Study on the Characterization of a Novel Chitinolytic Thermophilic Anaerobic Bacterium and Its Syntrophic Relationship in Chitinolytic Bacterial Community

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Study on the Characterization of a Novel Chitinolytic Thermophilic Anaerobic Bacterium and Its Syntrophic Relationship in Chitinolytic Bacterial Community

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ACRONYMS AND ABBREVIATIONS

ANI	Average Nucleotide Identity
CBC	Chitinolytic Bacterial Community
CBM	Carbohydrate-binding Module
CDSs	Protein- coding Sequences
CID	Chitin Insertion Domain
COGs	Clusters of Orthologous Groups
dDDH	DNA-DNA hybridization
GC	Gas Chromatography
GH	Glycoside Hydrolase Family
GlcNAc	N-acetyl-D-glucosamine
HPLC	High-performance Liquid Chromatography
IMG-ER	Integrated Microbial Genomes-Expert Review
NCBI	National Center for Biotechnology Information
PGAP	Prokaryotic Genome Annotation Pipeline
SDS-PAGE	Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis
TCD	Thermal Conductivity Detector
TIM	Triosephosphate Isomerase

Chapter 1

Introduction

1.1 Background

A contemporary increase of human population with a high resource consumption generates an enormous waste burden inevitably. According to the World population prospects 2019, United Nation reported that the number of populations continues to rise 2.0 billion people for 30 years (from 2019 to 2050) (Nations, 2019). Subsequently, a growing need for food worldwide become more pressing. For example, the World production of farmed shrimp was up to around 4 million tonnes in 2018, which Asia countries play the major market such as China, India, Vietnam, Indonesia and Thailand. Additionally, the global crab market in 2018 was approximately 0.5 million tonnes via Canada, Russia and China as the largest crab exporters (Fao, 2018). Consequently, an expansion of food production following a global demand has led to an uncontrollable disposal of food waste especially in the seafood industry. In general, the contents of shrimp or crab such as cuticle and heads of shrimp are removed before processing and packaging. Around 45 to 75 % by weight of whole raw shrimp is a waste disposal relying on its species and peeling conditions (Sachindra and Mahendrakar, 2005), (Kandra et al., 2012), (Synowiecki and Al-Khateeb, 2003)). The complex exoskeleton of shellfish contains mineral salts (30 – 50%), protein (30 – 40 %), and

chitin (13 – 42%), while the percentage of shell contents is depending on age and reproductive cycle. Older shellfish includes high ratio of minerals (phosphates and carbonates) and low chitin fraction. Due to the complexation of structural crustaceans (shrimps and crab) composing of chitin cross-linked with minerals and proteins via aspartate and histidine moieties, the natural degradation is difficult and time-consuming (Stankiewicz et al., 1997); (Stankiewicz et al., 1998). The gigantic seafood waste remaining, caused by the repercussion of these huge aquatic industry and the decay resistance, have been excluded by a marine dumping, landfill, an incineration and a chemical treatment. The improper processes result in an environmental concern such as an unpleasant odor, harmful compounds to inhabitants of ecosystem, a global warming from greenhouse gas emission and so on (Mathew and KG, 2006), (Morgan and Chuenpagdee, 2003). The trend of the world emerging markets considers an earth-friendly production such as a use of versatile microorganisms, instead of the traditional ways, decomposes the marine waste providing beneficial for the sustainable environment.

Recently, the temperature has been rising mainly caused by the human exploitation that emits greenhouse gases and land surface changes. As a result of terrestrial impacts, the high temperature ecosystem drastically changes and the debuts the thermal-tolerance microorganism (Manucharova et al., 2008) such as thermophilic bacteria and archaea such as *Serratia marcescens* (Sikorski et al., 2006) and *Cohnella* sp.A01(Aliabadi et al., 2016). Additionally, these thermophiles present in organic compost containing chitin and biopolymer, while their protein secretion have been a high thermo-stability and suitable for biotechnological industries (DeCastro et al., 2016) such as chitinase. To emphasize the

chitinase production, chitinases (EC 3.2.1.14) for a hydrolysis of chitin and produce a polymer chain of *N*-acetyl-D-glucosamine, GlcNAc. Chitinases are categorized into four glycoside hydrolases (GH) families (GH 18, 19, 23 and 48) which GH family 18 is commonly found in most chitinolytic bacteria. These chitinolytic enzymes contribute to the applications in medicine, agriculture and renewable energy (Stoykov et al., 2015). For example, the prospect of marine waste converts to biofuel and biogas (Gorrasi et al., 2014). As above mentioned, the thermophilic chitinolytic community belongs to aerobic bacteria, archaea and fungi; besides, the anaerobic bacteria that have chitinolytic potential rarely grow optimally at 50°C or higher (Manucharova et al., 2008). Consequently, we aim to study the chitinolytic thermophilic anaerobic bacteria and understand the symbiotic relationship between our isolated bacteria, its genetic characteristics and its chitin degradation function.

1.2 Objectives and outline

In this study, we investigate the novel thermophilic anaerobic bacteria that enable chitin degradation and study its enzymatic mechanisms. The objectives are as follows.

1. To clarify the chitinolytic bacterial community with syntrophic relationship
2. To isolate and characterize a novel the chitinolytic thermophilic anaerobic bacteria

1.3 Literature review

Chitin is the second enormous polysaccharides after cellulose with an abundant global production each year around 10 – 100 billion tons (Gooday, 1990; Kaiser et al., 2008). This large crystalline polymer widely presents in an arthropod exoskeleton such as shrimps and insects. The chitin structure has a similarity of cellulose structure, besides the carbon-2 position of glucose unit at the chitin is an acetylated amino group instead of hydroxyl groups within the cellulose(Shahidi et al., 2005).

1.3.1 Discovery of chitin (Crini, 2019)

The chitin was firstly mentioned by Charles Hatchett, an English chemist, in 1799. He found a compound like a cartilage from immersion of shell and animal cuticles in acidic solution (Hatchett, 1799). In 1811, Henri Braconnot, a French chemist, extracted an insoluble compound (named *fongine*/fungine) from *Agaricus volvaceus* fungi treated with dilute warm alkali (Braconnot, 1881). Ten years later, Auguste Odier isolated an insoluble alkaline component, given it the name of *chitine,* from insect cuticle treated by sulfuric acid (Odier, 1823). After that, Lassaigne proposed that chitin contained nitrogen (JL, 1843).and Winterstein and Ledderhose clarified the presence of glucosamine when the chitin was decomposed in acid solution and heat (Ledderhose, 1876), (Winterstein, 1894)). Further studies, many researchers reported the characterization of the chitin such as structure of chitin polymer. All the results demonstrated that the composition of chitin was a chain of *N*-acetyl-D-glucosamine unit with β-(1→ 4) glycosidic linkage and discovered its derivative and

chitosan (Meyer and Mark, 1928), (Karrer and Schubert, 1928), (Karrer and v. François, 1929), (Zechmeister and Tóth, 1932), (Zechmeister and Tóth, 1933) and (Hackman, 1960)).

1.3.2 Structure and sources of chitin

The structure of chitin shares similarities with the chitosan and cellulose. In common with chitosan, it is polymeric chains of glucosamine which is a similar form of chitin without acetyl group at the carbon 2. In contrast to cellulose, the cellulose polysaccharide is composed of glucose monomers with β-1,4 linkages (Gooday, 1990; Shahidi et al., 2005). Chitin is a chain of β-1,4-*N*-acetylglucosamine (GlcNAc) including a 180° rotation of two *N*-acetylglucosamine units. Chitin is an insoluble in most common solvents because it has intermolecular hydrogen bonds (Minke and Blackwell, 1978). However, the derivative forms of chitin such as chitosan and carboxymethyl chitin are water-soluble (Kurita, 2001). The versatile chitin can be changed in the different features depending on application such as fibers, sponges, membrane, beads and bio-hydrogels (Khor and Lim, 2003, Di Martino et al., 2005; Guo et al., 2005). Even though the chitin has transformed into many industrial products, but the common natural chitin is covalent and non-covalent linkages to other compounds such as protein, which presents over a half of the chitin composition in living cells tissue (Attwood and Zola, 1967, Merzendorfer and Zimoch, 2003, Schaefer et al., 1987). In various of features and sources, the structural chitin can be arranged differently. The conformation of chitin from an x-ray diffraction studies demonstrated that there are three patterns termed α, β and γ due to an arrangement of piles linked (1→4) glycosidic bonds. The pack of piles constructs the crystalline chitin structure. Firstly, the piles of the α chitin – structure order in

the opposite direction called as "antiparallel" or "up" and "down" (↑↓) (Rudall and Kenchington, 1973). The most stable formation is α chitin due to strong hydrogen bonding in the anti-parallel arrangement (Sikorski et al., 2009). Secondly, the orientation of β chitin is aligned in parallel manner or all "up" (↑↑). Lastly, the γ chitin is a mixed arrangement of antiparallel and parallel as two "up" and one "down" (↑↓↑). These three patterns of chitin have a difference of the degree of hydration, the total number and size of chitin chain per unit cell. The β and γ chitin can be transformed to α chitin by chemical treatment. For example, γ chitin change to α chitin using lithium thiocyanate solution at the room temperature. In case of β chitin, it dissolved in a cold hydrochloric acid solution. Naturally, the chitin in α form can be widely found from crab and shrimp shells. By contrast, the groups of β chitin are obtained from animal tissues such as pogonophore tubes in cocoon, annelid chaetae, cephalopod pen and the mid-gut secretions of some insects (Blackwell, 1969). The γ chitin structure was found in the cuticle lining a stomach of Loligo (Bidder, 1966). On the other hand, the composition of chitin resembles structure of chitosan and cellulose that differ in a linkage of acetyl group and a sugar unit. The removal of acetyl group in the chitin is subjected to chitosan, and deamination in chitosan creates cellulose. Overall, a hydrolysis of chitin uses chitinase to break β-1,4-glycoside bond between the chitobiose, by existing creatures.

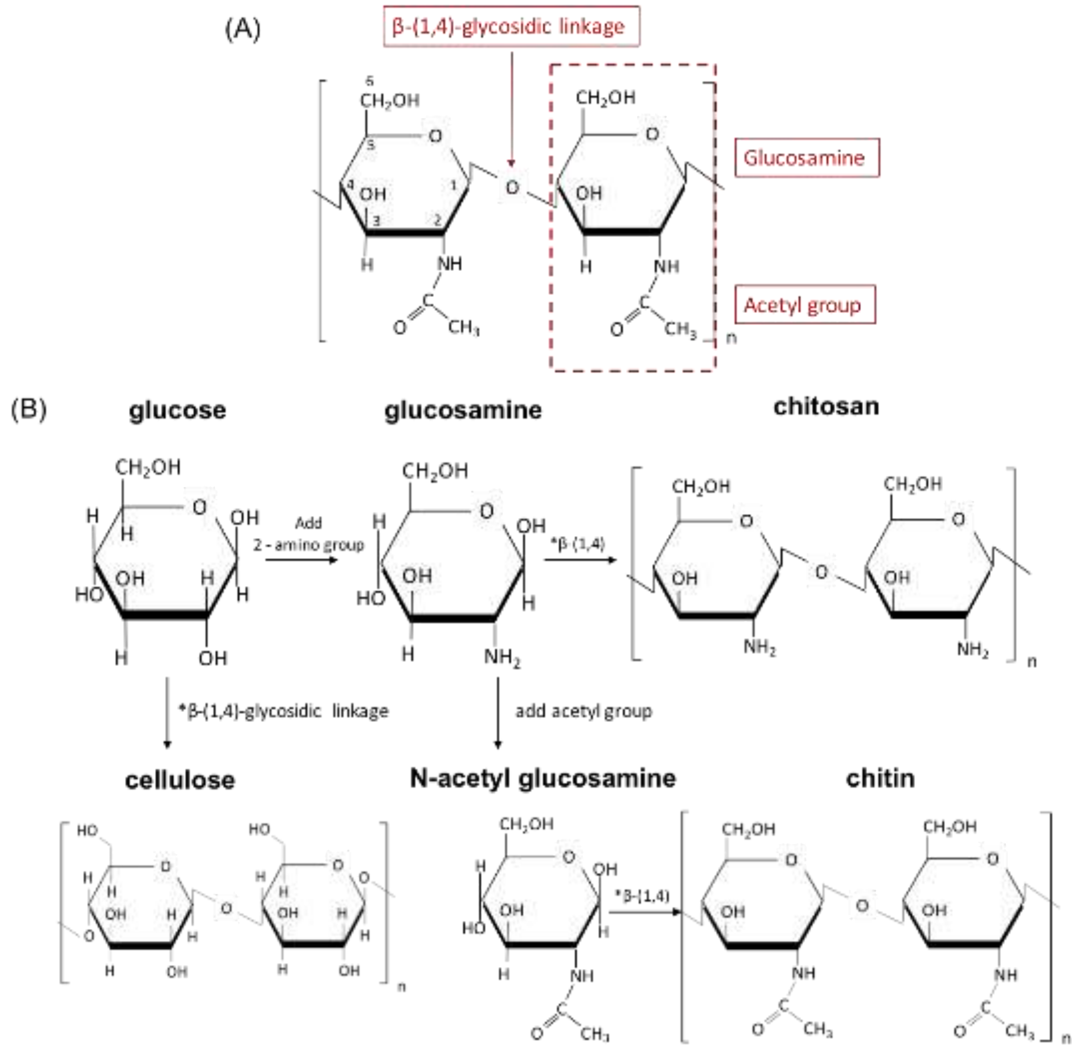

Fig 1.1 Structures of polymers (A) chitin chain (B) Structure of glucose, glucosamine, chitosan, cellulose, *N*-acetyl glucosamine and chitin

1.3.3 Chitinase

Chitinoclastic organism has an ability of chitin degradation by releasing chitinase. In nature, chitinase serves as virulence factors for defense mechanism against invasive pathogens, and hydrolyzing for nutrient uptake Moreover, chitinases are involved in morphogenesis and proliferation found in every hierarchy living levels such as plants, microorganisms, arthropods, vertebrates (Merzendorfer and Zimoch, 2003). In response to plenty of chitin, chitinases exist in bacteria, archaea and fungi as main chitin degrading-players in both land and aquatic systems (Huber et al., 1995b). For bacteria, the chitin-degrading mechanism between aerobic and anaerobic bacteria are different. Chitinases in form of exo- and endo enzyme from aerobic bacteria hydrolyze chitin into monomer GlcNAc and the cells will consume monomers. While anaerobic bacteria will release chitinase, complex structural chitin is hydrolyzed into short-chain oligomers. In the insoluble chitin polymer, there are three mechanism steps including cleavage of polymers into oligomers, oligomers into dimers, and dimers into monomers (Beier and Bertilsson, 2011), whereas they take up oligomer inside their body and cut oligomers to monomer and disaccharides resulting in the enzyme activity of anaerobic bacteria leading to lower than the actual rate from outside the cell (Wörner and Pester, 2019).

Measurement of the chitinase activity consists of various methods such as radioactivity, paper chromatography, colorimetric assay, plate-clearing assay, and running gel. The plate-clearing assay and turbidimetric assay are a basic measurement to detect chitinolytic enzyme, which is the disappearance of chitin substrate in culture medium. This

method has been used for screening chitinolytic microorganisms (Wirth and Wolf, 1990; Kuddus and Ahmad, 2013). To examine the size of chitin-oligomers, a thin-layer chromatography is an old-fashioned analysis (Powning and Irzykiewicz, 1967), however, recently the chito-oligosaccharides has been detected using a high performance liquid chromatography that is much more accurate and acceptable(Chang et al., 2000). The colorimetric assay using *p*-Nitrophenyl-*N*-Acetyl-*β*-D-glucosaminide (Roberts and Selitrennikoff, 1988) and hydrolysis of colloidal chitin measuring the concentration of reducing sugar, based on redox reactions and changes in cation level, such as Nelson-Somogyi (Somogyi, 1930; Nelson, 1944). For estimation of chitinase size, polyacrylamide gel electrophoresis (PAGE) containing soluble glycol chitin or zymogram has been used extensively (Trudel and Asselin, 1989; Reguera and Leschine, 2001).

The functional chitinase have been categorized into endochitinase (E.C.3.2.2.14) and exochitinase. The endochitinases cleave glycosidic bond randomly at internal site of chitin chain, while the exochitinases have been divided into chitobiosidase (E.C.3.2.1.29) hydrolyzing at the non-reducing end sites, and 1-4-*β*-glucosaminidases (E.C.3.2.1.30) cleaving at the reducing end sites of the chitin, chito-oligomers and chitobiose, thereby releasing *N*-acetylglucosamine (GlcNAc) as the final product.

Glycoside hydrolase (GH) is the most common enzyme classification depending on the substrate specificity, their catalytic mechanism and their domain structure, currently GH are categorized into over 130 families. Chitinases are belonged to GH families 18 and 19 mainly, GH20 includes chitobiase and *β*-N-acetylhexosaminidase for dimer breakdown such

as chitobiose, and a few members of family GH20, GH23 and GH48 have been determined and (Itoh et al., 2013). The GH 18, chitinase plays as an endogenous chitin-degrader and mostly found in both microorganisms, plants, and animals, with multi-functions such as tissue degradation and immune defense. However, GH19 chitinase is distributed in plants widely. The structures of GH18, depending on sequences homology in each organism, have been divided into many subgroups A, B, C, D, E, H, I, J, K, and L, which missing ChiF, G are family of GH19 (Kawase et al., 2006). Most of bacterial chitinases mentioned ChiA, B and C widely, the ChiA, ChiB and ChiC differ in the domain arrangement, shape, and functions. ChiA and ChiB catalytic domain includes a triosephosphate isomerase (TIM) barrel $(\beta/\alpha)_8$ at an insertion of $\alpha + \beta$ fold region but only ChiA has a presence of chitin insertion domain (CID) that is absent on subgroup B and C. Based on the 3 dimensional structure, ChiA was classified into ChiA1 and ChiA2, which ChiA1 contains a deep substrate-binding cleft on the top of its TIM barrel (Suzuki et al., 2002). Environmental studies on degradation of chitin involved in aerobic condition and few mentioned on anaerobic studies. Most chiA is abundant marker (beier et al 2011). GH18 are produced from several types of bacteria such as *Serratia marcescens* (Monreal and Reese, 1969)*, Vibrio harveyi* (Suginta et al., 2016)*, Bacillus licheniformis* (Laribi-Habchi et al., 2015)*, Chromobacterium violaceum* (Lobo et al., 2013) *and Yersinia entomophagy* (Busby et al., 2012).

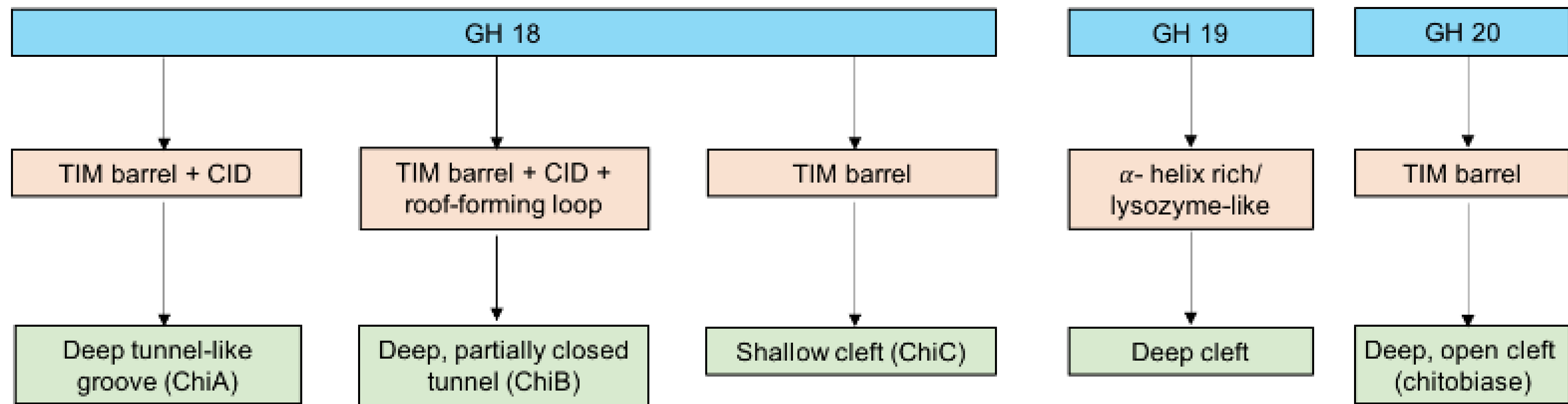

Fig 1.2 Classification of chitinase

In general, bacterial GH18 features consist of signal peptide, catalytic domain, carbohydrate-binding module (CBM) and /or a fibronectin type III-like (FnIII) module. The presence of catalytic domain, including substrate-binding sites lined with multiple aromatic residues, plays a role in conformational changes and enzymatic cleavage crystalline chitin. CBM and FnIII domains, located at the N- or C- terminal, are additional proteins for improving the interaction with insoluble chitin and hydrolysis of colloidal chitin effectively. (Yan and Fong, 2015; Chen et al., 2020).

