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Purification and Characterization of Strong Simultaneous Enzyme Production of Protease and α -Amylase from an Extremophile-*Bacillus* sp. FW2 and Its Possibility in Food Waste Degradation

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Abstract: Microbial enzymes such as protease and amylase are valuable enzymes with various applications, widely investigated for their applications in degradation of organic waste, biofuel industries, agricultural, pharmaceuticals, chemistry, and biotechnology. In particular, extremophiles play an important role in biorefinery due to their novel metabolic products such as high value catalytic enzymes that are active even under harsh environmental conditions. Due to their potentials and very broad activities, this study isolated, investigated, and characterized the protease- and amylase-producing bacterial strain FW2 that was isolated from food waste. Strain FW2 belongs to the genus *Bacillus* and was found to be closest to *Bacillus amyloliquefaciens* DSM 7^T with a similarity of 99.86%. This strain was able to degrade organic compounds at temperatures from -6 °C to 75 °C (but weak at 80 °C) under a wide pH range (4.5–12) and high-salinity conditions up to 35% NaCl. Maximum enzyme production was obtained at 1200 ± 23.4 U/mL for protease and 2400 ± 45.8 U/mL for amylase for 4 days at pH 7–7.5, 40 – 45 °C, and 0–10% NaCl. SDS-PAGE analysis showed that the molecular weights of purified protease were 28 kDa and 44 kDa, corresponding to alkaline protease (AprM) and neutral protease (NprM), respectively, and molecular weight of α -amylase was 55 kDa. Degradation food waste was determined after 15 days, observing a 69% of volume decrease. A potential commercial extremozyme-producing bacteria such as strain FW2 may be a promising contributor to waste degradation under extreme environmental conditions.

Keywords: organic waste-degrading bacteria; multiple enzyme-producing bacteria; extremophiles; amylase-producing bacteria; protease-producing bacteria



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1. Introduction

For several decades, numerous studies on extremophilic protease and amylase produced from extremophiles have been drastically increasing to aim at improving their ability to adapt to specific conditions [1,2]. It is thus not surprising that extremophiles have gained immense interest and have developed mechanisms to survive in severe conditions in terms of temperature, pH, pressure, and poor-nutrient conditions. Moreover, extremozymes isolated from extremophiles have now become an alternative to chemical catalysts in a broad range of applications, including textiles, detergents, leather, pharmaceuticals, agricultural chemicals, and eco-friendly indicators for nature [3]. The protease and α -amylase produced

by *Bacillus* spp. are important and valuable industrial enzymes, contributing more than 50% of the total protein market [4,5].

Being one of the most widely used and applied examples, proteases are considered the most powerful enzymes, accounting for around 60% of global enzyme usage due to their ability to break down complex protein compounds into amino acids and peptides [6–9]. Bacteria were considered as a great source for the production of three major types of proteases: acidic, neutral, and alkaline. Among them, neutral and alkaline proteases have a great potential for the application in the enzyme-based detergents and leather industries due to the increasing trend of developing eco-friendly technologies [10,11]. Thus, the investigation of microbial protease that can tolerate harsh conditions aims to enhance the efficiency and stability of enzyme activity. Prominent bacterial producers of protease are *Photobacterium* sp., *Bacillus* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Vibrio* sp., and *Aeromonas* sp. Among different producers of proteases, *Bacillus* sp. are recognized commercially exploited microbes for proteases production [3]. Multiple *Bacillus*-derived alkaline protease bacteria with significant activity, stability, broad pH, temperature, short fermentation time, and simple but high efficiency are gaining more consideration for their isolation and facilitation of the study of their produced enzymes.

Besides proteases, amylases are also industrial enzymes that are applied in food fermentation, paper, textile, and biofuel industries [12,13]. There are various genera that produce amylase, including *Bacillus*, *Micrococcus*, *Streptomyces*, *Pseudomonas*, and *Arthrobacter* [14–19]. *Bacillus* genus is well known and famous for α -amylase production. Numerous bacteria belonging the *Bacillus* genus such as *B. licheniformis*, *B. pumilus*, and *B. subtilis* are able to produce multiple extracellular enzymes and are adapted under the extreme growth conditions [20]. Members from *Actinomyces* genus were recently explored as a large source of amylase production [21,22]. However, there are limited numbers of enzymes that have been investigated from extremophiles that have illustrated activities at both low and high temperatures [18]. Additionally, some commercially available α -amylases do not meet industrial reaction conditions and not provide enough for a large industrial demand [23–25].

Therefore, it is necessary to investigate the novel protease and amylase enzymes produced from extremophiles for further valuable applications. This study aimed to coax the fastidious bacterial candidates that are capable of growing and producing enzymes at severed conditions of pH and temperature. The strongest strain was chosen for enhancing the composting process. Concentration and molecular weight of produced enzymes were determined, purified, and characterized.

