Study on Xylan Degradation System of New Isolated Thermophilic, Facultatively Anaerobic Bacterium *Paenibacillus* sp. Strain DA-C8

July 2021

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A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Bioresource Engineering

(Doctoral Program in Appropriate Technology and Sciences for Sustainable Development)

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Acronyms And Abbreviations

ANI	Average Nucleotide Identity
AAI	Average Amino acid Identity
BM	Basal Medium
dDDH	DNA-DNA hybridization
CDSs	Protein- coding Sequences
CBM	Carbohydrate-Binding Module
COGs	Clusters of Orthologous Groups
Fn3	Fibronectin type 3 domain
GGDC	Genome-to-Genome Distance Calculator
GC	Gas Chromatography
GH	Glycoside Hydrolase Family
HPLC	High-performance Liquid Chromatography
NCBI	National Center for Biotechnology Information
PCR	Polymerase Chain Reaction
SLH	Surface-layer Homology
SEM	Scanning Electron Microscopy
SDS-PAGE	Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis
TEM	Transmission Electron Macroscopy

Abstract

Xylan is an abundant and renewable resource from many biomass materials, including empty fruit, oil palm, corn hull/straw, rice straw, and birch wood. To expand and add value on this biomass resources, microbial xylanase production has been considered for bioconversion of the plant biomass and used in various biotechnological applications like biofuel production, pulp, and paper industry, baking and brewing industry, food and feed industry, and deinking of waste paper.

Currently, researchers revealed the xylanase produced from microbial used for commercial-level production of enzymes is limited due to the low yield and do not meet the demand of industries level as well as stability of pH value and temperature. Additionally, xylanase microbial from the thermophilic condition is less information. In this research, screening, identification, and study of xylanolytic bacteria's functional properties were conducted. To obtain a bacterium exhibiting efficient xylan-degradation ability - a thermophilic, facultatively anaerobic, xylanolytic bacterial strain DA-C8 (=JCM34211=DSM111723) as a pure culture was isolated from compost. Strain DA-C8 was observed under light microscopy after gram staining and by SEM and TEM. The strain was Gram-negative, and cell morphology was straight or slightly straight curve rod shape, a non-motile bacterium with 0.4-0.5 μm in diameter and 2-7 μm in length. Physiological characteristic of strain DA-C8 was studied. In terms of optimum condition for cell growth, it was at 55ºC with pH value 9.0 under anaerobic condition, 1% of xylose or glucose was used as a carbon source in BM. Strain can utilize variety of carbon sources, including L-arabinose, D-fructose, D-mannose, raffinose, D-glucose, D-xylose, cellobiose, maltose, sucrose, ribose, L-galactose. Strain DA-C8 can hydrolyze xylan, aesculin, arabinoxylan, galactan, pectin but could not hydrolyze Cellulose, gelatin, casein, chitin, CMC. To confirm the ability of xylan degradation with strain DA-C8, strain can degrade completely 1% beechwood xylan in 4 days under anaerobic conditions. From 16S rRNA and ANI sequence analysis, strain DA-C8 was closely related to many *Paenibacillus* sp and to *Xylanibacillus composti* K-13, known to utilize xylan as carbon sources. The observation and parameters below were conducted to confirm that strain DA-C8 belongs to a member of the genus's *Paenibacillus sp* or *Xylanibacillus sp*,

To understand the xylan degradation system, the genes related to the xylan degradation in DA-C8 and K-13 were compared; beechwood xylan and corn hull biomass degradation were investigated, cell mass and protein of cells of both strains were measured, and extracellular enzymes from anaerobic or aerobic growth conditions were analyzed. The result revealed that

the predicted genes regarding β-1,4-xylanases and endo-β-1,4-glucanases belonging to GH family-5, 9, 10, and 11 were found in the genome sequence of both strains. Meanwhile, genes of β-glucosidases and β-xylosidases belonging to GH family-3 and 16 were abundantly confirmed in genome sequence of stain DA-C8. For debranching genes, called the accessory enzymes such as α-L-arabinofuranosidase, β-galactosidase, α-glucosidase, and polygalacturonase were also confirmed to the genome of strain DA-C8. Strain DA-C8 was revealed to equip various genes regarding lignocellulose degradation more than *X. composti* K-13. Extracellular fraction of strain DA-C8 showed relatively high degradation activity of 33.00±0.03 U/mg toward xylan but low activity for crystalline cellulose. Optimum pHs and temperatures of the extracellular fractions of strain DA-C8 and *X. composti* K-13 were observed at pH 5.0-8.0 and pH 6.0-8.0, and 50-70°C and 50-55°C, respectively.

Extracellular fraction of *X. composti* K-13 was two times lower xylanase ability than that of strain DA-C8. Furthermore, extracellular fraction from strain DA-C8 possessed high β-xylosidase, β-glycosidase, and α-L-arabinofuranosidase activities compared with those of *X. composti* K-13. These results were an agreement to high xylan and plant biomass degradation abilities with strain DA-C8, indicating that high β-xylosidase, β-glycosidase and α-L-arabinofuranosidase activities of DA-C8 can enhance the degradation ability because these enzymes be able to avoid not only enzymatic feedback inhibition by xylobiose and cellobiose, but also debranching enzyme can remove arabinose substituents from arabinoxylan and arabinoxylooligomers, thereby, improving bioconversion of lignocellulosic biomass. When strain DA-C8 was grown on BM using xylose as the sole carbon source, the growth speed and cell densities under anaerobic growth conditions were slightly slower than aerobic growth conditions; however, both states were not a big difference and showed good growths. In contrast, growth of strain DA-C8 was drastically decreased on BM medium supplemented xylan as sole carbon sources under aerobic growth conditions. The cell density as seen from total proteins was two times lower than that of the anaerobic culture condition. These results indicate that strain DA-C8 especially prefers anaerobic conditions when xylan is used as carbon source and needs to shift the metabolisms from aerobic to anaerobic conditions to obtain an efficient xylan degradation ability. As a result, xylan degradation ability of strain DA-C8 decreased only 27% under aerobic culture conditions as well as its growth curves, but strain can degrade 100% until four days under anaerobic conditions. These results indicated that the xylan degradation system of strain DA-C8 might depressed by aerobically growth conditions. The xylanase activity in extracellular fraction prepared from a culture of strain DA-C8 under aerobic growth condition was observed 1.24±0.01 unit/mg protein as xylanase activity. Strain DA-C8 indicated

significantly high xylan degradation ability and enzymatic activity under anaerobic culture conditions. However, the degradation ability was deficient in aerobic growth conditions. These results suggest that the xylan degradation activity of DA-C8 may be strictly regulated by aerobically growth conditions.

Based on the above result, a new isolated strain DA-C8 should be represented in *Paenibacillus* sp.

Chapter 1

General Introduction

1.1 Background

Nowadays, the 7.8 billion people on the earth are consuming more of its resources than it can provide because the population growth rate is increasing every day; every new person is a new consumer then demand is increasing. We are now using renewable resources at almost twice the speed that the earth can renew them. To meet the demand for living, shelter, clothes, food, and water are needed. Primarily, food production is necessary for daily life consumption. Increasing agricultural production comes at a cost to nature because when food products are increasing, it causes agricultural waste also produced. Globally, 998 million tonnes of agricultural waste is made in a year. Agricultural waste produced, including rice husk, banana peel, sugarcane fibers, oil palm empty fruit bunch, oil palm stems, rubber wood, onion skin, groundnut husk, bagasse. By each country was described by Agamuthu, 2009. In 2008, 63 million tonnes of oil palm empty fruit bunch and palm oil mill effluent from Malaysia because this country is the largest producer of palm oil. In the southern Asiatic regions, including Thailand, Indonesia, the Philippines, and Vietnam, more than 38 million tonnes of rice husk and 34 million tonnes of bagasse are produced every year. Malaysia, Indonesia, and Thailand are also responsible for having more than 90 percent of global palm oil, which consequently causes 27 million tonnes of waste per annum from fruit bunches (EFBs), fibers, shells, and liquid effluent. Although Southern regions in Asia are major worldwide agriculture producers, farming practices are sometimes antiquated and often environmentally harmful. Every year, thousands of tons of biomasses, including stems, leaves, and seed pods, are destroyed, waste from crops is commonly left in the field to decompose or, much worse, burned. The total annual quantity of the biomass potential from the agriculture and forest sector in the region was estimated at more than 500 million tons per year in southeast Asian countries; (Wang et al., 2016; Tun et al., 2019).

From the above views, there are available agricultural biomass for renewable energy with technology advancement and research finding by using agricultural waste as a raw material for another process via fermentation. Agricultural waste can convert liquid biofuels from sugar cane, sugar beet, sweet sorghum through fermentation and distillation. Using microorganisms or microbial communities, including fungi and bacteria, is being considered for plant biomass bioprocessing without conventional pretreatment. So, waste is no longer an environmental issue

or negative effects to human health but a resource for energy production.

1.2 Problem statement

Lignocellulosic biomass is the most abundant renewable resource in nature; they have been considered as a starting material for many processes due to their polysaccharide composition. Lignocellulose is comprised of three classes of polymers consisting of cellulose microfibrils embedded in a matrix of cross-linked hemicellulose and lignin. A variety of lignocellulosic materials are known such as sugarcane bagasse, rice straw, rice bran, wheat straw, wheat bran, and corn cob (Alokika & Singh, 2019). Hemicellulose is the second-most abundant renewable polysaccharide after cellulose; xylan is the main component of the hemicellulose (Bajpai, 2014). Biological and commercial chemical processes are used to expand and add value to biomass materials because of their low cost with high availability. The process has also solved the issue of solid waste management. Using microbial enzymes such as xylanase is eco-friendly, but in the pulp and paper industry, the chemical-based bleaching process causes environmental pollution (Maalej-Achouri et al., 2012; Zhao et al., 2017). Xylanase enzymes from microbes play the primary role in the paper and pulp industry, food and brewery, textile, bleaching, deinking of waste paper, and production fuels (Bhardwaj et al., 2019; Alokika et al., 2018).

It is useful for the paper and pulp industry because the stability and activity of xylanase produced from bacterial sources is a wide range of pH and temperature, such as pH from 5.0-9.0, and temperature from 30-60˚C (Akhavan Sepahy et al., 2011). Xylanolytic thermophilic anaerobic have been interested because of their ability to utilize the xylan. Additionally, the bacterium with intense xylan degradation, which was able to grow under anaerobic thermophiles, is little known. Most of them were not isolated from animal feces but from hot-springs or oil-producing wells (Wagner & Wiegel, 2008). From the later half of the twentieth century, there has been a focus on xylanases from extremophiles that can withstand high temperatures and extreme pH found during industrial processes (Zhang et al., 2011; Baramee et al., 2015). According to the above views, microbial xylanase is used for many biotechnology applications like biofuel production, pulp and paper industry, baking and brewing industry, food and feed industry, and deinking of waste paper. In this research, to obtain the bacterium having xylanolytic enzymes to degrade xylan under anaerobic thermophilic conditions efficiently and to study its genetic characteristics and xylan degradation function.

1.3 Objective

In this study, we aimed to characterize the properties and function of the xylanolytic

bacterium from strain DA-C8.

To achieve this aim, the specific objective of this study is below:

1.3.1 To screen, isolate and characterize the xylanolytic bacterium with strong xylan-degradation ability.

1.3.2 Characterization of new isolated thermophilic, facultatively anaerobic bacterium *Paenibacillus* sp. strain DA-C8 with xylan degradation ability activated by anaerobic condition.

1.4 Scope of study

1.4.1 Isolation and characterization of the xylanolytic bacterium with strong xylan-degradation ability:

1) Screening of the xylanolytic thermophilic bacterium with strong xylan-degradation ability using pure beechwood xylan as carbon source.
2) Isolation of a novel xylanolytic bacterium using the Roll-tube technique.
3) Investigate the purity of bacterium using 16S rRNA, DNA amplification and sequencing, and cell morphology using a light microscope, gram-stain test, Scanning Electron Microscope (SEM), Transmission Electron Microscope (TEM).
4) Optimization pH and temperature for the cultivation of isolated strain using UV spectrophotometer OD_{600nm}.
5) Degradation of xylan under anaerobic growth condition of isolation strain

1.4.2 Draft genome sequence data of the facultative, thermophilic, xylanolytic bacterium Paenibacillus sp. strain DA-C8.

1) Investigation on whole-genome sequencing of novel strain DA-C8 using the Ion GeneStudio S5.

1.4.3 Characterization of new isolated thermophilic, facultatively anaerobic bacterium *Paenibacillus* sp. strain DA-C8 with xylan degradation ability activated by anaerobic conditions.

1) Measurement of mass cell and Protein content.
2) Measurement for xylan and corn hull degradation abilities of strain DA-C8 and *X. composti* K-13.
3) xylan degradation ability of strain DA-C8 under anaerobic and aerobic growth conditions.
4) Gel electrophoresis analysis and zymogram.
5) Quantitative real-time PCR.

1.5 Literature Review

1.5.1 Lignocellulosis biomass

Lignocelluloses or lignocellulosic biomass is the most bountiful crude material on earth. In nature, they regularly exist as unpredictable multicomponent polymers. The fundamental components of lignocellulosic biomass are cellulose 40-50% as polysaccharide making of β-1,4-linked D-glucose units, 20-30% hemicellulose as complex polysaccharide network of xylose, glucose, mannose, and 10-25% lignin a complex structural polyphenolic (Okeke et al., 2015). These polymers are tightly bound together into a robust network. In terms of agricultural biomass, such as corn fiber, corn stover, wheat straw, rice straw, and sugarcane bagasse, contain about 20–40% hemicellulose, and hemicellulose is the second most abundant polysaccharide in nature (Saha, 2003).

1.5.2 Source and structure of xylan

Xylan is a major structural polysaccharide of plant-cell wall being the second generally predominant in nature after cellulose.

Xylan is a homopolymeric backbone of β-(1,4)- D-xylose subunits, subbed by a variety of side chains depending on its source, predominantly α-L-arabinosyl and α-D-glucuronosyl units (Knob et al., 2013). The substituents of the main chain include 4-O-methyl-D-glucuronic acid, D-glucuronic acid, or L-arabinofuranose, and acetyl groups. Moreover, the substituents might be likewise addressed by oligosaccharides and esterified by cinnamic (phenolic) acids (Timell, 1967). It is available in various structures like as O-acetyl-4-O-methylglucuronoxylan in hardwoods constitute 10-35% of the hemicellulose, as arabino-4-O-methylglucuronoxylan in softwoods constitute 10-15% of the hemicellulose (Timell, 1967). In xylan composition, differ from its sources such as grasses, cereals, softwood, and hardwood. For instance, Birch wood xylan contains 89.3% xylose, 1.0% arabinose, 1.4% glucose, and 8.3% anhydrouronic acid (Kormelink & Voragen, 1993); rice bran neutral xylan contains 46% xylose, 44.9% arabinose, 6.1% galactose, 1.9% glucose, and 1.1% anhydrouronic acid; wheat arabinoxylan contains 65.8% xylose, 33.5% arabinose, 0.1% mannose, 0.1% galactose, and 0.3% glucose; corn fiber contains 48-54% xylose, 33-35% arabinose, 5-11% galactose, 3-6% glucuronic acid.

1.5.3 Classification and role of xylanolytic enzymes

Based on xylan structure and its chemical compositions, hydrolysis of xylan was a variety of enzymes including main- and side-chain acting enzymes to convert the complex polymers to monomer. In general, these are made out of two sorts: 1) Non-debranching compound (β-1,4-endo-xylanase, β-xylosidase), and 2)-Debranching compound (α-L-

arabinofuranosidase; α-D-glucuronidase; acetyl xylan esterase; and phenolic corrosive esterases). Various xylanolytic enzymes are:

Endo-β-1,4-xylanase (EC 3.2.1.8) and glucuronoarabinoxylan endo-1,4-β-xylanase (EC 3.2.1.136) is the crucial enzyme to catalyze the hydrolysis of the main backbone the internal β-1,4-glycosidic linkage of the xylan into xylooligosaccharides like xylobiose, xylotriose with a higher degree of polymerization. Additionally, based on the similarities of the amino acid sequences, most xylanases are confined into glycosyl hydrolase (GH) families 10 and 11 (http://www.cazy.org/Glycoside-Hydrolases.html). The attacked bonds was selected irregular because it depends on the degree of branching, chain length, and the presence of substituents in the substrate molecule. Moreover, Wong (Wong et al., 1988) reported that the difference of physiochemical properties such as molecular and isoelectric point of xylanolytic microbial can be separated into two groups: 1) it was made of high-molecular-mass enzymes (>30kDa) with acidic pI value, 2) it consisted of low-molecular-mass enzymes (<30kDa) with basic pI values. In general, classification of glycanases of hydrophobic cluster analysis and sequence similarities of xylanases were grouped (wong et al. reported). Hydrophobic cluster analysis is to predict protein folding based on hydrophobic/hydrophilic patterns and to compare members of a protein group of relative functions (Gaboriaud et al., 1987).

β-xylosidase

Xylan 1,4-β-xylosidase (EC 3.2.1.37) and oligosaccharide reducing-end xylanase (EC 3.2.1.156) attack the non-reducing end from xylooligosaccharides such as xylose residues. Generally, the best substrate for β-xylosidase was xylobiose and other substrates including p-nitrophenyl and o-nitrophenyl-β-D-xylopyranoside, but it could not hydrolyze xylan.

