Dilution rates and their transition modes influence organic acid productivity and bacterial community structure on continuous meta-fermentation using complex microorganisms

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Title: Dilution rates and their transition modes influence organic acid productivity and bacterial community structure on continuous meta-fermentation using complex microorganisms

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Key words: continuous meta-fermentation; complex microorganisms; organic acid; dilution rate; species-specific productivity; bacterial community structure

ABSRACT

We investigated the effect of dilution rates (D) (0.05, 0.15, and 0.4 h^{-1}) and its transition mode strategies (constant, up, and down modes) on organic acid productivity and bacterial community structure on continuous meta-fermentation using complex microorganisms. The number of bacterial species decreased with increasing D in the constant mode while up and down modes maintained high and low values, respectively, regardless of the changing D values. *Caldibacillus hisashii* was the predominant species in all modes at all D values, while other bacterial species, including *Anaerosalibacter bizertensis* and *Clostridium cochlearium* were predominant in only certain modes and D values. The highest total organic acid productivity of 3.16 $gL^{-1}h^{-1}$ was obtained with 82.2% lactic acid selectivity at D = 0.4 h^{-1} in constant mode. Heterofermentation occurred in the up mode, while the down mode exhibited the maximum butyric acid productivity of 0.348 $gL^{-1}h^{-1}$ with 43.8% selectivity at D = 0.05 h^{-1}. The constant, up, and down modes showed the distinct main products of lactic, acetic, formic, and butyric acids, respectively. In this study, we proposed a new parameter of species-specific productivity (SSP) to estimate which species and how much a bacterium

quantitatively contributes to the targeted organic acid productivity in continuous meta-fermentation. SSP was determined based on the abundance of functional genes encoding key enzymes from the results of 16S amplicon analysis. In conclusion, D values and their transition modes affect productivity by changing the bacterial community structure, and are a significant factor in establishing a highly productive process in continuous meta-fermentation.

INTRODUCTION

Organic acids as well as gasoline, alcohol, and ketones are valuable substances which can be used as themselves or their derivatives (1). For example, butyric acid, acetic acid, and their esters are used as flavor components in foods, such as beer and wine in the food industry. Lactic acid has attracted attention as a raw material for biodegradable plastics and as an alternative to petrochemical-derived plastics to alleviate the damage caused by environmental pollution (2–4). Compared with the petrochemical production process, anaerobic fermentation for organic acid production is considered to be a carbon-neutral and eco-friendly method because it can be performed under mild conditions through a single-step process (5) from many types of renewable resources, including pulp and dairy factory wastewater (3,6–8).

Generally, a pure fermentation process using a single microorganism is used for organic acid production (9). Recently, meta-fermentation has been proposed as a process for producing organic acids using complex microorganisms (6). Compared with the pure fermentation process, meta-fermentation has several advantages, such as the utilization of multiple substrates, low risk by contamination, feasible operations under open conditions, and low production costs (7,10). Our

previous studies have clarified that several parameters, such as pH, temperature, and inoculum, are significant factors affecting not only the microbial community structure but also efficiency of organic acid production in meta-fermentations (7,8,11). High-throughput microbial community structure analytical method using next-generation sequencers has been developed and are being applied worldwide, making it possible to elucidate the performance of meta-fermentation based on microbial community structure. (12). However, many studies have focused only on the relationship between fermentation factors and products and fermentation performance (13,14), and few studies have investigated the effects of fermentation factors on the bacterial community structure (15,16). Further research is required to elucidate the relationship among factors, products, and microorganisms to establish technical knowledge and theory for meta-fermentation.

Batch fermentation is generally performed owing to its simplicity, ease of operation, and low risk by contamination. However, batch fermentation has several disadvantages, including low productivity because of the accumulation of inhibitors and nutrient shortages, short operational stability, and additional procedures (ex. preparation of seed culture, washing fermentor, and exchange of new medium and fermentation broth) (17). In contrast, continuous fermentation has several advantages, such as higher productivity because of the dilution of inhibitors in broth, continuous supplementation of nutrients, and longer operational stability than batch fermentation (17). The dilution rate (D), calculated by dividing the medium inflow rate (=efflux rate) by the working volume, is a specific factor for continuous fermentation and can be manually controlled (18). The theory of continuous pure fermentation has been developed as follows: At steady state in continuous

fermentation, the D is known to be equivalent to the specific growth rate, whereas at a higher D than the specific growth rate (μ), the microorganism is washed out from the fermenter (19,20). In addition, an increase in D leads to not only a decrease in the concentration of products and cell density due to the dilution effect, but also an increase in productivity in pure fermentation (21). As mentioned above, D has a significant effect on product concentration, substrate consumption, productivity, and microbial proliferation (22,23).

As many bacterial species with different μ values exist in a broth of continuous meta-fermentation, the D would affect the microbial community structure and its metabolites. Thus, we hypothesized that a wide variety of bacteria with low and high μ proliferate at high cell densities under low D, whereas bacteria with high μ could be maintained in the fermenter at a low cell density under high D. Furthermore, it was hypothesized that a transition mode of D including constant (maintaining D constantly), up (increasing D stepwise), and down (decreasing D stepwise) modes would also affect bacterial community structures irrespective of the same D value, which may result in different performance in terms of metabolites and productivity. To confirm this hypothesis, this study investigated the effects of D and the transition mode of D on bacterial community structure and metabolites during continuous meta-fermentation. Significant knowledge on controlling the bacterial community structure of a targeted product was obtained, and high organic acid productivity with high lactic acid selectivity in continuous meta-fermentation was achieved by optimizing the D values and their transition modes.

MATERIALS AND METHODS

Inoculum and media

The marine-animal-resource compost produced by Japan Eco-science Co., Ltd. (Chiba, Japan) was used as a bacterial seed for continuous meta-fermentations. This compost has been reported to be a stable and useful bacterial seed for organic acid production because of the reproducibility of lactic acid production during batch fermentation, as reported in our previous studies (6,7,24). The production process and bacterial community structure of the compost have been previously reported (24). The compost (3.0 g) was suspended in 50 mL saline water, vortexed for 10 min, filtered with ADVANTEC No.2 filter paper (ADVANTEC, Tokyo) to remove large material, and inoculated with the filtrate containing the bacterial consortium. The medium used for the fresh culture was modified from a previous study and contained the following substances per liter of distilled water (25): 60 g Glucose, 21 g Corn steep solids (SIGMA-ALDRICH, MO, USA), 2 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 2 g NaCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot 5H_2O$, and 0.01 g $FeSO_4 \cdot 7H_2O$. The pH of the media was adjusted to 7.0, and the medium was sterilized in an autoclave at 121°C for 20 min.

Fermentation operation

A continuously stirred tank fermenter (1 L) with a working volume of 400 mL was used and the inoculum (50 mL) was added to 350 mL of sterilized medium in the fermenter. After inoculation, the medium was aerated with filtered oxygen-free nitrogen gas for 30 min to maintain strict anaerobic conditions, and the vents of the fermenter were closed. Meta-fermentation was performed at 50°C, with an agitation rate of 200 rpm. Batch fermentation was conducted for 24 h, and then switched to

continuous fermentation. The pH was adjusted to 7.0 every 6 h during batch fermentation according to our previous study (7) and then controlled constantly at 7.0, after switching to continuous fermentation.

