12-10-2020

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Recommended Citation

J. O'Hair, Q. Jin, D. Yu, J. Wu, H. Wang, S. Zhou, H. Huang, "Non-sterile fermentation of food waste using thermophilic and alkaliphilic Bacillus licheniformis YNP5-TSU for 2,3-butanediol production", Waste Management, Volume 120, 2021, Pages 248-256, ISSN 0956-053X, https://doi.org/10.1016/j.wasman.2020.11.029.

Non-Sterile Fermentation of Food Waste using Thermophilic and Alkaliphilic *Bacillus Licheniformis* YNP5-TSU for 2,3-Butanediol Production

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ABSTRACT

Conversion of food waste into 2,3-butanediol (2,3-BDO) via microbial fermentation provides a promising way to reduce waste disposal to landfills and produce sustainable chemicals. However, sterilization of food waste, an energy- and capital-costly process, is generally required before fermentation to avoid any contamination, which reduces the energy net output and economic feasibility of food waste fermentation. In this study, we investigated the non-sterile fermentation of food waste to produce 2,3-BDO using a newly isolated thermophilic and alkaliphilic *B. licheniformis* YNP5-TSU. Three unitary food waste samples (i.e., pepper, pineapple, cabbage wastes) and one miscellaneous food waste mixture were respectively inoculated with *B. licheniformis* YNP5-TSU under non-sterile conditions. At 50 °C and an initial pH of 9.0, *B. licheniformis* YNP5-TSU was able to consume all sugars in food waste and produce 5.2, 5.9, 5.9 and 4.3 g/L of 2,3-BDO within 24 hrs from pepper, pineapple, cabbage and miscellaneous wastes, respectively, corresponding to a yield of 0.40, 0.38, 0.41 and 0.41 g 2,3-BDO/g sugar. These 2,3-BDO concentrations and yields from the non-sterile fermentations were comparable to those from the traditional sterile fermentations, which produced 4.0 to 6.8 g/L of 2,3-BDO with yields of 0.31 to 0.48 g 2,3-BDO/g sugar. Moreover, *B. licheniformis* was able to ferment various food wastes (pepper, pineapple and miscellaneous wastes) without any external nutrient addition and produced similar 2,3-BDO quantities. The non-sterile fermentation of food waste using novel thermophilic and alkaliphilic *B. licheniformis* YNP5-TSU provides a robust and energy-efficient approach to convert food waste to high-value chemicals.

Keywords: non-sterile fermentation, food waste, 2,3-butanediol, *Bacillus licheniformis*, thermophile, alkaliphilic

1. Introduction

Nearly 30 to 40% of the total U.S. food supply becomes waste, causing a $240 billion economic loss and 3.3 gigatons of greenhouse gases annually (Yu and Jaenicke, 2020). Moreover, an estimated 1.3 billion tons of food is discarded every year globally and the majority of it ends up in landfills, causing potential environmental concerns (FAO, 2013). With the projected world population increase, accompanied with increased food production, the economic and environmental problems related to food waste will be more significant (FAO, 2009). Thus, it is urgent to develop appropriate strategies to valorize food waste. Food waste usually consists of substantial amounts of carbohydrates, proteins, lipids, and minerals (Paritosh et al., 2017; Jin et al., 2017), making it a potentially good feedstock for microbial fermentation to produce value-added chemicals (Raveendran et al., 2018). Studies have been conducted to convert food waste to various renewable chemicals such as lactic acid (Tang et al., 2003), succinic acid (Lam et al, 2014; Li et al., 2019), ethanol (Kim et al., 2011; Huang et al., 2015), butanol (Huang et al., 2015; Poe et al., 2020; Jin et al., 2018, 2020), and 2,3-butanediol (2,3-BDO) (Lee et al., 2019). Among all these chemicals, 2,3-BDO has recently gained much attention due to its expanded market ($43 billion per year) and its versatile application as a platform chemical to produce compounds including 2,3-butadiene, methyl ethyl ketone, acetoin, and diacetyl (Kim et al., 2017).

Sterilization of feedstock is usually required before fermentation to avoid any microbial contamination, especially in pure culture fermentation, because the indigenous microorganisms may outcompete the inoculated culture and fail the fermentation. However, sterilization is one of the most energy- and economic- costly steps in the whole fermentation

process (Tao et al., 2005). In most cases, the medium containing feedstock and nutrients is heated from room temperature to the desired sterilization temperature (e.g., 121 °C) and held for a fairly long time (e.g., 15 to 60 min) by high pressure steam to achieve a sterile condition; then the hot medium is cooled to the operating temperature (e.g., 30 to 40 °C) by cooling water or air (Clarke, 2013). A promising alternative strategy is to use thermophilic microorganisms to conduct non-sterile fermentation at temperatures above 50 °C, which could reduce contamination from mesophilic microorganisms (Jiang et al., 2017). The strategy of using thermophilic microorganisms to produce 2,3-BDO has previously been applied in several other studies (Xiao et al., 2012, Ge et al., 2016, Li et al., 2014). Xiao et al used a novel thermophilic *Geobacillus* strain for fermentation of acetoin and 2,3-Butanediol. Ge et al. genetically modified thermophilic *B. licheniformis* to produce 2,3-BDO from glucose at 50 °C (Ge et al., 2016). Li et al used thermophilic *Bacillus licheniformis* X10 to produce 2,3-BDO from corn stover hydrolysate and were able to show 2,3-BDO production at a fermentation temperature of 50 °C. However, these thermophilic fermentations have only been studied using pure sugars and lignocellulosic hydrolysates. It is unseen if 2,3-BDO could be produced through non-sterile fermentation of food waste. Compared with simple sugars and lignocellulosic hydrolysates, food waste is a much more complex feedstock and susceptible to microbial contamination because it usually contains substantial indigenous microorganisms. Moreover, the rich nutrient of carbohydrates, amino acids and minerals found in food waste, promotes the prosperous growth of indigenous microorganisms. Therefore, it is very possible that other thermophilic microorganisms, such as *Clostridium,*

89 *Thermoanaerobacterium* and *Lactobacillus*, found in food waste, could complicate 2,3-BDO
90 production (Lee et al., 2014).

91 Recently, we isolated a new thermophilic *B. licheniformis* strain, YNP-TSU, from
92 Yellowstone National Park's Whiterock spring (lat 44.7803, long -110.6981) (#YELL-2015-
93 SCI-6074). This strain can convert different types of carbohydrates to 2,3-BDO at high
94 temperatures of 50 to 55 °C with a high productivity (1.1 g/L/h) and a high yield (0.46 g
95 BDO/g glucose) (O'Hair et al., 2020). More importantly, YNP5-TSU can conduct
96 fermentation at alkaline conditions with an initial high pH of 9.0. The unique combination of
97 thermophilic and alkaliphilic characteristics of YNP-TSU grant this strain the capability to
98 potentially outcompete most contaminates in raw food waste, making it a potential candidate
99 for non-sterile fermentation. However, it is still unknown how this strain performs in the
100 fermentation of complex food waste under non-sterile conditions and whether the
101 combination of high temperature (>50 °C) and high pH (9.0) would cause the suppression of
102 contaminate microorganisms and enable non-sterile fermentation. Therefore, the objective of
103 this study is to investigate the feasibility of 2,3-BDO production via non-sterile fermentation
104 of food waste using the *Bacillus licheniformis* YNP5-TSU at thermophilic and alkaline
105 conditions with and without external nutrient addition. The outcome of this study will assist
106 in developing new strategies for non-sterile fermentation to reduce the capital and operating
107 costs for renewable chemical production.

