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Harnessing Co-Culture of Hydrolase–Producing *Bacillus* spp. to Boost Kitchen Waste Biodegradation

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ABSTRACT

In addressing the urgent challenge of organic waste management, our study investigates the use of hydrolase-producing bacteria (HPB) co-cultures as a targeted approach to enhance the biodegradation of kitchen waste (KW). This study aimed to isolate HPB with high enzyme activity to facilitate more efficient kitchen waste breakdown. The screening of 26 bacterial strains from fermented vegetables, agricultural soils, and in-house glycerol stock collections revealed *Bacillus licheniformis* 2D55 and *Bacillus xiamenensis* Y7 as standout candidates, exhibiting multiple hydrolase activities, including amylase, protease, lipase, and cellulase. In co-culture fermentation, these strains demonstrated superior hydrolase activities, microbial count, percentage of biodegraded total solids (TS), and gross degradation rate (GDR) during solid-state fermentation (SSF) compared to monocultures and controls. The co-culture treatments achieved 54.73% and 65% biodegraded TS in sterile and non-sterile SSF, respectively. Additionally, a 25.39% GDR in sterile SSF and 41.36% in non-sterile SSF were observed after the 14-day fermentation process. In the composting experiment, the inoculated compost exhibited a higher biodegraded TS (63.02%) compared to the control (29.80%). These findings underscore the potential of HPB co-cultures as an effective strategy to improve KW biodegradation, contributing to sustainable organic waste management solutions.

Keywords: Biodegradation, composting, hydrolase-producing bacteria, kitchen waste, solid-state fermentation

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INTRODUCTION

In developing countries like Malaysia, the disposal of household waste, a substantial component of municipal solid waste (MSW), predominantly occurs in landfills,

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many of which are anticipated to reach capacity within a decade (Abubakar et al., 2022). The complexity of managing MSW in Malaysia is exacerbated by escalating greenhouse gas (GHG) emissions from organic waste decomposition in landfills and the heightened demand for material and energy consumption to sustain the nation's growing population (Yong et al., 2019). Therefore, there is a pressing need for quick and practical ways to implement a more thorough and sustainable waste management strategy (Zainu & Songip, 2017).

Solid-state fermentation (SSF) and composting are widely recognized as effective, environmentally friendly approaches for treating organic waste, including kitchen waste (KW). SSF is particularly well-suited for KW due to its operational advantages, such as high microbial density, efficient enzyme production, and the ability to handle substrates with low moisture content, which aligns well with the high organic load in KW (Yafetto, 2022). SSF involves microbial growth on solid substrates, often producing a range of extracellular enzymes, such as hydrolases, that degrade complex organic matter into simpler compounds (Sarkar et al., 2016). Composting, on the other hand, has long been established as a natural, aerobic process for converting organic waste into stable humus-like substances through microbial activity (Storino et al., 2016). The composting process involves microbial hydrolysis and mineralization of organic matter, releasing fewer pollutants compared to other treatment options, and it has been shown to effectively reduce pathogenic microorganisms, promoting sanitary waste disposal (Wei et al., 2017). Given the challenges in landfill capacity and environmental concerns, composting also represents a sustainable alternative that contributes to a circular economy by recycling organic nutrients back into the soil.

The application of HPB in waste treatment has gained increasing attention, particularly in enhancing the biodegradation efficiency of composting and SSF processes. Previous studies have shown that HPB, including strains of *Bacillus* and other genera, contribute significantly to the degradation of complex organic molecules such as starch, proteins, lipids, and cellulose in organic waste (Cerda et al., 2017). For instance, Singh et al. (2021) demonstrated that *Bacillus* spp. with robust amylase and protease activities effectively degraded food waste in a composting setup, resulting in reduced total solids and improved compost quality. This suggests that targeted microbial inoculation could accelerate the composting process and reduce greenhouse gas emissions associated with the decomposition of organic waste. Besides, Drissi Kaitouni et al. (2020) also reported that HPB in SSF processes not only improves enzymatic activity but also enhances the overall microbial biomass, leading to more efficient substrate degradation. However, despite the apparent advantages, only a few studies have been conducted to examine the use of HPB to target KW. The specific data on the KW and the combined effect of HPB with multiple hydrolase activities in both SSF and composting remain sparse (Cerda et al., 2017).

Therefore, the main objective of this research is to isolate and evaluate HPB as an inoculant to improve the biodegradation of KW, which is mainly comprised of rice and vegetables. Rice and vegetables are staple foods in many diets worldwide, so they are often present in significant amounts in KW. Both rice and vegetables present diverse organic components, including carbohydrates, proteins, and fibers. This diversity allows for a comprehensive assessment of the effectiveness of HPB in degrading different substrates commonly found in kitchen waste (Wan et al., 2020). The selected HPB were then tested in sterile and non-sterile SSF. This study evaluates both sterile and non-sterile fermentation conditions to understand the biodegradation potential of monoculture versus co-culture HPB in controlled and natural microbial environments. The sterile fermentation provides a controlled baseline that eliminates external microbial influences, while the non-sterile fermentation introduces natural microbial communities that better mimic the actual environment where biodegradation would occur, such as in soil or waste treatment facilities. This allows us to assess the practical applicability of the co-culture in broader waste management contexts. Lastly, the research evaluated the effects of HPB on the composting of KW.

MATERIALS AND METHODS

Sample Collection

Fermented vegetables, specifically spinach, were obtained from a bioreactor (Biostat® B, Germany) set up in the laboratory experiment for undergraduate students at the Universiti Putra Malaysia (UPM). Agricultural soil, collected from the same research center at a depth of 5–10 cm, was sealed in polyethylene bags. Both samples were stored in 50 ml sterile centrifuge tubes in the chiller (4°C) until use. *Bacillus licheniformis* 2D55 was revived from the glycerol stock solution preserved at -80°C (Kazeem et al., 2016).

Isolation and Screening of Hydrolase-Producing Microorganisms

Isolation was carried out using the serial dilution technique. The samples were transferred to de Man, Rogosa, and Sharpe (MRS) agar (Oxoid, England), nutrient agar (Oxoid, England), yeast extract peptone dextrose (YEPD) agar (BD Biosciences, Canada), and incubated for 24 hr at 37°C. Morphologically distinct isolates were randomly selected and streaked onto freshly prepared agar plates. Each isolate underwent primary screening for hydrolytic enzyme production (amylase, protease, lipase, and cellulase). Starch, casein, tributyrin, and carboxymethylcellulose (CMC) were used in hydrolytic plate assay tests for amylolytic, proteolytic, lipolytic, and cellulolytic activities, respectively. Following incubation for 48 hr at 37°C, specific staining solutions were applied to the culture media. The presence of the corresponding enzymes was indicated by clear zones surrounding the colonies. For amylolytic activity, iodine solution was poured over the isolates after

they were inoculated onto starch agar media (HiMedia, India) (20 g/L soluble starch, 5 g/L peptone, 5 g/L sodium chloride [NaCl], and 20 g/L agar; pH 7) (Simair et al., 2017). Isolates were grown on skim milk agar (Oxoid, England) (28 g/L skim milk powder, 2.5 g/L yeast extract, 1 g/L dextrose, 5 g/L casein, 20 g/L agar; pH 7) to detect proteolytic activity (Tallapragada et al., 2018).

Similarly, isolates were inoculated on spirit blue agar (HiMedia, India) (10 g/L tryptone, 5.0 g/L yeast extract, 0.15 g/L spirit blue, 20 g/L agar; pH 7) mixed with 30 ml of a lipoidal solution (Merck, Germany) (1 ml of polysorbate 80, 100 ml of olive, and 400 ml of warm water at 60°C) to assess lipolytic activity (Privé et al., 2015). For cellulolytic activity, CMC-agar plates (HiMedia, India) (5 g/L CMC, 1 g/L peptone, 2 g/L monopotassium phosphate [KH_2PO_4], 1.4 g/L ammonium sulfate [$(NH_4)_2SO_4$], 0.3 g/L urea, 0.3 g/L magnesium sulfate heptahydrate [$MgSO_4.7H_2O$], 0.3 g/L calcium chloride [$CaCl_2$], 0.0016 g/L manganese (II) sulfate [$MnSO_4$], 20 g/L agar; pH 7) were used to inoculate the isolates. After incubation for 48 hr at 37°C, the plates were flooded for 5 min with Congo red solution (HiMedia, India; 2 g/L), followed by a 15-min wash with 1 M NaCl (Sethi et al., 2013). The existence of a clear zone enclosing the bacterial colony in all assays indicated positive hydrolytic activity (+), whereas the absence of such a zone indicated negative hydrolytic activity (–). The hydrolysis index (HI) was calculated by interpreting the ratio of the hydrolysis zone diameter to the colony diameter (Roslan et al., 2021). All experiments were conducted in triplicate to ensure the reproducibility of results, and the data presented reflect the mean values with standard deviations.

Quantitative Hydrolase Activity Assay

Isolates were introduced into a broth medium containing 1% (w/v) soluble starch (Merck, Germany), 1% (w/v) casein (Merck, Germany), p-nitrophenylpalmitate (p-NPP) (Thermo Scientific, USA), and 1% (w/v) CMC (HiMedia, India) as substrates for evaluating amylolytic, proteolytic, lipolytic, and cellulolytic activities, respectively. The broth underwent incubation for 24 hr at 37°C with shaking at 150 rpm. Subsequently, the culture was centrifuged for 10 min at 11,200 × *g*, and the supernatant was collected to serve as a crude enzyme. Hydrolytic enzyme activities were determined following standard methods. Hydrolase activity assays were performed in triplicate to ensure reproducibility. Identical conditions, such as temperature, pH, and substrate concentrations, were maintained across replicates. Data are presented as mean values with standard deviations to reflect observed variations. Cellulase and amylase activity were assessed using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). Using casein as a substrate, protease activity was determined (Tsuchida et al., 1986); whereas lipase activity was assessed by hydrolyzing p-NPP (Winkler & Stuckmann, 1979). Strains exhibiting more than one enzyme and higher enzymatic activities compared to others were selected and identified.

