Investigation of bacteria that degrade bacterial cells

（微生物細胞を分解する微生物に関する研究）

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LIST OF COMMONLY USED ABBREVIATIONS

BKPG.....*B. kururiensis* peptidoglycan

BSPG.....*B. subtilis* peptidoglycan

CFU.....Colony forming unit

CO_2.....Carbon di oxide

CH_4.....Methane

DW.....Distilled water

DAPA.....Diaminopimelic acid

ECPG.....*E. coli* peptidoglycan

GC.....Gas Chromatography

H_2.....Hydrogen

LB.....Luria Bertani

MLPG.....*M. luteus* peptidoglycan

NAG.....N-acetyl Glucosamine

OD.....Optical density

OTU.....Operational taxonomic unit

PCR.....Polymerase Chain Reaction

PG.....Peptidoglycan

qPCR.....Quantitative Polymerase Chain Reaction

R2A.....Reasoner's 2A agar

SDS-PAGE.....Sodium dodecyl sulfate-polyacrylamide gel

SL.....Sludge

SO.....Soil

TSA.....Tryptic Soy Agar

WM..... Inorganic media

WW.....Wastewater

Abstract

Bacterial biomass can be a great source of carbon and energy for other bacteria in both aerobic and anaerobic environments, and hence they contribute to the carbon cycle throughout their life cycle. Previously some studies reported that bacterial cell wall is difficult to be degraded and tends to be recalcitrant in the environment. Generally, most of the bacterial biomass comes from the bacterial cell wall especially from peptidoglycan (PG) which is a complex structure of polysaccharide and amino acids. Usually, in the aerobic environments, degradation of bacterial cells is initiated by activities of both eukaryotes and prokaryotes. Conversely, the contribution of prokaryotes (especially bacteria) to the bacterial cell degradation is expected to be greater in anaerobic environments, since the functional abundance of eukaryotes is quite small. However, the available knowledge of bacterial cell degradation by bacteria in the aerobic environment has been limited. Furthermore, the evidence of bacterial cell degradation is wholly lacking in the anaerobic environment. Investigation of bacterial cell degradation will expand the understanding of both environmental ecologies. Moreover, the degradation of bacterial cells is quite important in some anaerobic engineering environments where degradation of bacterial biomass is a well-established approach to reducing solid waste derived from municipal wastewater treatment in an environmentally friendly manner. Thus, this study focused on the identification of the bacterial cell degrader bacteria in both aerobic and anaerobic environments.

For the aerobic investigation, environmental samples (from pond, river and soil) were tested on purified peptidoglycan (from gram-negative *Escherichia coli* and gram-positive *Micrococcus luteus*) used as a substrate to identify the peptidoglycan (PG) degrader aerobic bacteria on an agar plate. In addition, anaerobic microbial communities were collected from anaerobic digester sludge, rice paddy soil and an anaerobic wastewater treatment system, and enriched under the methanogenic condition using bacterial whole-cell/ PG purified from gram-negative (*E. coli* and *Burkholderia kururiensis*) and gram-positive (*M. luteus* and *Bacillus subtilis*) as the sole substrate.

Results from the aerobic investigation showed some colonies produced a clear zone on the given PG lawn. Phylogenetic analysis, based on 16S rRNA gene sequence analysis, was performed to identify the clear zone producing colony and the result revealed a total of 32 different bacterial strains from the phylum Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. These findings indicate that there are many known bacteria in aerobic environments which are capable of degrading bacterial PG and were not reported before.

Only one colony belonging to the phylum Firmicutes showed low identity to known bacteria (*Aneurinibacillus* soil strain 94%) which indicate a novel PG degrader aerobic species isolated from the soil sample. Isolates were also tested in a zymogram gel containing extracted purified PG and the results revealed two protein bands with the PG lytic activity at 70 kDa and 100 kDa respectively from *B. pacificus* and *B. tyonensis* which had not been reported before.

Bacterial cell degradation investigation in the anaerobic environment showed that methanogenesis occur from bacterial whole-cell and PG enriched culture and the CH_4 production can vary not only depending on the distinctive microbial community but also depending on the types of the substrates (whole-cell/ PG) by the same community. The same scenario was also observed in the enriched microbial community analysis which was done using next-generation sequencing. The enriched community analysis revealed that a number of phylogenetically novel and uncultured bacteria, especially in the phyla Bacteroidetes, Chlorobi, Euryarchaeota, Firmicutes, Thermotogae, Verrumicrobia and WWE1 dominated the enrichment cultures, suggesting their possible involvement in anaerobic whole-cell and PG degradation. Species-level analysis also showed that some operational taxonomic units (OTUs) were only dominant in a specific type of enriched culture where others were present in both types (whole-cell and PG) even in the same community. This observation suggests that different microorganisms are involved in the anaerobic degradation of whole-cell and cell wall components (especially on PG) where some of them could be active on both parts of a cell. In addition, some OTUs with higher identity commonly have been found among three anaerobic communities indicating their strong involvement in bacterial cell degradation in nature. None of them have been reported as being enriched by bacterial cell before now.

1. GENERAL INTRODUCTION

1.1 Carbon cycle and energy source

Bacteria play an important role in organic compounds degradation releasing by-products such as carbon dioxide (CO_2), hydrogen (H_2) and methane (CH_4). Initially, photosynthetic organisms generate organic compounds from CO_2, these organic compounds are eventually broken down into CO_2 again by both aerobic and anaerobic microorganisms, this way they comprise the total carbon cycle (Fig. 1). Although microorganisms are generally regarded as final consumers/degraders, they are also significant contributors to the carbon cycles in natural environments (1, 2, 3). In addition, the organic compounds derived from bacterial cells can be used as carbon and energy sources by other bacteria. Many studies already have reported that bacteria contribute to an enormous amount of dissolved organic compounds in aquatic and terrestrial environments and thus making a huge impact on the global carbon cycle (4, 5, 6, 7, 8, 9, 10).

1.2 Bacterial death and contribution of organic compounds

Bacteria can contribute organic compounds during their whole life cycle, especially after their death. Usually, cell death occurred because of abiotic and biotic interference. When they need to adjust to a harsh environment they go through a process which is called autolysis (11, 12, 13, 14) and during this period they can contribute organic compounds in nature. Another important reason for bacterial death is predatory attack such as by protozoa, fungus, and even sometimes because of viral lysis (15, 16, 17). There are also many predatory bacteria which can cause the death of other bacteria. For example, *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus*can prey on other bacteria. They have the ability to prey on many of the multidrug-resistant pathogens related to human infection, decreasing their number. *Bdellovibrio exovorus* can degrade cell wall to consume (18, 19). Another predatory bacterium is *Vampirococcus* who prey on purple sulfur bacteria, and have been reported in many different lakes (20). Research on this form of predation is now considered as an important field in microbiology, and all the information are now being used in agriculture, industry and medicinal field (21, 22).

Besides, bacteria can stay dormant for a long time, as an inactive form, and other bacteria can consider them as dead and hence they become part of the diet of other bacteria (23). Additionally, matured bacterial cell uses extracellular enzymes to break their own cell wall (particularly peptidoglycan) to make a new daughter cell. This process is called peptidoglycan (PG) hydrolysis (24). During this time, they lose some organic compounds in the environment (25, 26, 27).

1.3 The bacterial cell as an organic compound

Organic compounds from the bacterial cell can be considered as carbon and energy source for other bacteria, since bacterial cells are composed of complex organic compounds, including proteins, nucleic acids, polysaccharides, and lipids (Fig. 2). PG is an abundant substance forming the cell walls of bacteria, giving structural strength, as well as counteracting the osmotic pressure of the cytoplasm (28. PG is composed of a polymer consisting of polysaccharides and peptides that form a mesh-like layer outside the cytoplasmic membrane. The polysaccharide chains consist of alternating residues of β-(1,4)-linked N-acetylglucosamine and N-acetylmuramic acid and are cross-linked by short peptides composed of four or five amino acids. The composition of the short peptides varies among bacterial species, in particular between gram-positive and gram-negative bacteria (Fig. 2) (29).

1.4 Peptidoglycan as a source of other bacterial diets

PG is a frequent source of organic compounds which could be used in other bacterial diets. Some studies investigated the fate of PG in aerobic environments, reporting that PG is eventually degraded, but its turnover rate is much lower than those of other cellular components (30, 31). Usually, bacteria have PG degrading abilities to modify and recycle their own PG but only some aerobic bacteria have been reported to utilize extracellular PG as their substrate and/or excrete PG hydrolyzing enzymes extracellularly (32, 33, 34). To date, few studies have been found on bacterial cell degradation by bacteria and only two novel bacteria (*Vogesell amureinivorans* and *Delftia lacustris*) were described, which can degrade bacterial PG in aerobic condition (32, 35). However, most of the study was done in the aquatic environment, especially in the marine surroundings. Despite its biological and biotechnological importance, little is known about how microbes degrade bacterial PG in

aerobic environments. Much of this gap in knowledge has resulted from lack of enough study. Moreover, no study has examined the fate of PG, and PG degrading microorganisms in anaerobic environments even though the degradation of bacterial cells assumes to contribute greatly to the carbon cycle in the anaerobic environments (36, 37).

1.5 Importance of bacterial cell degradation in the aerobic environment

Generally, aerobic digestion process can be performed on a small or big scale to remove hazardous compounds, sewage treatment and composting. However, this process became very popular in some industrial places to manage many types of waste. For example, food, cardboard and horticultural waste (38). The investigation of aerobic bacteria that degrade bacterial PG and the knowledge of their molecular mechanisms can be applied in agriculture, medicine as well as in the industrial field.

1.6 Anaerobic digestion and the importance of bacterial cell degradation in anaerobic environment

Anaerobic digestion is an arrangement of processes where organic compounds are broken down by different types of microorganisms in the absence of oxygen, and is often used for industrial or domestic purposes to manage waste or to produce biogas (production of CH_4) (Fig 3) (39). Generally, this digestion process is divided into four steps such as hydrolysis, fermentation, acetogenesis and methanogenesis, where different specialized microorganisms are usually involved. Biomethylation or methanogenesis is one of the most important processes in the anaerobic digestion process since methane (CH_4) is a potent greenhouse gas as well as a sustainable energy source (40, 41). Biogas or CH_4 can be converted to heat and/or electricity, and its purified derivative, biomethane, is suitable for every function for which fossil natural gas is used today. Methanogenesis occurs in diverse anaerobic environments such as ruminant, human gut, the soil of rice-paddy field, marine sediment, wastewater treatment plants and so on (42).

Unlike in an aerobic environment, there is limited life in an anaerobic environment. Bacteria and archaea are the most dominant life in anaerobic environments (43, 44). Being a dominant life in the anaerobic environment, bacteria supposedly contribute a significant number of organic compounds, and other bacteria can use those organic compounds as their carbon and energy source. In addition, these organic compounds could become part of the digestion

process and end up at methanogenesis. Besides, anaerobic bacteria have important roles in geo-elemental cycling, agriculture, and medicine (45).

In fact, the degradation of bacterial cells is quite important in some anaerobic engineering environments, where degradation of bacterial biomass is a well-established approach to reducing solid waste derived from municipal wastewater treatment in an environmentally friendly manner. Still, there is no study on bacterial cell degradation in anaerobic environments.

2. AIMS

Considering the importance of bacterial cell degradation and identification of bacterial cell degrader bacteria in both aerobic and anaerobic environments were investigated in this study.

In the aerobic environment, lake water, pond water and coastal soil were used as a microbial community and tested on *M. luteus* peptidoglycan and *E. coli* peptidoglycan to identify the PG degrader aerobic bacteria.

In the anaerobic environment, different methanogenic environmental samples were used to study bacterial cell degradation. Microbial communities were enriched under anaerobic, methanogenic conditions with bacterial whole-cell and peptidoglycan (PG) as the sole substrate using sludge of an anaerobic digester (SL), rice paddy soil (SO) and anaerobic wastewater treatment system (WW) as the microbial sources in order to investigate the capability of the whole cell and PG degradation by anaerobic microorganisms.

In the anaerobic digestion process, among the sequential metabolic flow, it is well known that the first step (hydrolysis of recalcitrant organic matters) and/or the last step (symbiotic degradation of volatile fatty acids) are the rate-limiting steps (46). Previous research has already proven that the addition of electrically conductive materials can accelerate symbiotic degradation of volatile fatty acids into CH_4, which is one of the rate-limiting steps in the methanogenic flow of the anaerobic digestion process, by enhancing interspecies electron transfer via electric currents flowing through conductive materials (47). Thus, activated carbon was used in this experiment to promote the CH_4 production from the whole cell and PG degradation in this study.

3. FIGURES LEGENDS

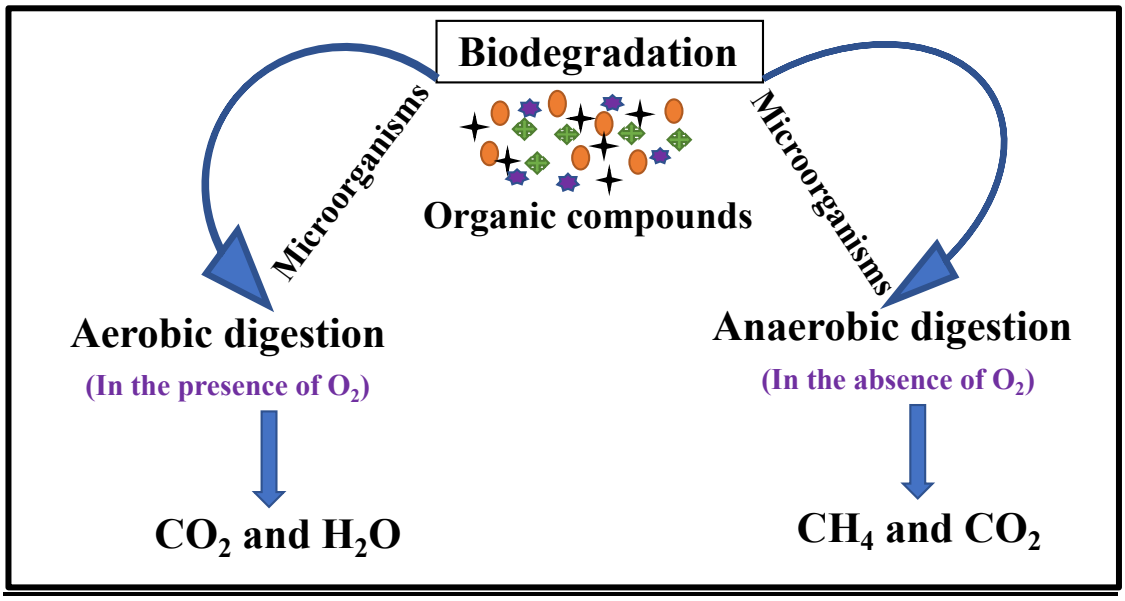


Fig 1: Biodegradation of organic compounds by microorganisms in the presence (aerobic digestion) and absence (anaerobic digestion) of oxygen.

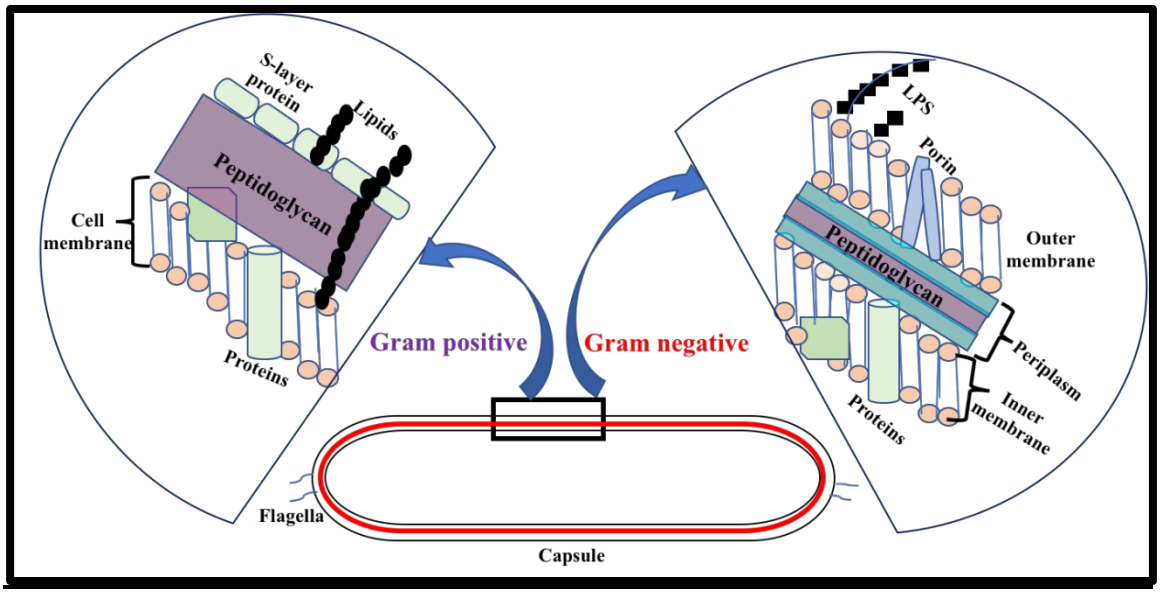


Fig 2: A typical bacterial cell structure categorized into gram positive and gram negative

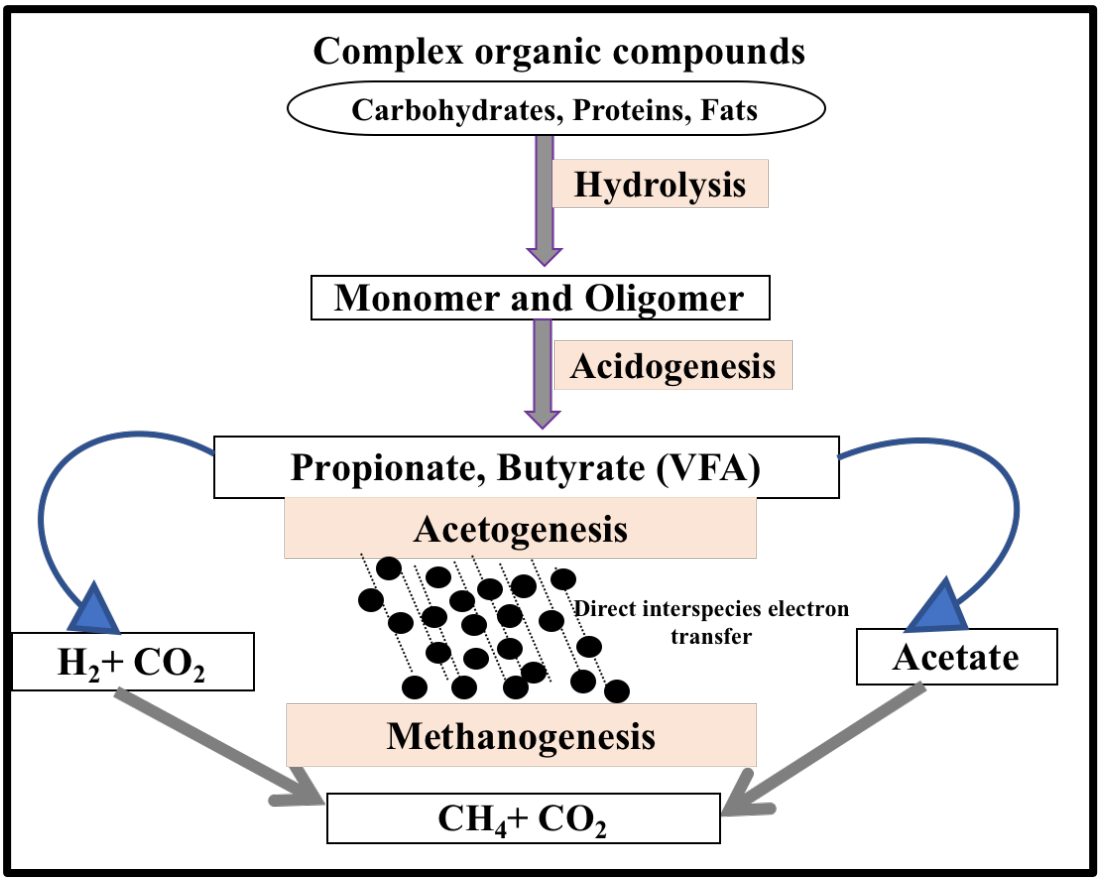


Fig 3: Steps of anaerobic digestion process.