108 **2. Materials and methods**

109 *2.1 Food waste collection and compositional analysis*

Three unitary food waste samples, i.e., cabbage, pepper, pineapple, and one miscellaneous waste mixture (a mixture of potato, pepper, strawberry, tomato, onion, cabbage, and pineapple) were collected from the Virginia Tech Dining Services in 2018 (Blacksburg, Virginia, USA). Food wastes in the Dining Service are discarded in 96-gallon plastic trash cans with a lid and are hauled away by a waste management company once a week. The collected food wastes were separately homogenized using a blender (UMS table top model, Stephan, Hameln, Germany), split into several packages, and stored at -20°C until further use.

Moisture, ash, crude fat, and protein contents of the food wastes were measured according to AOAC methods 925.10, 942.05, 920.39, and 990.03, respectively (AOAC, 2000). Starch content was measured using the Megazyme total starch assay kit (Megazyme Inc., Chicago, IL, USA). The ANKOM filter bag system (ANKOM 2000 automated fiber analyzer, ANKOM Technology, Macedon, NY, USA) was used to determine the neutral detergent fiber (NDF) content (Vogel et al., 1999). The extraction and determination of soluble sugars (glucose, fructose, sucrose) were according to a previous study (Jin et al., 2019). Briefly, each food waste sample, after oven drying (40 °C), was extracted by 85% (v/v) ethanol with a solid to liquid ratio of 1:50 in a constant shaking water bath at 50 °C for 30 min. After extracting three times, the liquid was combined and ethanol was removed by vacuum evaporation at 50 °C. The residue was then resuspended in water for the determination of glucose, fructose and sucrose using an Agilent 1200 high-performance liquid chromatograph (HPLC, Agilent Technologies, Santa Clara, CA, USA) with a refractive index detector (RID). The Bio-Rad Aminex HPX-87P column (Bio-Rad Laboratories, Hercules, CA, USA) was used for sugar separation at the temperature of 80 °C. Ultrapure

132 water was used as the mobile phase with a flow rate of 0.6 mL/min. The total running time
133 was 30 min with an injection volume of 5 µL.

134 *2.2 Culture maintenance and inoculation broth*

135 Isolate YNP5-TSU was grown in a two-stage (P_1 and P_2) seed culture inoculum. Stock
136 culture in 20% glycerol was thawed from -80 °C, and 1 mL was directly inoculated into 100
137 mL P_1 broth media (60 g/L glucose, 10 g/L yeast extract, and 5 g/L peptone, pH 7.5) and
138 incubated for 18 hrs at 50 °C and 150 rpm in a shaking incubator (New Brunswick Scientific
139 Inc, Edison, NJ, USA). The following day, 20 mL of the first stage culture (P_1) was added to
140 the P_2 broth media (40 g/L glucose, 10 g/L yeast extract, and 5 g/L peptone, pH 7.5) and
141 incubated at 50 °C and 150 rpm for 6 to 8 hrs until the optical density OD_{600} reached 1.0.

142 *2.3 Incubation of non-sterilized food waste without inoculation of B. licheniformis YNP5-TSU*

143 Homogenized pepper, pineapple, cabbage, and miscellaneous food waste mixture
144 were retrieved from -20 °C and thawed for 1 to 2 hrs at room temperature. With the purpose
145 to show the food waste degradation by the growth and metabolism of indigenous
146 microorganisms, all non-sterilized food wastes were incubated separately in 150 mL baffled
147 flasks at 20, 37, and 50 °C for 72 hrs in a shaking incubator at 50 rpm. The pH was left
148 unaltered at 6.5 or raised to 9.0 using 1 M NaOH. *B. licheniformis* YNP5-TSU was not added
149 to the food waste. The initial concentration (colony forming units (CFU)/mL) of bacteria in
150 the food waste slurry was determined by the surface plating method after serial dilution. In
151 short, homogenized waste was diluted ten-fold to a final dilution of 10^{-5}, spread plated on
152 Luria-Bertani (LB) agar, and incubated for 24 hrs at 37 °C, after which colonies were counted
153 (Ben-David et al., 2014). Liquid samples (1 mL) were collected at 0, 8, 24, 48 and 72 hrs of

154 incubation for sugar and fermentation products analyses. Each incubation was conducted in
155 duplicate.

156 *2.4 BDO production from fermentation of sterilized and non-sterilized food waste*

157 Homogenized pepper, pineapple, cabbage, and miscellaneous food waste samples
158 were retrieved from -20 °C and thawed for 1 to 2 hrs at room temperature. Food waste
159 slurries were prepared at 6% solids contents by mixing 25 g individual wet food waste
160 (containing 9.5 to 12.0 g of dry solids) with a calculated amount of deionized water (based on
161 the water content in each wet food waste) in 150 mL baffled flasks. Yeast extract and peptone
162 were then added at 0.5% (w/v) each. For the sterile fermentation, the food waste slurries were
163 sterilized in an autoclave at 121 °C for 60 min and cooled to room temperature. For the non-
164 sterile fermentation, the food waste slurries were not sterilized and used as is. All food waste
165 slurries were adjusted to pH 9.0 with 1 M NaOH and inoculated with 10% of the P_2 YNP5-
166 TSU culture (OD_{600} of 1.0) to start fermentation. Fermentations were carried out at 50 °C in a
167 shaking incubator at 150 rpm. Samples (1 mL) were taken at 0, 8, 24, and 48 hrs during
168 fermentation for sugar and fermentation product analysis. Each fermentation was conducted
169 in duplicate.

170 Since food waste already contains substantial amounts of protein and minerals, it is
171 possible that no external nutrient addition is needed for 2,3-BDO fermentation. To this end,
172 we also conducted non-sterile fermentation of food waste with the same experimental
173 procedures described above; however, no yeast extract or peptone were added to the food
174 waste slurries before or during fermentation. Moreover, it is speculated that there might be
175 some residual nutrients (yeast extract and peptone) may have supported the food waste

fermentation. An additional experiment was conducted using pelleted cells as a source of inoculum. In this experiment, the P2 culture (10% of the fermentation volume) was centrifuged at 2,400 rpm for 5 min to remove any residual nutrients (yeast extract and peptone), and the resulted pelleted cells were inoculated to the food waste media. Each fermentation was conducted in duplicate.

2.5 Analytical methods for fermentation samples

Glucose, sucrose, fructose, 2,3-butanediol, lactic acid, acetic acid, and ethanol concentrations in collected incubation/fermentation samples were quantified using an HPLC (Agilent Technologies, 1260, Santa Clara, CA) equipped with a refractive index detector (RID). Fermentation samples were centrifuged for 10 min at 16,639 × *g* (Eppendorf® Centrifuge 5424, Hamburg, Germany). Supernatants were syringe filtered through a 0.20 μm nylon filter (Acrodisc®, Pall Company, NY). A Bio-Rad organic acid Aminex® HPX-87H ion exclusion column (Bio-Rad Laboratories, Hercules, CA) was used with 0.005 M H_2SO_4 as the mobile phase (0.6 mL/min) at 50 °C. The total run time was 30 min and the injection volume was 5 μL. Multiple standard curves were created for each compound (measured in duplicate) to accurately measure the fermentation substrates and products for each of the collected samples. To be detailed, 5, 10, 12.5, 15, 20, 30, 40, 50, 60 g/L standards of glucose, sucrose, and fructose, along with, 0.5, 1, 2.5, 5, 10 and 20 g/L standards of acetic acid, lactic acid, and ethanol, and 0.35, 0.7, 1.75, 3.5, 7, and 14 g/L 2,3-BDO were used to develop linear regression equations (with $R^2 > 0.999$) for measuring fermentation samples. 2,3-BDO yield was calculated as total 2,3-BDO produced divided by total sugar utilized and expressed in g/g. According to the literature, the theoretical 2,3-BDO from sugars (glucose) is 0.5 (Sabra et al., 2015).