Characterization and Identification of Hydrolase-Producing Microorganisms

Observation of Gram's reaction and morphological characteristics of the selected strains was conducted using light microscopy (Olympus CH, Japan). The chosen bacteria were cultured overnight at 37°C in nutrient agar media to obtain a pure culture. DNA extraction was conducted using the GF-1 Bacterial DNA extraction kit (QIAGEN, Germany), and the amplification of the 16S rDNA gene fragment was achieved through polymerase chain reaction (PCR) using universal primers 1492R and 27F (Yadav et al., 2009). Using the QIA Quick PCR Purification kit (QIAGEN, Germany), the PCR product was purified. Sequencing of the PCR product was performed by the sequencing service center. To determine the species, the 16S rRNA gene sequences were run via the Basic Local Alignment Search Tool (BLAST). The construction of the phylogenetic tree was accomplished using MEGA X Alignment Explorer (version 10.2.6) (Kumar et al., 2018).

Biocompatibility Assay

The pour-plated method was used to evaluate the biocompatibility of the selected strains (Grossart et al., 2004). In this procedure, a 50 µl suspension of the target bacterial strain (1×10^8 CFU/ml) was combined with molten nutrient 1% agar (Oxoid, England) (2.5 ml). Once transferred onto a nutrient agar plate, the cell suspension agar solidified over a 10-min period. A 10 µl aliquot of the test strain (1×10^8 CFU/ml) was spotted onto the agar surface and incubated for 3 days at 37°C. The assessment of biocompatibility involved measuring the size of the inhibition zone formed on the plates post-incubation. Inhibition zones were monitored daily, and strains were tested in triplicate against one another. When the inhibition zone surrounding the spotted colony measured more than 4 mm in diameter, inhibitory activity was recorded.

Enzymatic Degree of Synergy (DS)

One ml aliquot of the starter culture, with the inoculum adjusted to 1×10^8 CFU/ml and grown in nutrient media, was utilized to inoculate specified production media for each enzyme. Prior to inoculation into the test media, individual inoculum cultures (0.5 ml each) were combined in an equal ratio of 1:1 (v/v) to create the co-culture. The cell-free culture supernatant, grown separately in those selective media, was shaken at 150 rpm for 24 hr at 37°C before the hydrolytic enzyme activity was determined. To obtain the cell-free culture supernatant, the culture was centrifuged for 10 min at 11,200 x *g*. By dividing the observed activity of the co-culture enzyme by the total of all the monocultural enzymes in the same media, the enzymatic DS is calculated (Van Dyk et al., 2013). To assess the effect of pH, each pure bacterial culture and a co-culture of the selected strain were incubated at different pH values ranging from 5.0 to 9.0, using standard assay methods as previously described. Each pure bacterial culture (pH 7) and co-culture of selected strains were incubated for 1

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day at temperatures ranging from 30 to 50°C, and the effect of temperature was evaluated. The enzymatic activities were also measured using similar methods.

Solid-State Fermentation

The SSF experiment was conducted in 250 ml Erlenmeyer flasks, each containing 100 g of KW, consisting of rice and vegetable waste collected from a restaurant near Sri Serdang, Selangor, Malaysia. The KW underwent washing, drying, and coarse grinding. The mixture's main characteristics are outlined in Table 1. Two types of pretreatments were employed: sterile KW samples (autoclaved for 15 min at 121°C) and non-sterile KW samples. Rice and vegetable wastes (50 g each) were weighed, mixed, and supplemented with sterilized water to maintain a 60-65% moisture content (Kaewlaoyoong et al., 2020). The mixture was then placed into a sterile conical flask. To prepare the inoculum for SSF, the chosen strains were grown separately in nutrient broth overnight. Subsequently, 1 ml (~1×10^8 CFU/ml) of the inoculum was added to each test flask and incubated statically for 14 days at 25 ± 2°C. For co-culture inoculation, the inoculum was prepared by combining equal volumes of different monocultures (1:1 ratio, v/v) before introducing it into the test flasks. Control flasks received 1 ml of sterile broth each.

Moisture and ash content were determined using the drying method (Kutsanedzie et al., 2012), while pH was monitored daily with a digital pH meter (Thermo Scientific Eutech Instruments, USA). Microbial population during KW degradation was assessed by mixing 9 ml of distilled water containing 0.85% (w/v) NaCl (Merck, Germany) with 1 g of the sample (Varma et al., 2017). Enumeration of the microbial population was conducted on nutrient agar using the standard plate count method (Sanders, 2012). The percentage of KW degradation based on total solids (TS) over the 14-day incubation period was determined following the standard procedure (Leege, 1998). Enzymatic activities (lipase, cellulase, protease, and amylase) were assayed using KW extracts in various solutions of buffer (Leow et al., 2018), and the hydrolase activities were evaluated as per standard procedures. The gross degradation rate (GDR) of KW during the 14-day digestion period was calculated using daily gross weight variations from the initial reading, following the method outlined by T. Li et al. (2014).

Table 1
Properties of the selected kitchen waste components

Parameter	Rice	Vegetables	Rice and vegetables
Moisture (%)	3.62 ± 0.12	15.54 ± 0.24	37.29 ± 0.30
Total solids (%)	97.30 ± 1.16	14.29 ± 2.20	76.31 ± 2.37
pH	6.34 ± 0.02	6.71 ± 0.03	6.68 ± 0.02

Note. Data represent mean ± SD; n = 3

Preparation, Monitoring, and Evaluation of Composting Process

A dual-chamber tumbling composter (Miracle-Gro®, United Kingdom) served as the experimental apparatus for this study. Composting materials, including vegetable waste, goat manure, and rice husk, were sourced from Pasar Borong Selangor, Fakulti Pertanian, UPM, and Kilang Beras Bernas Sekinchan, respectively. Each chamber was filled with 25 kg of feedstock (wet weight), comprising 50% vegetable waste, 25% goat manure, and 25% rice husk, establishing an initial carbon-to-nitrogen (C/N) ratio of 25-30:1. An amount of 1.5 L inoculum for the composting process was prepared by first growing the HPB in 250 ml of nutrient broth at 37°C with shaking for 24 hr. The culture was then diluted by taking 15 ml of the initial culture (~1×10^9 CFU/ml) and mixing it with 235 ml of sterile distilled water. This dilution was further scaled up by adding 1.25 L of sterile distilled water to achieve a final volume of 1.5 L.

The inoculum was mixed thoroughly using a magnetic stirrer to ensure uniform distribution of bacteria throughout the solution. To verify the final bacterial concentration, colony-forming unit (CFU) counts were performed by serial dilution and plating onto agar plates to confirm the final bacterial concentration, which was ~1×10^8 CFU/ml in the inoculum. Optical density (OD) measurements at 600 nm were also taken from aliquots of the inoculum to monitor the growth trend and ensure uniform bacterial distribution. In one chamber, 1.5 L of HPB was added, while in the control chamber, 1.5 L of distilled water containing 15 ml of sterile nutrient broth (prepared in the same manner as for the inoculated treatment) was used to achieve an initial moisture content of 40-60% (Wu et al., 2017). Throughout the two-month composting process, various parameters were monitored, including pH, temperature, odor, color, C/N ratio, hydrolase activities, bacterial population, and the percentage of degradation rate based on TS. Moisture, pH, temperature, hydrolase activities, bacterial population, and TS degradation rate were assessed using methods outlined in the Solid-state Fermentation section. For C/N ratio analysis, samples were dried at 70°C, ground, and analyzed using a carbon, nitrogen, and sulfur (CNS) elemental analyzer (TruMac CNS Macro Analyzer, USA) (Fan et al., 2018).

To evaluate phytotoxicity, a germination test using radish seeds was conducted on the final compost samples, both with and without HPB, over 8 weeks (Siles-Castellano et al., 2020). For each sample, 15 ml of distilled water was mixed with 1.5 g of compost (dry basis) for one hour at room temperature. The mixture was then centrifuged at 2,800 x *g* for 20 min, and after the supernatant was filtered, 5 ml of the extract was added to a container containing 20 radish seeds on tissue paper. Each sample was incubated in the dark for 72 hr at room temperature with two duplicates. As a control test, 20 radish seeds were placed in a container with 5 ml of distilled water. The germination index (GI) was determined based on the number of germinated seeds and the length of their roots (Wang et al., 2022).

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Statistical Analysis

Statistical analyses were conducted using two-tailed, unpaired t-tests at a confidence level of $P < 0.05$ and $P < 0.01$ to ascertain the relevance of variations in HPB's HI on screening plates and in the level of HPB synergism. Changes in hydrolase activity across treatments for HPB, TS, and GDR were assessed using one-way analysis of variance (ANOVA). Tukey's test was performed on the calculated mean at $P < 0.05$ and $P < 0.01$. The correlation coefficients between HI and hydrolase activity, hydrolase activity and pH, and hydrolase activity and temperature were measured using Pearson's correlation, all at $P < 0.05$ and $P < 0.01$ confidence ranges. By using SPSS software Package Version 25.0 (SPSS Inc., USA), all statistical analyses were performed.

