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Original article

Enhancing food waste biodegradation rate in a food waste biodigester with the synergistic action of hydrolase-producing *Bacillus paralicheniformis* GRA2 and *Bacillus velezensis* TAP5 co-culture inoculation

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ABSTRACT

Food waste (FW) minimization at the source by using food waste biodigester (FWBs) has a vast potential to lower down the impact of increasing organic fraction in municipal solid waste generation. To this end, this research sought to check the performance of locally isolated hydrolase-producing bacteria (HPB) to improve food waste biodegradation rate. Two under-explored HPB identified as *Bacillus paralicheniformis* GRA2 and *Bacillus velezensis* TAP5 were able to produce maximum amylase, cellulase, protease and lipase activities, and demonstrated a significant hydrolase synergy in co-culture fermentation. In vitro biodegradation analysis of both autoclaved and non-autoclaved FW revealed that the HPB inoculation was effective to degrade total solids (>62%), protein (>19%), total fat (>51), total sugar (>86%), reducing sugar (>38%) and starch (>50%) after 8-day incubation. All co-culture treatments were recorded superior to the respective monocultures and the uninoculated control. The results of FW biodegradation using batch-biodigester trial indicated that the 1500 mL and 1000 mL inoculum size of HPB inoculant reached a plateau on the 4th day, with gross biodegradation percentage (GBP) of >85% as compared to control (66.4%). The 1000 mL inoculum was sufficient to achieve the maximum GBP (>90%) of FW after an 8-day biodigestion in a FWB.

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1. Introduction

Accelerated urbanization and industrialization activities associated with rapid population growth are the key substantial aspects which drive the escalating pattern of municipal solid waste (MSW) generation in developing countries (Tan et al., 2014). In Malaysia, the MSW generation has inclined dramatically from 23,000 tons/-day in 2008 to 33,000 tons/day in 2012 (SWCorp, 2014). With over 31.6 million total population in Malaysia in 2018 (World Bank, 2018), the pattern of MSW generation has recorded a drastic increase of 1.00–1.49 kg per capita per day by September 2018 (Silpa et al., 2018). A national survey reported that 65% of MSW composition in Malaysia was contributed by the household sector in which it was largely dominated (45%) by food waste (FW) and organic waste (NSWMD, 2012). Unfortunately, 89% of the generated MSW ended up in landfills, while the rest 11% was managed via recycling and treatment (Environment Bureau, 2013). Thus, handling FW disposal is one of the major problems encountered in MSW management which is typically incurred by most households in densely populated cities.

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Most developing countries such as Malaysia have been actively incorporating incineration and landfilling to manage the increasing MSW generation. While those approaches are relatively faster, inexpensive and efficient in reducing the bulk quantity of MSW, they also cause toxic dioxin release, uninhibited leachates, diseases vectors, malodors and harmful greenhouse gasses production, affecting the environmental and human's health in a long run (Ferronato and Torretta, 2019). These drawbacks which are exacerbated by the large proportion of perishable FW in MSW could be reduced by restraining the amount of FW released as garbage. Therefore, to increase the diversion of putrescible FW from the assorted MSW, the minimization alternatives that are rather direct at the source and more environmentally-sound are required to resolve this matter.

Composting is an ancient eco-friendly approach to manage FW safely at the source. This method employs the conversion of a complex biodegradable organic material with the use of microorganisms into a stable end product such as soil conditioners (Toledo et al., 2018). Nonetheless, this long-used technology has some limitations that have refrained its extensive application such as phytotoxic presence, low nutrient content, lengthy process duration and malodor production (Ayilara et al., 2020). To alleviate these matters, numerous exploitations of composting techniques have been proposed to enhance its efficacy (Awasthi et al., 2015; Gu et al., 2017; Wang et al., 2016; Xue et al., 2013). However, none of these investigations managed to alter the inextricably lengthy process of composting in which was not able to concur with the amounting loads of FW generated. Besides, composting requires a basic setup of a controlled well-ventilated system and the incorporation of a suitable bulking agent, hence seemingly tedious and impractical particularly to the urban households.

Alternatively, FW minimization at the source using FW disposal units (FWDs) is comparatively faster, more versatile, and reliable to cope with the daily FW generated at all kinds of establishments. FWDs have been extensively adopted in the housing systems of modern countries mainly in the United States, as hygienic means to macerate FW at the source, which is subsequently discharged to the public sewer for treatment together with wastewater (Burguillos and Caldona, 2020). Even though this practice has been well-adopted by many countries worldwide, its potential as a component in waste management alternatives has not been fully considered (Iacovidou et al., 2012). This is due to the rising skepticism about the environmental and economic impacts of FWDs that hinder its broad application. However, a primary case study of FWDs implementation in the Greater Beirut Area revealed that FWDs were able to reduce 43% total solids of FW and provide 44% economic payback apart from the insignificant increase of domestic water consumption (Marashlian and El-Fadel, 2005). Additionally, Iqbal et al. (2020) reported a reduction of net carbon footprint by 37–63%, in the assessment of prospects of FW co-disposal into the sewer system and treatment with municipal sewage performed on Hong Kong's largest biological wastewater treatment plant. These findings provide clear evidence of the potential of FWD as a sustainable approach in FW management.

Over the years, the technology of FWDs have evolved from an exclusively grinding system to a more recent approach, biodigestion treatment, which are either built separately or in a dual integrated system. Biodigestion treatment employed in a food waste biodigester (FWB) differs from the conventional grinding wherein the former incorporates the efficient aerobic microorganisms aided with the recurrent mechanical agitation to avoid or reduce the use of electricity. In Malaysia, the current market of FWBs have been expanding recently with the active intervention of private sectors to penetrate the housing market, by proposing the installation of FWB as a standard item in the new home constructions. On account of that scenario, investigations on optimizing and improving the performance of FWBs are imperative to ensure the success of redirecting the organic fraction in MSW. Although FWBs' installation is typically supplied with so-called "efficient microbes" as the biological means to break down FW, to the best of our knowledge, there is yet scientific evidence to associate the role and efficiency of such microbes with FW biodegradation in FWBs which is principally different from traditional aerobic composting.

FW generally comprises of varied amount of complex polysaccharides such as cellulosic materials, sugars and starch, in addition to protein and fat (Suwannarat and Ritchie, 2015; Teck et al., 2010). To break down the complex FW components into soluble molecules, biological treatments of FW employ functional hydrolase-producing bacteria (HPB) which have been widely investigated in various experimental settings (An et al., 2018; Mekjinda and Ritchie, 2015; Suwannarat and Ritchie, 2015). HPB species that are commonly associated with FW treatments include *Bacillus* spp., *Brevibacillus* spp., *Paenibacillus* spp., *Pseudomonas* spp. and *Klebsiella* spp., as they are able to secrete manifold hydrolases, particularly amylase, cellulase, protease and lipase (Awasthi et al., 2018; Choi et al., 2002; Li et al., 2014; Msarah et al., 2020; Ren et al., 2020). The present study was designed to improve FW biodegradation using locally isolated HPB as an inoculant in a domestic FWB application for potential commercial purposes. To this end, HPB was primarily isolated from fermented foods and subsequently characterized based on respective hydrolase activities. To evaluate the ability of selected HPB to degrade protein, fat, total sugars and starch, a preliminary in vitro biodigestion and a batch-biodigester trial were performed thereafter.