3. Results and Discussion

3.1 Compositional analysis

All food waste types had a moisture content of 88% or higher (Table 1). The remaining 10 to 12% of solid waste was comprised of protein, sugars, fat, fibers, ash, and other solids. Soluble sugars are the most important constituents in food waste for fermentation to produce 2,3-BDO and comprised 35 to 45% of total dry weight of all tested food wastes (Table 1). These sugars (glucose, fructose and sucrose) are the main source of reducing power used for microbial fermentation (Doran-Peterson et al., 2008). Food waste sugar concentrations from our study were similar to those in a previous study, which reported that dry food waste had an average reducing sugar content of 46% (w/w, d.b.) (Gundupalli and Bhattacharyya, 2019). However, the sugar contents of pepper and miscellaneous wastes were below this average, indicating these two food wastes have less 2,3-BDO production potential. These two food wastes also lacked noticeable amounts of sucrose, 2.9% (pepper) and 4.5% (miscellaneous), when compared to cabbage and pineapple wastes (11.6% and 19.4%, respectively). Sucrose is the typical storage carbohydrate and the dominant sugar in pineapple followed by glucose and fructose (Cámara, 1996). Much like other sugars, sucrose is eventually converted to pyruvate once transported into the cell. From pyruvate, either mixed acids (i.e., lactate, acetate, formate) or 2,3-BDO precursors acetolactate, diacetyl, or acetoin are formed (Kandasamy et al., 2016). Besides soluble sugars, all food wastes contained high protein content (cabbage 11.4%, pepper 17.4%, pineapple 5.5%, miscellaneous 12.7%) indicating that food waste itself may contain sufficient nitrogen and minerals to support microbial fermentation. Therefore, we hypothesize that external nutrient

220 supplementation may not be needed for food waste fermentation; this hypothesis was tested
221 in section 3.4.

Table 1. Chemical composition of different food waste samples.

Parameters (%)	Cabbage waste	Pepper waste	Pineapple waste	Miscellaneous waste [a]
Moisture (wet basis, w.b.)	89.5 ± 0.3 [b]	90.8 ± 0.2	88.0 ± 0.3	90.5 ± 0.3
Ash (dry basis, d.b.)	6.0 ± 0.4	6.4 ± 0.3	1.4 ± 0.4	4.8 ± 0.2
Protein (d.b.)	11.4 ± 0.04	17.4 ± 0.2	5.5 ± 0.1	12.7 ± 0.2
Fat (d.b.)	0.6 ± 0.005	3.4 ± 0.07	0.9 ± 0.08	1.2 ± 0.04
NDF (d.b.)	15.2 ± 0.01	22.9 ± 0.9	33.8 ± 0.8	12.5 ± 0.09
Starch (d.b.)	0.7 ± 0.4	2.1 ± 0.03	1.0 ± 0.2	18.7 ± 0.4
Sucrose (d.b.)	11.6 ± 0.2	2.9 ± 0.0006	19.4 ± 0.3	4.5 ± 0.5
Glucose (d.b.)	19.7 ± 0.5	15.6 ± 0.2	12.7 ± 0.2	15.1 ± 0.3
Fructose (d.b.)	14.1 ± 0.5	20.4 ± 0.05	12.7 ± 0.3	15.5 ± 0.4
Total soluble sugars (d.b.)	45.4 ± 1.2	38.8 ± 0.3	44.8 ± 0.7	35.1 ± 1.1
Other solid (d.b.)	20.6 ± 1.2	9.0 ± 1.2	12.6 ± 0.4	15.0 ± 1.2

[a] Miscellaneous food waste mixture included potato, pepper, strawberry, tomato, onion, cabbage, and pineapple.

[b] Data expressed as mean ± S.D.

3.2 Incubation of unsterilized food waste without inoculation of YNP5-TSU

In order to investigate the effect of high temperature and high pH conditions on suppression of indigenous microorganisms in food waste, unsterilized miscellaneous waste was incubated at un-altered pH (pH 6.5) and mesophilic temperatures (20 and 37 ℃) to compare the effects of high temperature (50 ℃) and high pH (pH 9.0). Unsterilized food waste slurry had indigenous microorganisms at a concentration of 8.4×10^5 CFU/mL (Figure A2 in supporting information document). This is a typical amount for standard food waste as the range from 10^4 to 10^5 CFU/mL was also observed in raw food waste prior to treatment (Byungryul et al., 2018). When unsterilized food waste was incubated at 20 and 37 ℃, growth of indigenous microorganisms was observed, which was confirmed by the consumed sugar and detected metabolites (Figure 1A, 1B). The pH, which was initially 6.5, reached a

final value of 4.5 after 72-hr incubation at 37 ℃ and remained unaltered when incubated at 20 ℃. At 37 °C, sugars in food waste slurries were completely consumed by indigenous microorganisms by 72 hrs and resulted in 4.2 g/L lactic acid, 3.3 g/L acetic acid, and 2.6 g/L ethanol (Figure 1A). The ethanol observed here is most likely from wild yeast as they are typically found in food waste and have optimal growth temperatures around 32 °C (Salvadó et al., 2011). When unsterilized food waste was incubated at 20 °C, 4.5 g/L of soluble sugars was consumed at 72 hrs, producing 0.5 g/L acetic acid and 0.3 g/L lactic acid (Figure 1B). These products are produced most commonly by acetic acid producing *Acetobacter pasteurianus* and lactic acid producing *Lactobacillus* (Sampaio et al., 2014).

When unsterilized food waste slurry was incubated at 50 °C for 72 hrs, no sugars were consumed and no metabolites were produced, indicating that the indigenous microorganisms in food slurry were suppressed (Figure 1C). Most contaminate microorganisms in food waste grow in the so called “danger zone” which is from 4.4 to 65.6 ℃ (Johnson et al., 1983). Incubating food waste at 50 ℃ still allows for contaminates such as *Bacillus cereus* (isolated from food waste at 55 ℃) to be able to interfere with 2,3-BDO production. In this case pH must also be used as a barrier against food borne microbes. When unsterilized food waste pH was elevated to an initial 9.0 at 37 ℃ no contaminate growth was detected. The pH in both fermentations at 50 °C and pH 9.0 (Figure 1C and 1D) also remained unaltered throughout the 72-hr incubation.

While current biochemical production from non-sterile open fermentation commonly uses thermo-tolerant bacteria (Tongpim et al., 2014), the novel approach using a combination of both a thermophilic and alkaliphilic environment provides a double security blanket to

eliminate contamination in 2,3-BDO fermentation. By raising the fermentation pH to 9.0 thermophilic contaminants such as *Acinetobacter baumannii*, *Enterobacter sp*. and *Erwinia cypripedii* which were all found on food waste, can be suppressed (Yi et al., 2006).

