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ORIGINAL ARTICLE

Isolation, characterization, and genomic analysis of *Bacillus halotolerans* C1 as a robust alkaline protease source

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Abstract

Proteases constitute a major class of industrial enzymes, with alkaline proteases garnering significant interest due to their catalytic efficiency and stability under alkaline conditions, which are paramount for applications in detergents, waste treatment, and bioremediation. The escalating demand for biocatalysts that maintain functionality under polyextremophilic conditions, such as concurrent high temperature, alkaline pH, and organic solvents, drives the exploration of microbial diversity in underexplored ecological niches. In this study, we report the isolation and multi-faceted characterization of *Bacillus halotolerans* strain C1, a novel isolate from Iranian extreme environments, exhibiting exceptional alkaline protease production. Through a rigorous screening of 70 bacterial isolates, strain C1 was selected as the superior protease producer based on both qualitative and quantitative analyses. The protease was partially purified to homogeneity via ammonium sulfate precipitation and gel filtration chromatography, yielding a single band on SDS-PAGE corresponding to a molecular mass of approximately 27 kDa. The enzyme demonstrated a remarkably high specific activity of 5300 U/g. Biochemical profiling revealed unparalleled stability, retaining significant activity across a broad pH spectrum (5.0–11.0) and a wide thermal range (40–90 °C), with optimum activity observed at pH 7.0 and 70 °C (7299.67 U/g). Whole-genome sequencing unveiled a 4.121 Mb circular chromosome encoding 4121 predicted coding sequences. Comparative genomic analysis confirmed species-level identity as *B. halotolerans* (dDDH value > 98%), and identified a subtilisin-like serine protease gene (aprE) exhibiting 100% amino acid identity to its *B. subtilis* homolog, yet residing within a unique genomic locus. Notably, genome mining elucidated 12 biosynthetic gene clusters (BGCs) for secondary metabolites, including non-ribosomal peptide synthetases (NRPS) for fengycin, bacillaene, and the siderophore bacillibactin, alongside two distinct bacteriocin clusters encoding the sactipeptide subtilosin A and a novel class IV lanthipeptide. Concurrently, antimicrobial resistance (AMR) profiling identified genes conferring resistance to multiple drug classes, including macrolides (mphK), rifamycins (rphB), and cationic antimicrobial peptides (mprF), mediated through efflux pumps (ykkCD, bmr) and ribosomal protection proteins (vmlR). Critically, the co-localization of these stress-responsive elements with the protease-coding region suggests a genetically encoded, coordinated adaptive strategy to environmental extremism. Our findings posit *B. halotolerans* C1 as a formidable source of a robust, multi-tolerant alkaline protease and provide a comprehensive genomic blueprint that underscores the imperative of integrating phenotyping with genomic mining for the discovery and rational engineering of next-generation industrial biocatalysts.

Keywords Bacillus halotolerans · Alkaline protease · Extremozymes · Genome mining · Biosynthetic gene clusters · Antimicrobial resistance

Introduction

Proteases are a diverse group of enzymes that catalyze the hydrolysis of peptide bonds in proteins and represent one of the most extensively utilized classes of enzymes worldwide (Gurumallesh et al. 2019; Nandan and Nampoothiri 2020). Their broad utility spans various industrial sectors, including detergents, textiles, leather processing, dairy and food

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production, feather degradation, pharmaceuticals, and cosmetics (Mechri et al. 2017a, b). Proteases can be grouped according to their optimal functional pH range into alkaline, neutral, and acidic proteases (Maruthiah et al. 2013). Among these, alkaline proteases, characterized by their optimal activity at pH 9 to 11, are especially effective in hydrolyzing protein substrates in alkaline conditions, making them highly suitable for industrial applications such as detergent formulations (Mirzapour Kouhdasht et al. 2018; Pathak et al. 2020a, b).

Bacterial alkaline proteases are among the most widely utilized enzymes in industrial applications, particularly in the detergent sector, due to their high catalytic efficiency and stability under alkaline conditions. Prominent examples such as subtilisin Carlsberg, savinase, and subtilisin BPN have become key components in commercial detergent formulations (Gupta et al. 2002). Compared to plant- and animal-derived enzymes, microorganisms offer several benefits, including ease of cultivation, rapid growth rates, genetic manipulability, and cost-effective large-scale production (Gupta et al. 2002; Joo et al. 2002; Fasim et al. 2021; Cedeno et al. 2025). Among microbial producers, *Bacillus* species are especially noteworthy for their exceptional capacity to secrete large quantities of extracellular proteases, making them the primary source of commercially available alkaline proteases.

In recent years, significant research efforts have focused on characterizing enzymes derived from extremophilic microorganisms, including those adapted to high temperatures (thermophiles) (Dai et al. 2022), low temperatures (psychrophiles) (Santiago et al. 2016), acidic environments (acidophiles) (Parashar and Satyanarayana 2018), alkaline conditions (alkalophiles) (Srivastava and Khare 2025; Tadesse et al. 2025), saline habitats (halophiles) (Mokashe et al. 2018), as well as radiation-resistant (radiophiles) and high-pressure environments (barophiles) (Raddadi et al. 2015). Despite these advances, the current repertoire of extremozymes remains inadequate to satisfy the escalating requirements of industrial applications (Mesbah 2022). To meet the growing industrial demand for robust proteases with enhanced functional characteristics, the exploration of diverse ecological niches for novel microbial strains remains a critical strategy. Microorganisms adapted to extreme environments often produce enzymes with remarkable tolerance to harsh industrial conditions, such as elevated temperature, extreme pH, and high salinity (Maruthiah et al. 2013). In this context, salt-tolerant or facultative halophilic bacteria are of particular interest. These organisms not only survive but thrive in both saline and non-saline environments, positioning them as promising sources of salt-tolerant enzymes with broad biotechnological potential.

Among various microorganisms (Ashaolu et al. 2025; Boukeroui et al. 2025; Singh and Mani 2025), *B. halotolerans* has gained recognition as a highly promising source of industrially valuable proteases, with numerous studies characterizing its exceptional enzymatic properties. Among various *B. halotolerans* strains, several have demonstrated the capacity to produce proteases with robust characteristics suitable for diverse biotechnological applications. Notably, Dorra et al. (2018) isolated and characterized a serine alkaline protease from *B. halotolerans* CT2, highlighting its potential for industrial use (Dorra et al. 2018). Further investigations revealed even more remarkable enzymatic properties in *B. halotolerans* strain DS5, which produces an alkaline protease (designated prot DS5) exhibiting extraordinary stability under extreme conditions. Kinetic studies demonstrated that prot DS5 achieves peak activity (279.74 U/mL) within 12 h of cultivation at neutral pH (7.0). This protease displays remarkable adaptability, maintaining structural and functional integrity across a broad pH spectrum (6.0–12.0) and temperature range (40–60 °C), with optimal catalytic activity observed at pH 9.0 and 50 °C. While several *B. halotolerans* strains have been genomically characterized for traits like fengycin production (Shu et al. 2024), antifungal activity (Suji et al. 2024), and stress tolerance (Zhang et al. 2018), their potential as sources of industrially robust proteases remains unexplored. Notably, despite the species' known ecological versatility, no studies have linked its genomic features to high protease activity under extreme conditions, a critical gap given the demand for enzymes stable in harsh industrial processes. This study addressed this unmet need by screening *Bacillus* strains from diverse environmental niches across Iran to identify high-performance protease producers. We isolated bacterial strains from extreme habitats including hot springs, salt marshes, and industrial effluents, environments with high potential for harboring protease-adapted microorganisms. Through systematic qualitative (skim milk hydrolysis) and quantitative (casein digestion) assays, we identified *B. halotolerans* C1 as the most potent protease producer, demonstrating exceptional stability across pH (5–11) and temperature (40–90 °C) ranges. Our findings not only establish *B. halotolerans* as a promising source of industrial proteases but also provide the first genomic blueprint linking specific genetic features to extreme protease stability in this species, a valuable resource for future enzyme engineering efforts.