α-L-Arabinofuranosidase (EC 3.2.1.55)

This enzyme hydrolysis of terminal non-reducing α-L-arabinofuranoside residues in α-L-arabinosides. The exo-acting α-L-arabinofuranosidase (EC 3.2.1.55), The enzymes are able to act on p-nitrophenyl α-L-arabinofuranoside and breached arabinans, and the endo-α-L-arabinase (EC 3.2.1.99) works on both α-1,5 backbone and α-1,3–side chains of arabinan. These catalysts are utilized in numerous biotechnological applications, including the wine industry, clarification of fruit juices, processing upgrade of animal feedstuffs, and a typical improver for bread (Numan & Bhosle, 2006). They are classified in GH families 43, 51, 54, and 62. According to positional specificity and requirement for a free vicinal hydroxyl group on Xylp residue, α-L-arabinofuranosidases can be divided into two groups (1)-The major group consists of enzymes active on Xylp residues monosubstituted by Araf at either position 2 or 3. They are marked as 2,3 α-L-arabinofuranosidases and are disseminated in all mentioned GH families,

(2)- The minor group includes enzymes referred to as 3 α-L- arabinofuranosidases that are specific for doubly arabinosylated Xylp residues from which they selectively liberate only the α-1,3-linked Araf, leaving the α-1,2-linked Arafon the main chain. These enzymes are exemplified by GH43 family α-L-arabinofuranosidases from Bifidobacterium adolescentis (Biely et al., 2016)

Ferulate esterases (EC 3.1.1.73)

According to substrate usage information and supported by primary sequence identity, four sub-classes of feruloyl esterases have been described and named type-A, B, C and D. Individuals from each sub-class show comparative action profiles against four model substrates [methyl 3-methoxy-4-hydroxycinnamate (MFA), methyl 3,4-dihydroxycinnamate (MCA), methyl 3,5-dimethoxy-4-hydroxycinnamate (MSA), methyl 4-hydroxycinnamate (MpCA) and similar capacities to generate (Crepin et al., 2004)

Acetylxylan esterases (EC 3.1.1.72)

The enzyme catalyzes the hydrolysis of acetyl groups from polymeric xylan, acetylated xylose, acetylated glucose, α-naphthyl acetate, p-nitrophenyl acetate but not from triacylglycerol. It does not act on acetylated mannan or pectin.

They are currently grouped in at least eight families, specifically in CE families 1–7 and 16, originally assigned as acetylxylan esterases, the enzymes acting on hardwood acetyl glucuronoxylan and its fragments generated by endo-β-1,4-xylanases (Biely, 2012).

Alpha-glucuronidases (EC 3.2.1.139)

- α-Glucuronidase is classified in GH67

substituted α-1,2-linked MeGlcA or GlcA residues bound to non-reducing end Xylp residue that is not replaced at position 3 or 4.

- GH115: Unsubstituted α-1,2-linked MeGlcA and GlcA residue. Bound to terminal or internal Xylp residue not substituted at position 3 (http://www.cazy.org/Glycoside-Hydrolases.html).

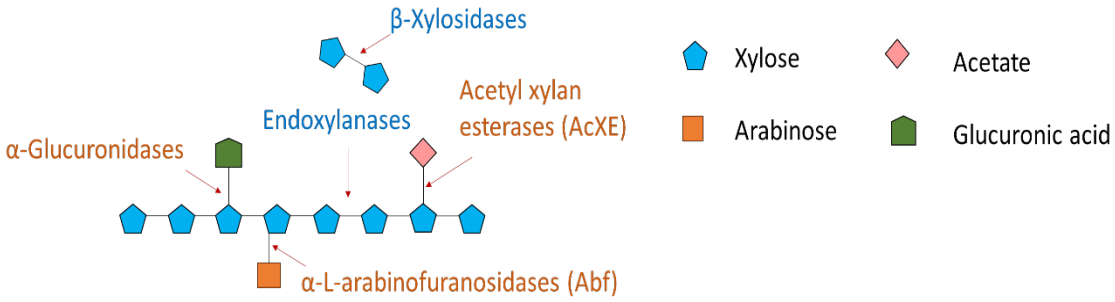

Figure 1.1. Xylan structure and xylanolytic enzymes are related to their degradation.

1.5.4 Anaerobic bacterial hemicellulose degradation

Anaerobic hemicellulolytic organisms are liable for the initial stage in the general conversion of lignocellulosic material to methane and carbon dioxide. There are made out of three steps continual processes of the anaerobic fermentation, which include hydrolysis of polymeric substrates to monomers, fermentation of monomers to organic acids, hydrogen and carbon dioxide, and conversion of organic acids, hydrogen, and carbon dioxide to methane (Schyns, 1997). The significant products of xylan fermentation were acetate and fumarate, and the minor effects were lactate and ethanol (Sizova et al., 2011). The hydrolysis of xylan by anaerobic microorganisms present in the rumen and gastro-intestinal tract plays a primary role in the nutrition of the herbivorous animal. A variety of anaerobic bacterial from rumen flora could utilize plant cell wall polysaccharide; their xylanolytic activities play a vital role in the overall degradation of plant cell wall material in the rumen system such as Ruminococcus, Butyhvibrio, Bacteroides, Prevotella, and Fibrobacter Bacteroides xylanolyticus X5-1 was investigated on xylan degradation and their regulation. For a thorough comprehension of anaerobic digestion was described; xylose enters the cell through an active uptake system and is converted via the pentose phosphate pathway followed by the glycolysis to acetate, ethanol, H_2, CO_2, and fumarate as the main fermentation products. Xylose-isomerase and xylose-kinase were activated in the xylose metabolism, whereas the xylose transport was constitutive. The regulation of product formation of some key enzymes by H was investigated. External electron acceptors could be used to shift the metabolic pathways. It was shown that it is possible to modulate the xylose metabolism by several methods and at different levels. (Schyns, 1997). *Clostridium thermocellum* was thermophilic anaerobic xylan degrading bacteria, some of *Clostridial* species which grow at pH 7 and 60°C. They can ferment cellulose, cellobiose, and several other monomeric sugars. Although *C. thermocellum* degrades xylan, the resulting xylose and xylobiose are not fermented. The organism produces a complex of cellulolytic enzymes organized in so-called cellulosomes. These complexes also may contain one or several xylanases.

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Chapter 2

Screening, Isolation and Characterization Xylanolytic Bacterium with Strong Xylan Degradation Ability.

Overview

A novel facultative, thermophilic, xylan-degrading bacterium was designated as DA-C8. The source of the isolated strain was from bovine compost on Ishigaki Island, Japan by enrichment culturing which beech wood xylan was used as a carbon source. Cells of strain DA-C8 stained Gram-negative. The cell morphology was straight or slightly straight rod-curved shape, non-motile, and spores could not observe during this research. The cell size (μm) was 2-7 in length and width 0.4-0.5. The growth condition of the strain was carried out at a temperature ranging 37-70°C (optimum 55 °C) and pH range 4-11 (optimum pH 9). The G+C content of the genomic DNA was 52.3%. The majority of cellular fatty acids of the strain were $C_{16:0}$ (42.94%), anteiso-$C_{15:0}$ (19.96%), anteiso-$C_{17:0}$ (16.74%). Its end-product included acetate, lactate; Fumarate; CO_2. Strain DA-C8 utilized many carbon sources including xylose; glucose; cellobiose; L-arabinose; fructose; mannose; raffinose; maltose; sucrose; ribose; galactose; corn starch; carboxymethyl cellulose (CMC); Aesculin; Wheat arabinoxylan; galactan, but not peptone, tryptone, D-mannitol, N-Acetyl-glucosamine, D-sorbitol, Adonitol, Glycerol, Glycitol, Arabinogalactan, Propionate, L-sorbose, Trehalose, Xylitol, chitin, casein, cellulose, gelatin. Based on its 16S rRNA gene sequences analysis, DA-C8 strain was closed (93.7%) to *Paenibacillus cisolokensis*, (92.8%) to the genus *Xylonibacillus composti*, and (92.7%) to *Paenibacillus pinstramenti*, but *Paenibacillus cisolokensis* was on a separate branch while the genus *xylanobacillus compotsi* was on the same brach in the phylogenetic tree. The average nucleotide identity (ANI) of strain DA-C8 was confirmed its closest relatives to *Paenibacillus chitinolyticus*, *Paenibacillus cisolokenesis*, and *Xylanibacillus composti* with 68.7% 68.33%, and 67.98% identity, respectively. Strain DA-C8 is considered in *Paenibacillus* sp. Based on phenotypic (chemotaxonomic characteristic, analysis of the 16S rRNA gene sequences) and ANI, we formally propose the strain DA-C8 as *Paenibacillus Thermoxylanovorax* sp. nov. The type strain is DA-C8 (= JCM 34211^T = DSM 111723^T).

2.1 Introduction

There are abundant of biomass from agricultural waste like straw are considered to be potential resources for renewable energy. The recycling and utilization of agricultural waste have been considered to be the important step in environmental protection, energy structure, and agricultural development due to disposition of agricultural waste improperly have caused environmental problems (Wang et al. 2016). Xylan is the most component in hemicellulose found in lignocellulosic biomass (Moreira and Filho 2016). Xylan is composed of the main linear chain β-1,4-linked xylopyranosyl residues, and it is usually substituted to various degrees by residues of 4-O-methyl-D-glucuronic acid D-glucuronic acid, or L-arabinofuranose, and acetyl groups (Scheller and Ulvskov 2010). The chemical structure of xylan differs in the character of their side chain and sources. Renewable energy from biomass materials as xylan by using microbial fermentation is environmentally friendly. Xylanase enzyme attack the main chain, and other accessory enzymes debranched the side chain of xylan (Bajpai 2014). Because of their ability to degrade xylan, xylanase production from microbes is used in various biotechnological applications like biofuel production, pulp, paper industry, baking and brewing industry, food and feed industry, and deinking of waste paper(Alokika and Singh 2019).

Xylanolytic thermophilic anaerobic have been interested because of their ability to utilize the xylan. Additionally, the bacterium with strong xylan degradation which was able to grow under anaerobic thermophiles, is little known, and most of them were not isolated from animal feces, but from hot-springs or oil-producing wells (Wagner and Wiegel 2008). In this study, screening, isolation, and characterization of a xylanolytic bacterium with strong xylan degradation ability in growth anaerobic thermophilic condition were investigated, and sources of microbes were described (Shikata et al. 2018; Widyasti et al. 2018). The morphological and physiological characters of a new strain isolated successfully as a pure culture were represented in this chapter.

2.2 Materials and methods

2.2.1 Source of DA-C8 and medium preparation

Source of microorganism:

Compost was used for screening collected since 2018 from Ishigaki island, Japan. Before inoculating, compost was kept at room temperature for at least 2 hours because it took from storage at –80˚C.

Medium preparation:

The medium was prepared for screening a novel thermophilic anaerobic bacterium with strong xylan degradation ability. Basal Medium (BM) and reagents to be used for

screening include: 2.9 g/L K_2HPO_4, 4.2 g/L urea, 2.0 g/L yeast extract, 1.0 g/L Na_2CO_3, 0.01 g/L $CaCl_2 \cdot 2H_2O$, 0.5 g/L cysteine-HCl, and 0.0005 g/L resazurin in water and 200 µL aqueous mineral solution (25.0 g/L $MgCl_2 \cdot 6H_2O$, 37.5 g/L $CaCl_2 \cdot 2H_2O$, and 0.312 g/L $FeSO_4 \cdot 7H_2O$). Pure beechwood xylan was used as a carbon source. The mixture of BM (15 ml) was pipetted and transferred into a Hungate tube. The gas inside the medium tube was removed by replacing them in the hot water bath and boiled for 15 min before being flushed with high purity nitrogen gas. The tubes were capped tightly and autoclaved at 121˚C for 15 min.

2.2.2 Screening of bacterium with high xylan degradation ability

To maintain strictly anaerobic conditions, high purity nitrogen gas was used during primary sampling and subculturing. Compost (2-3g) was added into the medium and then capped tightly before being incubated at 55ºC. 200µl culture was transferred into fresh medium and continued subculture for at least 4 generations. The bubbled formation, high turbidity, and high xylan degradation tubes were selected (Figure 2.5)

2.2.3 Pure culture isolation

The isolation process by the roll-tube method was carried out and described following (Hungate 1969).

BM was prepared, which contains 0.5% beechwood xylan as a carbon source. The mixture of BM (7ml) was transferred into the tube containing 0.1g agar inside. The culture was flushed with high purity nitrogen gas and capped before being autoclaved at 121ºC for 15 minutes. Medium (after autoclaved) was replaced in the water batch when the temperature was reached at 55˚C. 200µl of selected culture was transferred into the tubes with series dilution and vortex well. These tubes were kept in a water bath till the bubble disappeared and then placed on the rolling in cooling bath for 5 min. After solidified, roll tubes were kept at room temperature for at least 30 min before incubation at 55ºC. After incubation 2-3 days, the colony with surrounding clear zone was picked up and transferred into fresh BM with a needle under anaerobic condition and then inoculated at 55˚C (Figure 2.2).

Checking the purity of bacterium using 16S rRNA amplification and sequencing:

Genomic DNA of strain DA-C8 was extracted using the NucleoSpin Microbial DNA kit (Takara Bio, Shiga), according to the manufacturer's instruction. The process of 16S rRNA was described: the primers were used for PCR amplification: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The PCR amplification was performed under conditions: initial denaturation at 94 °C for 2 min followed by denaturing 30 cycles of 94°C for 60 s, 62°C annealings for 1 min 30 s, and 72 °C

for 1 min 30 s, and a final extension at 72°C for 5 min using real-time PCR Thermal cycler Dice Touch machine. For PCR purification, the QIAquick kit was performed according to the manufacturer's instructions. The sequences of 16S rRNA of DA-C8 strain were analyzed using GENETYX version 13.0. Regarding the similarity of the 16S rRNA sequences to DA-C8, the BLAST web (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used, and multiple sequences alignments by ClustalW were performed (https://www.genome.jp/tools-bin/clustalw). Kimura 2-parameter model was used (Tamura 1992), tree geographies and distances were assessed by playing out a bootstrap investigation with 1,000 re-samplings.

2.2.4 Physiochemical analysis

Cell morphology

The cells were observed by scanning electron microscopy (JSM-6320F; JEOL, Tokyo, Japan) and transmission electron microscopy (Hitachi H-7600. Tokyo, Japan). The cell was identified gram-stain using Gram stain kit S (BD Difco).

Cellular fatty acids were extracted according to the standard protocol (MIDI, USA) Sherlock Microbial Identification System (MIDI, Sherlock version 6.1). Calculation methods TSBA6 and library TSBA6 were used.

Oxidase and catalase measurement

Oxidase activity was studied using Cytochrome Oxidase Test kit Strips (Nissui, Tokyo, Japan). Catalase activity was investigated using the Megazyme Catalase Assay kit (K-CATAL 04/20), according to the manufacturer's instructions.

2.2.5 pH and temperature optimization for cell growth

In term of optimal growth condition, strain DA-C8 was performed at pH values from 5.5; 6.0; 6.5; 7.0; 7.5; 8.0; 8.5; 9.0; 10; and 11 with incubation at constant temperature 55˚C. The pH value was adjusted using 6N HCl and 5 NaOH, and 1% of xylose was added into BM, and cells have determined at the 1^{st}, 2^{nd}, 3^{rd}, and 4^{th} day. After optimal pH of growth rate was observed, strain DA-C8 was grown in the BM with constant optimum pH at a temperature ranging 37, 40, 45, 50, 55, 60, 65, 70˚C to understand the optimal temperature of growth condition. The cell was measured on the 1^{st}, 2^{nd}, 3^{rd}, and 4^{th} day. Moreover, the cell growth rate was tested in salinity range from 1-3%. The experience was carried out in triplicates. The cell was measured by using a spectrophotometer OD_{600nm} (UV-mini1240; Shimadzu, Kyoto, Japan).

A biochemical test of DA-C8 was conducted by adding 1% of each carbon source into BM. A variety of carbon sources were used such as L-Arabinose, D-Fructose, D-Mannose, Raffinose, D-glucose, D-xylose, Cellobiose, Maltose, sucrose, Ribose, L-galactose, Peptone,

Tryptone, D-mannitol, N-Acetyl-glucosamine, D-sorbitol, Adonitol, Glycerol, Glycitol, Arabinogalactan, Propionate, L-sorbose, Trehalose, Xylitol. Xylan, aesculin, arabinoxylan, galactan, pectin, cellulose, gelatin, casein, chitin, carbohydrate methylcellulose, microcrystalline cellulose were used to test the hydrolysis ability of strain DA-C8. Xylan was used as a carbon source during fermented production, and its products were measured 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).

2.2.6 Degradation level of beechwood xylan determination

The experience was prepared into triplicate, and the volume was 5 ml per tube of BM with 1% of beechwood xylan as a carbon source. 2% pure culture DA-C8 was transferred into the fresh BM with pH 9 and incubated at 55˚C for 4 days. The degradation level of xylan was measured:

1- The culture was centrifuged at 12,000 rpm for 10 min at 4˚C to collect the residues.
2- The residues were dried at 80˚C overnight.
3- The dried residue was cooled down in a jar with a desiccator inside it for 15 min before scaling.
4- Xylan residue was calculated by using the equation below:

$$\text{Xylan residue (\%)} = \frac{\text{Weight of residue (g)}}{\text{Weight of control sample (g)}} \times 100$$

Note:

- Weight of control sample: no inoculated sample after drying.
- Weight of residue: inoculated sample after drying.