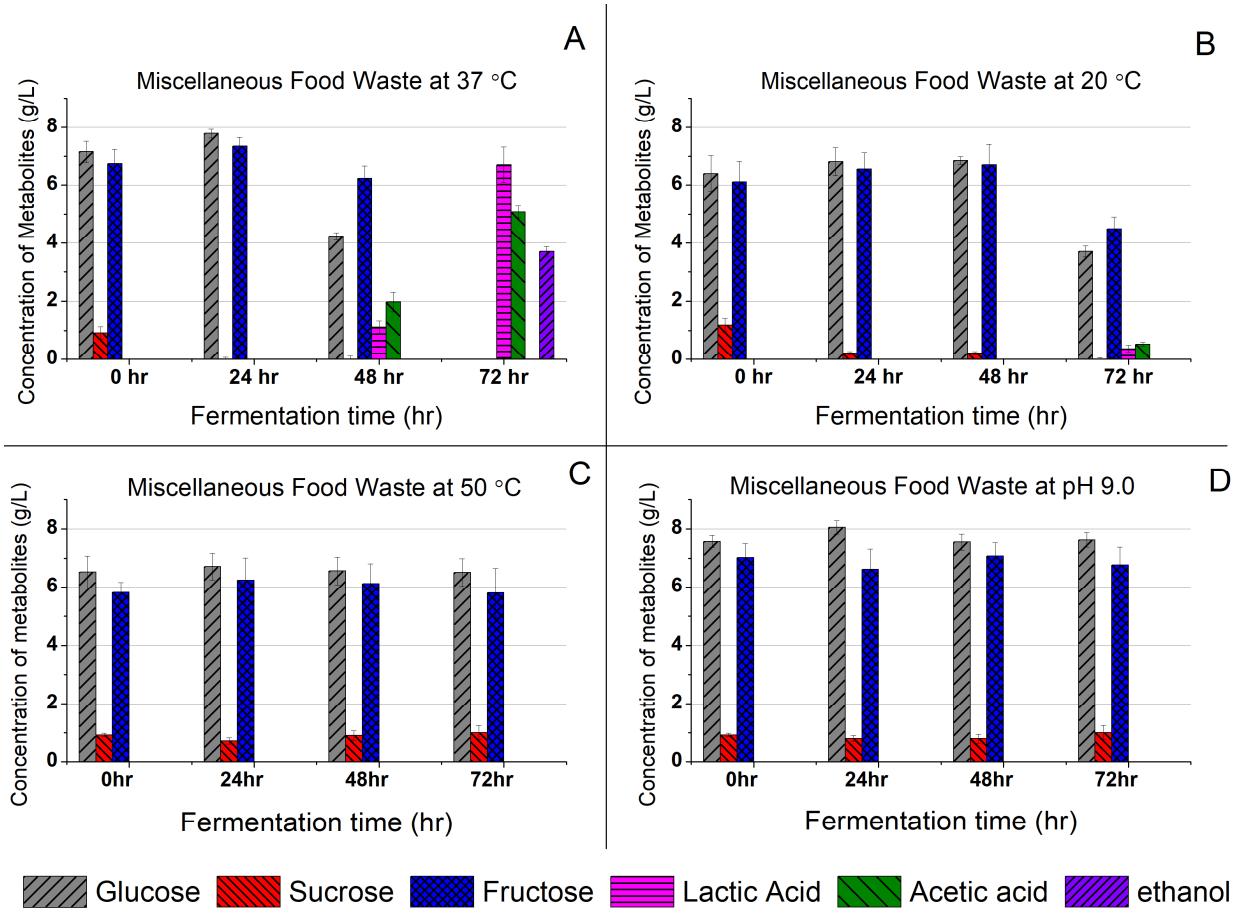

Figure 1. Incubation of unsterilized miscellaneous food waste without the inoculation of *B. licheniformis* YNP5-TSU. (A) Food waste slurry was incubated at 37 ℃ with its original pH of 6.5; (B) food waste slurry was incubated at 20 ℃ with its original pH of 6.5; (C) food waste slurry was incubated at 50 ℃ with its original pH of 6.5; (D) food waste slurry was incubated at 37 ℃ with a high pH at 9.0.

3.3 BDO production from fermentation of sterilized *and* unsterilized *food waste with nutrient addition*

When food waste slurries were sterilized and supplemented with yeast extract and peptone, sugars in all food wastes were completely consumed at 24 hrs, producing 4.2, 4.0,

6.8, and 4.2 g/L 2,3-BDO from pepper, pineapple, cabbage, and miscellaneous food waste, respectively (Figure 2). The 2,3-BDO yields at 24 hrs were 0.32, 0.31, 0.48, and 0.39 g BDO/g sugar for the fermentation of pepper, pineapple, cabbage, and miscellaneous wastes, respectively, which corresponds to 64%, 62%, 96%, and 78% of the theoretical yield of 0.5 g/g (Hakizimana et al., 2020). Cabbage waste produced the highest titer of 2,3-BDO (6.8 g/L) because the initial combined sugar concentration of glucose, sucrose, fructose was higher (14.2 g/L) than other waste types, pepper (13.2 g/L), pineapple (13.1 g/L), and miscellaneous (10.8 g/L). The majority of 2,3-BDO was produced within 8 hrs in the fermentations of pepper and pineapple wastes. In the fermentations of cabbage and miscellaneous waste, 2,3-BDO concentrations continued to increase until 24 hrs. All sterilized food wastes generated significant acetic acid when sugars (glucose, fructose and sucrose) in fermentation broth were depleted after 24 hrs. As shown in Figure 2, the increase in acetic acid from 24 to 48 hrs is accompanied by a decrease in 2,3-BDO. After 24 hrs with no sugars available, 2,3-BDO is most likely converted to acetyl-CoA through reversible pathways, generating waste acetic acid (Wang et al., 2013). Therefore, it is important to stop fermentation immediately after all sugars are consumed in order to harvest as much 2,3-BDO as possible.