To understand the chitin decomposition of bacteria, the microbial mechanism, enzymatic activities and genetic characteristics have been studied using whole genome sequencing technology. For example, the predicted chitin-degradation pathway of *Streptomyces diastaticu* CS1801 showed that chitinases (EC 3.2.1.14) hydrolyzing the insoluble chitin to oligosaccharides. The oligomers $(GlcNAC)_n$ are transformed to dimers $(GlcNAC)_2$ and monomer (GlcNAC) by endochitinase and *N*-acetylglucosaminidases (EC 3.2.1.52). In addition, acetyl-group in $(GlcNAC)_n$ is removed by chitin deacetylases (EC 3.1.1.72/ EC 3.5.1.41) and di-glucosamine $(GlcN)_2$ is spitted to GlcN by β-galactosidase or β-glucosidase. Specially, CS1801 can produce chitosanase (EC 3.21.132) to hydrolyse chitosan transforming from chitin. After that, GlcN is transformed into GlcN-6-phosphate by glucokinase (EC 2.7.1.12). Alternatively, some GlcN-6-phosphate are converted to fructose-6-phosphate by glucosamine-fructose- 6- phosphate aminotransferase (EC 2.6.1.16), undergoing to glycolysis cycles and produce CO_2 and H_2O at the end. Besides, GlcN-6-phosphate is changed to GlcN-1-phosphate by phosphoglucosamine mutase (EC 5.4.2.10). Then, GlcN-1-phosphate is added acetyl-group and transformed to UDP-N- GlcNAc by GlcN

-1- phosphate *N* - acetyltransferase and UDP-N- GlcNAc pyrophosphorylase (EC 2.3.1.157/ EC 2.7.7.23) respectively, which is used for reconstruction of skeletal structure by cellulose and chitin synthase (EC 2.4.1.12/ EC 2.4.1.16) (Xu et al., 2020).

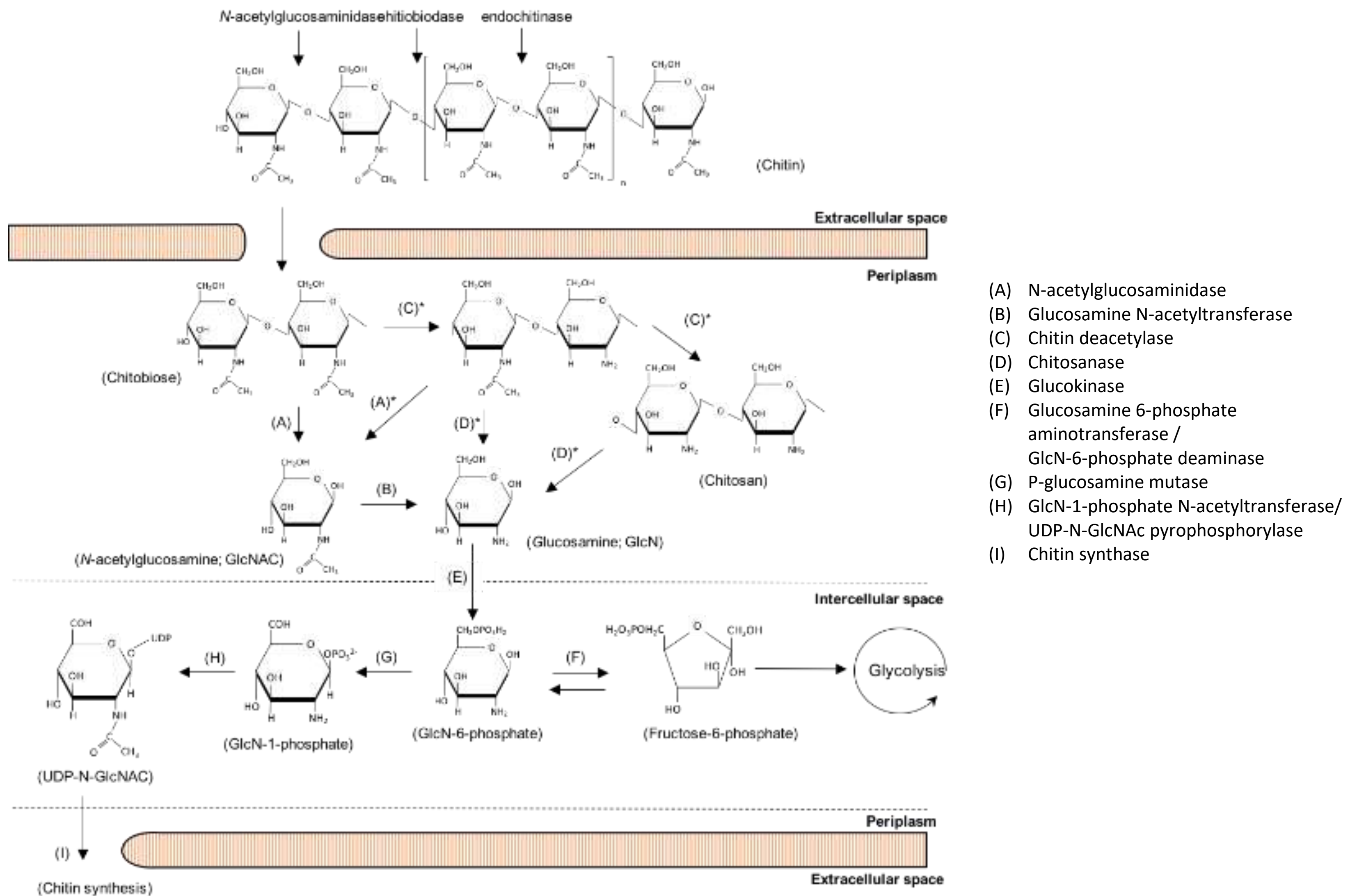

Fig 1.3 Pathway of chitin degradation in anaerobic bacteria

1.3.4 Chitinolytic microbial community and their microbial symbiosis

A total of isolated chitinolytic microorganism were discovered under either aerobic or anaerobic atmosphere. Commonly, the numbers of chitinolytic fungi (25- 60 %), found in the environment, quantitatively play main role of chitin degradation that are greater than that of bacteria. It has been reported that a total of chitinolytic fungi for 45 - 69% of *Actinomycetes*, are into the genus *Streptomyces* found in Lake Chełmżyńskie, Poland (Brzezinska et al., 2014). In case of bacteria studies, *Cytophaga-Flavobacteria*, mostly found in aquatic environment, are often identified as active chitin degraders (Cottrell and Kirchman, 2000). In contrast, 2% - 10% of bacteria isolated from sediment and water have chitin-degrading ability for biocontrol of pathogens such as fungi, besides only 1% of rhizobacteria has the actions of chitinases (Gooday, 1990; Cottrell and Kirchman, 2000). One of those reports mentioned about diversity of chitinoclastic bacteria from agricultural field found in Ibaraki prefecture, Japan. For metagenomics and phylogenetic analysis, the majority classes and phylum of chitin-degrading bacteria under aerobic condition were classified into *Betaproteobacteria* (the genus *Mitsuaria*), *Gammaproteobacteria* (the genera *Serratia*, *Stenotrophomon*as, and *Lysobacter*), and *Firimicutes* (*Paenibacillus* and *Bacillus*) (Someya et al., 2009). Another group of thermophilic bacteria was discovered from the Brown semi-desert soil in Mongolia. Thermophilic *Streptomyces roseolilacinus* and *Silanimonas lenta* were dominantly occupied and grown at 50ºC in this community (Manucharova et al., 2008).

However, most anaerobic bacteria that hydrolyze chitin are found in anaerobic extreme conditions. For example, *Chitinispirillum alkaliphilum*, is an anaerobic chitinolytic

bacterium found in the hypersaline alkaline lakes in Egypt (Sorokin et al., 2016), and *Thermococcus chitinophagus* is a chitinolytic hyperthermophilic bacteria growing at 60 - 93 ^{o}C (Huber et al., 1995a). Additionally, it has been reported that the chitin degradation under anoxic sediment with depth in Lake Constance Germany such as *Clostridia* and *Fibrobacteres* (the genera *Chitinivibrio* and *Chitinisprillum*) (Sorokin et al., 2014; Sorokin et al., 2016). Based on their dominant population of bacteria community, it divided into 3 phases: early phase (day 0 – day 9), intermediate phase (day 9 – day 21), and late phase (day 21 to day 43). The early phase was occupied by the genus *Chitinivibri*o, which has only a member in the group named *Chitinivibrio alkaliphilus*, an obligately alikaliphilic bacterium, isolated from an extremely alkaline lake. Next, the intermediate phase revealed a variety of dominant species not only *Chitinivibrio*, but also representatives of the phyla *Bacteroidetes*, *Proteobacteria* (the order *Myxococcales*, suborder *Sorangiineae*) (Garcia et al., 2010), *Spirochaetes*, and *Chloroflexi* (the family *Anaerolineaceae*) (Yamada et al., 2006). Finally, the late stage showed a high increase of *Firmicutes* (the genus *Ruminiclostrium*). In general, *Ruminiclostrium* sp. can utilize biopolymers such as cellulose and xylan, besides *Ruminiclostridium hungatei* is only member able to degrade chitin (Reguera and Leschine, 2001). Most of responding strains involve in uncultured bacteria and work in microbial community for chitin degradation and fermentation.

Symbiotic and syntrophic relationships among microbial community show a synergism for complex bio-substrate degradation such as lignocellulose and chitin. Symbiotic is an association among organisms of different species, regardless of advantage to each member, while syntrophic is subset of mutualistic symbiosis which all partners rely on

each other for nutritional benefit (Tipton et al., 2019). The combination of bacterial activities increases a surface area of polymer exposed to microbial hydrolysis. A digestive system of animal works with microorganisms symbiotically for food digestion such as cellulolytic and chitinolytic bacteria in a worm gut or arthropods. For example, a relationship of *Folsomia candida* and chitinolytic bacteria enhanced chitin degradation (Borkott and Insam, 1990). Regarding of Bacterial community, ISHI-3 was capable of lignocellulosic degrading ability, including a total of 4 members: *Herbivorax saccinocola* as a main active player, and *Pelotomaculum* (Imachi et al., 2002), *Tepidimicrobium* (Niu et al., 2009) and *Tepidanaerobacter* (Sekiguchi et al., 2006; Westerholm et al., 2011) as non-cellulolytic bacteria. Interestingly, these strains are known to utilize carbon and energy sources symbiotically and syntrophically for cell growth such as *Pelotomaculum thermopropionium* as a syntrophic propionate-oxidizing, *Tepidimicrobium ferriphilum, Tepidanaerobacter acetatoxydan* and *Tepidanaerobacter syntrophicus* as syntrophic organic acid- and alcohol-oxidizing.

Tepidanaerobacter syntrophicus and *Tepidanaerobacter acetatoxydan* are belonged to be anaerobic mesophilic-thermophilic syntrophic bacteria. *Tepidanaerobacter syntrophicus* co-cultivated with hydrogenotrophic methanogens, *Methanothermobacter thermautotrophicus* could accumulated a high concentration of acetate and hydrogen that converted from lactate. However, the pure culture of *T. syntophicus* could produce a high lactate concentration and less fermentation amount of acetate and hydrogen. Therefore, this reaction involved the shift from the lactic acidosis mechanism to syntrophic lactate-consuming between these two strains. Another member of this genus, *T. acetatoxydan* can

produce only acetate, meanwhile it showed acetate-oxidizing ability and generated methane and carbondioxide production in the culture associated with a hydrogen-consuming *Methanoculleus* sp. strain MAB2. For instances, other syntrophic acetate-oxidizing bacteria such as *Thermotoga lettinage*, *Methanobacterium bryantii*, *Thermacetogenium phaeum*, *Clostridium ultunense*, and *Syntrophaceticus schinkii* have similar behavior to utilize a few number of substrates: organic acids, alcohols and amino acids that can transform to other end products like carbondioxide, hydrogen and methane in the final degradation step in the anaerobic fermentation.

Anaerobic fermentation includes four main sequential processes: hydrolysis, acidogenesis, acetogenesis and methanogenesis. After getting volatile fatty acids, alcohol, and lactate from the acidogenesis, these compounds cleave to acetate, formate, hydrogen and carbondioxide in the acetogenic step. In presence of methanogenesis, there are two primary pathways; (1) acetoclastic: acetate as an intermediate product is metabolized into methane and carbondioxide and (2) hydrogenotrophic: carbondioxide and hydrogen transform to methane and water. Mostly, the methanogenic pathway in the thermophilic anaerobic fermentation work together with syntrophic acetate-oxidizing microbes along with increase of methane from substrate inhibition such as high acetate concentration due to increase of pH condition (Hao et al., 2011; Hershey et al., 2014).

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Chapter 2

Relationship of *Hydrogenispora* sp. UUS1-1 a novel thermophilic anaerobic chitinolytic bacterium and *Tepidanaerobacter* sp. GT38 a symbiotic bacterium

Overview

Chitin is the largest bio-compound in nature next to cellulose. Even though several scientists have reported an information of insoluble chitin-degrading mesophilic bacteria, there is a less knowledge of decomposition of crystalline chitin powder by bacteria in thermophilic anaerobic condition. In this study, a chitinolytic bacterial community (CBC), which came from cow manure isolation, presented an identification of this new symbiotic system for chitin decomposition under anaerobic thermophilic environment, i.e., 1% (w/v) crystalline chitin particle decompose at 60 °C in 7 days. Metagenomic evaluation showed that the population of CBC included dominant two bacteria which are *Hydrogenispora* sp., an uncultured taxon in cluster OPB54, and *Tepidanaerobacter* sp.. The population of *Hydrogenispora* bacterium occupied in the initial-to-mid stages of cultivation with crystalline chitin powder and colloidal chitin, but the increase of *Tepidanaerobacter* population during the late-cultivation stages. The isolated strains UUS1-1 and GT38, belonged to *Hydrogenispora ethanolica* and *Tepidanaerobacter acetatoxydans*, respectively, were isolated as a pure strain cultivation by the roll-tube technique using glucose *N*- acetyl-

D-glucosamine, and colloidal chitin as carbon sources. Strain UUS1-1 was the first chitin degrading player in the group of thermophilic anaerobic chitinolytic bacterium, meanwhile strain GT38 did not show chitinolytic activity. According to a phylogenetic evaluation, UUS1-1 should be proposed as novel genus and species, and GT38 should be proposed as a novel species. The evaluation of zymogram showed that UUS1-1 secretes at least two chitin enzymes with approximately molecular mass of 40 and 150 kDa. A co-culture of strain UUS1-1 and strain GT38 could show the higher speed of decomposition in chitin powder with lower accumulation of lactate and acetate comparing with only pure UUS1-1 culture, revealing that these strains maintain a symbiotic association through organic acids assimilation in crystalline chitin powder decomposition, meanwhile strain GT38 consumes organic acid products and thereby reduces inhibition from the end product and enhances chitin decomposition.

2.1 Introduction

Chitin is the second gigantic polysaccharide including a linear polymer chain of *β*-1,4 *N*-acetyl-D-glucosamine (GlcNAc) with a production of over hundred billion tons per year (Gooday, 1990). The structural chitin resembles the structural cellulose and chitosan except that the carbon-2-position of the D-glucose unit in chitin is an acetylated amino group (Shahidi and Abuzaytoun, 2005). This polymer is a main component of the arthropod's exoskeleton, like shrimps and insects (Keyhani and Roseman, 1999). Chitin is used in a broad range of agriculture and food industry products, including pesticide agents, oligosaccharides

and food preservatives (Kurita, 2006; Rinaudo, 2006; Prado and Matulewicz, 2014), and is also transformed to medical active products such as chitosan, chitin oligosaccharides and GlcNAc (Kurita, 2006; Prado and Matulewicz, 2014; Hamed et al., 2016).

The degradation of crystalline chitin necessarily requires multiple enzymes function together. Chitinases (EC 3.2.1.14), hydrolyzed a polymer chain of chitin, are produced by various microorganism, including fungi, archaea, and bacteria, and by higher plants, insects, and animals. Based on the Uniprot database, approximately 63% of the chitinolytic enzyme sequences are belonged to among bacteria of the genera *Actinobacteria*, *Cyanobacteria*, *Chloroflexi*, *Deinococci Firmicutes*, *Proteobacteria*, and *Tenericutes* (Le and Yang, 2019). Chitinases are major enzymes for chitin hydrolysis and are classified into main 3 types: endo-chitinases (EC 3.2.1.14), β-N-acetylglucosaminidases (EC 3.2.1.52) and exo-chitinases (EC 3.2.1.29) (Chavan and Deshpande, 2013). In accordance with the database of Carbohydrate-Active enZYmes (http://www.cazy.org/), most chitinases from bacteria are under the glycoside hydrolase (GH) family 18, but a few chitinases assigned to the GH19 family found in a smaller number of prokaryotic and plants. These GH families have specific structures of three-dimension and patterns of substrate binding (Sikorski et al., 2006; Tian et al., 2014). Chitinases from bacteria often have multi-auxiliary-domains, including a carbohydrate-binding module (CBM) that increase efficiently insoluble substrate binding by these enzymes (Bai et al., 2016). Chitinases from bacteria are versatile tools for use applications in several commercial food and medicine (Haki and Rakshit, 2003; Prado and Matulewicz, 2014; Hamed et al., 2016; Le and Yang, 2019). For example, the production of GlcNAc, chito-oligosaccharides for commercial applications using chitinases derivatives from *Salinivibrio*

sp. (Le and Yang, 2018), *Penicillium monoverticillium* (Suresh and Anil Kumar, 2012) and *Vivrio alginolytics* (Murao et al., 1992), respectively, have been undertaken (Le and Yang, 2019). Thermostable chitinases from bacteria should be applied into many applications for a reduction of costs in the operation (Haki and Rakshit, 2003; Yeoman et al., 2010); however, current chitinases have been applied in industrial applications are typically from mesophilic bacteria (Tom and Carroad, 1981; Le and Yang, 2019). Many chitinases are produced by thermophiles, including *Bacillus licheniformis* (Waghmare and Ghosh, 2010), *Thermomyces lanuginosus* (Guo et al., 2008) and *Paenibacillus thermoaerophilus* (Ueda et al., 2013) have been applied into industrial applications; however, there are few reports describing chitinases from thermophilic anaerobic bacteria. Therefore, screening, identifying, studying the properties and elucidating the functions and synergies of enzyme systems from anaerobic thermophilic chitinolytic microbes represents an effective resource of new enzymes for advances our understanding of microbial diversity and industrial tools.

Previously, ISHI-3 microbial community was isolated from cow manure biocompost and characterized (Shikata et al., 2018). This microbial community showed high decomposition activity toward rice straw and corn stover composed of lignocellulolytic and non-lignocellulosic and relationship of symbiotic bacteria that degrade polysaccharides efficiently (Shikata et al., 2018; Zhou et al., 2019; Ozbayram et al., 2020). In this study, we used the same manure compost as latest studied (Shikata et al., 2018; Widyasti et al., 2018b) to screen and characterize new chitin-decomposing and thermostable bacterial community that presented high chitin decomposition activity under thermophilic and anaerobic conditions. Two main genera were isolated successfully as a pure culture from the bacterial

community. The morphological and physiological characters of two identified bacteria are presented.

2.2 Materials and Methods

2.2.1 Materials

Cow manure compost was taken from the facility of Ishigaki Island compost (Ishigaki Taihi Center, Ishigaki City, Japan) in May 2015 (Aikawa et al., 2018; Shikata et al., 2018; Widyasti et al., 2018a). The samples are maintained in strictly anaerobic environment by bubbling with CO_2 into sampling and inoculation respectively.

2.2.2 Culture media, cultivation and enrichment procedures

BM7CO enrichment medium (Nakazono-Nagaoka et al., 2019) as a basal medium was consisted of (per 1 liter): 1.5 g KH_2PO_4, 2.9 g K_2HPO_4, 3.0 g yeast extract, 2.1 g urea, 4.0 g Na_2CO_3, 0.01 g $CaCl_2.2H_2O$, 0.0005 g resazurin, 0.5 g cysteine-HCl, and 200 µl mineral solution (0.312 g/l $FeSO_4.7H_2O$, 37.5 g/l $CaCl_2.2H_2O$ 25.0 g/l, and $MgCl_2.6H_2O$,) (all the buffers and chemicals were bought from FUJIFILM Wako Pure Chemicals, Osaka, Japan). The gas inside enrichment medium tube was removed in hot water (≈100 °C) and flushed with high concentration of carbon dioxide (CO_2) and autoclaved at 121°C respectively (Nakazono-Nagaoka et al., 2019). Hungate anaerobic culture methods were performed.

Roughly 1–2 g of biocompost was put into the tubes composing of 10 mL of BM7CO, pH 8.0, and 1 g of crystalline chitin powder (FUJIFILM Wako Pure Chemicals). After aliqouting, each tube was immediately bubbled with CO_2 and statically incubated at 60 °C. After six days of incubation, aliquots (200 µl) of the cultures were transferred to 5 mL of autoclaved medium comprising of chitin powder every six days. The enrichment cultures presented fast growth, turbidity, small bubbles, and a visual decrease in the chitin substrate. The culture was proceeded repeatedly with enrichment medium composing of 0.5% crystalline chitin powder. During the enrichment process, ineffective and unstable chitinolytic capacity of cultures was discarded. After transferring new cultures for over five generations, the enrichment cultivations with stable and high decomposition activity toward crystalline chitin powder were chosen to measure stable bacterial community (Fig. 2.1).