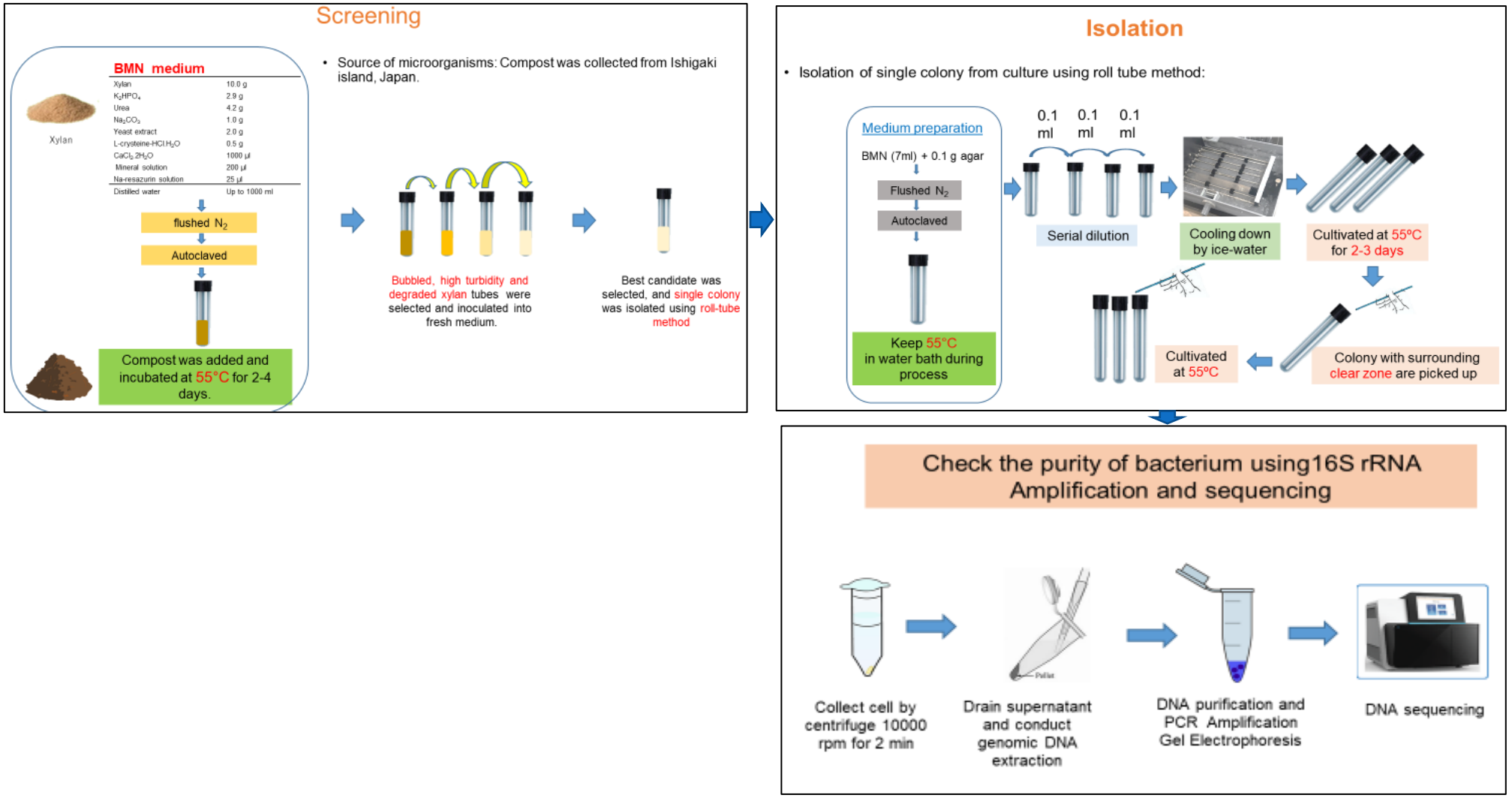

Figure 2.1. Screening, isolation flowchart of a novel thermophilic anaerobic bacterium with strong xylan degradation ability.

2.3 Result and discussion

2.3.1 Pure culture of strain DA-C8

The 1449 bp 16S rRNA gene sequence of strain DA-C8 was closely related to *P. cisolokensis* UICC B-42 (93.7% identity; accession no. NR_151901) was a new isolated from cisolok geyser, aerobically thermophilic grown condition with gram-positive type (Yokota et al. 2016); *Xylanibacillus composti* K-13 (92.8% identity; NR_159899), was a new isolated from the manure compost pile, aerobically mesophilic grown condition with the gram-positive type (Kukolya et al. 2018); *P. pinistramenti* ASL46 (92.7% identity; LC102482) was a new isolated from pine forest soil, aerobically mesophilic grown condition with the gram-positive type (Lee, Shin, and Whang 2020).

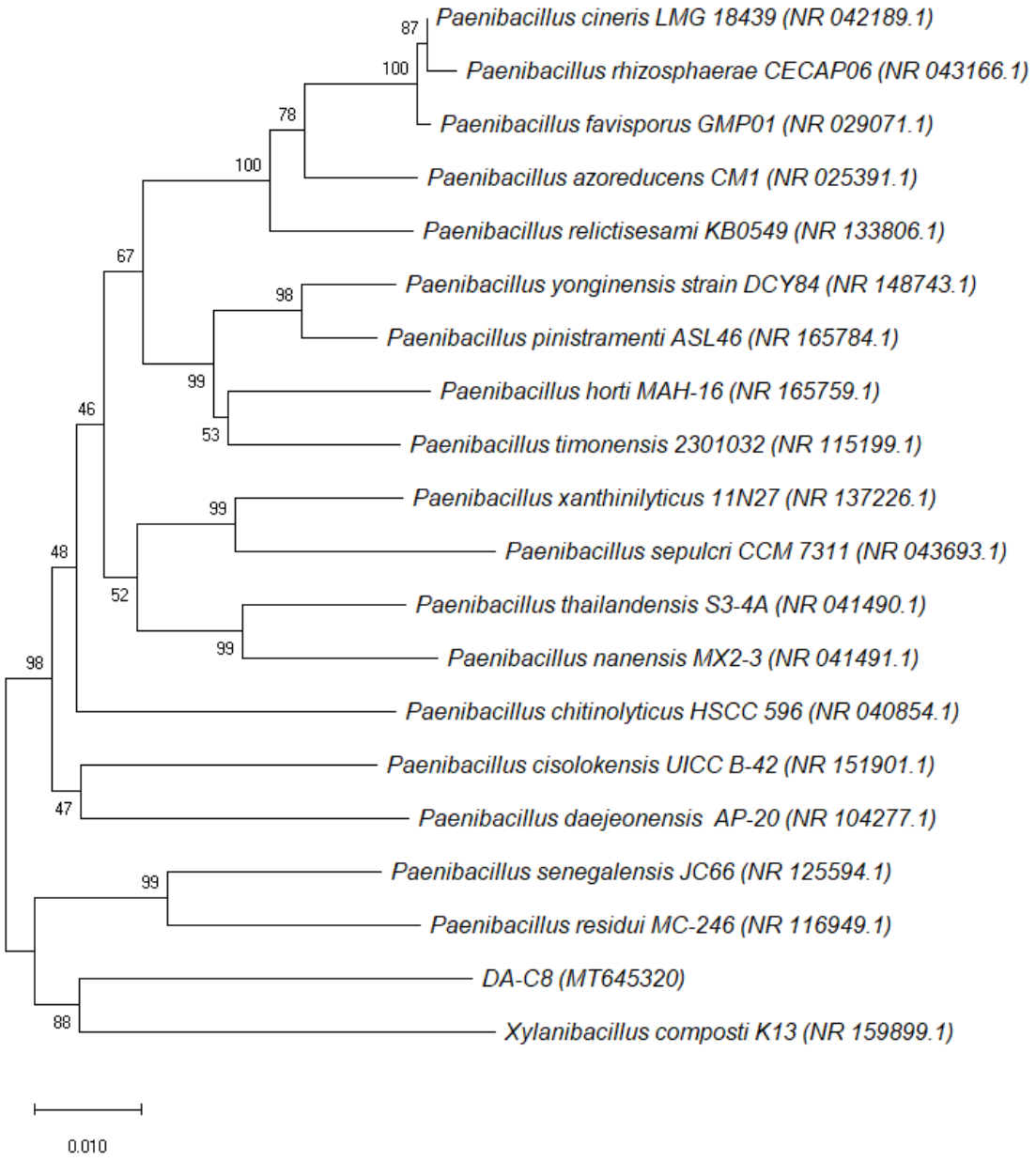

Figure 2.2. A neighbor-joining tree depicting the relationships of 13 *Paenibacillus* isolates, *Xylanibacillus composti*, and strain DA-C8 based on 16S rRNA sequences. Numbers at nodes are bootstrap support percentages based on 1,000 replicates. The bar represents 0.01 substitutions per nucleotide position.

The closest relatives of strain DA-C8 based on average nucleotide identity (ANI) values with nine other *Paenibacillus* strains were *P. chitinolyticus* KCMM41400 (68.7%), followed by *P. cellulositrophicus* KACC16577 (68.1%), *P. yonginensis* DCY 84 (68.1%), and *P. larvae* (67.7%) (Figure 2.2, and supplementary Tables 2.2. and 2.3.). ANIs between DA-C8 and all additional strains included in the analysis were < 70% (Table 2.1).

Based on phenotypic (chemotaxonomic characteristic, analysis of the 16S rRNA gene sequences) and ANI, we formally propose the strain DA-C8 as *Paenibacillus Thermoxylanovorax* sp. nov. The type of strain is DA-C8 (= JCM 34211^{T} = DSM 111723^{T}).

Table 2.1. Average nucleotide identities (%) between strain DA-C8, *X. composti* K-13, and other *Paenibacillus* strains.

	DA-C8	*X. composti* K-13	*P. cisolokensis*	*P. nanensis*	*P. pinistramenti*	*P. senegalens*	*P. yonginensis*	*P. chitinolyticus*	*P. daejeonensis*	*P. favisporus*	*P. larvae*
DA-C8	---	67.73	68.33	67.50	67.14	66.85	68.11	68.67	67.25	67.14	67.71
X. composti K-13	67.98	---	69.61	67.89	67.28	68.42	67.80	68.76	67.24	67.52	68.78
P. cisolokenesis	68.17	69.28	---	70.11	68.37	67.84	68.98	68.64	68.76	68.48	67.78
P. nanensis	67.65	67.70	70.43	---	67.94	66.86	68.91	66.75	68.19	67.79	68.11
P. pinistramenti	67.53	67.17	68.59	68.37	---	67.24	77.09	69.14	67.66	70.22	68.75
P.senegalensis	66.80	68.34	67.9	66.69	66.97	---	69.17	67.83	66.54	66.45	68.79
P. yonginensis	68.06	67.95	69.46	68.81	77.48	68.40	---	74.14	67.35	70.69	73.63
P. chitinolyticus	68.77	68.29	69.43	67.55	67.58	67.90	74.00	---	66.60	68.61	72.95
P. daejeonensis	66.83	67.03	68.37	66.69	67.36	66.19	66.68	65.11	---	66.62	66.01
P. favisporus	67.46	67.48	69.07	68.19	70.12	66.73	69.83	68.13	67.19	---	67.73
P. larvae	67.17	67.46	67.36	66.80	66.76	67.96	72.81	73.15	66.11	67.28	---

2.3.2 Physiochemical analysis

Cell morphology of strain DA-C8

The colony was round with a halo surrounding, size 0.2-0.5 (mm), and strain DA-C8 was confirmed as gram-stain-positive using Gram stain kit (BD Difco), and it was captured under the light microscope, shown in Figure 2.3 and Table 2.2.

Cells were observed by scanning electron microscopy (JSM-6320F; JEOL, Tokyo, Japan) and transmission electron microscopy (Hitachi H-7600. Tokyo, Japan). The cell was straight or slightly curved rods shape, motile and flagella are absent, and spores could not observe during this research. The cell size (μm) was 2-7 in length and width 0.4-0.5. Transmission electron microscope (TEM) images of a thin section of strain DA-C8, the periplasmic space contains no cellular material, and the peptidoglycan layer (PG), outer membrane (OM), and plasma membrane (PM) were observed in Figure 2.4.

Xylan was used as a carbon source during fermented production, and strain DA-C8 produced lactate, acetate, Fumarate, and CO_2 (Table 2.2).

Oxidase and catalase of strain DA-C8

Strain DA-C8 was negative for oxidase, while strain was grown under anaerobic and aerobic conditions. In terms of catalase measurement, it was positive while the strain was grown under aerobic conditions, but it was negative for oxidase while it was produced under aerobic conditions.

2.3.3 pH and Temperature optimization for cell growth

pH optimization for cell growth

The cell growth was performed at pH values from 4.0 to11 at a constant temperature 55˚C. After incubation at 55˚C, cell was observed OD_{600} measurement of > 0.30 at pH values 9.0 and 10.0 for 3rd and 4th day while OD_{600} measurement of < 0.28 at pH values 7.5 and 8.0. The optimal condition for cell growth rate was found at pH 9.0, shown in Table 2.2. Moreover, Cell was grown well in salinity range from 1-2%, while shown weak growth at 3% (w/v) NaCl, shown in Table 2.2.

Temperature optimization of cell growth

The cell growth rate was carried out at temperatures 37, 45, 50, 55, 60, 65, 70˚C at optimal pH 9.0. The result showed that the cell growth occurred at 45-60˚C while the optimal temperature was at 55˚C. The cell was not grown at 65 and 70˚C (Table 2.2).

2.3.4 Fatty acid compositions of strain DA-C8 and its relative closed strains

The G+C content of the genomic DNA was 52.3% (Table 2.2). The majority of cellular fatty acids of the strain were $C_{16:0}$ (42.94%), anteiso-$C_{15:0}$ (19.96%), anteiso-$C_{17:0}$ (16.74%) while *P.cisolokensis* contains high iso $C_{16:0}$ (23.40%), $C_{16:0}$ (23.20%); *X.composti* and *P.pinistramenti* contain anteiso $C_{15:0}$ (34.40%) as the majority content, shown in Table 2.3. This result showed that strain DA-C8 differs from its closed relative strain, according to the cellular fatty acid content analysis.

2.3.5 Substrate utilization of strain DA-C8

The biochemical test revealed that DA-C8 could use a variety of carbon sources including L-Arabinose, D-Fructose, D-Mannose, Raffinose, D-glucose, D-xylose, Cellobiose, Maltose, sucrose, Ribose, L-galactose, but it was negative in Peptone, Tryptone, D-mannitol, N-Acetyl-glucosamine, D-sorbitol, Adonitol, Glycerol, Glycitol, Arabinogalactan, Propionate, L-sorbose, Trehalose, Xylitol (Table 2.4). Strain DA-C8 can hydrolyze xylan, aesculin, arabinoxylan, galactan, pectin but could not hydrolyze Cellulose, gelatin, casein, chitin, CMC (Table 2.5).

2.3.6 Degradation level of beechwood xylan determination

After investigated the optimum temperature and pH value of the growth rate of a new strain, the degradation ability of a new strain on beechwood xylan was determined. Strain can degrade xylan completely after 4 days of cultivation. Residual xylan, calculated by subtracting weights from the initial xylan weights as 100%, and remaining substrate were collected after incubation every day, shown in Figure 2.5.

Table 2.2. Phenotypic characteristics of strain DA-C8 and its relative closed strains.