2. Materials and Methods

2.1. Isolation of Organic Compound Degrading-Bacteria

Bacterial strain FW2 was isolated from kitchen food waste collected from the Jowon Industry in South Korea using the selective medium for the isolation of the target organic degrading bacteria. Five grams of food waste was added to 50 mL of distilled water to make an original isolation solution. One milliliter of sample was then added to the culture medium, including (g/mL) skim milk, starch, and carboxymethylcellulose (CMC) 10 (for each); MgSO_4 , 0.024, NaCl 10, K_2HPO_4 0.3, and 10 mL of trace element mixture. The medium was adjusted to a pH of 7.0 at 25 °C. Vitamin solution 10 mL and 10 mL of autoclaved soil extract (ASE) was added as the final step after autoclaving for 20 min (121 °C, 103 kPa). The vitamin solution contained (g/L) citric acid 0.02, folic acid 0.01, riboflavin 0.025, and para-amiobenzoic acid 0.01. ASE was prepared following the previous study by adding 100 g of soil into 1000 mL of distilled water and adjusted to pH 7 before autoclaving [26]. These samples were incubated at different temperatures of -6 °C, 10 °C, 30 °C, 35 °C, 50 °C, 65 °C, and 80 °C on a rotary shaker at 200 rpm (1 d interval for each temperature change). The surviving bacterial culture in the medium (1 mL) was transferred to a fresh medium prepared as above and incubated at 30 °C for 5 d. Well-separated

colonies were sub-cultured to obtain pure cultures, and they were subsequently examined for enzyme production and waste degradation ability in the subsequent steps [18,19,27].

2.2. PCR Amplification, 16S rRNA Gene Sequencing, and Phylogenetic Analysis

The bacterial strain FW2 was identified using 16S rRNA gene by PCR amplification. Genomic DNA from the strain was extracted according to the manufacturer's instructions using the InstaGene Matrix kit (Bio-Rad). Amplification of the 16S rRNA gene was then performed by PCR using primers 27F and 1492R [28]. PCR products were purified using a multiscreen filter plate (Millipore Corp, Bedford, MA, USA), which was then sequenced using primers 518F (5'-CCA GCA GCC GCG GTA ATA CG-3') and 800R (5'-TAC CAG GGT ATC TAA TCC-3') with a PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). This process was conducted at 95 °C for 5 min and then cooled on ice for 5 min and analyzed using an ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA). Finally, the nearly full-length 16S rRNA sequence was assembled using SeqMan software (DNASTAR Inc., Madison, WI, USA). Sequence similarity was determined by comparing with the sequence available in the GenBank database using EZBioCloud server [29].

Sequence of the 16S rRNA genes related to strain FW2 were obtained from the GenBank database in order to construct a phylogenetic tree. The MEGA 7 program was used to align sequences and reconstruct the phylogenetic trees [30]. The best fit model used in this study for neighbor-joining (NJ) analysis was Tamura two-parameter model with gamma-distributed rates plus invariant site based on the minimum Bayesian information 140 criterion value (gamma parameter = 0.6 in this study) [31]. The reliability of 141 of the phylogenetic trees was estimated by bootstrap values of 1500 replications [32].

2.3. Primary Screening of Potential Alkaline Protease- and Amylase-Producing Bacterial Isolate

To screen the extracellular enzyme production, we incubated bacterial strains in the basal medium with 1% KH₂PO₄, 0.25% Na₂HPO₄, 1% NaCl, 0.2% (NH₄)₂SO₄, 0.005% MgSO₄·7H₂O, and 0.005% CaCl₂ with an added 1% each of skim milk and starch, and 5 g Yeast Extract. The growth and enzyme generation of strain FW2 were tested first at −6 °C and 80 °C as the minimum and maximum tolerant temperatures, respectively.

After 4 d of incubation, formation of zones of clear halo surrounding the colonies in skim milk agar plate indicated the presence of protease enzyme. The plate containing starch was flooded with 1% iodine solution, and transparent zones surrounding the colonies indicated amylase production [33].

2.4. Determination of Food Waste Degradation by Pure Bacterial Culture

The degradability of strain FW2 was examined in lab-scale trials with 1000 g of small heaps of food waste. Each heap was inoculated with 1–5% of bacterial culture (1% interval) compared to the control without inocula. All trials were set up under temperature ranging from 14 to 16 °C. The heaps were mixed thoroughly to maintain aeration in the period. The volume of the waste was calculated accordingly on 7 days, 15 days, 21 days, and 28 days with the following equation:

$$\text{Volume} = \pi \cdot r^2 \cdot h$$

where h is the height of the small heap, and r is the radius of the bottom of the heap.

2.5. Optimization of Enzyme Production

2.5.1. Optimization of Physio-Chemical Parameters

The enzyme production process was examined with different experimental conditions such as various pH values (4–12.5), temperatures (−6 °C–80 °C), and levels of NaCl (0–35%). Different pH was maintained using appropriate buffers: 0.1 M citrate buffer (pH 4–5), 0.2 M phosphate buffer (pH 6–8), and phosphate–NaOH buffer (pH 8–12.5). Each experiment was performed in triplicate and averaged.

2.5.2. Effect of Carbon and Nitrogen Sources on Enzyme Production

Fermentation medium for strain FW2 was prepared in supplement of 1% (*w/v*) glucose, maltose, starch, dextrose, and lactose as various carbon sources. Other trials added 1% (*w/v*) ammonium nitrate, potassium nitrate, yeast extract, casein, skim milk, and peptone as different nitrogen sources. The bacterial inoculated cultures were incubated at optimal temperature of 45 °C, 120 rpm for 36 h. The supernatant included enzymes were collected in order to examine the enzyme production after a centrifugation at 10,000 rpm for 10 min at 4 °C. Each experiment was performed in triplicate and averaged.

2.6. Enzyme Assay

Bacterial strain was inoculated in the medium for protease fermentation included (per 1 L) peptone 15 g, yeast extract 3 g, NaCl 5 g, and glucose 5 g. Amylase production medium was composed of (per 1 L) peptone 10 g, beef extract 5 g, NaCl 5 g, and starch 2 g. Bacterial culture was incubated at 45 °C and 150 rpm for 5 d. Enriched bacterial cultures were then centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was used for the enzymatic assay. All trials were conducted in triplicate, and standard error was subsequently calculated.

