Brazilian Journal of Microbiology (2023) 54:725–737
https://doi.org/10.1007/s42770-023-00944-0

BIOTECHNOLOGY AND INDUSTRIAL MICROBIOLOGY - RESEARCH PAPER

Isolation and characterization of detergent-compatible amylase-, protease-, lipase-, and cellulase-producing bacteria

Kadriye Inan Bektas[1] · Aleyna Nalcaoğlu[1] · Esma Ceylan[2] · Disat Nigar Colak[3] · Pınar Caglar[1] · Sevda Agirman[1] · Nur Sena Sivri[1] · Sueda Gunes[1] · Alanur Kaya[1] · Sabriye Canakci[2] · Ali Osman Belduz[2]

Received: 12 July 2022 / Accepted: 28 February 2023 / Published online: 9 March 2023
© The Author(s) under exclusive licence to Sociedade Brasileira de Microbiologia 2023

Abstract

Detergent-compatible enzymes are the new trend followed by most in the detergent industry. Cellulases, lipases, proteases, and amylases are among the enzymes frequently used in detergents. Detergent-compatible enzymes can be obtained from many organisms, but the stability, cheapness, and availability of microbial enzymes make them preferable in industrial areas. In the present study, soil samples contaminated with household waste were collected from different regions of Trabzon (Turkey) for amylase-, cellulase-, protease-, and lipase-producing bacteria. A total of 55 bacterial isolates differing in colony morphology were purified from the samples and 25 of the isolates gave positive results in enzyme screening. The enzyme screening experiments revealed that 10 isolates produced amylase, 9 produced lipase, 7 produced cellulase, and 6 produced protease. While 2 isolates showed both protease and lipase activity, for 2 different isolates cellulose and amylase activity were detected together. It was also observed that one isolate, C37PLCA, produced all four enzymes. The morphological, physiological, and biochemical analyses of the bacteria from which we obtained the enzymes were performed and species close to them were determined using 16S rRNA sequences. Based on the results obtained, our enzymes show tremendous promise for the detergent industry.

Keywords Amylase · Cellulase · Detergent compatible · Lipase · Protease

Introduction

Bacteria have a wide distribution in the soil and soils typically contain 10^9 to 10^{10} microorganisms per gram (dry weight) [1]. The species of bacteria, their living conditions, and the enzymes produced by them may vary according to their ecological environment. Bacterial species with different growth conditions prefer different temperatures. While thermophilic bacteria generally grow at temperatures above 45 °C [2], the optimum temperature for mesophilic bacteria is between 20 and 45 °C [3]. Thermophilic and mesophilic microorganisms are promising for use in industrial applications because of the biotechnological potential of their enzymes such as for cellulases, hemicellulases, amylase, pectinases, xylanases, and proteases [4, 5].

Detergent enzymes constitute 25–30% of the enzyme market worldwide. Enzymes are considered "green chemicals" and they play a very important role in the detergent industry [6]. Their use in detergent formulations not only increases stain removal efficiency, but also makes these types of detergents more environmentally friendly and more reliable [7]. Detergent enzymes make an extra contribution to removing difficult stains. The main advantages of using an enzyme-containing biological detergent are its effectiveness at lower temperatures and faster washing performance, saving time, energy, water, and money. Detergent enzymes include four main classes and each of them has a different area of use. Of these enzymes, proteases are used to remove protein stains, lipases to break down oil residues in materials, amylases to remove starch-based food stains, and

Responsible Editor: Gisele Monteiro

✉ Kadriye Inan Bektas
kadriyensis@gmail.com

[1] Department of Molecular Biology and Genetic, Faculty of Sciences, Karadeniz Technical University, 61080 Trabzon, Turkey

[2] Department of Biology, Faculty of Sciences, Karadeniz Technical University, 61080 Trabzon, Turkey

[3] Department of Biology, Faculty of Sciences, Giresun University, Giresun, Turkey

cellulases to remove dirt by eliminating small cotton fibers that form on the fabric surface during use, thus helping to make the fabric smooth [8].

The current trend in the detergent industry is to save energy and preserve the quality of fabrics by using enzymes that are compatible with detergent formulations to produce detergents that act at lower temperatures. The importance of adding detergent-compatible enzymes to the content of detergents is obvious. Enzymes used as detergent components must be stable and effective under the desired conditions (alkaline pH, wide temperature range, etc.), and should be compatible and stable with various detergent components (organic solvents, surfactants etc.) [9].

Due to the importance of using detergent-compatible enzymes in the global enzyme market, the demand for these types of enzyme has rapidly increased [10, 11]. In general, most of the enzymes used in the detergent industry have some limitations, such as low activity and stability against anionic surfactants (SDS, chaotropic agents, and oxidants) [12]. Most of the commercial detergent-compatible enzymes are unstable at desired levels in the presence of detergent components such as surfactants and oxidizing agents [13]. Although various detergent-compatible proteases, lipases, amylases, and cellulases have been obtained during the last decade, intensive studies are still needed for enzymes with better industrial properties [14, 15].

Detergent-compatible enzymes can be produced by plants, animals, and microorganisms. However, the stability, cheapness, and availability of microbial enzymes make them preferable in industrial areas [16, 17]. Many studies concerning the isolation, production, purification, and characterization of microbial enzymes and their industrial applications have been reported. However, most of them focused on one or two enzymes. In this work, bacterial isolates from soil samples were screened for all four enzymes simultaneously. The aim of this study was to isolate and identify new bacteria from soil samples for the detergent industry and to determine their enzyme production potential.

Materials and methods

Sample collection

Soil samples contaminated with sink and washing machine waste were collected from the Besikduzu and Yomra districts of Trabzon (Turkey). The soil samples were collected aseptically in sterile culture tubes and transported to the laboratory as soon as possible. Soil samples were separately inoculated in TSB medium (1 g/100 ml) and incubated aerobically in shakers at 37 °C and 55 °C for 24–72 h. Growing cultures were diluted 10^{-1} to 10^{-10} and streaked directly onto TSA agar plates. The plates were incubated for 2 to 5 days at 37 °C and 55 °C. After bacterial growth was seen on the plates, single colonies with divergent morphologies were transferred onto new TSA plates and purified. The purified bacterial isolates were maintained in 20% glycerol at − 80 °C for further analyses [18].

Screening the enzyme activity of the bacterial isolates by plate assay

The bacterial isolates were grown on TSA plates for 24 h for preliminary qualitative screening of the four hydrolytic enzymes. Enzyme activity screening was performed on agar plates each including enzyme-specific substrates by the drop plate technique after incubation at 37 °C and 55 °C for 1–5 days. All experiments were performed in duplicate according to the standard protocols. The isolates that showed high activity were selected for further study.

The amylase activity of the isolates was determined using LB agar plates including 1% (w/v) starch as substrate. After incubation at 37 °C and 55 °C for 3 days, starch hydrolysis was detected by flooding the plates with Lugol's solution. Large clear zones around the colonies indicated starch consumption by the bacteria [19]. The lipase activity of the bacterial isolates was tested using the diffusion agar method on tributyrin agar medium containing 1% tributyrin (glycerol tributyrate). After incubation at 37 °C and 55 °C for 2 days, the colonies with a very visible clear zone were identified as lipase producers [20, 21]. Proteolytic activity was tested on agar medium containing 10% (w/v) skim milk. The bacterial isolates were incubated at 37 °C and 55 °C for 2 days. The observation of clear zones around the colonies indicated positive proteolytic activity [22]. Screening of cellulase activity was performed by incubating the bacterial isolates on carboxymethyl cellulose (CMC) agar plates containing 5% (w/v) CMC for 3 days at 37 °C and 55 °C. After this period, the plates were stained with Congo red (1% w/v) solution for 15 min and washed with 1 M NaCl for 15 min to remove the dye. The formation of clear zones around the colonies was considered to indicate cellulose activity [23].

Production of the crude enzymes

Based on plate screening, the bacterial isolates exhibiting enzyme activity were further analyzed by spectrophotometric assay. For enzyme production, the isolates were seeded in NB medium at pH 7.0 containing skim 10% milk, 10% starch, 5% olive oil, and 1% CMC. The bacterial cultures were incubated at 37 °C and 55 °C for 3 days. The cell-free supernatants obtained after centrifugation at 11,000 rpm for 7 min were used as crude enzyme extracts and assayed for respective enzymatic activity and partial characterization.