Characteristic	DA-C8	*X. composti* K-13	*P. cisolokensis*	*P. pinistramenti*
16S rRNA identity (%)	100	92.8%	93.7%	92.7%
Isolation source	bovine manure compost	manure compost pile	cisolok geyser	pine forest soil
Growth condition	facultative anaerobic	aerobic	aerobic	aerobic
Cell morphology	slightly curved rods	rod	rod	ND
Gram	-	+	+	+
Motility	-	+	+	+
Endospore formed	ND	+	+	+
Cell length/width (μm)	2.0-7.0/ 0.4-0.5	2.0-3.0/ 0.5-0.8	2.0-2.4/ 0.8-0.9	ND
Temperature/optimum (°C)	37-60/ 55	30-50/ 35-40	37-55/ 45-50	20-45/ 37
pH range/ optimum	5.5-11/ 9.0	7.0-11.0/ 10.0	6.0-9.0/ 6.0-7.0	6.0-11.0/ 7.0
Colony size (mm)	1-2	1-2	2-3	ND
Colony color/ shape	Pale white	Pale brownish/irregularly	White/circular, flat, smooth	ND
Salinity (%w/v NaCl)	0-3.0	0.5-2	0-2.0	0-2.0
C+G content (mol%)	52.3	52.3	56.6	52.1
Oxidase	-	-	+	-
Catalase	+	-	ND	-
Products	Lactate, Acetate, Fumarate, CO_2	ND	ND	ND
Reference	This study	(Kukolya et al., 2018)	(Yokota et al., 2016)	(Lee et al., 2020)

ND, Not detected

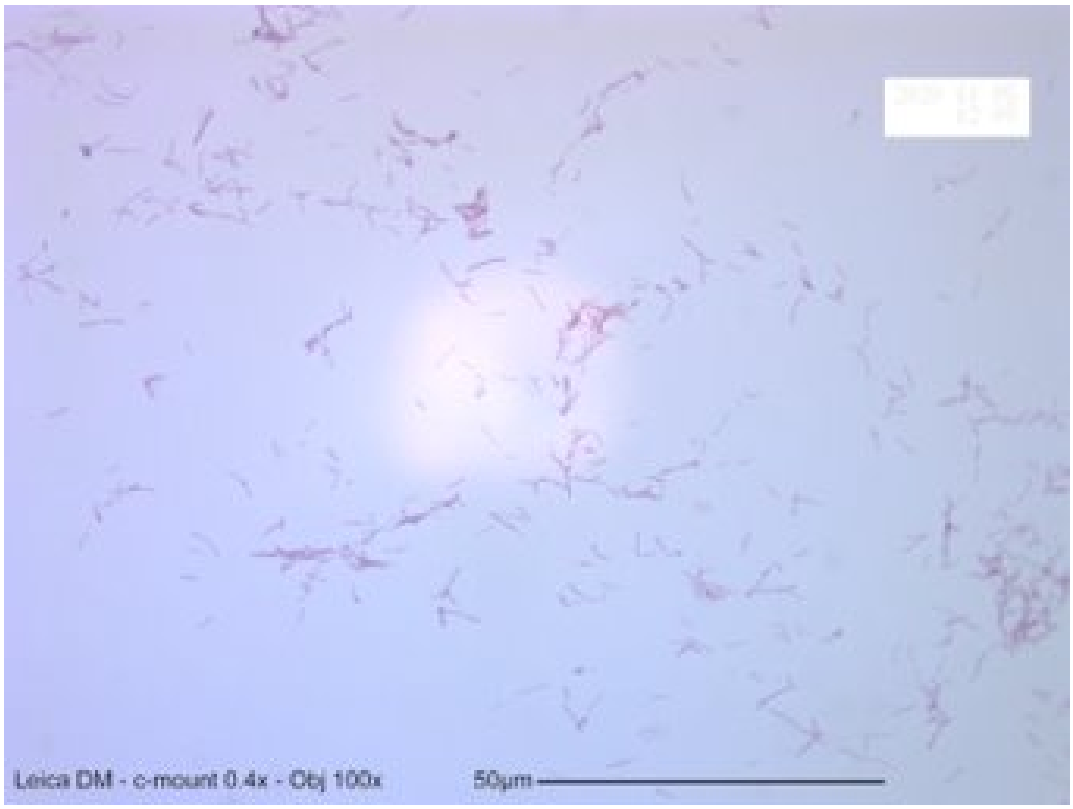

Figure 2.3. Gram stain of strain DA-C8 under the light microscope (50 µm).

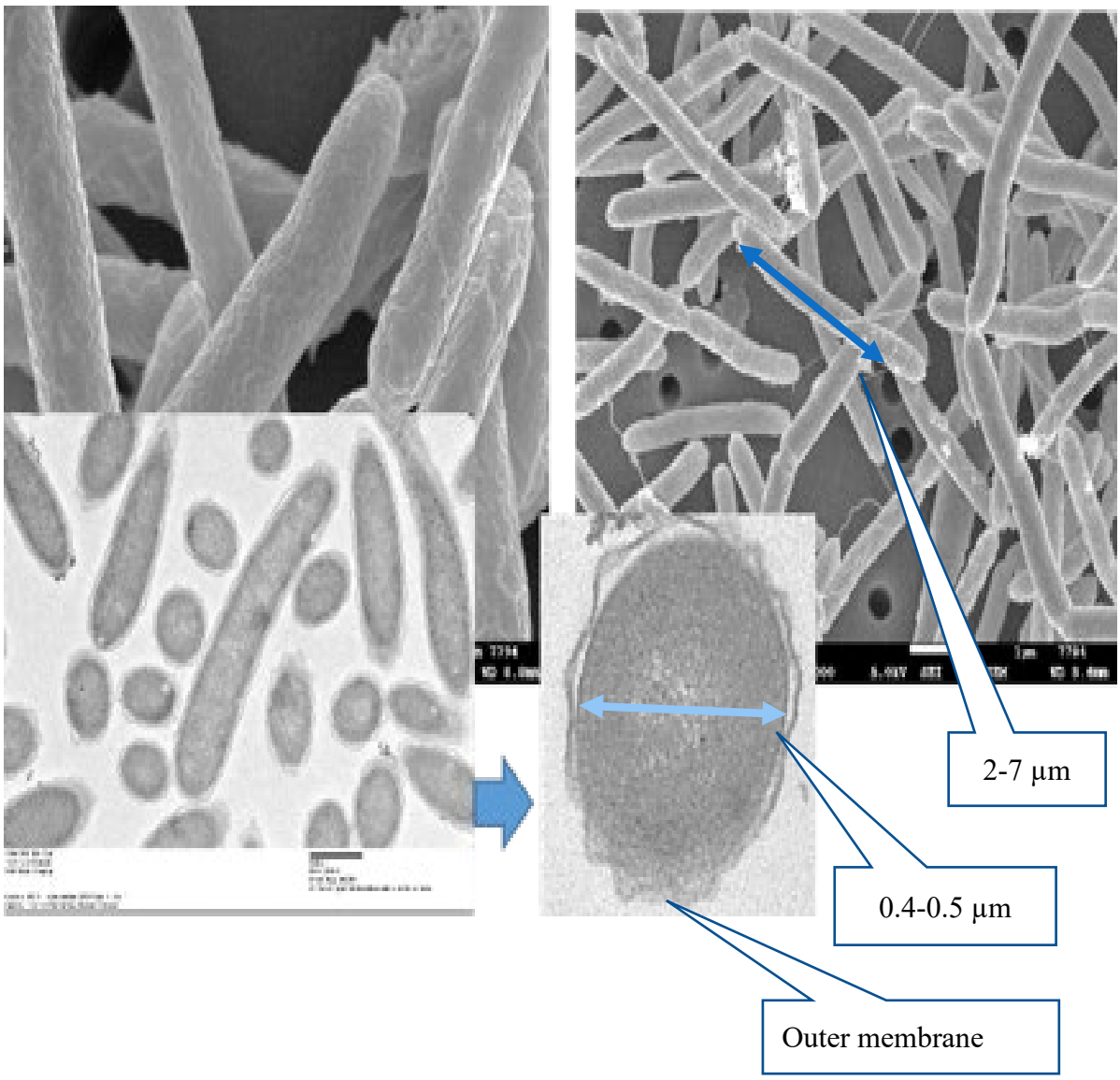

Figure 2.4. A scanning electron micrographs (SEM) and Transmission electron microscope (TEM) images of strain DA-C8, cell grown at 55ºC on BM with xylose as carbon source.

Table 2.3. Cellular fatty acid compositions of strain DA-C8 and its relative closed strains.

Fatty acid (%)	DA-C8	*X. composti*	*P. cisolokensis*	*P. pinistramenti*
anteio$C_{13:0}$	**0.14**	ND	ND	ND
iso $C_{14:0}$	**0.15**	1.90	4.50	2.7
$C_{14:0}$	**2.65**	1.50	3.50	5.5
iso $C_{15:0}$	**3.69**	9.80	11.40	7.6
anteiso $C_{15:0}$	**19.96**	34.40	21.50	47.7
$C_{15:0}$	**1.22**	2.70	2.50	ND
iso $C_{16:0}$	**4.82**	17.30	23.40	9.1
$C_{16:0}$	**42.94**	10.00	23.20	14.7
iso $C_{17:0}$	**4.67**	7.80	4.20	2.4
anteiso $C_{17:0}$	**16.57**	9.50	-	6.2
Reference	This study	(Kukolya et al., 2018)	(Yokota et al., 2016)	(Lee et al., 2020)

ND, not detected.

Table 2.4. Effect of growth with various substrates of strain DA-C8 and its relative closed strains.

Carbon source	DA-C8	*X. composti*	*P. cisolokensis*	*P. pinistramenti*
Arabinose	+	-	+	+
Fructose	+	-	+	+
Glucose	+	-	+	+
Mannose	+	-	-	+
Raffinose	+	-	-	+
Ribose	+	-	-	+
Xylose	+	+	+	+
Cellobiose	+	-	+	+
Maltose	+	+	+	+
Sucrose	+	-	+	+
N-acetyl glucosamine	-	ND	-	-
Adonitol	-	ND	+	-
Mannitol	-	-	+	+
Sorbitol	-	ND	+	-
Reference	This study	(Kukolya et al., 2018)	(Yokota et al., 2016)	(Lee et al., 2020)

+, Positive; –, negative; DN, not detected.

Table 2.5. Phenotypic characteristics of strain DA-C8 and its relative closed strains.

Substrate utilization	DA-C8	*X. composti*	*P. cisolokensis*	*P. pinistramenti*
Xylan	+	+	+	ND
Carboxymethyl cellulose (CMC)	+	ND	ND	-
Cellulose	-	ND	+	ND
Aesculin	+	+	+	+
Casein	-	+	+	+
Chitin	-	ND	+	ND
Gelatin	-	-	+	-
Starch	-	-	+	-
Reference	This study	(Kukolya et al., 2018)	(Yokota et al., 2016)	(Lee et al., 2020)

+, Positive; –, negative; DN, not detected.

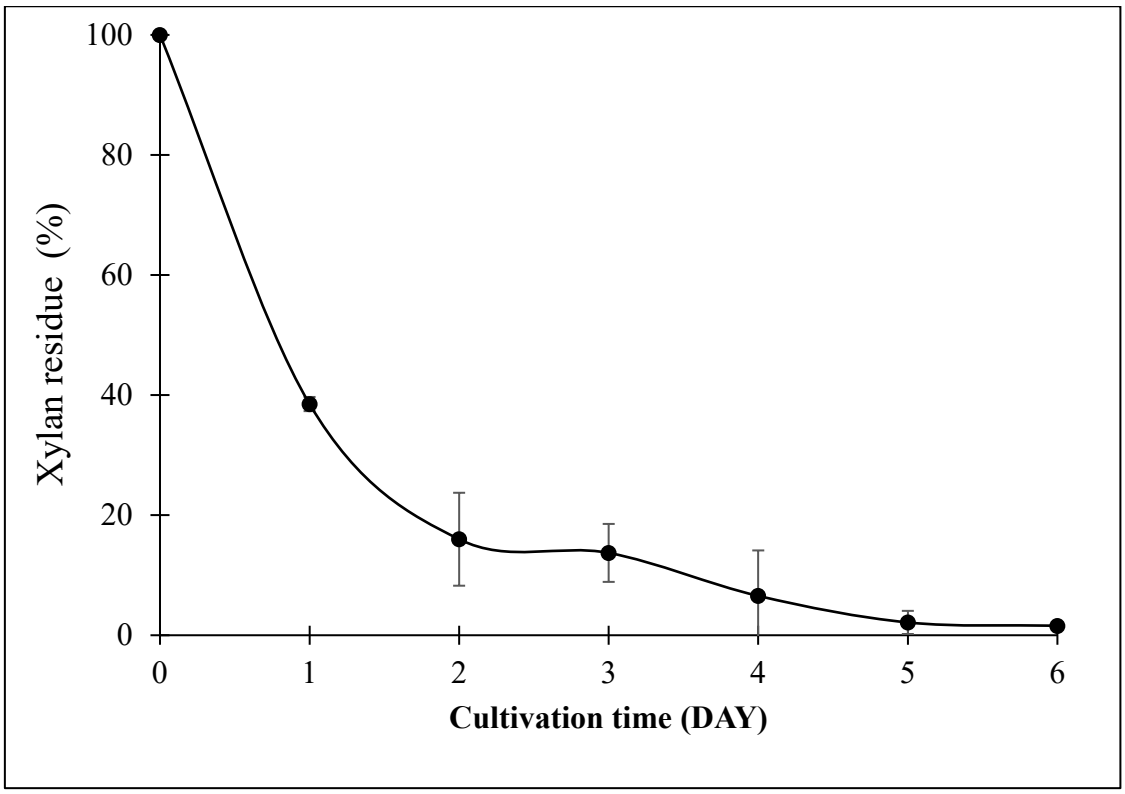

Figure 2.5. The percentage of weechwood xylan residue under anaerobic growth condition decreased from the beginning substrate weight of 1% dry (w/v) against cultivation period (day). Error bars showed ± standard deviation with triplication.

2.4 Conclusion

Strain DA-C8 is a new novel bacterium isolated from bovine compost using an enrichment culture containing beechwood xylan as a carbon source. Strain DA-C8 has high xylan degradation ability under the anaerobic thermophilic condition at 55ºC and pH value 9.0. Oxidase and catalase activity was negative while strain grows under anaerobic conditions. In terms of catalase activity, it was positive when strain grows under aerobic conditions while negative for oxidase when grown in aerobic conditions. The average nucleotide identity (ANI) of strain DA-C8 was confirmed its closest relatives to *Paenibacillus chitinolyticus*, *P. cisolokenesis*, and *Xylanibacillus composti* with 68.7% 68.33%, and 67.98% identity, respectively. This was considering strain DA-C8 in *Paenibacillus* sp. Based on phenotypic (chemotaxonomic characteristic, analysis of the 16S rRNA gene sequences) and ANI, we formally propose the strain DA-C8 as *P. thermoxylanovorax* sp. nov. The type strain is DA-C8 (= JCM 34211^T = DSM 111723^T).

2.5 References

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Chapter 3

Draft Genome Sequence Data Analysis of the Facultative, Thermophilic, Xylanolytic Bacterium *Paenibacillus* sp. Strain DA-C8

Overview

Strain DA-C8 (=JCM34211 =DSM111723) is newly isolated from compost, thermophilic, facultatively anaerobic, a xylanolytic bacterium with strong beechwood xylan degradation ability. The cell morphology of strain DA-C8 was the straight or slightly straight rod-curved shape, non-motile, and the strain was 2-7μm in length and width 0.4-0.5 μm. Whole-genome sequencing of strain DA-C8 on the Ion GeneStudio S5 system yielded 69 contigs with a total size of 3,110,565 bp, 2,877 protein-coding sequences, and a G+C content of 52.3 mol%. Genome annotation resulted that strain DA-C8 possesses debranching enzymes, such as β-L-arabinofuranosidase and polygalacturonase, that are vital for efficient degradation of xylan. As inferred from 16S rRNA sequences and average nucleotide identity values, the closest of strain DA-C8 are *Paenibacillus cisolokensis* and *P. chitinolyticus*. The genomic data have been deposited at the National Center for Biotechnology Information (NCBI) under accession number BMAQ00000000.

Table 3.1 Specifications table.

Subject	Microbiology
Specific subject area	Genomics, Bacteiology,
Type of data	Table, Figure
How data were obtained	Whole-genome sequencing using the Ion GeneStudio S5 System
Data design	Raw, Analyzed
Boundaries for data collection	Genomic DNA was extracted from strain DA-C8 (DSM 111723=JCM34211). Ion GeneStudio S5 system and de novo were used for genomic sequencing and assembled using CLC Genomics Workbench 20.0.1. The genome of strain DA-C8 was annotated using the DDBJ Fast Annotation and Submission Tool (DFAST).
Explanation of data collection	Genomic DNA was removed from strain DA-C8. A sequencing library with an addition size of 30 0–40 0 bp was arranged utilizing an Iron Express Plus Fragment Library kit (Thermo Fisher Scientific, Waltham, MA, USA). Around 200–300 bp fragments size were chosen by electrophoresis on E-Gel SizeSelect II agarose gels (Invitrogen, Thermo Fisher Scientific) before library arrangement. Whole-genome was sequencing, assembly, and annotation after the genomic library of strain DA-C8 was exposed.
Information source area	Japan International Research Center for Agricultural Sciences (JIRCAS), Tsukuba, Ibaraki, Japan
Information availability	The draft genome succession has been saved at DDBJ/ENA/GenBank under promotion number BMAQ00 000. The information can be accessed through: https://www.ncbi.nlm.nih.gov/nuccore/BMAQ00000.1. BioProject and BioSample IDs in GenBank are PRJDB10171 (https://www.ncbi.nlm.nih.gov/bioproject/PRJDB10171) and SAMD00235398 (https://www.ncbi.nlm.nih.gov/biosample/SAMD00235398).

3.1 Introduction

Productive hydrolysis of lignocellulosic biomass are required the support of β-1,4-glycosidic chain-separating enzymes, for example, endo-β-1,4-glucanase, cellobiohydrolases, and β-glycosidase, the participation of numerous hemicellulosic enzymes (e.g., xylanolytic enzymes) and also side chain-debranching enzymes (e.g., β-1,4-xylanase and α-L-arabinofuranosidase) (Moreira & Filho, 2016). Cellulolytic and xylanolytic enzymes have gained importance in the biotechnology and industries due to their potential applications such as food and feed industries, the production of supplements, bio-ethanol, and pulp (Bajaj & Mahajan, 2019; Kumar et al., 2018). The majority of consumers of industrial enzymes production are the laundry and dish detergent industries (Bajaj & Mahajan, 2019; Alokika & Singh, 2019). Xylanolytic enzymes production was including amylases, cellulases, xylanases, other hemicellulases, and lipases, which were produced from *Paenibacillus* strains, used in food, paper, biofuels, and cleansers industries (Grady et al., 2016). Production enzymes from *Paenibacillus* strains are strongly active under industrially relevant conditions, and their cost of production can be made is lower than available alternatives due to their high-density culture (Grady et al., 2016).