To evaluate the optimal pH for growth of strains UUS1-1 and GT38, the pH of the enrichment medium composing of 0.5% (w/v) glucose or 0.5% (w/v) GlcNAc was adjusted to pH 5.0–10.0 by adding diluted HCl or NaOH at room temperature under high-purity CO_2 gas prior to inoculation. Growth and substrate utilization by those strains were observed by measuring a turbidity, which involved measuring the optical density of the cultures at 600 nm.

2.2.3 Stable culture of the chitinolytic bacterial community

To get the stable and minimum chitinolytic bacterial community, the candidates of enrichment cultivation were carried out on agar medium containing colloidal chitin after five

subsequent inoculations in enrichment medium composing of colloidal chitin, with the following changes (Shikata et al., 2018): 1.5% of agar was filled in and concentration of the colloidal chitin was 1%. Concentrated colloidal chitin was treated from crystalline chitin powder (Hsu and Lockwood, 1975) and stored at 4 °C. Colloidal chitin was dried for overnight at 80 °C. Chitinolytic bacterial communities (CBC) were inoculated and serially diluted into agar medium containing colloidal chitin in the serial order. After the agar gel in the roll tubes was solidified and incubated under anaerobic environment at 60 °C. CBC formed a clear halo around small colonies after incubated for five days. Selected single colonies were picked up using a needle and transferred to enrichment colloidal chitin medium.

The selected cultures were tested again for their decomposition activity in chitin. This isolation of single colony step was conducted five times repeatedly following the roll tube method and liquid enrichment medium containing colloidal chitin; in contract, unstable chitinolytic decomposition activities were observed for several isolated single colonies (Fig. 2.1). Thus, we continued to isolate colonies again several times using colloidal chitin. To screen a higher stable chitinolytic bacterial community, the roll tube method using 1% GlcNAc (FUJIFILM Wako Pure Chemicals) as a soluble carbon source was carried out. Isolated single pure colonies were picked up into enrichment medium containing GlcNAc. The selected cultures were proved again for their substrate decomposition activity against colloidal chitin and crystallized chitin powder. This isolation of single colony process using GlcNAc was proceeded five times repeatedly. A stable chitinolytic bacterial community (CBC) with efficient decomposition of crystalline chitin particles was screened and isolated from this following enrichment culture procedures (Fig. 2.1).

2.2.4 Measuring crystalline chitin powder and colloidal chitin decomposition

A bacterial chitin decomposition test was carried out in 300 mL of enrichment medium composing of 1% substrate (crystalline chitin powder or 1% colloidal chitin). An inoculum (2% v/v) in the medium with same substrates was incubated from four-day-old culture with shaking at 150 rpm at temperature 60°C. Remaining mass was centrifuged at 3,800 g for 10 min using a swing rotor before drying for two days and final weighing at 80 °C. Eight samples at day 0th –5th, 8th and 9th were collected during the chitin degradation period. The dry weight of the remaining substrate was evaluated and differentiate with a non-inoculated control culture. The weight loss percentage in the substrate was defined as the ratio of weight loss compared with the initial weight (%) as evaluated by the following equation: chitin degradation (%) = $[(a-b)/c] \times 100$, where a is the weight of remaining control chitin, b is the weight of remaining chitin and c is the total weight of chitin. Data were the average values of experiments performed in triplicate.

2.2.5 Metagenomics sequencing using the Ion Torrent S5 system

The extraction of total genomic DNA from the CBC was performed with a NucleoBond® AXG column and NucleoBond® Buffer Set III (Takara Bio Inc., Kusatsu, Japan), according to the instructions. The purity and quantity of the isolated genomic DNA was performed using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher

Scientific, Waltham, MA, USA). The amplification of specific 16S rRNA gene hypervariable region (V4) was constructed by the universal primers, 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso et al., 2011). The amplified fragments were purified using the Agencourt AMPure® XP beads (Beckman Coulter, Brea, CA, USA), and the quantification of amplicons were performed using the Qubit Fluorometer with the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). The preparation of DNA libraries was carried out using the Ion Plus Fragment Library Kit (Thermo Fisher Scientific) and Ion Xpress Barcodes Adapters 1–16 Kit (Thermo Fisher Scientific), following the manufacturer's instructions. The purification DNA libraries were performed with Agencourt AMPure® XP beads. Library concentrations were analyzed by qPCR using the Ion Universal Library Quantitation Kit (Thermo Fisher Scientific). After dilution to a concentration of 25 pM and processed with the Ion Chef™ systems using the Ion 510™, Ion 520™ and Ion 530™ Chef Reagents, Each library was combined. The amplicon libraries were sequenced on an Ion 510™ Chip using the Ion GeneStudio S5 system (Thermo Fisher Scientific) for 850 flows. Each sequence read was analyzed using CLC microbial Genomics Module v20.0 (Qiagen, Valencia, CA, USA). Sequences were trimmed between size of >50 bp and <400 bp and size of >150 bp and <300 bp. The operational taxonomic units (OTUs) were aligned to the reference 16S rRNA database, SILVA v.132 (http://www.arb-silva.de/) (Quast et al., 2012) at 97% similarity. The identification of chimeric sequences was removed by the software. The read sets were categorized into operational taxonomic units with 0.02 distance unit cutoffs measured by the

BLASTN algorithm in the National Center for Biotechnology Information (NCBI) GenBank database.

2.2.6 Pure culture isolation

Each pure bacterium from the CBC was isolated by the roll tube method using 1% glucose as the carbon source. The colonies on glucose-consuming bacteria was appeared after 4-5 days of incubation. Single colonies were chosen with a needle and inoculated into enrichment medium containing glucose. This isolation step was repeated ~10 times. Further confirmation of the isolated purity was observed by inspecting light microscopic images and morphology of colony and identified by 16S rDNA gene sequencing (Fig 2.1).

2.2.7 Physiochemical testing

Deposition of isolated pure bacteria, *Hydrogenispora* sp. UUS1-1^T (= JCM 33882^T = DSM 111537^T) and *Tepidanaerobacter* sp. GT38^T (DSM 111783^T) as type strains in the open culture collection of the RIKEN Bioresource Research Center (JCM) and the Leibniz Institute DSMZ-German collection of microorganisms and cell cultures GmbH (DSMZ) were identified by 16S rDNA sequencing. The preparation of total DNA was collected from 5 mL of pure isolated cultures, as described in Section 2.6. The amplification of 16S rRNA gene was carried out using the bacterial sequence-specific primers 27F and 1492R. The alignment and comparison of sequences with the reference 16S rDNA gene sequences

available in the NCBI GenBank database to identify their closest phylogenetic neighbors was performed by a BLASTN search. The analysis of 16S rRNA gene sequences using BLAST program were manually aligned with sequences in the GenBank database using CLUSTAL_X v.1.81. The creation of phylogenetic trees was used by the neighbor-joining method using the program MEGA version 6.06 (Tamura et al., 2007). Tree topologies and distances were calculated by bootstrap analysis based on 1000 resamplings

Gram-strain kit Gram-stain kit (BD Biosciences, Difco, NJ, USA) and a Fulton spore staining kit (Fluka, Buchs, Switzerland) were used for examination of strains UUS1-1 and GT38. The morphology of bacterial cell was investigated using a scanning electron microscopy (SEM; Jeol JSM-6320F, Tokyo, Japan). The plasma osmium coating (OPC80T, Filgen, Nagoya, Japan) was used for the preparation of specimens for SEM. Each strain was incubated in enrichment medium composing of 1% (w/v) of various carbon sources under anaerobic environment at 60 °C and pH 7.0, and growth was examined with D-glucose, D-xylose, D-arabinose, arabinan, arabinogalactan, cellobiose, D-galactose, galactan, D-fructose, D-mannose, D-sucrose, maltose, GlcNAc, xylan (beechwood; Sigma-Aldrich, St. Louis, MO, USA), microcrystalline cellulose, chitosan, chitin, sodium L-lactate, sodium acetate, ethanol, butanol, n-propanol, glycerol, propionate (all chemicals were purchased from FUJIFILM Wako Pure Chemicals) and casamino acid (BD Biosciences). After seven days of incubation, cell growth was determined visually by the optical density of the sample at wavelength of 600 nm using a spectrophotometer (UV-mini 1240; Shimadzu, Kyoto, Japan). The criteria of substrate use were gas and bubble production, turbidity, acidification of the culture, by

gravimetric determination of remaining chitin materials, and by subtracting the dry weight of the remaining mass from the beginning weight of the substrate.

2.2.8 Culture characterization

Chitinase activity of the CBC, strain UUS1-1 and strain GT38 was characterized, the extracellular enzyme fractions were collected from 300 mL enrichment culture containing 1% colloidal chitin or glucose at 60 °C for seven days. Cell pellets of each grown cultivation were separated by centrifugation at 8,000 g for 10 min at 4 °C. Each culture supernatant was concentrated to ~10 mL by using Amicon Ultra centrifugal filters (Merck Millipore Corp., Darmstadt, Germany) and was purified to remove the small molecular compounds using an Econo-Pac 10DG column equilibrated with distilled water according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA). The proteins from each bacterium cultivation were used as the extracellular enzyme fractions. The protein concentration in the supernatant was measured using the Pierce BCA assay kit (Thermo Fisher Scientific) with bovine serum albumin as the standard reference. The total proteins' bands were observed by SDS-PAGE performing on 5 to 20% gradient polyacrylamide gels (ATTO, Tokyo, Japan). The zymograms for chitinases were carried out by using a 0.2% (w/v) concentration of ethylene glycol chitin (FUJIFILM Wako Pure Chemicals) merged into the polyacrylamide. After gel electrophoresis, the enzymes were activated by 25% (v/v) 2-propanol, and the gel was incubated at 50 °C for 30 min in 50 mM sodium phosphate buffer (pH 7.0). Zymograms were stained for remaining ethylene glycol chitin with a Congo red solution (1 mg/mL),

destained with 1 M NaCl and fixed with 5% (vol/vol) acetic acid. The appearance of clear halo in the zymograms indicated the area of enzyme activity. Molecular mass standards were from Bio-Rad Laboratories.

The ethanol and organic acids in the supernatants from the cultivation were detected by HPLC (Shimadzu) (model LC-20AD; Shimadzu) using a Shim-pack SCR-102H column with an electric conductivity monitor (CDD-10Avp). at 40 °C using a 5 mM p-toluenesulfonic acid solution and a 5 mM p-toluenesulfonic acid (FUJIFILM Wako Pure Chemicals) solution containing 20 mM bis-Tris (DOJINDO, Kumamoto, Japan) and 0.1 mM EDTA (DOJINDO) as a mobile phase at a flow rate of 0.8 mL/min, and by gas chromatography (GC; model GC-2014, Shimadzu) equipped with a flame ionization detector using Chromosorb 103 (60/80 mesh) (GL Science, Tokyo, Japan), respectively (Shikata et al., 2018). GC was used to detect oxygen (O_2), methane (CH_4), hydrogen (H_2) and CO_2 (model GC-2014; Shimadzu, detector type: TCD; column: SHINCARBON ST column (50/80 mesh) (GL Science); column temperature: 40 °C) with argon at a flow rate of 50 mL/min. The data values are shown as the average of triplicate experiments ± standard deviations.

2.2.9 Cell mass and enzyme assay measurement

The hydrolysis of chitin, cellulose and xylan activities were analyzed by evaluating the amount of GlcNAc or reducing sugar released from microcrystalline cellulose (Sigmacell type-20; Sigma-Aldrich), beechwood xylan (Sigma-Aldrich), carboxymethylcellulose and

colloidal chitin (Okada and Shinmyo, 1988). The reaction mixture included 0.9 mL of 0.5% (w/v) substrate in 0.1 M sodium acetate buffer at pH 7.0 and 30–40 μg of extracellular enzyme fractions (in 0.1 mL). Released reducing sugars (GlcNAc) were measured by the Somogyi–Nelson protocol with glucose, GlcNAc or xylose as the standard substrate (Wood and Bhat, 1988). One unit of polysaccharide hydrolysis activity was defined as the amount of enzyme that liberated 1 μmol of GlcNAc, glucose or xylose in 1 min under the conditions described above.

2.3 Results

2.3.1 Characterization of isolated chitinolytic bacterial community in anaerobic and thermophilic condition

A cultivation using chitin powder combining with bovine compost was carried out to discover bacteria with new strong anaerobic thermophilic chitin-decomposing activities. The biocompost is not involved in resources associated with chitinolytic materials (Aikawa et al., 2018; Shikata et al., 2018; Widyasti et al., 2018b). The chitin powder degradation culturing in the enrichment cultivations after five transferring times were observed visually. Eventually, one thermophilic, anaerobic, and chitinolytic culture with a high activity of chitin decomposition at 60 °C was provided (Fig. 2.1). To investigate the stable chitinolytic bacterial communities in their structure and function, the enrichment cultures were carried out to get single colonies using the agar medium containing colloidal chitin by the roll-tube method (Fig. 2.1). After doing roll tube and picking up colonies, many colonies provided

high chitin decomposition activity and lost the chitin decomposition ability later after subsequent cultivations using *N*-acetyl-D-glucosamine (GlcNAc). This lost chitin decomposition ability phenomenon indicated that the dominant bacterial communities in the single colonies were non-chitinolytic bacteria when use of GlcNAc immediately as the carbon source in the enrichment cultures caused the chitin decomposition activity. Therefore, to isolate a high stable chitinolytic bacterial community with high chitin decomposition activity, another screening step was used to prove the crystalline chitin decomposition activity by colony isolation using GlcNAc as a carbon source. GlcNAc was used for the enrichment cultures because it was an easy way to get rid of the member in the community that did not assist the main chitin degrading player decomposing chitin and increased the stability of bacterial community. After single colonies isolated from enrichment agar containing colloidal chitin medium were inoculated into the crystalline chitin powder medium and then single colony isolation using GlcNAc agar medium again was carried out (Fig. 2.1). The high chitin-decomposing single colonies were given a name as a CBC. Stable chitin decomposition by a CBC was finally confirmed by sub-culturing to the next culture of media containing crystalline chitin powder and GlcNAc. Gram-staining analysis using a light microscopy illustrated that a CBC, as the selected single colony, was a mixed cultivation composed of two major bacteria. The CBC at 60 °C showed high decomposition activity toward chitin and colloidal chitin (Fig. 2.2). Remaining chitin, calculated by subtracting weights from the beginning chitin weights as 100%, were decomposed after 4 days and 8 days of incubation with crystalline chitin powder and colloidal chitin solution, respectively (Fig. 2.2).

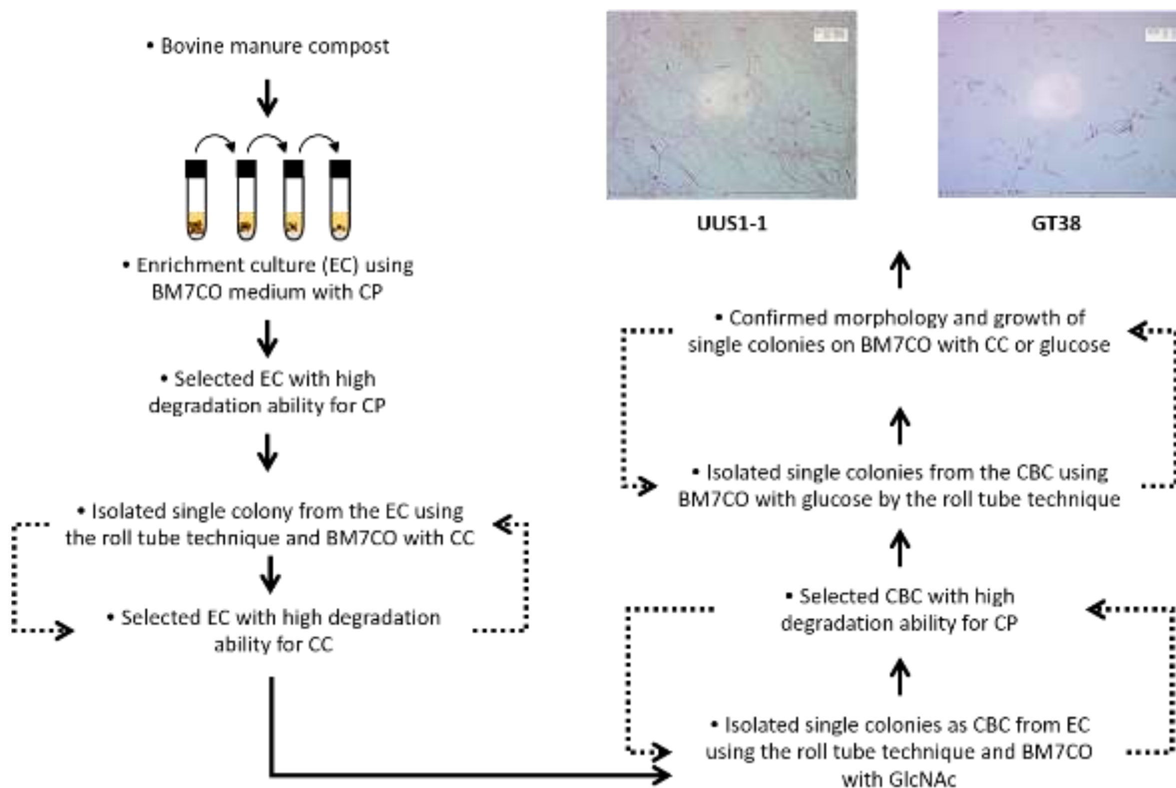


Fig. 2.1 Enrichment culture screening flowchart. Manure compost was added into enrichment cultures (EC). The chitinolytic bacterial community (CBC) colonies were picked up from the roll tubes with strong decomposition activity toward chitin powder (CP) and colloidal chitin (CC). Pure clones of strains UUS1-1 and GT38 were picked up from the medium using CC, GlcNAc and glucose by the roll tube method.

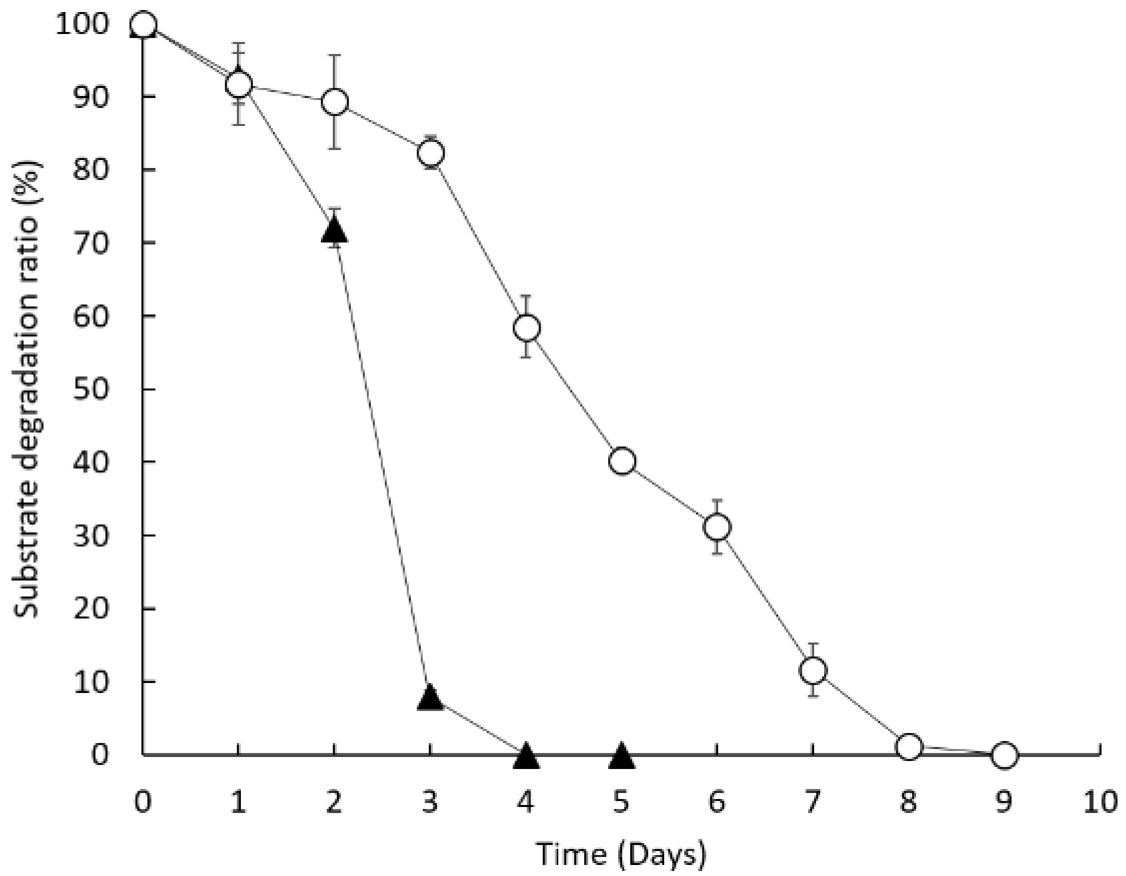


Fig. 2.2 CBC decomposition activity toward crystalline chitin (closed squares) and colloidal chitin (closed circles). The percentage of crystalline chitin and colloidal chitin declined from the beginning substrate weight of 1% dry (w/v) against cultivation time. Error bars showed ± standard deviation with triplication.