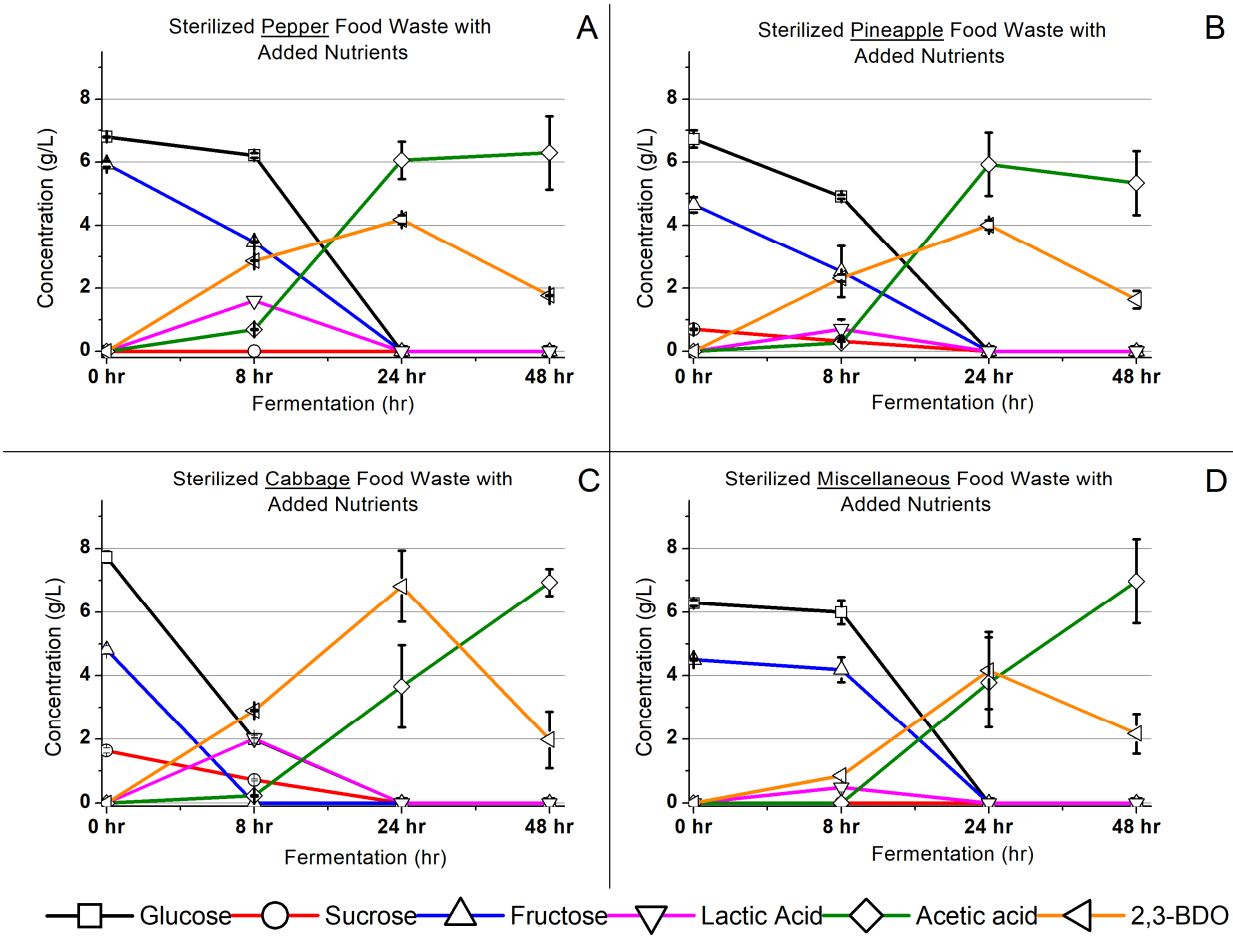

Figure 2. 2,3-BDO production from sterilized food waste fermentation with *B. licheniformis* YNP5-TSU and nutrient addition at 50 °C, initial pH 9.0, and 150 rpm.. (A) pepper food waste, (B) pineapple food waste, (C) cabbage food waste, (D), miscellaneous food waste.

In the fermentation of unsterilized food waste supplemented with yeast extract and peptone, soluble sugars (glucose, fructose and sucrose) were consumed with negligible sugars left (<1.0 g/L) by 24 hrs for all types of food wastes (Figure 3). Fermentation of pepper waste produced a maximum 2,3-BDO concentration of 5.2 g/L at 24 hrs, whereas fermentation of pineapple waste, cabbage waste, and miscellaneous food waste produced a maximum 2,3-BDO concentration of 5.9, 5.9, and 4.3 g/L at 24 hrs, respectively. The 2,3-BDO yields were 0.40, 0.38, 0.41, and 0.41 g/g for the fermentation of pepper, pineapple, cabbage and miscellaneous waste at 24 hrs, respectively, with an average yield of 0.4 g/g. Concentrations of mixed acids in non-sterile fermentations at 24 hrs were different from those in sterile

fermentations as only miscellaneous waste had noticeable acetic acid (4.5 g/L) (Figure 3D). Pepper, pineapple, and cabbage had acetic acid concentrations below 0.2 g/L at 24 hrs (Figure 3A, B, C).

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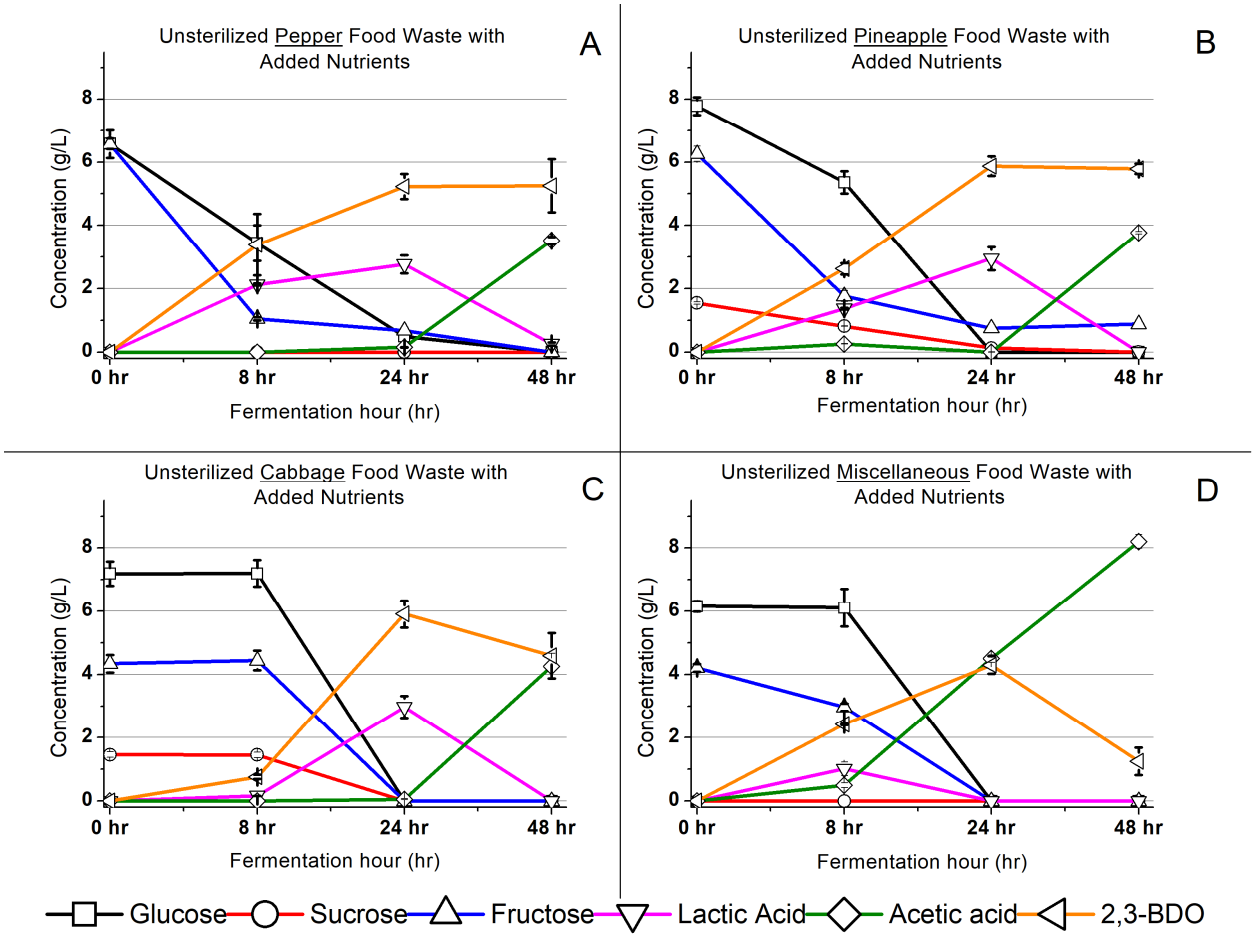

Figure 3. 2,3-BDO production from unsterilized food waste fermentation with *B. licheniformis* YNP5-TSU and nutrient addition at 50 °C, initial pH 9.0, and 150 rpm. (A) pepper food waste, (B) pineapple food waste, (C) cabbage food waste, (D), miscellaneous food waste.