The screening, identification, and characterization of the functional properties of strongly xylanolytic bacteria plays important role for the development of applicable bioprocesses. To get a bacterium displaying proficient xylan-degradation ability under anaerobically thermophilic conditions, we recently detected strain DA-C8, assigned to the genus *Paenibacillus*, as a pure culture from manure. The strain DA-C8 was gram-stained negative type, straight and slightly curved rod shape, non-motility (Figure 3.1). This strain was stored at the RIKEN BioResource Research Center and the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) with bacterium repository number JCM 34211 and DSM111723. Strain DA-C8 has strong xylan-degradation ability under thermophilic anaerobic conditions. Comparison of xylan degradation ability of strain DA-C8 and *P. curdlanolyticus* B-6 was investigated. *P. curdlanolyticus* B-6 was isolated strain, which has high xylan degradation ability due to its high production of an extracellular multienzyme complex (Pason et al., 2010). Strain B-6 was inoculated in Berg's mineral salt medium and grown under aerobic condition at 37°C, 150 rpm (Pason et al., 2010). For strain DA-C8, incubated at 55°C, 150 rpm, under anaerobic condition in BM medium. The medium and cultivation condition was reported (Aikawa et al., 2018). Beechwood xylan was hydrolyzed

earlier by strain DA-C8. Strain DA-C8 can used beechwood xylan more efficiently than can xylanolytic *P. curdlanolyticus* B-6.

Whole-genome of strain DA-C8 was sequenced in order to understand the differences of xylan degradation system. Draft genome of strain were analyzed using the Ion GeneStudio S5 system generated 11,760,377 reads. *De novo* genome assembly using CLC Genomics Workbench 20.0.1 (CLC Bio, Qiagen, Valencia, CA) yielded 69 contigs with an N50 of 108,510 bp and a mean contig length of 45,081 bp. The result of the draft genome of strain DA-C8 was generated 3,110,565 bp and had a G+C content of 52.3 mol%. Strain DA-C8 encodes 2,877 protein-coding sequences, classified in 2 rRNA genes, 55 tRNA genes, and 5 CRISPR genes. In term of the genome coverage depth, we got 650-fold.

Comparison of the 16S rRNA sequence of strain DA-C8 (GenBank accession number MT645320) with *Xylanibacillus* and other *Paenibacillus* strains (Figure 3.2) revealed that strain DA-C8 shared highest sequence similarity with *P. cisolokensis* UICC B-42 (93.7% identity; accession no. NR_151901), *Xylanibacillus composti* K-13 (92.8% identity; NR_159899), *P. pinistramenti* ASL46 (92.7% identity; NR_165784.1), *P.* senegalensis JC66 (92.6% identity; NR_125594), *P. yonginensis* DCY84 (92.5% identity; NR_148742), *P. chitinolyticus* HSCC 596 (92.4% identity; NR_040854), *P. favisporus* GMP01 (92.4% identity; NR_02971.1), *P. residui* MC-246 (92.2% identity; NR_116949), *P. xanthinilyticus* 11N27 (92.0% identity; NR_137226), *P. sepulcri* CCM 7311 (91.9% identity; NR_043693), *P. daejeonensis* AP-20 (91.7% identity; NR_104277), and *P.nanensis* MX2-3 (91.6% identity; NR_041491), *P.cellulositrophicus* P2-1 (91.4% identity; FJ178001.2) (Supplementary Table 3.1). Based on the 16s rRNA sequence, strain DA-C8 shared higher identity to *P.cisolokensis* (93.7%) than *Xylanibacillus composti* (92.8%), respectively. So, strain DA-C8 should be belonged in *Paenibacillus* genes group even the position of the strain DA-C8 was on the same branch with *Xylanibacillus composti*, according to the phylogenetic tree.

Additionally, the similarity relatives of strain DA-C8 based on average nucleotide identity (ANI) values between *Xylanibacillus composti* K-13, and other *Paenibacillus* strains were *P. chitinolyticus* KCMM41400 (68.77%), *P. cisolokenesis* LC2-13A (68.17%), *P. cellulositrophicus* KACC16577 (68.1%), *P. yonginensis* DCY 84 (68.08%), *P. composti* K-13 (67.98%), *P. nanensis* DSM 22867 (67.65%), *P. pinistramenti* ASL46 (67.53%), *P. favisporus* Y7 (67.46%), *P. larvae* ATCC-9545 (67.17%), and *P. daejeonensis* DSM 15491 (66.83%), *P. senegalensis* JC66 (66.80%) (Figure 3.3 and supplementary Tables 3.2 and 3.3). ANIs between DA-C8 and all additional strains included in the analysis were < 70%.

A new isolated strain DA-C8 should belong in the genus of *Paenibacillus* species based on comparing the 16S rRNA sequence and ANI values with the closest relative strains.

According to the result of genome sequencing and annotation revealed the predicted crucial enzymes which are necessary for xylan and lignocellulosic biomass degradation abilities from strain DA-C8 including endo-β-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37)/β-glucosidase (EC 3.2.1.21), acetyl xylan esterase (EC 3.1.1.72), α-L-arabinofuranosidase (EC 3.2.1.55), β-L-arabinofuranosidase (EC 3.2.1.185), α-xylosidase (EC 3.2.1.177), endo-β-glucanase (EC 3.2.1.4), α-glucoamylase (EC 3.2.1.3), polygalacturonase (EC 3.2.1.15), and α-1,2-glucuronosidase (EC 3.2.1.131) (shown in Table 3.3). Specifically noteworthy, β-L-arabinofuranosidase and polygalacturonase enzyme were for debranching side chain, are not found in the genome sequence of *P. curdlanolyticus* B-6 (Baramee et al., 2020). The contigs and annotated data of strain DA-C8 can be accessed at Mendeley Data (Chhe et al., 2021).

3.2 Materials and Methods

3.2.1 Genomic DNA purification and sequencing

Strain DA-C8 was incubated for 4 days at 55°C under the anaerobic condition with 1% of xylose as a carbon source. A cell of strain was collected by centrifugation for 8000 rpm for 10 minutes at 4°C, genomic DNA of strain DA-C8 was extracted by the phenol/chloroform method (Baramee et al., 2020). DNA fragmentation purification and library preparation were performed using an Ion Xpress Plus Fragment Library kit (catalog no. #4471269, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. DNA fragments roughly 200 to 300 bp in size were arranged through electrophoresis on Invitrogen E-Gel SizeSelect II agarose gels (catalog no. #G661012, Thermo Fisher Scientific) before library preparation. Genomic DNA sequences of strain DA-C8 were acquired using the Ion GeneStudio S5 system and then processed (Baramee et al., 2020).

3.2.2 Phylogenetic analysis

The 16S rRNA gene sequences of strain DA-C8 (accession number MT645320) and the relative strains acquired through BLAST searching in the GenBank database, and the 16S rRNA sequence of strain DA-C8 were manually aligned with the close strains using CLUSTAL_W (Thompson et al., 1994). A phylogenetic tree was created by the neighbor-joining method based on the Tamura-3 parameter model (Tamura, 1992) in MEGA X v10.1 (Kumar et al., 2018).

3.2.3 Genome assembly, annotation, and analysis

de novo genome assembly were performed in CLC Genomics Workbench v20.0.1, after removal of low-quality raw sequences. The genome assembly was annotated using DDBJ DFAST (https://dfast.nig.ac.jp). Protein families of predicted genes of essential xylan degradation enzymes were proved through CAZymes (http://www.cazy.org/).

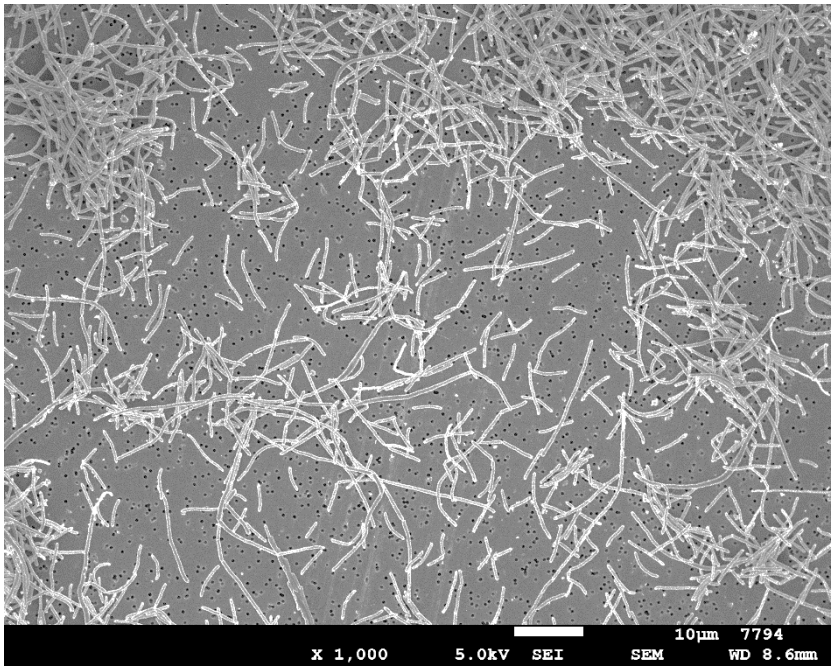
3.2.4 Genomic ANIs

Pairwise ANI values of whole-genome sequences between strain DA-C8, *Xylanibacillus composti* K-13, and close relative strains in the genus *Paenibacillus* were calculated using GENETYX NGS v4.1.1. The matrix created from the analyzed ANI values was turned into a genetic dendrogram using algorithms described previously (Baramee et al., 2020).

Table 3.2. Features of the *Paenibacillus* sp. DA-C8 genome.

Feature	Description
Number of reads used in the assembly	11,760,377
Genome size	3,110,565 bp
Number of contigs	69
G+C content (%)	52.3
N50 contig length	108,510 bp
Mean contig length	45,081 bp
Number of CDSs	2,877
Number of rRNAs	2
Number of tRNAs	55
Number of CRISPRs	5
Genome coverage depth	650-fold

(A)



(B)

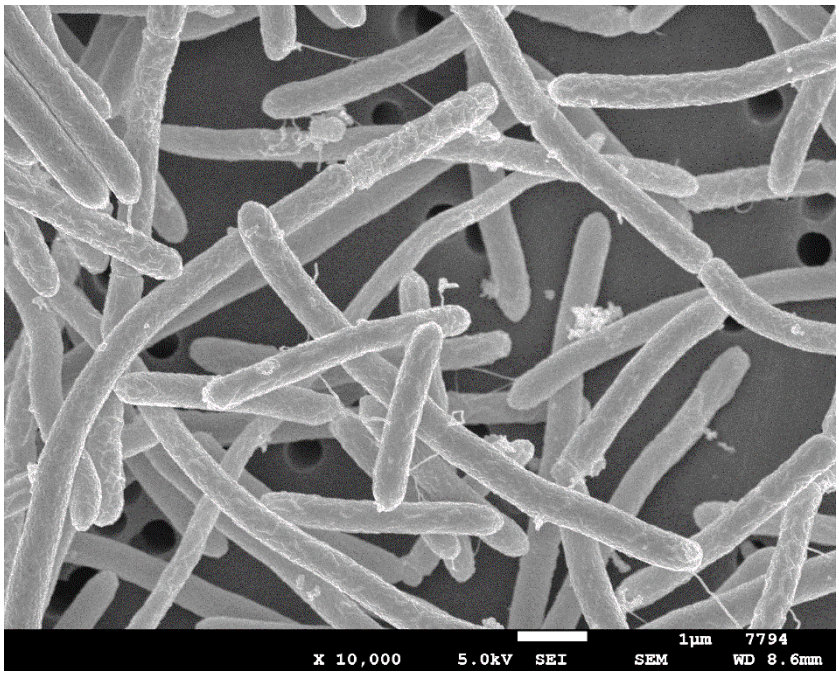


Figure 3.1. Scanning electron micrographs of *Paenibacullus* sp. Strain DA-C8 in BM medium with 1.0% xylose for three days. The white bars are 10 µm (A) and 1 µ (B).

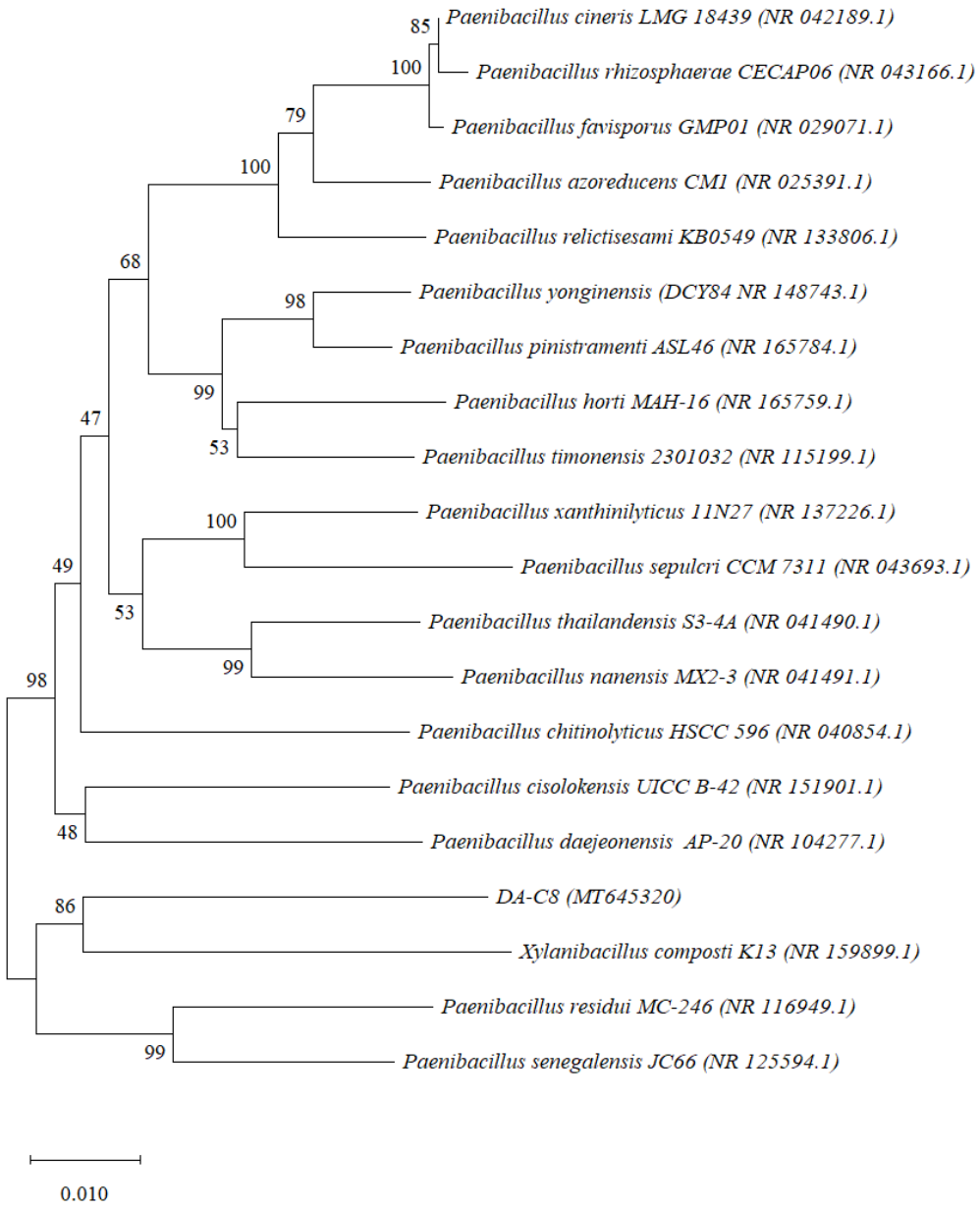


Figure 3.2. Comparative sequence analysis of 16S rRNA from strain DA-C8 and its closest relative strains from Genbank, using a neighbor-joining tree. Numbers at nodes are bootstrap support percentages based on 1,000 replicates. The bar represents 0.01 substitutions per nucleotide position.

Supplementary Table 3.1. List of phylogenetic tree strains based on their 16S rRNA sequence, strain DA-C8, and the nearest members.