2. Materials and methods

2.1. Sample collection

Bacterial strains were isolated from several types of homemade fermented foods which were fermented tapioca; fermented red grape; yogurt and cheese. All fermented foods were purchased from local wet markets in Bangi, Selangor, Malaysia, on the first and second weeks of October 2017. Food samples were transported to the laboratory and aseptically transferred into sterile 50 mL centrifuge tubes. Until used, they were stored in a 40L cooler box at 4 °C not longer than a week.

2.2. Isolation and screening for hydrolase producing bacteria

About 4 g of the food sample was homogenized in a 40 mL sterile phosphate-buffered saline (pH 7.4) using vortex at full speed. The sample was centrifuged at 5000 rpm for 10 min and the supernatant was serially diluted prior to plating onto nutrient agar (R&M) and MRS agar (Oxoid) separately. The agar plates were incubated at 30 °C for 24 to 48 h. Bacterial colonies with distinctive morphology were selected and streaked onto a fresh nutrient agar to obtain pure a culture.

The pure bacterial colonies were further screened for their hydrolase producing activity in vitro which were amylase, cellulase, protease and lipase using starch agar (soluble starch 20 g; sodium chloride 5 g; peptone 5 g; agar 20 g; distilled water 1 L; pH 7.0 ± 0.2) (Simair et al., 2017), Mandels-Reese agar (peptone 1 g; ammonium sulfate 1.4 g; urea 0.3 g; potassium dihydrogen phosphate 2 g; calcium chloride 0.3 g; magnesium sulfate 0.3 g; ferrous sulfate 0.005 g; manganese sulfate 0.0016 g; zinc chloride 0.0017 g; carboxymethyl cellulose 5 g; agar 20 g; distilled water 1 L; pH 7.0 ± 0.2) (Mandels and Reese, 1957) skim milk agar (peptone 5 g; beef extract 3 g; skim milk 5 g; agar 15 g; distilled water 1 L; pH 7.0 ± 0.2) (Bhowmik et al., 2015), and tributyrin agar (peptone 5 g; yeast extract 3 g; tributyrin 10 mL; phenol red 1 g; 15 g agar;

distilled water 1 L; pH 7.0 ± 0.2) (Ramnath et al., 2017) respectively.

The screening plates were incubated at 30 °C for 24 h. After incubation, the starch agar was flooded with 1% iodine in 2% potassium iodide while the Mandels-Reese agar was stained with Congo red dye solution (1%). The hydrolytic activity of an isolate was classified qualitatively as high activity (++) when the halo zone was >5 mm or low activity (+) when the halo zone was <5 mm. Negative hydrolytic activity (−) was determined when the halo zone was not observed around the bacterial colony. The ratio of hydrolysis zone diameter to the colony diameter was calculated and thereafter interpreted as hydrolysis index (HI).

2.3. Characterization and identification of hydrolase producing bacteria

Light microscopy (Olympus CH, Japan) was used to examine morphological characteristics and Gram's reaction of the HPB. The biochemical characteristics of the HPB were evaluated according to Bergey's Manual of Determinative Bacteriology (Bergey and Holt, 1994). The bacterial 16S rRNA gene was analyzed by employing a DNA barcoding procedure using the BigDye® Terminator v3.1 cycle sequencing kit and sequenced by Applied Biosystems genetic analyzer platform. The sequence generated was trimmed using BioEdit version 7 (Hall, 1999) and compared with the closest strains in the GenBank database using the Basic Local Alignment Search Tool (BLAST) in terms of percent identity. The sequences were aligned by ClustalW and the phylogenetic tree was reconstructed using MEGA X Alignment Explorer (Kumar et al., 2018) and inferred by using the Neighbour-Joining method (Saitou and Nei, 1987) with 1000 replicates of bootstrap values (Felsenstein, 1985). The evolutionary distances among strains were computed using p-distance (Nei and Kumar, 2000). Sequences were deposited into National Center for Biotechnology Information (NCBI) GenBank database via online sequence submission tool BankIt to retrieve the accession numbers.

2.4. Antagonistic interaction assay

Antagonistic interaction assay between the selected HPB was evaluated according to the pour-plate method (Grossart et al., 2004). A molten nutrient 1% agar (2.5 mL) was mixed with 50 µL target bacterial strain suspension (1×10^8 CFU/mL). The cell suspension agar was poured onto a nutrient agar plate and left to solidify for 10 min. An aliquot (10 µL) of test strain (1×10^8 CFU/mL) was spotted onto the lawn and incubated at 30 °C for 3 days. The HPB was tested in triplicate against each other and observed daily for inhibition zones. Inhibitory activity was recorded when the inhibition zone was more than 4 mm of the diameter of the spotted colony.

2.5. Quantitative hydrolase activity assay

An aliquot (1 mL) of starter culture (inoculum adjusted to 1×10^8 CFU/mL) grown in nutrient media was used to inoculate 100 mL starch, Mandels-Reese, skim milk and tributyrin liquid media respectively. Co-culture was prepared by mixing individual inoculum culture (0.5 mL) at an equal ratio of 1:1 (v/v) before inoculating the test media. The hydrolase activity of each HPB was analyzed using cell-free culture supernatant grown separately in those selective media at 30 °C with shaking at 150 rpm for 3 days. The HPB culture was centrifuged at 10,000 rpm for 10 min to obtain the cell-free culture supernatant. The synergistic effect of co-culture was interpreted as the enzymatic degree of synergy (DS) which was calculated by dividing the observed activity of the multicultural enzyme by the sum of every monocultural enzyme in the same media (Van Dyk et al., 2013). The hydrolase activities were determined according to the standard methods: amylase activity was determined using 3,5-dinitrosalicylic acid (DNS) method (Bernfeld, 1955); cellulase activity was measured by the standard DNS method (Miller, 1959); protease activity was determined using casein as a substrate (Tsuchida et al., 1986); lipase activity was assayed by p-nitrophenylpalmitate (p-NPP) hydrolysis (Winkler and Stuckmann, 1979).

2.6. In vitro food waste biodegradation analysis

The effect of monoculture and co-culture inoculation of HPB on biodegradation of FW was initially investigated via in vitro setting. Artificial FW was set up in this experiment with predetermined composition to mimic the actual FW found in these localities. The artificial FW was prepared based on the general composition of food and kitchen waste commonly found at local houses and eateries which comprises different classes of nutrient components such as fiber, protein, fat and carbohydrate. A guideline on the composition of general FW found in Bandar Baru Bangi (Selangor, Malaysia) was employed to create the artificial FW in a systematic manner (Teck et al., 2010). The individual component of the artificial FW and the size preparation were presented in Table 1.