RESULTS

Isolation and Screening of Hydrolase-Producing Microorganisms

A total of 26 bacterial cultures, comprising 10 isolates from fermented vegetables, 15 isolates from soil samples, and one isolate from a glycerol stock solution, were selected based on diverse colony morphology for further analysis. Screening on plates containing starch, casein, tributyrin, and CMC agar revealed that 24 isolates exhibited significant hydrolase activity (Table 2). Out of these, 16 isolates could produce protease, 8 isolates demonstrated lipase activity, 7 isolates showed cellulase activity, and 6 isolates exhibited amylase activity. Some strains displayed more than one hydrolytic activity, with only strains 2D55 and Y7 showcasing more than two hydrolytic activities. Strain 2D55 displayed all four hydrolytic activities, while strain Y7 produced amylase, protease, and lipase. On average, the HI of 2D55 was consistently higher than that of Y7 in the amylase and lipase agar assays ($P < 0.05$). However, for the protease assay, the HI of 2D55 and Y7 showed no significant difference. Among all hydrolases, 2D55 exhibited the highest HI for cellulase (5.24), while Y7 demonstrated the highest HI for lipase (3.17).

Table 2
Qualitative screening of hydrolytic enzymes produced by the isolates

Source	Isolate	Hydrolysis index			
		Amylase	Protease	Lipase	Cellulase
Fermented vegetables	N1	1.07 ± 0.02a	nd	nd	nd
	N2	nd	1.07 ± 0.02a	1.07 ± 0.02a	nd
	N3	nd	nd	nd	1.07 ± 0.02a
	N4	nd	nd	1.12 ± 0.07a	nd
	N5	1.12 ± 0.07a	1.12 ± 0.07a	nd	nd
	N6	nd	1.08 ± 0.03a	nd	nd
	N7	1.08 ± 0.03a	nd	nd	1.12 ± 0.07a
	N8	nd	nd	nd	3.17 ± 0.11c

Table 2 *(continue)*

Source	Isolate	Hydrolysis index			
		Amylase	Protease	Lipase	Cellulase
	N9	1.21 ± 0.11a	nd	nd	nd
	N10	nd	1.21 ± 0.11ab	nd	nd
Soil sample	Y1	nd	1.38 ± 0.07ab	nd	nd
	Y2	nd	nd	nd	1.21 ± 0.11a
	Y3	nd	nd	nd	nd
	Y4	nd	2.04 ± 0.26c	nd	nd
	Y5	nd	nd	nd	1.38 ± 0.07a
	Y6	nd	nd	nd	nd
	Y7	1.38 ± 0.07a	2.02 ± 0.03c	3.17 ± 0.11c	nd
	Y8	nd	1.53 ± 0.03b	nd	2.04 ± 0.26b
	Y9	nd	1.36 ± 0.14ab	1.21 ± 0.11a	nd
	Y10	nd	1.21 ± 0.09ab	1.38 ± 0.07a	nd
	Y11	nd	1.40 ± 0.21ab	nd	nd
	Y12	nd	1.23 ± 0.08ab	2.04 ± 0.26b	nd
	Y13	nd	1.17 ± 0.04a	nd	nd
	Y14	nd	1.06 ± 0.01a	2.02 ± 0.03b	nd
	Y15	nd	1.11 ± 0.08a	nd	nd
Glycerol stock collection	2D55	2.04 ± 0.26b	2.20 ± 0.02c	4.12 ± 0.24d	5.24 ± 0.24d

Note. Data represent mean ± SD. According to Tukey's test, values in the same column that are followed by different lowercase letters indicate significant differences at $P < 0.05$; nd = Not detected; n = 3

Quantitative Assay of Microbial Hydrolase Activities

In the liquid-based assays (Table 3), strain 2D55 exhibited the highest amylase potential with an enzyme activity and specific activity of 211.06 U/ml and 222.17 U/mg, respectively. The highest protease activity was observed in strain Y1, but no significant difference was found compared to strains 2D55 and Y7. Both strain 2D55 and Y7 showed equally high lipase activity ($P > 0.05$), recording enzyme activities of 303.89 and 313.39 U/ml, respectively. In terms of cellulase activity, strain 2D55 demonstrated the highest enzyme activity, almost double the value of strain N3, at 169.04 U/ml. Additionally, the HI of amylase ($r = 0.887$, $P < 0.05$), protease ($r = 0.578$, $P < 0.05$), lipase ($r = 0.595$, $P < 0.05$), and cellulase ($r = 0.699$, $P < 0.05$) showed significant correlations with hydrolase activity in liquid media. Therefore, strains 2D55 and Y7 were chosen for additional analysis based on the overall outcomes.

Bacterial Characterization and Identification

By conducting biochemical analysis and sequencing the 16S rRNA gene of each strain, the phenotypic and genotypic characteristics of the selected strains were determined (Table 4). The results revealed that both HPBs were capable of thriving in high-temperature

Table 3
Quantitative screening of hydrolytic enzyme-producing isolates

Strain ID		Enzyme activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)
Amylase	N1	23.01 ± 7.26a	0.65 ± 0.04b	35.40a
	N5	87.07 ± 3.16c	0.55 ± 0.02a	159.27c
	N7	61.58 ± 5.47b	0.84 ± 0.04c	73.02b
	N9	62.27 ± 3.16b	0.64 ± 0.02b	97.81b
	Y7	149.07 ± 7.82d	0.87 ± 0.02cd	171.34c
	2D55	211.06 ± 6.64e	0.95 ± 0.04d	222.17d
Protease	N2	17.09 ± 0.60a	0.32 ± 0.02a	52.87c
	N5	25.10 ± 6.80a	0.47 ± 0.03bc	53.79cd
	N6	31.37 ± 5.75a	0.60 ± 0.02cd	51.99c
	N10	90.90 ± 3.36b	0.96 ± 0.02g	94.69g
	Y1	118.76 ± 2.76c	1.24 ± 0.02i	95.52g
	Y4	117.71 ± 8.92c	1.35 ± 0.05j	87.41f
	Y7	105.53 ± 6.38bc	0.85 ± 0.03ef	123.67h
	Y8	103.90 ± 14.62bc	1.40 ± 0.02j	73.46e
	Y9	112.84 ± 3.95c	1.53 ± 0.03k	73.91e
	Y10	117.02 ± 18.40c	1.17 ± 0.02h	100.30g
	Y11	23.71 ± 1.04a	1.19 ± 0.04i	19.87a
	Y12	25.45 ± 5.26a	0.54 ± 0.02c	47.42c
	Y13	27.89 ± 2.76a	0.46 ± 0.03b	60.19d
	Y14	39.73 ± 9.48a	0.46 ± 0.01b	86.36f
	Y15	27.89 ± 2.76a	0.78 ± 0.02e	35.60b
	2D55	100.65 ± 3.19bc	0.86 ± 0.02f	117.49h
Lipase	N2	104.46 ± 37.99a	1.36 ± 0.04de	76.62a
	N4	284.90 ± 18.99c	1.44 ± 0.04e	198.31d
	Y7	313.39 ± 9.50d	1.29 ± 0.01cd	242.94e
	Y9	212.09 ± 5.48b	1.36 ± 0.04de	155.57c
	Y10	120.29 ± 29.01a	1.03 ± 0.03a	116.79b
	Y12	319.72 ± 19.77d	1.20 ± 0.02bc	265.70f
	Y14	218.42 ± 18.99bc	1.16 ± 0.04b	187.76d
	2D55	303.89 ± 37.99d	1.27 ± 0.06cd	238.66e
Cellulase	N3	88.45 ± 7.45d	0.98 ± 0.07d	90.56d
	N7	27.14 ± 3.16ab	0.75 ± 0.04ab	36.03a
	N8	20.25 ± 5.47a	0.72 ± 0.02a	28.26a
	Y2	26.45 ± 5.47ab	0.74 ± 0.04a	35.91a
	Y5	40.92 ± 7.45b	0.85 ± 0.03bc	48.14b
	Y8	65.03 ± 5.20c	0.94 ± 0.03cd	68.93c
	2D55	169.04 ± 6.02e	1.43 ± 0.03e	118.21e

Note. Data represent mean ± SD. According to Tukey's test, values in the same column that are followed by different lowercase letters indicate significant differences at $P < 0.05$

conditions (60°C), exhibiting growth even in low pH environments down to pH 3. They also demonstrated moderate survivability at a high pH condition, up to pH 12. Strain Y7 exhibited 100% homology with *B. xiamenensis* strain MCCC 1A00008 (NR 148244.1), while strain 2D55 showed a 99% similarity to *B. licheniformis* strain 1-13AIA (FN 397486). Data for strain 2D55 has been previously published by Kazeem et al. (2016).

The evolutionary relationships between the enzyme-producing isolates and their closest type strains were analyzed using partial 16S rRNA gene sequences. A maximum-likelihood phylogenetic tree was constructed with the Tamura-Nei model after sequence alignment by ClustalW. As shown in Figure 1, the isolate *Bacillus xiamenensis* strain Y7 clustered closely with *B. xiamenensis* strain MCCC 1A00008, confirming its taxonomic placement among gram-positive rods.