For the protease assay, sample volume (1 mL) was prepared by adding different dilutions of enzyme samples to 50 mM glycine–NaOH buffer (pH 9). The mixture was incubated at 60 °C for 10 min after adding 1 mL of 1% casein (*w/v*). The reaction was terminated by adding 0.5 mL of 20% trichloroacetic acid (TCA) (*w/v*). To remove the precipitated pellets, we filtered the mixture after being kept at room temperature for 30 min, and 5 mL of 0.5 M Na₂CO₃ solution was added to 1 mL of the filtrate. The sample was covered with aluminum foil and kept in dark after adding 0.5 mL of Folin and Ciocalteu's reagent (phenol reagent) for color development. The absorbance was measured spectrophotometrically at 660 nm using tyrosine as a standard. One unit of protease activity was determined as the amount of enzyme required to release 1 g of tyrosine per mL in 1 min (U/mL/min).

The α -amylase assay was performed using 3,5 dinitro salicylic acid (DNSA) method. One milliliter of 1% starch and 1 mL of citrate-phosphate buffer (pH 6.0) were mixed with different dilutions of enzyme samples and incubated at 50 °C for 30 min. The mixture was placed in a boiling water bath for exactly 10 min after adding 2 mL of DNS solution. The absorbance was recorded at 540 nm using glucose as a standard. The enzyme activity was determined as the amount of enzyme that releases 1 μ mol of reducing sugar as glucose per minute (U/mL/min) [34].

2.7. Purification of Protease and Amylase Enzymes

The quantitative estimation of the protein content of protease was determined following a traditional method of previous study [35]. Ammonium sulfate (10–100% saturation) was slowly added to precipitate the enzyme. Precipitation was done at 4 °C. The pellets were collected by centrifugation at 10,000 rpm, 4 °C for 15 min, and then dissolved in the glycine–NaOH buffer (pH 10). The sample was continued in the next step of purification using DEAE–Cellulose Ion exchange chromatography and gel filtration on Sephadex G-100.

Purification of α -amylase enzyme was carried out by ammonium sulphate precipitation followed by dialysis. The cell-free supernatant containing enzyme was saturated from 40–80% (*w/v*) ammonium sulphate at 10,000 rpm, 4 °C for 15 min. The precipitate was suspended in 25 mM Tris–HCl buffer, pH 8, and dialyzed overnight at 4 °C. The enzyme fractions were then obtained after separation using DEAE–Cellulose Ion exchange chromatography and gel filtration on Sephadex G-100.

2.8. Estimation of Protein Content and Molecular Weight Determination of Enzymes

The purity and molecular weight of the sample at each step was examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [36].

3. Results

3.1. Physiology and the 16S rRNA Gene Analysis

The 16S rRNA gene analysis illustrated that the bacterial strain FW2 in this study belonged to the *Bacillus* genus. Its sequence was deposited in the GenBank database with Accession No. MW652625. On the basis of the 16S rRNA sequences, we found that the highest pairwise similarity of the strain FW2 was with *Bacillus amyloliquefaciens* DSM 7^T (99.86%) (Figure 1).