FW samples were divided into two types of pretreatment which were autoclaved samples (121 °C, 15 min) and non-autoclaved (raw) samples in triplicate for each treatment. The food components were weighed (100 g), mixed and transferred into a sterile 250 mL conical flask. The HPB were grown overnight separately in the nutrient broth before 1 mL (1×10^8 CFU/mL) single inoculum or mixed inoculum (co-culture of 1:1 ratio, v/v) cell suspension was centrifuged down at 5000 rpm for 10 min and resuspended in 1 mL sterile saline water (0.85%) as an inoculum. The inoculum was introduced to each test flask and incubated in a rotary shaker aerobically for 8 days at 30 °C, shaking at 150 rpm. Control flasks were introduced with an equal volume of sterile saline water without bacterial cells. The bacterial population during FW biodegradation was determined via the standard plate count method on nutrient agar, while pH change was measured using a digital pH meter on daily basis.

The biodegradation percentage of the FW was estimated based on the daily remaining total solids (TS) and the change in nutrient composition. The TS of the FW was determined daily throughout the 8-day fermentation period by following the standard method (APHA, 1998). The nutrient component analysis was performed on the treated FW on day 0 and day 8 of post-inoculation to find out the nutrient fraction of protein, total fat, total sugar, non-reducing sugar, reducing sugar and starch by following standard protocols on a dry weight basis. Protein, total fat and moisture content were assayed using the standard method of AOAC, 16th Edi-

Table 1
Artificial food waste composition and preparation per 100 g.

Food class	**Food item**	**Weight (g)**	**Preparation**
Grain & carbohydrate	Cooked rice	30	–
	Eggshell	10	Coarsely blended to 5 ± 2 mm
Fruit & vegetable	Potato	10	Diced to 5 ± 2 mm
	Apple	10	Diced to 5 ± 2 mm
	Banana peel	10	Diced to 5 ± 2 mm
	Spinach	15	Cut to 10 ± 3 mm
Meat & fish	Raw chicken gizzard	5	Cut to 1/8 size
	Canned sardine	10	Cut to 10 ± 3 mm

tion 981.10 (AOAC International, 1998) while total sugar, non-reducing sugar, reducing sugar and starch were assayed using standard methods of APHA (1998).

2.7. Preliminary batch-biodigester trial

A commercial FWB and a full set of charcoal cubic polyvinyl alcohol (PVA) sponge as an inoculant carrier were contributed by Eco Ecotech Sdn. Bhd. (Malaysia). HPB co-culture was prepared using the same method as described in the previous section and grown overnight before the experiment, in molasses media (sugarcane molasses 30 g; NaCl 10 g; yeast extract 10 g; distilled water 1000 mL; pH 7.0 ± 0.2) in a rotary shaker at 30 °C. Different volumes (0 mL, 500 mL, 1000 mL or 1500 mL) of overnight HPB co-culture (adjusted to ~1×10^{10} CFU/mL) were transferred into separate sterile 3 L beaker and mixed with sterile uninoculated media to a final volume of 2000 mL. The carrier sponge (200 g) was soaked in the adjusted inoculum size until liquid media was fully absorbed. FW was collected beforehand from a cafeteria in Universiti Kebangsaan Malaysia in three different batches of more than 5 kg each. Toothpicks, plastics, tissues, chopsticks and large bones were separated from the drained FW prior to use. The FW was transferred into a plastic container and stored in a −20 °C freezer prior to further analysis.

Nutrient component analysis of the FW was performed on a dry weight basis before the experiment started using standard methods as described previously. About 1 kg of FW was thawed at room temperature and mixed sufficiently with the HPB-presoaked charcoal cubic sponge before transferring into the FWB. The operational cycle mode of the FWB was 5 min mixing while washing with drizzling tap water and draining through an exhaust pipe, and 20 min in static mode. At the beginning of every treatment, FW residue in the internal space of the FWB was washed off completely and sterilized thoroughly using a mixture of 70% ethanol and 3% Clorox® solution. Gross biodegradation percentage (GBP) was measured based on daily gross weight differences from the initial reading as described by (Li et al., 2014) throughout the 8-day digestion period. To monitor the temperature dynamic of the FW matrix, a digital probe thermometer was inserted through the top air hole of the FWB cover every day until it reached the center of the pile without opening the bin.

2.8. Statistical analysis

To determine the significance of the differences between HI of the HPB on screening plates and the degree of synergism between HPB, two-tailed, unpaired t-tests were performed at a confidence level of $P < 0.05$. The differences between treatments on hydrolase activity between HPB and the nutrient analysis of FW were assessed through one-way analysis of variance (ANOVA), and the calculated means were subjected to the Least Significant Difference (LSD) test at $P < 0.05$. Pearson correlation was conducted to quantify correlation coefficients between HI and hydrolase activity, and GBP and temperature dynamic at $P < 0.01$ confidence interval. All statistical analyses were performed using SPSS software Package Version 25.0 (SPSS Inc., USA).

3. Results

3.1. Isolation, screening and identification of hydrolase producing bacteria

The isolation of natural bacteria from the fermented foods was primarily conducted to isolate non-pathogenic and good bacteria that was generally safe for human handling and showed potential in hydrolase activities. As a result, 115 pure bacterial colonies with distinctive phenotypes were successfully isolated and maintained on nutrient agar. Out of 115 isolates, 25 isolates demonstrated a high potential of hydrolase activity on screening plates which were starch, Mandels-Reese, skim milk and tributyrin agar respectively (Table 2). Based on the screening results, strain GRA2 and TAP5 were able to demonstrate high hydrolytic activities which were amylase, cellulase, protease and lipase, hence selected for further analysis.

The phenotypic and genotypic properties of the HPB were assessed through biochemical characterization and the 16S rRNA gene sequencing of each strain respectively (Table 3). Based on the results, both HPB were able to grow in a low pH condition up to pH 4 but only strain GRA2 was able to survive at a moderately high temperature condition (65 °C). BLAST result and phylogenetic analysis (Fig. 1) revealed that strain GRA2 was closely related to *Bacillus paralicheniformis* sp. with 100% homology similarity to *Bacillus paralicheniformis* strain KJ-16 (NR137421.1) while strain TAP5 was closely related to *Bacillus velezensis* sp. with 99.87% similarity homology to *Bacillus velezensis* strain FZB42 (NR075005.2).

3.2. Quantitative estimation of hydrolytic activities

The hydrolytic capacity of the HPB was evaluated in two different sets of experiments which were agar-based and liquid-based assays (Fig. 2). In the agar-based assay, the HPB were individually grown on starch, Mandels-Reese, skim milk and tributyrin agar respectively for an extended incubation time of 48 h to obtain clearer halo zone formation around the bacterial colony (Fig. 2B). On average, the HI of GRA2 was recorded higher than TAP5 in amylase, protease and lipase agar assays, although only lipase showed a significantly higher index ($P < 0.05$). In contrast, TAP5 exhibited significantly higher ($P < 0.05$) HI for cellulase (4.44) as compared to GRA2 (3.46). The highest HI amongst all hydrolases was cellulase for both HPB. Surprisingly, the agar-based assay showed that the irregular-shaped colony of TAP5 grew more rapidly in size than any plates of GRA2 (data not shown).