Chapter 1

INVESTIGATION OF PEPTIDOGLYCAN DEGRADING AEROBIC BACTERIA

1. INTRODUCTION

Bacteria play fundamental role in the global nutrient cycling (48, 49). They contribute with a significant number of dissolved organic compounds into the environment during their life cycle, because of biotic and abiotic interaction. Protozoal grazing and viral lysis are two important reasons for bacterial cell lysis in the aerobic environment (15, 16, 17), which help them to actively contribute with their organic compounds into the environment. Many studies have reported about the numerous available parts of bacterial cell wall components in aquatic environments, which suggested their participation in the total organic compounds of nature. For example, D-amino acids, glucosamine and Diaminopimelic acid (DAPA) (27, 50) are available compounds derived from the bacterial cell, they are often reported, but in the aquatic environment since most of the studies focus on this environment. After becoming available, these organic compounds undergo degradation by the prokaryotes, especially by other bacteria, as their nutrition source. Previous studies stated that most of the organic compounds coming from bacterial cell wall are considered as a recalcitrant part to be degraded by other bacteria and tend to be remain in environment (30, 31, 34). Bacterial cell wall is composed of peptidoglycan (PG), which is made of polysaccharide chains cross-linked by unusual peptides containing D-amino acids (29) which is also a complex structure and could vary depending on the bacterial species. Some previous reports also stated that gram-negative bacterial PG is not easily degraded by other bacteria in aerobic environments (30, 32). In addition, bacterial PG degradation by bacteria also could be varied depending on the degrader microbial community. To illustrate, gram-negative bacteria are abundant in marine environments whereas gram-positive bacteria are abundant in fresh water environments, indicating that in those particular environments organic compounds will be different since they are derived from the dominant bacteria (51, 52, 53, 54). Therefore, a particular degrader is assumed to be abundant in each environment. In this study, I have focused on both terrestrial (soil aerobic community) and aquatic environments (pond water and river water aerobic community) to investigate the bacterial PG degradation and to

identify the aerobic PG degrader. PG of both gram-negative (*E. coli*) and gram-positive (*M. luteus*) were extracted and used as a substrate due to the difference in structure and composition of their PG structure.

2. MATERIALS AND METHODS

2.1 Sampling of microbial community

Soil aerobic community was collected from the coastal area of Uraichinai river, Sapporo, Japan and water aerobic community was collected from the Ono pond of Hokkaido University and Shiroishi river water, Sapporo, Japan (Figure 1).

Figure 1. Sampling location of aerobic peptidoglycan degrader investigation. (A) Soil near to Uraichinai river (B) Water from Ono pond (C) Shiroishi river water sample were used as an aerobic community for PG degrader investigation.

All samplings were done between May- September in the year 2019. Each community sample individually was carried in a plastic open tube to allow air in so that the community can get oxygen. After arriving in the laboratory, the Shiroishi soil sample was observed under a light microscope to observe any eukaryotes. Other debris such as leaves, grasses and stones were excluded from all samples before starting the experiment. Distilled water (DW) was used for making a suspension of soil sample (100 ml DW/1 gm soil) and then followed by

dilution series by using distilled water. In the case of both water samples, the direct sample was taken for making a dilution series.

2.2 Extraction and preparation of purified peptidoglycan

Two different bacteria were used in this study and cultured in Luria Bertani (LB) broth media for 18-24 hours at 37°C. *Escherichia coli* (*E. coli*) as a representative of gram-negative and *Micrococcus luteus* (*M. luteus*) as a gram-positive representative were used in this study. Cells were separated by centrifugation for 10 min at 4000×*g* from the broth culture (each time 40 ml) to get a homogenized bacterial cell for further preparation. After centrifugation, the bacterial cell was collected from the bottom of the vial and washed three times by using an inorganic media (WM) with the following ingredients (per liter): 0.54 g NH_4Cl, 0.13 g KH_2PO_4, 0.24 g $MgCl_2 \cdot 6H_2O$, 0.15 g $CaCl_2 \cdot 2H_2O$, 2.52 g $NaHCO_3$, 0.1 g Bacto yeast extract, 0.1 g Bacto proteose peptone, and 1 mL each of a trace element solution and vitamin solution (55). Along with the inorganic media the pellet was centrifuged again to remove the media components associated with cell. After centrifugation, the homogenized bacterial cell was used for the peptidoglycan (PG) preparation. PG was prepared according to (56) and modified according to my purpose. Briefly, the preparation included: (a) Treatment with 30% aqueous phenol and stirred for 20 minutes at 65˚- 68˚C. At this stage, lipopolysaccharides (LPS), proteins, nucleic acids and other bacterial cell wall components non-covalently associated with PG were removed. (b) cooling and centrifugation three times at 47000 turns/min for 10 mins of each, (c) Treatment with 100% acetic acid and stirred at 100 ˚C for three hours. At this phase, trace amount of Lipopolysaccharides were eliminated from the suspension, (d) cooling the suspension and centrifugation for 20 min at 47000 turns/ min. This time the precipitate was washed three times by sterile distilled water, (e) performing dialysis for three days at 22 ˚C to remove low molecular weight compounds. A 0.05 M sodium acetate (CH_3COONa) solution of pH 5.8 was used as a buffer for the dialysis and this buffer solution was changed every day. After three days of dialysis, extracted PG was washed three times by the same WM media for the culture and centrifuged for 10 min at 47000 turns/min. Finally, the prepared PG was mixed with WM media to make a liquid homogenized suspension and used for the PG degrader aerobes isolation.

2.3 Isolation of peptidoglycan degrader aerobic bacteria

To isolate a single colony, soil and water were diluted by 10-fold serial dilution method (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, $10^{-6,}$ 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10}). Extracted PG of both substrates bacteria was spread separately onto a plastic 8cm Petri dish containing medium of Reasoner's 2A agar (R2A), Tryptic Soy Agar (TSA) and inorganic media (WM), separately to make a lawn for the degrader isolates. Ten percent of R2A and TSA were used to investigate the PG degrader aerobes. In the case of WM media, it was not diluted as it is already a minimal media. After preparing the media agar plate, 500 µl of extracted PG was spread on the plate to make a lawn. Then, 100 µl of the community (soil/ water) suspension from each dilution was spread on Petri plates and let them dry for 10 min. 100 µl of each dilution community (soil and water) was also spread separately only on the agar plate without PG was considered as control. When all the plate dried enough then it was sealed by parafilm and incubated at 25ºC for 24 hours. The test was performed in triplicates (n=3). After incubation, colonies that showed a clear zone on the PG agar plate were considered as an aerobic PG degrader and selected for pure culture preparation and isolation. Pure culture of the clear zone produced isolates were obtained after 3-4 consecutive sub-culturing on to fresh diluted same media plates with the specific PG which was used for the initial isolation followed by quadrant streaking procedure. Then the pure isolates were subjected to the 16S rRNA gene sequence analyzing.

2.4 Identification of peptidoglycan degrader aerobic bacteria

Direct colony PCR (Polymerase chain reaction) of the pure culture isolates were performed for the species-level identification. A single colony was collected using a sterile toothpick and transferred to the PCR tube. PCR was performed with universal bacterial primers complementary to conserved regions of the 5′ and 3′ ends of the 16S rRNA gene; 27F (forward) 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R (reverse) 5'-GGHTACCTTGTTACGACTT-3' (57). PCR was performed using AmpliTaqGold® (Applied Biosystems). After initial denaturation for 10 min at 95 °C, target DNA was amplified in 30 cycles. Each cycle consisted of denaturation for 30 s at 93 °C, annealing for 30 s at 55 °C and extension for 2 min at 72 °C. The final extension was 5 min at 72 °C. In accordance with the manufacturer's instruction, the PCR products were purified with QIAquick® PCR purification kit (Qiagen). The purified 16S rDNA was sequenced directly

using the ABI PRISM® BigDye® Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an ABI PRISM model 377 genetic analyzer (Applied Biosystems). The obtained 16S rDNA sequences of isolated bacteria were compared with those from the NCBI nucleotide sequence database, using the program BLAST.

2.5 Preparation and concentration of isolate's lysate

To check the hydrolyses activity of the isolated strains on the extracted PG, lysate suspension of isolated strains was prepared. *M. luteus* extracted PG was used as a substrate for this experiment. The same lysate preparation was followed for both enzymatic activity and zymogram experiment. Some clear zone producing isolates were chosen randomly for the enzymatic activity testing against the extracted PG. Selected strains were cultured in a diluted TSA media with the extracted purified PG for approximately 16-18 hours at 25ºC under a shaking condition to get the expected enzymes in the culture suspension. Then, the culture was centrifuged and the supernatant was filtered through a 0.22 mm pore size filter (membrane HA; Millipore). The filtrate corresponding to the lysate was recovered and concentrated 10-fold using an Amicon ultrafiltration cell (DIAFLO; YM10 membrane; 40 lb/in^2 at 4ºC). The protein content of the lysate was determined by the Bradford procedure (58) using the Bio-Rad protein assay kit, with bovine serum albumin (Sigma) as a standard. Then the extracted purified PG was added with the lysate and the optical density (OD_{600}) of the suspension was measured every 3 hours for a duration of 72 hours (at 25ºC under a shaking condition). The suspension which gave the maximum OD reduction was chosen for the zymogram experiment and in this case, *Bacillus pacificus* and *Bacillus toyonenesis* were selected.

2.6 SDS-PAGE and renaturing SDS-PAGE (Zymogram)

The lysates or extracted proteins were separated by electrophoresis using a Sodium dodecyl sulfate (SDS) gel containing extracted purified PG and then the proteins were renatured in the gel. The renatured PG hydrolases in the gel hydrolyzed the material around the lysates. The PG in the gel was stained by methylene blue and the hydrolyzed material could not be stained, allowing for the detection of PG hydrolytic activities of the enzymes on the gel. Lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) for protein content and by zymogram assay to detect proteins showing lytic activity. In order to prepare SDS-treated walls, 20 mg of purified PG was resuspended in 100 ml of distilled

water. SDS-PAGE was carried out as described by Lortal et al. (59) with an SDS-12% polyacrylamide separating gel (pH 8.8) and a constant voltage (180 V) at room temperature. Renaturing SDS-PAGE was performed according to the methods of Foster et al. (60), with some modifications. SDS-polyacrylamide separating gel (12% acrylamide, pH 8.8) containing 0.2% (wt/vol) PG was used to detect the lytic activities. After electrophoresis (1 h, 180 V, constant voltage at room temperature), the gels were soaked for 30 min in distilled water at room temperature (25ºC). The gels were then transferred into the renaturing buffer (50 mM Tris-HCl [pH 8.0] containing 1% [vol/vol] Triton X-100) and shaken gently for 2h at 30ºC to allow renaturation. The renatured hydrolysis appeared as clear translucent bands on the opaque background. The contrast was enhanced by staining the gels in 0.1% (wt/vol) methylene blue in 0.01% (wt/vol) potassium hydroxide. In order to determine the apparent molecular weight, the standard provided by Pharmacia was used. One lane was systematically loaded with the standards in all the gels for SDS-PAGE. No difference in the migration of the standards due to the presence of added PG in the gels was noted. Photographs of the gels were taken by using a luminous table; the translucent bands appeared as white bands in the gel. The results shown in figure 3 are representative, and all the observations were confirmed in at least three separate experiments.

3. RESULTS AND DISCUSSION

3.1 Investigation of aerobic bacterial PG degradation

Different environmental samples were studied to isolate the PG degrader aerobic bacteria. After 24 hours of incubation, it was observed that some colonies appeared on the PG lawn which made a clear or partial clear zone around the given PG (Figure 2).

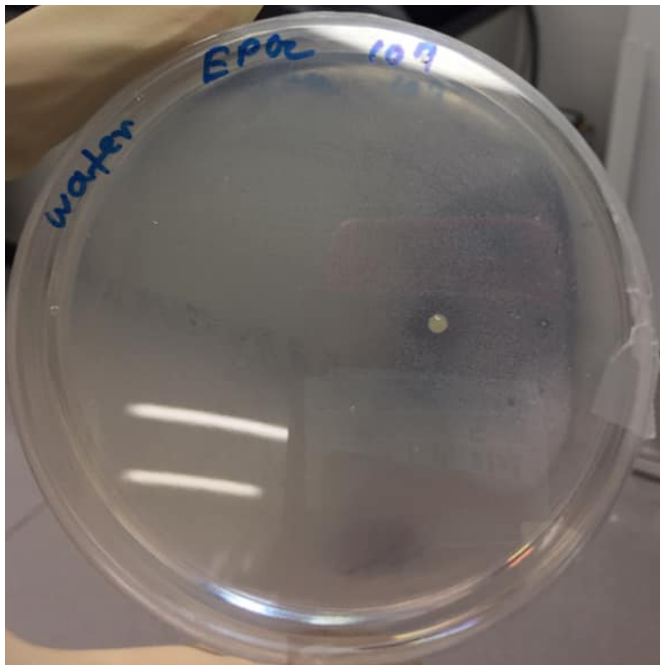


Figure 2. Observation of clear zone producing bacteria on *E. coli* peptidoglycan (EPG) from the Shiroishi river water sample.

The clear zone indicated the complete degradation whereas the partial clear zone probably indicated the fractional degradation ability of the colonies on PG. As the concern was initially to investigate the PG degrader, the size of the clear zone was not measured, and the remaining part of the given PG was also not investigated yet in this initial study. However, to identify the PG degrader aerobic bacteria, clear zone producing colonies were subject to further investigation. A pure culture of the isolates was prepared to avoid any misinterpretation of the taxonomic identification. The phylogenetic analysis of 16S rRNA gene sequence analysis revealed a total of 32 different bacterial strains from the three different samples (Table 1) which showed activity on *M. luteus* peptidoglycan (MPG) and *E. coli* peptidoglycan (EPG), individually.