2.3.2 CBC population during the process of chitin decomposition

The microbial population of the CBC was observed by proceeding the DNA metagenomes from the cultivation of CBC in crystalline chitin powder and colloidal chitin, and the extracted DNA was amplified as templates for the partial 16S rRNA genes. The amplified genes were called amplicon. The amplicon sequences of 440 bps were analyzed by pyrosequencing (a replication-based sequencing method). The reads from pyrosequencing were removed short sequences and categorized following their attached tag. In the configuration throughout the cultivation period, two bacteria which were genus *Hydrogenispora* (an unidentified taxonomic culture at the order- or class-level of OPB54 group), and genus *Tepidanaerobacter* were grew on both substrates (colloidal chitin and crystalline chitin). The relative abundance of the *Hydrogenispora* bacterium presented the highest population in the fermentation period of colloidal chitin and crystalline chitin powder. However, the ratio of *Tepidanaerobacter* bacterium was a smaller population when compared with that of the *Hydrogenispora* bacterium population throughout the fermentation period and especially in the crystalline chitin fermentation period. The ratio of *Tepidanaerobacter* population increased slightly at the period of complete fermentation (Fig. 2.3A and B). In particular, the population change of genus *Hydrogenispora* and genus *Tepidanaerobacter* ratio changed from 82.4% to 63.7% and from 17.1% to 36.0%, respectively, during the mid to later stages of cultivation on colloidal chitin (Fig. 2.3A). A similar phenomenon was observed when crystalline chitin was utilized as the substrate. Therefore, t*Hydrogenispora* populations were dominant during the initial stages of crystalline chitin decomposition; however, the relative abundance of *Tepidanaerobacter*

population gradually increased from 0.74% to 13.7% during the middle to late stages of fermentation (Fig. 2.3B). This result strongly suggests that the two bacteria belonged to the *Hydrogenispora* bacterium may be mainly responsible for decomposing chitin. Another bacterium assigning to the *Tepidanaerobacter* bacterium probably consumed fermented products produced by the genus *Hydrogenispora*. Both bacteria exist in a symbiotic association to provide strong the activity of chitin decomposition in the CBC.

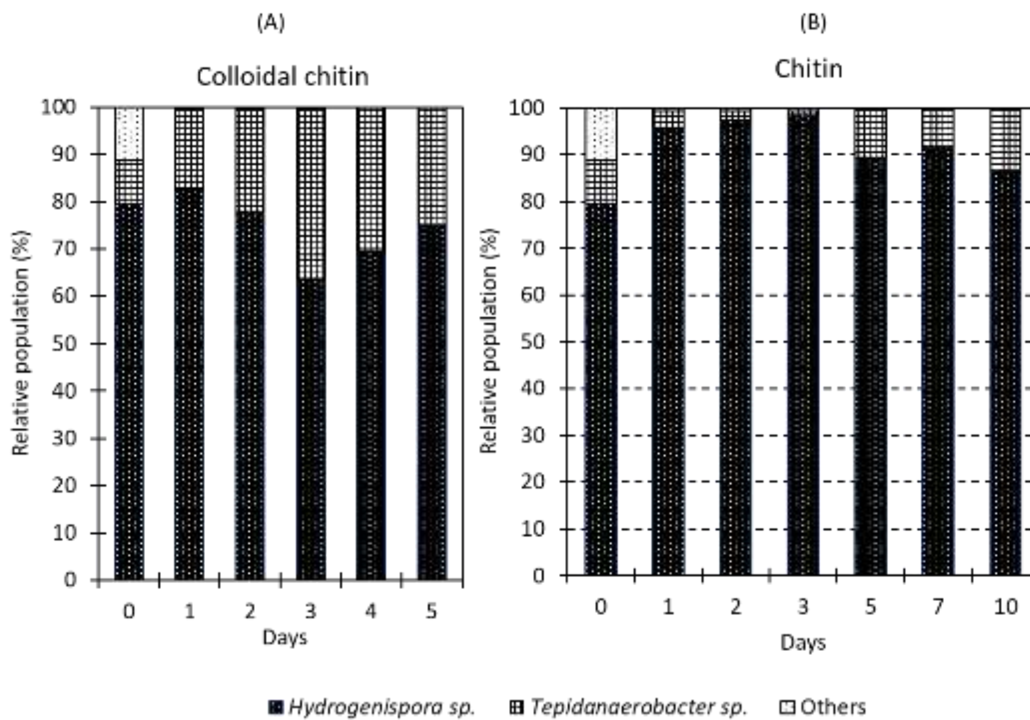


Fig. 2.3 Relative population of CBC profiles between colloidal chitin (A) and chitin powder (B). The change in the CBC population cultured in colloidal chitin and chitin powder was evaluated by the data of 16S rRNA gene sequences. Experiment was conducted in triplicate. The composition error was < 2%, and classes with >2% relative abundance in all these data were inputted.

2.3.3 Single pure isolation and characterization of chitin decomposing bacterium from the CBC

The bacterium assigning to the genus *Hydrogenispora* belonged into the OPB54 cluster under an unidentified taxonomic culture at the order- or class-level, and based on the ARB-SILVA reference database (http://arb-silva.de) only a few reports and their phylogeny have been described (Lee et al., 2013). The OPB54 members, who were belonged to class *Clostridia* (https://lpsn.dsmz.de/) (Parte, 2018) at the class level, are discovered differently in ecosystems and are phylogenetically (Dunfield et al., 2012). To identify the novel chitinolytic bacteria from the community, a single pure bacterium was isolated from the CBC by the roll-tube method using glucose-agar medium following GlcNAc. The structure of glucose is similar to GlcNAc without acetyl group. Many colonies appeared on the BM7CO-glucose agar medium. Isolated colonies were transferred into the same medium supplemented with glucose, which can be grown in various carbon sources. Single colony isolation was repeated at least ten times on the glucose agar medium using the roll-tube technique. To proceed a same method for isolation of GT38, the xylose was used as a carbon source, which UUS1-1 could not utilize it. Finally, bacterial strains UUS1-1 as long rod shape and GT38 as a short rod shape were successfully isolated as pure clones (Fig. 2.1). Strain UUS1-1 has long rod shape in contrast to strain GT38, its shape is sausage rod-shaped under a light microscope. UUS1-1 and GT38 bacteria were separately inoculated in enrichment medium containing colloidal chitin to clarify the bacterium consuming chitin decomposition. The UUS1-1 bacterium could utilize the colloidal chitin in the medium under thermophilic anaerobic conditions, but strain GT38 showed no growth on the colloidal chitin medium, showing that

strain UUS1-1 decomposes chitin in the CBC. The detection of lactate, acetate, H_2 and CO_2, as end products from chitin decomposition by strain UUS1-1 was performed using HPLC and GC (Table 2.1).

Bacterial morphology of strain UUS1-1 and strain GT38 were investigated by the scanning electron microscopy (SEM). Long rod strain UUS1-1 morphology was an apparent long rod like hair-shaped bacterium of 28–50 µm in length and 0.3–0.4 µm in width (Fig. 2.4A) and Gram-negative staining (Fig. 2.1 and Table 2.1). The location of spores was located sub-terminally and terminally and the spores formed a bulged sporangium (Fig. 2.4A). Strain GT38 was a short rod-shaped with 0.3–0.4 µm in width and 3.0–4.0 µm in length (Fig. 2.4B), and Gram-negative staining (Fig. 2.1 and Table 2.1). The sequencing fragments in the metagenome evaluation were related to the 16S rRNA gene sequences of UUS1-1 bacterium (GenBank accession number: MN602556) and GT38 bacterium (GenBank accession number: MT533891). The phylogenetic tree evaluation showed that the close relation between strain UUS1-1 and *Hydrogenispora ethanolica* strain LX-B^T (90.4% identity), isolated from an anaerobic herbicide wastewater as an hydrogen-ethanol-coproducing bacterium and classified uncultured taxon OPB54 group (Liu et al., 2014) (Fig. 2.5A). The result of 16S rRNA gene sequences of UUS1-1 compared with close neighbors revealed that a sulfate-reducing *Desulfotomaculum thermobenzoicum* TSB^T (<87.6%), isolated from a methane fermentation reactor (Tasaki et al., 1991), an anaerobic propionate-oxidizing *Pelotomaculum thermopropionicum* SI^T (<87.3%), isolated from a granular sludge (Imachi et al., 2002) and an anaerobic sulfate-reducing *Desulfotomaculum hydrothermale* Lam5^T (<87.1%), isolated from a hot spring (Haouari et al., 2008) showed similarities (Fig. 2.5A).

In addition to clade of *H. ethanolica*, UUS1-1 bacterium is the second isolated member in the OPB54 clade.

According to 16S rRNA gene sequence of GT38 bacterium, the result of identity revealed 95.5% similarity with: (i) an anaerobic syntrophic acetate-oxidizing *Tepidanaerobacter acetatoxydans* strain Re1^T, isolated from two different mesophilic methanogenic environment (Westerholm et al., 2011); (ii) an anaerobic, moderately thermophilic, syntrophic *T. syntrophicus* strain JL^T (94.9% identity), isolated from digested thermophilic sludges(Sekiguchi et al., 2006); (iii) a thermophilic anaerobic *Thermosediminibacter oceani* DSM 16646^T (89.8% identity), isolated from deep sea sediments of the Peru Margin (Lee et al., 2005); and (iv) a thermophilic anaerobic *Caldanaerovirga acetigignens* strain JW/SA-NV4^T (88.8 % identity), isolated from a xylan-enrichment medium from the Trego hot spring (Wagner et al., 2009) (Fig. 2.5B). Observation of UUS1-1 bacterium and GT38 bacterium in physiological characteristics were observed using various pHs and temperatures growth. Both of UUS1-1 bacterium and GT38 bacterium were strict anaerobic cultivation. UUS1-1 bacterium grew anaerobically on glucose and GlcNAc mediums at pH 6.5–8.0 (optimum growth, pH 7.0), and 40–65 °C (optimum growth, 55 °C), but did not grow at a temperature < 40 °C or >65 °C. UUS1-1 bacterium grew several carbon sources such as cellobiose, GlcNAc, glucose, galactose, mannose, maltose, sucrose, β-glucan, casamino acids, colloidal chitin, galactan, crystalline chitin, and peptone. The following substrates were not utilized by strain UUS1-1: acetate, lactate, pyruvate arabinose, xylose, arabinan, chitosan, cellulose, and xylan. Besides, GT38 bacterium grew anaerobically on glucose and GlcNAc mediums at 40–70 °C (60 °C as an optimum growth), at pH 6.0–9.2

(pH 8.0 as an optimum growth), but showed no grow at a temperature < 40 °C or > 70 °C. Strain GT38 could also utilize cellobiose, maltose, glucose, galactose, GlcNAc, sucrose, lactate, peptone, casamino acids, and pyruvate for growth; however, the complex structures i.e. acetate, arabinan, mannose, xylose, cellulose, chitosan, colloidal chitin, crystalline chitin, and, xylan were not assimilated (Table 2.1), suggesting that strain GT38 may be a kind of methanogenesis bacterium. *T. syntrophicus* strain JL^T and *T. acetatoxydans* strain Re1^T closely to GT38 bacterium on the phylogenetic tree, are syntrophic lactate- and primary alcohol- utilizing microorganisms in co-cultivation with *Methanothermobacter thermautotrophicus* as a hydrogenotrophic methanogen respectively.

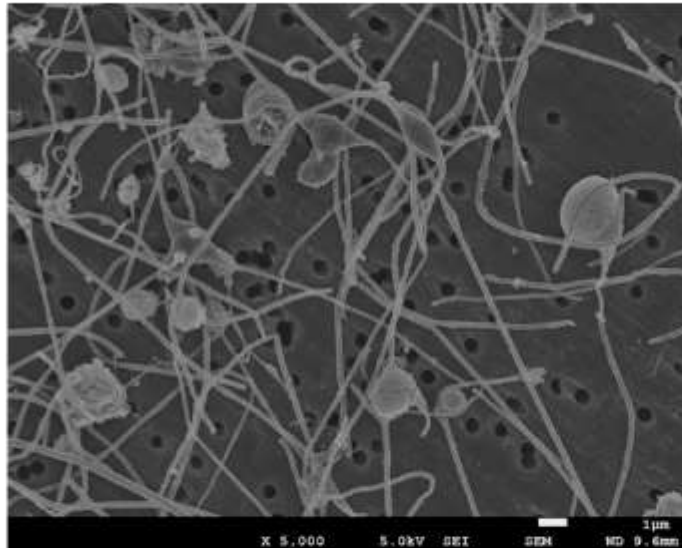
GT38 bacterium in enrichment medium composing lactate and >3.0 g/L yeast extract could grow. In addition, strain GT38 grew in the presence of 20 mM thiosulfate, 20 mM lactate and 0.1 g/L yeast extract. Therefore, GT38 can metabolize lactate and may synergize with methanogens. The fermented products of strain UUS1-1 are lactate as short-chain fatty acids during chitin decomposition and primary aliphatic alcohols, and the products from fermentation may inhibit the chitinases activity. GT38 assimilated the fermented products in chitin decomposition from UUS1-1 bacterium to remain the activities of the chitinases.

Table 2.1
Characteristics of UUS1-1 bacterium and GT38 bacterium, and their closest 16S rRNA gene neighbors.

Characteristics	1[a]	2	3	4	5	6
Culture environment	Biocompost	Anaerobic sludge treating herbicide wastewater	Kraft pulp sludge from a methane reactor	Biocompost	Ammonium sludge	Sewage sludge
Cell morphology	Long rod-shaped	Rod-shaped	Spindle-shaped	Sausage-shaped	Rod-shaped	Rod-shaped
Cell width and length (μm)	0.3 - 0.4 × 28.0 – 50.0 μm	0.3 - 0.5 × 3.0 - 18.0 μm	1.5 - 2.0 × 5.0 – 8.0 μm	0.35 - 0. 45 × 2.0 – 8.0 μm	0.3 - 0.5 × 1.5 - 15.0 μm	0.6 – 0.8 × 1.5 – 10.0 μm
Motility	Peritrichous flagella	-	+	-	+	-
Spore formation	+	+	+	-	+	-
Temperature range (°C)	45 - 65	37 - 45	62	45 - 70	20 - 55	25 - 60
pH range	6.5 – 8.0	6.0 - 7.7	7.2	6.0 - 9.2	4.0 – 9.5	5.5 – 8.5
Fermented products	Lactate, acetate, H_2, CO_2	Ethanol, H_2	Acetate	Acetate, H_2, CO_2	Acetate	Acetate, H_2, CO_2
References	This study	Liu et al. 2014	Tasaki et al. 1991	This study	Westerholm et al. 2009	Sekiguchi et al. 2006

[a] Strains: 1, *Hydrogenispora* sp. UUS1-1; 2, *Hydrogenispora ethanolica* strain LX-B^T; 3, *Desulfotomaculum thermobenzoicum* TSB^T; 4, *Tepidanaerobacter* sp. GT38; 5, *Tepidanaerobacter acetatoxydans* strain Re1^T; 6, *Tepidanaerobacter syntrophicus* strain JL^T.

(A)



(B)

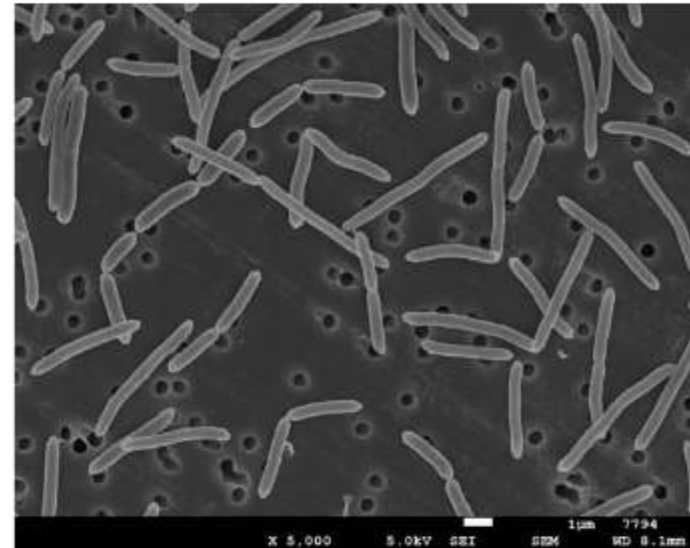
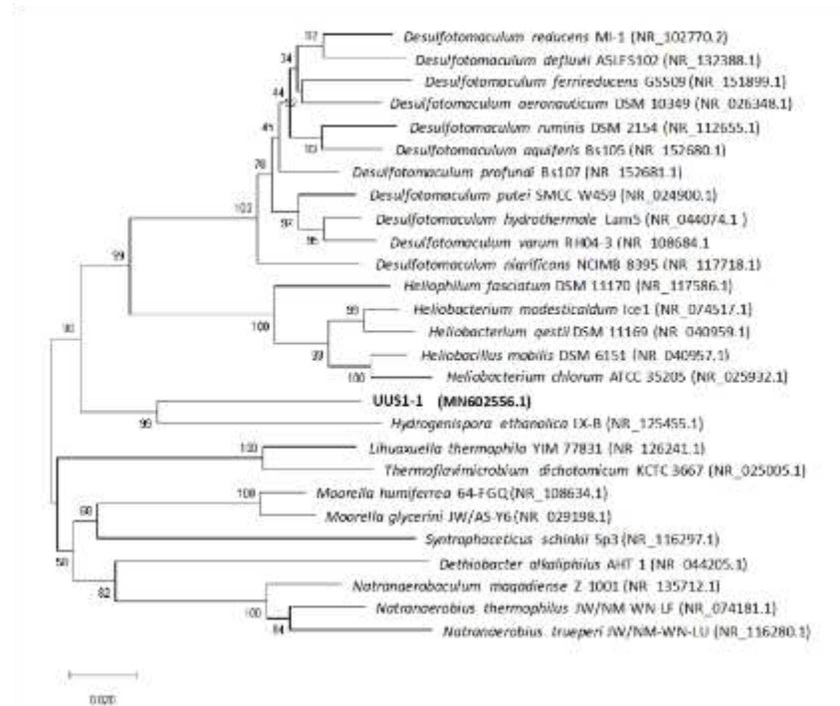


Fig. 2.4 Scanning electron images of UUS1-1 bacterium (A) and GT38 bacterium (B). The cultivation of UUS1-1 bacterium and GT38 bacterium in enrichment medium containing 1.0% glucose was performed for three days. The white bars represent 1 µm.

(A)



(B)

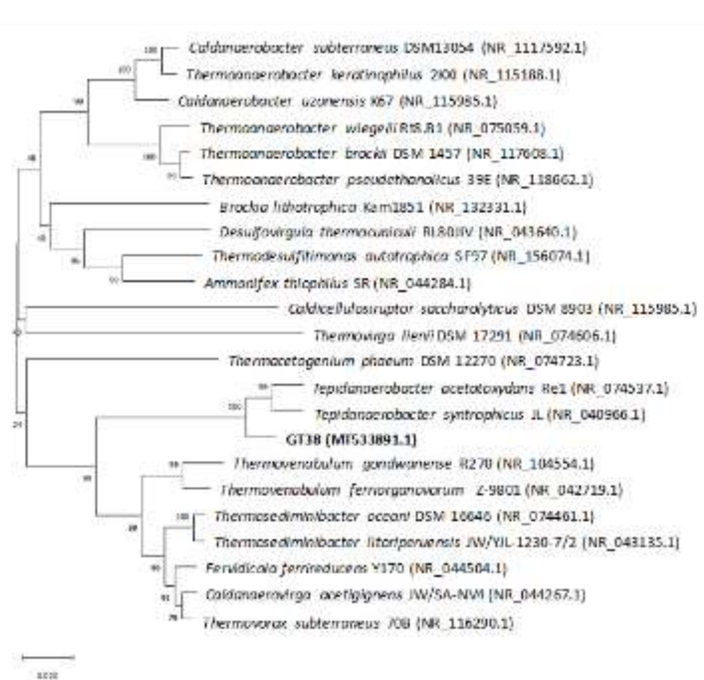


Fig. 2.5. Phylogenetic trees of UUS1-1 bacterium (A) and GT38 bacterium (B) relative to closely members of the phylum *Firmicutes* using Neighbor-joining method based on an analysis of distance-matrix for 16S rRNA gene sequences. The percentage of bootstrap provided from 1000 resamplings. Bars presents 0.020 nucleotide changes per sequence position.