Since both HPB showed no signs of antagonistic interaction against each other in the previous pour-plate assay, the quantitative hydrolase activities through liquid-based assay were determined in both monoculture and co-culture experiments in vitro (Fig. 2C). In terms of monoculture, TAP5 recorded significantly higher activities than GRA2 for amylase, cellulase and lipase activities except for protease activity ($P < 0.05$). Interestingly, co-culture significantly recorded overall higher activities of hydrolase ($P < 0.05$) with cellulase activity (109.77 U/mL) being the highest of all types. Closer inspection of Fig. 2D shows that the DS of all hydrolase activities was more than one time the cumulative monoculture activity thus validated the hydrolase synergy of the co-culture. Pearson correlation revealed that the HI results on agar were not correlated with the hydrolase activity in liquid media for both HPB. A negative correlation was recorded between the HI of amylase ($r = -0.026$, $P > 0.05$), protease ($r = -0.150$, $P > 0.05$) and lipase ($r = -0.990$, $P < 0.05$) on agar with the amylase, protease and lipase activity in liquid media respectively. On the contrary, the HI of lipase on agar was positively correlated ($r = 0.692$, $P > 0.05$) with the lipase activity in liquid media.

3.3. Effects of HPB inoculation on food waste biodegradation

A preliminary FW biodegradation experiment was conducted in vitro for eight days to study the effect of HPB inoculation on the biodegradation of FW. The nutrient components of the artificial FW (100 g) on dry weight basis were protein 15.3%, total fat 2.9%, non-reducing sugar 49.5%, reducing sugar 21.5% and starch 10.8%. Throughout the experiment, the growth dynamics of the culturable

Table 2
Qualitative screening of hydrolase producing bacteria isolated from fermented foods.

Source	Isolate ID	Amylase	Cellulase	Protease	Lipase
Fermented cassava	TAP1	++	+	++	+
	TAP2	++	+	+	+
	TAP3	+	++	++	–
	TAP4	+	++	++	–
	TAP5	++	++	++	++
	TAP6	++	+	+	+
	TAP7	++	+	+	–
Fermented glutinous rice	TAG1	+	+	+	+
	TAG2	++	++	+	–
	TAG3	++	+	+	+
	TAG4	++	+	+	+
	TAG5	++	+	++	–
	TAG6	++	+	+	–
Fermented red grape	GRA1	+	–	+	–
	GRA2	++	++	++	++
	GRA3	+	–	+	–
	GRA4	+	–	+	–
	GRA5	++	+	++	–
Cow's milk yogurt	BCY1	–	++	+	–
	BCY2	–	++	++	–
	BCY3	–	++	+	–
Cow's milk cheese	PPC1	–	–	++	++
	PPC2	+	++	++	+
	PPC3	–	++	++	–
	PPC4	–	+	++	–

The hydrolytic activity was classified qualitatively as high activity (++) when the halo zone was >5 mm or low activity when the halo zone was <5 mm. Negative hydrolytic activity (–) was determined when the halo zone was not observed.

Table 3
Cell and colony morphology, biochemical characteristics and molecular identification of HPB.

Characteristics	GRA2	TAP5
Cell morphology	Single rod, Gram-positive	Single rod, Gram-positive
Colony morphology	Translucent, white, irregular, smooth, raised, mucoid, 3–4 mm in diameter	Translucent, white, circular, smooth, raised, viscid, 4–5 mm in diameter
Catalase production	+	+
Oxidase production	–	–
Voges Proskauer (VP)	–	+
Methyl red	–	–
Nitrate reduction	+	+
Citrate utilization	–	–
Mannitol salt agar	+	–
Indole production	–	–
Casein hydrolysis	+	+
Anaerobic growth	+ (facultative anaerobic)	+ (facultative anaerobic)
Growth at 45 °C	+	+
Growth at 65 °C	+	–
Growth in 7% NaCl	+	+
Growth in different pH	4 < 8 < 5 < 6 < 7	4 < 5 < 8 < 7 < 6
Motility	+	+
Triple sugar iron	yellow slant; yellow butt; Glucose fermentation: +; sucrose fermentation: +; H_2S production: +	yellow slant; yellow butt; Glucose fermentation: +, sucrose fermentation: + H_2S production: +
Closest relatives in NCBI GenBank	*Bacillus paralicheniformis* strain KJ-16 (NR137421.1)	*Bacillus velezensis* strain FZB42 (NR075005.2)
Percentage similarity	100.00%	99.87%
Designated species name	*Bacillus paralicheniformis* strain GRA2	*Bacillus velezensis* strain TAP5
NCBI Accession no.	MK861160	MK861159

+ or – symbol indicates positive or negative reaction respectively.

bacterial population and the change of pH were recorded daily as illustrated in Fig. 3. The plate count of autoclaved samples solely consisted of a pure strain of the bacterial inoculant while the non-autoclaved samples consisted of the assorted indigenous bacteria in addition to the bacterial inoculant (picture not shown). In the autoclaved sample, the culturable bacteria in the inoculated flasks inclined dramatically up to 10.3 log CFU/g (co-culture) on the 4th DPI and slightly declined to 7–9 log CFU/g on the 8th DPI. The samples inoculated with co-culture showed notably higher cell counts on both autoclaved and non-autoclaved samples as compared to monoculture and control. Whereas in the non-autoclaved sample, the culturable bacterial population started at higher CFU which was around 10 log CFU/g due to the addition of HPB inoculum, and slightly inclined to around 11–12 log CFU/

Fig. 1. Molecular identification of HPB via 16S rRNA gene sequencing. The optimal phylogenetic tree of HPB (indicated with an asterisk) and their closest strains was reconstructed by the Neighbor-joining method with the sum of branch length of 0. 23,647,485 using MEGA X Alignment Explorer.

g before gradually decreasing to 9 log CFU/g at the end of the experiment. The pH dynamic of the biodegraded FW showed an almost similar pattern for both autoclaved and non-autoclaved samples. The pH of the inoculated samples dropped dramatically from a nearly neutral level (above pH 6) to mildly acidic (around pH 4) after just 2 DPI before steadily inclined to around pH 5 towards the 8th DPI.

The ability of the HPB to grow in organic FW media corresponded to the FW analysis results in which the overall biodegradation rate was improved efficiently based on the remaining TS and the breakdown of nutrient components in both autoclaved and non-autoclaved FW (Fig. 4). It is apparent from Fig. 4A and B that the inoculated FW was degraded more efficiently than the uninoculated control. A strong evidence of synergistic effects of the co-culture inoculation was found when both autoclaved and non-autoclaved FW showed a higher percentage of biodegraded TS up to 49.3% and 62.7% respectively as compared to the monoculture treatments. Further analysis of the nutrient composition of the FW revealed the efficiency of HPB to biodegrade a high percentage of protein, total fat, total sugar, reducing sugar and starch at the end of the experiment (Fig. 4C and D). In the autoclaved samples, the HPB-inoculated FW recorded a significantly higher percentage ($P < 0.05$) of biodegraded protein, total fat, total sugar, reducing sugar and starch as compared to the uninoculated control. The co-culture treatment showed a significantly higher reduction ($P < 0.05$) of all nutrient compositions as compared to the other treatments except for reducing sugar and starch ($P > 0.05$). Unlike the non-autoclaved samples, the HPB-inoculated samples recorded significantly higher ($P < 0.05$) removal of protein, total sugar, reducing sugar and starch as compared to the untreated control except for total fat ($P > 0.05$).