In order to investigate the effectiveness of non-sterile fermentation of food waste using YNP5-TSU, sugar utilization, 2,3-BDO titer and yield, as well as acid concentrations in sterile and non-sterile fermentations at 24 hrs (when the highest 2,3-BDO concentration occurs) are summarized in Figure 4. When food wastes inoculated with YNP5-TSU were

316 incubated at an initial pH of 9.0 and temperature of 50 °C, sterile and non-sterile
317 fermentations had very similar 2,3-BDO titers and yields. In both cases the pH after 24 hrs
318 for each food waste was as follows: pepper waste pH 4.9, pineapple waste pH 3.8, cabbage
319 waste pH 5.2, and miscellaneous waste pH 6.6. The 2,3-BDO titers for the non-sterile
320 fermentation of different types of food waste at 24 hr were in the ranges of 4.3–5.9 g/L with
321 an average of 5.3 ± 0.8 g/L; whereas these values for the sterile fermentation were in the
322 ranges of 4.0–6.8 g/L with an average of 4.8 ± 1.3 g/L. In terms of 2,3-BDO yields, non-
323 sterile fermentation of different types of food waste resulted in yields of 0.38–0.41 g/g with
324 an average of 0.40 ± 0.0 g/L; whereas sterile fermentation of different types of food waste
325 resulted in yields of 0.31–0.48 g/g with an average of 0.38 ± 0.1 g/L. 2,3-BDO titers and
326 yields were consistent between the nonsterile and sterile fermentations, even though food
327 wastes are highly complex and very different in composition (Paritosh et al., 2017). Sugar
328 utilization between sterile and non-sterile fermentations was almost identical for each type of
329 food waste. This result is apparent because all sugars were completely utilized at 24 hrs by
330 YNP5-TSU. We also used 10 g/L of glucose solution as a control feedstock to conduct non-
331 sterile fermentation and found that the 2,3-BDO yield is 0.40 (Figure 4), which was very
332 close to the ones from the fermentations of food waste, indicating the robustness of the 2,3-
333 BDO fermentation using YNP5-TSU. Overall, based on the 2,3-BDO titer, yield, and sugar
334 utilization in sterile and non-sterile fermentations of food wastes, we could conclude that the
335 thermophilic and alkaliphilic fermentation using YNP5-TSU allows the elimination of the
336 costly sterilization of food waste and supports the non-sterile fermentation without affecting
337 2,3-BDO production.

However, differences were observed in mixed acid production between sterile and non-sterile fermentation (Figure 4). Sterile fermentations of pepper, pineapple and cabbage had acetic acid concentrations of 6.0 g/L, 5.9 g/L, and 3.7 g/L, respectively at 24 hrs while non-sterile fermentations of pepper, pineapple, and cabbage had less than 0.1 g/L acetic acid each. As for lactic acid production at 24 hrs, all sterile fermentations had 0 g/L (Figure 4), while non-sterile fermentations of pepper, pineapple, and cabbage produced 2.8 g/L, 3.0 g/L, and 3.0 g/L lactic acid, respectively. The lactic acid concentrations, nonetheless, did reduce to negligible levels (0.3 g/L) at 48 hrs (Figure 3). Mixed acids such as lactic acid and acetic acid compete for carbon and NADH utilization. In a study by Cho et al., they found dissolved oxygen content played a large role in metabolite production and increasing the agitation speed from 300 to 400 rpm to increase dissolved oxygen eliminated lactic acid production. Non-sterilized miscellaneous food waste was the only non- sterilized food waste that did not produce any lactic acid by 24 hrs, similar to sterilized food wastes, which might be due to the lowest percentage of non-dissolvable fibers (NDFs) in miscellaneous food waste. NDFs can form an insoluble layer on the surface of media and decrease oxygen's capability to mix with media (Lourenco et al., 2013). Therefore, the low NDFs in miscellaneous food waste might have caused higher oxygen dissolution, thereby lowering the lactic acid production. Autoclaving could also have a role in breaking down NDFs which may explain little lactic acid production in fermentation of sterilized food waste. The lactic acid concentration reduced to a negligible level at 48 hr in all cases because many gram-positive *Bacillus* species carry highly conserved genes (LutABC operon) for lactate utilization (Chai et al., 2009). Temperatures of 121 °C have been shown to reduce specific amino acids (e.g., lysine)

depending on the length of sterilization (del Cueto et al., 1960), and could have an effect on acid production. Although differences existed in acid production between sterile and non-sterile fermentations, 2,3-BDO production was unaffected.

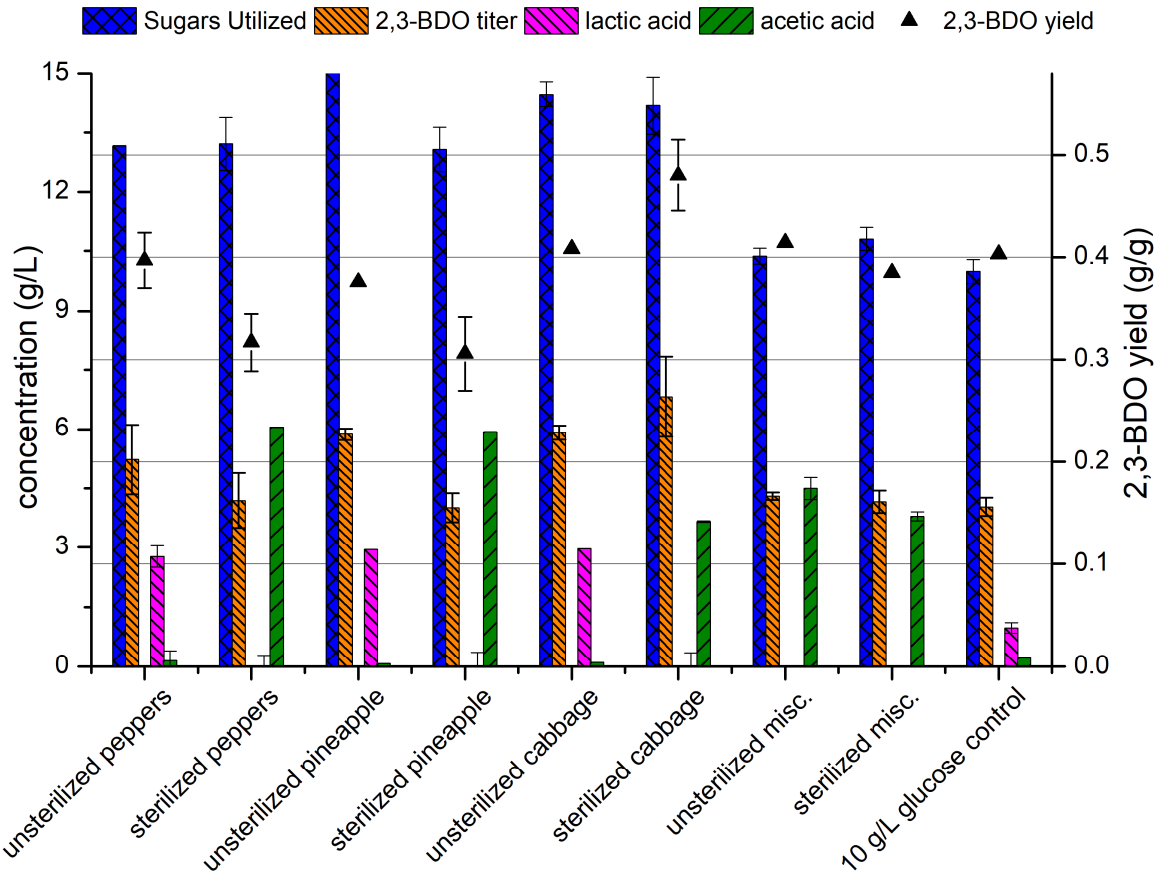

Figure 4. A summary of sterilized and unsterilized food waste fermentation at 24 hrs with *B. licheniformis* YNP5-TSU at 50 °C and initial pH 9.0 to produce 2,3-BDO.