Clear zone produced Bacteria (blast identity in %)	Shiroishi river water		Uraichinai soil		Ono pond water		Media	Phylum
	MPG	EPG	MPG	EPG	MPG	EPG		
Microbacterium oleivorans (99%)	X	X	X	✓	X	X	T& R	Actinobacteria
Streptomyces cacaoi (98%)	X	X	✓	X	X	X	TSA	Actinobacteria
Rhodococcus jostii (99%)	X	X	✓	X	X	X	R2A	Actinobacteria
Nocardia coeliaca (100%)	X	X	✓	X	X	X	WM	Actinobacteria
Pseudoarthrobacter defluvii (99%)	X	X	✓	X	X	X	TSA	Actinobacteria
Pseudoarthrobacter equi (99%)	X	X	X	✓	X	X	WM	Actinobacteria
Micrococcus aloeverae (99%)	X	X	X	✓	X	X	TSA, R2A	Actinobacteria
Mucilaginibacter calamicampi (99%)	X	X	X	✓	X	X	R2A	Bacteroidetes
Spirosoma aerophilum (100%)	X	X	X	✓	X	X	TSA	Bacteroidetes
Mucilaginibacter paludism (98%)	X	X	X	✓	X	X	R2A	Bacteroidetes
Bacillus mobilis (99%)	X	✓	✓	✓	X	X	R2A, WM	Firmicutes
Bacillus aryabhati (100%)	X	X	✓	✓	X	X	R2A	Firmicutes
Bacillus wiedmannii (100%)	X	X	X	✓	X	X	R2A	Firmicutes
Bacillus toyonensis (100%)	X	X	✓	✓	✓	X	R2A& TSA	Firmicutes
Bacillus pacificus(100%)			✓	✓	✓		R2A & TSA	Firmicutes
Bacillus megaterium (99%)	X	X	X	✓	X	X	R2A	Firmicutes
Bacillus cereus (100%)	X	X	X	X	✓	X	R2A	Firmicutes

Bcillus firmus (99%)	X	X	X	X	✓	X	R2A	Firmicutes
Bacillus thuringensis (100%)	X	X	X	X	✓	X	R2A	Firmicutes
Bacillus badius (99%)	X	X	✓	✓	X	X	TSA, R2A	Firmicutes
Bacillus subtilis (100%)	X	X	✓	X	X	X	TSA, R2A	Firmicutes
Brevibacillus reuszeri (99%)	X	X	✓	X	X	X	R2A	Firmicutes
Brevibacillus choshinensis (99%)	X	X	✓	X	X	X	R2A	Firmicutes
Lysinibacillus sphaericus (98%)	X	X	✓	✓	X	X	R2A	Firmicutes
Aneurinibacillus soil strain (94%)	X	X	X	✓	X	X	TSA	Firmicutes
Acinetobacter pitti (100%)	✓	X	X	X	X	X	R2A	Proteobacteria
Acinetobacter proteolyticus (99%)	✓	X	X	X	X	X	R2A	Proteobacteria
Acinetobacter junii (98%)	✓	X	X	X	X	X	R2A	Proteobacteria
Acinetobacter johnsonii(99%)	X	✓	X	X	X	X	WM	Proteobacteria
Massilia pinisoli (98%)	X	X	✓	X	X	X	TSA	Proteobacteria
Rugamonas rubra (99%)	X	X	X	✓	X	X	R2A	Proteobacteria
Rhizobium nepotum (99%)	X	X	✓	X	X	X	WM	Proteobacteria

Table 1: Isolates of peptidoglycan degrading aerobic bacteria from different environment. Bacterial types written in red and violet are gram-negative and gram-positive bacteria respectively.

16S rRNA gene sequence analysis blast result of all the isolates pure culture showed a maximum identity to known bacteria except one pure culture isolates which showed clear zone on EPG (from the Shiroishi soil sample). The blast result showed that the pure culture isolates was closely related to *Aneurinibacillus* soil strain (94%). From the identity

percentage, it is assumed that the isolates close to the *Aneurinibacillus* soil strain (94%) is a novel PG degrader aerobic bacterial strain; however, this needs further investigation to conclude. Description of this new strain could help us to know more about PG degradation and could add a new bacterium in the aerobic PG degrader list (32, 35).

Additionally, phylum level investigation of the pure culture isolates was considered to find out more about the aerobic PG degrader and to speculate more about the degradation in environments. Phylum level investigation revealed that different bacteria from the Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria were involved on *M. luteus* and *E. coli* PG degradation in different environmental samples, even though there was a specification of the phylum in an individual sample. For example, the phylum Actinobacteria and Bacteroidetes were found only from the Uraichinai soil sample, the phylum Firmicutes was found commonly in three individual samples whereas the phylum Proteobacteria was found in Shiroishi river water and Uraichiani soil sample.

In case of the phylum Bacteroidetes, only three isolates (*Mucilaginibacter calamicampi*-99%, *Spirosoma aerophilum*-100% and *Mucilaginibacter paludism*-98%) were found which showed specific activity only on EPG in the Uraichinai soil sample. This result indicates that the phylum Bacteroidetes from the soil aerobic environment may have the specificity only on gram-negative (*E. coli*) PG even though other different aerobic soil environments should be checked to confirm this speculation. However, the phylum Actinobacteria was only observed dominantly in the Uraichinai soil sample. In this study, though Actinobacteria can be found in terrestrial and aquatic both environments, it was only observed in the terrestrial sample (Uraichinai soil). Actinobacteria has great importance in agriculture as they can decompose the organic matter of dead organisms and this way they help plants to uptake the degraded molecules (61). Hence, these degradation characteristics of the phylum Actinobacteria also support them as PG degrader.

It was also clearly observed that isolates from the Ono pond water sample was only from the phylum Firmicutes. It should be mentioned that the other two environments (Shiroishi and Uraichinai sampling site) are more natural than the pond environment and therefore, some factors may influence the microbial structure in that particular environment. Bacterial mortality rate and their degradation could vary depending on the seasonal changes as well (62, 63). Sampling was done in a different time (May-September) in the year and therefore, this different timing could affect the bacterial community structure and ultimately on the PG degradation in this study. Besides that, different media composition could be another factor in the obtained result. Three different media were used in this study to

compare the results among three samples and it seems that the highest amount of clear zone produced isolates was observed from the R2A media and the lowest was observed from the WM media (Table 1). However, the media composition may have a slight influence on this different result.

To date, two novel (*Vogesella mureinivorans* and *Delftia lacustris*) PG degrader bacteria were reported in some studies (32, 35) which investigated the aquatic environment and both of the novel bacteria belong to the phylum Proteobacteria. In my research, the phylum Actinobacteria, Bacteroidetes and firmicutes are for first time reported as a gram-negative (EPG) and gram-positive (MPG) bacterial PG degrader in the aerobic environment.

In this study, the bacterial community from each dilution factor was also tested on the agar plate without using any substrate (PG) which were considered as controls plates. From the result, it was observed that most of the isolates which showed a clear zone around the PG did not grow on the control plates except for the three following strains; i) *Acinetobacter* soil strain (100%) also showed activity on MPG from Shiroishi water sample which was found from the 10^5 dilution factor of Shiroishi water sample, ii) *Brevibacillus choshinensis* (99%) also showed activity on MPG from Uraichinai soil sample which was found from 10^7 dilution factor of Uraichinai soil sample and iii) *Lysinibacillus sphaericus* (98%) showed activity on both types of PG in Ono pond water sample which was found from the 10^8 dilution factor of the pond water sample. It is assumed that these isolates might have the ability to grow in lower metabolism or are capable of surviving using only the limited source of metabolism from the diluted R2A media.

Among all investigated isolates, it was clearly observed that each strain was specifically active on a particular PG. However, some isolates showed activity on both PG in the same sample. For example, *Bacillus mobilis* (99%), *Bacillus aryabhati* (100%), *Bacillus toyonensis* (100%), *Bacillus pacificus* (100%), *Bacillus badius* (99%), *Lysinibacillu ssphaericus* (98%) from the Uraichinai soil sample showed activity on both PG. It was also observed that the same isolates did not show the same activity in three community samples. *Bacillus mobilis* (99%) was active on both PG in Uraichinai soil but was active only on EPG in Shoroishi river water sample, *Bacillus toyonensis* (100%) and *Bacillus pacificus* (100%) were active on both PG in Uraichinai soil but was only active on MPG in Ono pond water. This observation indicates that the bacterial activity may depend both on the community and substrate.

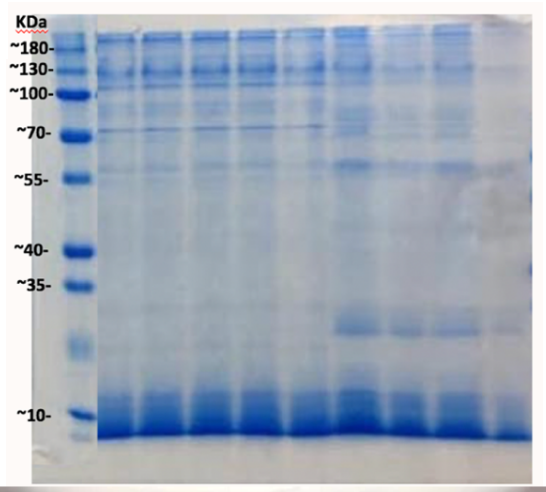
From this analysis, it is also clear that there are many known aerobic bacteria which have the ability to degrade PG and these isolates (species) were never reported before on PG degradation. The activity could be different in the natural environment because of many

biotic and abiotic factors such as pH, temperature, competition with other living things, availability of the substrate, amount of substrate. However, analyzing these strains and investigating their enzymes could help us to understand the molecular mechanism of PG degradation and will contribute a huge impact in aerobic microbial ecology as well as some possible application in agriculture or medicine.

3.2 Zymogram of the hydrolysis of isolated strains

Zymography of PG hydrolases is a simple technique to specifically detect cell wall or PG hydrolytic activity. The zymographic method can be used for assessing the hydrolytic activities of purified target proteins, cell surface proteins, and proteins secreted to culture. Using the zymogram assay (renaturing SDS-PAGE), the hydrolytic activity of two clear zone producing isolates *B. pacificus and B. tyonensis* were tested. The zymogram gel made with PG as the substrate, revealed one translucent zone of each isolate's lysates, corresponding to the hydrolytic activities, after 10 hours of incubation at 30ºC in the renaturing buffer (Fig. 3). The translucent zone of *Bacillus pacificus*, (orange bar) had an evident molecular mass in the range of 70kDa and the translucent zone of *Bacillus toyonensis* lysates showed the protein band (green bar) in the range of 100kDa. According to the molecular weight of the protein, it is clear that both enzymes are distinct from each other. From this result, we assumed that both of these proteins could be used for the same activity as we have used the same types of substrates (peptidoglycan). It is also possible to get more hydrolytic enzymes depending on the AMICON tube as we have used only one size (12K) in this study. This study revealed the first (observational) detection of PG hydrolytic protein band of *B. pacificus and B. tyonensis* on *M. luteus* PG although the identification of the protein is not done yet. Therefore, further investigation such as, protein sequencing and the whole genome analysis of *B. pacificus* and *B. tyonensis* could help us to know more about the detected proteins in the zymogram gel.

(A)


KDa
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~100-
~70-
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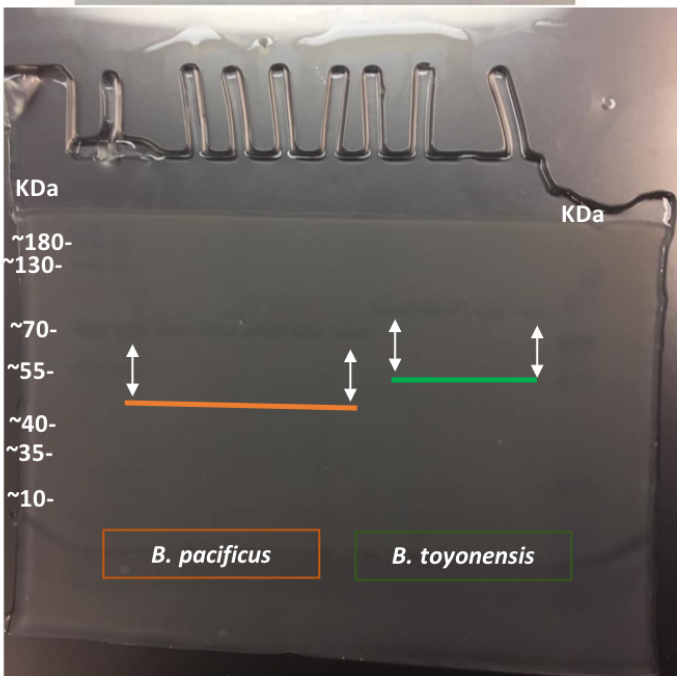
~40-
~35-
~10-

(B)


KDa
KDa
~180-
~130-
~70-
~55-
~40-
~35-
~10-
B. pacificus
B. toyonensis

(C)


KDa
KDa
~180-
~130-
~100-
~70-
~55-
~40-
~35-
~10-
~180-
~130-
~100-
~70-
~55-
~40-
~35-
~10-

Figure 3. (A) Protein profile of *B. pacificus* and *B. tyonensis* SDS extract without PG; (B) Variation of the hydrolytic activity in buffered solution and of the activity profiles in zymogram assays. The orange color bar represents the standard of *B. pacificus* protein bands and the yellow color bar represents the standard of *B. tyonensis*protein bands; (C) Zymogram gel with the hydrolytic activity protein band after staining.

4. CONCLUSION

Peptidoglycan (PG) is a very common occurred components of the bacterial cell in many aerobic environments (64, 65) which is hardly degradable by bacteria and contributes to the pool of recalcitrant organic compounds. Previously, the aerobic PG degradation by bacteria was investigated mainly in the aquatic environments but this study also focused on the terrestrial environment. Moreover, this study reported a broad-spectrum of investigation of PG degrader aerobic which was not done before. Through this investigation, it also observed that there are many known bacteria in nature which may degrade *E. coli* and *M. luteus* PG but was not reported before. In addition, the zymogram analysis of clear zone produced isolates against PG showed two distinct protein bands of *B. pacificus* and *B. toyonensis* which showed a hydrolytic activity on *M. luteus* PG and was not reported before. Additional investigation is needed to examine the recalcitrant part of the given PG, identification of the clear zone producing protein band as well as the mechanism of the degradation to deepen the PG degradation process.

Chapter 2

MICROBIAL COMMUNITY ANALYSIS OF ANAEROBIC ENRICHMENT CULTURES SUPPLEMENTED WITH BACTERIAL WHOLE CELL AND PEPTIDOGLYCAN AS THE SOLE SUBSTRATE

1. INTRODUCTION

Reason for bacterial cell lysis and contribution of organic compounds by bacterial cell have been studied very well in aerobic environments. Contrarily, the anaerobic environments have always been neglected even though bacteria are one of the most important and abundant lives there. Usually, bacterial cell lysis occurs by the eukaryotes in aerobic environments where eukaryotes are less abundant in anaerobic environments. So, it is assumed that bacterial cell lysis also could occur by other bacteria in that environment better and therefore, their organic compound's contribution is expected much higher compared to other lives contribution. Usually, it is assumed that most of the organic compounds only come from bacterial cell wall components but a whole cell also could be also considered as a diet for other bacteria. Generally, in nature morphologically intact but dead bacterial cell originates from mortality that only causes minor or no damage on the cell wall. For example, starvation, antibiosis and possibly predatory attack/ viral infection (30, 66, 67). Many times, it is observed that nucleoid-less bacteria or empty sell sac have also been reported in the aquatic environment (68, 69). It is assumed that these conditions may occur because of either prokaryotic or eukaryotic attack. Therefore, I decided to enrich methanogenic microbial community by using both whole-cell and cell wall fractionated components (extracted peptidoglycan) of four different substrate bacteria to investigate bacterial cell degradation and to speculate the scenario in anaerobic environments.

Anaerobic digestion is an eco-friendly process which is often used to manage waste and to produce a renewable energy source where organic compounds are broken down into smaller molecules and end up at methanogenesis. In addition, enormous wastewater activated sludge development is a big problem in the artificial wastewater treatment process where the bacterial cell has a great contribution to this sludge progress. Degradation of these bacterial cells can minimize the activated sludge efficiently and also can enhance the biofuel generation. Bacteria are primarily responsible for removing organic nutrients from the

wastewater in aerobic and anaerobic both environments where organic compounds resultant from bacterial cells also can be used as carbon and energy sources by other organisms. This degradation also may promote methane (CH_4) production. In this study, I enriched microbial communities from the different anaerobic environment and using different bacterial whole cells and extracted peptidoglycan as the sole carbon and energy sources. This investigation could help us to know more details about anaerobic ecology and let us find a possible way of application in the wastewater treatment process system.

2. MATERIALS AND METHODS

2.1 Cultivation and preparation of substrate bacteria

Four different bacteria were used in this study and cultured in Luria Bertani (LB) broth media for 18-24 hours at 37°C. *Escherichia coli* and *Burkholderia kururiensis* as representative of gram-negative and *Micrococcus luteus* and *Bacillus subtilis* as gram-positive representative were used in this study. Cells were separated by centrifugation for 10 min at 4000 ×*g* from the broth culture (each time 30 ml/50 ml tube) to get a homogenized bacterial cell for further preparation. After centrifugation, bacterial cell was collected from the bottom of the vial and washed three times by using the inorganic media (WM) and centrifuged again to remove the media components associated with cell. After centrifugation, the homogenized bacterial cell suspension was collected and directly used as whole-cell substrate.

To check the survivability, substrate bacteria was also enriched individually in a glass bottle only filled with 15ml of WM media with the following ingredients (per litre): 0.54 g NH_4Cl, 0.13 g KH_2PO_4, 0.24 g $MgCl_2 \cdot 6H_2O$, 0.15 g $CaCl_2 \cdot 2H_2O$, 2.52 g $NaHCO_3$, 0.1 g Bacto yeast extract, 0.1 g Bacto protease peptone, and 1 mL each of a trace element solution and vitamin solution (55) at 30 °C to check the cell viability (CFU/ml) and to quantify the DNA present at a particular time (qPCR) in anaerobic condition without the addition of microbial community until 40 days. The enriched culture was sealed under an atmosphere of N_2/CO_2 (80/20) without shaking. All the experiments were done in triplicate at the same time to confirm the reproducibility of the results. 1ml of the incubated sample in the different time interval was taken and diluted in sterile 9 ml of WM media to check the colony-forming unit

(CFU). 0.1ml of each sample from dilutions, 10^{-1}-10^{-10} was introduced onto the LB agar media by means of spreading plate technique respectively (70). Sterile WM media was also spread separately onto the LB agar plate as a control. Plates were incubated at 37°C for 24 hours. 37 °C temperature was decided to provide an optimum growth temperature and to get the maximum cell viability on the agar plate.

Quantitative PCR (qPCR) was also performed for checking the quantitative assurance of four substrate bacteria (whole-cell enrichment experiment only) under anaerobic condition until 45 days by using a LightCycler 96 real-time PCR system (Roche, Basal, Switzerland) and THUNDERBIRD SYBR Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The qPCR temperature profile consists of the initial denaturation at 95°C for 1 min targets amplified by 40 cycles of denaturation for 15 sec at 95 °C followed by annealing and extension for 45 sec at 60 °C. At the end of the extension step, fluorescence was measured and then the PCR amplicons were passed via a melting curve analysis to check for successful amplification. Two separate trials were conducted for each DNA sample's triplicates. Finally, the standard curves were generated with serially diluted PCR products amplified using respective primer sets.