2.3.4 The relationship in chitin decomposition potential of *Hydrogenispora* sp. UUS1-1 and *Tepidanaerobacter* sp. GT38

To clarify the relationship of UUS1-1 bacterium and GT38 bacterium in the chitin decomposition, extracellular enzymes from the cultivation of UUS1-1 bacterium and GT38 bacterium cultured in enrichment medium containing 1% (w/v) colloidal chitin solution or glucose were collected. Extracellular enzymes of strain UUS1-1 revealed relatively high decomposition potential of 5.75 ± 0.05 U/mg for colloidal chitin and 1.26 ± 0.04 U/mg for crystalline chitin powder respectively, but chitosan potential did not appear. In contrast, extracellular enzymes of strain GT38 did not show the decomposition activity toward crystalline chitin powder or colloidal chitin. Extracellular enzymes from both strain UUS1-1 and strain GT38 did not show potential toward other complex polymers like xylan and cellulose. UUS1-1 bacterium decomposed chitin with extreme potential (100% relative activity) at range of 60 -70 °C and at pH 7.0. After incubation, approximately 90% of the potential was retained with substrates for 1 h over at the pH between 6.0–8.0 at 70 °C. Evaluation of zymogram including soluble ethylene glycol chitin was performed using the extracellular enzyme of strain UUS1-1 to show clear halo for the chitin-decomposing potential (Fig. 2.6A and B). Active chitinolytic bands were mainly shown at ~40 and 150 kDa (Fig. 2.6B). The extracellular fraction of strain GT38 did not show bands in the zymogram gel (Fig. 2.6B). The strong band of chitinase activity detected with a molecular mass of 150 kDa probably have a strong interaction with the zymogram gel containing chitin. Many chitinases from bacteria included chitin-binding domains or the carbohydrate-binding module (CBM) such as CBM family 3, 14 and 49 (http://www.cazy.org/). The function of

chitin-binding has been described in eukaryotes, bacteria and plants. These results revealed that he GT38 strain does not contribute any chitin decomposing- activity, but UUS1-1 produces an important chitinase in chitin decomposition.

The relationship of symbiotic bacteria was evaluated from the function and association of GT38 in the CBC. Chitinase activities from a single cultivation of strain UUS1-1 and a mixed-cultivation of UUS1-1 bacterium and GT38 bacterium using colloidal chitin and crystalline chitin powder as carbon sources were examined. Single cultivation of UUS1-1 bacterium and co-cultivation of UUS1-1 bacterium and GT38 bacterium incubated with colloidal chitin showed a complete decomposition of substrate and fermentation of lactate and acetate was observed in the both cultivations (Fig. 2.7). Interestingly, the crystalline chitin decomposition ability was improved by mixed cultivation of UUS1-1 bacterium and GT38 bacterium comparing with a single UUS1-1 cultivation's activity. The mixed cultivation of UUS1-1 and GT38 could decompose the crystalline chitin powder within nine days, in contrast a single cultivation of UUS1-1 bacterium spent longer time for 12 days to completely decompose chitin powder (Fig. 2.7). According to the results, strain GT38 as co-supporter assisted strain UUS1-1 for crystalline chitin decomposition. Following the phenotypic characterization of both strains, UUS1-1 bacterium cannot grow in acetate and lactate, meanwhile GT38 bacterium can utilize these organic acids as carbon substrates. In the single culture of UUS1-1 bacterium for crystalline chitin powder and colloidal chitin, these organic acids produced to maximum amount of 3.46 ± 0.06 g/L and 6.0 ± 0.07 g/L (crystalline chitin powder), and 3.46 ± 0.02 g/L and 6.2 ± 0.1 g/L (colloidal chitin). In contrast to the co-cultivation of UUS1-1 bacterium and GT38 bacterium, the maximum yield of

lactate and acetate accumulation were 1.95 ± 0.10 and 6.98 ± 0.15 g/L (crystalline chitin powder), and 1.93 ± 0.23 g/L and 7.2 ± 0.17 g/L (colloidal chitin). Following to the results, lactate from UUS1-1 probably is assimilated through GT38. In addition, excess acetate and lactate have a negative effect on chitinase performance of UUS1-1 due to inappropriate environment such as a decrease in pH from an optimal pH (< pH7.0). Additionally, the decomposition activity of UUS1-1 toward crystalline chitin powder at pH 6.0 reduced (0.74 ± 0.02 U/mg) by around 41% comparing with the maximum yield (1.26 ± 0.04 U/mg) at an optimal pH. These experimental results indicated that effective chitin decomposition activity of UUS1-1 can be provided through a symbiotic association with the GT38 strain.

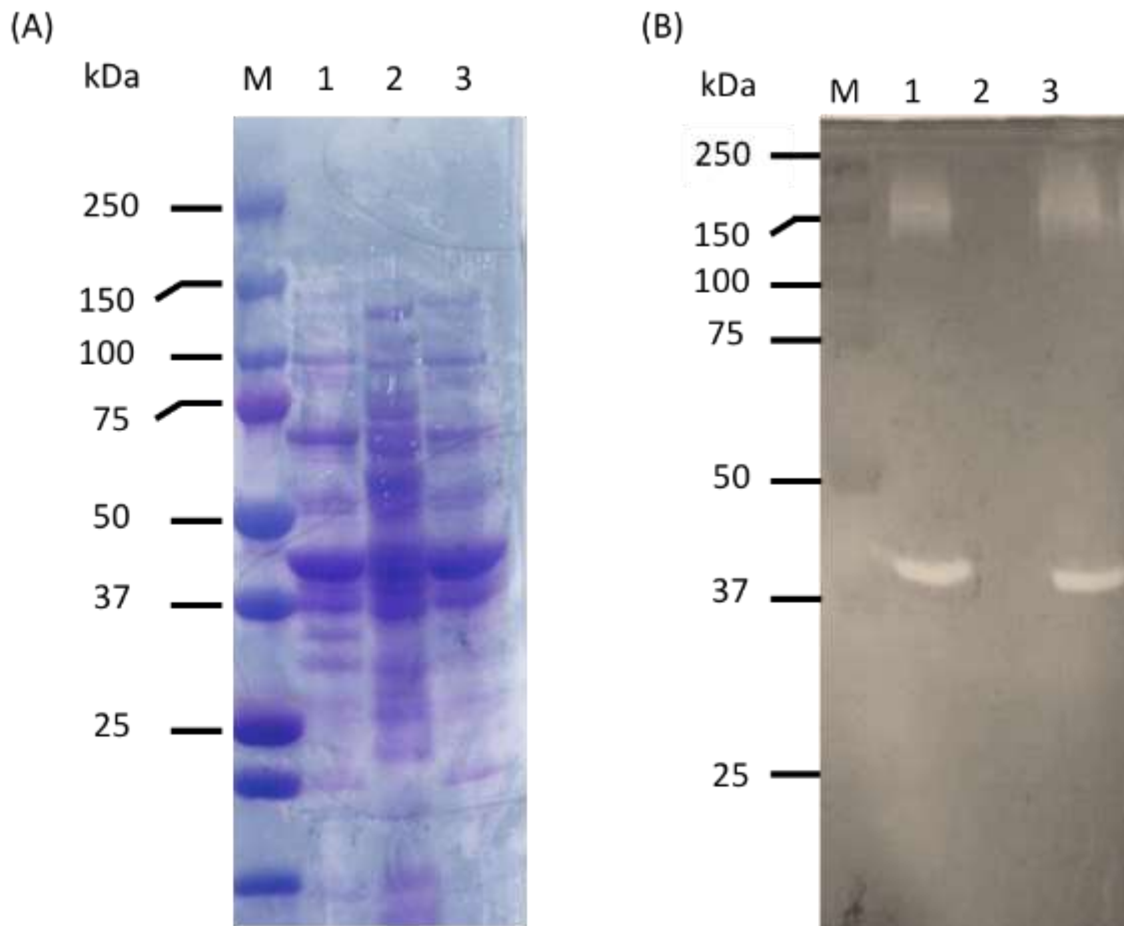


Fig. 2.6 Extracellular enzymes activity on zymogram using UUS1-1 bacterium, GT38 bacterium and the CBC for chitin decomposition. SDS-PAGE (A) and zymogram (B) Extracellular enzymes (10 μg protein) from UUS1-1 bacterium (lane 1), GT38 bacterium (lane 2) and the CBC (lane 3) cultured in an enrichment medium containing colloidal chitin or glucose. Lane M is a standard protein molecular mass marker.

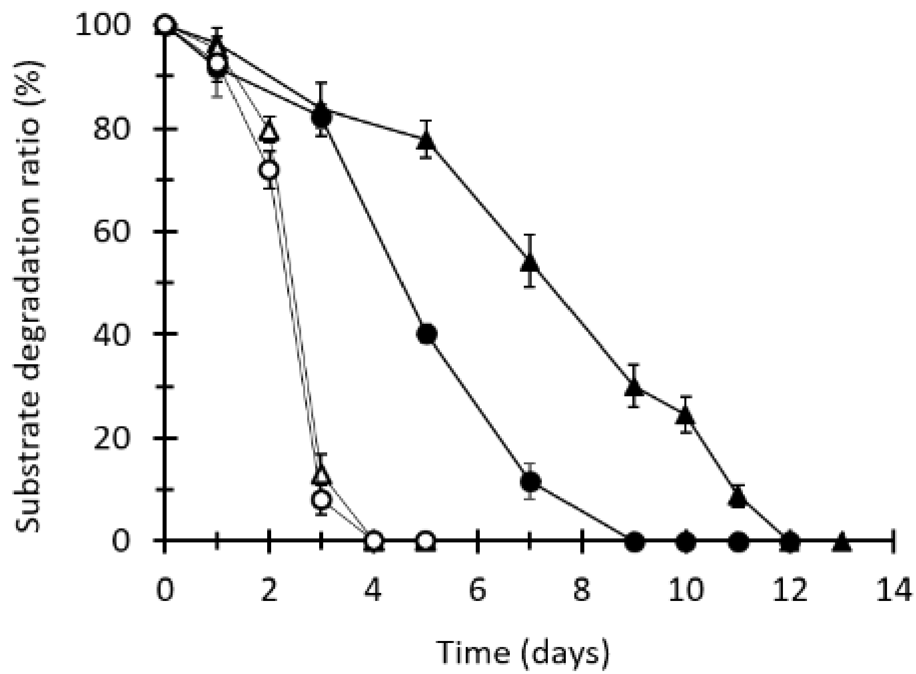


Fig. 2.7 Decomposition profiles of the single cultivation UUS1-1 bacterium (circles) and mixed cultivation UUS1-1 bacterium and GT38 bacterium (square) toward crystalline chitin (closed symbols) and colloidal chitin (open symbols). The mean values for the triple experiments are presented. Error bars show ± standard deviation.

2.4 Discussion

Strain UUS1-1 as a novel chitinolytic thermophilic anaerobic bacterium and strain GT38 as a symbiotic thermophilic anaerobic bacterium discovered in the current work were isolated achievably from a chitinolytic bacterial community (CBC). CBC was selected by the efficient chitin decomposition performance from enrichment culture containing biocompost and chitin powder at the beginning. In particular, the strain UUS1-1 as a new bacterium, originally came from the manure with no relation to marine and chitinolytic compounds, is the first description of a chitinolytic anaerobic thermophilic bacterium. In addition, UUS1-1 bacterium is classified in the OPB54 linkage as the second affiliated member besides *H. ethanolica* is the ethanol-hydrogen-contributing bacterium (Liu et al., 2014). Taxonomy of OPB54 included several uncultured taxa in the phylum *Firmicutes* and has been firstly investigated from a Yellowstone Park hot spring in the United State, but it was unsuccessful a pure clone isolation (Dunfield et al., 2012). The microorganisms that belong to OPB54 are found in a diverse environment. *H. ethanolica* strain LX-B^T from OPB54 group only has been reported (Liu et al., 2014). Following the analysis of phylogenetic tree relied on sequences of 16S rRNA gene, the strain UUS1-1 showed closely similar to strain LX-B^T; but several physiological and morphological characteristics of strain UUS1-1 are totally different comparing with that of strain LX-B^T (Table 1). Main differences distinguishing UUS1-1 bacterium from LX-B^T bacterium are chitin-decomposing activity, the long thin rod-shaped like hair and the optimal growth range between 55 and 60 °C. Besides chitin degradation, UUS1-1 bacterium shows similar metabolism properties in fermentation to that of LX-B^T bacterium, even though growth on pentoses such as arabinose and xylose (Liu et al., 2014)

were not observed for the UUS1-1 bacterium. Deposition of draft whole genome sequence data of strain UUS1-1 have been at the NCBI under the accession number JAAKDE00000000. Average nucleotide identity (ANI) evaluation revealed a 65.5% sequence identity between UUS1-1 bacterium and *H. ethanolica* strain LX-B^T. In accordance with genetic, phylogenetic and physiological properties, strain UUS1-1 reveals a new genus and new species in the phylum *Firmicutes*.

GT38 bacterium, also screened from the CBC, did not present chitin activity toward crystalline chitin powder and colloidal chitin. In contrast, GT38 bacterium could assimilate several polysaccharides like pentose sugars i.e. xylose and arabinose, and carbohydrates and syntrophic substrates such as lactate, acetate and ethanol comparing with that of UUS1-1 bacterium. The 16S rRNA gene sequences of GT38 bacterium showed a high similarity to *T. syntrophicus* (Sekiguchi et al., 2006) and *T. acetatoxydans* (Westerholm et al., 2011). These two bacteria are syntrophic acetate-oxidizing bacteria isolated from sludge and biogas digesters. In pure cultures of *T. syntrophicus* and *T. acetatoxydans*, the bacteria do not show activity toward amino acids, primary alcohols, carboxylic acids and organic acids, and ferment acetate and H_2 as their major product from glucose medium supplemented with yeast extract. However, in a co-culture with *M. thermautotrophicus* and *Methanoculleus* sp., which were hydrogenotrophic methanogen. Their syntrophic acetate-oxidizing activity was examined during co-culture of *T. syntrophicus* and *T. acetatoxydans*, respectively (Sekiguchi et al., 2006; Westerholm et al., 2011). Acetate, a major fermentation product of many microbes, is usually available product because acetate is a central intermediate during the different organic compounds' degradation in anaerobic environment. Currently, only four

syntrophic acetate-oxidizing bacteria, *Clostridium ultunense* (SchnÜrer et al., 1996), *Thermacetogenium phaeum* (Hattori et al., 2000), *Syntrophaceticus schinkii* (Westerholm et al., 2010), and *T. acetatoxydans* (Westerholm et al., 2011) have been isolated from different anaerobic reactors and characterized. GT38 bacterium probably assist chitin decomposition by UUS1-1 bacterium and have a symbiotic association with UUS1-1 bacterium. Another qualitative property of CBC has been described in an aquatic ecology. For instance, *Cytophaga-Flavobacteria* take an advantage from chitin decomposition by assimilating chitin decomposition (Cottrell and Kirchman, 2000; Beier and Bertilsson, 2011). Additionally, many *Actinomyces* bacteria are often identified as an active chitinolytic player in soil environments and have also been found together with non-chitinolytic activity bacteria groups (Gomes et al., 2000). In general, specific extracellular hydrolysis enzymes are necessarily to contribute an efficient degradation of polysaccharides and cooperates with symbiotic or syntrophic bacteria that utilize the fermented products to avoid negative inhibition of decomposing enzymes.

As similar bacterial communities' examples, the group of heterotrophic and symbiotic thermophilic anaerobic bacteria revealed the decomposition process of lignocellulosic substrate. ISHI-3 as a bacterial community composed of lignocellulosic bacteria and non-lignocellulosic symbiotic bacteria given a high decomposition performance toward rice straw and corn stover, isolated from the same manure compost (Shikata et al., 2018). The three non-cellulolytic anaerobic bacteria (*Pelotomaculum*, *Tepidanaerobacter,* and *Tepidimicrobium*) and the cellulose-xylan decomposing anaerobic bacteria (*Herbivorax saccincola*) also found in a heterotrophic or symbiotic relationship supporting cellulose-

xylan decomposition. This bacterial community declined the organic inhibitors through cellulose- and xylan-decomposing reactions from *Herbivorax saccincola* by the three non-cellulose degrading anaerobic bacteria utilizing sugars, solvents and organic acids (Shikata et al., 2018). In a mixed cultivation containing crystalline chitin powder with UUS1-1 bacterium and GT38 bacterium, the lactate concentration in the culture medium was double less than the single cultivation of UUS1-1 bacterium. The fermented products like remaining chito-oligosaccharides and lactate probably be converted to acetate, H_2, and CO_2 by GT38 bacterium. Similar phenotypic species such as *Tepidimicrobium xylanilyticum* strain EN5CB1, screened from a polylactic acid system, uptakes lactate as organic acid source, producing the highest amount of acetate, H_2 and CO_2 [Tseng et al., 2020]. Even though it is unclarified about metabolism of the conversion of lactate to acetate, H_2 and CO_2, GT38 bacterium utilized organic acid, lactate in presence of yeast extract (>3 g/L). Future study will investigate the interaction between strain GT38 and strain UUS1-1 to clarify their symbiotic relationship.

In short, UUS1-1 bacterium should be grouped as a new genus or new species and strain GT38 should be proposed as a new species and may have a symbiotic association for chitinolytic activity. Moreover, UUS1-1 bacterium and GT38 bacterium play a crucial role in cycle of carbon and nitrogen through chitin transformation in anaerobic thermophilic environments.

In addition, the biomass technology has influenced waste management, minimize waste generation and turn to renewable energy such as bioethanol and biogas production.

The biogas production such as methane from digestion of lignocellulose waste has been required several steps of process starting from pretreatment, hydrolysis, fermentation and methanogenesis for microbial conversion due to the limitation of microbial growth condition such as temperature, pH, and culture medium (Fannin et al., 1980). The pretreatment in biomass such as chitin was performed in two steps to extract the chitin from shell wastes: demineralization and deproteination. Next, the fermentation process is to use microorganisms utilize the biomass and synthesize the organic compounds such as acetic acid and lactic acid (Novik et al., 2017). Finally, the methanogenesis is the formation of methane by microorganisms/ methanogens that mostly belong to archaea.Here, the symbiotic relationship in CBC from chitin powder cultivation can produced methane for 66.1 g/m^3 using GC detector. Interestingly, CBC can decompose the chitin and produce methane in the same culture, in addition, it is the first publication to describe the chitin-decomposing bacterial community works at the thermophilic anaerobic condition. However, the yield of methane synthesis from chitin powder (0.5% w/v) is only 1.32% which need to be developed and optimized the process for a higher performance (Detman et al., 2018). Moreover, the methane-producing microorganism is necessarily investigated and isolated from the CBC culture to understand its genotype and phenotype to clarify the symbiotic relationship among microorganisms in the CBC.

2.5. Conclusion

UUS1-1 bacterium and GT38 bacterium as novel bacteria were isolated from a CBC by method of enrichment culture containing chitin or glucose as substrates. Single cultivations of UUS1-1 bacterium and GT38 bacterium and the evaluation of DNA sequencing indicated that these two bacteria are closely related to *H. ethanolica* and *T. acetatoxydans*, respectively. The strain UUS1-1 has high chitin decomposition performance, but GT38 bacterium did not show chitin decomposition performance. Following phylogenetic and physiological analysis, UUS1-1 bacterium should be grouped into new genus and species, and GT38 should be proposed into new species. Interestingly, this is the first record that UUS1-1 is an anaerobic thermophilic chitinolytic bacterium and the second member in the OPB54 linkage besides *H. ethanolica*. Potential of a single UUS1-1 cultivation and mixed cultivation of UUS1-1 bacterium and GT38 bacterium indicated that both bacteria maintain a symbiotic relationship through organic acids assimilation in chitin decomposition. This is the first description about the association between a new chitinolytic anaerobic thermophilic bacterium and a symbiotic thermophilic anaerobic bacterium in the decomposition of chitin.

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Chapter 3

Data analysis and genome mining of draft genome sequence of chitinolytic thermophilic anaerobic bacterium strain UUS1-1 assigning to genus *Hydrogenispora* of the uncultured taxon in the OPB54 linkage

Overview

A novel chitinolytic anaerobic thermophilic bacterium strain UUS1-1 (=JCM33882 =DSM111537) is affiliated with the genus *Hydrogenispora* of the uncultured taxon in the OPB54 linkage, the phylum *Firmicutes*. UUS1-1 bacterium has a unique long rod shape like a long hair and strong degradation potential for crystalline chitin powder. The analysis of whole genome of this bacterium was performed on an Ion GeneStudio S5 system with resulted in 86 contigs with 2,482,547 bp, 2,235 protein-coding sequences, and a G+C content of 52.1%. The dendrogram of average nucleotide identity (ANI) values showed 65.5% for *H. ethanolica* and 82.9% for similarly *Hydrogenispora* sp., that was virtually identified by metagenome-assembled genomes from a large-scale anaerobic digestion microbial genome database, was closest bacterium for UUS1-1. Strain UUS1-1 should be the second isolated bacterium, belonging to OPB54 cluster besides *H. ethanolica* and classified into a new genus and species. The deposition of data has been into genomes database at the National Center for Biotechnology Information (NCBI) under the accession number JAAKDE00000000.