Effect of pH and Temperature on the Hydrolase Activity

In the pour-plate assay, there were no indications of antagonistic interaction between strains 2D55 and Y7. Additionally, the liquid-based assay (Figure 2) revealed that the initial pH

Table 4
Cell morphology, colony morphology, and molecular identification of strain 2D55 and Y7

Characteristics	2D55	Y7
Cell morphology	Rod, Gram-positive	Rod, Gram-positive
Colony morphology	Whitish, rough, hair-like outgrowth, 2-3 mm in diameter	Large-sized, flat, irregular, Off-white, smooth colonies
Aerobic growth	+	+
Anaerobic growth	+ (facultative anaerobic)	+ (facultative anaerobic)
Growth at 30°C	++	++
Growth at 40°C	++	++
Growth at 50°C	+++	++
Growth at 60°C	++	++
Growth in pH 3	+	+
Growth in pH 5	++	++
Growth in pH 7	+++	+++
Growth in pH 9	+++	++
Growth in pH 12	+	+
Motility	+	+
Closest relatives in NCBI Genbank	*Bacillus licheniformis* strain 1-13AIA (FN 397486) *	*Bacillus xiamenensis* strain MCCC 1A00008 (NR 148244.1)
Percentage similarity	99%*	100%
Designated species name	*Bacillus licheniformis* 2D55*	*Bacillus xiamenensis* strain Y7
NCBI Accession no.	KT799651*	OK090764

Note. + = Positive reaction; – = Negative reaction; ++ = Moderate growth; +++ = High growth; NCBI = National Center for Biotechnology Information; * = Adapted from Kazeem et al. (2016)

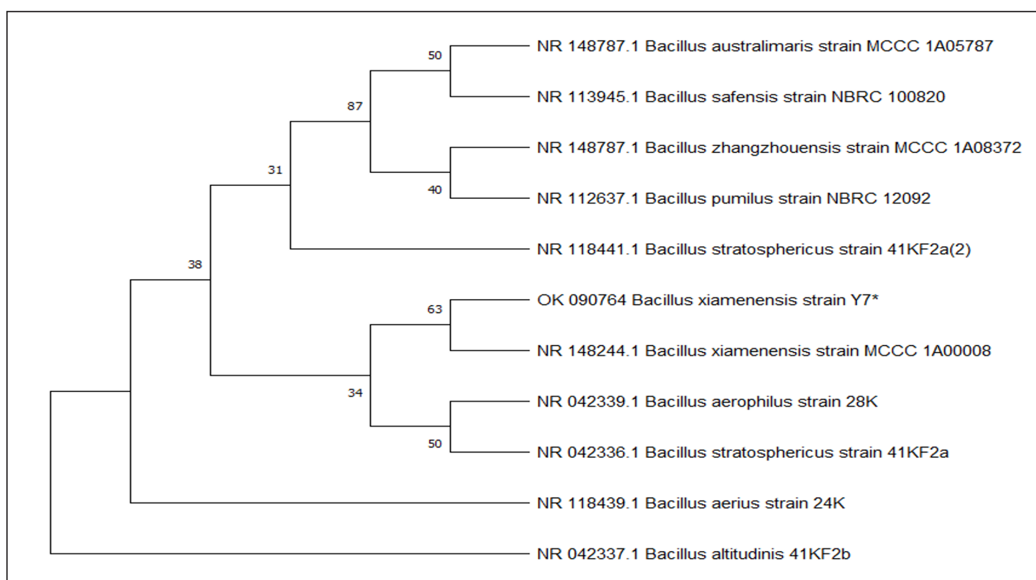

Figure 1. The strain names are preceded by the accession numbers of the type strains, enclosed in parentheses. The picture showed gram-positive rods of the *Bacillus xiamenensis* strain Y7

significantly influenced hydrolase production. In co-culture media, the highest hydrolase activities were observed at pH 7 ($P < 0.05$). While there was a notable decline in hydrolase production in co-culture at pH values below or above optimality, these productions surpassed those of both monocultures. Among these, amylase activity recorded the highest at 410.47 U/ml. Regarding monoculture, strain 2D55 consistently showed a notably higher activity ($P < 0.05$) than Y7 for amylase, lipase, and cellulase activity across all pH levels (5-9), except for protease activity. Upon closer examination, it was evident that the DS in total hydrolase activity was greater than the sum of all mono-culture activities, confirming the co-culture's hydrolase synergy.

Similarly, temperature had a significant impact on hydrolase activities, as shown in Figure 3. For amylase activity, both monocultures exhibited an increase from 35°C to optimal activity at 45°C, indicating the preferred temperature range for active amylase secretion. The co-culture maintained stable activity between 35 and 45°C, reaching its highest DS of 1.03 at 35°C. Strain 2D55 showed maximum protease activity at 35°C, decreasing at higher temperatures, while strain Y7 exhibited a gradual increase up to 45°C. However, the co-culture demonstrated significantly higher protease activities at all temperatures, peaking at 40°C with a DS value of 1.1. Both monocultures and the co-culture displayed optimal lipase activities at 40°C, with the co-culture exhibiting the highest activity at all temperature points, followed by strain 2D55 and Y7. Interestingly, contrary to the agar-based assay, strain Y7 exhibited cellulase activity in this liquid-based assay, albeit at a relatively low level. Meanwhile, strain 2D55 consistently showed higher

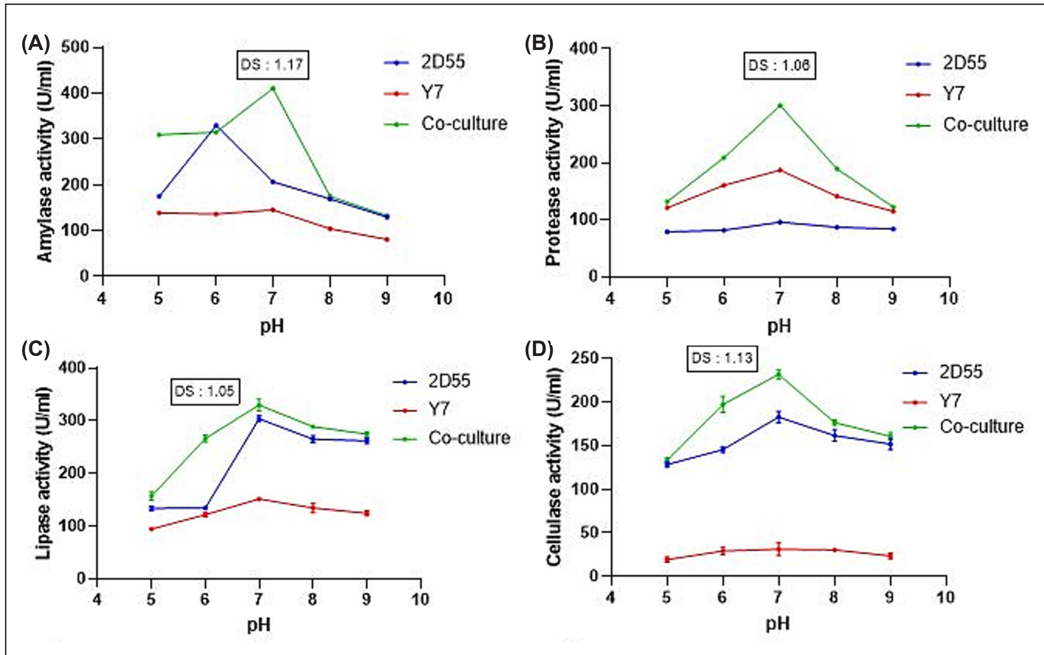

Figure 2. Effect of pH on the hydrolase activity of strain 2D55, Y7, and co-culture: (A) amylase, (B) protease, (C) lipase, and (D) cellulase
Note. DS = Degree of synergy; The standard errors (n = 3) are shown by error bars

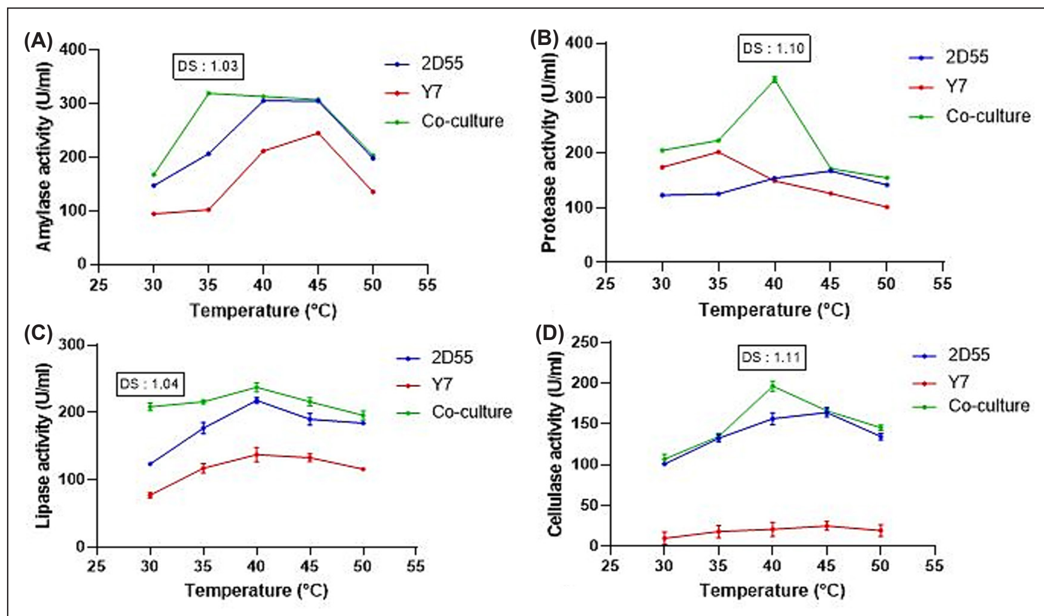

Figure 3. Effect of temperature on the hydrolase activity of strain 2D55, Y7, and co-culture: (A) amylase, (B) protease, (C) lipase, and (D) cellulase
Note. DS = Degree of synergy; The standard errors (n = 3) are shown by error bars

cellulase activity at all temperatures compared to strain Y7. The co-culture demonstrated similar cellulase activities to strain 2D55, reaching maximum synergy at 40°C with a DS value of 1.11.