A preliminary experiment showed different behaviors of main products and predominant bacterial species at D of 0.05, 0.15, and 0.4 h^{-1},and we, therefore, focused on these D values in this study. In a continuous fermentation, D values were set *via* the following three patterns: (I) constant mode: D of 0.05, 0.15, and 0.4 h^{-1} were carried out individually, (II) up mode: D was set at 0.05 h^{-1} and then increased to 0.15 and 0.4 h^{-1} step by step, and (III) down mode: D was set at 0.4 h^{-1} and then decreased to 0.15 and 0.05 h^{-1} step by step. With the up and down modes, D were changed to the subsequent D values after the steady state was considered to have been achieved at least three retention times (26).

Measurements of metabolites and substrates

Organic acids (citric acid, malic acid, pyruvic acid, succinic acid, propionic acid, lactic acid, acetic acid, butyric acid, and formic acid) in the broth supernatant were determined using a specific HPLC system (Organic Acid Analyzer; Shimadzu, Kyoto, Japan) equipped with an ion-exclusion column (Shim-pack SCR- 102H; Shimadzu) at 40°C and an electric conductivity detector (CDD-10AVP; Shimadzu). Mobile phases A (5 mM p-toluenesulfonic acid) and B (20 mM bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane, 5 mM p-toluenesulfonic acid, and 100 μM EDTA) were used, each at a flow rate of 0.8 mL min^{-1}. The glucose concentration was determined using a biosensor (BF-7, Oji Scientific Instrument, Hyogo, Japan). Metabolites and substrates were measured

in triplicate from different samples in steady state at the same D value.

Quantification of bacterial 16S rRNA gene copy number

Quantitative PCR (qPCR) was performed to determine the bacterial density in the fermentation broth. Total DNA was extracted from a 1 mL sample using a PowerSoil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The bacterial 16S rRNA gene copy number in the DNA extracts was quantified using a real-time PCR thermal cycler (CFX Connect System, Bio-Rad, CA, USA) with a universal primer set (357F [5' -CCTACG GGA GGC AGC AG-3'] and 518R [5' -ATT ACC GCG GCT GCT GG-3']) for a portion of the bacterial 16S rRNA gene. The qPCR mixture and cycling conditions were as previously described (27). The 16S rRNA gene was calculated as the copy number/mL of the fermentation broth.

Analysis of bacterial community structure and prediction of functional genes of predominant species

To analyze bacterial community structure, 16S rRNA gene amplicon analysis was performed using a next-generation sequencer. Two-stage PCR was performed to prepare the library for application to an Illumina MiSeq instrument. The partial 16S rRNA gene (V4 region) was targeted as described previously (28). In the first-stage PCR, a universal primer set with a universal primer region for the 16S rRNA gene and tailed sequences for the MiSeq instrument were used:1-515F, 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GTG CCA GCM GCC GCG GTA A-3'; 1-806R, 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGG ACT ACH VGG GTW TCT AAT-3'. In the second-stage PCR, a primer set with flow cell adapter sequences, index sequences,

and tailed sequences was used (forward primer, 5'-AAT GAT ACG GCG ACC ACC GAG ATC TACAC-Index sequence-TCG TCG GCA GCG TC-3'; reverse primer, 5'-CAA GCA GAA GACGGC ATA CGA GAT-Index sequence-GTC TCG TGG GCT CGG-3'). Bacterial community structures were analyzed from different samples under steady-state conditions at each D, at least in triplicate, except for the down mode. Bioinformatics analysis of the sequence data was performed using and QIIME 1.9.1 and QIIME2 (29). Phylogenetic investigation of communities by reconstruction of unobserved state 2 (PICRUSt2) (30) was used to count the number of functional genes (L-lactate dehydrogenase, butyrate kinase, acetate kinase, and formate C-acetyltransferase) possessed by a targeted and all bacterial species, which are the predominant species encoded using the calculations shown below.

Calculation

The following equation was used to calculate the organic acids volumetric productivity:

Volumetric productivity ($g\ L^{-1}\ h^{-1}$) = C × D

where C is the organic acid concentration ($g\ L^{-1}$) and D is the dilution rate (h^{-1}).

The species-specific productivity (SSP) was calculated as follows:

SSP ($g\ L^{-1}\ h^{-1}$) = C × D ×

(Number of functional genes possessed by the targeted bacterium)

/ (Number of functional genes possessed by all bacteria)

Selectivity for each organic acid (S) was calculated as follows:

$S\ (\%) = C_{OA}/(C_{LA}+C_{BA}+C_{AA}+C_{FA}) \times 100$

where $C_{OA,}$ $C_{LA,}$ C_{AA}, C_{BA}, and C_{FA}, are the respective concentrations (g L^{-1}) of targeted organic acid, lactic acid, butyric acid, acetic acid, and formic acid produced, respectively.

Accession number

Illumina raw read sequences were deposited under BioProject ID PRJDB11604 with accession numbers DRA015775 from the DNA Data Bank of Japan.

RESULTS

Continuous meta-fermentations rate with a constant mode strategy of dilution rate

The continuous meta-fermentations were conducted with a constant mode at D = 0.05, 0.15, and 0.40 h^{-1}, individually. The bacterial copy number and bacterial community structure were determined by qPCR and 16S rRNA gene amplicon analysis, respectively, as shown in Fig. 1. The batch fermentation after 24 h exhibited 9.7 of observed species on average and 1.2×10^7 copies mL^{-1}. In the continuous fermentations, values of observed species (19.7, 11.8, and 8.6, respectively) decreased with increasing D (0.05, 0.15, and 0.4 h^{-1}, respectively). In contrast, bacterial copy numbers were almost constant in the range of 1.3×10^7 to 8.0×10^7 mL^{-1} regardless of the D in the continuous fermentation. In batch fermentation, *Caldibacillus hisashii* (56.2%) and *Weizmannia coagulans* (42.7%) were the predominant species. The relative abundances of *C. hisashii* in continuous fermentation increased from 37.5% to 51.7% and 89.3% with increasing D from 0.05 h^{-1} to 0.15 h^{-1} and 0.4 h^{-1}, respectively. However, after initiation of continuous fermentation, the relative abundances of *W. coagulans* drastically decreased to 1.4% at D of 0.05 h^{-1} from 42.7% in batch fermentation for

24 h, and then gradually increased to 8.1% and 10.6% at D of 0.15 and 0.4 h^{-1}, respectively. Besides these two species, *Anaerosalibacter bizertensis* was predominant (38.6%) at D of 0.05 h^{-1} and *Clostridium cochlearium* (29.6%) at D of 0.15 h^{-1}. These results indicate that continuous fermentation would exhibit different bacterial community structures and bacterial diversity from batch fermentation, and that the D value would be a significant factor in determining these factors.