2.2 Extraction of purified peptidoglycan

For the peptidoglycan (PG) extraction, four substrate bacteria were cultured individually in the LB broth media at 37˚C and collected the cell suspension from the active growth phase. Cells were separated by centrifugation for 10 min at 4000×*g* from the broth culture (each time 40 ml) to get a homogenized bacterial cell for further preparation. After centrifugation, the bacterial cell was collected from the bottom of the vial and washed three times by using an inorganic media (WM) (55). Along with the inorganic media the pellet was centrifuged again to remove the media components associated with cell. After centrifugation, the homogenized bacterial cell was used for the peptidoglycan (PG) preparation. PG was prepared according to (56) and modified according to my study purpose. Briefly, the preparation included: (a) Treatment with 30% aqueous phenol and stirred for 20 minutes at 65˚- 68˚C. At this stage, lipopolysaccharides (LPS), proteins, nucleic acids and other bacterial cell wall components non-covalently associated with PG were removed, (b) cooling and centrifugation three times at 47000 turns/min for 10 mins of each, (c) Treatment with 100% acetic acid and stirred at 100 ˚C for three hours. At this phase, trace amount of Lipopolysaccharide was eliminated from the suspension, (d) Cooling the suspension and

centrifugation for 20 min at 47000 turns/ min. This time the precipitate was washed three times by sterile distilled water, (e) Performing dialysis for three days at 22 ˚C to remove low molecular weight compounds. A 0.05 M sodium acetate (CH_3COONa) solution of pH 5.8 was used as a buffer for the dialysis and this buffer solution was changed every day. After three days of dialysis, extracted PG was washed three times by the same WM media for the culture and centrifuged for 10 min at 47000 turns/min. Finally, the prepared PG was dried and used for the anaerobic culture.

2.3 Enrichment of microbial community

Anaerobic community from sludge, soil and wastewater were enriched in a glass bottle (68 ml incapacity) filled with 15 ml of WM media, 1 ml of substrate bacterial liquid suspension for the whole-cell experiment except for the peptidoglycan (PG), as it was added in dry condition 50gm/L,), 2 ml of activated carbon powder (diameters of 37 to 149 μm, Sigma-Aldrich, St. Louis, MO, USA) solution (W/V: 1gm/L) in each enrichment, culture bottle to enhance the interspecies electron transfer of microbial community, especially in the downstream reaction of anaerobic digestion and to stimulate the CH_4 production (72) and 2 ml of the anaerobic community was added as an inoculum. The enriched culture was sealed under an atmosphere of N_2/CO_2 (80/20) and enriched at 42ºC, 30 ºC and 25 ºC without shaking. All culture experiments were accompanied in triplicate to check the reproducibility. Each original community (sludge, soil and wastewater) and individual substrate (whole-cell and PG) were also incubated independently in a fresh WM media respectively at 42ºC, 30 ºC and 25 ºC to check the CH_4 production by themselves without addition of any substrate and to compare with the enriched community's CH_4 production. Then, weekly gas was measured by gas chromatography and recorded in a spreadsheet. CH_4 gas-phase was measured weekly from each culture (0 days to until 63 days) by using a gas chromatograph (GC-2014, Shimadzu, Kyoto, Japan) as described earlier in Kato et al. 2014 (73). Enough CH_4 produced (at least twice of the original community's CH_4 production) enriched culture was transferred three times and each time from the log phase (between 30-35 days) of triplicates culture, separately. The enriched microbial community was collected from the log phase of the third transference by centrifugation at 14,000Xg for 15 min to obtain the sample pellet for DNA extraction and at the same time a sample was also taken for the enriched bacterial isolation.

2.4 DNA extraction, PCR amplification and PCR product purification and Next-generation sequencing (NGS)

DNA was extracted using FAST DNA Spin Kit for Soil (MP Biomedicals, lrvine, US) and the 16S rRNA gene V4 region were amplified by PCR using Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) with the primer pair of 515′F (5′-GTGBCAGCMGCCGCGGTAA-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) (74, 75) with adaptor and index sequence tags under the following thermal conditions: initial thermal denaturation at 98°C for 30 sec, followed by 25 cycles of heat denaturation at 98°C for 10 sec, annealing at 55°C for 20 sec and extension at 72°C for 30 sec. The PCR products were subjected to agarose gel electrophoresis to confirm fragment length and then purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Purified PCR products were subjected to sequence the analysis with an Illumina MiSeq platform (Illumina, San Diego, CA, USA) by Hokkaido System Science Co. Ltd. (Sapporo, Japan) to generate Illumina shotgun paired-end (2 × 301 bp) sequence libraries. Raw 16S rRNA sequence data were adaptor trimmed at the 3′ end to remove adaptor sequences (cutadapt 1.1), quality trimmed (Trimmomatic v. 0.32; TRAILING:20 MINLEN:50), and the individual read pairs were overlapped to form single synthetic reads (fastq-join v. 1.1.2-537; 8 per cent maximum difference, 6 minimum overlap; https://github.com/brwnj/fastq-join). The obtained readings were clustered with the UCLUST algorithm using a ≥97% sequence identity cut-off with MacQIIME 1.9.1. Representative sequences of each operational taxonomic unit (OTUs) were aligned using PyNAST and chimeric sequences and were removed using ChimeraSlayer. Bacterial 16S rRNA reference sequences were compared to find out the closest identical species in the BLAST database where uncultured environmental samples were excluded (55).

2.5 Isolation and identification of potential bacterial cell degrader anaerobic bacteria

Each enriched community was used for isolation on the agar plate. 1ml of the incubated sample was taken and diluted in sterile 9 ml of WM media. 0.1ml of each sample from dilutions, 10^{-1}-10^{-10} was introduced onto the gellan gum agar plate adding the same substrate (which was used to enrich them) in an anaerobic pack. All plates were kept according to the original community's incubation temperature (sludge- 42 ºC, soil- 30 ºC and wastewater- 25ºC) for 24 hours. Some colonies were observed on the agar plate of each

dilution factor and direct colony PCR was performed of the colony appeared on the PG agar plate. The colony was collected using a sterile toothpick and transferred to the PCR tube. PCR was performed with universal bacterial primers complementary to conserved regions of the 5′ and 3′ ends of the 16S rRNA gene; 27F (forward) 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R (reverse) 5'-GGHTACCTTGTTACGACTT-3' (57). PCR was performed using AmpliTaqGold® (Applied Biosystems). After initial denaturation for 10 min at 95 °C, target DNA was amplified in 30 cycles. Each cycle consisted of denaturation for 30 s at 93 °C, annealing for 30 s at 55 °C and extension for 2 min at 72 °C. The final extension was 5 min at 72 °C. According to the manufactures' instruction, the PCR products were purified with QIAquick® PCR purification kit (Qiagen). The purified 16S rDNA was sequenced directly using the ABI PRISM® BigDye® Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an ABI PRISM model 377 genetic analyzer (Applied Biosystems). The obtained 16S rDNA sequences of isolated bacteria were compared with those from the NCBI nucleotide sequence database, using the program BLAST.

3. RESULTS AND DISCUSSIONS

3.1 Methanogenesis from enriched anaerobic community supplemented with bacterial cell

Each original community and substrates were enriched only in inorganic media separately which was used as a control in this study. Weekly CH_4 production of these controls was then observed. To observe the bacterial cell degradation, three communities were enriched in the anaerobic condition in an inorganic media (WM) by addition of whole-cell and PG of *M. luteus, B. subtilis, E. coli* and *B. kururiensis* as a substrate as well as the main carbon source of energy. Activated carbon (AC) as a conductive mineral was added along with the substrates and anaerobic community to enhance the downstream interspecies electron transfer. The enriched culture which produces higher CH_4 than the original was considered as a successful enriched culture and measure weekly CH_4 production (Figure 1).

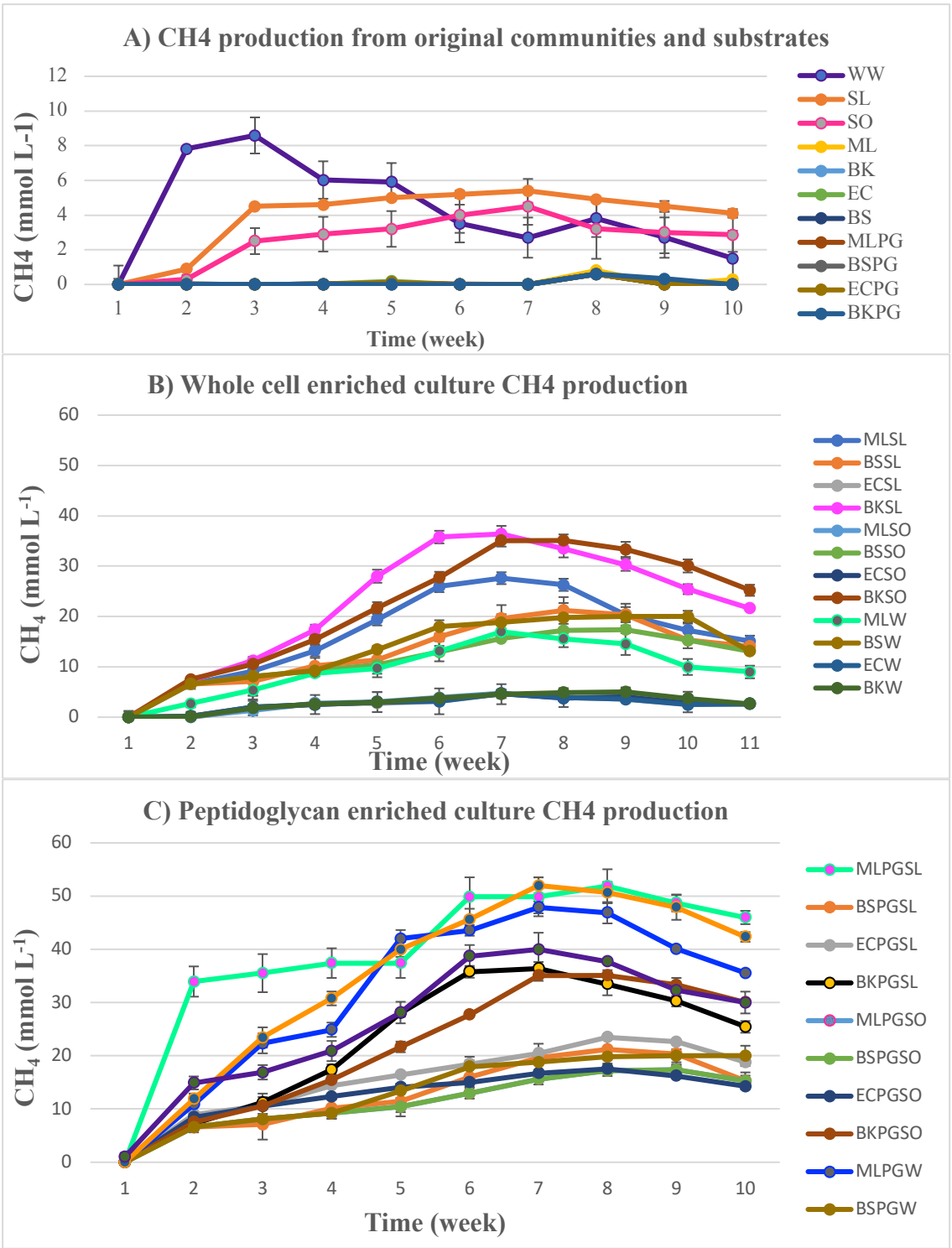


Fig: 1. Weekly observation of methanogenesis by gas chromatograph from rice paddy soil (SO), sludge of an anaerobic digester (SL) and from an artificial anaerobic wastewater treatment system (WW) by different bacterial whole-cell and fractionated parts as a substrate. This graph shows methanogenesis results from A) three individual original communities and substrate's methane production as a control, B) whole cell enriched community's methane

production, and C) extracted peptidoglycan enriched community's methane production after three successful transference. Data are presented as the means of three independent cultures. Error bars represent standard deviations.

Methanogenesis result from the enriched culture showed 5 mM of CH_4 from sludge, soil and wastewater original community and for this reason more than 10 mM of CH_4 was considered as a successful enrichment culture from each transference, which is at least double of the original community's CH_4 production for three communities. It is possible that original communities (especially sludge and soil) initially were mixed with some other organic compounds derived from the natural environment, which assumed to supply the initial carbon source for anaerobic bacteria to proceed the methanogenesis in this experiment. However, the original community's initial total carbon amount and the given substrate's carbon amount were not measured before starting the experiment. Therefore, it is difficult to explain the exact amount of carbon utilization by the anaerobic community. Other anaerobic micro eukaryotic life including protozoa, protist, metazoa were also not detected in the original sludge and soil community before starting the experiment. Although the number of eukaryotes is very low in the anaerobic environments their presence and influence cannot be neglected in the culture. Besides that, the presence of the virus also cannot be neglected as the virus can also degrade some bacteria (76, 77). Moreover, growing bacterial PG hydrolyses also cannot be neglected. This might have a part of the contribution to the total organic compounds during enrichment. This is also needed to mention that the addition of yeast extract and peptone of the inorganic media could have a small contribution in the initial methanogenesis.

About the wastewater original community, it was initially enriched by using bacterial cell and therefore, the methanogenesis could have occurred from the previously given substrate. Thus, it was decided to transfer all enrichment culture thrice to avoid misapprehension in the result. Methanogenesis results from most of the enriched culture produced higher amounts of CH_4 than the control's CH_4 production. In this study, figure 1 represents only the third transference of the enrichment culture except controls (original community's and substrates CH_4 production). Original communities and substrates (controls) were not transferred like enriched culture. Methanogenesis result showed that the substrate cannot produce CH_4 without the addition of the community. Actually, CH_4 was measured in this study as methanogenesis is the terminal step of anaerobic digestion process (46). Production of CH_4 also indicates the successful degradation of given substrates (bacterial whole-cell or PG) by

the anaerobic community and further investigation of the enriched culture can help us get some useful information about the possible anaerobic bacterial cell degrader.

Hence, variation in methanogenesis was observed from bacterial whole-cell and PG by three different anaerobic communities. In the sludge degrading anaerobic community, the highest CH_4 production was observed (52 and 28 mM) respectively from *M. luteus* peptidoglycan (MLPGSL) and *M. luteus* whole cell (MLSL) enriched culture. The lowest CH_4 (21 and 5 mM) correspondingly was observed from *B. subtilis* peptidoglycan (BSPGSL) and *E. coli* whole-cell (ECSL) enriched culture (Figure 1).

Similarly, in the soil community, the highest CH_4 (56 and 35mM) was detected respectively from the *M. luteus* peptidoglycan (MLPGSO) and *B. kururiensis* whole-cell (BKSO). The lowest CH_4 (17, 5 and 5 mM) was produced respectively from *B. subtilis* peptidoglycan (BSPGSO), *M. luteus* whole cell (MLSO) and *E. coli* whole-cell (ECSO).

In the wastewater enriched anaerobic community, the highest CH_4 (52 and 20 mM) was observed respectively from *E. coli* peptidoglycan (ECPGW) and *B. subtilis* whole cell (BSW). On the other hand, the lowest (20,5 and 5 mM) CH_4 was observed respectively from *B. subtilis* peptidoglycan (BSPGW), *E. coli* whole-cell and *B. kururiensis* whole cell (BKW). However, the highest methanogenesis by three anaerobic communities was observed between 42 to 56 days of the incubation period for all enrichment cultures and CH_4 production dropped after 65 days. The increase in pH might inhibit the growth of methanogens and as a result, CH_4 dropped (46, 79).

Among the three enriched communities' CH_4 production, it was commonly observed that *E. coli* whole cell-enriched culture could not produce more CH_4 than any of the original individual community's CH_4 production. These results also support the CFU count and qPCR results (Figure 2 and 3) where it was observed that *E. coli* DNA stability was consistent during the incubation time in anaerobic condition.

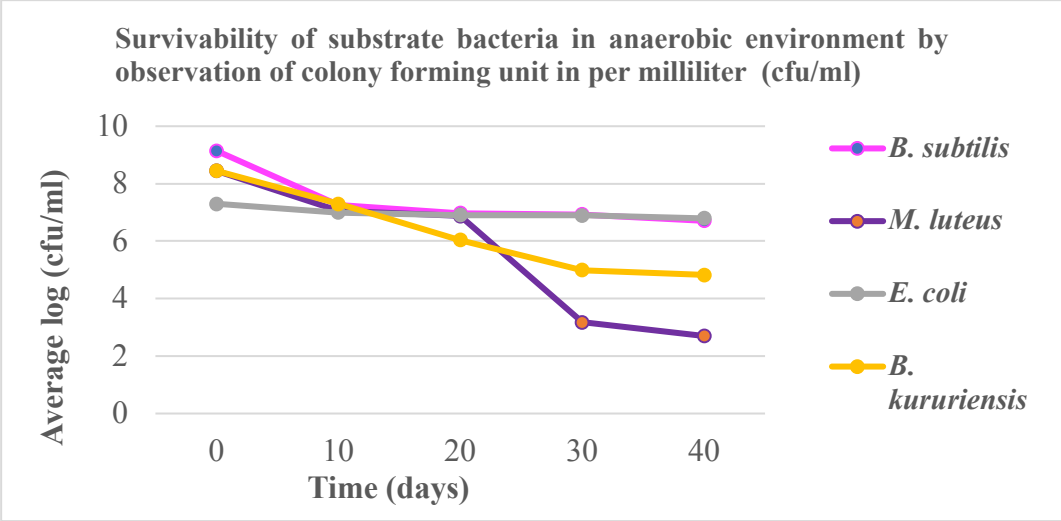


Fig: 2. A graph of log CFU/ml against incubation time showing the effect of extended incubation time separately in anaerobic condition on the survivability of substrate bacteria in WM minimal media.