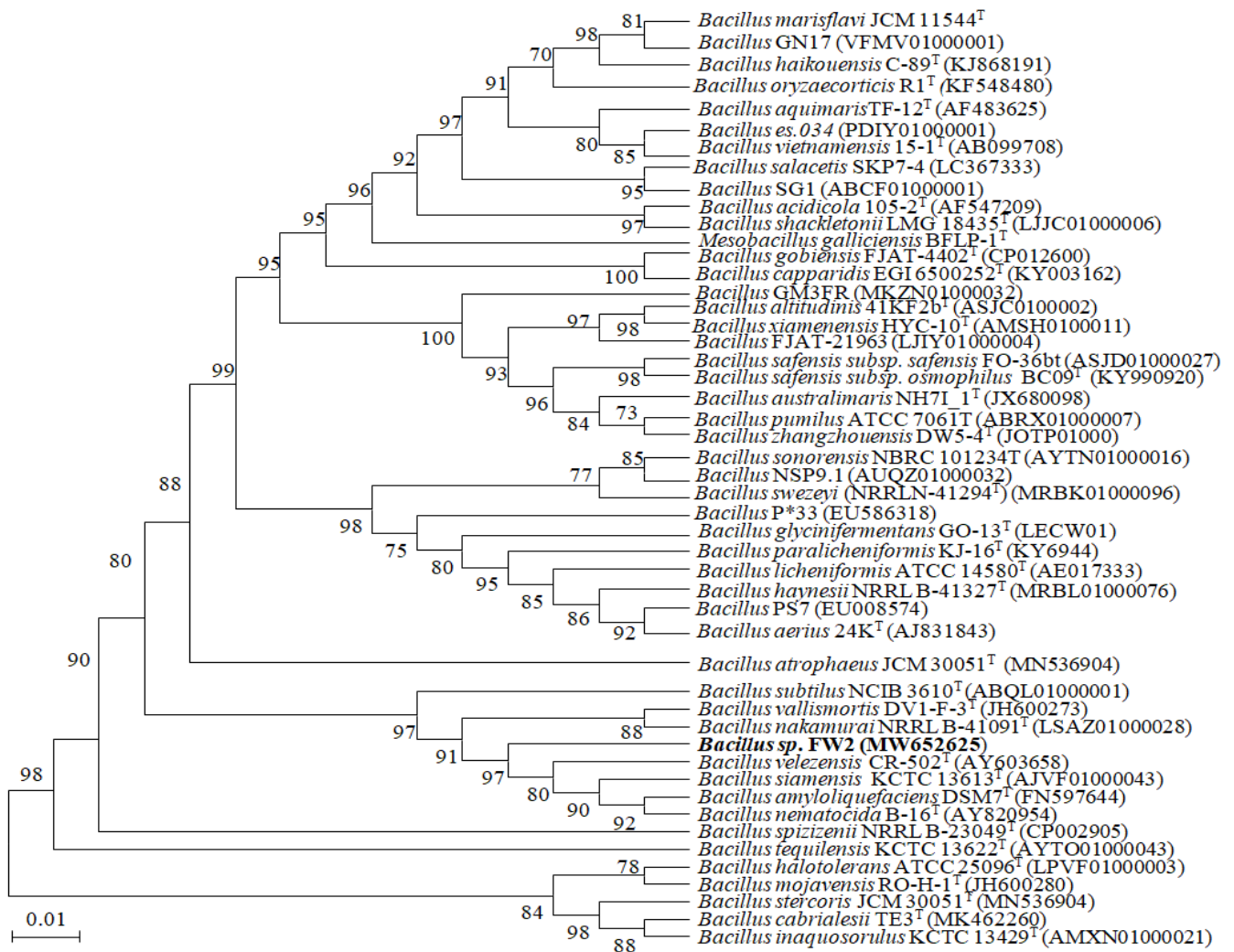


Figure 1. Phylogeny of 16S rRNA gene sequences of strain FW2 and related *Bacillus* genus members. The tree was constructed using the neighbor-joining method. Bootstrapping was carried out with 1500 replicates.

3.2. Optimization of Fermentation for Enzyme Production

Optimization of Physio-Chemical Parameters

Enzyme activities were determined by the size of a clear zone for protein while Gram's iodine was flooded; a clear distinct zone around bacterial colonies indicated amylase production (Figure 2).

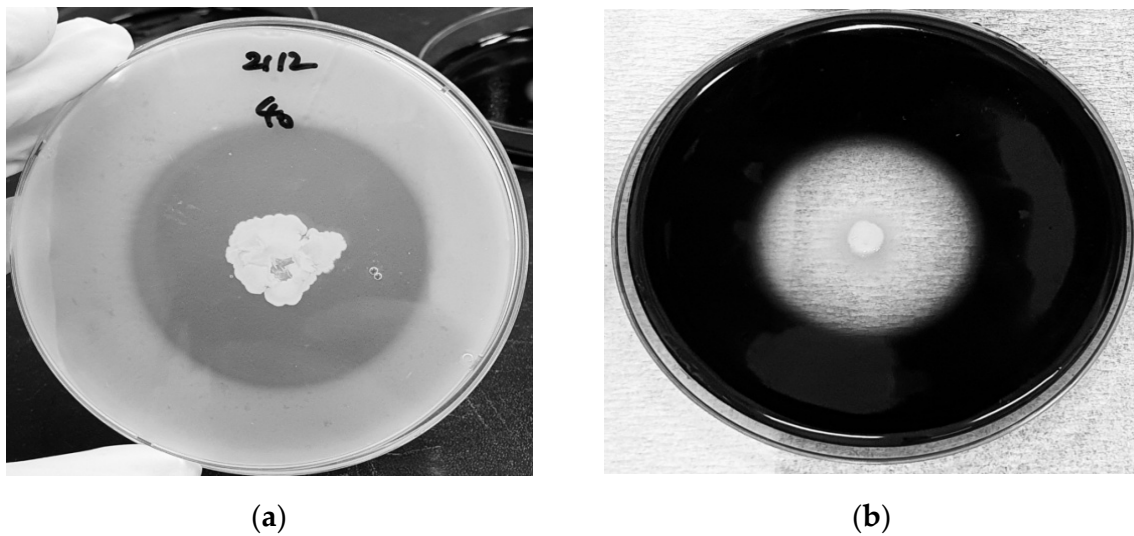


Figure 2. The protein (a) and starch (b) degradation of strain FW2 incubated at 45 °C for 4 days.

The largest sizes of halo zones were 52 mm and 43 mm for protein and starch degradation, respectively, at 45 °C for 4 days (Figure 3).