In batch fermentation, lactic acid (6.29 g L^{-1}) was mainly produced with the productivity of 0.262 g L^{-1} h^{-1} and the selectivity of 81.8%, and small amounts of acetic acid (0.617 g L^{-1}) and formic acid (0.746 g L^{-1}) were produced (Table 1). *C. hisashii* was considered to produce lactic acid, acetic acid, and formic acid, while *W. coagulans* would contribute to lactic acid production homofermentatively. In continuous fermentation, the concentrations of consumed glucose and produced total organic acids decreased with increasing D, which suggests that a lower D can lead to higher organic acid conversions because of the longer contact time between the substrate and the microorganisms. Moreover, 3.16 g L^{-1} h^{-1} of total acid productivity at D = 0.4 h^{-1} would be 9.9 times higher than 0.320 g L^{-1} h^{-1} in batch fermentation. Lactic acid was the most produced organic acids at all D, and 10.2, 12.2, and 6.60 g L^{-1} of lactic acid was produced at D = 0.05, 0.15, and 0.4 h^{-1}, respectively. Lactic acid selectivity (48.5–82.2%) and lactic acid productivity (0.510–2.64 g L^{-1} h^{-1}) increased with increasing D. At D = 0.4 h^{-1}, no butyric acid was produced similar to batch fermentation, while butyric acid was the second highest metabolite at D = 0.05 h^{-1}. It was produced at a concentration of 4.32 g L^{-1} with 20.5% selectivity and 0.216 g L^{-1} h^{-1} of productivity, and it would result from the function of the closest strain to *C. cochlearium*. These results suggest that continuous

meta-fermentations with a constant mode strategy would show higher productivity than batch fermentation, and that the D would affect not only bacterial community structures, but also type of metabolites and productivity.

Continuous meta-fermentations with an up mode strategy of dilution rate

After batch fermentation, continuous fermentation was initiated with an up mode strategy while increasing D from 0.05 h^{-1} to 0.15 and 0.4 h^{-1}. Figure 2 shows the results of the bacterial copy number and bacterial community structure using the up mode strategy. The number of observed species at D = 0.05 h^{-1} was 19.7, while 18.0 and 20.5 species were observed at D = 0.15 and 0.4 h^{-1}, respectively, which was much higher than those observed in the constant mode strategy (11.8 and 8.6, respectively) (Figs. 1A and 2A). In addition, the bacterial copy number was constant at the level of 10^7 copies/mL, similar to that obtained using the constant mode strategy. If several bacterial species were washed out at high D values, the observed species and copy numbers would decrease. These results suggest that several bacterial species would acclimatize to the conditions required for proliferation while maintaining the bacterial copy numbers at a high D using the up mode strategy. With the up mode strategy, *C. hisashii* was the predominant species at relatively constant abundances of 37.5-48.9% at the three D, while the relative abundances of *W. coagulans* were less than 1% in continuous fermentation. *A. bizertensis* was also predominant species with 38.6% at D = 0.05 h^{-1}, but drastically decreased to 2.7% and 8.7% with increasing D to 0.15 and 0.4 h^{-1}, respectively. Interestingly, the relative abundances of *X. thermophila*, which were not the predominant species with a constant mode

247 strategy, were 45.9% and 34.0% at D = 0.15 and 0.4 h^{-1}, respectively. Gradually increasing D with
248 the up mode strategy was found to be a new method to change the bacterial community structure
249 differently from the constant mode strategy.

250 Table 1 shows the production and consumption in continuous meta-fermentation at several D
251 using the up mode strategy. Concentrations of produced total organic acid (21.0, 12.2, and 6.74 g L^{-1})
252 and consumed glucose (43.4, 29.0, and 13.7 g L^{-1}) decreased but total organic acid productivities
253 (1.05, 1.83, and 2.69 g L^{-1} h^{-1}) increased with increasing D. Compared with the constant mode strategy,
254 the up mode strategy showed greater tendency to produce organic acids heterofermentatively,
255 depending on the D as follows: 0.05 h^{-1}, 10.2 g L^{-1} lactic acid (48.5%) and 4.32 g L^{-1} butyric acid
256 (20.5%); 0.15 h^{-1}, 4.12 g L^{-1} acetic acid (33.8%) and 3.87 g L^{-1} formic acid (31.8%); 0.4 h^{-1}, 2.31 g
257 L^{-1} lactic acid (34.2%) and 2.17 g L^{-1} (32.1%). These results indicate that the gradual change in the
258 D is a new strategy to modify the bacterial community structure and metabolite patterns in continuous
259 culture.

260

261 **Continuous meta-fermentations with a down mode strategy of dilution rate**

262 After batch fermentation, continuous fermentation was initiated with D of 0.4 h^{-1}, followed by
263 decreasing to 0.15 and 0.05 h^{-1}, called the down mode strategy. Figure 3 shows the results of the
264 bacterial copy number and bacterial community structure using the down mode strategy. The number
265 of observed species were relatively maintained at low levels of 8.6–11.1 at D = 0.05–0.4 h^{-1}, which
266 were much lower than 18.0–20.5 with up mode strategy (Fig. 2A). However, the bacterial copy
267 numbers were constant at approximately 10^7 copies/mL, similar to those obtained with the constant

mode and up mode strategies. These results suggest that the bacterial species with a low specific growth rate would be washed out at the initial D = 0.4 h^{-1}, and then would disappear at low D, which resulted in low diversity of bacterial species. In fact, at D = 0.05 h^{-1}, several bacterial species, including *Schnuerera ultunensis* observed in the up mode, were not observed in down mode.

C. hisashii was predominant with relative abundances of 60.6–89.3% at D = 0.05–0.4 h^{-1}, while the relative abundance of *W. coagulans* decreased from 10.6% to 0.43–3.91% with decreasing D from 0.4 h^{-1} to 0.15 and 0.05 h^{-1}. In contrast, *C. cochlearium* became the predominant species with 34.0% relative abundance after decreasing D to 0.05 h^{-1} with down mode strategy, though *C. cochlearium* was a minor species at 0.05 h^{-1} with the constant and up mode strategies. These results indicate that not only the D value but also the D transition affected the bacterial community structure as well as the up mode strategy.

Table 1 shows the production and consumption of meta-continuous fermentation using the down mode strategy. Concentration of produced total organic acids and consumed glucose increased from 7.90 g L^{-1} to 15.6 and 15.9 g L^{-1} and from 16.9 g L^{-1} to 32.3 and 45.3 g L^{-1} with decreasing D from 0.4 h^{-1} to 0.15 and 0.05 h^{-1}, respectively. On the other hand, total organic acid productivities (3.16, 2.35, and 0.785 g L^{-1} h^{-1}) decreased with decreasing D. Contrary to the up mode strategy, the down mode strategy showed less tendency to produce organic acid heterofermentatively, depending on D as follows:0.4 h^{-1}, 6.60 g L^{-1} lactic acid (82.2%); 0.15 h^{-1}, 8.95 g L^{-1} lactic acid (57.6%); 0.05 h^{-1}, 6.96 g L^{-1} butyric acid (43.8%). It was notable that the highest butyric acid concentration (6.96 g L^{-1}) and productivity (0.348 g L^{-1} h^{-1}) were obtained at D = 0.05 h^{-1} with the down mode strategy among all

the modes. Thus, these results indicate that the down mode strategy by initiating a high D would have a positive effect on continuous meta-fermentation because it prevents hetero-fermentation by decreasing the number of bacterial species.