Materials and methods

Sample collection and strain isolation

Samples were collected from diverse geographical regions across Iran, encompassing the northern provinces, Isfahan, Kurdistan, and Hormozgan. Sampling sites included hot springs, paddy fields, forests, fish farm effluents, compost, grape juice, vinegar, and marshlands (Table 1). Following collection, all samples were transported under controlled conditions (4 °C) to the laboratory at Isfahan University of Technology for further processing. To facilitate the isolation of single bacterial colonies, serial dilutions of the collected samples were prepared. For cultivation, 100 µL aliquots from dilutions 10^{-3} to 10^{-7} were spread onto Luria-Bertani (LB) agar plates and incubated at 37 °C for 24 h. Following incubation, well-isolated single colonies from higher dilutions (10^{-5} to 10^{-7}) were selected and subcultured onto fresh LB plates to obtain axenic cultures.

Screening of protease-producing strains

Primary screening for protease production was conducted by streaking isolated colonies onto skim milk agar plates, followed by incubation at 37 °C for 48 h. Proteolytic activity was indicated by the formation of clear zones surrounding bacterial growth. Strains exhibiting the largest hydrolysis zone diameters were selected for subsequent enzymatic characterization (de Veras et al. 2018; Masi et al. 2021).

Table 1 Geographic coordinates of sampling locations across different regions of Iran

Sampling location	Geographic coordinates
Water of Cheshmeh Abgarm (Mahallat, Isfahan Province)	34.00784°N, 50.54971°E
Paddy field soil (Babol, Mazandaran Province)	36.529817°N, 52.720684°E
Sisangan forest soil (Mazandaran Province)	36.576177°N, 51.812767°E
Sarvelat forest soil (Gilan Province)	36.956474°N, 50.545023°E
Tea field soil (Kelachay, Gilan Province)	37.047424°N, 50.420184°E
Abgarm-e Genu spring (Hormozgan Province)	27.44863°N, 56.30408°E
Rainbow trout farm effluent	32.80039°N, 50.91794°E
Cattle manure compost	32.79508°N, 50.91354°E
Homemade grape syrup (Tiran vineyards)	32.71243°N, 51.12022°E
10-year grape vinegar (Tiran vineyards)	32.71243°N, 51.12022°E
10-year verjuice (Tiran vineyards)	32.71243°N, 51.12022°E
20-year grape vinegar (Kashan vineyards)	33.98371°N, 51.16188°E
Gavkhuni wetland soil (Isfahan Province)	32.29017°N, 52.8104°E
Nowshahr forest soil (Mazandaran Province)	36.61225°N, 51.54416°E

Quantitative assay of protease activity

Prior to inoculation into the fermentation medium, bacterial pre-cultures were prepared in LB broth and incubated at 37 °C for 18 h. For protease activity analysis at three time points (24, 48, and 72 h), 10% (v/v) of the pre-culture was transferred into 50 mL of four distinct media: skim milk, casein, glucose-based, and LB. The medium comprised 1% casein, peptone, $(NH_4)_2SO_4$, KH_2PO_4, $CaCl_2$, and $MgSO_4$. The glucose-based medium contained 0.7% casein, 7.5% peptone, NaCl, KH_2PO_4, $CaCl_2$, $MgSO_4$, and $FeSO_4$. The skim milk medium was formulated with 30 g/L skim milk and solidified with 15 g/L agar. LB solid medium consisted of 10 g/L NaCl, 10 g/L peptone, 5 g/L yeast extract, and 1.5% (w/v) agar. The precise composition of the casein medium was (g/L): casein (10), peptone (4), $(NH_4)_2SO_4$ (3), KH_2PO_4 (3), $CaCl_2$ (1.5), $MgSO_4$ (0.5). The glucose-based medium contained (g/L): casein (7), peptone (7.5), NaCl (2), KH_2PO_4 (3), $CaCl_2$ (2), $MgSO_4$ (2), $FeSO_4$ (1). The skim milk agar contained (g/L): skim milk (30), agar (15). Cultures were incubated in 2-L Erlenmeyer flasks at 37 °C with shaking at 160 rpm (Yang et al. 2021; Majeed et al. 2024). Following incubation, culture purity was first assessed by examining morphological consistency and Gram staining aliquots to check for obvious bacterial contamination. Selected strains were then re-streaked onto fresh LB agar to confirm axenic purity based on colony morphology before further analysis. The cultures were subsequently transferred into 50 mL centrifuge tubes and centrifuged at 5000×g for 20 min at 4 °C. The resulting supernatant was collected and used for enzymatic assays.

Protease activity was quantified using casein as the substrate. Specifically, 1 µL of 1% (w/v) casein in 50 mM potassium phosphate buffer (pH 7.0) was incubated with 200 µL of enzyme extract at 37 °C for 20 min in a water bath. The reaction was terminated by adding 1 mL of 110 mM trichloroacetic acid (TCA), followed by incubation at 37 °C for 30 min. The mixture was then centrifuged at 5,000×g for 5 min to remove precipitated proteins. An aliquot of 400 µL of the resulting supernatant was mixed with 1 mL of 0.05 M sodium carbonate and 200 µL of Folin-Ciocalteu's phenol reagent. The mixture was incubated at 37 °C for 30 min, centrifuged briefly (5000×g, 1–2 min, 4 °C), and the absorbance of the developed blue color was measured at 660 nm. Tyrosine was used to generate a standard curve. One unit of protease activity (U/mL) was calculated by the amount of enzyme required to produce 1 µmol of tyrosine per minute under the specified assay conditions. Finally, the enzyme activity in units per gram (U/g) of dry cell weight

was calculated using a two-step conversion: first determining activity per milliliter (U/mL) by dividing micromoles of tyrosine produced by reaction time and enzyme volume, then normalizing to biomass concentration by dividing U/mL by mg biomass per mL enzyme solution and multiplying by 1000 (convert mg to gr for improved readabillity) (Rostami and Nouri Inanlou 2022; Noskova et al. 2025).

$$enzyme\ u/ml = \frac{(\ maltose\ librated\ (u\ mol))\ (dilution\ factor)}{(time\ of\ assay\ (min))\ (\ volume\ of\ enzyme\ used(\ ml\ ))}$$

$$enzyme\ u/mg = \frac{enzyme\ u/ml}{\frac{mg\ solid}{ml\ enzyme}}$$

Commercial Savinase (Novozymes), served as a positive control. For each sample, a corresponding blank was prepared where enzyme was added to the reaction mixture after the addition of TCA, thus correcting for background absorbance.