3.4 BDO production from non-sterile fermentation of food waste without nutrient addition

Food waste already contains substantial amounts of protein, vitamins and minerals that might be sufficient to support 2,3-BDO fermentation without addition of yeast extract and peptone. To this end, we further investigated the non-sterile fermentation of food waste using YNP5-TSU without any nutrient addition. As shown in Figure 5, this fermentation

resulted in more variance when compared to the fermentations of food waste with nutrient addition.

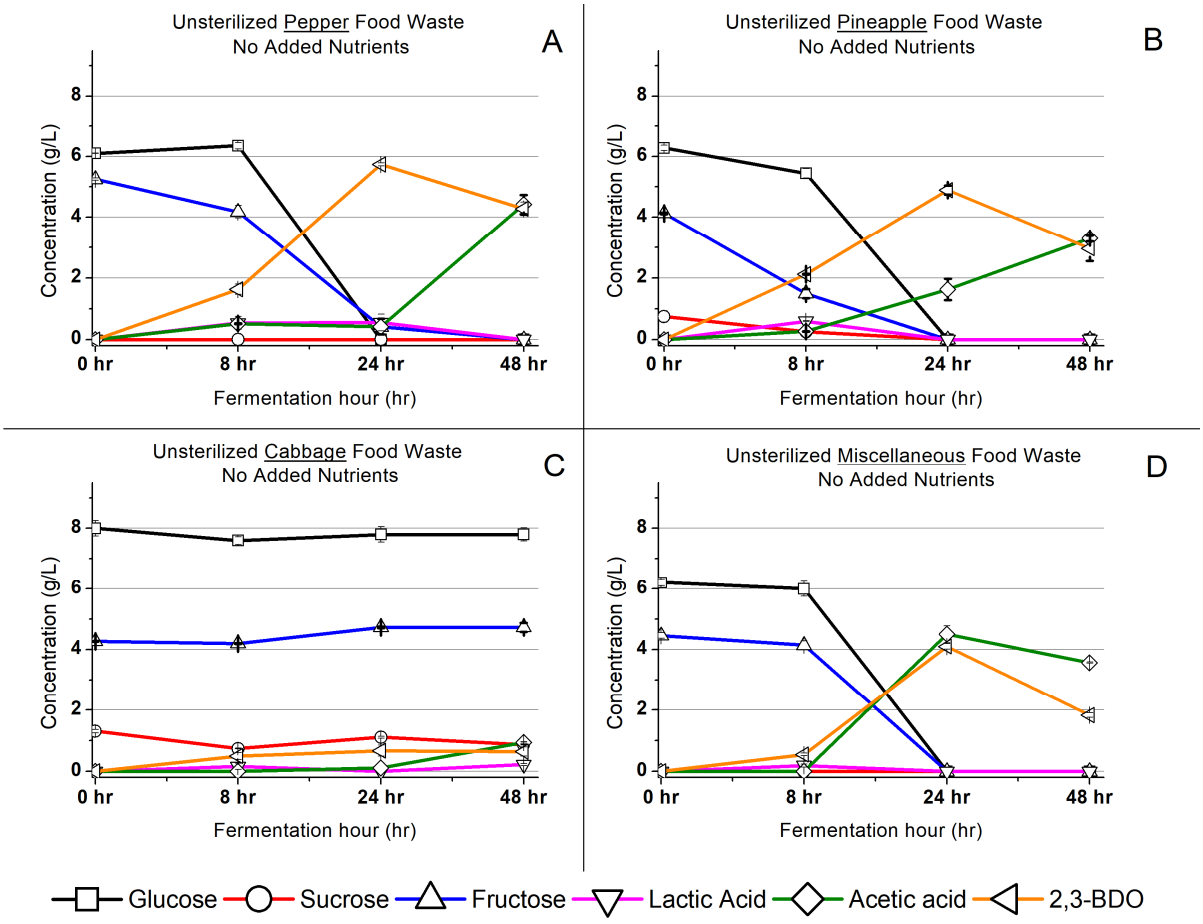

Figure 5. 2,3-BDO production from unsterilized food waste inoculated with *B. licheniformis* YNP5-TSU without external nutrient addition. Homogenized food waste containing 6% solids was added in 100 mL aliquots to baffled flasks. Fermentation was carried out at 50 °C at 150 rpm and an initial pH of 9.0 for 48 hrs.

Fermentations of pepper, pineapple, and miscellaneous wastes successfully produced 5.7, 4.9, and 4.1 g/L 2,3-BDO, respectively, at 24 hrs, corresponding to a yield of 0.49, 0.44 and 0.39 g/g. Pepper waste produced the most 2,3-BDO with the highest yield, while also having the lowest lactic acid concentrations at 24-hrs fermentation. Looking closer at pepper waste, it has the highest percent composition of proteins (17.4%) (Table 1). In the case of cabbage waste, negligible 2,3-BDO (0.67 g/L) was produced and only 1-2 g/L sugars were

385 utilized during the 48 hrs fermentation. While cabbage waste had a protein composition of
386 11.4% (Table 1) it has minimal to nonexistent free amino acids, such as phenylalanine,
387 histidine, and tryptophan, valine and isoleucine, in external and internal leafs (Oliveira et al.,
388 2008). The stringent amino acid requirements by YNP5-TSU may explain the lack of sugar
389 utilization in cabbage waste. Through our previous whole genomic sequencing it was
390 predicted that YNP5-TSU is an auxotrophic organism and requires the amino acids of lysine,
391 phenylalanine, tyrosine, tryptophan, histidine, arginine, isoleucine, leucine, serine, and valine
392 (O'Hair et al., 2019). Since 2,3-BDO production was successful with unsterilized
393 fermentation of cabbage waste supplemented with 0.5 g/L peptone and yeast extract (Figure
394 3), the most likely explanation is cabbage waste itself does not contain all essential amino
395 acids needed for *Bacillus* growth and metabolism. The fermentation of miscellaneous food
396 waste was successful, as all sugars consumed and 4.1 g/L 2,3-BDO produced at 24 hrs.
397 Because the miscellaneous food waste is a mixture of a variety of wastes (potato, pepper,
398 strawberry, tomato, onion, cabbage, and pineapple), it is unlikely that the miscellaneous food
399 waste fermentation will fail due to lack of essential amino acids.