Amylase and cellulase assay

Amylase and cellulase activity were assayed using 1% (w/v) CMC and 1% (w/v) soluble starch as substrate, respectively. The reaction mixture included 100 µl of substrate solution and 100 µl of enzyme solution (cell-free supernatant) was incubated for 30 min at 50 °C. Then 500 µl of 3,5-dinitrosalicylic acid (DNS) was added to the mixture, which was boiled in a water bath for 10 min for color development. The color change in the samples was measured by spectrophotometer at 540 nm [24].

Lipase activity assay

Lipase activity was determined using p-nitrophenyl butyrate (pNPB) as substrate based on the spectrophotometric methodology. The substrate solution was prepared by dissolving 10 mM of pNPB in an acetonitrile, ethanol, and Tris–HCl buffer (50 mM, pH 8.0) mixture (1:4:95 (v/v/v) ratio, respectively). Next 0.9 ml of substrate solution and 0.1 ml of crude enzyme were mixed and incubated at 50 °C for 30 min. The enzyme activity was monitored at 410 nm spectrophotometrically [25].

Protease activity assay

The caseinolytic method described by Kunitz was modified for determination of protease activity. Azocasein was used as substrate. Crude enzyme extract (0.5 ml) was added to 1 ml of 1% azocasein solution in 50 mM Tris–HCl pH 8.0. The reaction mixture was incubated at 55 °C for 30 min. After incubation, 100 µl of 10% trichloroacetic acid (TCA) was added to the reaction mixture to terminate the reaction. The mixture was kept at room temperature for 15 min and centrifuged at $10{,}000 \times g$ for 5 min. The clear supernatant (300 µl) was removed in a clear microcentrifuge tube and 300 µl of 1% NaOH was added. Proteolytic activity was recorded at 440 nm using a spectrophotometer [26].

Characterization of enzymes

Effect of temperature on the activity and stability of the crude enzymes

To evaluate the optimal temperature of the enzyme activity, reactions were performed at different temperatures, ranging from 30 to 80 °C, using CMCase, protease, amylase, and lipase standard assays. The effect of temperature on enzyme stability was determined by pre-incubating the crude enzyme extracts at 30 °C and 50 °C without substrate. Aliquots were withdrawn at regular intervals to assay the residual activity under defined conditions. The untreated enzyme was considered the control (100%).

Effect of pH on activity and stability of the crude enzymes

The effect of pH on the activity of the crude enzymes was tested by using substrate solutions prepared in 50 mM of the following buffers: sodium citrate buffer (pH range 3.0–5.0), sodium phosphate buffer (pH range 6.0–8.0), and glycine–NaOH buffer (pH 9.0–10.0). Starch, casein, pNPB, and CMC were used as substrates for amylase, protease, lipase, and cellulase, respectively. The activity of the crude enzymes was determined under standard assay conditions at optimum temperatures. To determine the stability of the crude enzymes at pH 8.0, they were pre-incubated at pH 8.0 and 30 °C. The enzyme activity was measured at regular intervals to assay the residual activity under standard conditions.

Compatibility with detergents

Detergent compatibility of the selected enzymes was determined by standard enzyme assay. Four different commercial detergents were used at a concentration of 5 mg/ml ((A) Ariel, Procter & Gamble; (B) OMO Matik, Unilever; (C) Bingo, Hayat Kimya; and (D) Boron, Eti Maden). First, all of the commercial detergents were incubated at 60 °C for 3 h to inhibit the probable enzyme activity in the detergents; then they were mixed with the same concentration of crude enzymes (protease, amylase, lipase, and cellulase). The enzyme–detergent solutions were incubated at 50 °C for 60 min, followed by a standard enzyme assay for determination of the remaining activity [27].

Conventional analysis of the bacterial isolates

Conventional tests of the isolates were performed in duplicate. Gram and endospore staining were performed using the methods described by Harley and Prescott [28]. The temperature, pH, and sodium chloride (NaCl) demands of bacterial growth were detected by incubating strains in tryptic soy broth (TSB) medium at different temperatures (30–70 °C), pH (4.0–10.0), and NaCl concentrations (0–12%). The optimal temperature, pH, and NaCl concentration of bacterial growth were determined by measuring the absorbance of bacterial culture densities at 600 nm; periodical measurements were carried out between 12 and 76 h. The catalase and oxidase activity of the bacterial isolates were investigated as described by Maugeri et al. [29]. The atmospheric O_2 demands of the bacterial isolates were determined by incubating in brain heart infusion agar.

Molecular identification by 16S rRNA gene sequencing and phylogenetic analysis

The genomic DNAs of the bacterial isolates were extracted using a Wizard® Genomic DNA Purification Kit (Promega, USA).

Table 1 The results of the physiological tests of isolates

Isolates	Temperature (°C)		pH		NaCl (%)
	Growth range	Optimal	Growth range	Optimal	Growth range
1NP5A	30–50	37	5–9	7	0–5
C37-13	30–50	37	5–9	7	0–7
C55-8	40–60	50	4–10	8	0–10
C37-8	30–50	37	6–8	7	0–10
C37-5	30–40	37	5–10	8	0–5
C37-3	30–50	37	5–7	7	0–7
1NP7	30–50	37	5–9	7	0–5
4NL7C1	30–50	37	4–9	7	0–8
A37-3	30–40	37	5–8	8	0–5
A37-4	30–50	37	5–10	7	0–5
A55-3	30–60	45	5–9	7	0–10
A55-4	30–55	45	5–10	7	0–10
A55-8	30–50	45	5–10	7	0–10
C37-1	30–50	37	5–9	7	0–5
C37-4	30–60	37	4–10	8	0–7
1NP6	30–50	37	4–9	7	0–5
2NL3B	30–45	37	5–10	7	0–5
2NP4	30–45	37	5–10	7	0–8
2NL1	30–55	37	5–8	7	0–10
5NP2B2-3	30–45	37	5–9	8	0–8
5NP3D-1	30–50	37	5–9	7	0–5
5NP2B2-2	30–50	37	4–10	7	0–10
1NL100B	30–50	37	4–10	7	0–10
5NP2A3	30–50	37	5–10	8	0–8
C37PLCA	30–50	37	5–9	7	0–10

Table 2 The optimum temperature and pH of the crude amylase, cellulase, lipase, and protease

Amylase			Cellulase			Lipase			Protease		
	pH_{opt}	$Temp._{Opt}$		pH_{opt}	$Temp._{Opt}$		pH_{opt}	$Temp._{Opt}$		pH_{opt}	$Temp._{Opt}$
C37-1	7.0	50 °C	C37-1	9.0	60 °C	1NP6	10.0	60 °C	1NP6	9.0	50 °C
C37-4	6.0	50 °C	C37-3	9.0	40 °C	1NP7	10.0	60 °C	2NP4	8.0	50 °C
C37-5	8.0–9.0	50 °C	C37-4	7.0	50 °C	5NP2B2-2	8.0	30 °C	5NP2A3	9.0	60 °C
C37-8	8.0–9.0	50 °C	C55-8	7.0	50 °C	2NL3B	8.0	40 °C	5NP2B2-3	7.0	50 °C
C37-13	8.0–9.0	50 °C	A55-3	8.0	50 °C	1NL100B	8.0	40 °C	5NP3D-1	10.0	60 °C
A37-3	7.0	50 °C	4NL7C	9.0	50 °C	2NL1	9.0	50 °C	C37PLCA	8.0	50 °C
A37-4	7.0	50 °C	C37PLCA	8.0	60 °C	2NP4	8.0	60 °C			
A55-4	6.0	50 °C				1NP5A	10.0	40 °C			
A55-8	6.0	50 °C				C37PLCA	8.0	60 °C			
C37PLCA	8.0	50 °C									

The 16S rRNA sequences were amplified from the extracted DNA samples by PCR with the universal primers UNI16S-L (5′-ATTCTAGAGTTTGATCATGGCTCA-3′) and UNI16S-R (5′-ATGGTACCGTGTGA CGGGCGGTGTGTA-3′) [30]. The following PCR amplification conditions were used to amplify the 16S rRNA gene: initial denaturation at 95 °C for 2 min, denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 2 min, a total of 36 cycles, and a final polymerization step of 72 °C for 5 min. The PCR products were cloned in a cloning vector system (pGEM-T Easy, Promega, UK) and 16S rRNA gene sequences were determined with an Applied Biosystems model 373A DNA Sequencer (Macrogen, Netherlands). The isolates were identified by analyzing the 16S rRNA gene sequences using the GenBank Advanced Blast Program provided in National Center for Biotechnology Information resource and the EzBioCloud server (http://www.ezbiocloud.net) [31]. The

16S rRNA gene sequences of closely related type strains were downloaded from the EzBioCloud server and edited using the program BioEdit. Phylogenetic trees based on 16S rRNA gene sequences were reconstructed by three algorithms with neighbor-joining [32], using MEGA version 7.0 [33]. The evolutionary distance matrix was calculated according to Kimura's two-parameter model [34]. Bootstrap analysis based on 1000 replicates was also conducted in order to obtain confidence levels for the branches [35].