Partial purification of protease and SDS-PAGE

Partial purification of protease enzyme was carried out using ammonium sulfate precipitation. Bacterial strains were first cultured in a selective medium containing 1.5% starch and incubated for 24 h. Following incubation, the cultures were centrifuged at 10,000 rpm (~12,000 × g) for 10 min at 4 °C to separate the cell-free supernatant. For precipitation, solid ammonium sulfate (ACS grade, Sigma-Aldrich) was added slowly to the supernatant on an ice bath with constant stirring to achieve 100% saturation (760 g/L). Ammonium sulfate was then gradually added to the supernatant with constant stirring to achieve saturation (100%) based on the volume, thereby inducing protein precipitation. The mixture was stirred continuously for 2 h at 4 °C and subsequently centrifuged at 15,000 rpm for 40 min at 4 °C. The resulting protein pellet was collected, while the supernatant was discarded. The pellet was re-suspended in 1 mL of deionized water and stored at 4 °C for immediate use or at – 20 °C for long-term storage. To eliminate residual ammonium sulfate, the enzyme solution was subjected to dialysis overnight at 4 °C against 20 mM Tris-HCl buffer (pH 8.0) using a 10 kDa MWCO membrane and applied onto a Superdex-75 column equilibrated with the same buffer. Each 2 ml fraction was collected and analyzed for the protease activity. Each step of purification was checked by 12% SDS-PAGE. SDS-PAGE was performed according to Laemmli (1970), with proteins visualized using Coomassie Brilliant Blue R-250 (Laemmli 1970). Destaining was carried out using a methanol: acetic acid: water (1:1:8) solution. The molecular weight of the purified protease was estimated by comparison with a BIO-RAD protein marker (#1610374).

Effect of pH and temperature on protease activity and stability

The stability and activity of protease enzymes were evaluated under varying pH and temperature conditions. Initially, the bacterial growth curves were established to determine the optimal growth phase, with strains cultured in nutrient-rich media at pH 7.0 and 37 °C for 72 h in triplicate. To assess the effect of pH on bacterial growth, cultures were incubated at pH values ranging from 6.0 to 9.0, adjusted using HCl and NaOH. Protease activity was assessed using a quantitative casein-based assay, and enzyme stability was examined across a pH range of 5.0–12.0 and temperatures between 40 and 100 °C. For pH stability tests, the enzyme was incubated in 100 mM buffer systems tailored to specific pH intervals: sodium acetate (pH 5.0–5.8), potassium phosphate (pH 6.0–7.0), Tris-HCl (pH 8.0–9.0), and glycine-NaOH (pH 9.0–13.0), following standard protocols. Temperature-dependent activity was evaluated by incubating the enzyme in its optimal pH buffer across incremental temperatures, and residual activity was recorded.

Genome sequencing and annotation

Genomic DNA was extracted from an overnight culture of strain C1 utilizing the DNeasy UltraClean Microbial Kit (Qiagen LLC, Germantown, MD, USA), following the manufacturer's instructions. The isolated DNA was subsequently employed for whole-genome library construction. Sequencing was executed by Novogene (UK) on the NovaSeq X Plus Series platform, utilizing paired-end 150 bp reads (PE150), which produced approximately 1 Gb of raw data. The initial quality evaluation of the sequencing reads was performed using FastQC v0.11.2 (Brown et al. 2017) to verify data integrity. High-quality reads were then assembled de novo with SPAdes v3.12 (Bankevich et al. 2012; Prjibelski et al. 2020). The resulting scaffolds were compared against existing *Bacillus* species genomes through BLAST to identify phylogenetically related strains. For comprehensive taxonomic and genomic similarity analysis, digital DNA-DNA hybridization (dDDH) and average nucleotide identity (ANI) were computed using various tools: GGDC 2.0 (https://ggdc.dsmz.de) (Meier-Kolthoff et al. 2013), the orthoANI function on the EzTaxon-e platform, and the ANIb algorithm via the JSpeciesWS web server (https://jspecies.ribohost.com/jspeciesws/#home). To enhance the contiguity and structural precision of the assembly, scaffolds were organized and refined using the CSAR-web server (Chen and Lu 2018). To provide robust phylogenetic placement and confirm the taxonomic assignment of strain C1, whole-genome-based phylogenetic analysis was performed using the Type (Strain) Genome Server (TYGS)

(Meier-Kolthoff and Göker 2019), a recommended platform for automated prokaryotic taxonomy. Branch support was assessed via 100 pseudo-bootstrap replicates. The visualization of the tree was accomplished using the iTOL online tool (https://itol.embl.de/) (Letunic and Bork 2021). Genome annotation and the identification of target genes were performed using the Rapid Annotation using Subsystem Technology (RAST) webserver (Aziz et al. 2008) and Prokka (Seemann 2014), both executed within the Galaxy platform with default parameters. To detect antimicrobial resistance (AMR) genes in the assembled genome, we performed computational screening using ABRicate with stringent parameters (minimum 80% identity and coverage) against multiple databases, including the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al. 2020) and ResFinder (Bortolaia et al. 2020). Additionally, we conducted genome mining to identify secondary metabolite biosynthetic gene clusters (BGCs) and bacteriocin-coding regions using AntiSMASH8 (Blin et al. 2025) and BAGEL5 (van Heel et al. 2018).

Statistical analysis

All experiments were performed in triplicate using three independent replicates ($n=3$). Prior to analysis, data were tested for assumptions of normality and homogeneity of variances using the Shapiro-Wilk test and Levene's test, respectively. Data meeting these assumptions were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post-hoc test for multiple comparisons. For data violating parametric assumptions, the non-parametric Kruskal-Wallis test with Dunn's post-hoc correction was applied. Results are presented as mean ± standard error (SE). Differences were considered statistically significant at $p<0.05$.

Results

Screening, production, and purification

In total, 70 strains were screened from various samples to produce clear hydrolysis zones on milk plates and then rescreened with fermentation culture. Seven strains exhibited higher enzyme activity. Comprehensive analysis of seven experimental strains (S1-S6, C1) and a Savinase control revealed three distinct pH-dependent activity profiles across 24 h, 48 h, and 72 h incubations. Under acidic conditions (pH 5, Fig. 1A), strains C1 and S5 demonstrated superior performance with significant early-phase activation (24 h→48 h). Strains S2 and S6 exhibited consistently low activity (all timepoints), suggesting either acidic pH inhibition or constitutive low-expression protease systems. Transitioning to neutral pH (7), all strains showed elevated absolute activity compared to acidic conditions (Fig. 1B). C1 maintained temporal consistency with sustained 24 h→48 h activation, while S3 emerged as a neutral-pH specialist, matching C1's performance. The Savinase control showed complete temporal invariance. Strikingly, all strains exhibited non-monotonic activity curves, with 48 h peaks declining by 72 h, suggesting potential protease instability or substrate depletion. Alkaline environments (pH 9) produced also divergent strain behaviors (Fig. 1C). While C1, S3, and S5 maintained 24 h→48 h activation, their early-phase (24 h) activities exceeded 72 h values by 12–15%.