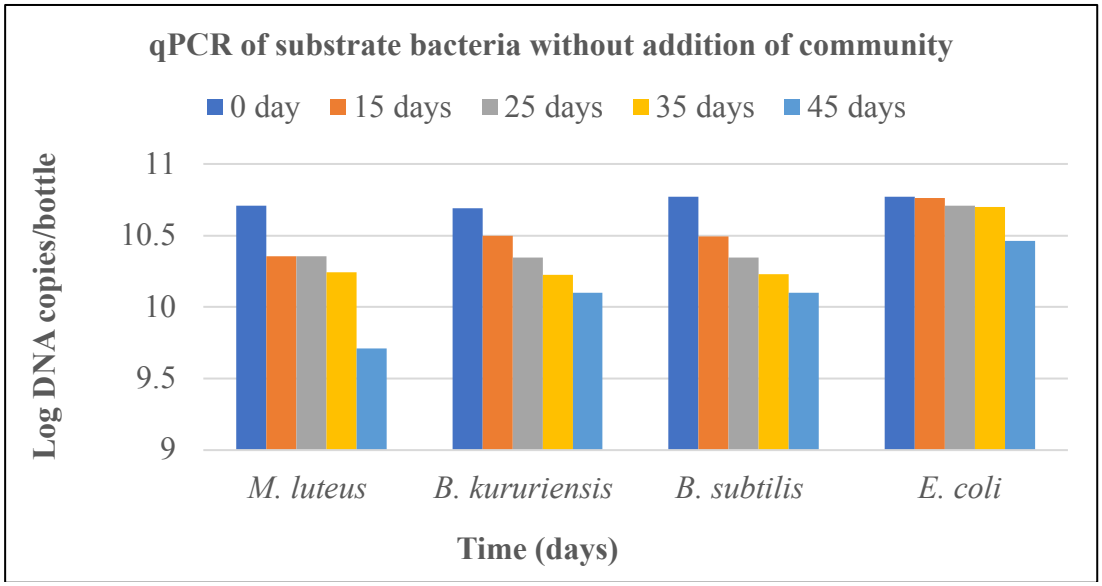


Fig: 3. A graph of log DNA copies/bottle against incubation time showing the effect of extended incubation time separately in anaerobic condition on the survivability of substrate bacteria in WM minimal media by quantitative polymerase chain reaction (qPCR) analysis.

It is assumed that *E. coli* whole-cell was less easily degraded by all the anaerobic community investigated in this study. The possible reason could be the outer membrane layer which is hard to be degraded. This has also been reported previously in some aerobic study (31, 32). It is also assumed that *E. coli* can survive in an anaerobic condition (79) as it is a facultative anaerobe and thus it is possible the whole biomass of the given substrate did not go through the degradation process. So, the following reasons are assumed to explain the *E. coli* survivability; the whole cell of *E. coli* should be damaged or broken to be degraded by the given community but in this study, any kind of physical disruption was not followed to kill the substrate bacteria especially for the whole-cell enrichment to correlate with the natural condition. Contrarily, another gram-negative bacterium (*B. kururiensis*) whole-cell enriched culture showed higher CH_4 production in soil and sludge anaerobic community. It is also noted that the qPCR result showed that the reduction of *B. kururiensis* DNA supports their low survivability in the anaerobic condition which possibly supported them to die and allowed the community to feed on it.

However, this initial study on bacterial cell degradation in anaerobic environment successfully observed methanogenesis from the whole cell and PG by three different anaerobic communities. Though the reason of having these differences was not revealed as I could not analyze the structure of the substrate before using and even after the enrichment culture to speculate the degraded part and the integral part of the substrate bacterial cell. But from this study, it is clear that methanogenesis varies depending on the substrate, especially different cell wall structure. However, it is possible that the given substrate was not degraded completely by the degrader bacteria during the enrichment period in this study (based on visual observation only) as I observed some clotted residues from outside of the culture bottle. It is assumed that the membrane-associated proteins of whole-cell substrate could be a source of methanogenesis with the other part of the substrate bacterial cell wall. This phenomenon was also reported previously in many studies that showed that the membrane-associated proteins are more easily degraded than the other parts of the bacterial cell. In case of PG degradation, one important reason could be the Diaminopimelic acid (DAPA) which may possibly remain (mostly in gram-negative bacteria) even after several days of enrichment and may not be degraded by anaerobic communities in this experiment. For example, though *E. coli* is a gram-negative bacterium but comparing to another gram-negative, their PG is thicker (80). This is also important to clarify that another gram-positive substrate bacteria *B. subtilis* also contain DAPA in their PG even though methanogenesis was observed from the whole cell of *B. subtilis* enriched culture by three community individually.

From the qPCR result analysis, it is observed that the DNA of *B. subtilis* decreased in anaerobic condition (Figure 3) but not like as another gram-positive substrate bacteria (*M. luteus*) in this study. Besides, it cannot be neglected that *B. subtilis* is a spore-forming bacterium and it is formed when their growth conditions become unfavorable (81). During this time, *B. subtilis* undergoes a complex morphological transformation when they can synthesize both thin and thick PG layers like gram-negative bacteria and gram-positive bacteria, respectively (82). The continuous transformations from thick to thin and back to thick during sporulation suggest that the status of a cell may have an effect on the *B. subtilis* whole-cell degradation by the community in the culture bottle. It is thought that some part of the cell can be broken throughout this sporulation formation time and those available broken parts of the cell may serve as a source of the organic compound in the *B. subtilis* whole cell-enriched culture which ultimately let the methanogenesis process in this study. However, the structural differences between a spore-forming cell and a non-spore forming cell of *B. subtilis* could not be investigated in the whole-cell enriched culture in this study. Therefore, further investigation is needed to explain it more clearly.

Additionally, the amount of peptide bond and polysaccharide could be different. However, in both types of (gram-positive and negative) bacteria, PG structure can vary in terms of their length of glycan strands, the cross-linkage position of the peptide bond or interpeptide bridge, integrity of the structure, types of species, position or number of amino acids and the presence of DAPA etc. (83). Structural and quantitative variation of PG also can vary because of the growth condition in the laboratory and natural environments both. Previously it was reported that the PG of a matured cell is much more integrated than the PG of a new cell (84). Hence, the status of PG could be an important factor even in the environmental degradation as in the environment bacteria can die at any stage of their growth and thus PG structure also can vary.

Another possible recalcitrant component could be N-acetyl Glucosamine (NAG) as other studies also reported (85). But in this study, PG (both types of bacteria) components were not measured before and after the enrichment culture as the main purpose was to investigate the methanogenesis from PG degradation in the anaerobic environment and identify the degrader anaerobes. So, further investigations of enrichment cultures using a ^{13}C-labeled particular part of peptidoglycan are required to determine the detailed behaviors of microbial PG degrader.

3.2 Enriched microbial community analysis

Successful CH_4 producing enriched communities were analyzed based on the amplicons of the 16s rRNA gene sequencing by next-generation sequencing to investigate the possible bacterial cell degrader anaerobic microorganisms especially the hydrolytic bacteria who initiate the degradation of whole-cell and PG and proceed with the methanogenesis. 16S rRNA was chosen because it is widely used for taxonomic and phylogenetic studies due to its highly conserved sequences in both bacteria and archaea (reference). However, a total of 58,274 16S rRNA gene readings (3052–5334 per sample) were retrieved and classified into 2376 operational taxonomic units (OTUs) with ≥97% sequence identity cut-off. Original communities individually (as control) were also analyzed to observe the differences between with the addition of substrate and without the addition of substrate. According to a principal component analysis (Figure 4) of the microbial community patterns, the community structures of the triplicate enrichment cultures were very similar (except MLSL, BSSL, MLPGSL, MLPGSO and BKPGSO) and were clearly separated from the original inoculum.

Disintegrated triplicates enriched community might originate during the DNA extraction step. However, in fig. 4A, SL seems close to both BSSL and BKSL enriched culture where another original community SO seems only close to BSSO but far from BKSO. In the case of WW, MLW and BSW showed approximately the same distance from the original WW. In the case of PG enriched community (Fig. 4B), all individual ECPG enriched community seems very far from the original community. All these instances of longer distance of enriched community from the original community, indicate either highly enriched or very functional whole-cell/ PG degrader community.

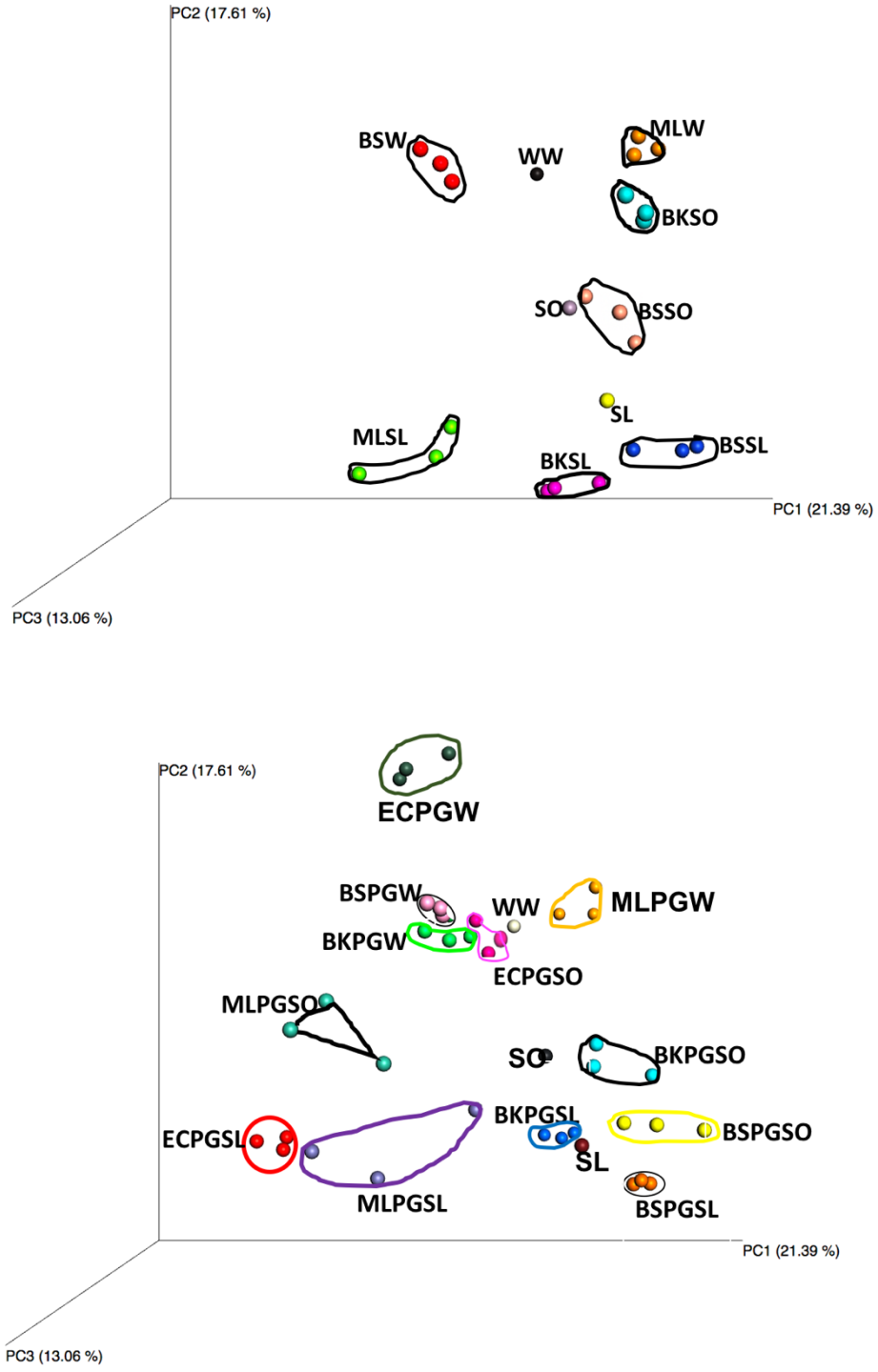


Fig. 4. The result of weighted principal component analysis using all the original communities and substrate enriched community's analysis data. A) Whole cell enriched culture B) Peptidoglycan enriched culture. Triplicate enrichment cultures are enclosed by a circles, triangles and ovals.

i) Phylum based comparisons

>1% of value was considered in at least in one sample of triplicates for this analysis and a phylum-level analysis based on whole-cell (Fig. 5) and PG (Fig. 6) enriched community analysis showed total of 22 phyla which were found inconsistently dominant among all enriched cultures including the individual original community.

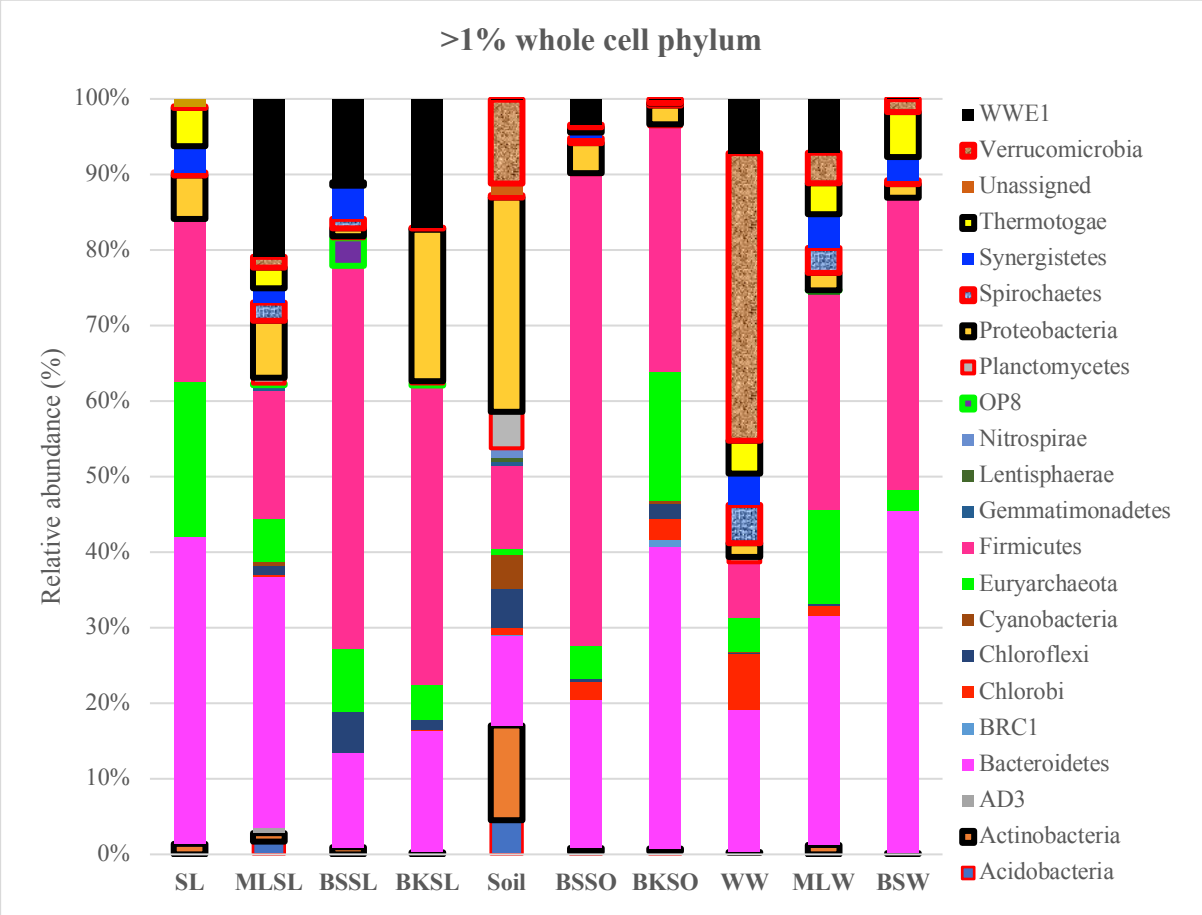


Fig 5: Phylogenetic distribution of the enrichment cultures (and the original microflora) derived from an anaerobic digester sludge (SL), rice paddy soil (SO) and from an artificial anaerobic wastewater treatment system (WW) supplemented with whole cell from *Micrococcus luteus* (ML), *Bacillus subtilis* (BS) and *Burkholderia kururiensis* (BK) at the phylum level. Only the dominant phyla (>1% in at least one condition) are shown. *Escherichia coli* whole enriched culture by three anaerobic community was not analyzed as the enriched culture did not produce higher CH_4 than the individual original community.