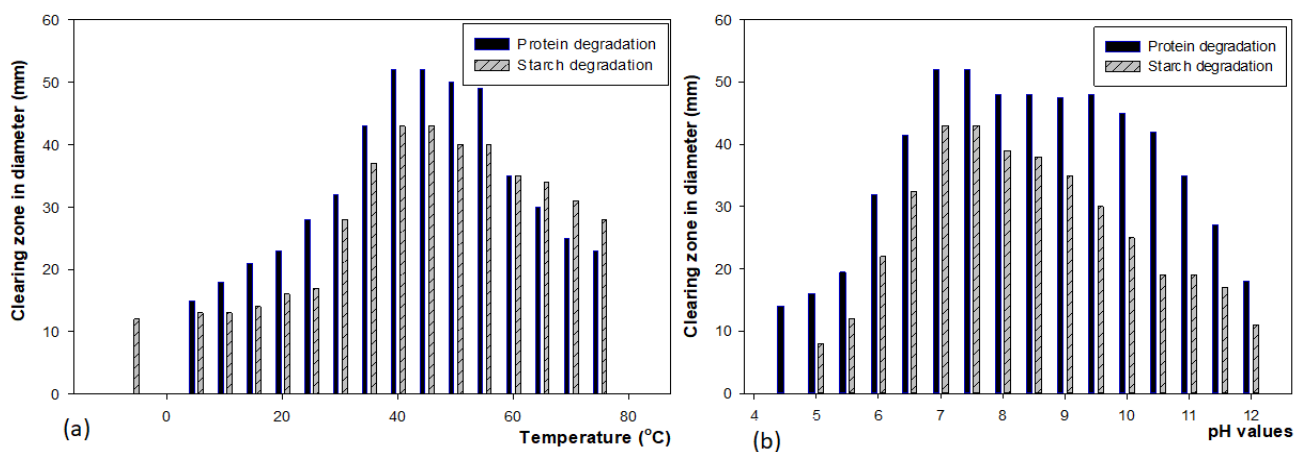


Figure 3. The effect of temperature (a) at pH 7–7.5 and pH values (b) at 45 °C for 4 d on protein and starch degradation.

Protease was produced strongly in the pH range of 5–10 and observed even at pH 4.5 and 35% NaCl but weakly at pH 4 and 40% NaCl. Optimal conditions for both protease and amylase generation were pH 7–8, NaCl 0–10%, and temperature 40–45 °C. Protease enzyme synthesis was found in a wide range of temperatures but not at –6 °C and 80 °C, and was repressed by rapid metabolism at 25 °C after 12 h. Enzyme production was investigated in a pH range of 4.5–12, but optimal production was observed between 7 and 8. A similar profile was obtained at 40–55 °C, which decreased rapidly from 60 °C after fixing the optimum pH value (Figure 3).

This enzyme demonstrated high stability at optimal temperature of 45 °C retaining 83%, decreasing to 32% at 75 °C and 12% at –6 °C after an incubation of 12 h (Figure 4).

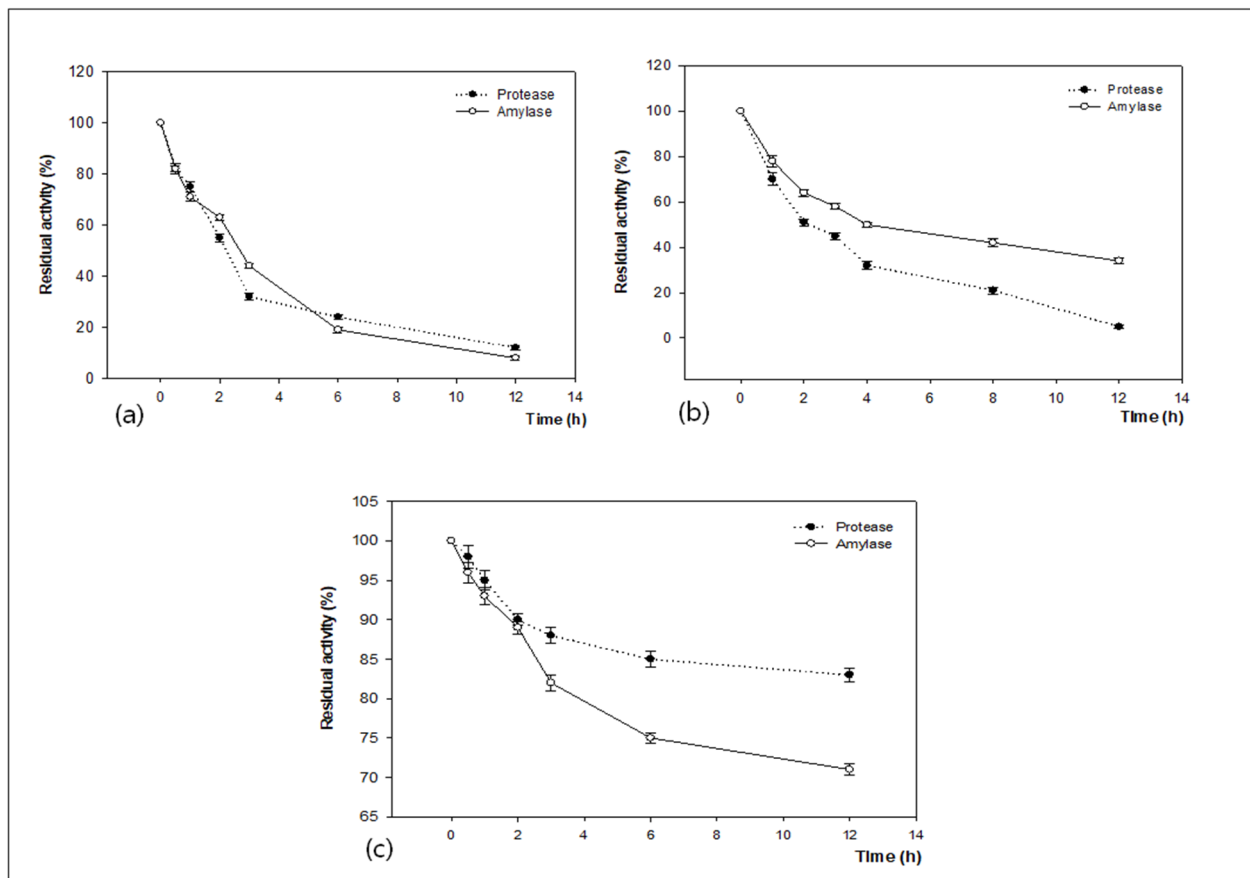


Figure 4. The stability of enzymes at different extreme and optimal temperatures (a) $-6\text{ }^{\circ}\text{C}$, (b) $75\text{ }^{\circ}\text{C}$, and (c) $45\text{ }^{\circ}\text{C}$.