Purification analysis indicated that following ammonium sulfate precipitation and subsequent Superdex-75 gel filtration chromatography, the target enzyme was obtained in a purified state with a yield of ~18% and a 5.7-fold purification. The specific activities of the purified enzyme were determined to be 4400 U/g, 4650 U/g, and 5300 U/g for samples S3, S5, and C1, respectively. SDS-PAGE analysis confirmed the enzyme's homogeneity, demonstrating a single band corresponding to a molecular mass of approximately 27.0 kDa (Fig. 2). Although SDS-PAGE analysis of strains C1, S3, and S5 showed bands migrating at similar positions (~27 kDa), this outcome is consistent with the fact that all tested isolates are *Bacillus* species, which typically produce subtilisin-like serine proteases of conserved molecular size. The observed similarity in molecular mass does not imply identical enzymatic performance; rather, it reflects the conserved nature of protease genes within the genus. Indeed, despite comparable banding patterns, strain C1 exhibited markedly higher specific activity (5300 U/mg) and superior pH and thermal stability compared to S3 and S5. Thus, the SDS-PAGE confirms homogeneity in protease molecular weight, while the biochemical assays reveal the functional differences that distinguish C1 as the superior producer.

Effect of temperature and pH on the activity and stability

The enzymatic activity of various strains was assessed across a wide range of pH values and temperatures, revealing distinct patterns of performance and stability. At pH 5.5, under weakly acidic conditions, strain C1 consistently exhibited significantly higher enzymatic activity compared to strains S3 and S5 (Fig. 3). For instance, at 40 °C for 20 min, strain C1 achieved an activity of 3065.00 ± 84.52 U/g, outperforming S3 (1910.67 ± 84.27 U/g) and S5 (1433.33 ± 30.10 U/g), with the differences confirmed statistically. Similar trends were observed at elevated temperatures, such as 60 °C and 70 °C, where C1 maintained superior activity, indicating

Fig. 1 Temporal protease activity profiles of bacterial isolates under acidic (**A**; pH 5), neutral (**B**; pH 7), and alkaline (**C**; pH 9) conditions. Strains are denoted by different colors (see key). Activity is expressed in units per gram of dry cell weight (U/g DCW). Data points represent the mean of three independent biological replicates ($n = 3$); error bars indicate ± standard error (SE). Statistical significance between strains at each time point was determined by one-way ANOVA with Tukey's HSD post-hoc test. Asterisks above bars denote significant differences for the indicated strain compared to others at that time point (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

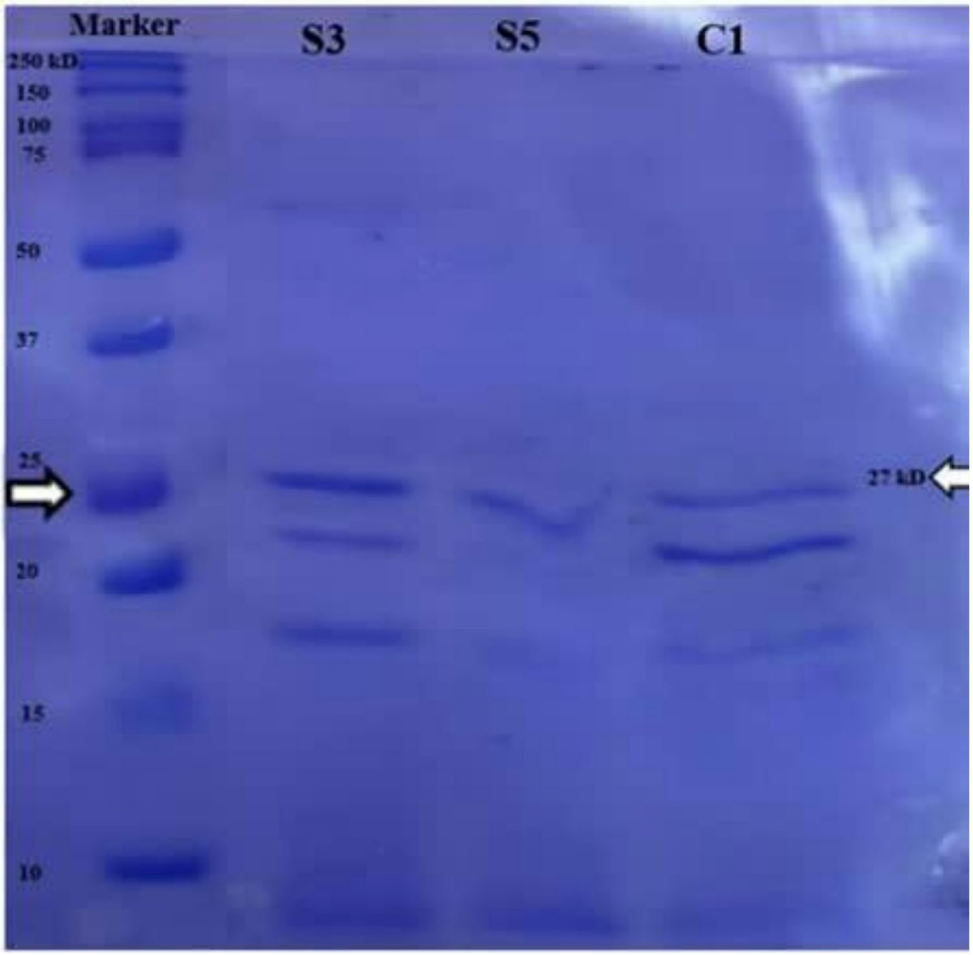

Fig. 2 SDS-PAGE analysis of partially purified proteases from strains S3, S5, and C1. Lane M: protein molecular weight marker (kDa); Lane S3: partially purified protease from strain S3; Lane S5: partially purified protease from strain S5; Lane C1: partially purified protease from strain C1. All three strains showed dominant band at ~27 kDa, consistent with subtilisin-like proteases typically secreted by Bacillus species. Although strains S3 and S5 were included for comparison at the screening stage, only strain C1 was selected for detailed characterization in this study due to its markedly higher specific activity and superior stability

enhanced thermostability. Notably, at 70 °C, C1's activity (3325.67 ± 348.76 U/g) was 2.7 and 3.4 times greater than that of S3 (1230.00 ± 160.53 U/g) and S5 (985.33 ± 57.68 U/g), respectively. Under thermal shock at 90 °C for 1 min, C1 retained significantly higher activity (2643.67 ± 24.01 U/g) than S5 (1364.33 ± 22.33 U/g), but did not significantly differ from S3 (2296.67 ± 36.61 U/g). Savinase, a commercial enzyme, surpassed all strains in this condition, reaching 4095.33 ± 327.91 U/g. However, at 80 °C for 20 min, all strains exhibited minimal or negative activity, suggesting substantial thermal denaturation. At neutral pH (7.0), enzymatic activity profiles shifted, with strain C1 again demonstrating exceptional performance. The highest activity recorded for this strain across all conditions occurred at 70 °C for 20 min (7299.67 ± 414.22 U/g), greatly exceeding S3 (2039.00 ± 47.47 U/g) and S5 (3100.00 ± 260.88 U/g). This suggests that pH 7 and 70 °C represent the optimal conditions for strain C1. At 60 °C (5510.00 ± 248.22 U/g) and 40 °C (5084.67 ± 302.15 U/g) for 20 min, C1 maintained significantly higher activity than S3 (2254.67 ± 51.96 U/g, 1627.67 ± 36.86 U/g) and S5 (2140.33 ± 191.00 U/g, 2226.67 ± 134.93 U/g). Following thermal shock at 90 °C, C1 retained high enzymatic activity (2875.00 ± 43.13 U/g), significantly outperforming S3 (2085.00 ± 90.38 U/g) and S5 (1440.33 ± 31.07 U/g). These findings highlight the remarkable thermal resistance of the C1 enzyme under neutral pH conditions. At pH 8, representing weakly alkaline conditions, strain C1 maintained superior enzymatic activity under most temperature treatments. At 90 °C for 1 min, C1 recorded 3814.00 ± 251.38 U/g, significantly higher than S3 (2302.33 ± 63.64 U/g), S5 (1484.00 ± 49.60 U/g), and Savinase (2177.67 ± 76.21 U/g). At 60 °C for 20 min, C1 again showed strong performance (6223.33 ± 216.06 U/g), though Savinase (7113.67 ± 318.58 U/g) surpassed all strains under this specific condition. Interestingly, S5 displayed no activity at 80 °C for 1 min (−38.33 ± 27.02 U/g), indicating particular sensitivity to this temperature and pH combination, unlike other strains which maintained substantial activity. At a strongly alkaline pH of 10, enzymatic activity patterns further diverged. Strain C1 remained the most robust, recording 2499.00 ± 91.96 U/g at 90 °C for 1 min, far exceeding S3 (1071.00 ± 75.40 U/g) and S5 (870.33 ± 67.14 U/g). At 80 °C for 1 min, C1 again demonstrated superior activity (3684.00 ± 139.22 U/g). Under the most alkaline condition