Results

Isolation of enzyme-producing bacterial strains and screening of the enzymes

In the present study, soil samples contaminated with household waste were screened for amylase-, cellulase-, protease-, and lipase-producing bacteria; enzyme-producing bacterial isolates were partially characterized; and the suitability of the enzymes for the detergent industry was investigated. Soil samples were collected from different regions of Trabzon (Turkey) and a total of 55 bacterial isolates differing in colony morphology were purified from the samples. Forty-eight of the isolates easily grew at 37 °C and the remaining seven isolates at 55 °C. The isolates were screened for protease, amylase, cellulase, and lipase production. It was observed that 25 of the isolates were positive for one or more of the enzymes screened. Ten of the isolates produced amylase (C37PLCA, C37-1, C37-4, C37-5, C37-8, C37-13, A37-3, A37-4, A55-4, and A55-8), 9 were positive for lipase (C37PLCA, 1NL100b, 2NL1, 2NL3b, 5NP2b2-2, 1NP6, 1NP7, 1NP5a, and 2NP4), 7 showed cellulase activity (C37PLCA, C37-1, C37-3, C37-4, C55-8, A55-3, and 4NL7c), and 6 produced protease (C37PLCA, 5NP203, 5NP3d-1, 5NP2b2-3, 2NP4, and 1NP6). While 2NP4 and 1NP6 showed both protease and lipase activity, C37-1 and C37-4 were positive for cellulase and amylase together. However, C37PLCA was able to produce all four enzymes. The optimum growing temperatures of the enzyme-producing isolates were mostly 37 °C except for A55-3, A55-4, and A55-8 (45 °C) and C55-8 (50 °C) (Table 1).

Effect of temperature and pH on the activity and stability of the crude enzymes

Among the screened isolates, the enzyme producers were inoculated into enriched media for enzyme production. The cell-free supernatants obtained after centrifugation were

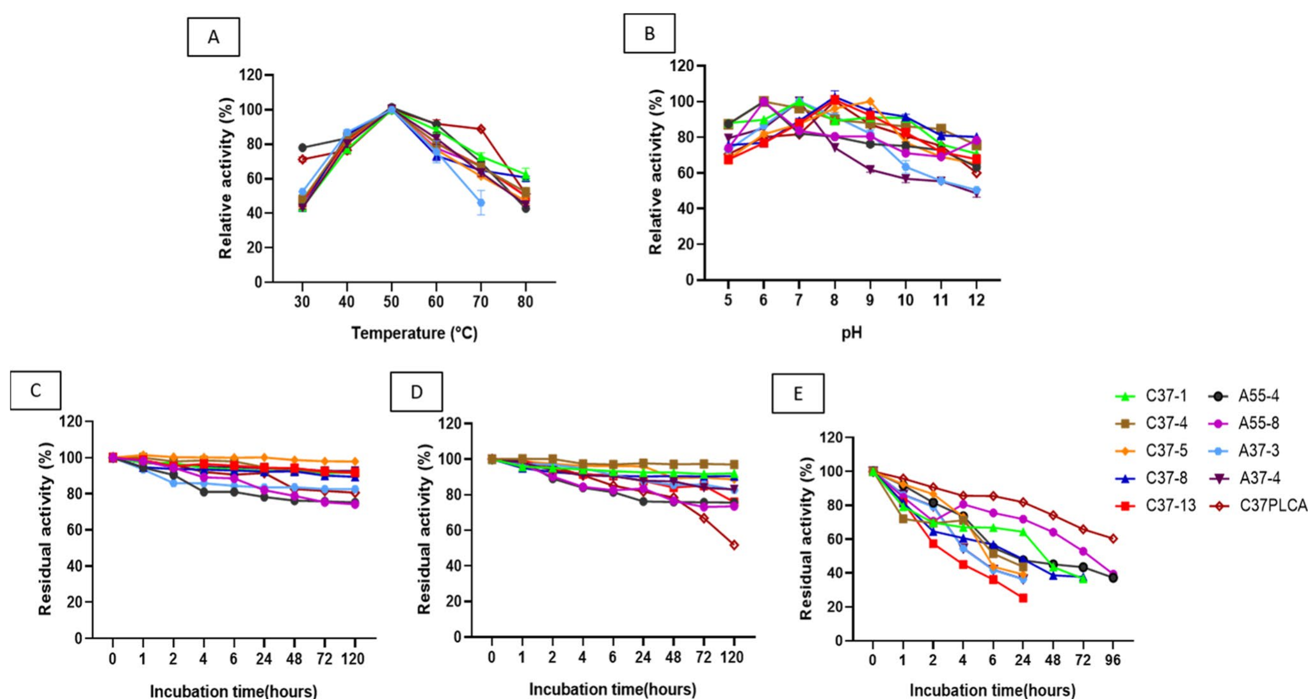

Fig. 1 **A** Effect of temperature and **B** pH on the crude amylase extracts from C37PLCA, C37-1, C37-4, C37-5, C37-8, C37-13, A37-3, A37-4, A55-4, and A55-8 strains. **C** Effect of temperature on the stabilities of the crude amylase extracts from the 10 strains. The enzyme stability was determined by pre-incubating enzyme at 30 °C and residual activities were calculated. **D** Effect of temperature on the stabilities of the crude amylase extracts from the 10 strains. The enzyme stability was determined by pre-incubating enzyme at 50 °C and residual activities were calculated. **E** Effect of pH on the stabilities of the crude amylase extracts from the 10 strains. For determining the stability of the crude amylase at pH 8.0, the enzymes were pre-incubated at pH 8.0 and residual activities were calculated

used as crude enzyme extracts. The optimum temperature and pH of the enzymes are given in Table 2. The temperature stability of the crude enzymes produced from bacteria was determined by pre-incubating the crude enzymes at 30 °C and 50 °C.

Optimum amylase activity was observed at 50 °C for all 10 amylase-positive isolates and high enzyme activity at 40 and 60 °C, which is close to optimum. The amylases obtained from C37-5, C37-8, C37-13, and C37PLCA showed optimum activity at alkaline pH values (8.0 and 9.0), while the others showed optimum activity at pH 6.0 and 7.0 (Table 1). The thermostability profile showed that all crude amylases obtained from the 10 isolates retained more than 75% of their catalytic activity at 30 and 50 °C for 120 h. At 50 °C, the crude amylase produced by C37-5 was fully heat stable. Further, the crude amylase produced by C37-4 was fully heat stable at 30 °C. The A37-3, A37-4, C37-4, C37-5, and C37-13 amylases retained 50% of their amylase activity after 24 h, while C37-1 and C37-8 retained it after 72 h and A55-4 and A55-8 after 96 h at pH 8.0 (Fig. 1).

All 7 cellulases showed optimum activity at 40 °C and above. All of them showed optimum activity at alkaline pH (Table 1). The catalytic activity of the A55-3, C37-4, C55-8, 4NL7C, and C37PLCA cellulases was less than 40% at 30 and 50 °C after 4 h. Under the same conditions, C37-1 retained 80% of its activity. The cellulase of C37PLCA showed 80% activity at pH 8.0 after 6 h, whereas the relative activity levels of all other cellulases decreased below 50% (Fig. 2).