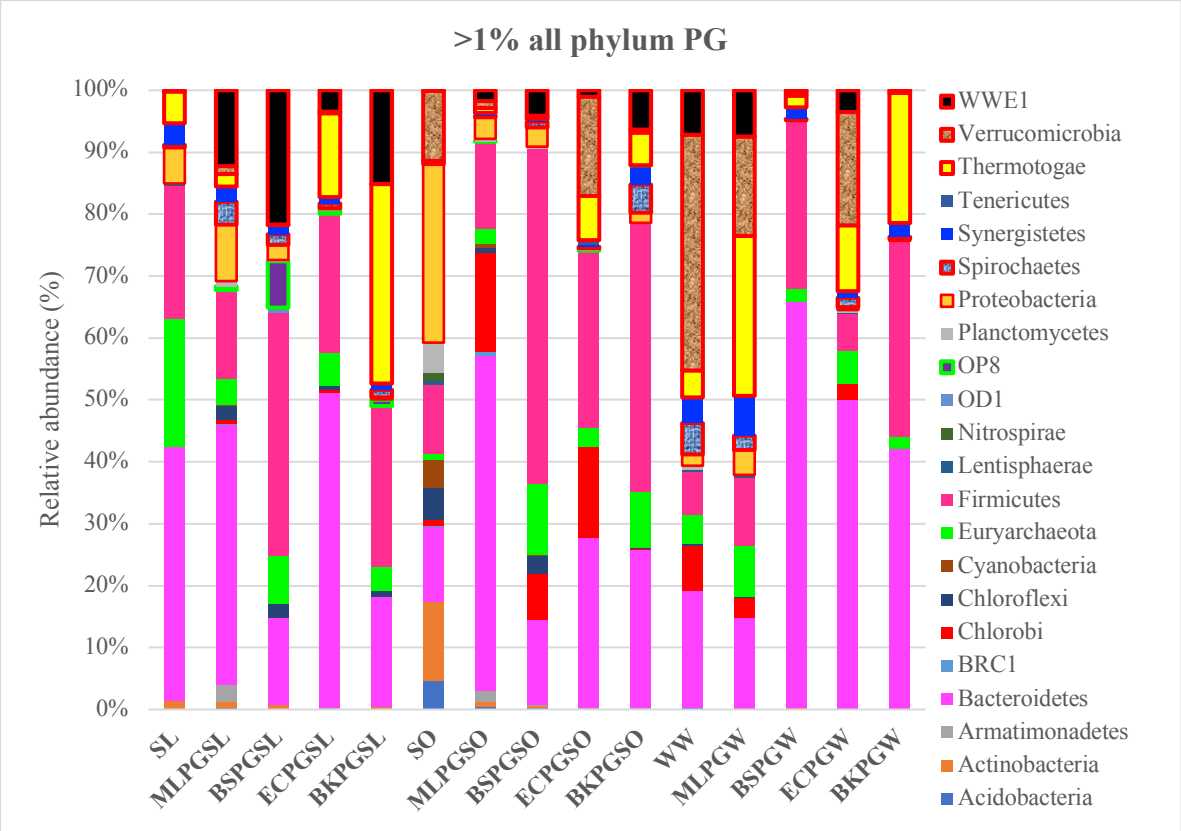


Fig 6: Phylogenetic distribution of the enrichment cultures (and the original microflora) derived from an anaerobic digester sludge (SL), rice paddy soil (SO) and from an artificial anaerobic wastewater treatment system (WW) supplemented with peptidoglycan (PG) extracted from *Micrococcus luteus* (MLPG), *Bacillus subtilis* (BSPG), *Escherichia coli* (ECPG) and *Burkholderia kururiensis* (BKPG) at the phylum level. Only the dominant phyla (>1% in at least one condition) are shown.

In sludge whole-cell enriched community, the following phylum (Bacteroidetes, Euryarchaeota, Firmicutes and WWE1) were observed to be dominant among three enriched communities except for the phylum Proteobacteria which was lower in BSSL comparing to MLSL and BKSL. The entire dominant phylum in the whole-cell enriched community was also dominant in the original sludge (SL) but during enrichment moderately changed in terms of their percentage. In addition, the phylum Thermotogae which was abundant in the original sludge (SL) seemed to be decreased during whole-cell enriched culture except for MLSL. Some phyla seemed very specific only in a particular enriched culture and some are common between two enriched cultures. For example, the phylum OP8 was only abundant in BSSL

and the phylum Spirochaetes was dominant only in MLSL where the phylum Synergestetes was common in both MLSL and BSSL. On the other hand, versatility was seen between two whole-cell enriched soil anaerobic community where a notable decline was observed in case of the phylum Actinobacteria, Planctomycetes, Proteobacteria and Verrucomicrobia during the enrichment. However, phylum Bacteroidetes Chlorobi and Firmicutes were also found dominant in both BSSO and BKSO like sludge anaerobic community. Dissimilarities were seen in the case of phylum Euryarchaeota (which was dominant only in BKSO) and WWE1 (which was dominant only in BSSO. Like other two enriched communities, Bacteroidetes and Firmicutes were also found dominant in wastewater whole-cell enriched community. Besides, other dominant phyla such as phylum Proteobacteria, Synergestetes and Thermotogae were also observed. However, a significant difference was also observed in the case of phylum Euryarchaeota and WWE1 between two different gram-positive substrates bacterial (MLW and BSW) whole-cell enriched community where both of the phyla were higher in MLW and lower in BSW. Among three different anaerobic whole-cell enriched community, it was observed that the phylum Spirochaetes (BSSL and MLW) and the phylum Synergestetes (MLSL and BSSL; MLW and BSW) were mostly dominant in gram-positive bacterial cell wall enriched culture even though it is difficult to demonstrate based on only two gram-positive substrate bacteria. Therefore, more different gram-positive bacteria should be investigated as a substrate. However, some previous studies reported that the phylum Synergestetes is one of the recently investigated phyla which are commonly found in sludge and wastewater anaerobic environment. Besides, most of the species described of this phylum can also produce hydrogen gas from the carbohydrate metabolism and is one of the best candidates for renewable energy production (86). The information on their carbohydrate metabolism and hydrogen production suggest them as a hydrolytic bacterium on bacterial cell substrate and the findings from this study also suggest them specifically as a gram-positive bacterial whole-cell degrader even though further study is needed.

In the case of PG enriched community (Figure 6), Bacteroidetes, Euryarchaeota, Firmicutes and WWE1 were also dominant with some variation among three community. In sludge PG enriched community, some phylum was found dominant unambiguously in the individual community, such as OP8 in BSPGSL; Thermotogae in ECPGSL and BKPGSL and Proteobacteria in MLPGSL and BSPGSL. Comparing to the *B. subtilis* whole-cell enriched sludge community, the phylum OP8 also was only found dominant in BSPGSL. This result indicates that the phylum OP8 may have the ability to be active on *B. subtilis* PG. Some previous studies reported that the phylum OP8 (also called Aminicenantes) was mostly found

in the deep marine and underground water environment (87)where they act as destructors of buried organic matter and produce hydrogen and acetate which may support the methanogens for producing CH_4 in the anaerobic digestion and even in this study. Moreover, this phylum was not reported before on bacterial whole-cell and PG degradation. In the PG enriched soil community, Bacteroidetes and Firmicutes were also found dominant similarly like PG enriched sludge community with some distinction. Moreover, the following phylum such as Thermotogae (in ECPGSO and BKPGSO) and Verrucomicrobia (in ECPGSO) were only dominant specifically on gram-negative bacterial PG enriched soil community. The phylum Chlorobi was observed dominantly in both whole-cell and PG enriched soil community except the BKPGSO. Moreover, some phylum (Cyanobacteria, Planctomycetes, Proteobacteria and Veruucomicrobia (except ECPG) distinctively were decreased during enrichment comparing to the original soil community (SO). However, Bacteroidetes and Firmicutes (except ECPGW) were also observed as the most abundant phylum similarly in wastewater PG enriched community. Additionally, the phylum Euryarchaeota was also found dominant but mostly in MLPGW and ECPGW which may indicate the higher CH_4 production from these two enriched cultures compared to BSPGW and BKPGW in wastewater community (Figure 1). Apart from this, phylum such as Chlorobi, Spirochaetes, Synergistetes and Verrucomicrobia were similarly found dominant in all PG enriched wastewater community.

All these dominant phylum variations in both whole-cell and PG enriched different anaerobic community also support dissimilar CH_4 production among all enriched culture and support their possible involvement in the bacterial cell wall and PG degradation.

ii) OTU based/ Species-level comparisons

To demonstrate the possible degrader bacteria, individual OTU of the enriched community was also analyzed. Thus, >5% of cut off value was considered in at least one of the replicates and all phylotypes are summarized in figure 7 and 8.

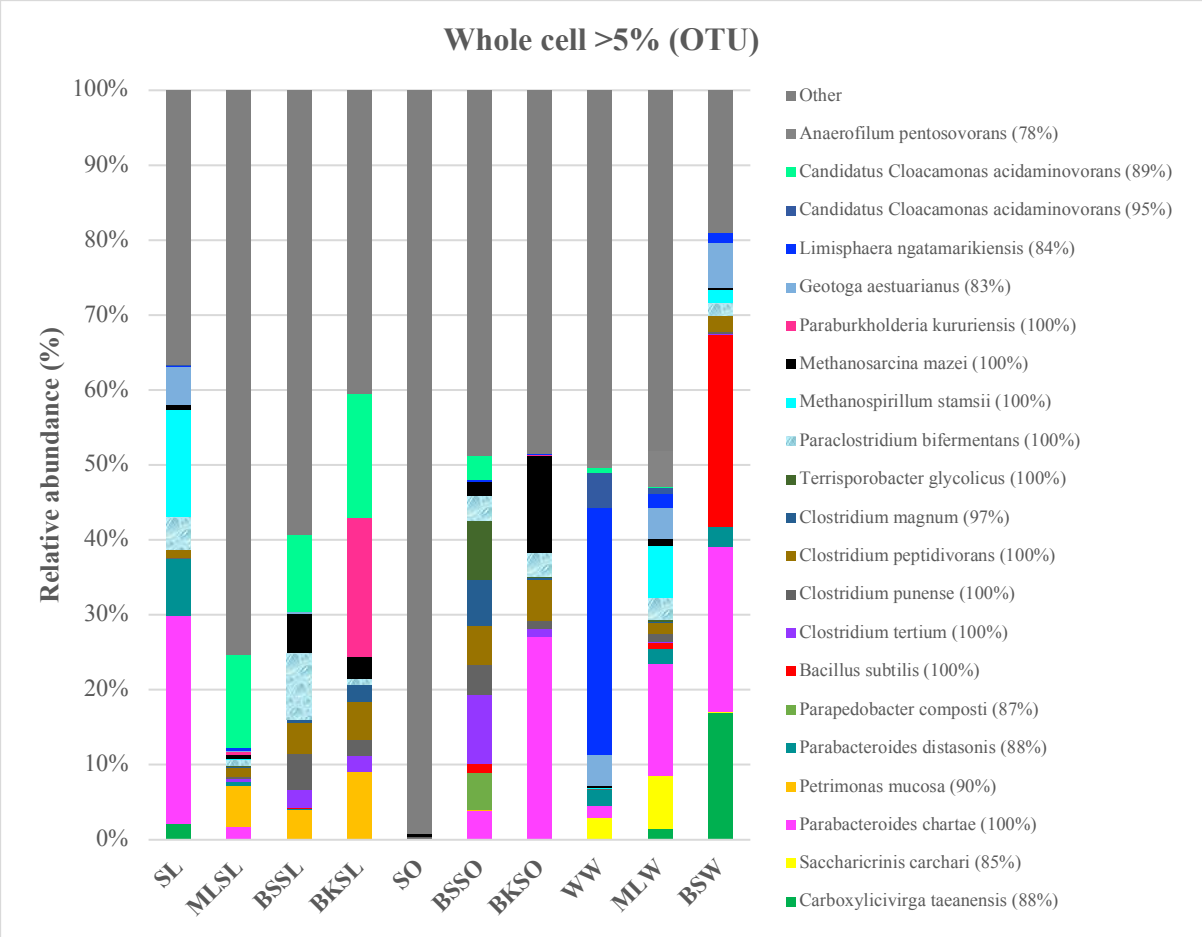


Fig. 7. Phylogenetic distribution of the enrichment cultures OTUs (and the original microflora) derived from an anaerobic digester sludge (SL), rice paddy soil (SO) and from an artificial anaerobic wastewater treatment system (WW) supplemented with whole cell from *Micrococcus luteus* (ML), *Bacillus subtilis* (BS) and *Burkholderia kururiensis* (BK) at the species level. OTUs >5% in at least one replica were used to make the graph and are shown here.

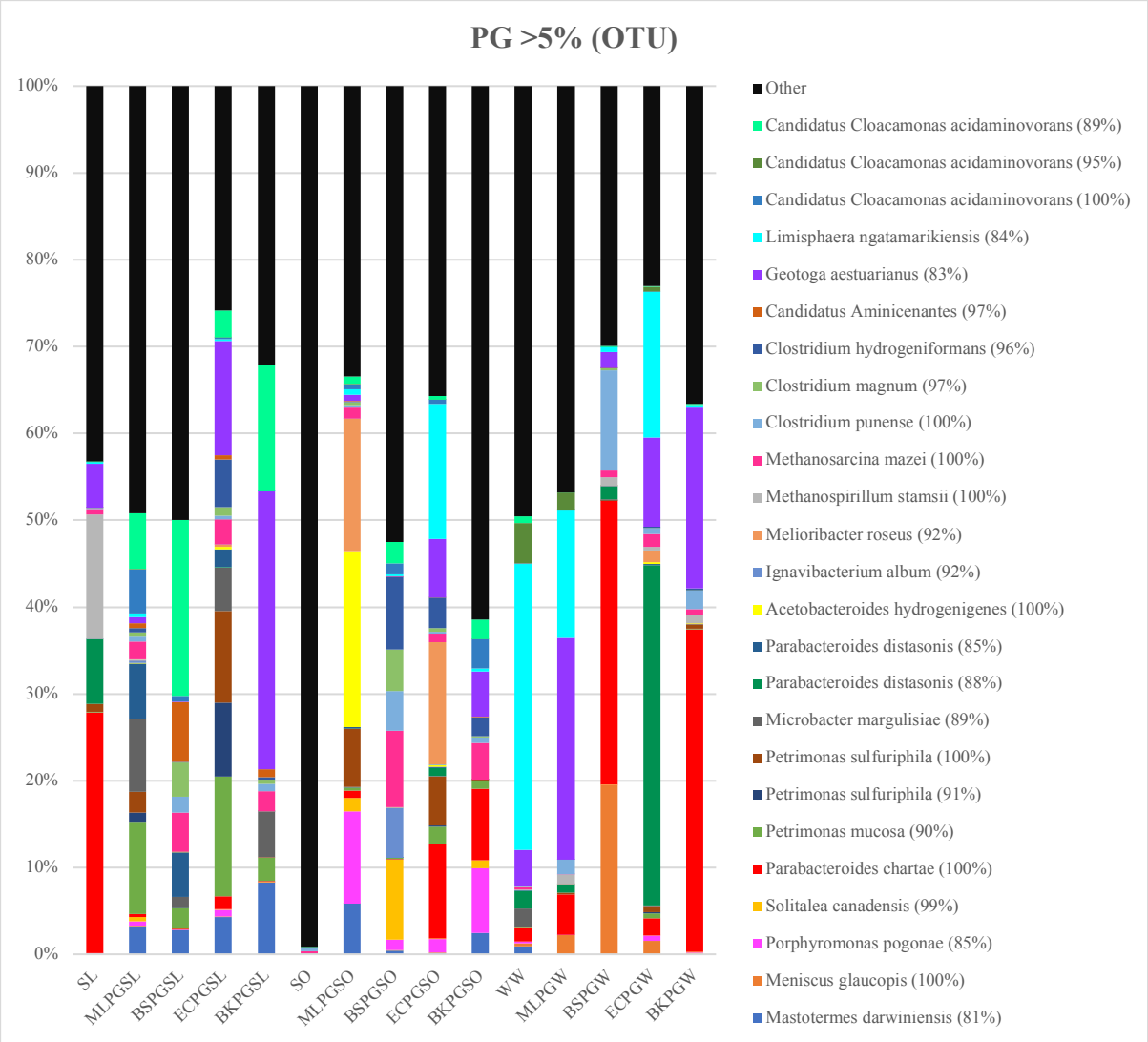


Fig. 8. Phylogenetic distribution of the enrichment cultures OTUs (and the original microflora) derived from an anaerobic digester sludge (SL), rice paddy soil (SO) and from an artificial anaerobic wastewater treatment system (WW)supplemented with peptidoglycan (PG) extracted from *Micrococcus luteus* (MLPG), *Bacillus subtilis* (BSPG), *Escherechia coli* (ECPG) and *Burkholderia kururiensis* (BKPG) at the species level. OTUs >5% in at least one replica were used to make the graph and are shown here.

Table 1: Phylogenetic distribution of the enrichment cultures OTUs (and the original microflora) derived from an anaerobic digester sludge (SL), rice paddy soil (SO) and from an artificial anaerobic wastewater treatment system (WW) supplemented with whole cell from *Micrococcus luteus* (ML), *Bacillus subtilis* (BS) and *Burkholderia kururiensis* (BK) at the species level. OTUs >5% in at least one replica were used to make the graph and are shown here.

Blast bacteria (Identity %)	SL	ML SL	BS SL	BK SL	SO	BS SO	BK SO	WW	ML W	BS W	Phylum
Carboxylicivirgataeanensis (88%)	2	0	0	0	0	0	0	0	1	17	Bacteroidetes
Saccharicrinis carchari (85%)	0	0	0	0	0	0	0	3	7	0	Bacteroidetes
Parabacteroides chartae (100%)	28	1	0	0	0	4	27	2	15	22	Bacteroidetes
Petrimonas mucosa (90%)	0	6	4	9	0	0	0	0	0	0	Bacteroidetes
Parabacteroides distasonis (88%)	7	1	0	0	0	0	0	2	2	3	Bacteroidetes
Parapedobactercomposti (87%)	0	0	0	0	0	5	0	0	0	0	Bacteroidetes
Bacillus subtilis (100%)	0	0	0	0	0	1	0	0	1	26	Firmicutes
Clostridium tertium (100%)	0	0	2	2	0	9	1	0	0	0	Firmicutes
Clostridium punense (100%)	0	0	5	2	0	4	1	0	1	0	Firmicutes
Clostridium peptidivorans (100%)	1	1	4	5	0	5	5	0	2	2	Firmicutes
Clostridium magnum (97%)	0	0	0	2	0	6	0	0	0	0	Firmicutes
Terrisporobacterglycolicus (100%)	0	0	0	0	0	8	0	0	0	0	Firmicutes
Paraclostridiumbifermentans (100%)	4	1	9	1	0	3	3	0	3	2	Firmicutes

Methanospirillumstamsii (100%)	14	0	0	0	0	0	0	0	7	2	Euryarchaeota
Methanosarcinamazei (100%)	1	1	5	3	0	2	13	0	1	0	Euryarchaeota
Paraburkholderiakururiensis (100%)	0	0	0	18	0	0	0	0	0	0	Proteobacteria
Geotogaaestuarianus (83%)	5	0	0	0	0	0	0	4	4	6	Thermotogae
Limisphaerangatamarikiensis (84%)	0	0	0	0	0	0	0	33	2	1	Verrucomicbia
CandidatusCloacamonasacidaminovorans (95%)	0	0	0	0	0	0	0	5	1	0	WWE1
CandidatusCloacamonasacidaminovorans(89%)	0	12	10	17	0	3	0	1	0	0	WWE2
Anaerofilumpentosovorans (78%)	0	0	0	0	0	0	0	1	5	0	WWE3
Other	37	75	59	40	99	49	49	49	48	19	Other

Table: 2. Phylogenetic distribution of the enrichment cultures OTUs (and the original microflora) derived from an anaerobic digester sludge (SL), rice paddy soil (SO) and from an artificial anaerobic wastewater treatment system (WW)supplemented with peptidoglycan (PG) extracted from *Micrococcus luteus* (MLPG), *Bacillus subtilis* (BSPG), *Escherechia coli* (ECPG) and *Burkholderia kururiensis* (BKPG) at the species level. OTUs >5% in at least one replica were used to make the graph and are shown here.