3.4. Optimal inoculum size in batch-biodigester trial

The efficiency of the HPB co-culture to biodegrade FW was further evaluated using a domestic FWB. A preliminary batch-biodigester trial was set up where the optimal inoculum size of the HPB co-culture was determined using different volumes

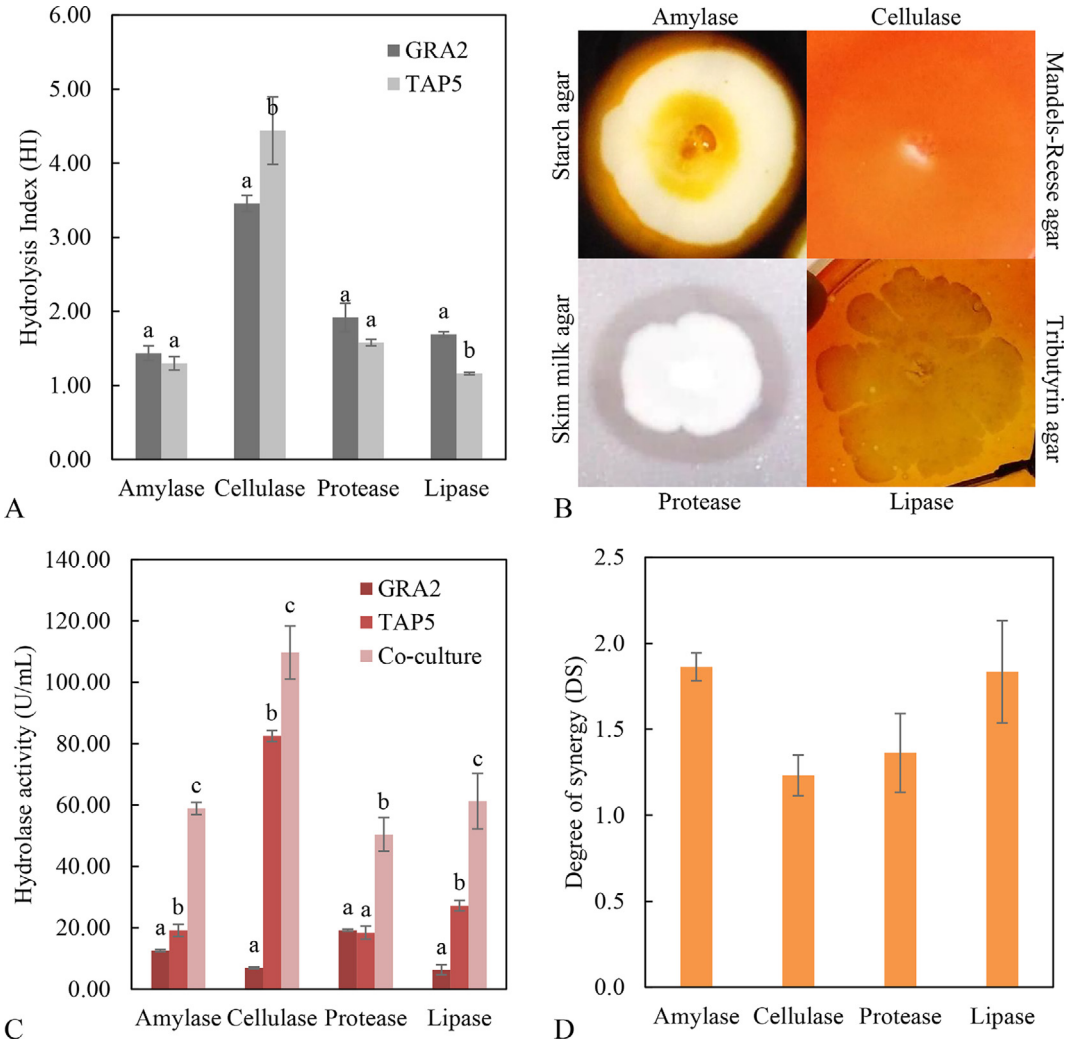

Fig. 2. Hydrolase activities of *B. paralicheniformis* GRA2 and *B. velezensis* TAP5. A: Hydrolysis index (HI) of GRA2 and TAP5 via agar-based assay of hydrolase. B: Hydrolytic activity of TAP5 on screening agar. C: Comparison of hydrolase activity of monoculture and co-culture. D: Degree of synergy of co-culture. Bars followed by different letters are significantly different according to the LSD test at $P < 0.05$. Error bars represent the standard error ($n = 3$).

(Control 0 mL, 500 mL, 1000 mL and 1500 mL). Nutrient composition analysis for every batch of the FW sample was profiled separately on a dry weight basis as illustrated in Fig. 5A. The effects of HPB inoculants on FW biodegradation were evaluated daily based on the GBP and temperature dynamic within the 8-day digestion period. The data of the three different FW batches were averaged and demonstrated in Fig. 5B. Based on the chart, the performance of the co-culture inoculant to drive the pattern of the GBP throughout the 8-day digestion period could be predicted from the 1st DPI. In this primary stage, the co-culture inoculum size of 1500 mL recorded the highest percentage biodegradation which was 52.7% followed by 1000 mL (47.3%), 500 mL (37.3%) and 0 mL (28.2%).

However, the inoculum size of 1000 mL and 1500 mL recorded a quite comparable pattern of GBP on the subsequent DPI and reached a plateau on the 4th DPI onwards at GBP of more than 85%. The inoculum size of 500 mL showed a slower rate of biodegradation in which it achieved a plateau on the 6th DPI at 87.4%. Contrariwise, the GBP of the uninoculated control (0 mL co-culture inoculum size) did not achieve a plateau and continually increased every day up to 86% on the 8th DPI. None of the treatments achieved nearly 100% biodegradation of FW at the end of the digestion period. The temperature dynamic for every co-culture inoculum size exhibited a distinctive pattern among each other except for 1000 mL and 1500 mL. All treatments showed a temperature spike on the 1st DPI, whereby the 1000 mL and 1500 mL inoculum treatments exhibited the highest rise during the first 4 DPI (up to 43.4 °C) before gradually decreased to about 32 °C at the end of the digestion. The temperature dynamic of FW biodegradation was positively correlated to the daily GBP ($r = 0.695$, $P < 0.05$).