Proposal of new parameter to estimate species-specific productivity in continuous meta-fermentation

Although which and how much a bacterial species would contribute to the productivity of a certain metabolite should be considered, there is no parameter to quantitatively estimate the productivity related to a targeted bacterial species in meta-fermentation. In this study, species-specific productivity (SSP) was newly proposed by multiplying the volumetric productivity by the relative abundance of functional genes encoding the key enzymes (lactate dehydrogenase, butyrate kinase, acetate kinase, and formate C-acetyltransferase) distributed from the targeted bacterial species estimated by PICRUSt2. Figure 4 shows the SSP for lactic acid (SSP_{LA}), butyric acid (SSP_{BA}), acetic acid (SSP_{AA}), and formic acid (SSP_{FA}). The SSP_{LA} of *W. coagulans* (0.146 g L^{-1} h^{-1}) was higher than that of *C. hisashii* (0.111 g L^{-1} h^{-1}) during batch fermentation, which suggested more contribution of *W. coagulans* to lactic acid production. In continuous meta-fermentation, 2.34 g L^{-1} h^{-1} SSP_{LA} of *C. hisashii* was the highest at D = 0.4 h^{-1} with constant and down mode strategies among all the runs; this SSP_{LA} was ca. 21 times higher than that in batch meta-fermentation, and SSP_{LA} of *C. hisashii* dominated more than 80% of volumetric lactic acid productivities at all D with all strategies. In contrast, the maximum SSP_{LA} of *W. coagulans* (0.297 g L^{-1} h^{-1}) was obtained at D = 0.4 h^{-1} with up mode strategy. These results suggest that *C. hisashii* may be contributing to lactic acid production

during continuous meta-fermentation.

Bacterial species and their contributions to butyric acid productivity depend on the D and mode strategies. With the constant mode strategy, 0.171 g L^{-1} h^{-1} SSP_{BA} of *A. bizertensis* and 0.115 g L^{-1} h^{-1} SSP_{BA} of *C. cochlearium* were the highest at D = 0.05 and 0.15 h^{-1}, respectively. In addition, the SSP_{BA} of *A. bizertensis* and *C. cochlearium* were the highest among the up mode and down mode strategies, respectively. Among these, 0.304 g L^{-1} h^{-1} of *C. cochlearium* was the largest SSP_{BA} at D = 0.05 h^{-1} with the down mode strategy, which was estimated to contribute 87% of the volumetric butyric acid productivity. These results indicated that *C. cochlearium* is useful for butyric acid production during continuous meta-fermentation. Similar bacterial species contributed to the production of acetic acid and formic acid in continuous meta-fermentation. The SSP_{AA} and SSP_{FA} of *C. hisashii* dominated ca. 50% and over of volumetric acetic acid and formic acid productivities at all D with all strategies. With only the up mode strategy, *X. thermophila* showed 0.265 g L^{-1} h^{-1} and 0.227 g L^{-1} h^{-1} SSP_{AA} and 0.214 g L^{-1} h^{-1} and 0.254 g L^{-1} h^{-1} SSP_{FA} at D = 0.15 h^{-1} and 0.4 h^{-1}, respectively. Thus, the parameter of SSP would be valuable to estimate a contribution to the productivities of a targeted bacterial species qualitatively, and their contributions would be dependent on not only D but also mode strategies.

DISCUSSION

Meta-fermentation is a sustainable process for the production of valuable organic acids because of its wide substrate utilization from renewable resources. Our previous studies on batch meta-fermentation have elucidated that factors, such as pH, temperature, and inoculum are significant in

controlling fermentation performance (6,7,26). However, little is known about continuous meta-fermentation compared to pure fermentation. Most studies have investigated the effects of factors, such as temperature and pH on the production performance (type of produced metabolites, concentration, and productivity) and only a few studies have evaluated them based on bacterial communities (Table 2) ; nevertheless, production performance affect the bacterial community and vice versa (32). In this study, the effects of D and their transition modes on production performance and bacterial community structure in continuous meta-fermentation were elucidated in detail.

Based on the theory of continuous pure fermentation that the D is equivalent to the specific growth rate (μ), and the following hypothesis was generated in continuous meta-fermentation: increasing the D would decrease the number of bacterial species (observed species) by washing out bacterial species with low μ, which would lead to a change in metabolites. Increasing D from 0.05 h^{-1} to 0.4 h^{-1} in continuous meta-fermentations with the constant mode strategy decreased the observed species from 19.7 to 8.6 (Fig. 1A) and increased lactic acid selectivity from 48.0% to 83.3% (Table 1), which supported this hypothesis. The predominant species of *W. coagulans* and *C. hisashii* at D = 0.4 h^{-1} were reported to show high μ of ca. 0.4 h^{-1} (31), while the closest species related to *A. bizertensis* and *C. cochlearium* with the μ of ca. 0.2 h^{-1} and 0.25 h^{-1} can be predominant at D = 0.05 and 0.15 h^{-1}, respectively, but would be washed-out at D = 0.4 h^{-1} (28,29). Regardless of high μ of ca. 0.4 h^{-1}, *W. coagulans* was minor species at D = 0.05 and 0.15 h^{-1} (Fig. 1B). These results indicated that D alone (equivalent to the μ of a bacterial species) could not determine the bacterial community structure and metabolites, and that another factor would be related to the relative abundance of a

bacterial species in continuous meta-fermentation.

In this study, the transition mode of the D in continuous meta-fermentation affected the bacterial community structure and their metabolites. The up mode strategy maintained the observed species at high values of 18.0–20.5 with D ranging from 0.05 to 0.4 h^{-1}, while relatively low values of 8.6–11.1 were stably obtained with the down mode strategy (Figs. 2A and 3A). Furthermore, the predominant bacterial species differed at the same D between the up mode and down mode strategies (Figs. 2B and 3B), which resulted in different metabolites and productivities (Table 1). Based on the theory of $D=\mu$ in pure continuous fermentation, the down mode strategy at high D washed out bacterial species with low μ, and then bacterial species with high μ proliferated even at low D. Although there are only a few reports on the investigation of μ between up mode and down mode strategies in continuous meta-fermentation, this study suggested that bacterial species with low μ would be adapted at high D with the up mode strategy. The highest butyric acid production (concentration, 6.96 g L^{-1}; selectivity, 43.8%) was achieved at D = 0.05 h^{-1} with down mode strategy with the highest relative abundance (34.0%) of *C. cochlearium* among all the transition modes tested (Table 1, Fig. 3B). Thus, it is possible that optimization of the transition mode of D would select the production of targeted metabolites by increasing the related bacterial species in continuous meta-fermentation.

To date, several parameters related to productivity, including volumetric productivity and specific productivity in pure fermentation, have been used to evaluate fermentation performance (33,34). These parameters can be used in continuous meta-fermentation as the overall productivity is related to all microorganisms. Although it is important to estimate which and how much bacterial species

would contribute to production in continuous meta-fermentation, there is no report on the development of this parameter. In this study, we proposed SSP and indicated a qualitative contribution to the productivity of targeted metabolites related to the bacterial species. SSP has the advantage of not requiring additional experiments to estimate the quantitative contribution simply because of the use of 16S amplicon analysis data, which would be a useful parameter for continuous meta-fermentation as well as batch and fed-batch meta-fermentations. In contrast, two disadvantages of SSP should be considered. First, a contribution would be overestimated or underestimated for a bacterial species with multiple copies (high or low number, respectively) of the 16S rRNA genes compared with a bacterial species with one copy number. Second, SSP values may be overestimated or underestimated in bacterial species with low or high expression levels of target genes and low or high enzymatic activities, respectively. To the best of our knowledge, a direct analytical method to measure quantitatively measure the contribution of organic acid productivity related to specific bacterial species has not yet been established; therefore, an evaluation of the validity of this parameter is required, and another useful parameter should be proposed.