Organisms	Strain	Accession no.
Paenibacillus	DA-C8	MT645320
Paenibacillus cisolokensis	UICC B-42	NR_151901.1
Xylanibacillus composti	K-13	NR_159899.1
Paenibacillus pinistramenti	ASL46	NR_165784.1
Paenibacillus senegalensis	JC66	NR_125594
Paenibacillus yonginensis	DCY84	NR_148743.1
Paenibacillus chitinolyticus	HSCC 596	NR_040854
Paenibacillus favisporus	GMP01	NR_02971.1
Paenibacillus residui	MC-246	NR_116949
Paenibacillus xanthinilyticus	11N27	NR_137226
Paenibacillus sepulcri	CCM 7311	NR_043693
Paenibacillus daejeonensis	AP-20	NR_104277
Paenibacillus nanensis	MX2-3	NR_041491
Paenibacillus cellulositrophicus	P2-1	FJ178001.2
Paenibacillus azoreducens	CM1	NR_025391.1
Paenibacillus relictisesame	KB0549	NR_133806.1
Paenibacillus rhizoshaerae	CECAP06	NR_043166.1
Paenibacillus thailandensis	S3-4A	NR_041490.1
Paenibacillus horti	MAH-16	NR_165759.1
Paenibacillus timonensis	2301032	NR_115199.1
Paenibacillus cineris	LMG18439	NR_042189.1

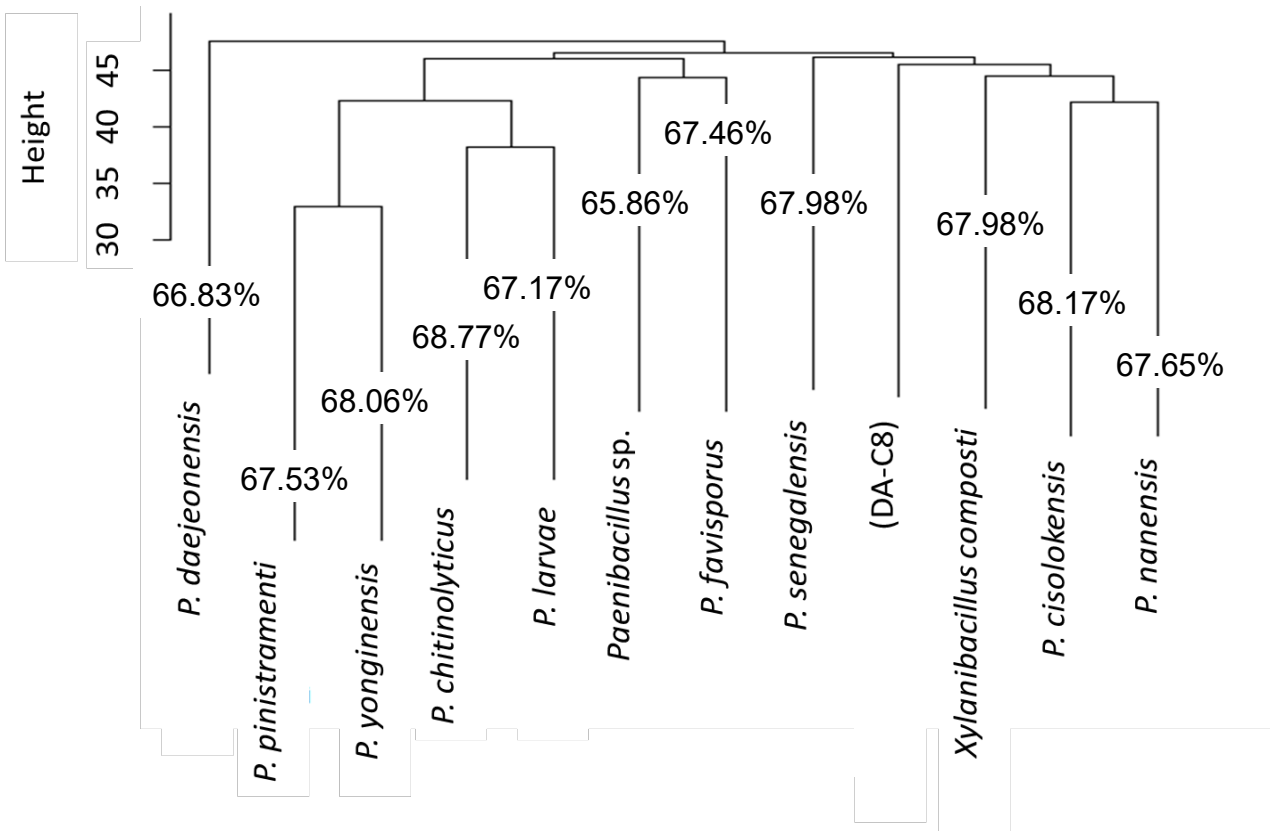


Figure 3.3. Dendrogram of average nucleotide identity (ANI) values. ANI values between strain DA-C8, *Xylanibacillus composti*, and 10 *Paenibacillus* strains were analyzed and used to build a dendrogram based on the unweighted pair group method arithmetic means. The following 12 strains were used: DA-C8, *Paenibacillus cisolokenesis*, *Xylanibacillus composti*, P. chitinolyticus (NZ_CP026520); *P. daejeonensis*, *P. favisporus, P. larvae*, *P. nanensis*, *P. pinistramenti*, *P. senegalensis*, *Paenibacillus* sp, and *P. yonginensis*.

Supplementary Table 3.2. Strains included in the dendrogram based on average nucleotide identity values in Table 3.3.

Organism	Strain	Accession
DA-C8	DA-C8	BMAQ00000000.1
Paenibacillus cisolokenesis	LC2-13A	BOVJ00000000.1
Xylanibacillus composti	K-13	BOVK00000000.1
Paenibacillus yonginensis	DCY84	CP014167
Paenibacillus cellulositrophicus	KACC16577	CP045295
Paenibacillus chitinolyticus	KCMM41400	NZ_CP026520.1
Paenibacillus favisporus	Y7	WIBG00000000.1
Paenibacillus senegalensis	JC66	CAES00000000.1
Paenibacillus nanensis	DSM 22867	QXQA00000000.1
Paenibacillus pinistramenti	ASL46	VAWG00000000.1
Paenibacillus larvae	ATCC-9545	NZ_CP019687
Paenibacilllus daejeonensis	DSM 15491	ARKE00000000.1
Paenibacillus	ALJ109b	JAAIFP000000000

Supplementary Table 3.3. Pair wise average nucleotide identities (%) between strain DA-C8, *Xylanibacillus composti* K-13, and other *Paenibacillus* strains.

	DA-C8	*Paenibacillus cisolokenesis*	*Xylanibacillus composti*	*Paenibacillus nanensis*	*Paenibacillus pinistramenti*	*Paenibacillus senegalensis*	*Paenibacillus yonginensis*	*Paenibacillus chitinolyticus*	*Paenibacillus daejeonensis*	*Paenibacillus favisporus*	*Paenibacillus larvae*	*Paenibacillus sp*
DA-C8	---	68.33	67.73	67.5	67.14	66.85	68.11	68.67	67.25	67.14	67.71	66.15
Paenibacillus cisolokenesis	68.17	---	69.28	70.11	68.37	67.84	68.98	68.64	68.76	68.48	67.78	66.71
Xylanibacillus composti	67.98	69.61	---	67.89	67.28	68.42	67.8	68.76	67.24	67.52	68.78	66.30
Paenibacillus nanensis	67.65	70.43	67.7	---	67.94	66.86	68.91	66.75	68.19	67.79	68.11	66.80
Paenibacillus pinistramenti	67.53	68.59	67.17	68.37	---	67.24	77.09	69.14	67.66	70.22	68.75	68.75
Paenibacillus senegalensis	66.8	67.9	68.34	66.69	66.97	---	69.17	67.83	66.54	66.45	68.79	66.12
Paenibacillus yonginensis	68.06	69.46	67.95	68.81	77.48	68.4	---	74.14	67.35	70.69	73.63	68.67
Paenibacillus chitinolyticus	68.77	69.43	68.29	67.55	67.58	67.9	74	---	66.6	68.61	72.95	66.99
Paenibacillus daejeonensis	66.83	68.37	67.03	66.69	67.36	66.19	66.68	65.11	---	66.62	66.01	66.41
Paenibacillus favisporus	67.46	69.07	67.48	68.19	70.12	66.73	69.83	68.13	67.19	---	67.73	69.00
Paenibacillus larvae	67.17	67.36	67.46	66.8	66.76	67.96	72.81	73.15	66.11	67.28	---	66.57
Paenibacillus sp	65.86	66.66	65.90	66.34	68.6	66.12	67.9	65.93	66.38	68.8	66.49	

Table 3.3. Predicted of genes encoding of xylanolytic enzymes from *Paenibacillus* sp. strain DA-C8.

Product name	Accession number and protein structure	Product name	Accession number and protein structure
Acetylxylan esterase (EC 3.1.1.72)	GFR38380.1(Axe1) GFR39008.1(Putative esterase) GFR37235.1(Putative esterase) GFR38723.1(2CBM9-SLH-esterase) GFR38721.1(CBM9-SLH-esterase)	**endo-β-1,4-galactanase (EC 3.2.1.89)**	GFR38027.1(GH53-3Car-SLH) GFR38725.1(GH53)
Acetylesterase	GFR38363.1 GFR38801.1 GFR39004.1(CBM9-Putative esterase) GFR39009.1	**β-galactosidase (EG 3.2.1.23)**	GFR38738.1 (GH2) GFR39515.1 (GH2) GFR37853.1 (GH2) GFR37864.1 (GH2-CBM6)
α-N-arabinofuranosidase (EC 3.2.1.55) **Glycoside hydrolase 43 family protein**	GFR38619.1(CBM4/9-AbfA) GFR38760.1(GH43-GH43) GFR36961.1(GH43-GH43) GFR37842.1(GH43) GFR38761.1(GH43-CBM6-CBD) GFR39512.1 (GH43-AbfA) GFR36960.1(GH43-GH43-GH43-CBM6/36-xylanase)	**α-galactosidase (EC 3.2.1.22)**	GFR37862.1 (GH4) GFR39540.1 (GH27)
Intracellular exo alpha-L-arabinofuranosidase 2 (EC:3.2.1.55)	GFR38054.1(AbfA)	**β-xylosidase (EC 3.2.1.37)** **Glycoside hydrolase 43 family protein**	GFR39117.1(GH3), GFR38678.1(GH43) GFR37272.1(GH43-GH43)
Intracellular exo-alpha-(1->5)-L-arabinofuranosidase 1	GFR38378.1(AbfA)	**β-xylanase** **Endo-1,4-beta-xylanase A** **β-xylanase (EC 3.2.1.8)**	GFR38137.1(GH10) GFR38732.1(GH10) GFR36972.1(GH10) GFR37282.1(CBM4/9-GH10-CBM9-SLHs) GFR37281.1(2CBM4/9-GH10-CBM9-SLH) GFR39210.1(GH11) GFR39349.1 (GH11-2CBM36-2CBM6)
β-L-arabinofuranosidase (EC 3.2.1.185)	GFR37345.1(GH127)	**Endoglucanase (EC 3.2.1.4)**	GFR37866.1(GH5), GFR38098.1(GH9-CBM3-CBM3) GFR38096.1(GH44-3FN3-2CBM3) GFR38097.1(GH48-3FN3-2CBM3) GFR38136.1(GH6-3FN3-2CBM3) GFR37117.1(GH8)
α-xylosidase (EC 3.2.1.177)	GFR37132.1(GH31)	**Glucan 1,4-alpha-glucosidase** **Glucoamylase (EC 3.2.1.3)**	GFR37009.1(GH15) GFR39124.1(GH15)
β-glucosidase (EC 3.2.1.21)	GFR36975.1(GH3-GH3) GFR36724.1(GH3-CBM6-xylanase) GFR37131.1(GH3+CBM6-xylanase) GFR36976.1(GH3) GFR37339.1(GH16) GFR37331.1(GH16)	**Polygalacturonase (EC 3.2.1.15)**	GFR38312.1(GH28)
α-amylase (EC 3.2.1.1)	GFR39119.1(GH13) GFR39563.1(GH13-GH13) GFR39005.1(GH13)	**Xylan alpha-(1->2)-glucuronosidase (EC 3.2.1.131)**	GFR38733.1(GH67M) GFR38734.1 (GH67M-GH115-GH115-CBM6)

3.3 References

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Chapter 4

Characterization of New Isolated Thermophilic, Facultative Anaerobic Bacterium *Paenibacillus* sp. Strain DA-C8 with Xylan Degradation Ability Activated by Anaerobic Condition

OVERVIEW

The screening, identification, and study of the functional properties of cellulolytic xylanolytic bacteria is of crucial importance for the construction of applicable bioprocesses. To obtain a bacterium exhibiting efficient xylan-degradation ability, thermophilic, facultatively anaerobic, xylanolytic bacterial strain DA-C8 (=JCM34211=DSM111723) was newly isolated from compost. Strain DA-C8 can degrade complete 1% beechwood xylan for 4 days under anaerobic conditions. In 16S rRNA and ANI sequence analysis, strain DA-C8 was closely related to *Xylanibacillus composti,* however, strain DA-C8 should be classified as new species of genus *Paenibacillus* rather than genus *Xylanibacillus*, because of sugar utilization properties. In the genome sequence between DA-C8 and *X. composti*, the numbers of xylanase and endoglucanase were not many different; however, strain DA-C8 possessed more abundance α-L-arabinofuranosidase, β-xylosidase, and β-glucosidase than *X. composti*. Strain DA-C8 drastically decreased xylan degradation ability and growth on xylan medium under aerobic conditions. Real-time PCR analysis clearly showed high expression of genes regarding xylan and cellulose degradation under anaerobic conditions, but the genes were repressed under aerobic conditions, indicates that strain DA-C8 controls xylan degradation depending on the growth conditions. This is the first report as genus *Peanicacillus* with a unique polysaccharide degradation system.

4.1 Introduction

The ability to deconstruct plant biomass is an essential characteristic for any organism being considered for the consolidated bioprocessing of plant biomass to fuels and chemicals (Ning et al., 2021). Plant biomass composes mainly of three different polymers, i.e., cellulose, hemicelluloses, and lignin. Among hemicelluloses, xylan is the major component of hemicellulose, which is one of the most abundant in plant polysaccharides in nature. The main chain of xylan composed β-1,4-D-xylosidic linkage of D-xylose is sometimes modified and branched by 4-O-methyl-D-glucuronic acid, D-glucuronic acid or L-arabinofuranose, and acetyl group (Holtzapple, 2003). Endo-β-1,4-xylanases (EC 3.2.1.8) are major glycoside hydrolases that hydrolyze xylan. Many characterized xylanases belong to glycoside hydrolase

families 10 and 11, according to the classification in the Carbohydrate-Active Enzymes (CAZy) Database (http://www.cazy.org). Recent studies have shown that the pretreatments of hemicellulose and lignin that surround crystalline cellulose polymer is enhanced the efficient decomposition of plant biomass, which is an important technology (Taherzadeh and Karimi, 2008; Agbor et al., 2011; Galbe and Wallberg, 2019). To obtain more efficient degradation for plant biomass, the screening, identification, and study of the functional properties of high xylanolytic bacteria are of crucial importance for the construction of applicable bioprocesses. To obtain microbes having more strong and effective plant biomass degradation, we recently reported the characterization of the thermophilic microbial community and isolation of the several microbes from cow manure compost (Shikata et al., 2018; Widyasti et al., 2018; Ungkulpasvich et al., 2021a).

In this study, we also screened to isolate new bacteria with high xylan degradation ability under anaerobic thermophilic conditions using the same compost as previously studied(Shikata et al., 2018; Widyasti et al., 2018; Ungkulpasvich et al., 2021a). One bacterium, strain DA-8C, was successfully isolated as a pure culture from the compost. Strain DA-C8 was appeared as new species of genus *Paenibacillus* according to the physiological, morphological properties. Strain DA-8C is interestingly able to activate xylan degradation under anaerobic conditions, but not aerobic conditions, which may possess a unique polysaccharide degradation system different from similar *Paenibacillus* species.

4.2 Materials and Methods

4.2.1 Materials

The composts involved bovine manure as the original compost samples were collected as an original sample from Ishigaki Taihi Center (Okinawa, Japan)(Shikata et al., 2018; Widyasti et al., 2018; Ungkulpasvich et al., 2021a). *Xylanibacillus composti* strain K-13 (DSM 29793^T) was purchased from DSMZ (the Leibniz Institute DSMZ-German collection of microorganisms and cell cultures GmbH). Corn hulls used in this study were purchased from the maize farm of the Institute of Livestock and Grassland Science, National Agriculture and Food Research Organization (NARO).

4.2.2 Media, cultivation methods

X. composti K-13 were cultured aerobically using trypticase soy yeast extract medium (DSMZ media 92) in a flask at 45 °C under aerobic culture condition with shaking at 150 rpm. Strain DA-C8 was grown on basal medium (BMN) supplemented with 1% (w/v) xylan or 1% (w/v) mono-sugars such as xylose. The BMN medium was composed of: 2.9 g K_2HPO_4, 4.2 g

Urea, 1.0 g Na_2CO_3, 2.0 g Yeast extract, 0.5 g L-cysteine-HCl·H_2O, 0.01 g $CaCl_2 \cdot 2H_2O$, 0.5 mg resazurin, and 200 μL mineral solution (Shikata et al., 2018) for 1 litter and adjusted at pH 9.0 by 1N HCl or NaOH. To prepare strict anaerobic culture conditions, BMN medium was degassed in boiling water and bubbled with high-purity N_2 before autoclaved. In case of aerobic culture condition for strain DA-C8, BMN medium eliminated L-cysteine-HCl, and resazurin was directly used in triangle flasks closed by silicone caps without degassing in boiling water bath and paging by N_2 gas with shaking at 150 rpm after autoclaved. The aerobic culture was carried out with shaking at 150 rpm at 55 °C. All chemicals were purchased from FUJIFILM Wako Pure Chemicals, Osaka, Japan. Hungate anaerobic culture methods were performed using 1% (w/v) beechwood xylan (FUJIFILM Wako Pure Chemicals).

4.2.3 Measurement of bacteria cell growth

Cell growth in the BMN medium with xylan and xylose or glucose was assayed by the increased protein concentration in the pellet or optical density at 600 nm. Cells were lysed in a NaOH/SDS solution (Shikata et al., 2018). The protein concentration in the supernatant was measured using the Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, U.S.) with bovine serum albumin as the standard.