4. Discussion

The impact of the amounting co-generated organic fraction in the municipal solid waste could be reduced through FW minimization at the source by using FWBs. To that end, this research sought to check the strategy of utilizing locally isolated HPB to improve food waste biodegradation rate by the action of an active synergy of hydrolytic bacteria. In the present study, a preliminary in vitro biodigestion and a batch-biodigester trial were performed to evaluate the ability of selected HPB to degrade protein, fat, total sugars and starch, hence improving the efficacy of FW biodegradation. Through primary isolation and screening of HPB from various fermented foods, two selected isolates namely GRA2 and TAP5 showed great potential as

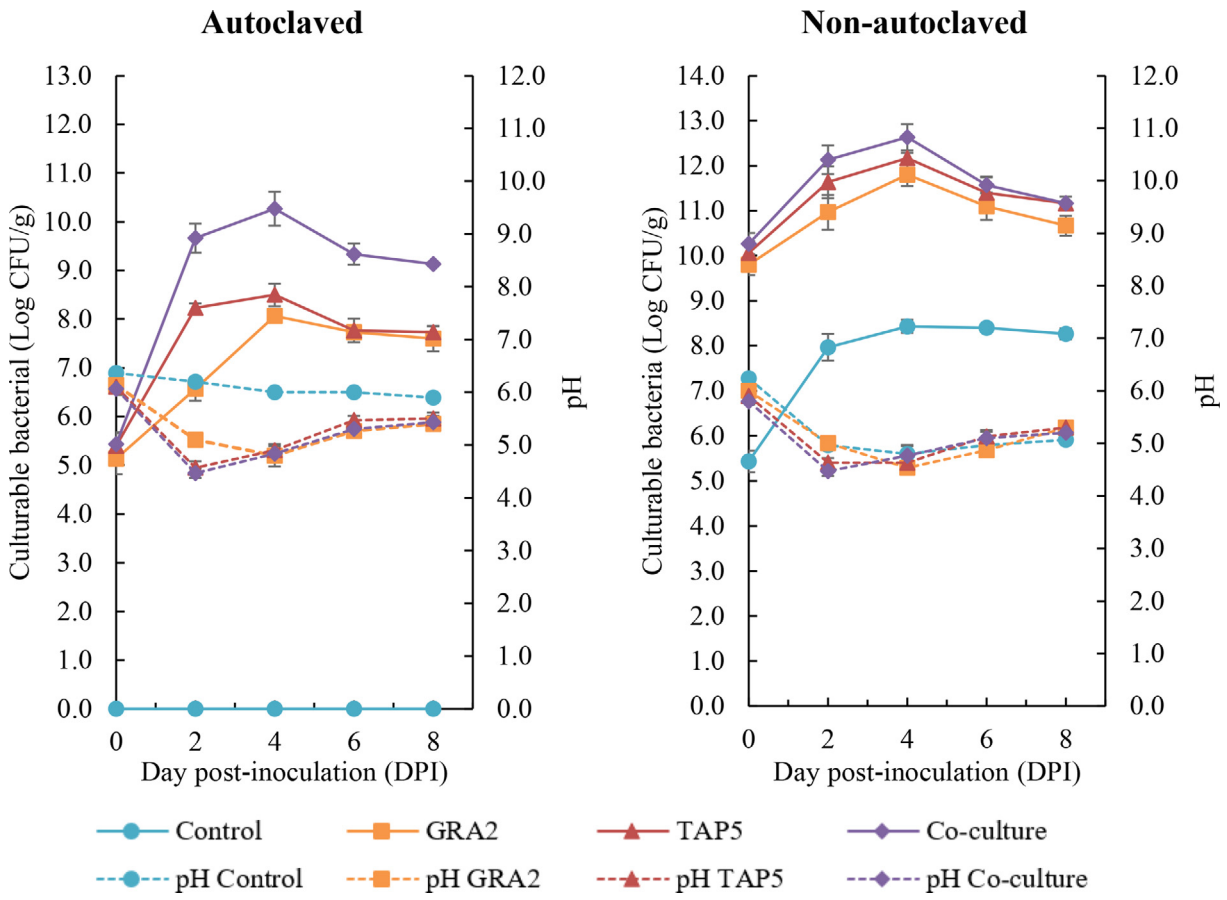

Fig. 3. Growth dynamic of the viable bacterial population (A) and pH dynamic (B) during FW biodegradation in vitro. Error bars represent the standard error (n = 3).

FW biodegradation agents where they were able to produce maximum amylase, cellulase, protease and lipase activities in the qualitative solid phase assay. The HI of GRA2 for amylase, protease and lipase on agars were higher than those of TAP5 except for cellulase. Since the colony of TAP5 showed more vigorous growth on plates than any plates of GRA2, the validity of the HI results as a preliminary indication of the hydrolytic capacity of HPB were supported by the subsequent liquid-based assay.

The biochemical characterization of HPB was also performed (Table 2) in which a growth test revealed that the HPB could survive in acidic and moderately high temperature conditions. The ability of the HPB to survive in this environment is one of the crucial characteristics as potential FW biodegraders since FW biodegradation in nature generally involves a wide array of organic acids built-up in relatively high temperature conditions (Sundberg et al., 2013). Results of molecular analysis validated that GRA2 belonged to *B. paralicheniformis* while TAP5 belonged to *B. velezensis* phylogeny. *B. paralicheniformis* was a novel species proposed by Dunlap et al. (2015) as they successfully isolated a new bacterial strain from a fermented soybean-based paste. This species has been attributed to various industrially significant potentials by virtue of its antimicrobial properties, enzyme and biomolecules production (Ahire et al., 2020). *Bacillus velezensis* sp., on the other hand, has been regarded as a plant-beneficial bacterium through the production of its secondary metabolites which trigger induced systemic resistance in plants as a defense mechanism against recurrent phytopathogen infestation (Rabbee et al., 2019). While the information on these species was scarce, to our knowledge, both species have never been associated with FW biodegradation potential.

Since the antagonistic interaction between both HPB was undetected, they were tested as co-culture to investigate the presence of bacterial synergistic interaction. The HPB co-culture surprisingly demonstrated a significant hydrolase synergy ($P < 0.05$) in co-culture fermentation as compared to those of respective monocultures. These results corroborate the findings of a great deal of the previous work in microbial co-culture synergism where the combination of *Bacillus* sp. and *Saccharomyces cerevisiae* culture improved α-amylase production by more than two folds as compared to respective monoculture (Fossi et al., 2014). Similarly, Cortes-Tolalpa et al. (2017) reported the enzymatic synergism in *Citrobacter freundii* and *Sphingobacterium multivorum* co-culture in the production of cellobiohydrolase, β-mannosidase, β-xylosidase assays which inclined dramatically up to 6.4 DS value after 72-h extended incubation period. The plausible microbial interactions such as commensalism and mutualism during concurrent cell growth were suggested as the general mechanism behind this synergistic action of the co-culture.

The negative Pearson correlation between the HI results on agar and the hydrolase activities in liquid media for both HPB implied that the HI data was not a reliable indicator of the hydrolytic capacity of HPB, and therefore must be supported with hydrolase activity analysis through liquid-based assay. Agar-based assay might exert some limitation for direct quantitative interpretation of the hydrolytic capacity of HPB in terms of the nutrient accessibility, species-specific bias and substrate suitability as compared to liquid-based assay, hence suitable to only represent a preliminary qualitative determination of HPB. However, with a small sample size, caution must be applied in this interpretation as the findings might not apply to all HPB species and fungal counterparts.