Table 3.1 Specifications Table

Subject	Microbiology
Specific subject area	Genomics, Bacteriology
Data type	Figures, Tables
How data were acquired	Whole-genome sequencing using Ion GeneStudio S5 System
Data format	Raw and Evaluated
Data collection Parameters	The extraction of genomic DNA was from pure strain UUS1-1 culture (=JCM 33882 =DSM111537) assigned to member of the genus *Hydrogenispora*. Strain UUS1-1 genome was performed by using Ion GeneStudio S5 System. The reads were trimmed, and *de novo* assembled using CLC Genomic Workbench 20.0.1, and annotated using NCBI Prokaryotic Genome Annotation Pipeline (PGAP).
Data collection description	Extracted genomic DNA of strain UUS1-1, following whole-genome sequencing, assembly, and annotation
Location of data source	Japan International Research Center for Agricultural Sciences (JIRCAS) and University of Tsukuba Tsukuba, Ibaraki, Japan
Data accessibility	Repository name: NCBI Data identification number: JAAKDE000000000. The data version in this paper is JAAKDE010000000.1 Direct URL to data: https://www.ncbi.nlm.nih.gov/nuccore/JAAKDE000000000.1 The BioProject ID in GenBank is PRJNA607398 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA607398) The BioSample ID in GenBank is SAMN13885186 (https://www.ncbi.nlm.nih.gov/biosample/SAMN13885186)

	Repository name: IMG ER Data identification number: Genome ID: 2882933831 Direct URL to data: https://genome.jgi.doe.gov/portal/IMG_2882933831 GOLD study ID: Gs0149361 (https://gold.jgi.doe.gov/study?id=Gs0149361) GOLD Project ID: Gp0502315 (https://gold.jgi.doe.gov/projects?id=Gp0502315)

3.1 Introduction

Chitin consists of insoluble complex polymer β-1,4-*N*-acetylglucosamine (GlcNAC) chain. This crystalline chitin produces the second enormous polymer following cellulose with gigantic global accumulation rate over hundred billion tons per year. In present, chitin is components of an arthropod exoskeleton, such as shrimps and insects (Gooday, 1990; Keyhani and Roseman, 1999). A conversion of chitin and its derivatives can be contributed to highly valuable components for multi-purposed applications in several industries i.e. pharmaceutics, food and agriculture. The decomposition of crystalline chitin powder requires the cooperation of multi-enzymes such as chitinase.

Chitinases (EC 3.2.1.14) is a chitin-hydrolyzing enzyme. Chitinase are found in the secretion of various microorganisms, including archaea, bacteria, and fungi, and high-level kingdom by higher plants, insects, and animals. Many chitinolytic microorganisms have been isolated from various environments such as marine and landfill. Among microbes, bacterial chitinases hold promises for numerous commercial applications. Chitinases with thermo resistance from bacteria should have many applications because thermostable chitinases can reduce expenses and easier to operate (Haki and Rakshit, 2003; Yeoman et al., 2010); however, recent chitinases used in applications are majorly produced by mesophilic bacteria (Tom and Carroad, 1981). Thus, isolating, identifying, and studying the functions and characteristics of thermophilic chitinases are an important research. In current, there is a few information in the function of anaerobic thermophilic bacteria in crystalline chitin decomposition systems.

In present, we screened cow compost to study new thermostable chitin decomposition activity and characterized a bacterial community under thermophilic conditions. The strain UUS1-1 that was affiliated to the genus *Hydrogenispora* classified into an unidentified taxonomic OPB54 cluster, was achievably isolated as a single cultivation from the bacterial community. UUS1-1 bacterium was deposited into RIKEN BioResource Research Center as JCM33882 and the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) as DSM111537, has a unique and an outstanding characteristic such as a long rod-shape like a long hair (Fig. 3.1) and high chitinase performance under thermophilic anaerobic environment. UUS1-1 bacterium is the first thermophilic anaerobic chitinolytic report and the second member next to *H. ethanolica* belonging to the OPB54 linkage (Liu et al., 2014).

In this work, the sequence of draft genome of UUS1-1 bacterium revealed a new knowledge on anaerobic thermophilic chitin decomposition in the genus *Hydrogenispora* classified OPB54 belong to an unidentified taxonomic culture at the order- or class-level. Characteristics of the genome are shown in Table 3.1 and 3.2. sequencing of DNA, processed using the Ion GeneStudio S5 System, output 11,760,377 single reads with an average read length of 187 bp. The genomic sequences were trimmed and assembled *de novo* using CLC Genomic Workbench 20.0.1 (CLC Bio, Qiagen, Valencia, CA), released 86 contigs including an N50 of 117,588 bp and a maximum contig size of 238,883 bp. The total genome of strain UUS1-1 consisted of 2,482,547 bp with a G+C content of 52.1%, nearly familiar to that of *H. ethanolica* strain LX-B^T (5,983,461 bp, with a G+C content of 54.2%). The distribution of genome size in strain UUS1-1 provided 2.4 times smaller than that in *H. ethanolica*.

The phylogenetic tree among closest relatives relied on 16S rRNA sequences is *H. ethanolica* strain LX-B (90.4% similarity, Accession no.: SLUN00000000) as the only candidate in the taxa OPB54 cluster, *Firmicutes* phylum (Fig. 3.2 and Suppl. Table 3.1) (Liu et al., 2014). The comparison of the properties between *H. ethanolica* and the strain UUS1-1 found that *H. ethanolica* as the mesophilic anaerobic bacterium with totally different phenotypic characteristics to strain UUS1-1. Neighbors of strain UUS1-1, such as *Desulfotomaculum thermobenzoicum* (Tasaki et al., 1991), *D. hydrothermale* (Haouari et al., 2008), *Pelotomaculum thermopropionicum* (Imachi et al., 2002), and *Moorella humiferrea* (Nepomnyashchaya et al., 2012) shared less than 87% identity. Moreover, the average nucleotide identity (ANI) (Goris et al., 2007) of strain UUS1-1 and other relative 8 strains, *Hydrogenispora*, *Desulfofundulus*, *Desulfotomaculum* and *Pelotomaculum* species, showed 65.5% to *H. ethanolica* strain LX-B, 82.9% to *Hydrogenispora* sp. (DUQQ01000000) that was identified by comprehensive genome-resolved metagenomics (Campanaro et al., 2020) and 65% less to other bacteria (Fig. 3.3 and Suppl. Table 3.2). According to the results, strain UUS1-1 can be proposed to the taxonomical classification as a novel genus and species.

Genome annotation was executed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016). In *silico* analysis, strain UUS1-1 possessed 2,337 total predicted genes, while the predicted protein-coding genes are 2,235 protein-coding sequences (CDSs), 4 rRNA genes (encoding two 5S rRNAs, one 16S rRNAs, and one 23S rRNAs), 47 tRNA genes and 4 CRISPR genes. The prediction of CDSs pattern to the functional 25 general Clusters of Orthologous Groups (COGs) categories were carried out a putative function under Genome ID 2882933831. The presence of predicted enzymes

regarding chitinolytic metabolism of strain UUS1-1 was proved for chitinases (EC 3.2.1.14), *N*-acetylglucosaminidases (EC 3.2.1.52), chitin deacetylase (EC 3.1.1.72/EC 3.5.1.41), glucosidase (EC 3.2.1.20/ EC 3.2.1.21), glucokinase (EC 2.7.1.2), glucosamine-fructose-6-phosphate aminotransferase (EC 2.6.1.16), phospho-glucosamine mutase (EC 5.4.2.10), glucosamine-6-phosphate deaminase (EC 3.5.99.6), and glucosamine-1-phosphate *N*-acetyltransferase/UDP-*N*-acetylglucosamine pyrophosphorylase (EC 2.3.1.157/EC 2.7.7.23) (Table 3.2). The predicted chitinases and chitin deacetylases are a crucial role in the efficient insoluble chitin decomposition in strain UUS1-1. The genome information of strain UUS1-1 will be useful for studies of a bacterium belonging to OPB54 cluster and application for chitin utilization and its derivatives products under thermophilic anaerobic environments (https://www.ncbi.nlm.nih.gov/nuccore/JAAKDE000000000.1).

3.2 Materials and Methods

3.2.1 Genomic DNA extraction and sequencing

Genomic DNA of strain UUS1-1 was extracted using phenol/chloroform from cells grown under anaerobic cultures at 60°C (Murray and Thompson, 1980). The fragmentation and library DNA preparation were using an Ion Xpress Plus Fragment Library kit (Thermo Fisher Scientific) following the manufacturer's protocol. Prepared fragments were generated with an average size length of 400 bp. After library preparation, the fragment size of 200- to 300-bp were selected by electrophoresis on E-Gel SizeSelect II agarose gels (Invitrogen,

Thermo Fisher Scientific). The genomic DNA of *Hydrogenispora* sp. UUS1-1 was performed using an Ion GeneStudio S5 System.

3.2.2 Genome assembly and annotation

The low-quality reads were removed, and *de novo* genome was assembled using CLC Genomic Workbench version 20.0.1. The annotation of genome was determined using NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016). The prediction and function of each annotated gene were computed using the Integrated Microbial Genomes-Expert Review (IMG-ER) system developed by the Joint Genome Institute Walnut Creek, CA, USA (Markowitz et al., 2009).

3.2.3 Genomic ANI

Pairwise ANI values of whole genome sequences of 2 *Hydrogenispora* strains, 2 *Desulfofundulus* strains, 3 *Desulfotomaculum* strains,1 *Pelotomaculum* strain, and 1 *Moorella* strain were evaluated using GENETYX NGS version 4.1.1 with the BLASTALL algorithm. The matrix constructed from ANI values among these 10 strains including UUS1-1 was represented to a dendrogram of genome using algorithms; the unweighted pair group method with arithmetic means and the single-linkage clustering method in the R statistical program (Goris et al., 2007).

Table 3.2 Features of the *Hydrogenispora* sp. UUS1-1 genome

Features	Description
Reads numbers used in the assembly	11,760,377
Mean read length	187 bp
Genome size	2,482,547 bp
Contigs number	86
G+C content (%)	52.1
N50 contig length	117,588 bp
Mean contig length	28,867 bp
Total number of genes	2,337
CDSs number	2,235
rRNAs number	10
tRNAs number	48
CRISPRs number	4
Genome coverage depth	1,469-fold

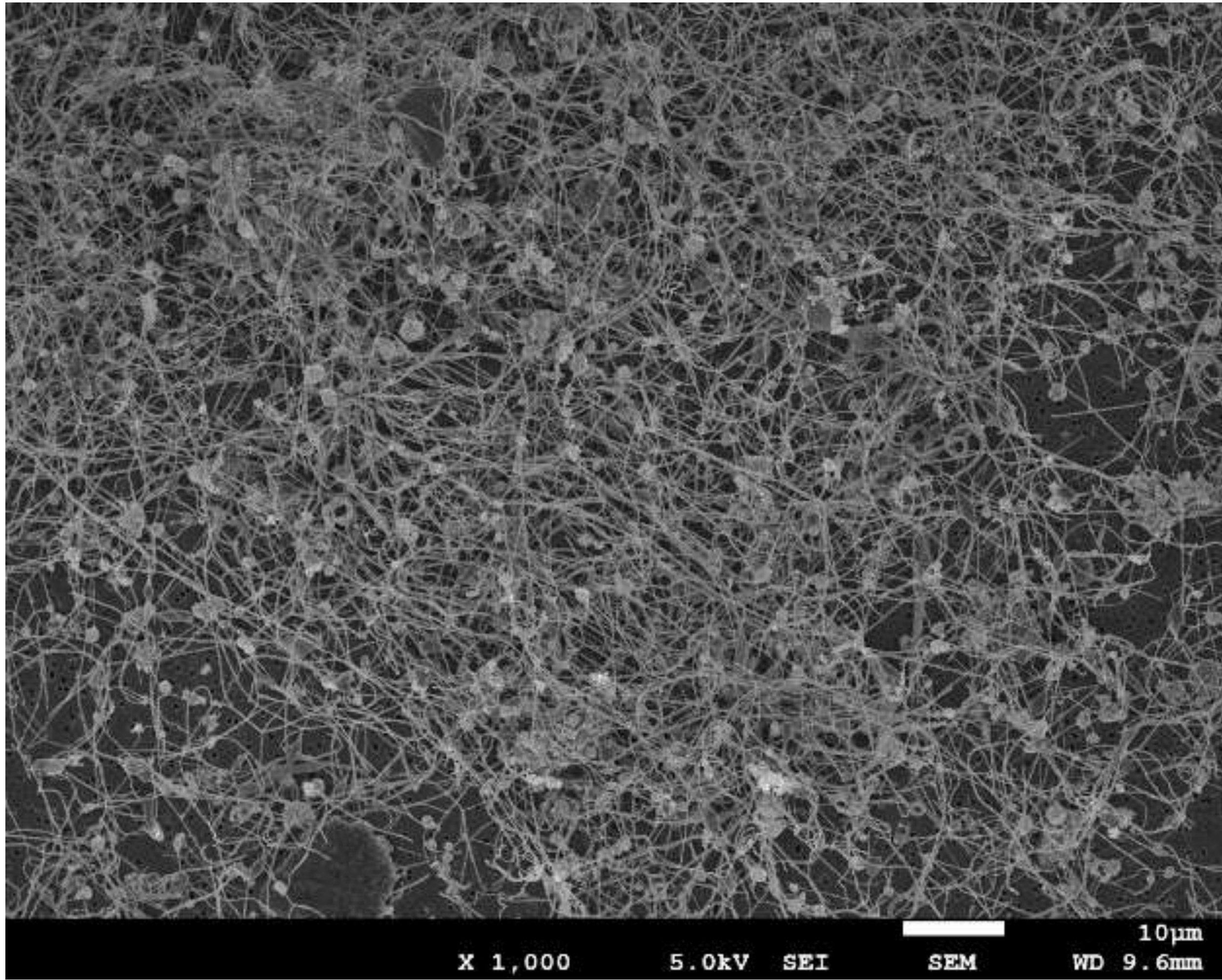

Fig. 3.1 Scanning electron micrographs of *Hydrogenispora* sp. UUS1-1 in BM7CO medium with 1.0% glucose for three days. The white bars are 1 µm.

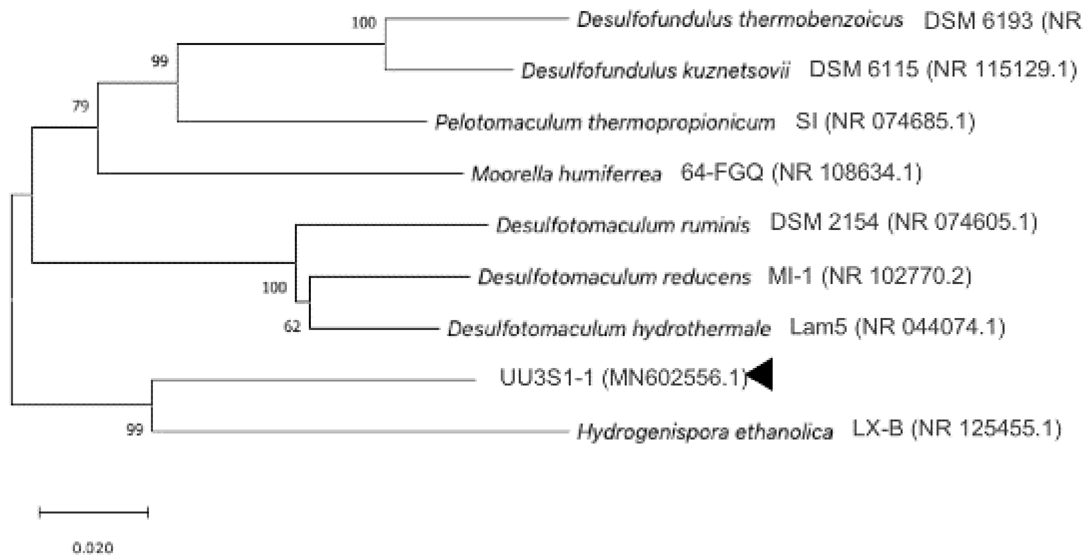

Fig. 3.2. Phylogenetic trees of *Hydrogenispora* sp. UUS1-1 relative to closely species of the phylum *Firmicutes* using Neighbor-joining method based on an analysis of distance-matrix for 16S rRNA gene sequences. Bootstrap percentages provided from 1000 resamplings. Bar presents 0.020 nucleotide changes per sequence position.

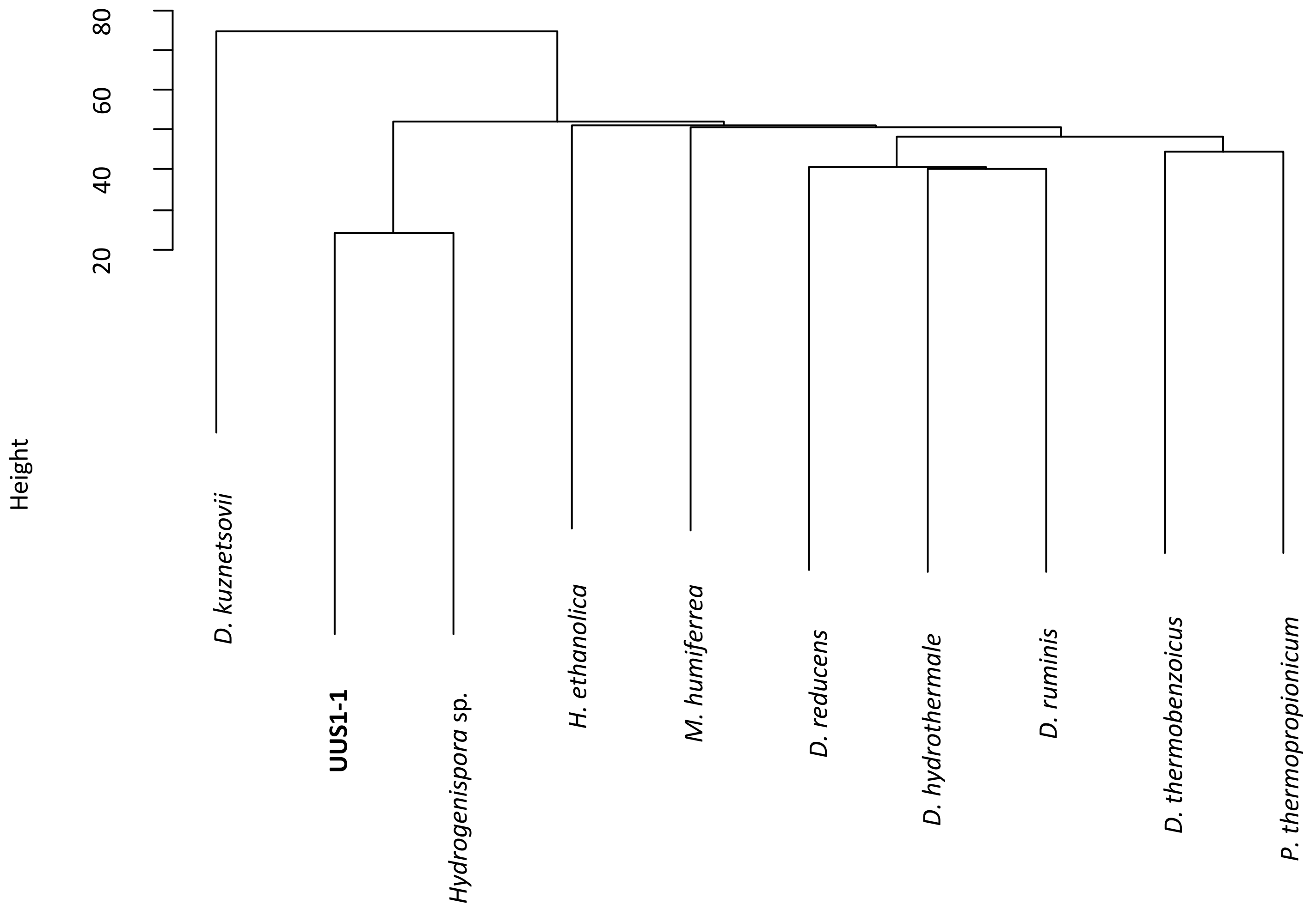

Fig. 3.3 Average nucleotide identity (ANI)-based dendrogram. The ANI value of each strain was accessed, and the ANI-based dendrogram was constructed using the unweighted pair group method with arithmetic means. Strains which were used for calculation and dendrogram were as follows: *Desulfofundulus kuznetsovii* (NZ_LGGU01000000), *Desulfofundulus thermobenzoicus* (NZ_WHYR01000000), *Desulfotomaculum hydrothermale* (NZ_FQXF01000000), *Desulfotomaculum reducens* (NC_009253), *Desulfotomaculum ruminis* (NC_015589), *Hydrogenispora ethanolica* (NZ_SLUN01000000), *Hydrogenispora* sp. (DUQQ01000000), and *Pelotomaculum thermopropionicum* (AP009389).