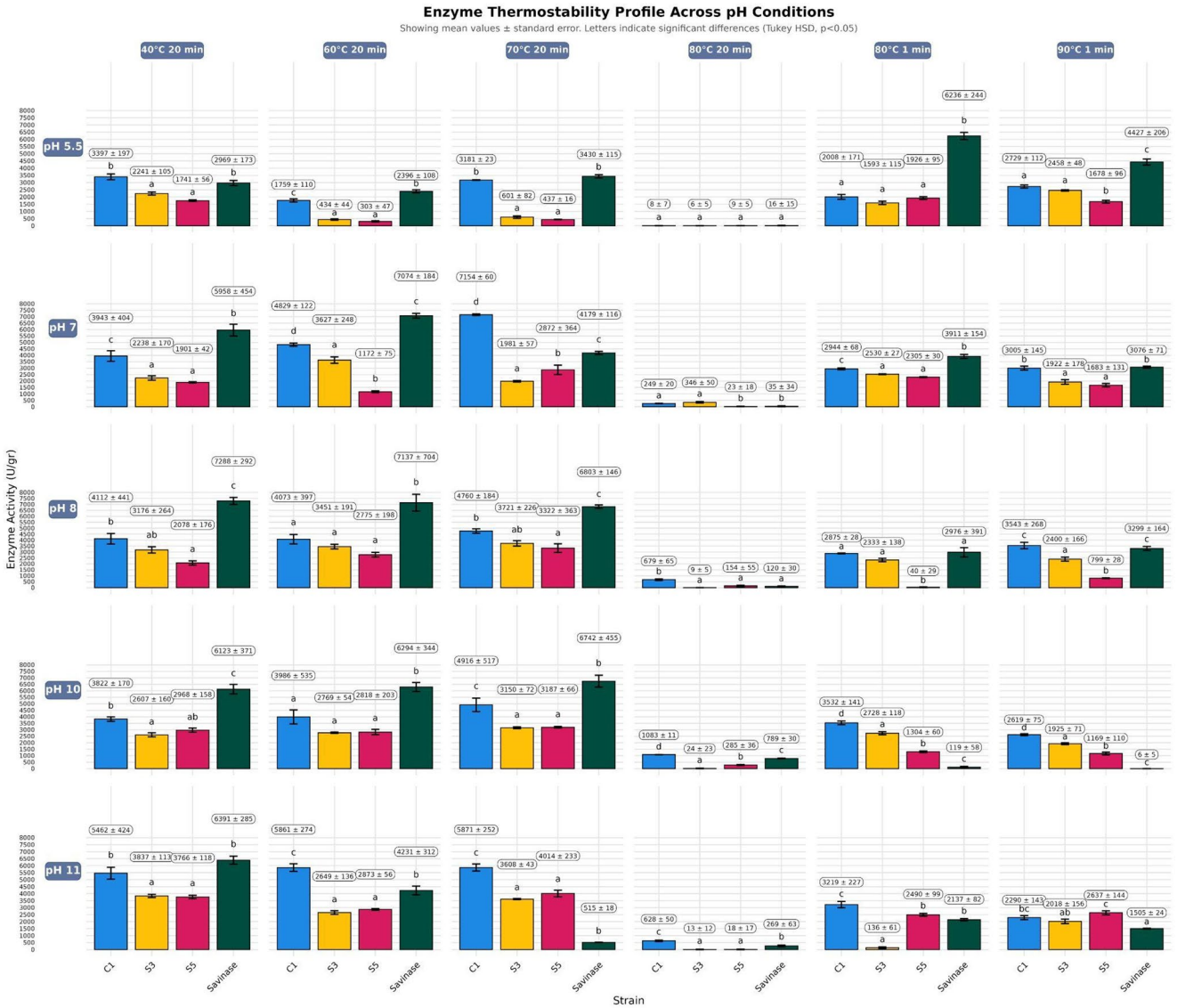

Fig. 3 Thermostability and pH tolerance of protease activity from strains C1, S3, and S5. Enzymatic activity (U/g DCW) was assessed across a pH gradient (5.5, 7.0, 8.0, 10.0, 11.0) and temperatures (40–90 °C; see x-axis). Data bars represent the mean of three independent biological replicates ($n=3$); error bars indicate ± standard error (SE). For each pH panel, bars labeled with different lowercase letters (a, b, c) are statistically significantly different ($p<0.05$, one-way ANOVA with Tukey's HSD post-hoc test). The absence of shared letters indicates a significant difference between strains under that specific condition

tested (pH 11), strain C1 maintained high enzymatic activity across most temperature treatments. At 40 °C for 20 min, its activity reached 5596.67 ± 158.15 U/g, surpassing S3 (2006.67 ± 121.66 U/g) and S5 (1955.00 ± 112.29 U/g). At 60 °C (4394.00 ± 81.97 U/g) and 70 °C (4142.33 ± 159.55 U/g) for 20 min, C1 again outperformed all other strains. A particularly notable observation was at 80 °C for 1 min, where strain S3 exhibited very low activity (564.33 ± 58.33 U/g), contrasting sharply with the robust activity levels of C1 (3180.33 ± 108.42 U/g) and S5 (3019.33 ± 29.87 U/g), indicating specific vulnerability of S3 to high pH and temperature combinations.

A comprehensive comparison of all strains highlights C1 as the most stable and efficient enzyme producer across diverse environmental conditions. This strain maintained enzymatic activity above 1000 units in 22 out of 25 experimental scenarios, with the highest activity observed at pH 7 and 70 °C (7299.67 U/g), suggesting these as optimal operating conditions. Furthermore, C1 uniquely sustains high activity in both strongly alkaline environments (pH 10 and 11) and under thermal stress (90 °C), a combination rarely achieved by other strains. Additionally, its capacity to function effectively under weakly acidic conditions further broadens its applicability. Although a reduction in activity

was noted during prolonged exposure to 80 °C, especially at pH 10, C1 still maintained substantial enzymatic performance, underscoring its remarkable thermal resistance and operational range. In contrast, strain S3 demonstrated moderate stability and maintained activity above 1000 units in 17 of 25 conditions. It performed particularly well under alkaline conditions, reaching its highest activity at pH 8 and 70 °C (3377.67 ± 122.07 U/g). However, it displayed significant sensitivity under more extreme environments, particularly at pH 11 and 80 °C. Overall, while S3 shows potential for use under moderately alkaline conditions, its application may be limited in more demanding industrial settings.