In-vitro KW Biodegradation via SSF

To assess the effectiveness of the selected HPB in degrading KW, a 14-day SSF experiment was conducted, and the biological and physicochemical changes throughout the degradation process were monitored, as illustrated in Figure 4. In the monoculture treatment, the count of culturable bacteria steadily increased, reaching 11.40 log CFU/g by day 14 in sterile SSF. In contrast, the co-culture exhibited a dramatic increase, reaching 10.78 log CFU/g by day 6 and continuing to rise to 13.54 log CFU/g by day 14. In non-sterile SSF, bacterial growth was higher compared to sterile SSF, starting at 11.22 log CFU/g and reaching 14.00 log CFU/g by the end of the experiment for monocultures. However, the culturable bacteria in the non-sterile control showed the lowest count, reaching 13.70 log CFU/g by day 14. Surprisingly, the co-culture treatment in non-sterile SSF had the highest number of culturable bacteria, with bacterial growth reaching 11.95 log CFU/g by day 6 and continuing to increase to 14.60 log CFU/g by the end of the experiment (day 14). The pH values in both treatments ranged from 4.5 to 7.5. In sterile SSF, the pH shifted from acidic (pH 4) to neutral (pH 6), while in non-sterile SSF, the pH transitioned from neutral to slightly acidic, showing opposite trends compared to sterile SSF.

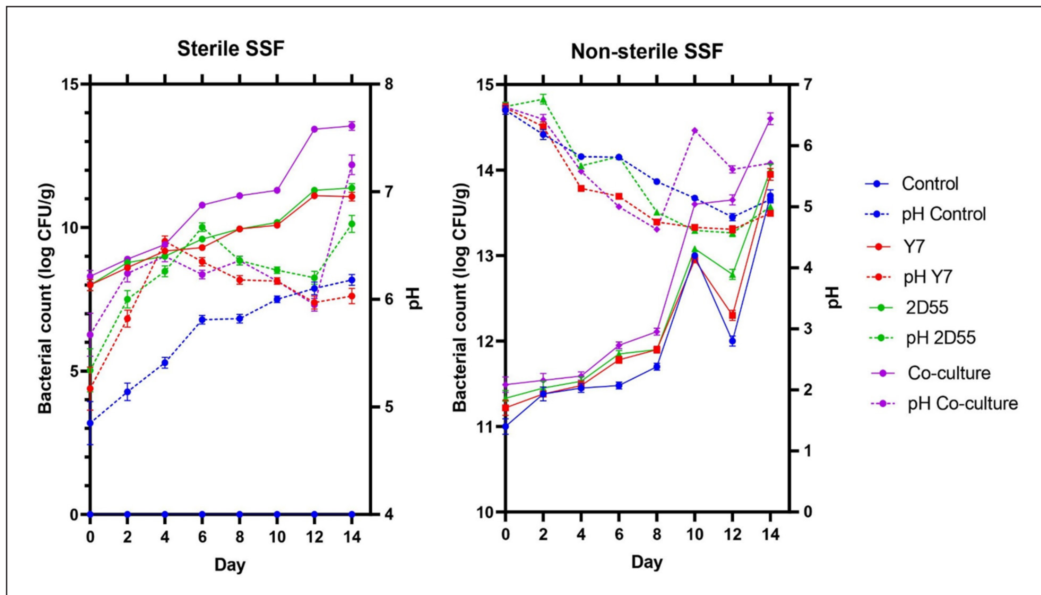

Figure 4. Bacterial count and the dynamics of pH during kitchen waste biodegradation via solid-state fermentation
Note. The standard errors (n = 3) are shown by error bars

The hydrolase activities observed in both sterile and non-sterile SSF are illustrated in Figure 5. In the inoculated flasks of both sterile and non-sterile SSF, amylase activity showed an overall increase up to day 10 of fermentation. Subsequently, amylase activity in sterile SSF began to decline towards day 14, while in non-sterile SSF, it exhibited a gradual increase during the same period. Protease activity in sterile SSF increased up to day 10, followed by a sudden decline towards the end of the experiment (day 14), whereas in non-sterile SSF, the decline started on day 8. In terms of lipase activity, the inoculated flasks in sterile SSF demonstrated an increase up to day 6, sharply declining after day 10. Conversely, in non-sterile SSF, lipase activity increased up to day 4 (except for strain Y7, where the increase was observed up to day 8) and gradually declined towards day 14. Cellulase activity in both sterile and non-sterile SSF increased up to day 12, followed by a gradual decline until day 14. The co-culture exhibited the highest amylase activity in sterile and non-sterile SSF, with optimal DS of 1.16 and 1.48, respectively. Similarly, for cellulase activity, the co-culture achieved the highest hydrolase activity with an optimal DS of 1.22 in sterile SSF and a DS of 1.03 in non-sterile SSF. Although the recorded lipase activity was comparatively lower than that of amylase and cellulase activity, the highest DS was found in lipase activity, with the co-culture achieving maximum synergy in both sterile and non-sterile SSF, with DS of 3.76 and 2.57, respectively.

A comprehensive analysis of the HPB's capacity to degrade KW was conducted based on the remaining TS and GDR, as illustrated in Figure 6. It is evident that inoculated KW exhibited higher degradation rates compared to the uninoculated control in both sterile and non-sterile SSF (Figures 6A and 6B). In sterile SSF, the TS degradation of the co-culture significantly increased up to 39% on day 2, reaching 54% by the end of the experiment (day 14). Similarly, monoculture treatments achieved slightly lower percentages, with Y7 and 2D55 reaching 48 and 51%, respectively, on day 14. In non-sterile SSF, the TS degradation of the co-culture also showed a significant increase, reaching 44% on day 2 and 65% by the end of the experiment, outperforming monoculture treatments, where Y7 and 2D55 achieved 49 and 53%, respectively. Co-culture inoculation resulted in significantly higher TS degradation compared to monoculture and uninoculated control ($P < 0.05$). Regarding GDR, co-culture inoculation in sterile SSF achieved a high percentage of 7% on day 2, rapidly increasing to 25% by the end of the experiment. In contrast, monoculture treatments reached lower percentages, with Y7 and 2D55 achieving 15 and 17%, respectively, on the final day. In non-sterile SSF, co-culture inoculation also exhibited a rapid increase in GDR, reaching 41%, whereas Y7 and 2D55 monoculture treatments achieved only 20 and 23%, respectively, by the end of the experiment. Co-culture inoculation resulted in significantly higher GDR percentages compared to monoculture and uninoculated control ($P < 0.05$). These findings demonstrate that co-culture inoculation in both sterile and non-sterile SSF can effectively enhance TS and GDR degradation of KW.

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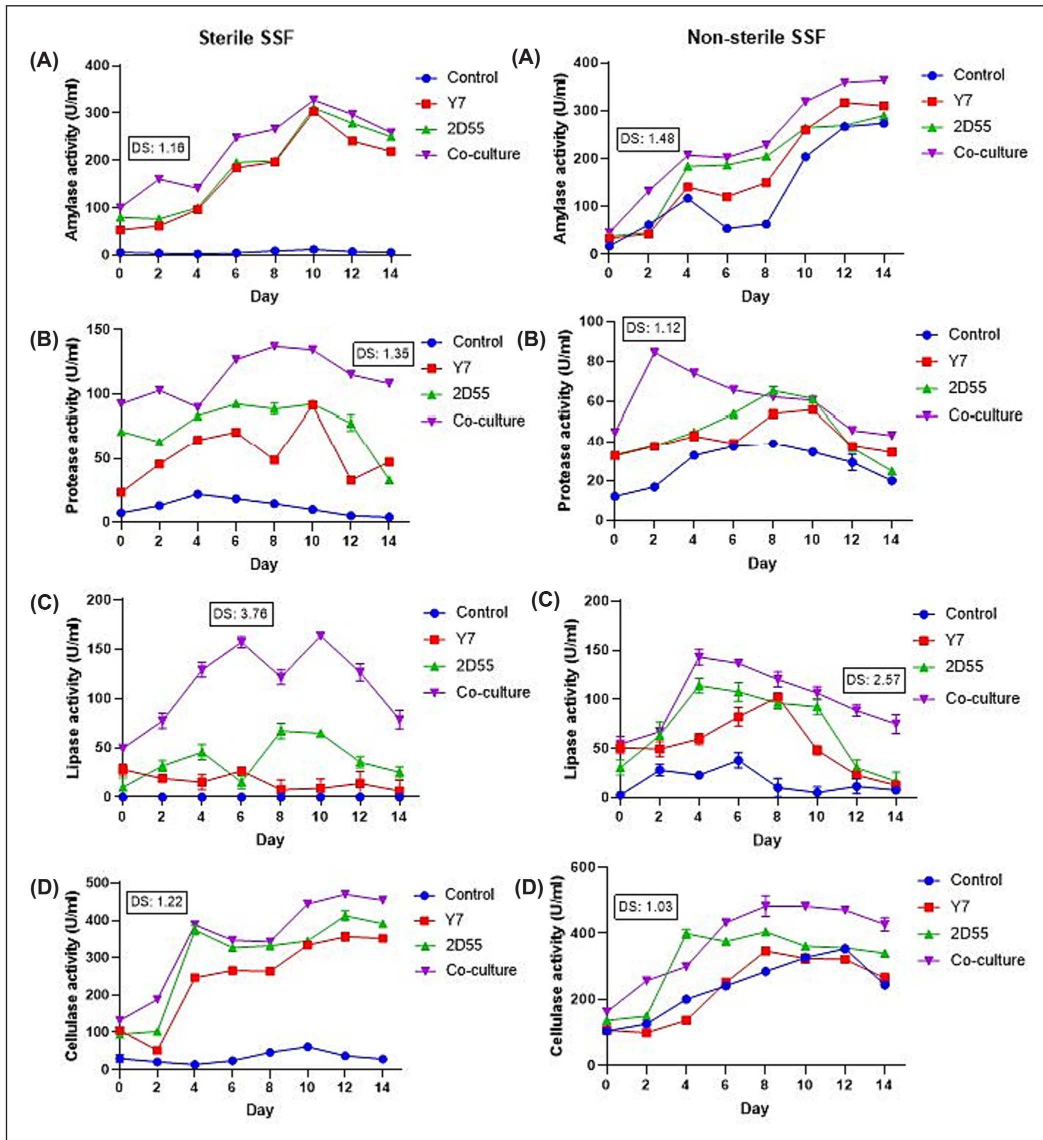