4.2.4 DNA extraction, PCR amplification, and phylogenetic analysis

Genomic DNA was isolated using a NucleoBond AXG column and a NucleoBond Buffer Set III (Takara Bio Inc., Kusatsu, Japan) following the manufacturer's protocol. The 16S rRNA gene was amplified using the bacterial domain-specific primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR products were purified using a QIAquick PCR Purification kit (Qiagen, Frederick, MD). The 16S rRNA gene sequence was analyzed by the NCBI BLAST program [https://blast.ncbi.nlm.nih.gov/] and manually aligned against sequences in the GenBank database using ClustalW version 2.1 (https://clustalw.ddbj.nig.ac.jp/). Phylogenetic trees were constructed by the neighbor-joining method using MEGA version 10.0.5 (Kumar et al., 2018).

4.2.5 Measurement for xylan and corn hull degradation abilities of strain DA-C8 and *X. composti* K-13

A microbial xylan degradation experiment was performed in 300 mL of BMN medium or DSMZ media 92 containing 1% (w/v) beechwood xylan and corn hull, respectively. Cultures were incubated at 55 °C and 45°C with anaerobic and aerobic conditions for strain DA-C8 and *X. composti* strain K-13, respectively. Residual biomass solids were collected by centrifugation for 10 min at 10,000 rpm at 4°C and dried at 80 °C for 2 days. Six samples (days 0, 1, 2, 4, 5,

or 6) were obtained during the process. The weight of the residual biomass was compared with the control treatment without the inoculum. The percentage weight loss (referred to below as the "ratio") was defined as the ratio of weight loss compared with the initial weight (%) calculated as described previously (Ungkulpasvich et al., 2021a).

To characterize the glucosyl hydrolysis activity of strain DA-C8 and *X. composti* K-13, extracellular enzyme fractions were prepared from each culture of 300 mL supplemented with 1% (w/v) beechwood xylan. Culture supernatant was concentrated using Amicon Ultra centrifugal filters (Merck Millipore Corp., Darmstadt, Germany) and loaded onto an Econo-Pac 10DG column equilibrated with distilled water following the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA).

The enzymatic activities regarding xylan degradation were basically measured by determining the amount of reducing sugars released from beechwood xylan and carboxymethylcellulose (CMC). The reaction comprised 0.5% (w/v) substrate in 0.1 M sodium phosphate buffer, pH 8.0, and 30–40 μg of extracellular fractions. The optimum pHs and temperatures of the extracellular fractions prepared from strain DA-C8 and *X. composti* K-13 were investigated in variety pH ranging from pH 4.0 to 10 (50 mM sodium acetate buffer for pH 4.0-6.0; 50 mM phosphate or Tris-HCl buffers for pH 6.0-8.0; 50 mM sodium carbonate buffer for pH 9.0-10.0) at 55˚C for 10 min, or at 40–70˚C on pH8.0, respectively. The released reducing sugars were quantified using the Somogyi–Nelson method with xylose or glucose as the standard (Wood and Bhat, 1988; Shikata et al., 2018). One unit of polysaccharide hydrolytic activity was defined as the amount of enzyme that liberated 1 μmol of xylose or glucose in 1 min. The activities of β-xylosidase, β-glucosidase, α-L-arabinofuranosidase, and acetyl esterase were measured using p-nitrophenyl-β-D-xylopyranoside, p-nitrophenyl-α-D-glucopyranoside, p-nitrophenyl-α-L-arabinofuranoside, and p-nitrophenyl acetate as the substrate (all from Sigma-Aldrich; St Louis, MO, USA), respectively (Wood and Bhat, 1988). One unit (U) of enzyme activity was defined as the amount of enzyme-producing 1 μmol of p-nitrophenol per minute under the assay conditions.

Proteins were analyzed using SDS-PAGE (5-20% polyacrylamide gradients) (ATTO, Tokyo, Japan). SDS-PAGE gel was stained with Comassie brilliant blue R-250. Molecular mass standard were from Bio-Rad Laboratories. Zymograms were performed on 10% polyacrylamide gels containing beechwood xylan as substrate as described previously (Widyasti et al., 2018). Clear bands in the zymograms indicated enzyme activity.

4.2.6 Quantitative real-time PCR

Total RNA was extracted from cultured DA-C8 in anaerobic or aerobic condition using

RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The quantity and purity of RNA were determined using NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Extracted total RNA was prepared using the SuperScript™ II Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific) following the instruction in a 20 µL total reaction volume to obtain the first strand of cDNA. Synthesized cDNA was diluted into 100 µL, and 2 µL of this dilution was used for quantitative real-time PCR (qRT-PCR). qRT-PCR was performed using CFX96 Touch™ Real-time system (Bio-Rad Laboratories Inc., Hercules, CA, USA) with a 10 µL reaction volume using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories Inc.) with the gene specific primers (supplementary Table 4.1). The housekeeping gene that encoded 16S rRNA was used as a control. The thermal cycler conditions were denaturation at 98 °C for 2 min; followed by 40 cycles of 10 sec at 98 °C and 1 min at 60 °C; and 65 °C to 95 °C for 5 sec with 0.5 °C increments for melt curve analysis. The fluorescent product was detected during the final step of each cycle. Amplification, detection, and data analysis were carried out on a CFX Maestro 1.1 software version 4.1 (Bio-Rad Laboratories Inc.). In duplicate, the Ct value for each gene expression was normalized to the Ct value for 16S rRNA and calculated to determine the ΔCt in template abundance for each sample.

- Heatmap generation

Heatmap of the expression levels of 17 genes was generated by Heatmapper (http://www.heatmapper.ca/) (Babicki et al., 2016). Each row represents a gene, and each column represents an average of double samples from cultured DA-C8 for 24h or 48h under anaerobic and aerobic conditions. The green and red gradients indicate an increase and decrease in gene expression, respectively.

- Genome analysis

Whole-genome sequence data sets for strain DA-C8 and *X. composti* K-13 were obtained from the National Center for Biotechnology Information databases (project accession numbers BMAQ01000000 (Chhe et al., 2021) and BOVK01000000, respectively) and used for genome annotation and bioinformatic analysis. Comparative amino acid sequence analyses were performed using the Local BLASTP and ATGC tools in GENETYX version 13 (GENETY Software Development, Tokyo, Japan), followed by manual curation.

4.3 Results and discussion

4.3.1. Characterization of newly isolated thermophilic, facultatively anaerobic, xylanolytic bacterium strain DA-C8

In previous research, strain DA-C8, newly isolated from compost by enrichment culture, shows high xylan degradation ability under thermophilic (55°C) anaerobic conditions (Chhe et al., 2021). The full 16S rRNA sequencing analysis (Genbank accession number; MT645320) of strain DA-8C was used for the subsequent phylogenetic analysis. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain DA-C8 branches from many *Paenibacillus* sp and belong to the lineage of *Xylanibacillus composti* strain K-13 (supplementary Figure 4.1). The highest 16S rRNA gene sequence identity values for closest relatives were as follows: *Paenibacillus cisolokensis* strain LC2-13A (95.5%) isolated from Cisolok geyser (Yokota et al., 2016), *X. composti* strain K-13 (92.5%) isolated from compost, *P. pinistramenti* strain ASL46 (92.7%) isolated from litter layer of a pine forest (Lee et al., 2020) and *P. senegalensis* strain JC66 (92.6%) isolated from the fecal flora of a healthy patient (Mishra et al., 2012), indicates that strain DA-C8 surely belongs to a member of the genus *Paenibacillus* or *Xylanibacillus*. Strain DA-C8 was observed under an optical microscope after Gram staining and by SEM and TEM. Gram staining also demonstrated that DA-8C was Gram-negative (Figure 1A). The cell morphology of strain DA-C8 was a typical rod-shaped bacterium of 0.4–0.5 μm in diameter and 30-50 μm in length (Figure 1B and 1C). Spores could not be observed during this research. Strain DA-8C possessed properties of a Gram-negative type cell, *i.e.*, the periplasmic space contains no cellular material, and the peptidoglycan layer (PG), outer membrane (OM), and plasma membrane (PM) were observed (Figure 1C). Physiological characteristics of strains DA-C8 were carried out using various growth temperatures and pHs under anaerobic growth conditions. Strain DA-C8 grew on BMN mediums with xylose at 30–60 °C (optimum, 55 °C), at pH 4.0–10.0 (optimum, 9.0), but did not grow at temperatures above 65 °C. Strain DA-C8 was grown well in salinity on 2% (w/v) NaCl. Strain DA-C8 grew vigorously with the following carbon sources: soluble starch, arabinoxylan, galactan, carboxymethyl cellulose (CMC), sucrose, cellobiose, arabinose, glucose, xylose, maltose, L-raffinose galactose, and mannose. The following substrates were not utilized: chitin, casein, crystalline cellulose, gelatin, glycerin, peptone, and tryptone. Meanwhile, *X. composti* K-13, phylogenetically most similar bacterium, cannot assimilate many carbon sources from results using API 50 CH test (Kukolya et al., 2018). The properties of *X. composti* strain K-13 were quite different from the properties of strain DA-C8 and cell wall structure, even though the closest species based on 16S rRNA sequences. The carbon utilization of *P. polymyxa* DSM36

and *F. aquaticus* DSM17643 as a comparison of *X. composti* K-13 (Kukolya et al., 2018) was more similar to the properties of strain DA-C8, indicated that strain DA-C8 should be classified into the genus of *Paenibacillus* rather than *Xylanibacillus*.

4.3.2. Comparison of xylan degradation abilities between strain DA-C8 and strain K-13

To compare xylan degradation abilities between strain DA-C8 and *X. composti* K-13, the degradation tests were carried out using beechwood xylan and corn hull as plant biomass. Strain DA-C8 and *X. composti* K-13 showed 100% and 80%, and 68% and 28% degradation for 1% (w/v) beechwood xylan and 1% (w/v) corn hull, respectively (Figure 4.2 A, B). Strain DA-C8 was observed higher degradation ability for lignocellulosic biomass than the ability of *X. composti* (Figure 4.2 B). Strain DA-C8 produced lactate, acetate, and CO_2 from beechwood xylan and corn hull. Although xylan degradation ability of *X. composti* K-13 was limitedly known, the other abilities regarding plant biomass degradation have never reported as well as strain DA-C8. To characterize differences between the degradation abilities of strain DA-C8 and *X. composti* K-13, extracellular fractions were prepared from the xylan grown cultures. Extracellular fraction of strain DA-C8 showed relatively high degradation activity of 33.00±0.03 U/mg toward xylan but low activity for crystalline cellulose such as Avicel. Optimum pHs and temperatures of the extracellular fractions of strain DA-C8 and *X. composti* K-13 were observed at pH 5.0-8.0 and pH 6.0-8.0, and 50-70°C and 50-55°C, respectively. Extracellular fraction of *X. composti* K-13 was 2 times lower xylanase ability than that of strain DA-C8. Furthermore, extracellular fraction from strain DA-C8 possessed high β-xylosidase, β-glycosidase, and α-L-arabinofuranosidase activities compared with those of *X. composti* K-13 (Table 4.1). These results were an agreement to high xylan and plant biomass degradation abilities with strain DA-C8, indicating that high β-xylosidase, β-glycosidase and α-L-arabinofuranosidase activities of DA-C8 can enhance the degradation ability because these enzymes be able to avoid not only enzymatic feedback inhibition by xylobiose and cellobiose but also debranching enzyme can remove arabinose substituents from arabinoxylan and arabinoxylo-oligomers, thereby, improving bioconversion of lignocellulosic biomass.

To more understand the lignocellulosic biomass degradation system of strain DA-C8. The comparison of the predicted genes regarding xylan-degradation was carried out using genome sequence data of strain DA-C8 and *X. composti* K-13. Many predicted genes regarding β-1,4-xylanases and endo-β-1,4-glucanases belonging to GH family-5, 9, 10, and 11 were found in the genome sequence of both strains (Table 4.2). Meanwhile, genes of β-glucosidases and β-xylosidases belonging to GH family-3 and 16 were abundantly confirmed in the genome sequence of stain DA-C8 (Table 4.2). The high activities of β-glucosidase and β-xylosidase in

extracellular fraction prepared from strain DA-C8 may reflect the existence of these genes. In addition, genes corded the accessory enzymes such as α-L-arabinofuranosidase, β-galactosidase, α-glucosidase, and polygalacturonase were also confirmed to the genome of strain DA-C8 (Table 4.2). Unsaturated β-glucuronyl hydrolase (Mori et al., 2003) catalyzes the hydrolytic release of unsaturated glucuronic acids from oligosaccharides produced through the reactions of polysaccharide lyases such as gellan, xanthan, hyaluronate, and chondroitin lyases, only found in strain DA-C8. It is well known that multiple hydrolase activities such as not only β-1,4-xylanases and β-1,4-glucanases but also β-xylosidase, α-L-arabinofuranosidase, β-galactosidase, and α-glucosidase, are necessary to achieve complete hydrolysis of lignocellulosic biomass. Strain DA-C8 was revealed to equip various genes regarding lignocellulose degradation more than *X. composti* K-13.

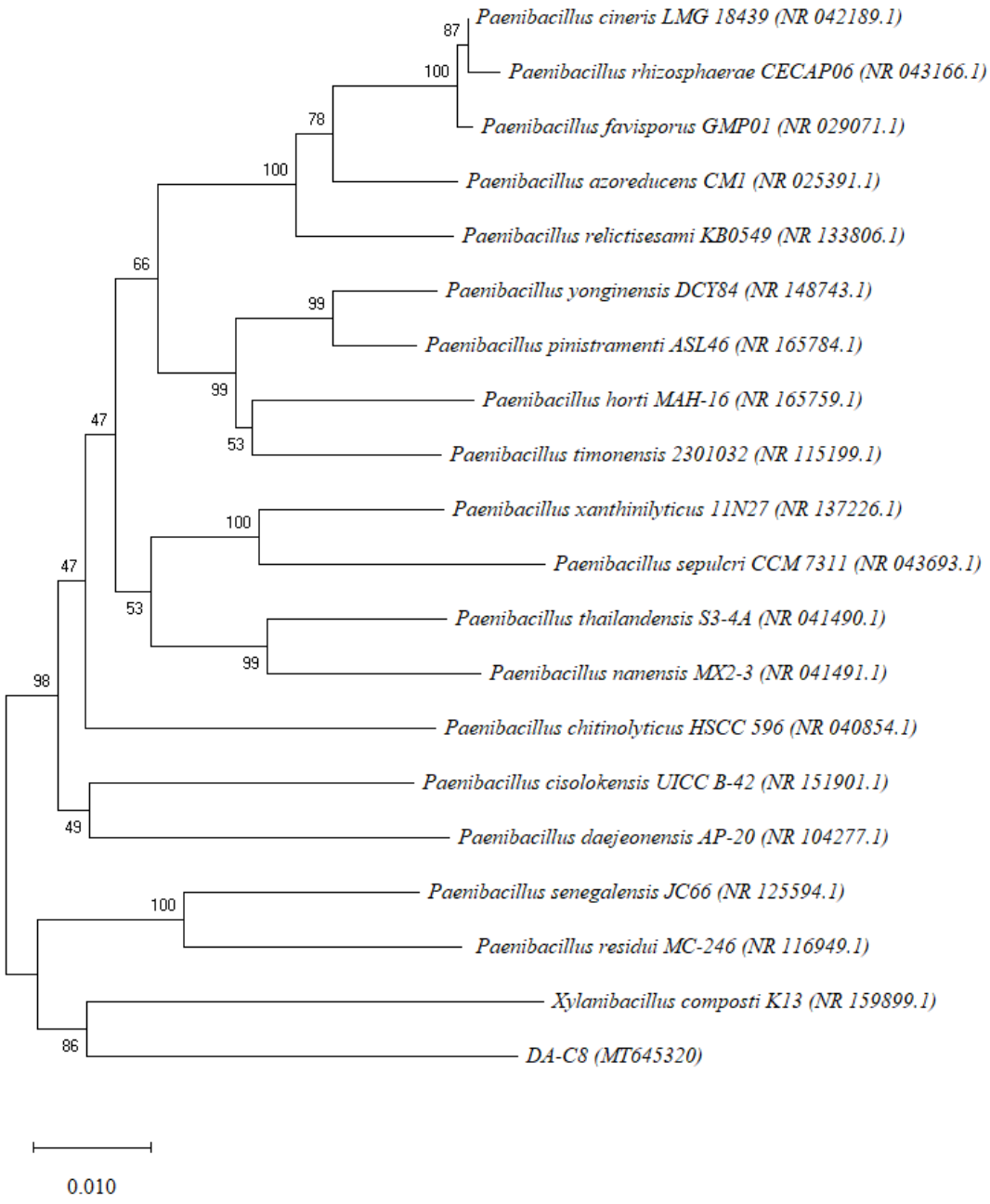
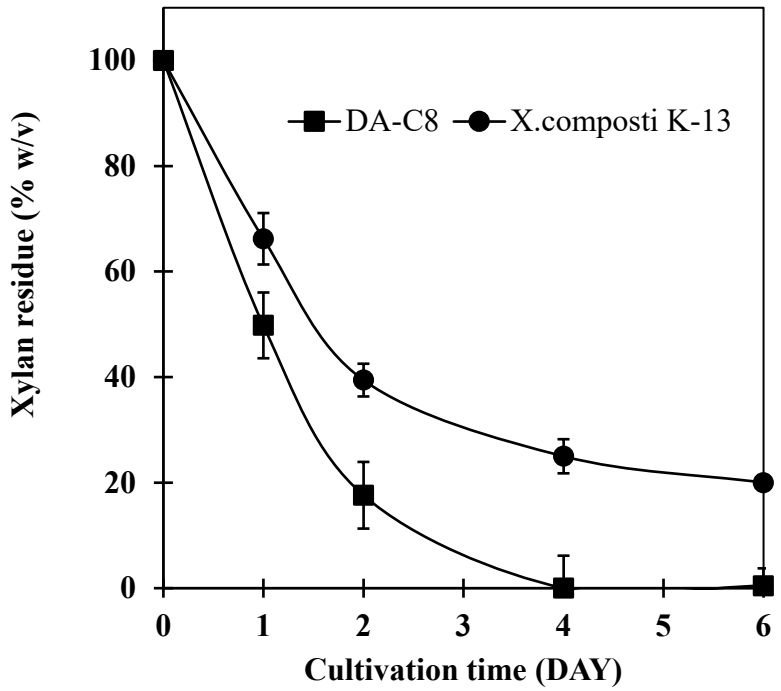


Figure 4.1. Phylogenetic tree based on 16S rRNA sequences of strain DA-C8, *Xylanibacillus composti* K-13, and other *Paenibacillus* sp.