Genome sequencing

Strain C1 was selected for genome sequencing due to its overall higher activity under different conditions, demonstrating superior performance across various assays. Whole-genome sequencing of strain C1 revealed a genome size of 4,121,629 bp. The final assembly resulted in a single scaffold (N50 = 4,121,629 bp) with ~242x coverage, and the complete genome sequence was deposited in GenBank under accession number CP195069.1. Comparative genomic analysis identified the strain as *Bacillus halotolerans*, supported by dDDH analysis with a 98.43% probability that the DDH value exceeded 70%, coupled with an ANI value of 98.25% against the *B. halotolerans* type strain, strongly confirming species-level identity. The resulting tree, constructed from genome-scale data using the Genome BLAST Distance Phylogeny (GBDP) method (Fig. 4), provided a robust phylogenetic framework for species assignment. Strain C1 formed a highly supported monophyletic clade (100% bootstrap support) with the type strains *B. halotolerans* ATCC 25,096 and *B. halotolerans* DSM 8802 (Fig. 6). This genomic-based classification is further strongly supported by previously reported dDDH values exceeding the 70% threshold for species delineation. The phylogeny also clearly delineates *B. halotolerans* from its closest relatives, including *B. axarquiensis* and *B. malacitensis*, confirming the distinct species status of strain C1. Furthermore, the tree recapitulates the established phylogeny of the *B. subtilis* group, with *B. halotolerans* forming a distinct lineage separate from the clades containing *B. subtilis*, *B. mojavensis*, and *B. inaquosorum*.

Functional annotation using RAST server identified 4,426 features (Fig. 5), while Prokka revealed 4,121 coding sequences (CDSs), 8 rRNA genes, and 97 tRNA genes. This difference in CDS count is a known phenomenon attributable to the distinct algorithmic approaches. The higher number predicted by RAST is typical, as it often employs a more sensitive prediction model, while Prokka's conservative parameters yield a more specific set of CDSs, a well-documented discrepancy in genomic annotation. Notably, a subtilisin E protease gene (*aprE*) was identified, spanning 1,146 bp and encoding a 381-amino acid protein (39.48 kDa, pI 8.91) that showed 100% sequence identity to *B. subtilis aprE*. The apparent molecular weight of the purified protein (~27 kDa) differs from the predicted mass of the full-length *aprE* product; this discrepancy may be attributed to post-translational proteolytic processing to generate a mature active enzyme, a common feature observed in subtilisin-like proteases.

Genomic analysis of AMR and secondary metabolites

Genomic screening using ABRicate against the CARD and ResFinder databases revealed multiple AMR genes in *B. halotolerans* C1 (Table S1). The CARD analysis identified several efflux pumps, including ykkCD (conferring resistance to aminoglycosides, phenicols, and tetracyclines), bmr (associated with fluoroquinolone, chloramphenicol, and puromycin resistance), and lmrB (implicated in lincosamide resistance). Additionally, a rifampin phosphotransferase (*rphB*) was detected, which likely inactivates rifamycin-class antibiotics. The genome also harbored mprF, a gene involved in cationic peptide resistance, and vmlR, an ABC-F ribosomal protection protein conferring resistance to lincosamides, macrolides, and streptogramins. Screening with ResFinder confirmed the presence of mph(K), a macrolide phosphotransferase gene associated with resistance to spiramycin and telithromycin.

The genome of *B. halotolerans* C1, was analyzed using antiSMASH to identify potential biosynthetic gene clusters (BGCs) involved in secondary metabolite production. A total of 12 BGCs were predicted (Fig. 6, Table S2), encompassing diverse classes of bioactive compounds, including nonribosomal peptide synthetases (NRPS), polyketide synthases (PKS), terpenes, lanthipeptides, and other specialized metabolites. Among the identified BGCs, several exhibited high similarities to known clusters with established bioactivities. Region 2 (134,536–208,610 bp) displayed high confidence in similarity to the fengycin NRPS cluster, a lipopeptide with antifungal and surfactant properties. Region 3 (299,242–414,349 bp) was highly similar to the bacillaene hybrid PKS-NRPS cluster, a polyene antibiotic known for its role in microbial competition. Additionally, Region 8 (2,482,380–2,523,798 bp) showed high similarity to the bacilysin cluster, a dipeptide antibiotic, while Region 9 (2,525,724–2,547,336 bp) was closely related to subtilosin A, a ribosomally synthesized and post-translationally modified peptide (RiPP) with antimicrobial activity. Notably, Region 10 (3,073,538–3,139,649 bp) exhibited high similarity to the bacillibactin NRPS-metallophore cluster,