Figure 5. Hydrolase activity of control, strain 2D55, Y7, and co-culture in sterile and non-sterile solid-state fermentation: (A) amylase, (B) protease, (C) lipase, and (D) cellulase
Note. DS = Degree of synergy; The standard errors (n = 3) are shown by error bars

Pearson's correlation coefficients were computed to assess the relationships between hydrolase activities, bacterial count, TS, and GDR (Table 5). Strong correlations (**$P < 0.01$) were observed between bacterial count and TS, GDR, and all hydrolase activities in sterile SSF. Conversely, negative correlations were noted between bacterial count and protease and lipase activity. In sterile SSF, robust correlations (**$P < 0.01$ and *$P < 0.05$) were found between TS and all hydrolase activities. However, in non-sterile SSF, no

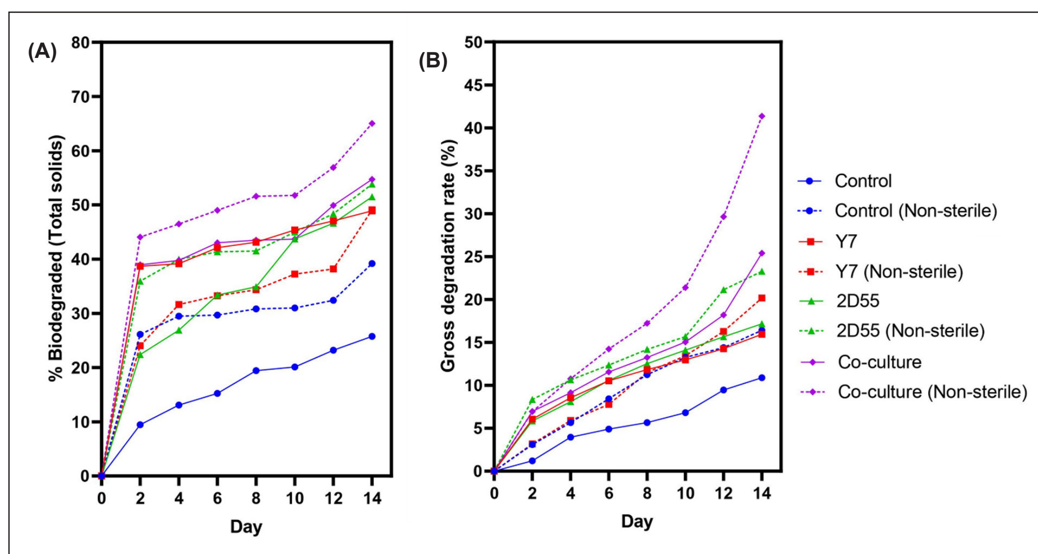

Figure 6. Effect of different hydrolase-producing bacteria on kitchen waste biodegradation. (A) Percentage of biodegradation based on total solids left within 14-day solid-state fermentation (SSF); (B) Gross degradation rate of KW within 14-day SSF

Table 5

Pearson's correlation coefficient between hydrolase activity, bacterial count, total solids (TS), and gross degradation rate (GDR)

	Bacterial count	TS	GDR	Amylase	Protease	Lipase	Cellulase
Sterile							
Bacterial count	1	0.691**	0.619**	0.861**	0.777**	0.572**	0.865**
TS		1	0.907**	0.797**	0.509**	0.432*	0.798**
GDR			1	0.777**	0.453**	0.403*	0.768**
Non-sterile							
Bacterial count	1	0.675**	0.875**	0.853**	-0.071	-0.071	0.568**
TS		1	0.850**	0.806**	0.434*	0.168	0.783**
GDR			1	0.885**	0.161	0.402*	0.768**

Note. Significant correlations ($P < 0.01$) are marked with ** or ($P < 0.05$) with *

significant correlations were observed between TS and lipase activity. Similar trends were observed for GDR, with strong correlations identified between GDR and all hydrolase activities in sterile SSF. In contrast, no significant correlation was found between GDR and protease activity in non-sterile SSF.

Analysis of the Composting Process

The kinetics of KW degradation during composting were elucidated by the observed hydrolase activity (Figure 7). In the inoculated compost, amylase activity exhibited an

overall increase until week 4 of composting, followed by a decline starting from week 5. Conversely, the control compost showed a slight increase in amylase activity during the first two weeks of composting, followed by a decline until the end of the process. Cellulase activity in the inoculated compost decreased after week four, mirroring the trend observed in amylase activity, while cellulase activity in the control compost began to decline after week two. For protease activity, the inoculated compost displayed a decline in the first two weeks, followed by a slight increase in week 3, whereas the control compost showed relatively stable protease activity until a slight increase towards the end of the composting period. Inoculated compost exhibited an increase in lipase activity until week 6, followed by a gradual decline towards the end of the experiment (week 8). Conversely, lipase activity in the control compost started declining after week 5. All hydrolase activities in the inoculated compost were significantly higher compared to the control compost ($P < 0.05$).

Figure 8 illustrates the changes in physicochemical and biological parameters during the composting process. In both inoculated and control composts, the moisture content remained within the range of 40 to 70% throughout the experiment (Figure 8A). The temperature profiles (Figure 8B) exhibited a similar trend in composting processes with and without HPB. There was a notable rise ($P < 0.05$) in temperature during the initial days of composting, followed by a gradual decrease after reaching the peak (5-10 days). Subsequently, the temperature stabilized between 24 and 30°C after 30 days, approximating

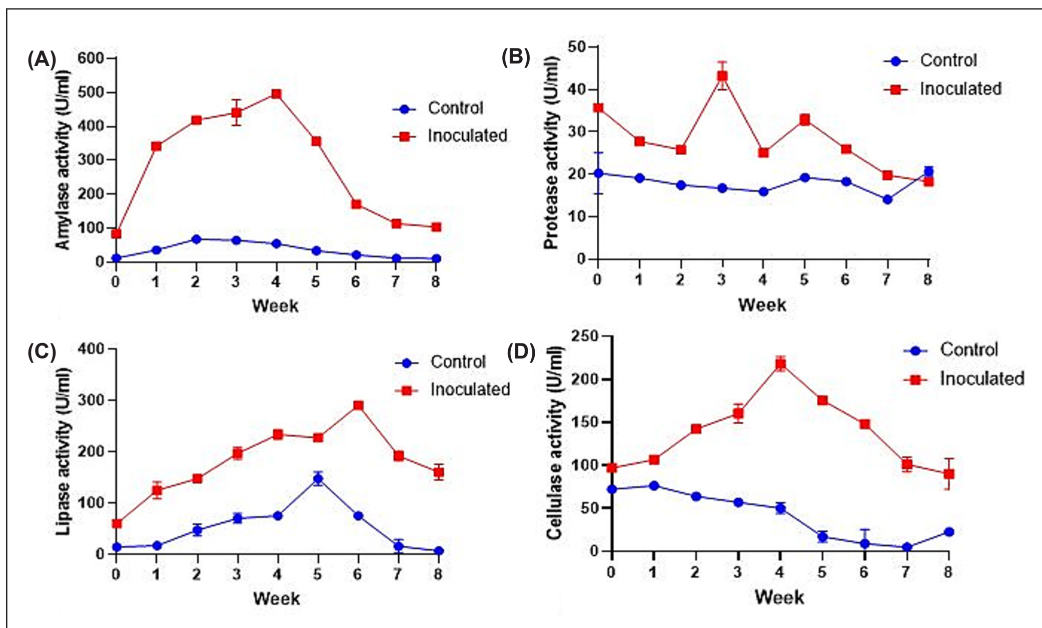

Figure 7. Hydrolase activity of inoculated and control compost during the composting process: (A) amylase, (B) protease, (C) lipase, and (D) cellulase
Note. The standard errors (n = 3) are shown by error bars