The optimum temperatures for lipase activity ranged from 30 to 60 °C; however, all of them showed optimum activity at alkaline pH. The lipases of 1NL100b, 2NL3b, and 5NP2b2-2 remained stable for 144 h; of 2NL1 for 168 h; and of 1NP6, 1NP7, 1NP5a, and 2NP4 for 192 h at 30 °C. The lipase of 1NP5a retained 80% of its activity after 48 h at 50 °C, while 40% lipase activity was observed for the other strains. pH stability analyses revealed that C37PLCA and 1NP7 had 90% residual activity at pH 8.0 after 48 h (Fig. 3).

The optimal reaction temperature for the proteases of 5NP2A3 and 5NP3D-1 was 60 °C. However, all of the other proteases showed optimum activity at 50 °C. All proteases had optimum activity at alkaline pH, except for 5NP2B2-3 (Table 1). The proteases of 1NP6, 2NP4, 5NP2A3, 5NP2B2-3, and 5NP3D-1 remained stable for 96 h at 30 °C. The proteases produced by 1NP6 and 2NP4

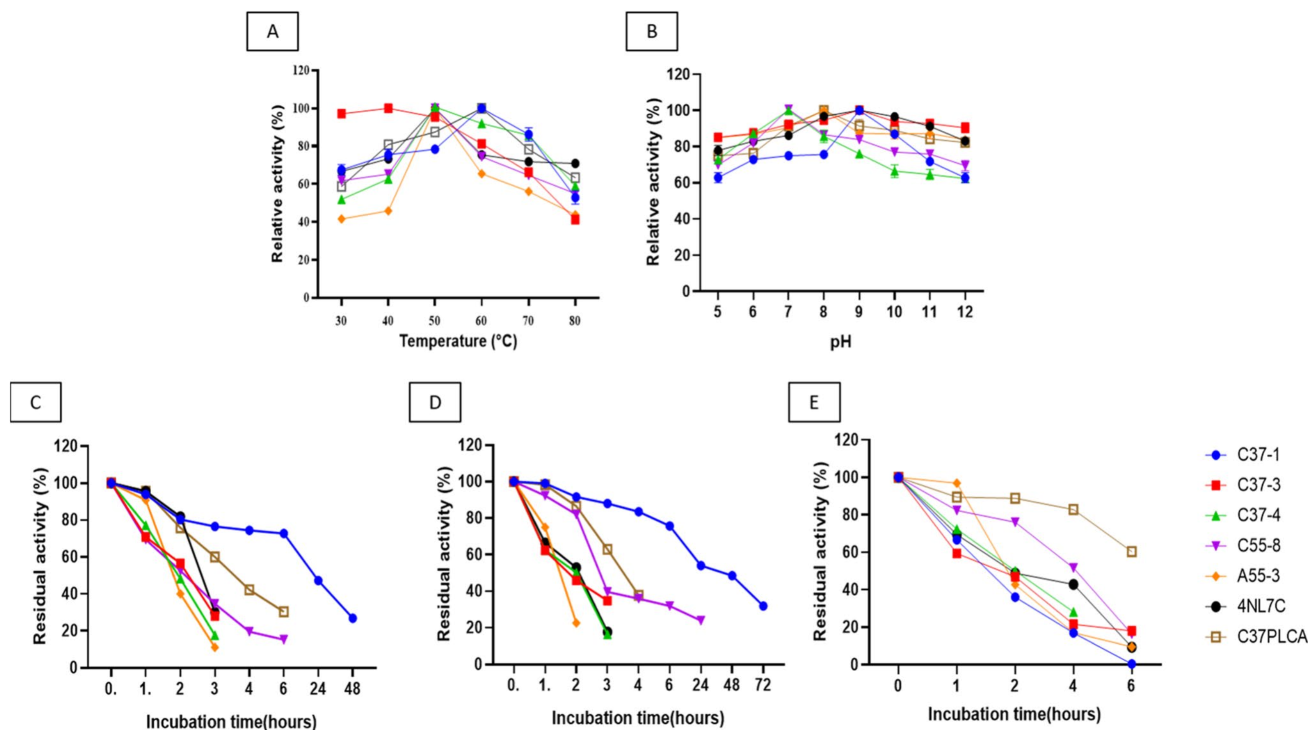

Fig. 2 **A** Effect of temperature and **B** pH on the crude cellulase extracts from C37PLCA, C37-1, C37-3, C37-4, C55-8, A55-3, and 4NL7c strains. **C** Effect of temperature on the stabilities of the crude cellulase extracts from the 7 strains. The enzyme stability was determined by pre-incubating enzyme at 30 °C and residual activities were calculated. **D** Effect of temperature on the stabilities of the crude cellulase extracts from the 7 strains. The enzyme stability was determined by pre-incubating enzyme at 50 °C and residual activities were calculated. **E** Effect of pH on the stabilities of the crude cellulase extracts from the 7 strains. For determining the stability of the crude amylase at pH 8.0, the enzymes were pre-incubated at pH 8.0 and residual activities were calculated

were stable almost 100% after 6 h of incubation at 50 °C. On the other hand, the proteases of 5NP2A3 and 5NP2B2-3 lost 50% of their activity after 4 h at 50 °C. The proteases lost 40% of their activity after 120 h at 30 °C, except for C37PLCA. All of the proteases had 80% activity remaining at pH 8.0 even after 216 h (Fig. 4).

Selection of enzymes according to their detergent compatibility

To determine the suitability of the enzymes for the detergent industry, their compatibility with four commercial detergents frequently used nowadays was studied. The lipase of 1NL100b was compatible with detergents A and D; the lipase of 2NL1 was compatible with B, C, and D; and the lipases of 2NL3B, 5NP2B2-2, 1NP6, 1NP7, 1NP5A, and 2NP4 were compatible with all of the detergents tested. The proteases of 1NP6, 2NP4, 5NP2A3, and 5NP2B2-3 were compatible with all of the detergents and the protease of 5NP3D-1 was compatible with A, B, and D. The protease, lipase, cellulase, and amylase produced by C37PLCA, which produces all four enzymes, were compatible with the detergents.

Phenotypic features of the isolates

The results of the physiological tests of the bacterial isolates are shown in Table 1. All of the bacterial isolates tested were Gram-positive. Microscopic analysis showed that only two (5NP2B2-2 and 1NL100B) of the isolates were cocci-shaped bacteria, while the others were rod-shaped. While 16 of the isolates were able to form endospores, the remaining 8 isolates (1NP5A, 1NP6, 1NP7, 1NL100B, 2NL3B, 2NP4, 5NP2B2-3, 5NP2B2-2, and C37-4) were not capable of forming spores. All of the isolates were catalase positive; 19 isolates were oxidase positive and the remaining eight (C55-8, C37-8, A55-8, C37-4, 2NL1, 5NP3D-1, 5NP2B2-2, and 1NL100B) were oxidase negative. All of the isolates were able to grow in media not supplemented with salt, so salt was not necessary for bacterial growth. All isolates were able to grow in salt concentrations ranging from 0 to 5%. Nine of the isolates were able to tolerate up to 5% NaCl concentration in growth media, 3 of the isolates could tolerate up to 7% NaCl, 4 of them were able to tolerate up to 8% NaCl, and only 9 of the isolates could grow in 10% NaCl. None of the strains was able to grow in a salt concentration of 15%. The optimum growing pH analyses revealed that

