (w/v) SDS caused a slight improvement of the stability of the enzyme. The enzyme showed 15–20% activity losses in the presence of the non-ionic surface active agents Tween-20, 40, 60 and 80 at 1.0% (w/v) concentration (Table 6). 51%, 43% and 31% Activity losses were observed on the enzyme activity at 5%, 10%, and 15% (w/v) H$_2$O$_2$ concentrations for 75 min incubation, respectively (Figure 5); however, the oxidant- and SDS-stable *B. clausii* alkaline protease reported by Joo et al.[21] retained more than 75% and 110% of its activity after 72h treatment with 5% SDS and 10% H$_2$O$_2$, respectively. Nevertheless, the alkaline protease of *B. clausii* GMBE 22 showed considerable stability towards SDS, non-ionic surfactants, and SPB, and thus can be considered as a potential additive for commercial detergents.

### Effect of Active Site Inhibitors on Alkaline Protease Activity

Among the active site directed irreversible inhibitors studied, only phenylmethylsulphonylfluoride (PMSF) at 1 and 10 mM concentrations caused complete activity loss of the enzyme AP22 within 2 hr at 30°C.

---

**Table 6.** Effect of metal ions and surface active agents on GMBE 22 alkaline protease activity (SPB: Sodium per borate, SDS: sodium dodecyl sulfate)

<table>
<thead>
<tr>
<th>Metal ions in alkaline protease solution</th>
<th>Residual enzyme activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free enzyme (FE)</td>
<td>100.00</td>
</tr>
<tr>
<td>FE + 5 mM CuSO$_4$</td>
<td>156.00</td>
</tr>
<tr>
<td>FE + 5 mM CaCl$_2$</td>
<td>82.00</td>
</tr>
<tr>
<td>FE + 5 mM MgCl$_2$</td>
<td>92.00</td>
</tr>
<tr>
<td>FE + 5 mM FeSO$_4$</td>
<td>54.00</td>
</tr>
<tr>
<td>FE + 5 mM CoCl$_2$</td>
<td>63.00</td>
</tr>
<tr>
<td>FE + 1.0% (w/v) SPB</td>
<td>97.70</td>
</tr>
<tr>
<td>FE + 0.2% (w/v) SDS</td>
<td>108.50</td>
</tr>
<tr>
<td>FE + 1.0% (w/v) Tween 20</td>
<td>87.50</td>
</tr>
<tr>
<td>FE + 1.0% (w/v) Tween 40</td>
<td>80.75</td>
</tr>
<tr>
<td>FE + 1.0% (w/v) Tween 60</td>
<td>84.00</td>
</tr>
<tr>
<td>FE + 1.0% (w/v) Tween 80</td>
<td>88.50</td>
</tr>
</tbody>
</table>
This indicates the existence of a serine residue at the active site of the enzyme; consequently, it can be considered as a serine alkaline protease.

Substrate Specificity and the Michaelis-Menten Kinetics Constants of Alkaline Protease

The substrate specificity of the enzyme was investigated by using various peptidyl-p-NAs. The highest specificity was observed for N-Suc-Ala-Ala-Pro-Phe-pNA (Table 7). The enzyme AP22 has relatively low specificity for L-leu-pNA, N-Suc-Ala-Ala-Ala-pNA, and Nα-Benzoil-L-Arginin-pNA compared to N-Suc-Ala-Pro-Phe-pNA, a specific substrate for chymotrypsin-like serine proteases.

The $K_m$ and $k_{cat}$ values of enzyme for hydrolysis of N-Suc-Ala-Ala-Pro-Phe-pNA were estimated as 0.347 mM and 1141 min$^{-1}$, respectively (Table 8). The $K_m$, $k_{cat}$ and $k_{cat}/K_m$ values for the hydrolysis of

![Figure 5. Effect of $H_2O_2$ as an oxidizing agent on alkaline protease activity.](image)

**Table 7.** Substrate specificity of *B. clausii* GMBE 22 alkaline protease towards oligopeptidyl p-nitroanilide (p-NA) substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (U mg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Suc-Ala-Ala-Pro-Phe-pNA</td>
<td>19.31</td>
</tr>
<tr>
<td>N-$\alpha$-Benzoil-L-Arginin-pNA</td>
<td>1.96</td>
</tr>
<tr>
<td>N-Suc-Ala-Ala-Ala-pNA</td>
<td>4.66</td>
</tr>
<tr>
<td>L-Leucine-pNA</td>
<td>1.27</td>
</tr>
</tbody>
</table>
Hammersten casein and N-Suc-Ala-Ala-Pro-Phe-pNA by AP22 are shown in Table 5. Comparison of the kinetic parameters indicates a higher specificity of the enzyme for the substrate N-Suc-Ala-Ala-Pro-Phe-pNA than that of Hammersten casein.

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**REFERENCES**


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