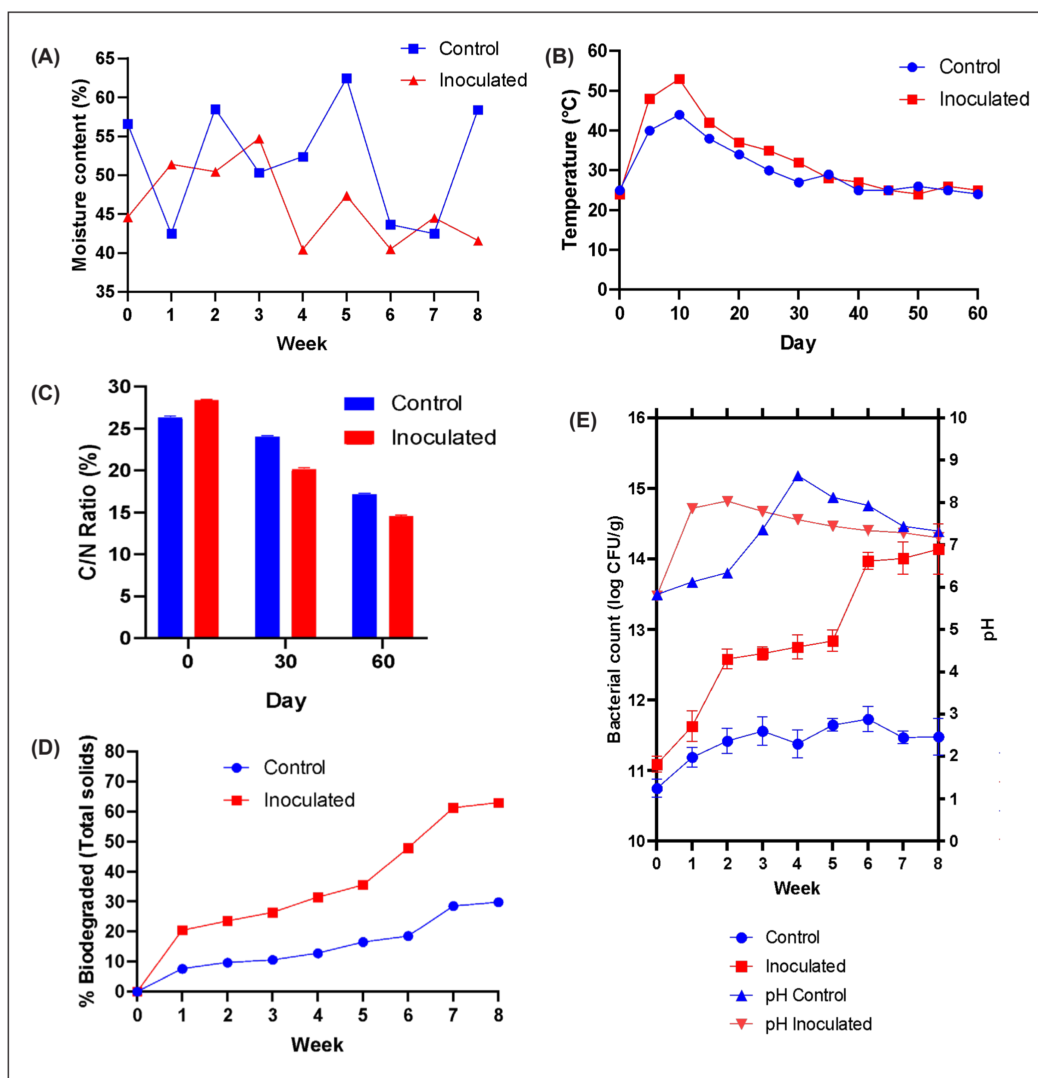

Figure 8. Biological and physico-chemical changes during the composting process: (A) moisture content, (B) temperature profile, (C) C/N ratio, (D) percentage of biodegradation based on the amount of total solids that remain, and (E) bacterial population and pH dynamics
Note. The standard errors (n = 3) are shown by error bars

room temperature. The carbon-to-nitrogen (C/N) ratio significantly decreased towards the end of the composting process in both inoculated and control composts (Figure 8C). The C/N ratio of inoculated compost (14.58) was substantially lower ($P < 0.05$) than the control compost (17.23). Both trials exhibited a final reduction in the C/N ratio ranging from 30 to 48%.

Additionally, the TS degradation in inoculated compost increased drastically up to 21% in the first week and continued to rise to 63% by the end of the experiment. In contrast, TS

degradation in the control compost was slightly lower, with the highest percentage achieved being 29% by the eighth week. In terms of bacterial growth, culturable bacteria steadily increased in the control compost, reaching 11.56 log CFU/g by the third week (Figure 8E). In contrast, the inoculated compost exhibited a drastic increase, reaching 12.58 log CFU/g by the second week and continuing to rise to 13.97 log CFU/g by the sixth week. The highest bacterial count was observed in the inoculated compost, reaching 14.14 log CFU/g by the end of analysis. The bacterial growth in the control compost was lower, recording 11.48 log CFU/g by the end of analysis. The pH values in both composts varied from 5.0 to 8.5, transitioning from acidic (pH 5) to neutral (pH 7) and weakly alkaline, demonstrating the organic matter's stability.

Table 6 presents Pearson's correlation coefficients calculated for various parameters, including hydrolase activities, C/N ratio, bacterial count, TS, and pH. Strong positive correlations (** $P < 0.01$) were observed between bacterial count and TS in both the inoculated and control composts. Conversely, negative correlations were found between the C/N ratio and bacterial count, as well as between the C/N ratio and TS in both compost types. Additionally, positive correlations were noted between pH and bacterial count, as well as between pH and TS. In the inoculated compost, strong correlations were discovered (** $P < 0.01$ and * $P < 0.05$) between lipase activity and bacterial count, hydrolase activity and TS, and TS and C/N ratio. However, no significant correlations were observed between protease activity and bacterial count, pH, or C/N ratio in the control compost. Similarly, there were no noteworthy correlations discovered between amylase activity and other parameters in both compost types, except for pH and TS. In the inoculated compost, a significant correlation (** $P < 0.05$) was found between cellulase activity and pH. In

Table 6
Pearson's correlation coefficient between hydrolase activity, carbon/nitrogen (C/N), bacterial count, total solids (TS), and pH

	C/N	**Bacterial count**	**TS**	**pH**	**Amylase**	**Protease**	**Lipase**	**Cellulase**
Inoculated								
C/N	1	-0.983**	-0.994**	-0.795*	-0.148	0.997**	-0.655	-0.057
Bacterial count		1	0.946**	0.248	-0.262	-0.586**	0.672**	0.000
TS			1	0.261	0.234*	0.689**	0.564**	0.113
pH				1	0.703**	-0.140	0.447*	0.413*
Control								
C/N	1	-0.663	-0.980**	-0.275	0.307	-0.249	0.368	0.965**
Bacterial count		1	0.539**	0.603**	0.171	-0.124	0.502**	-0.560*
TS			1	0.517**	0.416	0.210	0.058	0.831*
pH				1	0.087	-0.316	0.625*	-0.637*

Note. Significant correlations ($P < 0.01$) are marked with ** or ($P < 0.05$) with *

contrast, in the control compost, a significant correlation (**$P < 0.01$) was observed between cellulase activity and C/N ratio.

Table 7 summarizes the characteristics of composts made without HPB (control), including the suggested ranges for determining compost maturity and stability. After 8 weeks, both composts were well-matured, exhibiting a germination index (>100%), a pH of around 7, a C/N ratio of less than 20, a dark brown color, and an earthy smell. Both composts also showed non-phytotoxicity, as indicated by their pH, C/N ratio, and high germination index.

Table 7
Properties of final composts in week 8 and the typical range

Properties	Inoculated compost	Control	Typical range
GI (%)	178.25 ± 23.2	176.01 ± 31.6	> 100% (Palaniveloo et al., 2020)
C/N ratio	14.58 ± 0.14	17.23 ± 0.09	< 20 (Onwosi et al., 2017)
pH	7.17 ± 0.08	7.32 ± 0.04	6-8 (Varma et al., 2017)
Colour	Dark brown	Dark brown	Dark brown (Khalil et al., 2021)
Smell	Earthy smell (Start from week 3)	Earthy smell (Start week 7)	Earthy smell (Hubbe et al., 2010)

Note. Data represent mean ± SD; GI = Germination index; C/N ratio = Carbon/nitrogen ratio

DISCUSSION

In this study, SSF was utilized to assess the potential of various HPB to degrade starch, protein, fat, and total sugars, thereby enhancing KW biodegradation efficiency. After primary isolation and screening of HPB, two isolates, namely strain 2D55 and Y7, were selected. Molecular analysis confirmed that strain 2D55 belonged to the *B. licheniformis* species, whereas strain Y7 belonged to the *B. xiamenensis* phylogeny. *Bacillus licheniformis* is commonly utilized in the pharmaceutical industry for the production of biochemicals, enzymes, antibiotics, and aminopeptidase (Lee et al., 2017). On the other hand, *B. xiamenensis* was initially discovered in the intestines of a flathead mullet on Xiamen Island, China (Lai et al., 2014). According to Amna et al. (2020), this species exhibits plant growth-promoting capabilities, suggesting its potential to enhance plant development and act as a biological control against phytopathogens. While information on these species is limited, *B. xiamenensis* has not been previously associated with KW biodegradation capacity, and no research on the synergistic effect of both strains on KW degradation rate currently exists. This study aims to address this knowledge gap and explore the combined potential of these HPB strains in enhancing KW degradation. The biocompatibility assay confirmed that the selected strains, *B. licheniformis* 2D55 and *B. xiamenensis* Y7, demonstrated no inhibitory interactions when tested against each other. No inhibition zones exceeding 4 mm in diameter were observed for any of

the strains during the 3-day incubation period, indicating mutual compatibility. These results validated the feasibility of co-culturing these strains for subsequent experiments in the study.