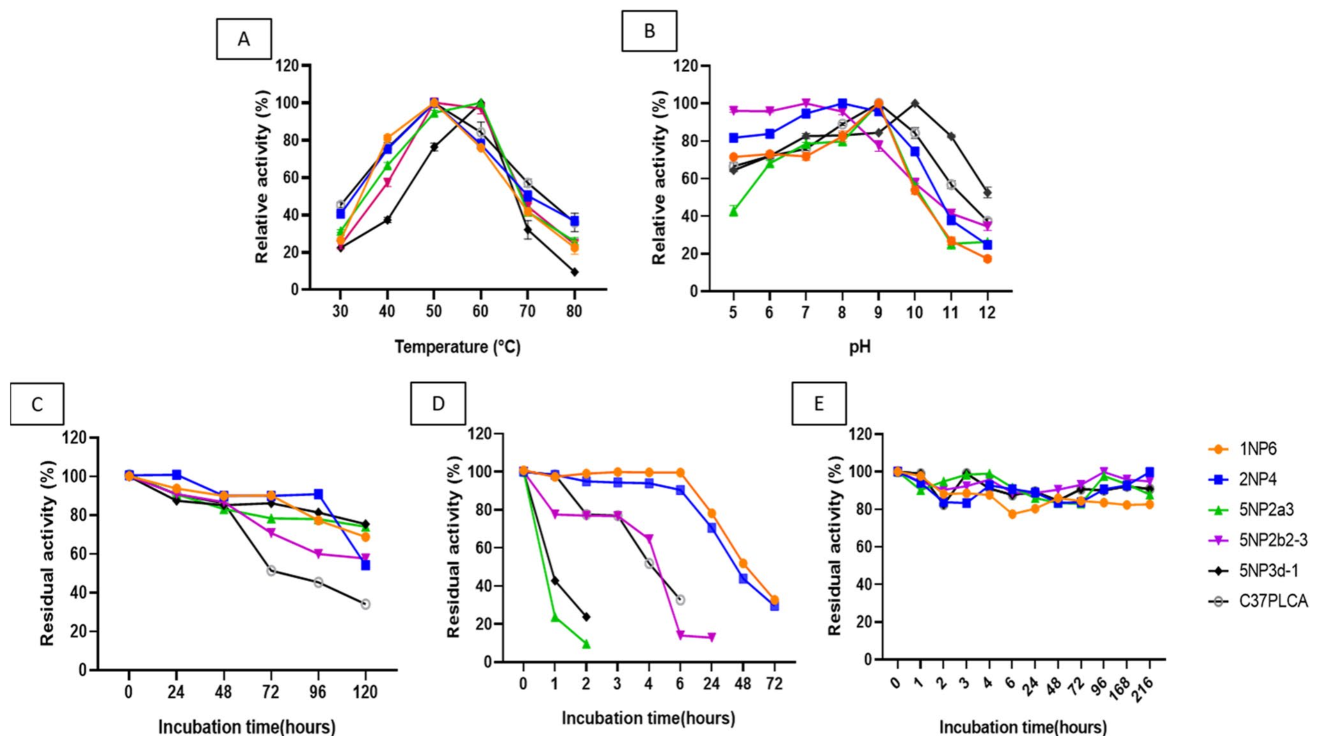

Fig. 3 **A** Effect of temperature and **B** pH on the crude protease extracts from C37PLCA, 5NP203, 5NP3d-1, 5NP2b2-3, 2NP4, and 1NP6 strains. **C** Effect of temperature on the stabilities of the crude protease extracts from the 6 strains. The enzyme stability was determined by pre-incubating enzyme at 30 °C and residual activities were calculated. **D** Effect of temperature on the stabilities of the crude protease extracts from the 6 strains. The enzyme stability was determined by pre-incubating enzyme at 50 °C and residual activities were calculated. **E** Effect of pH on the stabilities of the crude protease extracts from the 6 strains. For determining the stability of the crude amylase at pH 8.0, the enzymes were pre-incubated at pH 8.0 and residual activities were calculated

almost all strains grew in pH values ranging between 7.0 and 8.0. Of the 25 isolates, 10 isolates were able to tolerate up to pH 9.0, while 11 isolates were able to grow in pH 10.0. It was also determined that all isolates showed growth at pH 5.0 but only 4 isolates could grow at pH 4.0. The isolates were incubated at different temperatures between 30 and 70 °C with an increase of 5 °C. It was observed that all isolates could grow at between 30 and 45 °C.

Molecular characterization of the isolates

The 16S rRNA gene sequences of the 25 bacterial isolates producing the selected enzymes were analyzed. The sequences obtained were compared with the 16S database of EzBioCloud. The isolates were identified at the species level and the 16S rRNA gene sequences of the bacterial isolates were deposited in the GenBank database under accession numbers from OL308061 to OL308078. Blast analysis of the 16S rRNA gene sequences revealed that all isolates showed greater than 99.26% sequence similarity to closely related species. Two of the isolates (5NP2B2-2 and 1NL100B) belonged to the genus *Staphylococcus* (Fig. 5) and the others were *Bacillus* (Fig. 6). Phylogenetic trees based on the 16S rRNA gene sequences were constructed to determine the evolutionary relationships of the bacterial isolates. The constructed phylogenetic tree showed that three isolates (4NL7C, C37-1, and 2NL1) formed a cluster with *Bacillus siamensis* and two isolates (C37-8 and C37-3) shared a cluster with *Bacillus inaquosorum*. Isolates 1NP6, 1NP5A, 2NP4, 2NL3B, 1NP7, and C37-4 fell in a separate cluster within the main group of *Bacillus* type strains.

Discussion

Because of the harmful effects of chemicals on our ecosystem, environmentally friendly approaches are needed. In this context, the use of microbial enzymes in the detergent industry is an area of interest. Nowadays, enzymes used in the industrial field are generally obtained from microorganisms. Microbial enzymes are of prime importance due to their applications in the detergent, textile, feed, leather, waste treatment, and other sectors. The enzymes of microorganisms are preferable because they have higher catalytic activity than the enzymes obtained from other organisms, they are stable at broad ranges of industrial conditions and inexpensive, and do not form toxic by-products. Their other