Table 3.3 Predicted chitinolytic genes and related genes

Functions	EC	Predicted genes
Chitinase	EC 3.2.1.14	WP_181339859.1, WP_181339858.1, WP_181339894.1, WP_181340572.1, WP_181340429.1
N - acetylglucosaminidase	EC 3.2.1.52	WP_181339652.1
chitin deacetylase	EC 3.1.1.72/ EC 3.5.1.41	WP_181339645.1, WP_181340147.1, WP_181340352.1, WP_181339341.1, WP_181339805.1, WP_181339807.1, WP_181339869.1
Glucosidase	EC 3.2.1.21	WP_181340416.1, WP_181338805.1
Glucokinase	EC 2.7.1.2	WP_181340205.1, WP_181339717.1
Glucosamine – fructose – 6 - phosphate aminotransferase	EC 2.6.1.16	WP_013809510.1
Phosphoglucosamine mutase	EC 5.4.2.10	WP_007289355.1
Glucosamine -6- phosphate deaminase	EC 3.5.99.6	WP_013298094.1
Glucosamine -1- phosphate N-acetyltransferase/UDP - N-acetylglucosamine pyrophosphorylase	EC 2.3.1.157/ EC 2.7.7.23	WP_007506043.1

Supplementary Table 3.1 List of 16S rRNA gene-based phylogenetic tree of *Hydrogenispora* sp. UUS1-1 and related members

Organisms	Strain	Accession no.
Hydrogenispora sp.	UUS1-1	MN602556.1
Hydrogenispora ethanolica	LX-B	NR 125455.1
Desulfofundulus thermobenzoicus	DSM 6193	NR_119246.1
Pelotomaculum thermopropionicum	SI	NR_074685.1
Desulfotomaculum reducens	MI-1	NR_102770.2
Desulfotomaculum hydrothermale	Lam5	NR_044074.1
Moorella humiferrea	64-FGQ	NR 108634.1
Desulfotomaculum ruminis	DSM 2154	NR 074605.1
Desulfofundulus kuznetsovii	DSM 6115	NR_115129.1

Supplementary Table 3.2 *Hydrogenispora* and related isolates used in dendrogram of average nucleotide identity (ANI) values.

Organisms	Accession no.
Hydrogenispora sp. UUS1-1	JAAKDE00000000
Desulfofundulus kuznetsovii	NZ_LGGU01000000
Desulfofundulus thermobenzoicus	NZ_WHYR01000000
Desulfotomaculum hydrothermale	NZ_FQXF01000000
Desulfotomaculum reducens	NC_009253
Desulfotomaculum ruminis	NC_015589
Hydrogenispora ethanolica	NZ_SLUN01000000
Hydrogenispora sp.	DUQQ01000000
Pelotomaculum thermopropionicum	AP009389

Supplementary Table 3.3 Pairwise ANI values among *Hydrogenispora* sp. UUS1-1, *Desulfofundulus* spp., *Desulfotomaculum* spp., and *Pelotomaculum thermopropionicum*.

	UUS1-1	*Desulfofundulus kuznetsovii*	*Desulfofundulus thermobenzoicus*	*Desulfotomaculum hydrothermale*	*Desulfotomaculum reducens*	*Desulfotomaculum ruminis*	*Hydrogenispora ethanolica*	*Hydrogenispora* sp.	*Pelotomaculum thermopropionicum*
UUS1-1	100								
Desulfofundulus kuznetsovii	62.02	100							
Desulfofundulus thermobenzoicus	63.78	65.65	100						
Desulfotomaculum hydrothermale	63.14	64.23	66.93	100					
Desulfotomaculum reducens	63.84	62.79	65.18	71.25	100				
Desulfotomaculum ruminis	64.01	63.9	66.93	71.68	71.25	100			
Hydrogenispora ethanolica	65.47	61.6	64.45	63.32	62.61	63.64	100		
Hydrogenispora sp.	82.93	61.53	63.87	62.8	62.15	62.51	65.24	100	
Pelotomaculum thermopropionicum	63.06	64.67	68.74	65.9	65.62	65.78	63.43	62.87	100

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Chapter 4

Capillibacterium thermochitinicola gen. nov., sp. nov., a novel anaerobic thermophilic chitinolytic bacterium from manure compost

Overview

It is still few reports about anaerobic thermophilic bacterium which is degradable insoluble chitin. A novel, obligately anaerobic, thermophilic, chitin-degrading bacterium, strain UUS1-1^T, was isolated from manure compost on Ishigaki Island, Japan (24°23'09.9"N 124°11'28.1"E) by enrichment culture using chitin powder as carbon source. Cells were a Gram-negative, spore forming, flagella and unique long, hair-like rod-shaped. The strain was exhibited strong chitin degradation ability and was able to grow under anaerobic thermophilic condition. The novel strain grew at 45–65°C (optimum 55 °C) and pH 6.5 – 8.0 (optimum pH 7.0). The strain could grow not only chitin but also widely carbon sources including GlcNAc, glucose, maltose, cellobiose, galactose, galactan, fructose, and sucrose. The main end-products of chitin degradation were acetate, lactate, H_2 and CO_2. Phylogenetic tree analysis based on 16S rRNA gene sequences presented a clear affiliation of the proposed bacterium to the phylum *Firmicutes*; the most closely related species was *Hydrogenispora ethanolica* strain LX-B^T and *Desulfotomaculum thermobenzoicum* strain DSM6193^T with 90.4 and 87.8 % 16S rRNA gene sequences similarity, respectively. The G+C content of strain UUS1-1^T was 52.1 mol%. Major fatty acids of the strain were was $C_{16:0}$, anteiso-$C_{15:0}$,

$C_{14:0}$, $C_{12:0}$3-OH and dimethyl acetal-$C_{13:0}$. Biochemical, physiological, chemotaxonomic and phylogenetic analyzes indicated that strain UUS1-1^T could not be assigned to any known genus of the *Hydrogenispora* and *Desulfotomaculum*. Therefore, there properties of genomes and phenotypes suggest that strain UUS1-1^T should be classified within a novel genus and species, for which the name *Capillibacterium thermochitinicola* gen. nov., sp. nov. is proposed. The type strain of *Capillibacterium thermochitinicola* is UUS1-1^T (= JCM 33882^T = DSM 111537^T)

4.1 Introduction

Chitin is a crystalline polymer of β-1,4 *N*-acetylglucosamine (GlcNAc) which is the second largest polysaccharide in the global nature after cellulose production with around 10 billion tons per year (Gooday, 1990) (Kaiser et al., 2008). The structure of chitin exists in the exoskeletons of arthropods, and crustacean tightly linkage with protein polymer (Keyhani and Roseman, 1999). Additionally, chitin can be hydrolyzed into valuable products such as *N*-acetylglucosamine and chitin oligosaccharides widely applied in agricultural, pharmaceutical and food industries (Hamed et al., 2016). The compact crystalline chitin was decomposed by multi-enzymes such as chitinase. Chitinases (EC 3.2.1.14) are synthesized by various microbes, including archaea, bacteria and fungi, and by insects, higher plants and animals. Bacterial chitinases hold promises for use in several commercial applications. Thus, screening and characterizing the properties of thermophilic enzymes, and determining the function of chitinases are important related research areas; however, there remains a paucity

of information about chitin degradation systems from anaerobic thermophilic bacteria. In this study, we report the classification and taxonomy of a novel anaerobic, thermophilic, chitinolytic bacterium strain UUS1-1^T belonging to the second candidate in the uncultured taxonomic OPB54 cluster at the order- or class-level of the phylum *Firmicutes*.

4.2 Isolation and Ecology

Compost samples, including bovine manure were taken from the Ishigaki Island compost facility (Ishigaki Taihi Center, Ishigaki City, Japan) in May 2015. Originally, the compost included sugarcane bagasse, rice husk and bovine faeces. The samples were collected below than 10 to 20 cm from the surface at temperature between 60 and 75 °C and were packed in Ziploc bags (Johnson. Co., Ltd, Yokohama, Japan) (Shikata et al., 2018; Widyasti et al., 2018). The samples have stored at -80 °C until starting the enrichment culture for the isolation. To isolate chitinolytic anaerobic microbes, Hungate technique (Hungate, 1969) was performed for enrichment culture. The basal medium (BM7CO) was composed of the following (per liter) 1.5 g KH_2PO_4, 2.9 g K_2HPO_4, 2.1 g Urea, 3.0 g yeast extract, 4.0 g Na_2CO_3, 0.01 g $CaCl_2.2H_2O$, 0.5 g L-cysteine-HCl, 0.5 mg resazurin and 200 μL mineral solution (25.0 gL-1 $MgCl_2.6H_2O$, 0.312 gL-1 $FeSO_4.7H_2O$ and 37.5 gL-1 $CaCl_2.2H_2O$). The BM7CO (pH 7.0) with 0.5% (w/v) crystalline chitin powder was boiled under a stream of CO_2 gas and autoclaved. All the buffers and chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan. The initial culture was incubated at statically 60 °C. When the cultures were incubated for 5 days, the cultures with chitin

decomposition were sub-cultured again to flash same BM7CO with 0.5% (w/v) chitin powder and repeated enrichment culture of five times. To isolate single colony from the enrichment culture, the cultures were diluted with BM7CO and formed colony by roll tubes technic using the medium including 1.5% (w/v) agarose (BD Difco) with 1.0% (w/v) colloidal chitin. The preparation of colloidal chitin was conducted using chitin powder by Hsu and Lockwood method (Hsu and Lockwood, 1975) and stored at 4 ºC until preparing roll tubes. The total content of chitin was evaluated after drying at 70 °C overnight. Even the isolated colony exhibited the stable chitin decomposition ability, but the microscopic images of the colony showed the contamination of other strains. Therefore, next isolation performed in the BM7CO medium containing 1.0 % GlcNAc repeatedly for five times and executed in 1% glucose – BM7CO medium to confirm the pure colony respectively. So far, few reports are published about an anaerobic thermophilic bacterium with chitin decomposition ability otherwise extremophile bacteria and archaebacteria isolated from a deep-sea and a high salinity sample (Bale et al., 2019). Consequently, the organisms were subjected to taxonomic database determining its position as a member of a novel genus and species. Chitin decomposition ability of UUS1-1^T was measured to chitin residual weights subtracted from the initial chitin weight in media supplemented with colloidal chitin or chitin. The bacterium strain UUS1-1^T could completely decompose colloidal chitin and chitin powder under anaerobic thermophilic condition within 4 and 12 days respectively in 12 days (Fig 4.1).

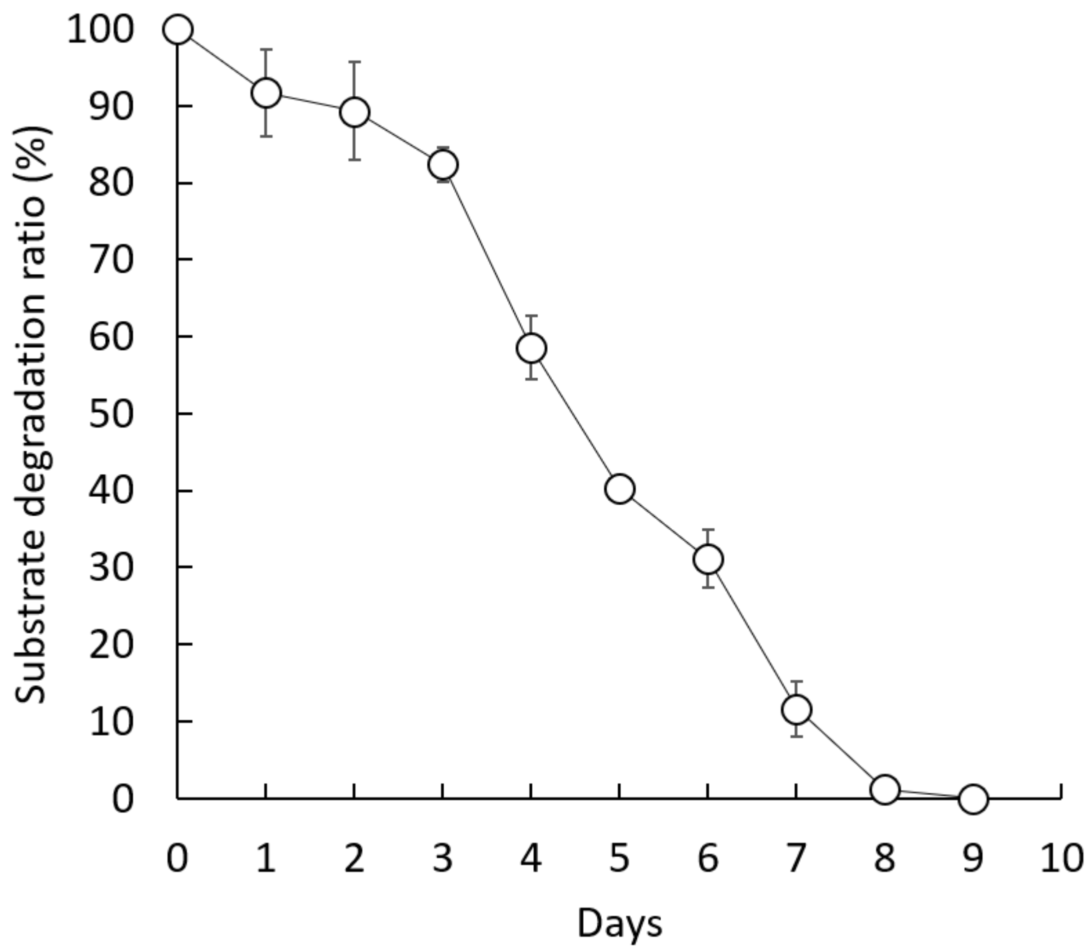

Fig. 4.1 Biodecomposition activity of strain UUS1-1^T toward chitin. The calculated percentage of crystalline chitin (i.e., residual) relative to the weight at day 0 is presented. Error bars represent standard deviations (n = 3).

4.3 16S rRNA gene phylogeny

Whole-genome DNA of stain UUS1-1^T was extracted and purified using NucleoSpin® Microbial DNA (Takara Bio, Shiga, Japan). Amplification of PCR and sequencing of the 16S rRNA gene were conducted as described previous report (Shikata et al., 2018). The PCR primers used in the amplification were the bacterial domain universal primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and the prokaryotic universal primer 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR product was sequenced directly on a DNA sequencer (ABI PRISM 310 instrument; Applied Biosystem) and sequences was assembled by using GENETYX software version 13. The complete 16S rRNA gene sequence of strain UUS1-1^T was compared with relative sequences taken from ribosomal sequence database in the National Center for Biotechnology Information (NCBI). Comparative 16S gene rRNA sequences phylogenetic tree was evaluated by the BLAST program and multiple alignments with sequences of closely related taxa were carried out by the GenBank database using CLUSTAL_X v.1.81 (Larkin et al., 2007). Phylogenetic tree was established by the neighbor-joining method (Saitou and Nei, 1987) and Tamura-3 parameter model (Tamura, 1992) using BioEdit version 7.1.9 (Hall, 1999) MEGA version X version 10.1 (Kumar et al., 2018). The topology of tree and distances was confirmed by bootstrap analysis based on 1,000 re-samplings (Felsenstein, 1985). Strain UUS1-1^T relied on 16S rRNA gene sequences was related to *Hydrogenispora ethanolica* LX-B^T (<90.4%) (Liu et al., 2014) in the unidentified taxonomy OPB54 in the phylum *Firmicutes* which was belonged to the order- or class-level relied on the ARB-SILVA analysis (http://arb-silva.de) (Quast et al., 2012). Strain UUS1-1^T is the second candidate in the OPB54 group, which was investigated firstly

from Obsidian Pool (OP) by Hugenholtz and colleagues (Hugenholtz et al., 1998). However, a relatively low similarity of the genus *Desulfotomaculum* (Tasaki et al., 1991; Haouari et al., 2008), *Pelotomaculum* (Imachi et al., 2002) and *Moorella* (Nepomnyashchaya et al., 2012) comparing to strain UUS1-1^T showed below 88.0% similarity. In addition, the differences in genetic, phenotypic and biochemical characteristics among strain UUS1-1^T and phylogenetic neighbors are summarized in Fig 4.2. Strain UUS1-1^T can be clearly apart from *Hydrogenispora ethanolica* LX-B^T by growth temperature, hydrolyze β-glucan, galactan and chitin.

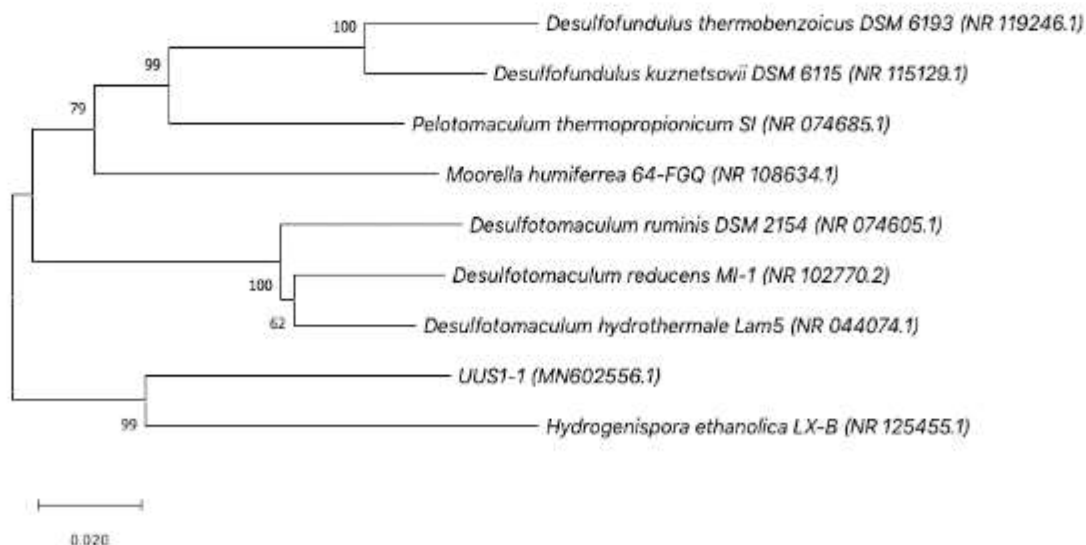

Fig. 4.2. Phylogenetic tree based on 16S rRNA gene sequences and evaluated by Neighbor-joining showing the relationship between strain UUS1-1^{T} and closely related type strains of species from the genus *Hydrogenispora*, *Desulfotomaculum*, *Pelotomaculum* and *Moorella* within the phylum *Firmicutes*. The construction of phylogenetic tree based on a distance-matrix was analyzed by 16S rRNA gene sequences. Accession numbers for 16S rRNA gene sequences are given in parentheses for each strain. Bootstrap percentages were calculated from 1000 resamplings. The bar represents 0.020 nucleotide changes per sequence position.

1 **4.4 Genome features**

2 The analysis of whole-genome sequence was performed using the Ion GeneStudio S5
3 system. In addition, the genomic assembly was evaluated *de novo* using CLC Genomics
4 Workbench 20.0.1 (CLC Bio, Qiagen, Valencia, CA, USA). The executed genome sequence
5 of strain UUS1-1^T resulted in 2,482,547 bp, contained 52.1% of G+C content and 86 contigs
6 with an N50 of 117,588 bp and a maximum size of 238,885 bp. The deposition of whole-
7 genome sequence was in the GenBank under accession number JAAKDE00000000. The
8 most closely related strain in this genus, *H. ethanolica* strain LX-B^T differs in its G+C content
9 but 2.4 times larger in size (5,983,461 bp, with a G+C content of 54.2%). The average
10 nucleotide identity (ANI) values and the digital DNA–DNA hybridization (dDDH) value
11 were calculated using the ANI calculator (www.ezbiocloud. net/tools/ani) (Yoon et al., 2017)
12 and the Genome-to-Genome Distance Calculator (GGDC)
13 (http://ggdc.dsmz.de/distcalc2.php) (Meier-Kolthoff et al., 2013), respectively. The ANI and
14 the dDDH values based on the genomic sequences between strain UUS1-1^T and the closest
15 relative strain, *H. ethanolica* strain LX-B^T (accession no. SLUN00000000) and
16 *Hydrogenispora* sp. isolate (accession no. DUQQ01000000), which was identified by
17 comprehensive genome-resolved metagenomics, gave ANI values of 65.5% and 82.9%, and
18 dDDH values of 21.0% and 26.0%, respectively, being lower than defined thresholds for
19 species 95%–96% for ANI and 70% for GGDC (Goris et al., 2007; Richter and Rosselló-
20 Móra, 2009). Based on the experimental results, strain UUS1-1^T reveals a novel species in a
21 new genus of the unidentified taxonomic group OPB54 in the phylum *Firmicutes*. The
22 annotation of genome sequences was performed using the NCBI Prokaryotic Genome

23 Annotation Pipeline platform (Markowitz et al., 2009). Strain UUS1-1^T provided 2,337 total
24 predicted genes, 2,235 protein-coding sequences, four rRNA genes (encoding two 5S rRNAs,
25 a 16S rRNA and a 23S rRNA), 47 tRNA genes and 4 CRISPR genes. The predicted protein-
26 coding sequences were assigned putative functions using the 25 Clusters of Orthologous
27 Groups under Genome ID 2882933831. The presence of predicted enzymes related to chitin
28 metabolism in strain UUS1-1^T was confirmed and included chitinase (EC 3.2.1.14), *N*-
29 acetylglucosaminidase (EC 3.2.1.52), chitin deacetylase (EC 3.1.1.72/EC 3.5.1.41),
30 glucosidase (EC 3.2.1.20/ EC 3.2.1.21), glucokinase (EC 2.7.1.2), glucosamine-fructose-6-
31 phosphate aminotransferase (EC 2.6.1.16), phospho-glucosamine mutase (EC 5.4.2.10),
32 glucosamine-6-phosphate deaminase (EC 3.5.99.6) and glucosamine-1-phosphate *N*-
33 acetyltransferase/UDP-N-acetylglucosamine pyrophosphorylase (EC 2.3.1.157/EC
34 2.7.7.23). The predicted chitinases and chitin deacetylases may be an important player for an
35 efficient decomposition of crystalline chitin by strain UUS1-1^T.