Continuous meta-fermentation for organic acid production has been reported in the literature (Table 2) using several substrates and inoculums under several conditions, including temperature, pH, and D. Most studies performed lower D of <0.1667 h^{-1} except for 0.5 h^{-1} reported by Kumar et al., 2016 (16). The organic acid productivity of 3.16 g $L^{-1}h^{-1}$ at high D = 0.4 h^{-1}, in this study, was much higher than those (0.01325–1.5 g $L^{-1}h^{-1}$) at D<0.1667 h^{-1}, and very similar with 3.29 $L^{-1}h^{-1}$ at D = 0.5 h^{-1}. These results suggest that an increase in D would improve productivity in continuous meta-

fermentation.

Microbial engineering using a single microorganism has been systematically developed; however, theoretical and technical knowledge is insufficient in the new field of ‘Complex Microbial Engineering’ using several microorganisms. This study demonstrated the effect of D values and their transition mode on not only production performance (type of metabolites, concentration, and productivity) but also bacterial community structure using 16S amplicon analysis in detail in continuous meta-fermentation. However, the selectivity of the target metabolite remains to be improved, and further research is needed to establish an efficient meta-fermentation process by elucidating another factor that determines the predominant bacterial species and their metabolites in continuous meta-fermentation.

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The authors declare no conflict of interest.

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Figure legends

Figure 1. Number of observed species and bacterial copy number (A) and relative abundance of bacterial species (B) in continuous meta-fermentation at 3 D values with the constant mode.

Figure 2. Number of observed species and bacterial copy number (A) and relative abundance of bacterial species (B) in continuous meta-fermentation at 3 D values with the up mode.

Figure 3. Number of observed species and bacterial copy number (A) and relative abundance of bacterial species (B) in continuous meta-fermentation at 3 D values with the down mode.

Figure 4. Species specific productivity of lactic acid (PSS_{LA}) (A), butyric acid (PSS_{BA}) (B), acetic acid (PSS_{AA}) (C), and formic acid (PSS_{FA}) (D) in continuous meta-fermentations with 3 modes.

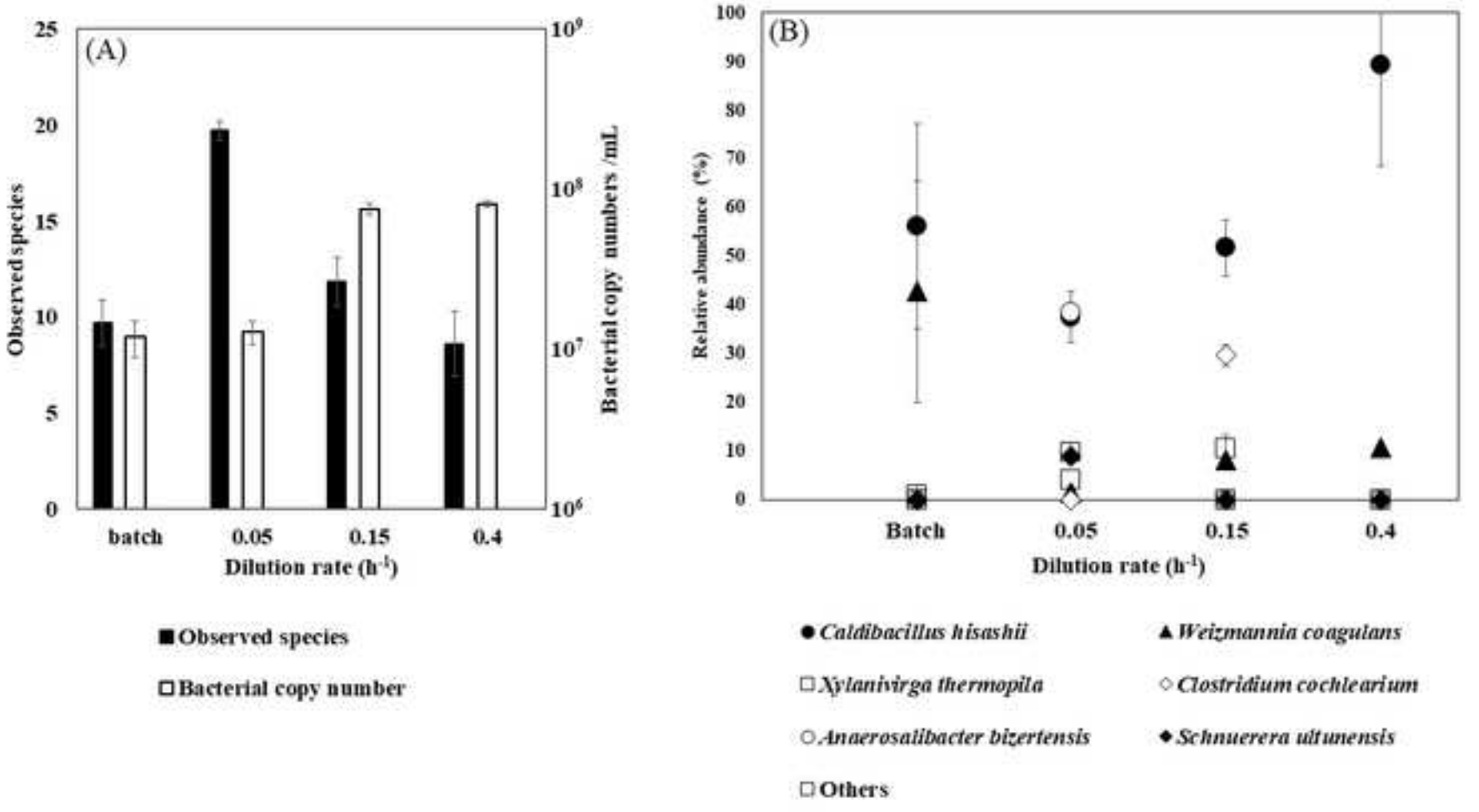
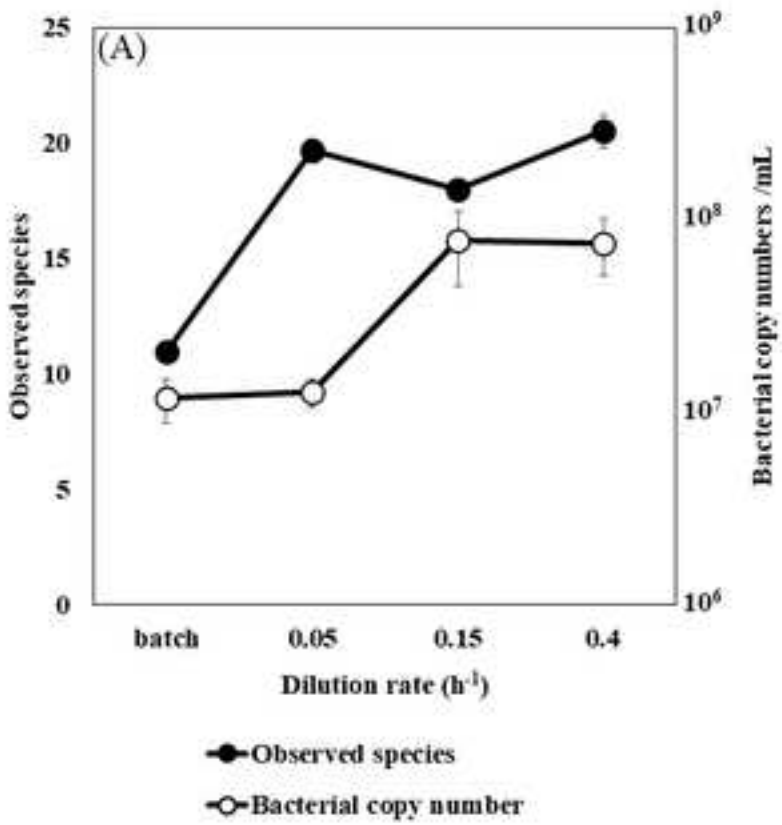


Fig. 1, Koga et al.