Under acidic of pH 4.5 and alkaline condition of pH 12, the stabilities were 75% and 52% after 2 h incubation, then dramatically downed to 46% and 12% after 12 h, respectively (Figure 5).

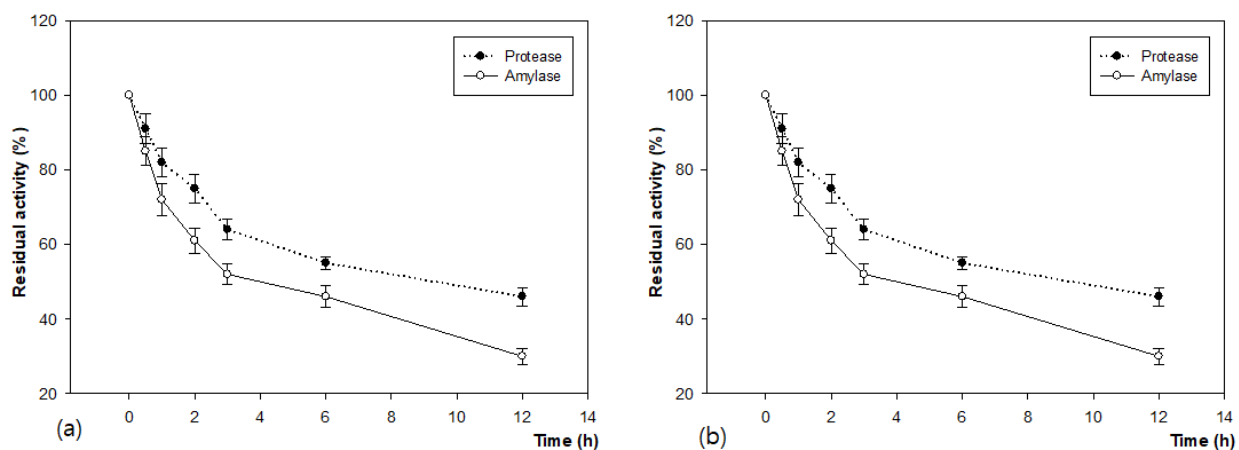


Figure 5. The stability of enzymes at acid and alkaline conditions: (a) pH 4.5 and (b) pH 12.

A clearing zone of protein degradation on skim milk agar plate was the highest at 52 mm in diameter, and the maximum concentration was optimized at 1200 U/mL mL^{-1} , at optimal conditions of 40 and $45\text{ }^{\circ}\text{C}$, pH 7–7.5, glucose (1% *w/v*) as the carbon source, and yeast extract powder (1% *w/v*) within 48 h of incubation compared to other carbon and nitrogen sources. Figure 6a shows that glucose was the most suitable carbon source

(1180 U/mL), followed by cellulose, starch, dextrose, and maltose. Minimum protease was reported in trials supplemented with lactose (980 U/mL). Additionally, among the six nitrogen sources, yeast extract was the most effective for protease production at 1150 U/mL, while the addition of ammonium chloride showed the lowest enzyme production level (850 U/mL) (Figure 6b).

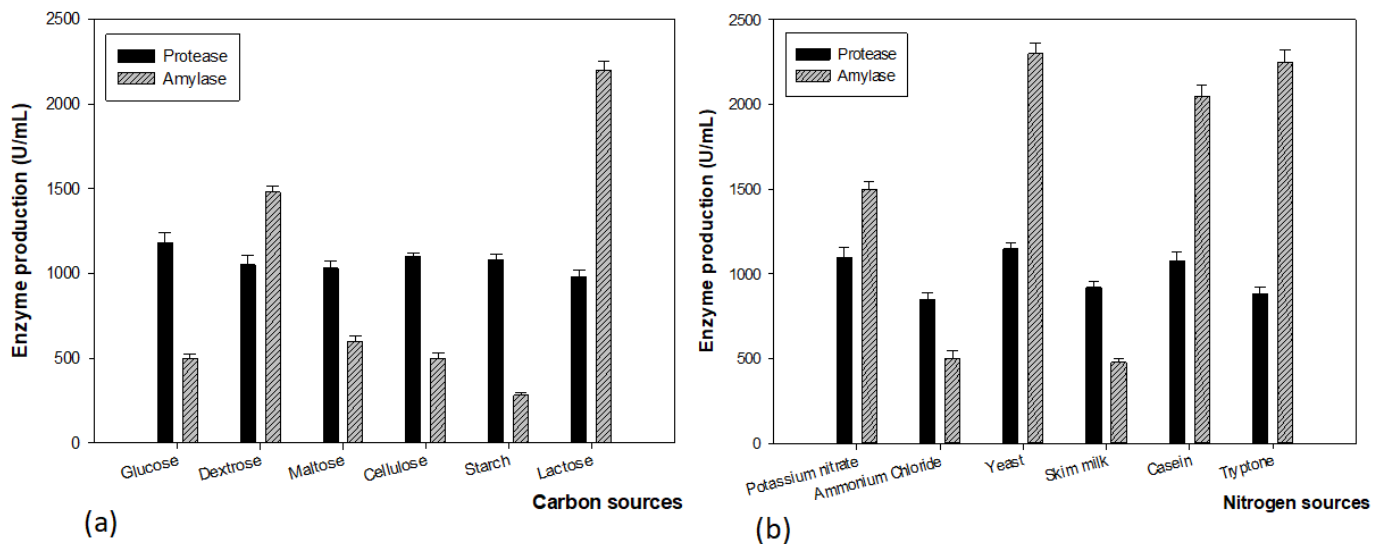


Figure 6. The effect of the carbon (a) and nitrogen sources (b) (1% *w/v*) on protease and amylase production of strain FW2 at 45 °C, initial pH 7–7.5 for 72 h. The experiments were carried out in triplicate. Error bars demonstrated standard deviation.