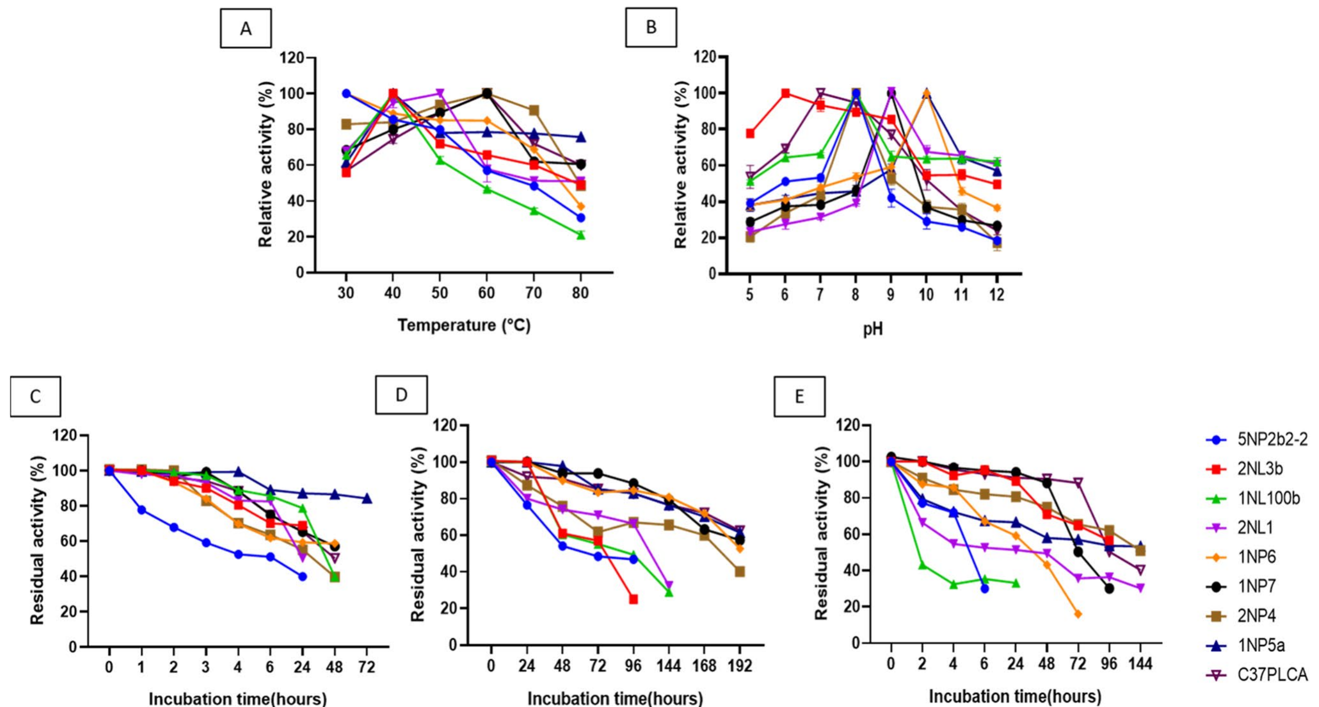

Fig. 4 **A** Effect of temperature and **B** pH on the crude lipase extracts from C37PLCA, 1NL100b, 2NL1, 2NL3b, 5NP2b2-2, 1NP6, 1NP7, 1NP5a, and 2NP4 strains. **C** Effect of temperature on the stabilities of the crude lipase extracts from the 9 strains. The enzyme stability was determined by pre-incubating enzyme at 30 °C and residual activities were calculated. **D** Effect of temperature on the stabilities of the crude lipase extracts from the 9 strains. The enzyme stability was determined by pre-incubating enzyme at 50 °C and residual activities were calculated. **E** Effect of pH on the stabilities of the crude lipase extracts from the 9 strains. For determining the stability of the crude amylase at pH 8.0, the enzymes were pre-incubated at pH 8.0 and residual activities were calculated

advantages include ease of modification and optimization and being easily obtained [31, 36]. Due to their industrial importance, interest in the isolation of new bacterial strains producing useful enzymes is increasing day by day [37]. There are many reports in the literature about the bacterial enzymes obtained from soil and water that possess desired properties for industrial applications. In the present study, household waste-contaminated soil samples were screened for enzyme-producing bacteria. We focused on four industrial enzymes, namely, protease, lipase, cellulase, and amylase, which have been used in industry especially as detergent ingredients for a long time. Although commercial detergents contain some microbial enzymes, there is still a need to identify new bacterial enzymes that are highly active and stable. We also aimed to obtain bacterial samples that produce two or more of these enzymes at the same time; in this way the production of commercial enzymes will be more cost effective. Therefore, our findings are important for filling the gap in this field; in particular, the strain C37PLCA (*Bacillus* sp.), which synthesizes 4 enzymes at the same time, can offer promising solutions to develop efficient processes.

Protease, lipase, amylase, and cellulase are important enzymes in terms of industrial value. These enzymes have a wide area of use in industry such as in detergents, food, bioremediation, and cosmetics [38]. Proteases, which constitute 60% of the global enzyme market, are used in detergents, leather, food, and cosmetics [16, 39]; starch-degrading amylases are used for wastewater treatment [40]; cellulases are used for improved malting and clarification in the fermentation industry [41]; and lipases are used in large-scale industrial areas such as food, cosmetics, and perfumery [38, 42]. Industrial enzymes are required to be resistant to high temperatures [43]. Studies have shown that bacterial lipases show high activity at 37–40 °C. Based on some studies in the literature, the activity of alkaline proteases is halved within 1.5 to 6.5 h at 50–55 °C, but they are generally resistant to high temperatures. Detergent-compatible bacterial amylases have different optimum temperatures, ranging from 22 to 70 °C [27] and the optimum temperature of cellulases is 50–55 °C [44]. Our enzymes are industrially important with their ability to show activity at an average temperature of 40–60 °C. Besides temperature resistance, the activity and

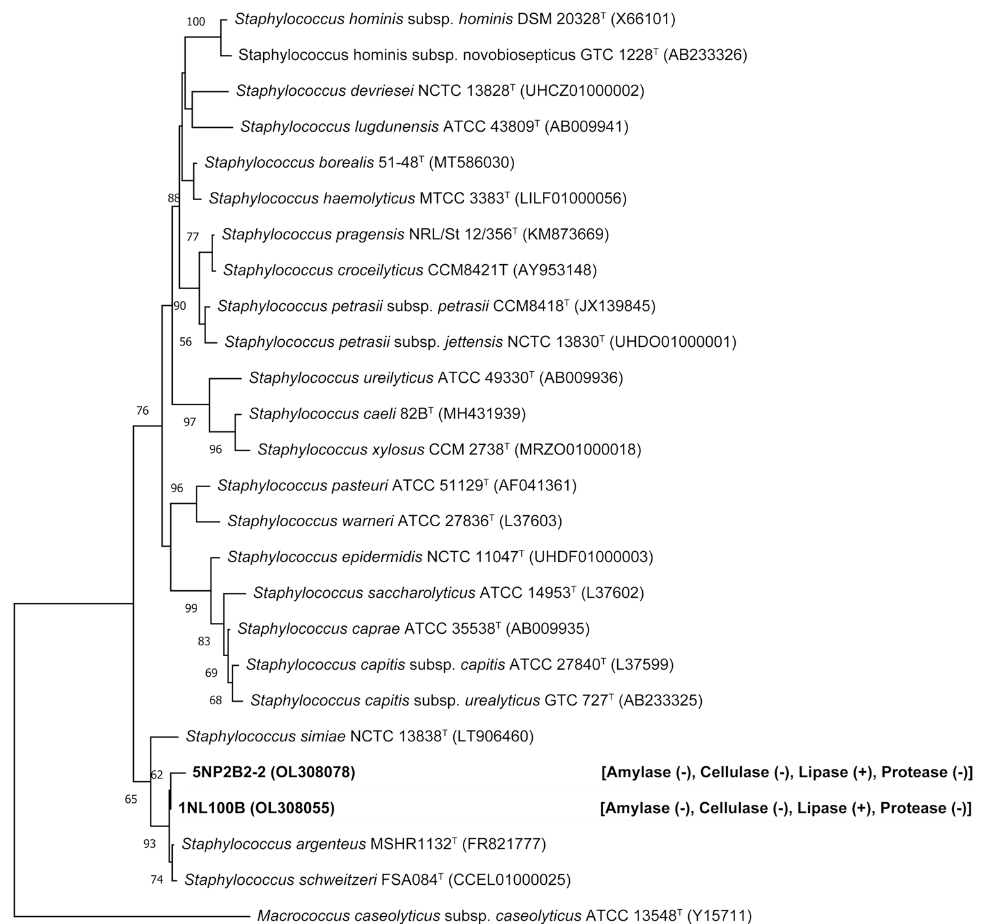

Fig. 5 Based on the 16S rRNA gene sequences of two isolates and other similar strains, a neighbor-joining tree was created. Each nucleotide location has 0.01 substitutions. At the branch locations, bootstrap percentages greater than 50% based on 1000 replications are displayed. As an outgroup, *Macrococcus caseolyticus* subsp. *caseolyticus* ATCC 13548 ^{T} was used