400 Because food waste media was inoculated with 10% (v/v) P2 culture, it was
401 speculated that the residual yeast extract and peptone in the P2 culture, but not the indigenous
402 nutrients in food waste, have supported the 2,3-BDO fermentations. To this end, we have
403 conducted another experiment to remove the residual nutrients from the P2 culture through
404 centrifugation of the culture (2,400 rpm, 5 min), discarding of the supernatant, and
405 resuspension of the cell pellet in food waste media. The prepared resuspended culture was
406 then inoculated to the miscellaneous food waste to start fermentation and the fermentation is

called ‘pellet fermentation’. For comparison purpose, P2 culture without the residual nutrient removal was also directly inoculated to the miscellaneous food waste media to start fermentation and it is called ‘non-pellet fermentation’. The results showed that there is minimal difference between the ‘pellet fermentation’ and ‘non-pellet fermentation’ (Figure 6). The ‘pellet fermentation’ had a similar 2,3-BDO titer (3.72 g/L) and yield (0.35 g/g) when compared to the ‘non-pelleted fermentation’, which had a 2,3-BDO titer of 4.02 g/L and yield of 0.37 g/g at 24 hr. For both the ‘pellet fermentation’ and ‘non-pellet fermentation’, all soluble sugars were consumed and similar amounts of acids (lactic acid and lactic acid) were produced at 24 hours. It was noticeable that, at 8 hrs, the 2,3-BDO concentrations as well as glucose and fructose were different between the ‘pellet’ and ‘non-pellet’ fermentations; however, the differences disappeared at 24 and 48 hrs. Therefore, it is concluded that the indigenous nutrients in food waste is sufficient for the growth and metabolisms of *B. Licheniformis* YNP5-TSU, and no additional nutrients are needed to support the food waste fermentations to produce 2,3-BDO.

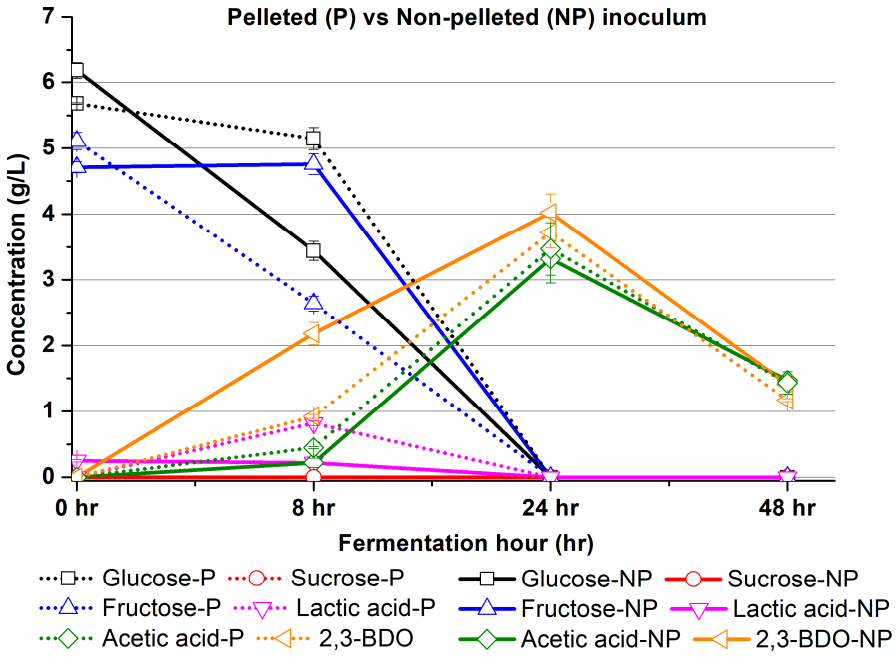

Figure 6. 2,3-BDO production from miscellaneous food waste inoculated with pelleted (P) and non-pelleted (NP) inoculum. Cells were grown at 50 °C, initial pH 9.0, and 150 rpms from a two-stage (P1, P2) stock culture where a 10% inoculum was either pelleted (P) (centrifuged at 2,400 rpm, for 5 min to remove residual nutrients in P2 culture) and resuspended in food waste media or directly added from P2 media (NP). Fermentation was carried out at 50 °C at 150 rpm and an initial pH of 9.0 for 48 hrs.

The microbial production of 2,3-BDO has previously been investigated in several studies using various feedstocks. The maximum yield of 2,3-BDO from batch fermentation of whey waste, cassava hydrolysates, seaweed hydrolysates, glycerol, was 0.47 g/g, 0.30 g/g, 0.43 g/g, and 0.41 g/g, respectively (Kandasamy et al., 2016; Lee and Seo, 2019; Mazumdar et al., 2013; Priya and Lal 2019). The microorganisms used in these studies were all genetically engineered strains of *Escherichia coli*, *Lactococcus lactis, or Saccharomyces cerevisiae*, except in the study by Priya and Lal, 2019, which used a wild-type *Enterobacter cloaca* to produce 2,3-BDO from glycerol. However, in all studies the preferred growth temperature was around 37 °C, and required extra energy to perform autoclave sterilization. Liakou et al. fermented sterilized fruit and vegetable wastes using *Enterobacter ludwigiia* at

30 °C and produced 2,3-BDO with a yield of 0.4 g/g. Other food wastes such as cheese whey powder (CWP), wheat straw hydrolysate (WSH) and sugarcane molasses have also been used as a feedstock for 2,3-BDO fermentation with yields of 0.23 to 0.42 g/g (Alvarez-Guzmán et al., 2020). In Alvarez-Guzmán et al's study, feedstocks were steam sterilized. One of the highest 2,3-BDO yields to date was achieved by Li et al. using *Bacillus licheniformis* X10 to ferment corn stover hydrolysate at 50 ℃. Yields from fed-batch reached 0.47 g/g after 80 hours of fermentation. Other thermophilic 2,3-BDO producers like *B. licheniformis* X10, however, have not been shown to produce these yields in unsterile food waste. To our best knowledge, *B. licheniformis* YNP5-TSU is the first strain to be added to a food waste feedstock without sterilization prior to fermentation. Until this study, heterogeneous food waste had also not been used without nutrient supplementation to produce 2,3-BDO.

4. Conclusions

Our research shows *B. licheniformis* YNP5-TSU is highly suited for the implementation of non-sterile fermentation of food waste to produce 2,3-BDO at thermophilic and alkaline conditions. We have shown that high amounts of indigenous microorganisms were present in raw food waste but were not effective in disrupting the thermophilic and alkaliphilic fermentation using YNP5-TSU. Under the thermophilic and alkaline condition, the fermentation of unsterilized food waste using YNP5-TSU resulted in consistent sugar utilization and 2,3-BDO production compared with fermentation of sterilized food waste. A 2,3-BDO yield of 0.38–0.41 g BDO/g sugar was consistently produced in the fermentation of different unsterilized food wastes. By using miscellaneous food waste, 2,3-

BDO production can be successful without the addition of costly nutrient supplements, which further improves fermentation economics. Conclusions reached from this research will save processing time, reduce energy consumption, and increase 2,3-BDO process profitability.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work is supported by the [UADS-NIFA-AFRI Postdoctoral Fellowship], grant # [2019-67012-29601] project accession no# [1019354] from the U.S. Department of Agriculture, National Institute of Food and Agriculture and the Virginia Agriculture Experiment Station and the Hatch Program of the National Institute of Food and Agriculture (NIFA), USDA. We would like to thank the Yellowstone National Park Service for access and collection of specimens from hydrothermal areas under permit# YELL-2015-SCI-6074 and YELL-2017-SCI-6074. Stacey Gunther, Sarah Haas, and Annie Carlson were invaluable for their help in permit coordination and field sampling guidance at Yellowstone National Park. We would like thank Mrs. Kim Waterman for helping review this manuscript.

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