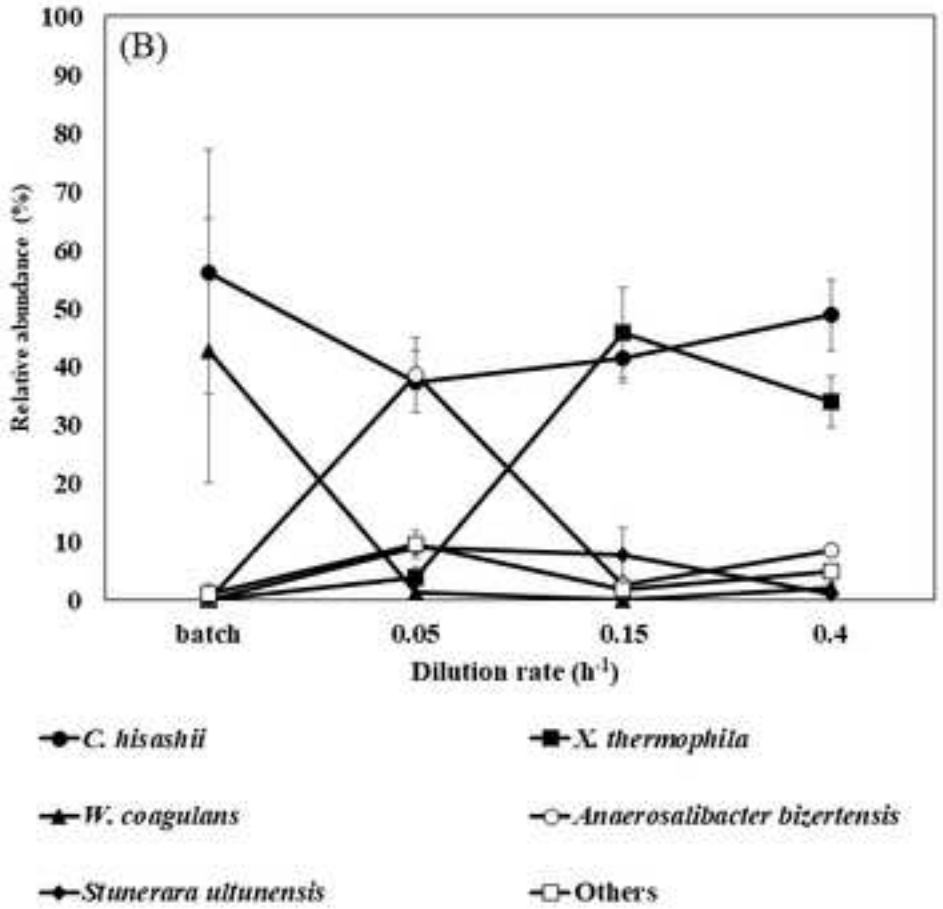


Fig. 2, Koga et al.

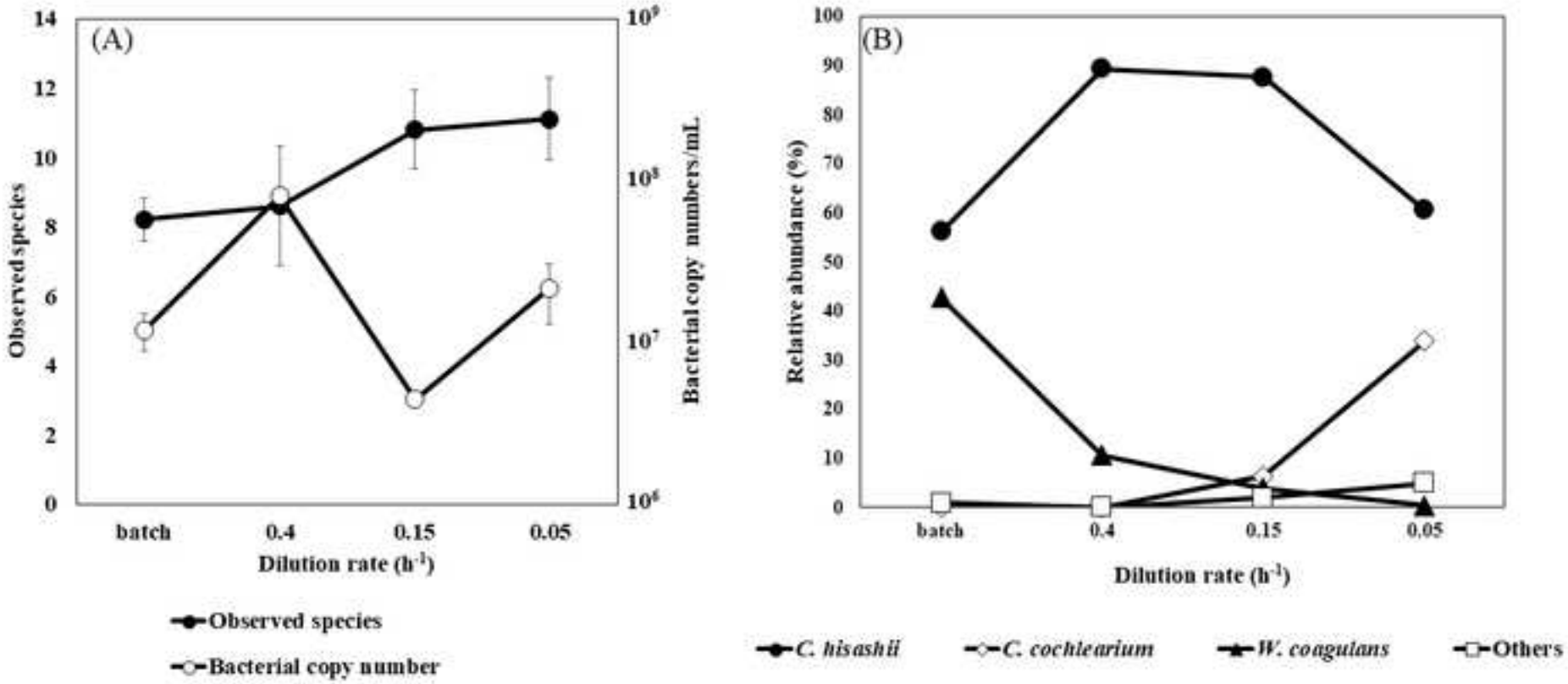
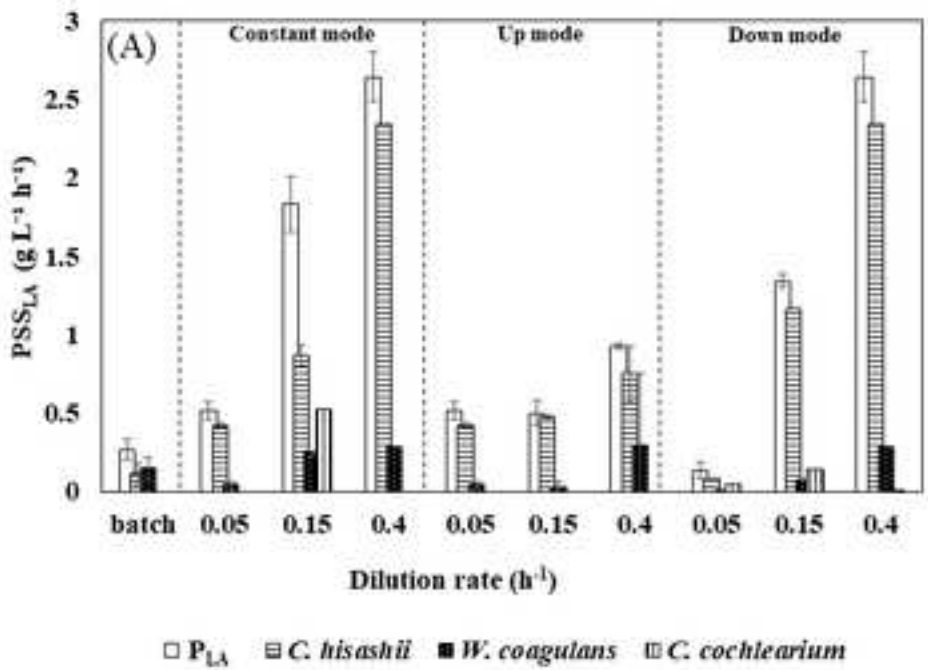