In the quantitative assay, it was intriguing to observe that the hydrolase activity of the HPB co-culture surpassed that of the corresponding monocultures, as evidenced by the significantly higher DS value ($P < 0.05$). This observation aligns with previous studies where co-cultures of *B. velezensis* and *B. paralicheniformis* exhibited greater reductions in various components of FW, including total solids, fat, sugar, reducing sugar, protein, and starch, compared to monocultures (Roslan et al., 2021). Similarly, another study reported that a bacterial co-culture was able to eliminate up to 98% of 17-estradiol, a common contaminant in water and soil, within 7 days of incubation, whereas monoculture achieved only 77% removal (M. Li et al., 2018). These findings suggest that microbial symbiotic relationships during fermentation may play a pivotal role in generating such significant synergistic effects.

Throughout the 14-day SSF process for KW biodegradation, bacterial proliferation was highly active, especially in the co-culture treatment, where the total culturable bacteria reached the highest level of 14.6 log CFU/g in the non-sterile SSF system (Figure 4). The decrease in bacterial population on day 12 for monocultures and control under non-sterile conditions could be due to the available nutrients in the substrate being significantly depleted, especially under non-sterile conditions, where native microbial communities compete with the inoculated bacteria for resources. This competition could lead to a temporary decline in the population of the inoculated strains as they experience nutrient limitations. Monocultures and control do not benefit from the synergistic interactions present in the co-culture, making them more susceptible to nutrient depletion or the inhibitory effects of accumulated metabolic byproducts (Bernal et al., 2009). The pH levels in both SSF systems fluctuated between 4.5 and 6.5 over the 14-day fermentation period. According to Chen et al. (2015), optimal pH values for degradation typically range from 5.5 to 8, facilitating microbial decomposition of organic materials. The initial decrease in pH during the starting point of degradation may be attributed to the volatilization of early ammonia and the release of organic acids during the degradation of simple organic substrates (Azim et al., 2018). However, as the co-culture consumes these acids or as their production declines in the later stages of fermentation, the buffering effect diminishes, allowing the pH to increase. Under sterile conditions, the absence of competing microbial populations that might utilize ammonia or other alkaline compounds could further amplify this effect (Zhang & Sun, 2017).

The unexpected trends in the pH and temperature optima for the enzymes in the co-culture system may be attributed to several factors related to microbial interactions and environmental dynamics within the co-culture. In SSF, localized microenvironments within

the solid substrate can lead to variations in pH and temperature gradients, particularly in a co-culture system where the metabolic activities of multiple strains interact. These microenvironmental factors may explain why the co-culture exhibited different enzymatic optima compared to the monocultures. In the co-culture, the interaction between *B. licheniformis* 2D55 and *B. xiamenensis* Y7 may have led to changes in the extracellular environment, such as shifts in pH or the production of secondary metabolites. These interactions could modulate enzyme activity and stability, potentially altering the observed optima for enzymatic functions. Previous studies have shown that co-cultures often produce synergistic effects that enhance enzymatic activity or change the optimal conditions for enzyme performance (Cerda et al., 2017). Therefore, careful selection of appropriate members for co-culture systems is crucial for establishing an effective long-term waste disposal strategy (Mujtaba & Lee, 2016).

Our findings indicate that during the initial phases of fermentation, amylase and cellulase exhibited significant activity in both sterile and non-sterile SSF co-culture inoculations, aligning with prior research documenting elevated levels of these enzymes at the onset of decomposition (Fan et al., 2018; Khalil et al., 2021). Conversely, protease and lipase displayed comparatively lower activity, possibly due to the predominance of rice and vegetable components in KW, which necessitate greater amylase and cellulase activity for breakdown. Compared to monoculture treatments, both sterile and non-sterile SSF demonstrated the highest percentage of biodegraded TS, reaching up to 54.73 and 65%, respectively, showing that co-culture inoculation has significant synergistic effects on TS degradation. These findings resonate with recent reports by Roslan et al. (2021), showing a reduction of FW solid composition by over 60% with *Bacillus* spp. co-culture inoculation, and another study reporting a 23.92% reduction in FW mass with microbial consortium addition (Zhao et al., 2017). The observed synergy in the co-culture fermentation of *Bacillus* spp. can be attributed to the complementary enzymatic activities provided by each strain. *Bacillus licheniformis* 2D55 contributes strong cellulase and lipase activities, while *B. xiamenensis* Y7 enhances protease and amylase activity, together forming a robust enzymatic profile capable of degrading a range of complex organic compounds found in kitchen waste. This complementarity allows for a more thorough breakdown of proteins, carbohydrates, lipids, and cellulose compared to monoculture treatments, where a single strain's enzymatic activity is more limited (Kapoore et al., 2021).

Co-culture treatments also exhibited the highest GDR percentages for both sterile (25.39%) and non-sterile (41.36%) SSF, underscoring the efficacy of co-culture inoculation in enhancing KW degradation and biomass reduction ($P < 0.05$). The observed difference in GDR between sterile and non-sterile conditions may be attributed to the role of native microbial communities in the non-sterile environment. Under non-sterile conditions, indigenous microorganisms interact with the introduced *Bacillus* spp. strains, potentially

creating synergistic effects that enhance enzymatic activity and accelerate organic matter breakdown (Niu & Li, 2022). This additional microbial contribution likely accounts for the higher GDR observed in non-sterile SSF compared to sterile conditions, where only the *Bacillus* spp. co-culture was active. The positive correlation between GDR and hydrolase activities suggests that robust hydrolase activity accelerates KW degradation, potentially shortening the initial lag time of the biological process (Rastogi et al., 2020). Overall, these results highlight the potential of the isolated HPB in the KW degradation process, showcasing their ability to effectively break down KW and reduce its biomass.

During the composting of KW, the hydrolase activities exhibited a similar trend to those observed in SSF, with amylase and cellulase displaying the highest activity during the initial stages of composting, followed by protease and lipase. Both the inoculated and control composts maintained moisture levels ranging from 40 to 70%, ideal for fostering biological activity during composting (Abdel-Rahman et al., 2016). However, unlike the inoculated compost, the control compost lacks the addition of HPB, which enhances microbial activity and regulates the degradation process. Metabolic heat generated by inoculated microbes during active degradation can facilitate consistent evaporation and maintain a more stable moisture balance. In the absence of these inoculants, the control compost becomes more susceptible to moisture fluctuations caused by uneven degradation or environmental factors. Studies have shown that inoculated compost tends to exhibit more uniform moisture distribution due to enhanced microbial metabolism and substrate breakdown (Zhang & Sun, 2017). Throughout the thermophilic phase of composting, the inoculated compost notably exhibited a more rapid temperature rise to thermophilic levels (> 45°C) in the early stages of composting compared to the control. Moreover, the thermophilic phase in the inoculated compost persisted for a longer duration, spanning from day 5 to day 10, with temperatures ranging from 48 to 53°C, respectively. These observations suggest that the inoculated compost enhanced microbial population and activity during composting. The elevated temperatures are believed to result from increased biological activity, stemming from the breakdown of carbon bonds during the biodegradation process (Azim et al., 2018).

Both treatments exhibited a decrease in the C/N ratio as composting progressed, with the inoculated compost showing a higher reduction in C/N ratio (48.71%) compared to the control (34.73%). Research indicates that as waste gradually degrades, the C/N ratio tends to decrease (Onwosi et al., 2017). The inoculated compost demonstrated a significantly higher percentage of biodegraded TS ($P < 0.05$), reaching 63.02%, in contrast to the control, which achieved 29.80%. The positive correlation observed between TS and hydrolase activities suggests that robust hydrolase activities facilitate KW degradation. A similar trend was noted between the bacterial count in the co-culture SSF and that in the inoculated compost, with the highest culturable bacteria (14.14 log CFU/g) recorded

throughout the two-month composting process. In both composts, the pH fluctuated from acidic to neutral to mildly alkaline toward the end of the process. The initial acidity of the pH in the early stages of composting may be attributed to the breakdown of simple organic compounds and the initial ammonia's volatilization, resulting in the formation of organic acids. Subsequently, the pH became alkaline due to mineralization and the decomposition of quickly biodegradable organic components (Bashir et al., 2021).

The properties of the final composts were evaluated, with a particular focus on odor emission, a common issue in composting processes. Typically, the rapid transition from acidic to alkaline conditions initially results in unpleasant odors. However, in our study, the inoculated compost began emitting an earthy odor after only three weeks, whereas the control compost took six weeks to achieve the same. Both samples exhibited values exceeding 100% for non-phytotoxicity, with the inoculated compost's germination index slightly higher than that of the control. Research suggests that substances with GI values below 50% are highly phytotoxic, those between 50% and 80% are moderately phytotoxic, values above 80% are non-phytotoxic, and values over 100% have a phytonutrient effect (Siles-Castellano et al., 2020).

CONCLUSION

The co-culture of *B. licheniformis* 2D55 and *B. xiamenensis* Y7 exhibited heightened hydrolase activities and demonstrated remarkable synergism, surpassing the levels observed in monocultures. These findings imply that employing HPB in composting accelerates vegetable waste (VW) breakdown, enhances hydrolase activity, yields compost with an earthy scent, and mitigates phytotoxicity. While this study highlights the enhanced hydrolase activity observed in co-culture conditions, the exact biological mechanisms driving this synergy remain to be explored. Detailed molecular studies, such as transcriptomics or metabolomics, could provide insight into these specific pathways and metabolic exchanges, offering a deeper understanding of the cooperative dynamics that enhance biodegradation rates. Large-scale trials are also necessary to assess the practical feasibility and economic viability of employing these bacterial co-cultures in waste management. Besides, future research should assess the efficacy of these *Bacillus* spp. co-cultures on a broader spectrum of organic waste types, including higher-protein and lipid-rich waste, to ensure wider applicability. Such insights could pave the way for developing a valuable tool to offer an alternative environmental approach and enhance waste treatment options holistically.

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