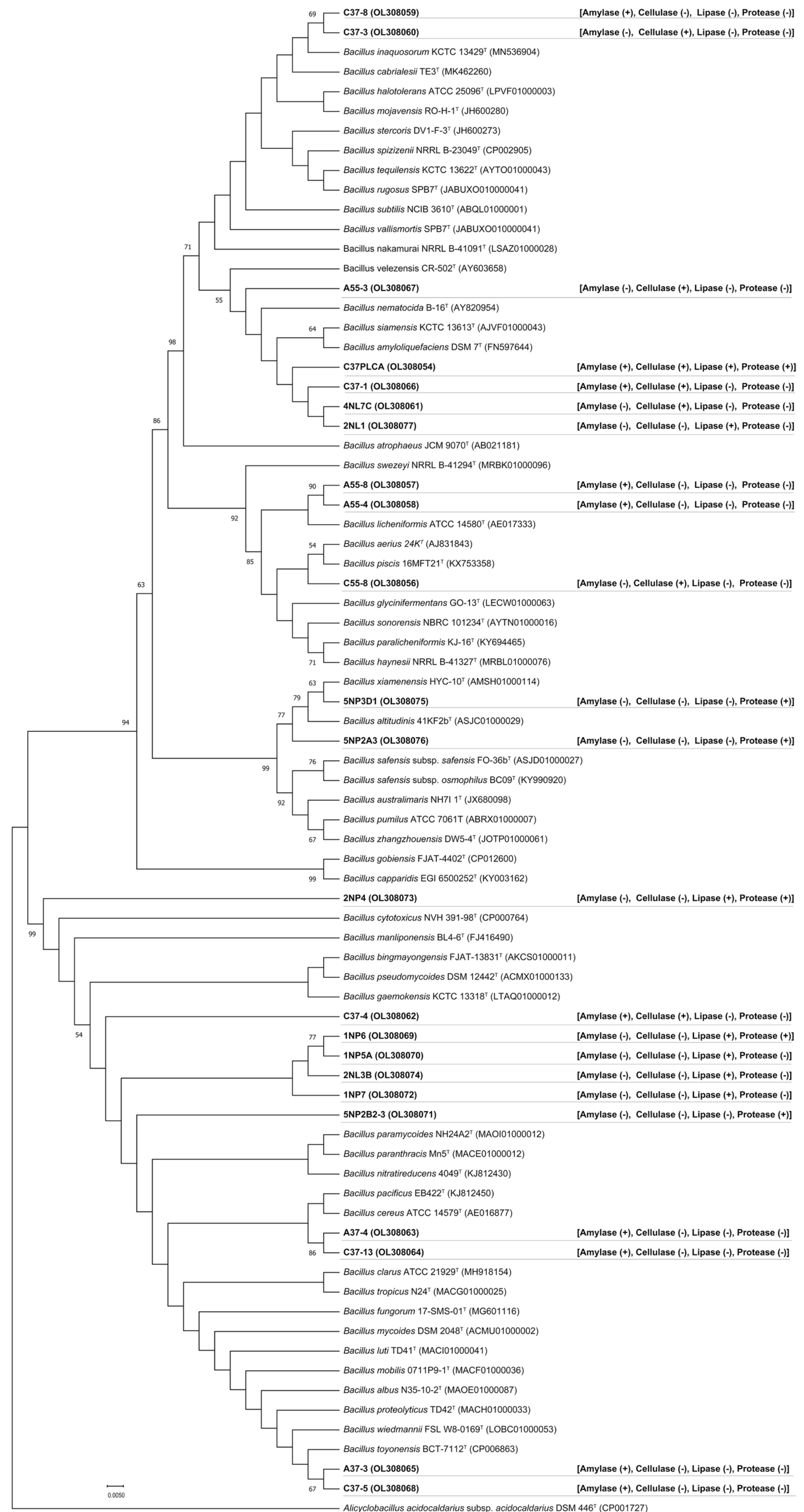

Fig. 6 Based on the 16S rRNA gene sequences of two isolates and other similar strains, a neighbor-joining tree was created. Each nucleotide location has 0.01 substitutions. Each nucleotide location has 0.01 substitutions. At the branch locations, bootstrap percentages greater than 50% based on 1000 replications are displayed. As an outgroup, *Alicyclobacillus acidocaldarius* subsp. *acidocaldarius* DSM 446 ^{T}

stability of proteolytic activity over a pH range enable these enzymes to withstand harsh environmental conditions in industrial processes such as for detergents, food, and leather, and the synthesis of biologically active peptides [45]. Our enzymes showed high activity between pH 6 and 10 and are stable at alkaline pH. The fact that the enzymes we obtained have activity at high pH is a demonstration that they are suitable for this sector because commercial detergents usually have an alkaline pH.

The compatibility of the enzymes obtained in the present study with commercial detergents (Ariel, OMO Matik, Bingo, and Boron) was also investigated. Fourteen partially characterized enzymes showed sufficient stability with the detergents. All four enzymes produced by C37PLCA were active when used with detergents at 50 °C after 60 min. Similar results were reported in the literature for other *Bacillus* strains. The protease of *Bacillus* sp. retained 65% of its activity after 30-min incubation with commercial detergents at 60 °C [46]. The lipase of *Bacillus smithii* BTMS11 showed more than 90% activity after 3 h of incubation in the presence of detergents [47]. In another study, a cellulase produced by *Bacillus* SMIA-2 was tested with commercial detergents, and after an incubation period of 30 min at 40 °C it was still 70% active [48]. Moreover, an amylase of *Bacillus subtilis* AS-S01a retained 69–100% of its activity after 1 h at room temperature [49]. These findings are compatible with our results and we can conclude that our enzymes can be included in commercial detergents. The strain C37PLCA produced all of the enzymes screened. The enzymes were compatible with the commercial detergents. They were also active and stable at alkaline pH and the temperatures needed for the cleaning industry. These features make this enzyme a promising tool for the detergent industry.

The morphological, physiological, and biochemical analyses of the bacteria from which we obtained the enzymes were performed and species close to them were determined using 16S rRNA sequences. Homology analysis of the gene sequences showed that they were over 99% the same as *Bacillus* species, except for 2 of the bacteria. Those two bacteria belong to the genus *Staphylococcus*. Half of the total global enzyme market is derived from *Bacillus* species. As stated before, in this field, enzymes obtained from *Bacillus* species are important for large-scale biotechnological processes due to their thermostability and efficient expression systems [36]. Although not all our bacteria are thermophilic, the enzymes they synthesize are thermostable, as seen in studies of *Bacillus* species [50]. In addition, lipases obtained from *Staphylococcus* species are suitable for use in the detergent industry and lipase enzyme studies of these species are of interest [51–53]. According to the results obtained, our enzymes are suitable candidates for the detergent industry.

Conclusions

Soil samples contaminated with household waste were screened for protease-, lipase-, cellulase-, and amylase-producing bacteria. A total of 25 isolates were determined producing one or more of the enzymes. Among these isolates, 10 of them produce amylase, 9 of them lipase, 7 of them cellulase, and 6 of them protease. The strain C37PLCA is capable of producing all four enzymes. Optimum temperatures of the enzymes range from 37 to 55 °C and they are active at alkaline pH values. 16S rRNA analyses revealed that two of the strains belong to the genus *Staphylococcus* and the rest belong to *Bacillus*. The stability of the enzymes at high temperatures, alkaline pH, and in commercial detergents make them promising.

Acknowledgements This study was supported by the Scientific and Technological Research Council of Turkey (TUBITAK)-2209 A (2019/2; 2020/1) and Karadeniz Technical University (KTU BAP FBA-2021-9731).

Author contribution KIB designed the study. KIB, PC, SA, NSS, SG, and AK isolated strains. KIB, AN, and ES performed physiological analysis of bacterial strains. KIB, PC, SA, NSS, SG, AK, AN, and ES performed characterization of amylase, protease, lipase, and cellulase. AOB and SC performed 16S rRNA analysis. KIB and DNC analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

References

1. Eichorst SA, Breznak JA, Schmidt TM (2007) Isolation and characterization of soil bacteria that define Terriglobus gen. nov., in the phylum *Acidobacteria*. Appl Environ Microbiol 73(8):2708–2717
2. Panda AK, Bisht SS, De Mandal S, Kumar NS (2019) Microbialdiversity of thermophiles through the lens of next generationsequencing. In: Panda AK, Bisht SS, De Mandal S, Kumar NS (eds) Microbial diversity in the genomic era. Academic Press, Cambridge, pp 217–226
3. Schiraldi C, De RM (2014) Mesophilic organisms. Encycl Membr 2014:1–2
4. Gomes J, Steiner W (2004) The biocatalytic potential of extremophiles and extremozymes. Food Technol Biotechnol 42:4
5. OztasGulmus E, Gormez A (2019) Characterization and biotechnological application of protease from thermophilic *Thermomonashaemolytica*. Arch Microbiol 202(1):153–159
6. Rai SK, Mukherjee AK (2010) Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent-stable subtilisin-like serine protease (Alzwiprase) from *Bacillus subtilis* DM-04. Biochem Eng J 48(2):173–180