Table 4. 1
Phenotypic characteristics of strain UUS1-1^T and its close 16S rRNA gene neighbors.

Characteristic	1[a]	2	3	4	5	6
16Sr RNA similarity (%)	100	<90.4	<87.6	<87.3	<87.1	<86.9
Cultivated environment	Compost	Anaerobic sludge treating herbicide wastewater	Kraft pulp sludge reactor	Methanogenic granular sludge	Hot spring in northeast Tunisia	Terrestrial hydrothermal spring
Cell morphology	Hair-like, long rod	Rod	Spindle rod	Sausage rod	Rod	Rod
Cell size (μm)	0.15–0.40 × 15.0–80.0 μm	0.3–0.5 × 3.0–18.0 μm	1.5–2.0 × 5.0–8.0 μm	0.7–0.8 × 1.7–2.8 μm	0.5 × 2.0–5.0 μm	0.3–0.5 × 2.0–5.0 μm
Motility	+[b]	−	+	−	+	+
Spore location	+/Terminal and subterminal	+/Terminal	+/ND[c]	+/Center	+/Terminal and subterminal	+/Terminal
Gram	Negative	Viable	Positive	Viable	Viable	Positive
Optimum temp. (°C)	55/45–65	37–45/20–50	62/40–70	55/45–65	50/40–60	65/46–70
Optimum pH	7.0/6.5–7.5	6.0–7.7/5.0–8.0	7.2/6.0–8.0	7.0/6.7–7.5	7.1/5.8–8.2	6.8–7.0/5.5–8.5
NaCl tolerance (%w/v)	0–4.5	0–2.5	ND	0–0.4	0–1.5	0–1.5
Products	Acetate, lactate, H_2, CO_2	Acetate, ethanol, H_2	Acetate	Acetate, malate, propionate, succinate	Acetate, CO_2	Acetate
DNA G+C content (mol%)	52.1	56.1	52.8	52.8	46.8	51.0
References	This study	Liu et al. 2014	Tasaki et al. 1991	Imachi et al. 2002	Haouari et al. 2008	Nepomnyashchaya et al. 2012

[a] Strains: 1, UUS1-1T; 2, *Hydrogenispora ethanolica* LX-B^T; 3, *Desulfotomaculum thermobenzoicum* TSB^T; 4, *Pelotomaculum thermopropionicum* SI^T; 5, *Desulfotomaculum hydrothermale* Lam5^T; 6, *Moorella humiferrea* 64-FGQ^T.
[b] +, Positive; –, negative.
[c] ND, not detected.

4.5 Physiology and Chemotaxonomy

Bacterial cells of strain UUS1-1^{T}were observed by a scanning electron microscope (SEM; JSM-6320F; JEOL, Tokyo, Japan) and a transmission electron microscopy (TEM; Hitachi H-7600. Tokyo, Japan) as described by Shikata et al., 2018b. Cell length was calculated using ImageJ software (Schneider et al., 2012). Gram staining was conducted using Gram stain kit S (BD Difco). Cell morphology of strain UUS1-1^T was long, hair-like rod with 0.15–0.40 µm in width and 15.0 - 80.0 µm in length (Fig 4.2A, B). Spores were located terminally and formed a bulged sporangium (Fig 4.2B). type cell, i.e., the periplasmic space contains no cellular material, and the peptidoglycan layer (PG), plasma membrane (PM) and outer membrane (OM) were observed (Fig. 4.2C and D) (Beveridge, 1999). Gram staining was demonstrated to be negative.

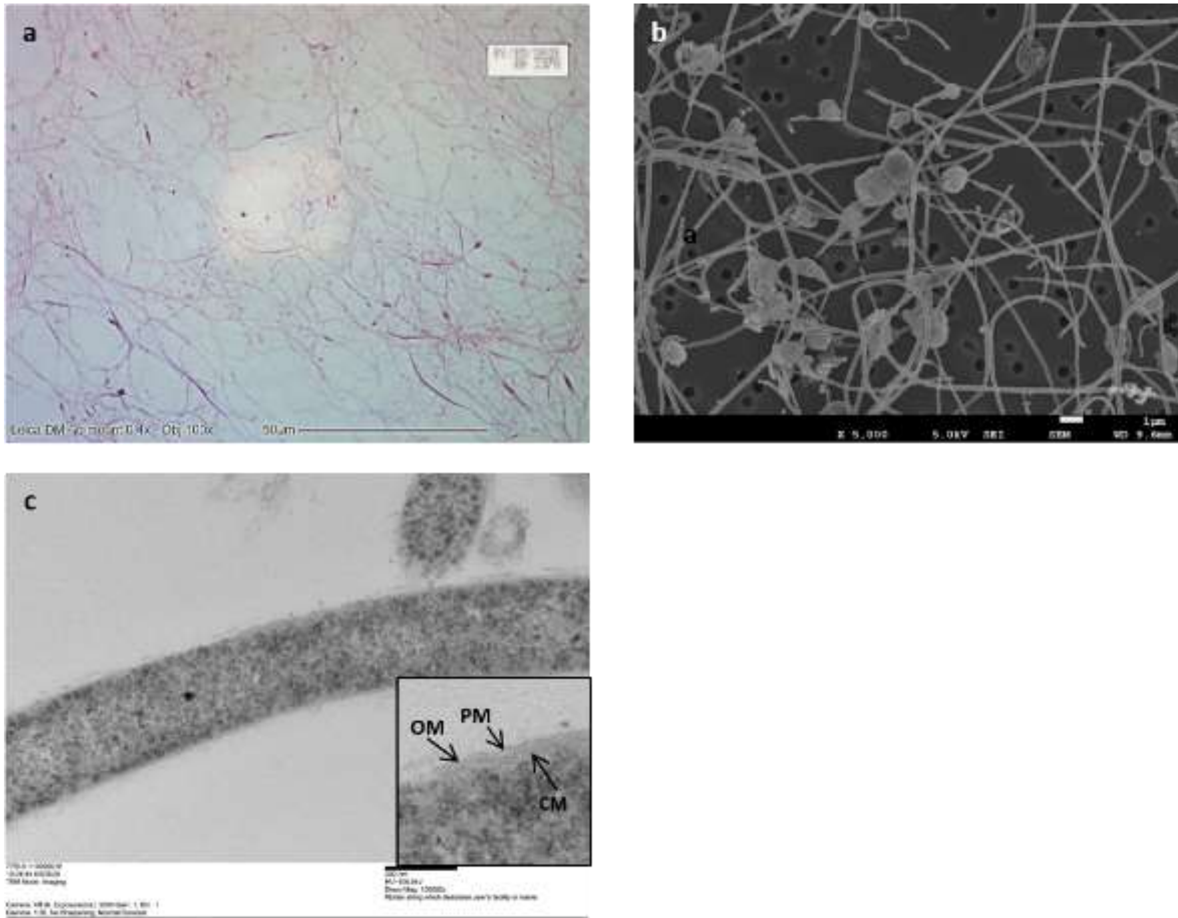

Fig. 4.3 Morphology of cells from the UUS1-1^{T} strain grown on medium with colloidal chitin. (a) A phase-contrast microscope photograph of strain UUS1-1^{T} after Gram staining. Bar, 50 µm. (b) A scanning electron microscope image of strain UUS1-1^{T}. Bar, 1 µm. (c) Transmission electron microscope (TEM) images of a thin section of strain UUS1-1^{T} and cell wall ultrastructure with the outer membrane (OM), the cytoplasmic membrane (CM) and plasma membrane (PM) indicated in the inset. Bar, 0.2 µm.

Physiological characteristics of UUS1-1^T were carried out using various growth temperatures and pHs on the medium containing 1% GlcNAc. The temperature range was grown between 45 and 70 °C (5 °C intervals), the growth of pH range was determined at 60 °C and range of pH 6.0 - 9.0 (0.5 pH unit interval) and NaCl tolerance was assessed at 0 – 5.0% (w/v, 0.5% intervals). The cell growth was evaluated visually and by examining the OD600 using a spectrophotometer (UV-mini 1240; Shimadzu, Kyoto, Japan) after 7 days of incubation. UUS1-1^T grew on the medium at optimum 55°C, pH 7.0, and maximum 4.5 % of NaCl concentration. Test of aerobic growth was examined in a same medium under aerobic conditions without reducing agents. UUS1-1^T was shown strictly anaerobic microorganism. It could grow after CO_2 purging only with reducing agents. Vigorous growth was observed with following carbon sources (1% w/v final concentration): arabinose, cellobiose, casamino acid, fructose, galactose, GlcNAc, glucose, mannose, maltose, peptone, and sucrose. The complex structural compounds such as arabinan, arabinogalactan, cellulose, chitosan and xylan are not utilized for the growth except for chitin, galactan, and β-glucan. Yeast extract was required enhancing growth of UUS1-1^T. In BM7CO medium supplemented with GlcNAc, glucose and colloidal chitin, UUS1-1^T produced acetate, lactate, H_2 and CO_2 as the end products were detected by HPLC (Shimadzu model LC-20AD; Shimadzu, Kyoto, Japan) using a Shim-pack SCR-102H column with an electric conductivity monitor (CDD-10Avp) and by gas chromatography (model GC-2014; Shimadzu) equipped with a thermal conductivity detector and a SHINCARBON ST column (50/80 mesh) (GL Science, Tokyo, Japan). The DNA G+C content of strain UUS1-1^T was determined using ACQUITY UPLC system (Water, USA) with LaChrom C18-AQ column (Hitachi High-Tech Fielding, Japan)

by DNA-GC kit (Yamasa Corporation, Chiba, Japan) (Katayama-Fujimura et al., 1984). Non-methylated lambda phase DNA was used as the calibration reference. The experiment was performed in triplicate and the data are shown as the mean of triplicate experiments. The DNA G+C content of strain UUS1-1^T was calculated to be 52.1 mol%.

The extraction of cellular fatty acids was performed according to the standard protocol of the MIDI Sherlock Microbial Identification System (MIS) (MIDI, Sherlock version 6.1). Fatty acids and its methyl esters analyzed by GC were identified by using the MIS and the Anaerobic Bacteria Library (MOORE6) for peak identification. The main cellular fatty acid composition of strain UUS1-1^T were C10:0 3-OH (6.43%), summed feature A (C12:0 3-OH and/or dimethyl acetal-C13:0; 12.49%), iso-C14:0 (2.33%), C14:0 (8.21%), iso-C15:0 (8.12%), anteiso-C15:0 (20.87%), iso-C16:0 (5.82%) and C16:0 (24.10%). The fatty acid composition of strain UUS1-1^T and its relative phylogenic neighbors are shown in Table 4.2. The fatty acid profile of UUS1-1^T was different from those of relative strains *H. ethanolica* strain LX-B^T and *D. thermobenzoicum* TSB^T, which contained dominantly anteiso-C15:0 and iso-C15:0, respectively.

According to the phylogenetic, physiological, chemotaxonomic and genotypic features, strain UUS1-1^T likely reveals a novel species of a new genus in the phylum *Firmicutes*, for which the name *Capillibacterium thermochitinicola* sp. nov. is proposed.

Table 4.2

Cellular fatty acid composition of *Capillibacterium thermochitinoanaerocola* and its relatives. Strains: 1, *Capillibacterium thermochitinoanaerocola* UUS1-1^T; 2, *Hydrogenispora ethanolica* strain LX-B^T; 3, *Desulfotomaculum thermobenzoicum* TSB^T; 4, *Pelotomaculum thermopropionicum* SI^T; 5, *Desulfotomaculum hydrothermale* Lam5^T; 6, *Moorella humiferrea* 64-FGQ^T

Fatty acid	**1**[a]	**2**	**3**	**4**	**5**	**6**
$C_{10:0}$ 3-OH	**6.43**	ND	ND	ND	ND	ND
Summed feature A[b]	**12.49**	ND[c]	ND	ND	ND	ND
iso-$C_{14:0}$	**2.33**	11.1	ND	ND	ND	1.19
$C_{14:0}$	**8.21**	4.2	2.0	3.2	14.0	0.97
iso-$C_{15:0}$	**8.12**	4.3	79.4	76.4	14.6	20.58
Anteiso-$C_{15:0}$	**20.87**	58.6	ND	ND	14.7	ND
iso-$C_{16:0}$	**5.82**	6.9	ND	ND	15.6	ND
$C_{16:0}$	**24.10**	9.6	7.4	10.7	16.0	21.65
References	This study	Liu et al. 2014	Tasaki et al. 1991	Imachi et al. 2002	Haouari et al. 2008	Nepomnyashchaya et al. 2012

[a] Strains: 1, UUS1-1^T; 2, *Hydrogenispora ethanolica* LX-B^T; 3, *Desulfotomaculum thermobenzoicum* TSB^T; 4, *Pelotomaculum thermopropionicum* SI^T; 5, *Desulfotomaculum hydrothermale* Lam5^T; 6, *Moorella humiferrea* 64-FGQ^T.
[b] Summed feature A contained one or more of the following fatty acids: $C_{12:0}$ 3-OH and/or dimethyl acetal $C_{13:0}$.
[c] ND, not detected.

The similarities of 16S rRNA gene sequences between two bacteria of less than 95% can be indicated to different genera (Ludwig et al., 1998). Strain UUS1-1^T most likely presents a novel genus, due to 16S rRNA gene sequence similarity with other species belonging to order *Clostridiales* and *Thermoanaerobacterales* of the phylum *Firmicutes* was less than 90%. The G+C content (53.2 mol%) is lower than those of *Hydrogenispora ethanolica* LX-B^T (56.1 mol%), but it is higher than those of *Desulfotomaculum hydrothermale* Lam5^T (46.8 mol%), and *Moorella humiferra* (51.0%). In addition, strain UUS1-1^T can be readily separated from its closest phylogenetic neighbors, *Hydrogenispora ethanolica* LX-B^T and *Desulfotomaculum thermobenzoicum* TSB^T, by fatty acid compositions. Thus, based on the overall topologies, the phenotypic and biochemical characteristics, we propose that the strain UUS1-1^T should be assigned to a novel species of a new genus in the phylum *Firmicutes* and novel species, for which the name *Capillibacterium thermochitinicola* sp. nov. is proposed.

According to lactic acid synthesis by strain UUS1-1, polylactic acid is a polymer consisting of lactic acid and lactide monomers. Due to the biodegradable property, polylactic acid has become the second highest uses for bioplastic the versatile applications of polylactic acid are biocompatible medical implants and disposable packages. The lactic acid production from sugars or starch hydrolysates using microbial fermentation (the genus *Lactobacillus*) has been reported (Datta and Henry, 2006, and Zhou et al., 2006). Most of lactate-producing are anaerobic and convert pyruvate to lactate (Singhvi et al., 2019). For a pure strain UUS1-1, the conversion of chitin powder (0.5% w/v) to lactic acid was around 34.7%. To improve the lactic acid production, the suggestion is to block acetic acid formation pathway using genetic modification. For example, Yang et al showed that *pta* and *ack* genes introduced to

Thermoanerobacterium aotearoensewith strain SCUT27 increased a yield in lactic acid meanwhile the acetic acid pathway was suppressed (Yang et al., 2013).

Chito-oligosaccharides are composed of GlcNAc units up to 20 units in a sequence with an average molecular weight lower than 3.9 kDa (Liaqat and Eltem, 2018). Industrial applications of chito-oligosaccharides have found in compositions for agriculture, cosmetic, foods, pharmaceutical etc. The polymer chain of chitin has been decomposed using chemicals and enzymes such as chitinase by microorganisms. The most commonly used chemical treatment of chito-oligosaccharides production consists of depolymerization of chitin and chitosan and purification. The problem in the purification of chito-oligosaccharides using chemicals method is a mixture of products. Therefore, the enzymatic treatment has been a good alternative tool due to usage of specific enzymes (chitinases and chitosanases) and non-specific enzymes (carbohydrases and proteases). The specific enzymes such as chitinase and chitobiase will particularly hydrolyze the position of polymer, resulting in the high yield of the pure product (Kaczmarek et al., 2019). The future study of strain UUS1-1 will be clarified the types and amount of chito-oligosaccharide product using chitinase assay evaluation and understand exact chitinases' activity. Even though the enzymatic depolymerization is easy to control desired products, but it is high expense of enzyme preparations. Recently, the development of enzymatic methods is very challenging starting from the pretreatment and extraction of chitin from waste and the microbial fermentation especially in modification of enzymes and cost reduction in enzymatic preparation (Kaczmarek et al., 2019).

4.6 Description of *Capillibacterium* gen. nov.

Capillibacterium (Ca.pil.li.bac.te'ri.um. L. masc. n. *capillus* hair; L. neut. n. bacterium a small rod; N.L. neut. n. *Capillibacterium* a hair-like rod). The cells are Gram-stain-negative. Flagella. Strictly anaerobe. Thermophilic and fermentative metabolism. Long hair-shape. Produce terminal, spherical spores. Chitin is fermented to acetate, lactate, H_2 and CO_2.

4.7 Description of *Capillibacterium thermochitinicola* sp. nov.

Capillibacterium thermochitinicola (ther.mo.chi.ti.no.co.la. Gr. masc. adj. *thermos* hot; N.L. neut. n. *chitinum* chitin; L. masc. suff. – cola (from L. n. *incola*) a dweller, inhabitant; N.L. masc. n. *thermochitinicola* dweller that can live in a hot and anaerobic environment with chitin decomposing activity).

Displays the following characteristics in addition to those given for the genus description. Cells are rod-shaped, 0.15–0.40 µm in diameter by 15.0–80.0 µm in length. Grows at 45–65 °C (optimum 55 °C), at pH 6.5–7.5 (optimum pH 7.0) and with 0–4.5% NaCl (w/v). Utilizes the carbon substrates chitin, starch, galactan, β-glucan, cellobiose, fructose, galactose, GlcNAc, glucose, mannose, maltose and sucrose. Fatty acid composition is C10:0 3-OH, summed feature A (C12:0 3-OH and/or dimethyl acetal-C13:0), iso-C14:0, C14:0, iso-C15:0, anteiso-C15:0, iso-C16:0 and C16:0. The genome of the type strain exhibits 52.1 mol% DNA G+C content. The 16S rRNA gene and genome sequences of the type strain were deposited in the Genbank under accession number MN602556.

The type strain is JCM 33882^T = DSM 111537^T, which was isolated from manure compost on Ishikagi Island (Japan) with chitin powder. The genomic DNA G+C content of strain UUS1-1^T is 52.1 mol%

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Chapter 5

General conclusion

Chitin is the second gigantic polysaccharide following cellulose, and mostly found in marine and landfill as the exoskeleton of shrimp, crab and arthropod. In present, the numerous seafood remaining has been discarded in several inappropriate ways causing an environmental concern. One of an earth-friendly method is a use of microorganism to decompose those waste. The microorganism such as bacteria and fungi secreted chitinases that hydrolyzed chitin structure into N-acetylglucosamine as its monomer or its derivatives as its oligomers. Recently, chitinase used in the biotechnology industry are produced by mesophilic bacteria, however the thermostable chitinase should have more versatile for easy operation and cost reduction. Thus, chitinase from thermophilic bacteria have been a high-thermo-stability and suitable for production. However, less information mentioned thermophilic bacteria with chitin-decomposing ability, the study of chitinolytic thermophile should contribute an advantage to the further research.

The isolation of the chitinolytic thermophilic anaerobic bacteria named UUS1-1 is the initial key to unlock the limitation of chitin–microbial research. Activities of a co-culture of isolated UUS1-1 and another isolated novel GT38 provided more efficient crystalline chitin decomposition when compared to activity of a pure UUS1-1 culture and no chitinolytic activity for GT38. The syntrophic relationship between UUS1-1 and GT38 maintain assimilation of organic acids in chitin decomposition.

The strain UUS1-1 is the first bacterium under anaerobic thermophilic condition was and belongs to the genus *Hydrogenispora* of the uncultured taxonomic OPB54 cluster within the phylum *Firmicutes*. On the basic of genome, the result of strain UUS1-1 is different from *Hydrogenispora ethanolica* (closest 16S rRNA gene identification) and other closest neighbors. Not only the genome analysis but only the morphology, characterization and function of UUS1-1 are unique and outstanding from its relatives. This bacterium is Gram-negative, spore forming, flagella and unique long, hair-like rod-shaped and is able to grow in widely carbon sources except pentose sugar and complex structure compounds. Therefore, the strain UUS1-1^T should be proposed to a novel species of a new genus in the phylum *Firmicutes* and novel species, as *Capillibacterium thermochitinoanaerocola* gen. nov. sp. nov.

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