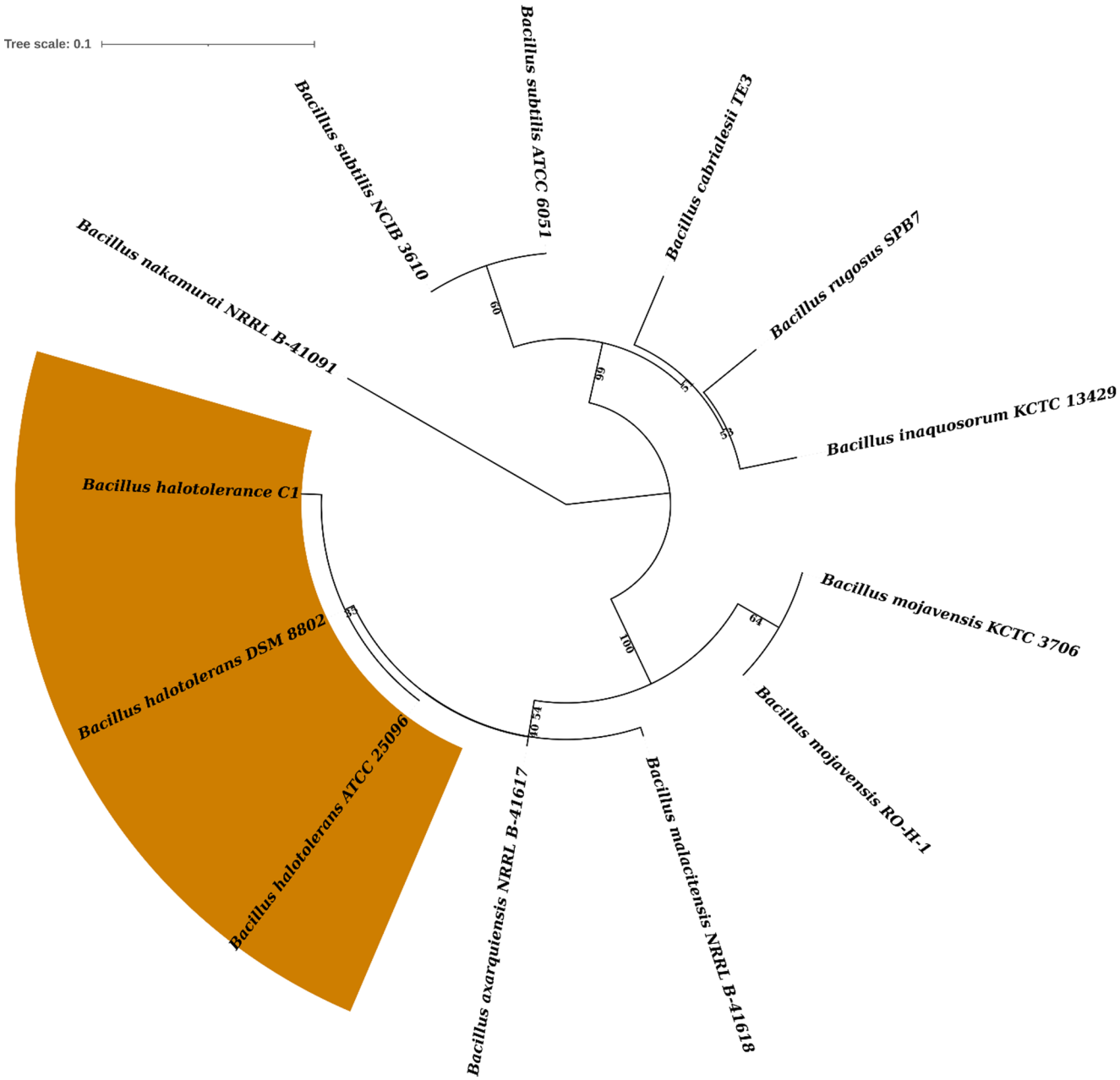

Fig. 4 Phylogenomic tree of *Bacillus halotolerans* C1 and related type strains. The tree was inferred from whole-genome sequences using the Genome BLAST Distance Phylogeny (GBDP) method on the TYGS platform. Branch support values (100 pseudo-bootstrap replicates) are shown at the nodes. The position of strain C1 is highlighted

involved in iron acquisition, whereas Region 6 (1,741,081–1,784,908 bp) showed low similarity to surfactin, a well-known biosurfactant. Other clusters, including terpenes (Regions 1, 4, 11), a class III lanthipeptide (Region 5), an epipeptide (Region 7), and a type III PKS (Region 12), were also detected.

Besides, BAGEL5 identified two Areas of Interest (AOIs) encoding potential antimicrobial peptides: AOI_01 (2,526,698–2,547,309 bp) harbors genes associated with Subtilosin A (145.1) and Subtilosin (SboX) (216.2), including an ABC transporter, BmbF, and multiple small ORFs, suggesting a functional cyclic bacteriocin cluster with thioether cross-links and self-immunity mechanisms, while AOI_02 (1,308,443–1,328,443 bp) contains a Class IV lanthipeptide biosynthesis system featuring a LanKC bifunctional enzyme, a protease for leader peptide cleavage, and an ABC transporter, indicative of a rare lanthipeptide with post-translational modifications.

Fig. 5 Nuclear genome circle diagram of *Bacillus halotolerans* C1

Discussion

This study presents the isolation, characterization, and genomic analysis of *B. halotolerans* C1, a bacterial strain exhibiting exceptional protease production with remarkable pH and thermal stability. Through comprehensive screening of 70 bacterial isolates from diverse ecological niches across Iran, strain C1 demonstrated superior protease activity across a wide range of conditions (pH 5–11 and 40–90 °C). The protease was partially purified, yielding a 27 kDa enzyme with specific activity of 5,300 U/g. Genomic characterization identified a subtilisin E protease gene (aprE) with 100% sequence identity to *B. subtilis* homologs, alongside multiple antimicrobial resistance genes and biosynthetic clusters that may contribute to the strain's industrial potential.

Importantly, the broad pH and temperature stability of the C1 protease distinguishes it from many existing enzymes. The enzyme's ability to maintain high activity levels at both acidic (pH 5) and strongly alkaline (pH 11) conditions, coupled with thermostability up to 90 °C, positions it as a versatile biocatalyst for diverse industrial processes. This multi-condition tolerance is particularly valuable for industries requiring robust enzymes that can withstand fluctuating or harsh processing environments (Gupta et al. 2002; Srivastava et al. 2025). Similar to our findings, Dorra et al. (2018) characterized a high molecular weight alkaline protease from *B. halotolerans* strain CT2 that demonstrated considerable stability at alkaline pH, though our C1 strain exhibits superior performance at both pH extremes (Dorra et al. 2018). The remarkable thermal stability of the C1 protease (maintaining > 70% activity at 90 °C) significantly exceeds the thermal tolerance reported for many commercial enzymes, including Savinase, which typically shows substantial activity loss above 60 °C (Mechri et al. 2017a, b). The molecular weight of the purified C1 protease (27 kDa) falls within the range typically observed for industrially relevant serine proteases from *Bacillus* species (25–45 kDa) (Vadlamani and Parcha 2011). Notably, this is considerably smaller than the 200 kDa protease reported from *B. halotolerans* CT2 (Dorra et al. 2018), suggesting structural differences that may contribute to the enhanced stability of our enzyme. The C1 protease's specific activity (5,300 U/g) indicates higher catalytic efficiency compared to other reported *B. halotolerans* proteases, such as the DS5 strain, which reached a peak activity of 279.74 U/mL (Wen et al. 2022). This high specific activity aligns with findings from other thermostable proteases, where compact protein structures often correlate with enhanced catalytic efficiency (Joshi and Satyanarayana 2013).

The distinctive temporal expression pattern of the C1 protease, with peak activity at 48 h followed by decline at 72 h, indicates specific regulatory mechanisms similar to those observed in other *Bacillus* species (Gupta et al. 2002; Joo et al. 2003). This characteristic expression pattern will be valuable for determining optimal harvesting times in commercial production systems, a critical factor for industrial enzyme production (Contesini et al. 2018). The decline in activity after 48 h suggests potential feedback inhibition or proteolytic degradation, mechanisms previously