Fig. 3, Koga et al.



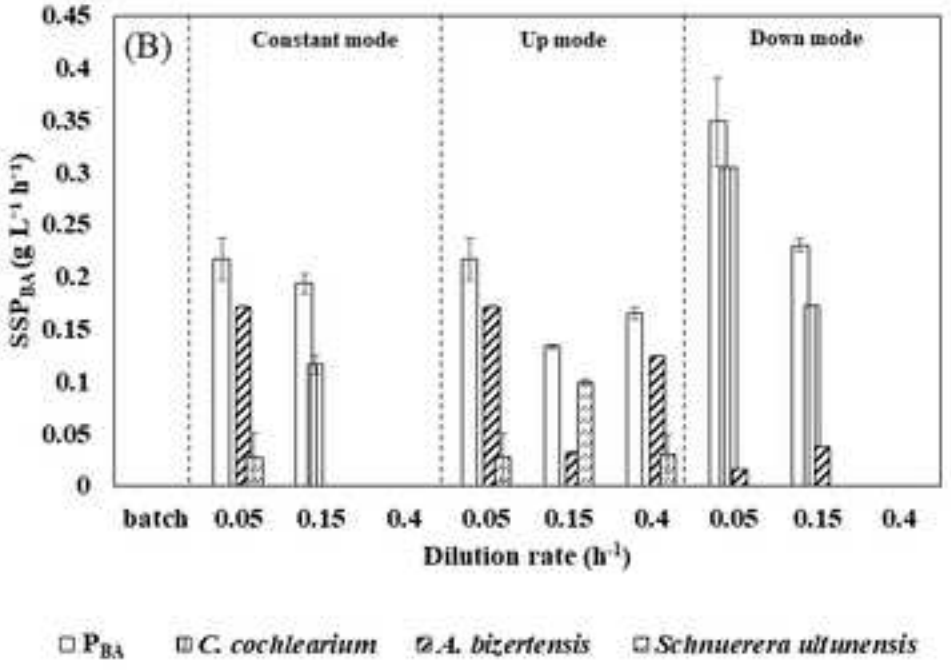
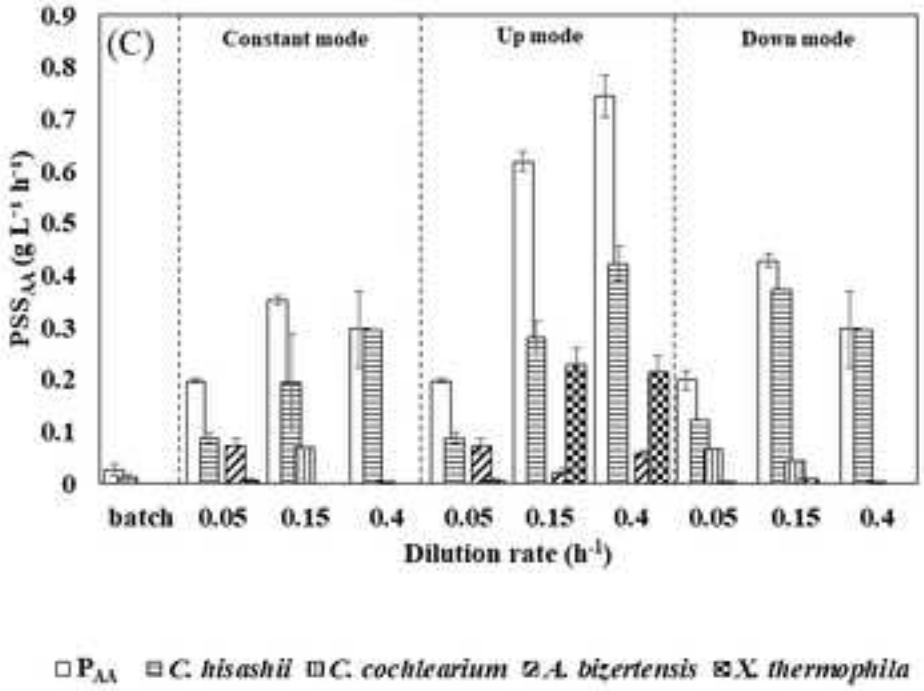


Fig. 4, Koga et al.



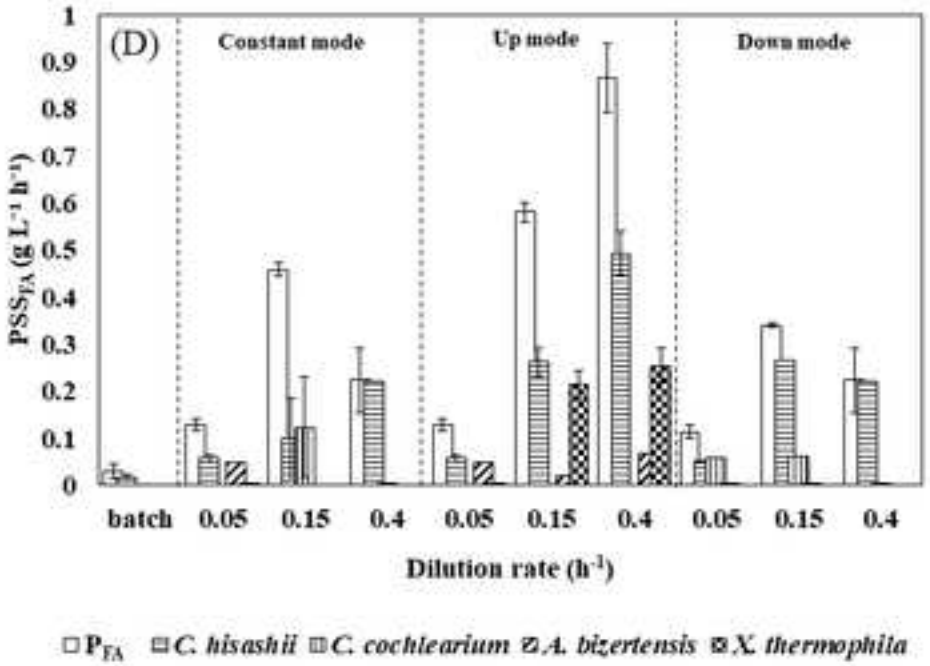


Fig. 4, Koga et al.