Blast bacteria identity (%)	SL	MLPGSL	BSPGSL	ECPGSL	BKPGSL	SO	MLPGSO	BSPGSO	ECPGSO	BKPGSO	WW	MLPGW	BSPGW	ELPGW	BKPGW	Phylum
Mastotermesdarwiniensis (81%)	0	3	3	4	8	0	6	0	0	2	1	0	0	0	0	Bacteroidetes
Meniscus glaucopis (100%)	0	0	0	0	0	0	0	0	0	0	0	2	19	2	0	Bacteroidetes
Porphyromonaspogonae (85%)	0	1	0	1	0	0	11	1	2	7	0	0	0	1	0	Bacteroidetes
Solitalea canadensis (99%)	0	0	0	0	0	0	2	9	0	1	0	0	0	0	0	Bacteroidetes
Parabacteroides chartae (100%)	28	0	0	2	0	0	1	0	11	8	2	5	33	2	37	Bacteroidetes
Petrimonas mucosa (90%)	0	11	2	14	3	0	0	0	2	1	0	0	0	1	0	Bacteroidetes
Petrimonassulfuriphila (91%)	0	1	0	9	0	0	0	0	0	0	0	0	0	0	0	Bacteroidetes
Petrimonassulfuriphila (100%)	1	2	0	11	0	0	7	0	6	0	0	0	0	1	1	Bacteroidetes
Microbacterm argulisiae (89%)	0	8	1	5	5	0	0	0	0	0	2	0	0	0	0	Bacteroidetes
Parabacteroides distasonis (88%)	7	0	0	0	0	0	0	0	1	0	2	1	2	39	0	Bacteroidetes
Parabacteroides distasonis (85%)	0	6	5	2	0	0	0	0	0	0	0	0	0	0	0	Bacteroidetes
Acetobacteroideshydrogenigenes (100%)	0	0	0	0	0	0	20	0	0	0	0	0	0	0	0	Bacteroidetes
Ignavibacterium album (92%)	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	Chlorobi
Melioribacter roseus (92%)	0	0	0	0	0	0	15	0	14	0	0	0	0	1	0	Chlorobi

Methanospirillumstamsii (100%)	14	0	0	0	0	0	0	0	0	0	0	1	1	0	1	Euryarchaeota
Methanosarcinamazei (100%)	1	2	5	3	2	0	1	9	1	4	0	0	1	1	1	Euryarchaeota
Clostridium punense (100%)	0	1	2	0	1	0	0	5	0	1	0	2	12	1	2	Firmicutes
Clostridium magnum (97%)	0	0	4	1	0	0	0	5	0	0	0	0	0	0	0	Firmicutes
Clostridium hydrogeniformans (96%)	0	1	0	5	0	0	0	8	4	2	0	0	0	0	0	Firmicutes
CandidatusAminicenantes (97%)	0	1	7	1	1	0	0	0	0	0	0	0	0	0	0	OP8
Geotogaaestuarianus (83%)	5	1	0	13	32	0	1	0	7	5	4	26	2	10	21	Thermotogae
Limisphaeragatamarikiensis (84%)	0	0	0	0	0	0	1	0	16	0	33	15	1	17	0	Verrucomicrobia
CandidatusCloacamonasacidaminovorans (100%)	0	5	1	0	0	0	1	1	0	3	0	0	0	0	0	WWE1
CandidatusCloacamonasacidaminovorans (95%)	0	0	0	0	0	0	0	0	0	0	5	2	0	1	0	WWE2
CandidatusCloacamonasacidaminovorans (89%)	0	6	20	3	14	0	1	2	0	2	1	0	0	0	0	WWE3
Other	43	49	50	26	32	99	33	52	36	61	50	47	30	23	37	Other

Significant differences were observed between control and enriched cultures in three distinct anaerobic communities (Table 1 and 2), which indicate the effect of different substrate utilization and these differences also supported the variation of distinctive CH_4 production (Figure 1). Single OTU investigation also revealed that many unknown bacteria (with low identity to known bacteria) are involved in both whole-cell and PG enriched community.

However, the species-level analysis also revealed that the phylum Bacteroidetes and Firmicutes are also dominant in both whole-cell and PG enriched culture like the phylum level analysis among three anaerobic communities. Both phyla were also reported before on the biomass polymers degradation (88, 89, 90). Therefore, the phylum Bacteroidetes and Firmicutes are considered to contribute mainly to the fermentation of bacterial cell biomass for the first time through this investigation. This is also needed to inform that the phylogenetically novel species from the phylum, Bacteroidetes (>5%) was found dominant especially in PG enriched culture in three anaerobic community and indicate their specific functional activity on PG degradation comparing to another dominant phylum. Bacteroidetes was also reported previously on several carbohydrates and suggest them as fermenting bacteria (88, 89, 90). For example, the phylum Bacteroidetes often resides in human and animal intestines so that they exhibit symbiotic relationship with other species. Most of the species of this phylum are also involved in the fermentation of dietary polysaccharides and other substrates in different environments and obtain carbon and energy through the hydrolysis of carbohydrates (91, 92, 93). So, abundance of the phylum Bacteroides in this study highly suggests their hydrolytic activity on bacterial cell and passes the metabolites (H_2, CO_2 and Acetate) to the downstream microbial community which may help for the observed methanogenesis. Besides, the phylum Bacteroidetes, phylum Firmicutes especially the genus Clostridium were also found dominant with higher identity in both whole-cell and PG enriched community. Many earlier studies also conveyed that the genus Clostridium are capable of a direct cellulosic substrate conversion into ethanol with high efficiency and are a good candidate for the degradation of cellulosic materials from plant biomasses (94, 95, 96). Similarly, Clostridium can be also found in manure-treated soil and animal faeces (95), and some grow anaerobically using ethanol and acetate as sole energy sources to produce butyrate, caproate, and H_2 (97). In the natural soil anaerobic environment, Clostridium was also identified from canal mud and are capable of producing the same by-products (ethanol, acetate, CO_2, and H_2) through fermentation (94, 95, 96, 97). Therefore, in this study abundance of both phyla (Bacteroides and Firmicutes) highly suggest their hydrolytic activity on bacterial cell and passing the metabolites (H_2, CO_2 and Acetate) to the downstream microbial community which may be helpful for the observed methanogenesis. However, it also important to mention that some dominant OTUs of enriched culture were classified into phyla with no or only a few isolated strains, namely candidate or rare phyla, respectively (Figure 7 and 8; table 1 and 2). For example, WWE1 is a candidate phylum with no isolated strain. Although WWE1 bacteria are frequently found in various anaerobic environments, in

particular anaerobic digesters (98, 99), their function is still unknown. Two OTUs in the phylum WWE1, OTU6988 and OTU16779 related to related to '*Candidatus Cloacamonas acidaminovorans*' with 100% and 89% identity, respectively, were dominantly detected from all sludge enrichment cultures supplemented with PG. The OTU16779 with 89% identity was also found dominant in whole-cell enriched sludge community, suggesting their involvement in both cell wall and PG degradation.

Another OTU6882 related to '*Limisphaera ngatamarikiensis*' with 84% belong to the phylum Verrucomicrobia which is also a rare phylum. This OTU was found dominant in ECPGSO, MLPGW and ECPGW including the original wastewater community (WW). However, this rare phylum has been dominantly detected also in anaerobic soil and digester sludge (89, 100). Although most Verrucomicrobia isolates are obligate or facultative aerobes, several anaerobic species have also been isolated. Some isolates were also detected from human faeces, and fermentatively degrades mucin (glycosylated proteins produced by animals) under anaerobic conditions. The substrate utilization of this rare phylum in this study supports them to be in the group of hydrolytic bacteria and their possible function on polymer/ protein degradation derived from a bacterial cell. Another phylum Chlorobi, especially the family Ignavibacteriaceae, was found abundant in species-level mainly in PG enriched soil community (MLPGSO, BSPGSO and ECPGSO). Specifically, OTU15478 related to *Melioribacter roseus* (92%) was 15% in MLPGSO and 14% in ECPGSO while OTU2500 related to *Ignavibacterium album* (92%) was 6% in BSPGSO. However, Vitaly et al. (101) reported that Ignavibacteriaceae have the ability to use ferric iron as an electron acceptor; that is, the microorganism of Ignavibacteriaceae has a potential iron-reducing function. Besides, Ignavibacteriaceae is often found in the microbial layer formed on the surface of plants in aquatic environments and its carbon source often comes from debris that has fallen off the plant, which means that the microbes in this group may have the ability to use plant biomass-type organic carbon directly or its decomposition products for growth and reproduction (102, 103). Since this family was found mostly in soil-enriched samples, the family Ignavibacteriaceae may have the ability to degrade the plant-associated bacterial biomass in soil anaerobic environment. Hence, further investigation on this is needed to explain more elaborately. However, certain OTUs seem to be changed rapidly comparing to their original communities during the whole cell and PG enrichment period. For example, OTU10961 related to *Parabacteroides chartae* (100%) was 28% in the original sludge community which was drastically decreased during all PG enrichment culture in sludge anaerobic community. Conversely, OTU10961 increased in the PG enriched soil (ECPGSO-

11% and BKPGSO-8%) and wastewater (MLPGW-5% and BSPGW-33%) community even though it was very lower in the original community. Another OTU10827 related to *Methanospirillum stamsii* (100%) was also dominant (14%) in the original sludge (SL) but decreased during whole-cell and PG enriched sludge community. Even though the identity of those particular OTUs were higher, the reduction indicates the nonfunctional activity of the particular OTU specifically on specific substrate utilization. Apart from this, OTU19500 related to *Geotoga aestuarianus* (83%) from the phylum Thermotogae was one of the dominants OTUs in the original sludge community which decreased specifically in the whole-cell enriched sludge community but conversely increased in PG enriched sludge community especially in ECPGSL-13% and BKPGSL-32%. The same OTU (OTU19500) was also found dominant in ECPGSO-7%, BKPGSO-5%, MLPGW-26%, ECPGW-10% and BKPGW-21%. This significant dominance indicates OTU19500 as expressly functional on the bacterial cell especially on gram-negative bacterial PG in sludge and soil in both communities. Hence, it is assumed that OTU19500 might be a PG degrader and depending on the environment and substrate, a particular bacterium can act differently. OTU19500 was also found in whole-cell enriched wastewater (4% in MLW and 6% in BSW) community which is contradictory to/with PG enriched community analysis as they showed dominant in the gram-positive whole-cell as like as gram-negative PG. So, it is assumed that OTU19500 may be also involved in both parts of substrate bacterial cell depending on the environment. On the other hand, OTU6292 related to *Parabacteroides distasonis* (88%) seems especially functional only on gram-positive bacterial (MLPGSL and 5% in BSPGSL) PG in sludge community. Therefore, from this study, it seems that OTU19500 and OTU6292 have the ability to degrade gram-negative and positive bacterial PG respectively.

Some OTUs appear particularly in the different whole-cell enriched culture among three anaerobic community even though most of them have a very low identity to known bacteria. Specifically, OTU14053 related to *Saccharicrinis carchari* (85%) was 7% in MLW, OTU13415 related to *Carboxylici virgataeanensis* (88%) was 17% in BSW, OTU13237 related to *Parapedobacter composti* (87%) was 5% in BSSO, OTU13721 related to *Clostridium magnum* (97%) was 6% in BSO, OTU17419 related to *Terrisporobacter glycolicus* (100%) was 8% in BSO, OTU17047 related to *Anaerofilum pentosovorans* (78%) was 5% in MLW, OTU9476 related to *Clostridium tertium* (100%) was 9% in BSSO and OTU10827 related to *Methanosarcina mazei* (100%) was 7% in MLW. These OTUs may have the ability to be functional on that particular bacterial cell wall part or in the membrane protein part but isolation is needed to clarify their function. One exception was observed in

the whole-cell enriched culture. For example, OTU7941 related to *Paraburkholderia kururiensis* (100%) was very dominant in BKSL (18%), even though it was not considered in the dominant enriched list as it was assumed that the OTU7941 might originate from the given substrate bacteria. This assumption was also supported by the qPCR result (Figure 3). However, based on the amplicon sequence analysis it is very difficult to define the actual number of intact cells and damaged cells in the enriched culture. Still, considering the cut off value >5%, some OTUs were observed commonly in between or among three whole-cell enriched communities with some specification and this observation indicates them as a versatile degrader. Specifically, OTU2525 related to *Clostridium punense* (100%) was 5% in BSL, 2% in BKSL, 4% in BSO, 1% in BKSO and 1 % in MLW; OTU12885 related to *Clostridium peptidivorans* (100%) was 1% in MLSL, 4% in BSSL, 5% in BKSL, 5% in BSSO, 5% in BKSO, 2% in MLW and 2% in BSW; OTU4868 related to *Paraclostridium bifermentans* (100%) was 1% in MLSL, 9% in BSSL, 1% in BKSL, 3% in BSSO, 3% in BKSO, 3% in MLW and 2% in BSW. However, some OTUs were also found dominant specifically in individual PG enriched culture among three communities. For example, OTU14862 related to *Meniscus glaucopis* (100%) was 19% in BSPGW, OTU2516 related to *Solitalea canadensis* 99% was 9% in BSPGSO, OTU13461 related to *Petrimonas sulfuriphila* (91%) was 9% in ECPGSL, OTU3027 related to *Parabacteroides distasonis* (88%) was 39% in ECPGW (though this OTU was also dominant in the original sludge but decreased during PG enriched sludge), OTU15460 related to *Acetobacteroides hydrogenigenes* (100%) was 20% in MLPGSO, OTU2500 related to *Ignavibacterium album* (92%) was 6% in BSPGSO, and OTU14828 related to *Candidatus aminicenantes* (97%) was 7% in BSPGSL. This information will help us with future investigation especially of those OTUs which have a higher identity and it may give some additional information to know the detail of the specific (PG) substrate degradation.

Some OTUs were found commonly in both soil and wastewater community enriched by PG. For example, OTU10961 related to *Parabacteroides chartae* (100%) was 11% in ECPGSO, 8% in BKPGSO, 5% in MLPGW, 33% in BSPGW and 37% in BKPGW. Differently, some OTUs were found particularly versatile in a certain community. Specifically, OTU12323 related to *Petrimonas mucosa* (90%) was 6% in MLSL, 4% in BSSL and 9% in BKSL where OTU16779 related to *Candidatus Cloacamonas acidaminovorans* (89%) was 12% in MLSL, 10% in BSSL and 17% in BKSL. These different activities indicate them as a versatile whole-cell degrader in sludge enriched community. On the other hand, OTU10961 related to *Parabacteroides chartae* (100%) seems dominant in both soil

(BSSO- 4%, BKSO- 27%) and in wastewater (MLW- 15% and BSW- 22%) enriched whole-cell community. Diversely, OTU15478 related to *Melioribacter roseus* (92%) (MLPGSO- 15% and ECPGSO- 14%) seems a versatile degrader only in PG enriched soil anaerobic community and OTU19500 related to *Geotoga aestuarianus* (83%) was dominant in whole-cell enriched wastewater (MLW4%- and BSW 6%) community though it was originally dominant (4%) in original wastewater community (WW). These versatilities indicate that these OTUs are commonly involved on either bacterial whole-cell or PG degradation in different anaerobic communities.