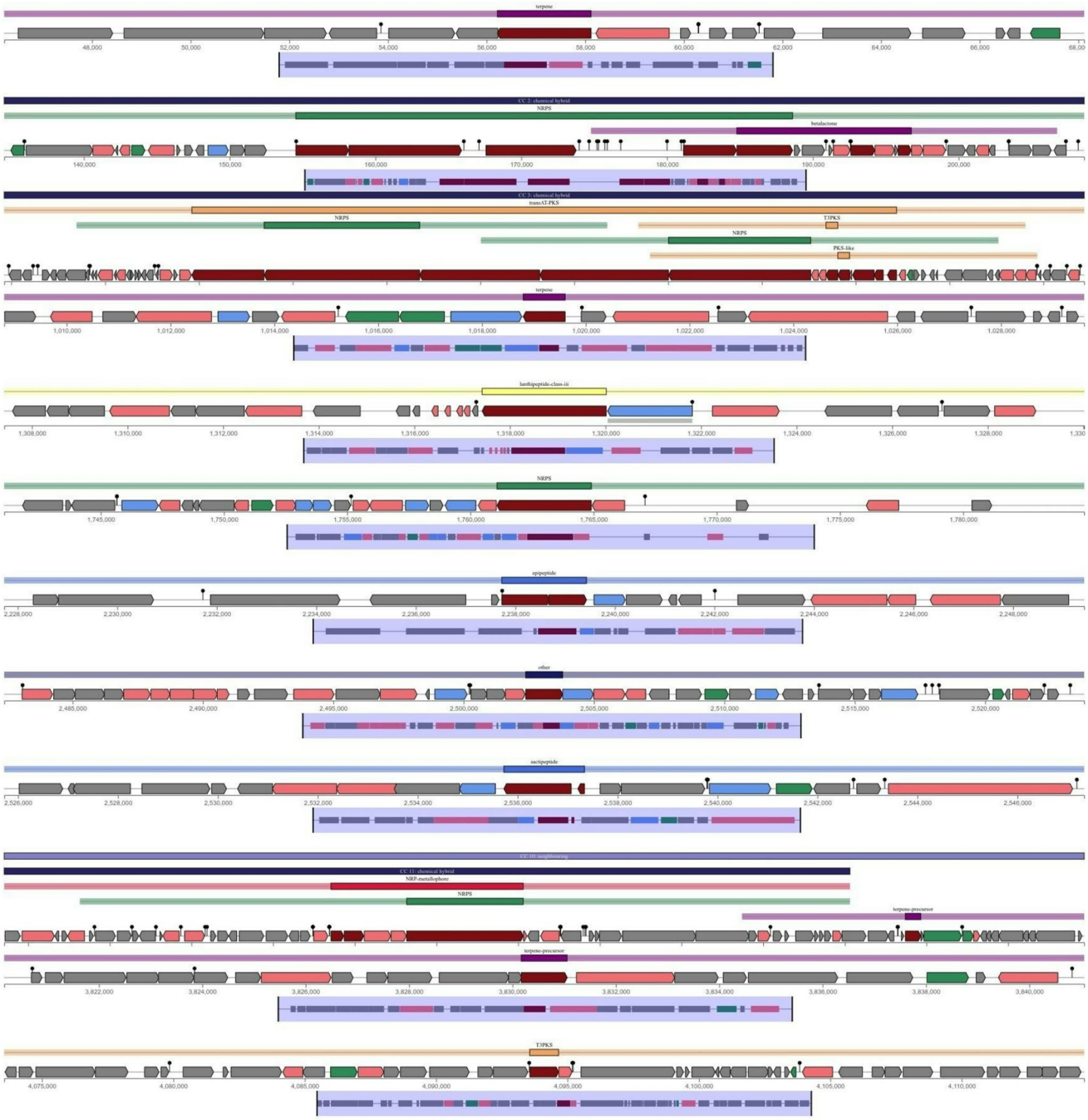

Fig. 6 Predicted secondary metabolite biosynthetic gene clusters (BGCs) in *Bacillus halotolerans* C1 identified using antiSMASH 8.0.1. 12 distinct BGCs encoding diverse metabolites, including non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS), terpenes, lanthipeptides, and RiPPs. In the diagram, dark red arrows represent core biosynthetic genes, pink arrows represent additional biosynthetic genes, blue arrows represent transport-related genes, green arrows represent regulatory genes, and gray arrows represent other genes in the cluster

documented in other *Bacillus* proteases (Perez-Garcia et al. 2011).

Our genomic analysis of *B. halotolerans* C1 provides critical insights into the genetic basis of its exceptional protease capabilities. The identification of the subtilisin E gene (*aprE*) with 100% sequence identity to *B. subtilis* homologs suggests potential horizontal gene transfer events in the evolutionary history of this strain, a phenomenon frequently observed among *Bacillus* species (Jers et al. 2025; Vesel et al. 2025). While the coding sequence shows high conservation, the exceptional stability of the C1 protease likely stems from unique regulatory mechanisms or post-translational

modifications influenced by other genomic elements (Sharma et al. 2017). The genomic analysis revealing multiple bacteriocin-related gene clusters, particularly the subtilosin A and lanthipeptide clusters, provides insight into the ecological role of this strain. The presence of these antimicrobial peptide biosynthetic pathways suggests a competitive strategy involving both enzymatic degradation of proteins and direct antimicrobial activity against competing microorganisms, a common ecological strategy among soil *Bacillus* species (Abriouel et al. 2011). Furthermore, the concomitant identification of AMR genes in the strain C1 genome underscores a multifaceted adaptive strategy for survival in competitive niches; however, it also necessitates careful attention to biosafety and the potential risks of horizontal gene transfer if the live organism were to be considered for applications in sensitive environments like food or feed. This combination of enzymatic and antimicrobial capabilities may explain the ecological success of *B. halotolerans* across diverse environments (Shu et al. 2024).

Despite the promising results, several limitations warrant consideration. While the *aprE* gene was identified, the regulatory elements controlling its expression under different environmental conditions remain unexplored. Future studies should investigate the regulatory architecture controlling C1 protease expression through transcriptomic analyses under various stress conditions (Kumar et al. 2008; Sharma et al. 2017). Additionally, the partial purification approach used in this study provides limited insight into the structural features contributing to the enzyme's remarkable stability. Advanced structural analyses employing X-ray crystallography or cryo-electron microscopy would elucidate the molecular basis of the observed multi-condition stability (Sarmiento et al. 2015; Elleuche et al. 2015). Future research should focus on heterologous expression and protein engineering to further enhance the industrial applicability of the C1 protease (Contesini et al. 2018), immobilization studies to enhance reusability and stability for industrial applications (Gupta et al. 2002; Paul et al. 2014), and application testing in specific industrial processes, including detergent formulation, waste treatment, and bioremediation (Shahid et al. 2016; Sharma et al. 2017). Additionally, exploration of potential synergistic applications leveraging both the proteolytic activity and antimicrobial properties of *B. halotolerans* C1, such as biofilm removal in industrial settings (Abriouel et al. 2011; Zheng et al. 2015), could reveal novel applications for this versatile organism.

Conclusion

This study establishes *B. halotolerans* C1 as a valuable microbial resource, producing a robust alkaline protease with exceptional stability across a broad pH (5–11) and temperature (40–90 °C) range. The enzyme's high specific activity and performance under polyextremophilic conditions position. Beyond its biochemical characterization, our genomic analysis provides a critical foundation for future work, revealing not only a highly conserved *aprE* gene but also a rich repertoire of biosynthetic gene clusters and stress-response elements that likely contribute to the strain's resilience. To translate this potential into practical applications, future research must address key experimental gaps. First, the precise regulatory mechanisms controlling the exceptional expression and stability of the protease require elucidation through transcriptomic and proteomic analyses under various stress conditions. Second, the enzyme's structure-function relationship must be deciphered via X-ray crystallography to identify the molecular determinants of its thermostability and pH tolerance, guiding rational protein engineering efforts. Finally, the hypothesized synergistic effects between the protease and co-produced antimicrobial metabolites (e.g., subtilosin A, lanthipeptides) need validation in real-world systems, such as in biofilm-based bioreactors or formulated detergents. By pursuing these specific directions, this research can transition from a promising in vitro discovery to a validated, next-generation biocatalyst for harsh industrial processes.

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Data availability The complete genome sequence of *Bacillus halotolerans* C1 has been deposited in the NCBI GenBank database under the accession number CP195069. The associated BioProject and BioSample accessions are PRJNA1273322 and SAMN48941539, respectively.

Declarations

Competing interests The authors declare no competing interests.

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

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