7. Hmidet N, El-Hadj Ali N, Haddar A, Kanoun S, Alya SK, Nasri M (2009) Alkaline proteases and thermostable α-amylase co-produced by *Bacillus licheniformis* NH1: characterization and potential application as detergent additive. Biochem Eng J 47(1–3):71–79
8. Haddar A, Agrebi R, Bougatef A, Hmidet N, Sellami-Kamoun A, Nasri M (2009) Two detergent stable alkaline serine-proteases from *Bacillus mojavensis* A21: purification, characterization and potential application as a laundry detergent additive. Biores Technol 100(13):3366–3373
9. Kumar CG, Takagi H (1999) Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol Adv 17(7):561–594
10. Niyonzima FN, More SS (2015) Minireview coproduction of detergent compatible bacterial enzymes and stain removal evaluation. J Basic Microbiol 55:1149–1158
11. Gopinath SCB, Anbu P, Arshad MKM et al (2017) Biotechnological processes in microbial amylase production. Biomed Res Int 2017:2017
12. Joo HS, Kumar CG, Park GC, Paik SR, Chang CS (2003) Oxidant and SDS-stable alkaline protease from *Bacillus clausii* I-52: production and some properties. J Appl Microbiol 95(2):267–272
13. Niyonzima FN, More SS (2015) Purification and characterization of detergent-compatible protease from *Aspergillus terreus* gr. 3 Biotech 5(1):61–70
14. Niyonzima FN, More S (2014) Purification and properties of detergent-compatible extracellular alkaline protease from *Scopulariopsis* spp. Prep Biochem Biotechnol 44(7):738–759
15. Niyonzima FN (2019) Detergent-compatible bacterial cellulases. J Basic Microbiol 59(2):134–147
16. Gupta R, Beg Q, Lorenz P (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59(1):15–32
17. Niehaus F, Gabor E, Wieland S, Siegert P, Maurer KH, Eck J (2011) Enzymes for the laundry industries: tapping the vast metagenomic pool of alkaline proteases. Microb Biotechnol 4(6):767–776
18. Inan K, Canakci S, Belduz AO (2011) Isolation and characterization of xylanolytic new strains of *Anoxybacillus* from some hot springs in Turkey. Turk J Biol 201:529–542
19. Vieira De Carvalho R, Lívia T, Côrrea R et al (2008) Propertıes of an amylase from thermophilic *Bacillus* sp. Braz J Microbiol 39:102–107
20. Yoneda Y, Yoshida T, Yasuda H et al (2013) A novel thermophilic, hydrogenogenic, and carboxydotrophic bacterium *Calderihabitansmaritimus* gen. nov., sp. nov. from a marine sediment core of an undersea caldera. Int J Syst Evol Microbiol 63:3602–3608
21. Bae J, Rhee S, Park JR, Kim B, Park Y (2005) Isolation of uncultivated anaerobic thermophiles from compost by supplementing cell extract of *Geobacillustoebii* in enrichment culture medium. Extremophiles 9(6):477–485
22. Kembhavi AA, Kulkarni A, Pant A (1993) Salt-tolerant and thermostable alkaline protease from *Bacillus subtilis* NCIM no. 64. Appl Biochem Biotechnol 38:83–92
23. Islam F, Roy N (2018) Screening, purification and characterization of cellulase from cellulase producing bacteria in molasses. BMC Res Notes 11(1):1–6
24. Bernfeld P (1955) Amylases, α and β. Methods Enzymol 1(C):149–158
25. Palacios D, Busto MD, Ortega N (2014) Study of a new spectrophotometric end-point assay for lipase activity determination in aqueous media. LWT - Food Sci Technol 55(2):536–542
26. Tripathi VR, Kumar S, Garg SK (2011) A study on trypsin, *Aspergillus flavus* and *Bacillus* sp. protease inhibitory activity in Cassia tora (L.) syn Senna tora (L.) Roxb. seed extract. BMC Complement Alternat Med 11:56
27. Niyonzima FN, More SS (2014) Detergent-compatible bacterial amylases. Appl Biochem Biotechnol 174(4):1215–1232
28. Harley JP, Prescott LM (2002) Laboratory exercises in microbiology, 5th edn. In: The McGraw-Hill Companies. Microbiology, Inc., New York, pp 139–140
29. Maugeri TL, Gugliandolo C, Caccamo D et al (2002) A halophilic thermotolerant *Bacillus* isolated from a marine hot spring able to produce a new exopolysaccharide. Biotech Lett 24:515–519
30. Inan K, Kacagan M, Ozer A, Belduz AO, Canakci S (2015) Algoriphagus trabzonensis sp. nov., isolated from freshwater, and emended description of Algoriphagus alkaliphilus. Int J Syst Evol Microbiol 65(7):2234–40
31. Yoon SH, Ha SM, Kwon S et al (2017) Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int J Syst Evol Microbiol 67(5):1613
32. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454
33. Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874. https://doi.org/10.1093/molbev/msw054
34. Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120. https://doi.org/10.1007/bf01731581
35. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x
36. Prakash O, Jaiswal N (2010) α-Amylase: an ideal representative of thermostable enzymes. Appl Biochem Biotechnol 160(8):2401–2414
37. Ghorbel RE, Maktouf S, Massoud EB, Bejar S, Chaabouni SE (2009) New thermostable amylase from Bacillus cohnii US147 with a broad pH applicability. Appl Biochem Biotechnol 157(1):50–60
38. Hasan F, Shah A, Javed S, Hameed A (2010) Enzymes used in detergents: lipases. Afr J Biotech 9(31):4836–4844
39. Laxman RS, Sonawane AP, More SV et al (2005) Optimization and scale up of production of alkaline protease from Conidiobolus coronatus. Process Biochem 40(9):3152–3158
40. Aiyer V (2005) Amylases and their applications Prasanna. Afr J Biotech 4(13):1525–1529
41. Kuhad RC, Gupta R, Singh A (2011) Microbial cellulases and their industrial applications. Enzyme Res 2011;2011(1)
42. Baltacı MÖ, Tuysuz E, Ozkan H, Taskın M, Adıguzel A (2019) Lipase production from thermophilic bacteria using waste frying oil as substrate. Journal of Technical Sciences 9(3):23–27
43. Chang T, Yao S (2011) Thermophilic, lignocellulolytic bacteria for ethanol production: current state and perspectives. Appl Microbiol Biotechnol 92(1):13–27
44. Sriariyanun M, Tantayotai P, Yasurin P, Pornwongthong P, Cheenkachorn K (2016) Production, purification and characterization of an ionic liquid tolerant cellulase from *Bacillus* sp. isolated from rice paddy field soil. Electron J Biotechnol 19(1):23–28
45. Nur M, Karaboga S, Logoglu E (2019) Purification of alkaline serine protease from local *Bacillus subtilis* M33 by two steps: a novel organic solvent and detergent tolerant enzyme. GU J Sci 32(1):116–129
46. Nascimento WCA, Martins MLL (2006) Studies on the stability of protease from *Bacillus* sp. and its compatibility with commercial detergent. Braz J Microbiol 37(3):307–311
47. Lailaja VP, Chandrasekaran M (2013) Detergent compatible alkaline lipase produced by marine *Bacillus smithii* BTMS 11. World J Microbiol Biotechnol 29:1349–1360
48. Ladeira SA, Cruz E, Delatorre AB, Barbosa JB, LealMartins ML (2015) Cellulase production by thermophilic *Bacillus* sp. SMIA-2 and its detergent compatibility. Electron J Biotechnol 18(2):110–115
49. Roy J, Rai S, Mukherjee A (2012) Characterization and application of a detergent-stable alkaline α-amylase from *Bacillus subtilis* strain AS-S01a. Int J Biol Macromol 50(1):219–229

50. Vihinen M, Mantsiila P (1989) Microbial amylolytic enzymes. Crit Rev Biochem Mol Biol 24(4):329–418
51. Ben Bacha A, Al-Assaf A, Moubayed NMS, Abid I (2018) Evaluation of a novel thermo-alkaline *Staphylococcus aureus* lipase for application in detergent formulations. Saudi J Biol Sci 25(3):409–417
52. Cherif S, Mnif S, Hadrich F, Abdelkafi S, Sayadi S (2011) A newly high alkaline lipase: an ideal choice for application in detergent formulations. Lipids Health Dis 10:221
53. Horchani H, Mosbah H, Ben SN, Gargouri Y, Sayari A (2009) Biochemical and molecular characterisation of a thermoactive, alkaline and detergent-stable lipase from a newly isolated *Staphylococcus aureus* strain. J Mol Catal B: Enzym 56(4):237–245

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.