Screening of amylase activity of strain FW2 was determined by the clearing zone area using iodine solution on the starch containing agar plate. Figure 3 shows the effect of fermentation temperature on the synthesis of amylase from strain FW2 in a culture medium containing (per 1 L) starch 10 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1 g, NaCl 10 g, CaCl_2 2 g, and KH_2PO_4 2 g. The highest starch degradation was exhibited by amylase production in 43 mm diameter clearing zone at 40–45 °C, pH 7–7.5, after 4 d of incubation. In the enzyme assay, fermentation conditions were optimized for temperature, influence of initial pH, NaCl concentration, carbon source, and nitrogen source to enhance the growth and enzyme production. The produced amylase profile increased with time, reaching 1850 U/mL after 42 h, and gradually decreased thereafter, which might have been due to nutrient deficiency and death phase of bacterial growth. Lactose was determined as the optimal carbon source with 2200 U/mL of enzyme produced, and 2300 U/mL of enzyme was produced in the presence of yeast as a nitrogen source. This was followed by dextrose at 1480 U/mL and tryptone and casein at 2250 U/mL and 2050 U/mL, respectively, which were higher than the other carbon and nitrogen sources (Figure 6). The maximum amylase synthesis was found under all optimal conditions, reaching 2400 U/mL. The production of amylase retained 63% at –6 °C, 89% at optimal 45 °C, and decreased to 58% at 75 °C after 2 h incubation (Figure 4). This pattern was determined with 61% to 30% at pH 4.5 and 49% to 10% under alkaline conditions of pH 12 after 2 h and 12 h of incubation, respectively (Figure 5).

3.3. Determination of Molecular Weight of Produced Enzymes

Crude extract of proteases and amylases produced from strain FW2 after purification were subjected to SDS-PAGE analysis (Figure 7). The result revealed in Lane 1, as visualized by Coomassie brilliant blue staining, that there were two protein bands from distinct protease with molecular 28 kDa and 44 kDa, respectively. Lane 2 of purified amylase showed a single band with a mobility equivalent to the molecular weight of 55 kDa.

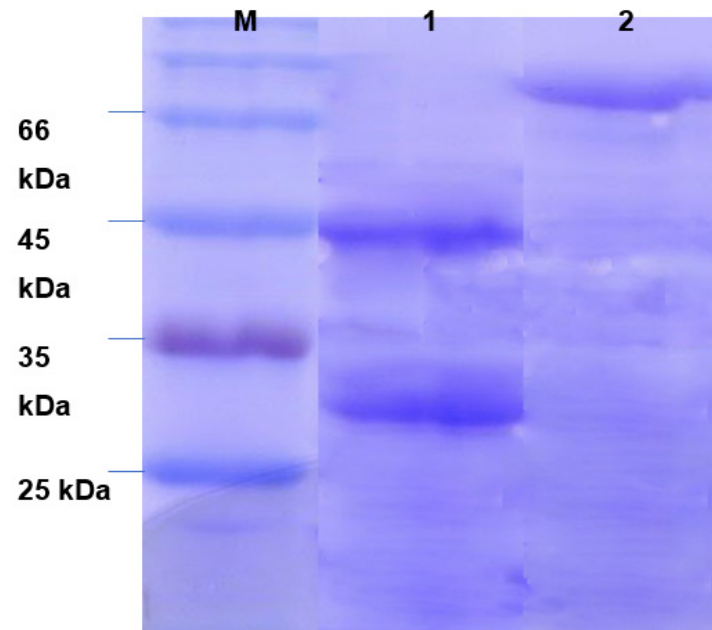


Figure 7. SDS-PAGE analysis of the crude extracellular proteases and amylase from *Bacillus* sp. FW2. Lane M, molecular weight of marker standard; lane 1: the crude extracellular proteases; lane 2: the crude extractcellular amylases.

3.4. Degradation of Food Waste in Volume

Compared to the control without bacterial inocula determined of 24% reduction after 28 days of composting process, all trials inoculated by bacterial strain FW2 exhibited the various efficiencies at different concentrations of bacterial culture under low and unstable temperatures ranging from 14 to 16 °C. An addition of 4% of inocula illustrated the highest degradation rate of food waste with 69%, followed by 3% and 5% of inocula with 51% and 50% of degradation, respectively. Inoculated trials with lower concentrations of 2% and 1% were observed the lower efficiencies with 42% and 39%, respectively (Figure 8).