Table 1. Organic acid production in continuous meta-fermentation with constant, up and down mode strategies at the different dilution rates and 50°C

Transition modes	D (h^{-1})	Lactic acid		Butyric acid		Acetic acid		Formic acid		C_{TA} (g L^{-1})	P_{TA} (g L^{-1} h^{-1})	P_{LA} (g L^{-1} h^{-1})	P_{BA} (g L^{-1} h^{-1})	C_{GLC} (g L^{-1})
		C_{LA} (g L^{-1})	S_{LA} (%)	C_{BA} (g L^{-1})	S_{BA} (%)	C_{AA} (g L^{-1})	S_{AA} (%)	C_{FA} (g L^{-1})	S_{FA} (%)					
Batch		6.29 ±0.26	81.8	0.00 ±0.00	0.00	0.617 ±0.236	8.02	0.746 ±0.333	9.70	7.69 ±0.32	0.320 ±0.057	0.262 ±0.065	0.00 ±0.00	12.7 ±2.3
Constant	0.05	10.2 ±1.1	48.5	4.32 ±0.40	20.5	3.94 ±0.06	18.7	2.59 ±0.28	12.3	21.0 ±1.0	1.06 ±0.05	0.510 ±0.056	0.216 ±0.020	43.4 ±1.3
	0.15	12.2 ±1.2	64.6	1.29 ±0.06	6.82	2.35 ±0.06	12.4	3.06 ±0.10	16.2	18.9 ±1.3	2.84 ±0.19	1.83 ±0.18	0.193 ±0.009	30.3 ±1.8
	0.4	6.60 ±0.39	82.2	0.00 ±0.00	0.00	0.740 ±0.185	9.36	0.560 ±0.170	7.93	7.90 ±0.56	3.16 ±0.22	2.64 ±0.16	0.00 ±0.00	16.9 ±2.9
Up	0.05	10.2 ±1.1	48.5	4.32 ±0.40	20.5	3.94 ±0.06	18.7	2.59 ±0.28	12.3	21.0 ±1.0	1.05 ±0.05	0.510 ±0.056	0.216 ±0.020	43.4 ±1.3
	0.15	3.31 ±0.54	27.2	0.883 ±0.015	7.25	4.12 ±0.13	33.8	3.87 ±0.13	31.8	12.2 ±0.5	1.83 ±0.08	0.497 ±0.080	0.133 ±0.002	29.0 ± 1.2
	0.4	2.31 ±0.04	34.2	0.413 ±0.015	6.12	1.86 ±0.10	27.6	2.17 ±0.19	32.1	6.74 ±0.33	2.69 ±0.13	0.923 ±0.016	0.165 ±0.006	13.7 ±1.6
Down	0.05	2.69 ±1.05	16.9	6.96 ±0.84	43.8	3.95 ±0.37	24.9	2.28 ±0.27	14.5	15.9 ±1.5	0.785 ±0.076	0.134 ±0.052	0.348 ±0.042	45.3 ±0.5
	0.15	8.95 ±0.26	57.6	1.53 ±0.05	9.81	2.85 ±0.08	18.3	2.27 ±0.04	14.5	15.6 ±0.2	2.35 ±0.03	1.34 ±0.04	0.230 ±0.007	32.3 ±0.5

0.4	6.60 ±0.39	82.2	0.00 ±0.00	0.00	0.740 ±0.185	9.36	0.560 ±0.170	7.93	7.90 ±0.56	3.16 ±0.22	2.64 ±0.16	0.00 ±0.00	16.9 ±2.9

D, dilution rate; C_{LA}, lactic acid concentration; S_{LA}, lactic acid selectivity; C_{BA}, butyric acid concentration; S_{BA}, butyric acid selectivity; C_{AA}, acetic acid concentration; S_{AA}, acetic acid selectivity; C_{FA}, formic acid concentration; S_{FA}, formic acid selectivity; C_{TA}, total organic acid concentration; P_{TA}, total organic acid productivity; C_{GLC}, Consumed glucose concentration.(Batch fermentation: n=5, Continuous fermentation: n=3)

Table 2. Factors affecting organic acids production, productivity, and predominant bacterial species in continuous meta-fermentation in literatures

Investigated factors	D (h^{-1})	Temp. (°C)	pH	Seed	Substrate	C_{LA} ($g\ L^{-1}$)	C_{BA} ($g\ L^{-1}$)	C_{AA} ($g\ L^{-1}$)	C_{FA} ($g\ L^{-1}$)	C_{EtOH} ($g\ L^{-1}$)	C_{TA} ($g\ L^{-1}$)	P_{TA} ($g\ L^{-1}\ h^{-1}$)	Predominant bacterial species	Reference
D	0.4	50	7	Compost	Glucose	6.60	0.00	0.740	0.560	-	7.93	3.16	*Caldibacillus hisashii*	This study
D	0.500	35	5.5	Granular from CSTR	Galactose	0.461	3.60	1.90	trace	-	6.58	3.29	Bacilli	32
D, pH	0.0417	37	3.5	Anaerobic sludge	Glucose	4.36	0.27	0.46	-	1.86	6.95	0.49	NA	33
pH	0.0833	55	3.0,5.5	Anaerobic sludge	Whey	5.7	trace	trace	-	trace	5.7	0.479	*W. coagulans*	34
pH	0.0104	35	4.2	Anaerobic Sludge	Food waste	12.5	2.04	5.49	-	0.74	22	0.07	*Lactobacillus*	35
pH, OLR	0.00833	18	5.5-5.9	Primary sludge	Papermill dairy & cheese whey	-	0.69	-	-	-	1.59	0.0133	*Coprothemobacter* sp.	3
OLR	0.125	37	5.5-6.5	Anaerobic sludge	Glucose	0	6.65	4.4	-	0.64	12	1.5	*Pseudomonas* sp.	36
Temp.	0.0833	50	5	Anaerobic sludge	Glucose	23	trace	trace	trace	-	23	1.39	*Bacillus* sp.	37
Inoculum	0.0208	35	Un-controlled	Activated sludge	Potato peel waste	6.41	trace	trace	-	trace	6.41	0.13	*Lactobacillus* sp.	8

D, dilution rate; Temp., temperature; C_{LA}, lactic acid concentration; C_{BA}, butyric acid concentration; C_{AA}, acetic acid concentration; C_{FA}, formic acid concentration; C_{EtOH}, ethanol concentration; C_{TA}, total organic acid concentration; P_{TA}, total organic acid productivity; OLR, organic loading rate.