From the above whole-cell and PG enriched community analysis, it was observed that both types of the substrate from *B. subtilis* bacteria can enrich very different community than the other substrates enriched community. OTU2525 related to *Clostridium punense* (100%) was dominant in both whole-cell (BSSL-5% and BSSO- 4%) and PG (BSPGSO- 5% and BSPGW- 12%) enriched three anaerobic communities. Another OTU13721 related to *Clostridium magnum* (97%) was (BSPGSL-4%, BSPGSO- 5% and BSSO-6%) also only dominant in a particular *B. subtilis* enriched culture. Therefore, it is anticipated that those dominant OTUs from the whole cell and PG enriched communities may be specialized on *B. subtilis* whole-cell protein or cell wall other parts or PG degrader and in case of *B. subtilis* whole-cell enriched culture, some of them may be *B. subtilis* spore degrader. Differentiation between a spore-forming and non-spore forming *B. subtilis* cell along with different PG structure, enriched community investigation could help us to explain in more detail about it. However, two OTUs were observed commonly as a versatile PG degrader in sludge and soil anaerobic community. OTU16549 related to *Petrimonas sulfuriphila* (100%) was 11% in ECPGSL, 7% in MLPGSO and 6% in ECPGSO; and OTU6988 related to *Candidatus Cloacamonas acidaminovorans* (100%) was 5% in MLPGSL and 6% in ECPGSO. These higher identities OTUs could be supportive of further PG degradation investigation in the Bacteroidetes and WWE1 phylum, respectively. OTUs from the phylum Bacteroidetes were found specifically dominant in sludge anaerobic community enriched by PG. OTU58 related to *Mastotermes darwiniensis*(81%) was 3% in MLPGSL, 3% in BSPGSL, 4% in ECPGSL and 8% in BKPGSL; OTU12323 related to *Petrimonas mucosa* (90%) was 11% in MLPGSL, 2% in BSPGSL, 14% in ECPGSL and 3% in BKPGSL; OTU12300 related to *Microbacter margulisiae* (89%) was 8% in MLPGSL, 1% in BSPGSL, 5% in ECPGSL and 5% in BKPGSL; OTU16779 related to *Candidatus Cloacamonasacid aminovorans* (89%) was 6% in MLPGSL, 20% in BSPGSL, 3% in ECPGSL and 14% in BKPGSL, though it seems that OTU58 and OTU12323 were also dominant in the whole-cell enriched culture of sludge

anaerobic community. This result indicates that these OTUs are capable of functioning on both whole-cell and PG. Some OTUs were found commonly in PG enriched soil and wastewater community. For example, OTU15113 related to *Porphyromonas pogonae* (85%) was abundant (11% in MLPGSO, 1% in BSPGSO, 2% in ECPGSO and 7% in BKPGSO) only in the PG enriched soil community mostly where OTU10961 related to *Parabacteroidetes chartae* 100% found commonly in both soil (ECPGSO- 11% and BKPGSO- 8%) and wastewater (MLPGW- 5%, BSPGW- 33%, ECPGW- 2% and BKPGW- 37%) community as a versatile. However, OTU10961 was also observed dominantly in whole-cell enriched soil (BSSO- 4% and BKSO- 27%) and wastewater (MLW- 15% and BSW- 22%) community which support them as functional on both side (Cell wall and PG) of those specific bacterial cells. Some OTUs were found commonly in between or among three PG enriched communities; specifically OTU16549 related to *Petrimonas sulfuriphila* 100% (ECPGSL- 11%, MLPGSO- 7% and ECPGSO- 6%); OTU13190 related to *Methanosarcina mazei* 100% (BSPGSL-5% and BSPGSO- 9%); OTU2525 related to *Clostridium punense* 100% (BSPGSO- 5% and BSPGW- 12%) and OTU14464 related to *Clostridium hydrogeniformans* 96% (ECPGSL- 5% and BSPGSO-8%); OTU6882 related to *Limiphaera ngatamarikiensis* 84% (ECPGSO-16%, MLPGW-15% and ECPGW-17%); OTU19500 related to *Geotoga aestuarianus* 83% (ECPGSL-13%, BKPGSL-32%, ECPGSO-7%, BKPGSO- 5%, ECPGW- 10% and BKPGW- 21%). These diverse activities of these particular OTU in different anaerobic environments indicate their strong participation in bacterial cell degradation. Also, between whole-cell and PG enriched three communities, some OTUs were found common which indicate both parts of that particular bacterial cell degrader; specifically, OTU10961 related to *Parabacteroides chartae* 100% (MLW- 15% and MLPGW- 5%; BSW-22% and BSPGW- 33%; BKSO- 27% and BKPGSO-8%) and OTU13721 related to Clostridium magnum 97% (BSSO-6% and BSPGSO-5%).

Besides, one methanogen was also found dominant in both sludge and soil enriched community in case of both types (whole-cell and PG) of substrate enriched community. OTU13190 related to *Methanosarcina mazei* (100%) was 5% in BSSL, 3% in BKSL, 13% in BKSO, 5% in BSPGSL, 3% in ECPGSL, 2% in BKPGSL, 1% in MLPGSO, 9% in BSPGSO, 1% in ECPGSO and 4% in BKPGSO. Further information and investigation on this methanogen (*Methanosarcina mazei*) could help us to understand the pathway of methanogenesis used on bacterial cell degradation although previously it was reported that *Methanosarcina mazei* had versatile metabolic activity on $H2/CO_2$, acetate, all methylamines, and methanol (104). However, it is very difficult to explain the pathway they have followed

for the methanogenesis from the given bacterial cell substrate, because from this the study it is detected that the different part of the given bacterial substrate could enrich different microbial community which obviously will have an effect on the survivable methanogen and eventually different pathway could be followed. Even though the abundance of OTUs (10827 and 13190) from the phylum Euryarcheota was comparatively low, the investigation of the phylum (especially the family *Methanospirillaceae*) could help us to understand the pathway of methanogenesis from bacterial cell degradation because they have higher identities of these two OTUs. For example, *Methanosarcina* includes many methanogens whose metabolic features are diverse and include both acetotrophic and hydrogenotrophic pathways. In particular, some strains in this genus are capable of utilizing methanol (105). Furthermore, most *Methanosarcina* are immotile and mesophilic, exhibiting multiple metabolic features with a strong advantage in survival. It is proposed that methanol is one of the major factors that influence methanogenesis (106). In sewage treatments, there are approximately 60% of the total mass containing complex organic matters and products of hydrolysis and acidogenesis which are most likely multiple since members of order Methanosarcinales have the widest substrate range among methanogens (107). The dominance of Methanosarcina demonstrates the relatively abundant nutrient sources and various metabolic pathways within this study's fermentation system. Therefore, both pathways of methanogenesis can be followed in this study for three communities. In this study, *E. coli* whole-cell enriched community could not produce higher CH_4 than the original community's CH_4 production, therefore it was excluded from the community analysis. However, investigation of that *E. coli* whole-cell enriched community could help us to compare the differences between gram-positive and gram-negative cell wall more strongly if there was any. From the community analysis, it is also anticipated that all the dominant OTUs with a high and low identity which were dominant in the individual enriched culture could be involved in the downstream reactions, such as, fermentation of monosaccharides and amino acids, oxidation of fermentation products such as organic acids, and methanogenesis. However, the cell wall structure of used four different substrate bacteria was not analyzed before starting the enrichment culture. Therefore, it is difficult to explain which part of the substrate bacterial whole-cell was degraded and what was the recalcitrant part which tend to remain after the 60 days of enrichment period. Therefore, additional analysis such as stable isotope probing analysis using ^{13}C-labeled whole-cell/ PG, identification of whole-cell/ PG hydrolases by metaproteomic coupled with activity staining and isolation of degraders will

contribute to the identification of both part degraders (whole-cell and PG) and elucidation of their molecular mechanisms.

Ordinarily, bacteria use their extracellular enzymes for their own cell wall (especially PG) hydrolysis for making a new cell where several enzymes are involved to complete the process. During PG hydrolysis, each enzyme has a specific function at a particular place of the cell wall PG. For example, cell wall amidase cleaves the amide bond between N-acetylmuramic acid and L-alanine residue at the N-terminal of the stem peptide, cell wall glycosidase catalyzes the hydrolysis of the glycosidic linkages, while cell wall peptidase cleaves amide bonds between amino acids within the PG chain (12, 23, 24). Consequently, it is assumed that the same kinds of enzymes are also involved in other bacterial cell degradation even though there is no scientific report yet on this hypothesis. However, through this study, I am proposing a schematic diagram for the bacterial cell degradation process by the anaerobic microbial community for methane production (Fig. 9).

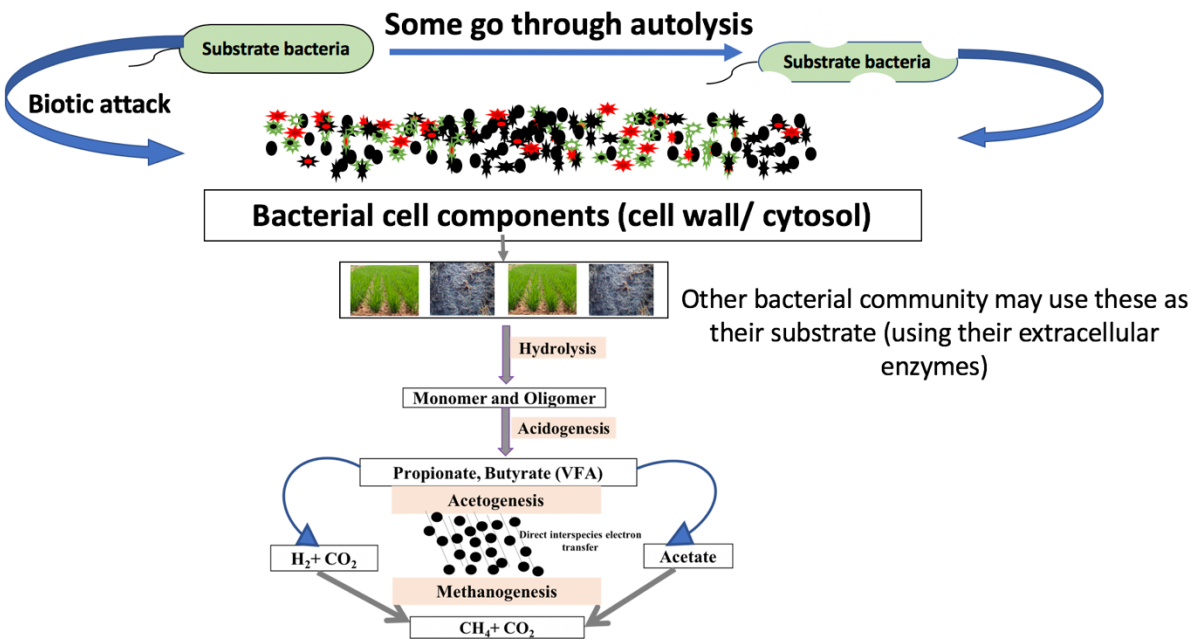


Fig. 9. A schematic diagram for the bacterial cell degradation process by anaerobic microbial community for methane production.

3.3 Isolation and identification of potential bacterial cell degrader anaerobic bacteria supplemented with bacterial cell

Enriched culture from the third transference was also tried to culture on the agar plate with the specific substrate that was used for the enrichment. The individual clear colony appeared on the gallan gum agar plate which was chosen for direct colony PCR of 16s rRNA gene sequencing. However, the isolation identification results and the community analysis of NGS came out contradictory to each other. Mostly dominant isolated bacteria from different enriched culture are presented in (Table 3).

Blast bacteria (identity %)	Phylum	Observation in community analysis	Enriched culture name
Caloramatormitchellensis (85%)	Firmicutes	NP	MLPGW
Bacillus thuringiensis (96%)	Firmicutes	NP	ECPGSO
Bacillus cereus (99%)	Firmicutes	NP	MPGSO
Bacillus paramycoides (99%)	Firmicutes	NP	BSPGSO
Enterococcus faecium (74%)	Firmicutes	NP	BSPGSL, BSSO
Clostridium pabulibutyricum(72%)	Firmicutes	NP	MLSL
Clostridium cylindrosporum (84%)	Firmicutes	NP	BKSO, BSPGW
Clostridium magnum (90%)	Firmicutes	Present	MLW
Clostridium amylolyticum (83%)	Firmicutes	Present	BKPGSO
Clostridium tunisiense (81%)	Firmicutes	NP	ECPGSL
Clostridium paraputrificum (94%)	Firmicutes	Present	BKPGSO, BKPGSL
Clostridium sartagoforms (99%)	Firmicutes	NP	MLPGSO
Paraclostridiumbifermentans (99%)	Firmicutes	NP	BSSL, BKSO
Fervidicellametallireducens (82%)	Firmicutes	Present	BKSL, BKPGSL, BSPGSL
Lutisphorathermophila (73%)	Firmicutes	Present	All
Romboutsiasedimentorum (80%)	Firmicutes	Present	BKSO
Sporacetigeniummesophilum (85%)	Firmicutes	Present	BSSO

Thermoanaerobacteriumsachharolyticum (75%)	Firmicutes	NP	ECPGW
Caloramatorboliviensis (86%)	Firmicutes	NP	BKPGW
Desnuesiellamassiliensis (87%)	Firmicutes	NP	BSW
Riemerellaanatipestifer (94%)	Bacteroidetes	NP	MLPGSL, ECPGSL
Proteinphilumacetatigenes (82%)	Bacteroidetes	NP	BSW, BSPGW, BSPGSL, BKPGSL
Salmonella enterica (83%)	Proteobacteria	NP	BSSO, BKSL, ECPGSL, BSPGW

Table 3: Isolation and identification of anaerobic bacteria from sludge, soil and wastewater community enriched by bacterial whole cell and peptidoglycan. NP represents the bacteria was not present in the enriched culture.

Most of the isolates have a very low identity to known bacteria except for *Bacillus cereus* (99%), *Bacillus paramycoides* (99%), *Clostridium sartagoforms* (99%), *Paraclostridium bifermentans* (99%). The reason of this low identity may be caused by the contamination during isolation. However, the phylum Bacteroidetes and Firmicutes were dominated from the NGS result in both whole-cell and PG enriched culture but direct colony isolation showed that mostly the phylum Firmicutes especially the genus *Clostridium* was dominant and appeared most on the agar plate. It is assumed that the isolated strains from the phylum Bacteroidetes and Firmicutes were present in the enriched culture but in a lower number that did not let them act as the dominant one and that is possibly why they were placed in the group 'other' phylum. Some isolated strains were found also in the NGS community analysis such as *Clostridium magnum* (90%) was 1% in MLW; *Clostridium amylolyticum* (83%) was 1% in BKPGSO; *Clostridium paraputrificum* (94%) was 1% in BKPGSo and BKPGSL; *Fervidicella metallireducens* (82%) was 1% in BKSL, BKPGSL and BSPGSL; *Lutisphora thermophila* (73%) was present >0% in almost all enriched culture; *Romboutsia sedimentorum* (80%) was 1% in BKSO; *Sporacetigeniumm esophilum* (85%) was 1% in BSSO. Among the isolates, there are some strict anaerobes and some belong to the

group of facultative anaerobes, for example, *Bacillus* species. Therefore, it is possible that those facultative anaerobes survived during the enrichment period by performing a lower metabolic activity.

Still, analyzing all the high identical isolated strains also could help us to understand their enzymatic activity used for bacterial whole-cell and peptidoglycan degradation. However, failure in isolation and identification of unknown bacteria revealed by NGS analysis indicates that the unidentified phylum needs more study to culture on the agar plate. Isolation of uncultured phylum especially species from the rare phylum (Verrucomicrobia and WWE1) and their enzyme analysis could bring an extra advantage to know more detail about bacterial cell degradation in anaerobic environment. Hence, it is possible in future to identify the extracellular hydrolyzing enzymes of enriched community or isolated strains by performing metagenomics and metatranscriptomes analysis for the next step. These approaches could allow us to know the real-time study of live consortia in various environments through the identification of the members of these enriched communities (108, 109) and/or determination of the relative abundances of particular physiological functions reflected in the occurrence of specific enzymes (110, 111). Also, advanced investigation of anaerobic whole-cell and peptidoglycan degradation will deepen our understanding of carbon cycles in natural environments and may lead to the development of more efficient methods especially for digestion of waste activated sludge.

4. CONCLUSION

In conclusion, this study provided the first observational evidence of methanogenesis from whole-cell and PG enriched anaerobic community. It was observed that CH_4 production and community can vary even in the same community depending on the part of the substrate bacterial cell. However, the scenario may be different in the natural environment as the structure of active/ inactive whole-cell and PG structure could be different than the laboratory preparation substrate. It is also possible that in the natural environment, number of organic compounds present in the original community, community structure may be responsible for the different results. Moreover, the amount of a particular substrate could be distributed differently in the original source of community whereas in the laboratory enrichment culture,

some factor might have influenced the community to function differently, for instance, incubation temperature.

From this study, it is implicit that phylogenetically diverse bacteria, especially uncultured bacteria with high phylogenetic novelty, would be involved in anaerobic whole-cell and peptidoglycan degradation. This initial study correspondingly revealed that some OTUs with a higher and lower identity, which were not reported before on bacterial cell degradation could be a potential for anaerobic microbial ecology. Though this study could not reveal their individual function on bacterial cell degradation, the information about the dominant phylum and OTUs with higher identity will help us to proceed further in the investigation of bacterial cell degradation, and isolation of this novel bacteria will be a great contribution to the anaerobic ecology as well as in the application.

GENERAL CONCLUSION

Bacterial cell degradation is very significant in the aerobic and anaerobic of both environments. Through this examination, it is observed that there are many known bacteria in the aerobic environment who can degrade bacterial cell part especially peptidoglycan. Further investigation of the isolated strain could help us to understand the molecular mechanisms of this degradation and their possible application in some fields.

On the other hand, bacterial cell degradation investigation in the anaerobic environments revealed that methanogenesis can occur by the different anaerobic community from the different bacterial whole-cell and their peptidoglycan which is the first report ever in anaerobic microbiology. The anaerobic microbial community analysis of bacterial cell and peptidoglycan enriched culture showed that different unknown bacteria from several phyla are involved in bacterial cell degradation in different anaerobic environments. It is presumed that the same scenario is also common in the natural anaerobic environments but further investigation such as metagenomics and metatranscriptomes analysis are needed to describe more elaborately the molecular mechanism of this kind of degradation process. Along with that, isolated strains from the anaerobic enriched culture are also could be examined more intensely to get additional detail information on the degradation process and their possible application in the anaerobic digester to reduce biomass.

However, two phyla (Bacteroidetes and Firmicutes) were found remarkably common on bacterial cell degradation in both environments which indicate their versatile involvement on this degradation as well in natural condition.

This initial study also brought a new approach in microbiology to culture unknown novel bacteria by using bacterial cell especially in the anaerobic environments. Moreover, this study will contribute a huge part in the microbial ecology especially in the anaerobic microbiology.

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