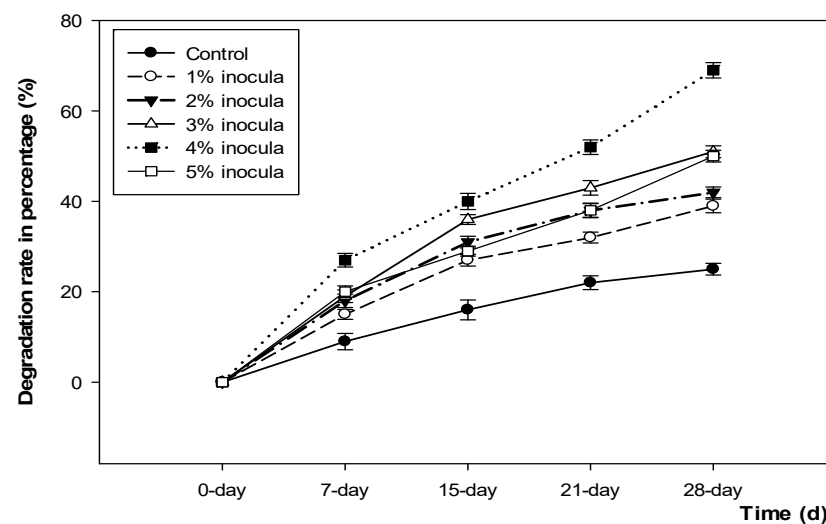


Figure 8. The degradation rate of food waste in a 28 day period was tested by 1% to 5% the culture of bacterial strain FW2 inoculated compared to control without bacterial inocula at pH 7 at a temperature of 14–16 °C. Experiments were performed in triplicate.

4. Discussion

To date, there have limited studies on enzyme production at low temperatures. Following the previous study, the related bacterial strains of strain FW2 grew within a limited temperature range of 15 to 45 °C, at pH 5–10, and were unable degrade protein [37]. The strain FW2 was halo-tolerant as it grew and showed enzyme activity in the presence of high NaCl concentrations up to 35% and was capable of affecting Tween 80, whereas it was not found in the closest strain *Bacillus amyloliquefaciens* DSM 7^T [38]. Sonika et al. reported that some halophilic isolates were capable of producing enzymes including protease and amylase at concentrations of NaCl up to 7.5%, pH 6–12, and at 25–70 °C. Some bacterial strains did not produce amylase even at 0% NaCl [39]. In another study, the enzyme production profile was obtained without amylase activity at concentrations below 5% NaCl, pH 8, and 55 °C [40]. However, protease activity of strain FW2 in this study was in the range of pH 5–10, consistent with the results of previous studies [41,42]. However, another thermophilic *Bacillus* strain investigated from other studies showed α -amylase production at a higher temperature of 90 °C compared to a maximum 75 °C for strains FW2 in this study and 40 °C for *B. subtilis* [43,44].

Recently, thermostable enzyme production from bacteria has attracted considerable attention, owing to its application in a wide range of fields. These thermo-enzymes are stable at high temperatures and active under other extreme conditions, such as varying pH values and salt concentrations [45–47]. However, these bacterial strains exhibited only one type of enzyme under thermophilic conditions.

The result from SDS-PAGE analysis in this study revealed that 28 kDa protein (AprM) is homologous to peptidase of *B. amyloliquefaciens* and *B. licheniformis* analyzed from previous studies [48–51]. However, the second band of 44 kDa band was investigated as neutral protease (NprM) of strain FW2, which was different from other *B. amyloliquefaciens* with 38 kDa [52], being 34 kDa from *B. subtilis* strain FP-133 [50]. The molecular weight of α -amylase extracted from strain FW2 in this study was 55 kDa, falling in the range of previous studies for members belonging *Bacillus* genus such as 56 kDa of *B. Bacillus subtilis* KIBGE HAS [53] while it was estimated of 68 kDa from *B. amyloliquefaciens* BH072 [54].

According to the obtained results in previous studies, many strains of *B. amyloliquefaciens* expressed the high application ability of extracellular amylase and protease such as *B. amyloliquefaciens* BH072, K11, and 7–6 [53,55,56]. Therefore, strain FW2 with high enzyme activities may have a significant contribution, not only in organic waste degradation but also in other industrial applications in the future.

In addition to the renewable energy production, saving energy has also been considered as a matter of concern, especially in winter. In South Korea, the ambient temperature drops to sub-low temperatures, which may cause the failure of composting process due to retarded bacterial growth. Moreover, the thermophilic phase has been recognized as a decidable period that affects efficiency and shortens the entire composting process. Therefore, the supplementation of the target bacterial culture to composting might increase the initial temperature to accelerate bacterial growth. Heat generated from microbial metabolism increases the compost temperature, thereby rapidly entering the thermophilic phase [55–59]. Thus, with a high food waste degradation rate in composting process in lab-scale, strain FW2 was found with strong adaptation in extreme environmental condition in this study to enhance the efficiency of food waste degradation.

5. Conclusions

As an important impressive point, many bacteria make resting forms such as spores in extreme environments in which bacteria are not active. In this study, we investigated extremophiles that could survive and produce multiple enzymes under fluctuating conditions. *Bacillus* sp. FW2 can be potentially applied in identifying enzymes that are important in terms of industrial value and have diverse applications in detergent, textile, leather, cosmetics, food, and paper industries. Moreover, the results obtained in our study could also facilitate the enhancement of waste treatment and optimize it to conserve energy in

winter. In particular, these types of bacteria need to develop the tolerance to improve their survival ability under extreme competitive conditions. Additionally, microbiologists can investigate new methods of culturing to coax fastidious organisms in future studies to explore the unknown bacterial functions.

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Conflicts of Interest: The authors declare no conflict of interest.

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