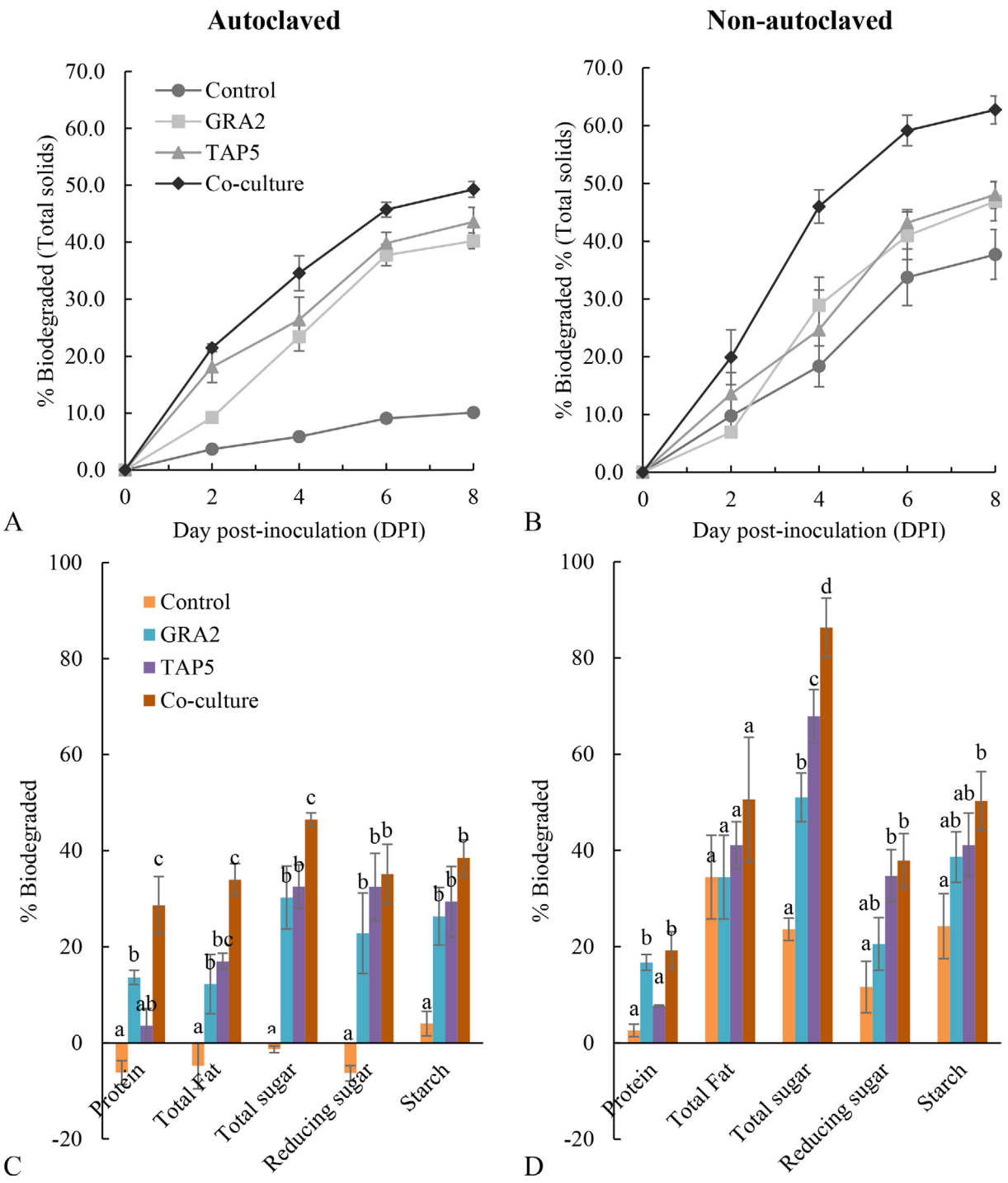

Fig. 4. Effects of different HPB treatments on FW biodegradation. A, B: Biodegradation percentage based on the remaining total solids. C, D: Biodegradation percentage of nutrient components of FW. Charts on the left and the right represent data of autoclaved and non-autoclaved FW respectively. Bars followed by different letters are significantly different according to the LSD test at $P < 0.05$. Error bars represent the standard error (n = 3).

In the preliminary FW biodegradation experiment in vitro for eight days, both HPB showed a persistent growth during the experiment where the highest CFU (12 log CFU/g) of culturable bacteria was detected by the co-culture treatment in the non-autoclaved FW (Fig. 3). The relatively high increase in cell counts of HPB indicated that they were able to grow and survive in FW media as the sole nutrient source. The ability of co-culture to thrive better as compared to monoculture was also documented by Cortes-Tolalpa et al. (2017) where the co-culture growth of *C. freundii* and *S. multivorum* revealed an 18.2-fold increase in cell density. An et al. (2018) also reported that *Bacillus* spp. was able to sustain growth at pH 5.0–5.5 (7–8 log CFU/g) in organic FW media after 8-day fermentation in vitro. A sudden drop in pH was also detected at the early stage of biodegradation from around pH 6 to 4 after 2 DPI and maintained around pH 5 until the end of the experiment. A similar phenomenon was reported by Awasthi et al. (2018) who observed a sharp decrease in pH in the early stage of FW biodegradation process by different species of *Bacillus* spp. Wong et al.

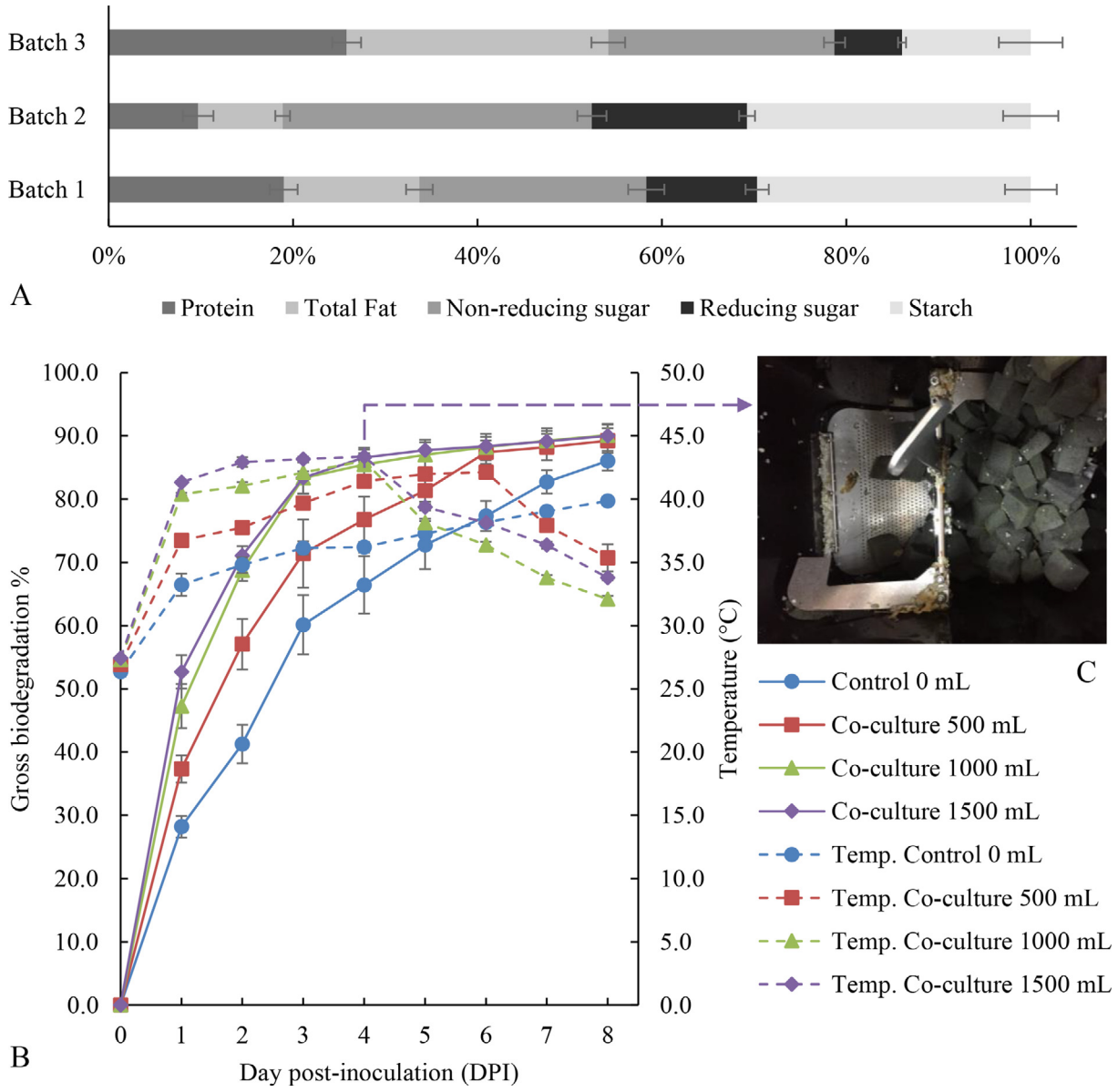

Fig. 5. Nutrient composition of dry FW (A) and gross biodegradation percentage of FW in a FWB within the 8-day digestion period for three FW batches combined, along with the recorded temperature dynamic (B). Barrel of FWB during FW biodegradation using 1000 mL co-culture inoculum size on the 4th DPI (C). Error bars represent the standard error (n = 3).

(2009) suggested that the pH drop in the initial stage of organic waste degradation was attributed to the accumulation of volatile fatty acids from the breakdown of complex organic matter.

The ability of the HPB to grow in the organic FW media reflected the performance of FW biodegradation of the inoculated FW during the experiment which was based on the remaining TS and the breakdown of nutrient components (Fig. 4). In particular, the highest percentage of biodegraded TS (>60%) was detected in the co-culture treatments. One of the possible mechanisms of the HPB to degrade food solids is through sugar biodegradation in which glucose is converted into carbon dioxide and water, thus reducing the TS of FW (Coker, 2014). These results are in agreement with those recently stated by An et al. (2018) and Msarah et al. (2020) who reported the reduction of the solid content of FW by more than 2% and 43% by *Bacillus* spp. inoculation. Additionally, Awasthi et al. (2018) also reported the decomposition of over 60% dry weight of pre-consumed and post-consumed FW inoculated with amylolytic *Bacillus* spp. consortium.

The HPB inoculation was also effective to degrade total solids (>62%), protein (>19%), total fat (>51), total sugar (>86%), reducing sugar (>38%) and starch (>50%) at the end of the experiment. One counterintuitive outcome from the co-culture treatment was that the protein reduction was observed higher in the autoclaved sample than in the non-autoclaved sample. It is difficult to explain this result, but it might be related to the higher bioavailability of simple nitrogen sources in the form of amino acids due to the heat pretreatment of FW prior to digestion, hence more readily accessible to be utilized by the HPB. Specifically, the metabolic assimilation between hydrolytic microorganisms and synergistic activity of cumulative enzymes secretion may allow for an efficient degradation process. Cortes-Tolalpa et al. (2017) stated that co-cultures perform division of labour to complement metabolic activities required for substrate utilization, hence superior to monocultures.

Finally, the batch-biodigester trial using a domestic FWB was performed to screen for the optimal inoculum size for this application. In principle, the FWB applies general mechanical mixing and

washing to break down the structural integrity of FW, while employing natural microbial activities to catalyze the biodegradation process. The disintegrated FW is filtered through a stainless-steel strainer and safely discharged as a wastewater run-off to the sewer. Thus, the HPB co-culture inoculation in this work is expected to outperform the natural FW biodegradation rate in the FWB by the synergistic action of hydrolase released. As shown in Fig. 5, the 1000 mL and 1500 mL inoculum size recorded a higher GBP (>85%) on the 4th DPI as compared to the 500 mL inoculum size and the uninoculated control. However, all treatments hardly reached 100% GBP of FW at the end of the biodigestion experiment. This discrepancy could be attributed to the residual amount of FW matrix which was stuck and remained on the upper wall lining and the edges of the mechanical rotator in the barrel of the FWB (Fig. 5C). This resulted in the inaccuracy of measurement of the actual biodegradation capacity of the FWB but not however, interfere with the performance of the inoculants to catalyse the breakdown of FW. As well, the temperature dynamic of FW biodegradation was positively correlated to the daily GBP ($r = 0.695$, $P < 0.05$). These results confirm the association between biodegradation of organic matter and temperature dynamic as described by (Li et al., 2014). However, the temperature range reported in this research is far below than the previous observation in the pre-thermophilic and thermophilic stage of anaerobic digestion or bio-composting which commonly ranged from 50 °C to 80 °C (Li et al., 2014; Liu et al., 2018; Palaniveloo et al., 2020). This inconsistency might be due to the default operational method of the FWB which rotates and rinses the FW matrix recurrently, thus regulating the internal ambient temperature.

According to Palaniveloo et al. (2020), the initial biodegradation of organic matters is driven by mesophiles that consume readily available carbon sources at the beginning of the digestion and results in the release of heat generated through wide-ranging microbial metabolic activities. During the subsequent thermophilic phase, various thermophilic microorganisms, such as *Bacillus* spp. will dominate the microbial population to catalyze the breakdown of substances with the assistance of high temperature. This leads to the exhaustion of nutrients and causes the temperature to decline gradually before entering the mesophilic phase again. Based on this biodigestion experiment, the inoculum size of 1000 mL is recommended for optimal use of FW biodegradation using domestic FWB. A higher volume of inoculum is not suggested since there was no significant difference when 1500 mL inoculum was incorporated.

5. Conclusion

B. paralicheniformis GRA2 and *B. velezensis* TAP5 co-culture demonstrated a notable hydrolase synergism and actively proliferate in FW media, outperforming the monoculture treatments. The HPB co-culture easily reduced a high percentage of TS and efficiently degrade protein, total fat, total sugar, reducing sugar and starch of FW in vitro. Additionally, the HPB co-culture achieved >85% GBP on the 4th DPI in a commercial domestic FWB using the optimal 1000 mL inoculum size. To the best of our knowledge, this is the first report to highlight the practicality and performance of locally isolated HPB co-culture inoculation in a commercial domestic FWB.

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