(A)



(B)

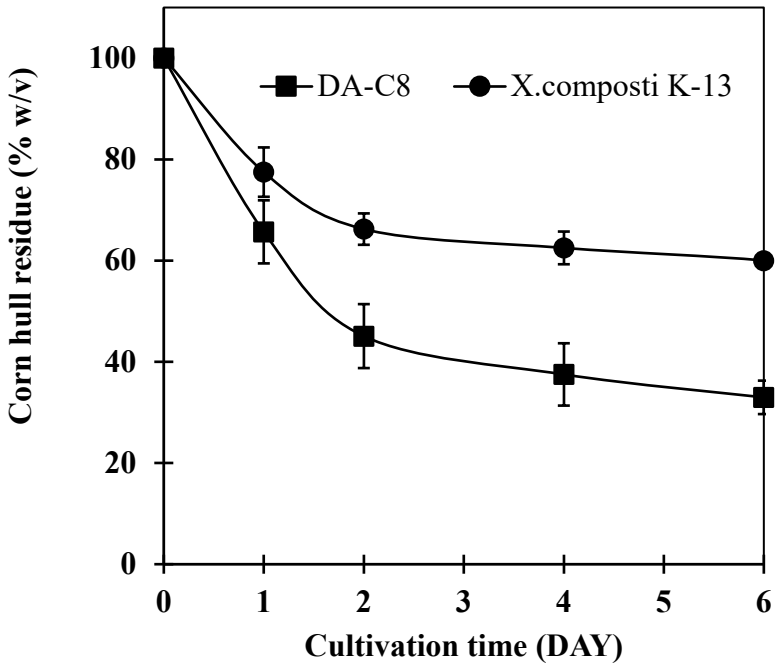


Figure 4.2. Degradation profiles of: (A) beechwood xylan and (B) corn hull of strain DA-C8 under anaerobic cultivation and *X.composti* K-13 under aerobic cultivation. The mean values for the triplicate experiments are presented. Error bars show ± standard deviation.

Table 4.1. Comparison of glycosyl hydrolase activities of extracellular enzymes prepared from strain DA-C8 and *X. composti* K-13.

Enzymes (u/mg protein)	**Strains**	
	Strain DA-C8	*X. composti* K-13
Xylanase	33.00±0.03	13.6±0.001
CMCase	1.37±0.012	0.8±0.002
Microcrystalin cellulose	0.0033±0.002	0.002±0.0003
β-xylosidase	0.29±0.001	0.03±0.001
β-glucosidase	0.65±0.002	0.12±0.001
α-L-arabinofuranosidase	0.12±0.002	0.01±0.001
α-glucosidase	0.03±0.002	0.03±0.0001
Acetyl xylan esterase	0.06±0.002	0.06±0.0001

Table 4.2. Comparing the predicted genes encoding glycosyl hydrolases and xylan degradation related enzymes of strain DA-C8 and *X. composti* K-13 (BLWM00000000).

Product name	Predicted gene(s)	
	DA-C8	**K-13**
Acetylxylan esterase (EC 3.1.1.72)	GFR38380.1(Axe1)	GIQ70188.1 (Axe1)
α-L-Arabinofuranosidase (EC 3.2.1.55)	GFR38619.1(CBM4-AbfA) GFR38760.1(GH43) GFR36961.1(GH43), GFR38678.1(GH43) GFR37272.1(GH43)	GIQ70614.1 (GH43) GIQ71518.1 (GH43-CBM6)
Intracellular α-L-arabinofuranosidase (EC:3.2.1.55)	GFR38378.1(GH43) GFR38054.1(GH43)	GIQ70617.1 (GH43) GIQ67909.1 (GH43)
β-L-Arabinofuranosidase (EC 3.2.1.185)	GFR37345.1(GH127)	N.D.
α-Xylosidase (EC 3.2.1.177)	GFR37132.1(GH31)	GIQ69911.1 (GH31)
β-Glucosidase (EC 3.2.1.21) β-Xylosidase (EC 3.2.1.37)	GFR36975.1(GH3-GH3) GFR36724.1(GH3+CBM6) GFR37131.1(GH3+CBM6) GFR36976.1(GH3) GFR39117.1(GH3) GFR37339.1(GH16) GFR37331.1(GH16)	GIQ69936.1(GH3) GIQ70591.1(GH43) GIQ70613.1 (GH3)
Glucoamylase (EC 3.2.1.3) α-Glucosidase (EC 3.2.1.20)	GFR37009.1(GH15) GFR39124.1(GH15)	GIQ70267.1(GH4) GIQ71073.1 (GH13)
α-Amylase (EC 3.2.1.1)	GFR39119.1(GH13) GFR39563.1(GH13) GFR39005.1(GH13)	GIQ71073.1(GH13)
β-Amylase (EC 3.2.1.2)	N.D.	GIQ70629.1(GH14-CBM25)
β-1,4-Galactanase (EC 3.2.1.89)	GFR38027.1 (GH53-3Carbox-SLH) GFR38725.1 (GH53-RICIN)	N.D.
β-Galactosidase (EG 3.2.1.23)	GFR38738.1 (GH42) GFR39515.1 (GH2) GFR37853.1 (GH2) GFR37864.1 (GH2-Ig-CBM6)	GIQ67232.1 (GH6) GIQ70532.1 (GH42)
α-Galactosidase (EC 3.2.1.22)	GFR37862.1 (GH4) GFR39540.1 (GH27)	GIQ68167.1(GH36) GIQ70267.1 (GH4)

		GIQ70384.1 (GH4)
β-1,4-Xylanase (EC 3.2.1.8)	GFR37282.1(CBM4-GH10-CBM9-SLH) GFR37281.1 (CBM4-GH10-CBM9-SLH) GFR38137.1 (GH10) GFR38732.1 (GH10) GFR36972.1 (GH10) GFR39210.1 (GH11) GFR39349.1 (GH11-2CBM6)	GIQ67218.1 (GH10) GIQ67238.1 (GH10) GIQ68060.1 (GH10-FM3-CBM3) GIQ70565.1 (GH11) GIQ70580.1 (GH11-CBM6)
Endo-β-1,4-glucanase/cellulase (EC 3.2.1.4)	GFR37866.1 (GH5) GFR38098.1 (GH9) GFR38096.1 (GH44-3FN3-CBM3) GFR38097.1 (GH48-3FN3-CBM3) GFR38136.1 (GH6-3FN3-CBM3) GFR37117.1 (GH8)	GIQ67658.1 (GH9-CBM3-2FN3-CBM3) GIQ69147.1 (CE2-SGNH) GIQ69528.1 (GH5) GIQ69905.1 (GH9) GIQ68056.1 (GH5-CBM46-CBM3)
Polygalacturonase (EC 3.2.1.15)	GFR38312.1 (GH28)	GIQ70534.1 (GH28)
α-Glucuronosidase (EC 3.2.1.131)	GFR38733.1 (GH67)	GIQ68892.1 (GH67)
Unsaturated β-glucuronyl hydrolase (EC 3.2.1.179)	GFR37895.1 (GH88) GFR38036.1 (GH88) GFR37539.1 (GH88)	N.D.
Chitinase (EC 3.2.1.14)	GFR39365.1 (GH18)	N.D.
α-Trehalase (EC 3.2.1.28)	GFR39510.1 (GH65) GFR36727.1 (GH65)	N.D.
α-L-Rhamnosidase (EC 3.2.1.40)	GFR37053.1 (GH106)	N.D.

4.3.3 Regulation of xylan degradation ability of strain DA-C8 under anaerobic and aerobic growth conditions.

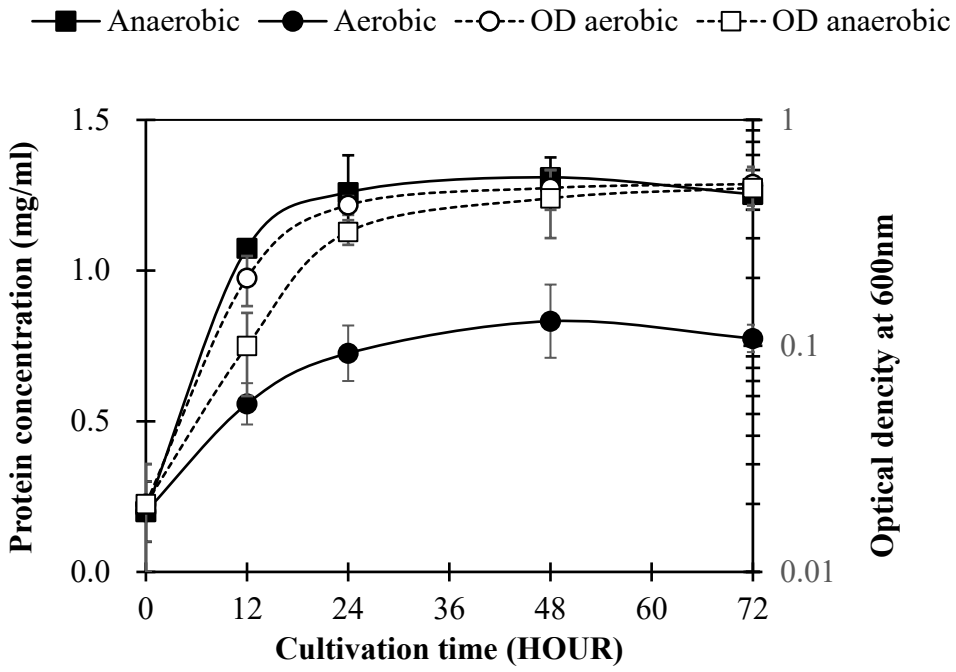
So far, in phylogenetic tree based on 16S rRNA and physiologic analysis, strain DA-C8 appeared as new species of the genus *Paenibacillus* which possesses properties such as rod-shaped cells of a Gram-positive or negative structure with oval endospores and aerobic or facultative anaerobic metabolism, as general characteristics (Vos et al.). The closest species *X. composti* K-13 and *P. cisolokensis* strain LC2-13A, were successfully isolated on aerobic conditions (Yokota et al., 2016; Kukolya et al., 2018). Meanwhile, strain DA-C8 was isolated on strict anaerobic growth conditions using compost (Chhe et al., 2021). When strain DA-C8 was grown on BM medium using xylose as the sole carbon source, the growth speed and cell densities under anaerobic growth conditions were slightly slower than aerobic growth conditions; however, both conditions were not a big difference and showed good growths (Figure 4.3 A). In contrast, growth of strain DA-C8 was drastically decreased on BM medium supplemented xylan as sole carbon sources under aerobic growth conditions. The cell density, as seen from total proteins, was 2 times lower than that of the anaerobic culture condition (Figure 4.3 A). These results indicate that strain DA-C8 prefers anaerobic conditions when xylan is used as a carbon source and needs to shift the metabolisms from aerobic to anaerobic conditions to obtain an efficient xylan degradation ability. To know whether the growth conditions are influenced by the degradation activities, xylan degradation ability was measured under both conditions. As a result, the xylan degradation ability of strain DA-C8 decreased under aerobic culture conditions as well as its growth curves (Figure 4.3 B). Strain DA-C8 can degrade 100% until 4 days under anaerobic conditions; however, only 27% of xylan is under aerobic conditions (Figure 4.3 B). These results indicated that the xylan degradation system of strain DA-C8 might be depressed by aerobically growth conditions. The xylanase activity in extracellular fraction prepared from the culture of strain DA-C8 under aerobic growth condition was observed 1.24±0.01 unit/mg protein as xylanase activity. Kim et al. reported that novel strain *Paenibacillus* sp. CAA11 harboring cellulase (168cel5) gene, which can degrade on cellulose under aerobic and anaerobic growth conditions, was enhanced 1.83 times of the ability even under strictly anaerobic conditions; however, the mechanism is still unclear for why capacity increasing (Kim et al., 2018). Although few reports on metabolic changes of anaerobic and aerobic cultures in genus *Paenibacillus*, this unique regulation system depending on growth conditions might be the first report among facultative microbes belonging to *Paenibacillus*.

To investigate the pattern of component of the xylanase enzymes isolated from strain DA-C8, gel electrophoresis, and zymograms was used. SDS-PAGE was used to analyze the

protein components. Zymogram analysis using xylan revealed that few or many xylanases were expressed into the extracellular fractions prepared from aerobically- and anaerobically-grown cultures, respectively (Figure. 4.4). These results strongly indicate that strain DA-C8 has a unique polysaccharides degradation system controlled by anaerobic or aerobic growth conditions. It is still unclear whether this regulation occurred by sensing of the dissolved oxygen concentration in the medium

To understand whether xylan degrading genes of strain DA-C8 are comprehensively regulated under aerobic growth conditions or not, real-time PCR (qPCR) was carried out for major xylan and lignocellulosic biomass-degrading genes. We selected 17 genes encoding predicted acetyl xylan esterase (GFR38380.1), α-L-arabinoflanosidase (GFR38619.1, GFR38760.1), intracellular α-L-arabinofuranosidase (GFR38054.1), β-L-arabinofuranosidase (GFR37345.1), β-glucosidase/β-xylosidase (GFR36724.1, GFR37339.1), β-1,4-galactanase (GFR38027.1), β-1,4-xylanase (GFR37282.1, GFR37281.1, GFR39210.1, GFR39349.1), endo-β-1,4-glucanase/cellulase (GFR37866.1, GFR38098.1), α-glucuronosidase (GFR38733.1), polygalacturonase (GFR38312.1), and unsaturated β-glucuronyl hydrolase (GFR37895.1), and analyzed whether there genes are expressed in strain DA-C8 under anaerobic and aerobic conditions. According to heatmap analysis using results from qPCR, strain DA-C8 showed significantly high expression on logarithmic phases for these genes under anaerobic growth conditions. On the other hand, the selected almost genes under aerobic growth conditions in strain DA-C8 were quite lower expression than stationary phases of the anaerobic conditions (Figure 4.5). These results strongly indicate that strain DA-C8 has a unique polysaccharides degradation system controlled by anaerobic or aerobic growth conditions. It is still unclear whether this regulation is occurred by sensing of O_2 concentration in the medium. It has been reported that anaerobic bacteria such as *Clostridium acetobutylycum* can recognize the favorable O_2 environment for their growth and has the ability to judge the start and stop of growth (Kawasaki et al., 2004). In addition, *C. acetobutylycum* and *C. butyricum* can induce large-scale expression of genes involved in O_2 metabolism immediately after O_2 aeration (Kawasaki et al., 2005). When *Bacillus subtilis* was exposed to low concentrations of hydrogen peroxide, a typical oxidative stress response is induced, including the scavenging enzymes such as catalase, a putative DNA binding, and protecting protein (Dowds, 1994). The repression of xylan degrading enzymes could be one of the oxidative stress responses in strain DA-C8. Furthermore, research is necessary to reveal this regulation.

(A)



(B)

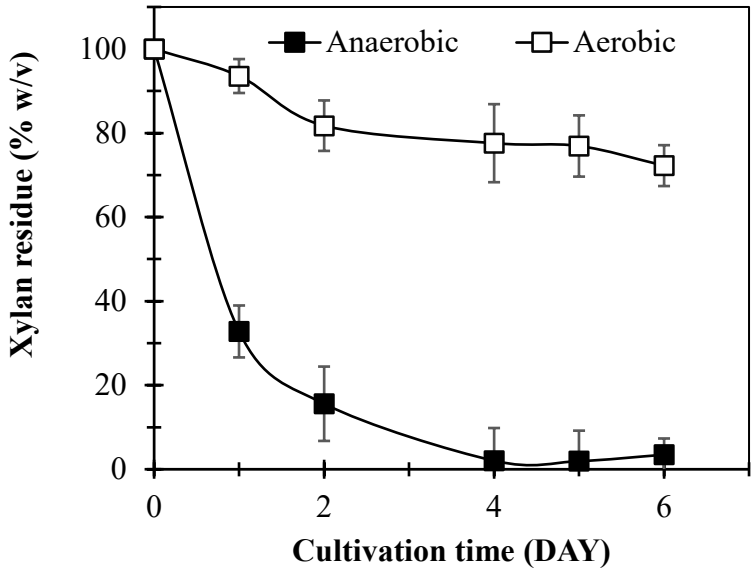


Figure 4.3. Measurement of bacteria cell growth and xylan degradation of strain DA-C8, (A) Protein content of cell using xylan as carbon source growth under anaerobic and aerobic; Cell growth rate using xylose as a carbon source growth under anaerobic, aerobic, and (B) Xylan residue of strain DA-C8 cultivation under anaerobic and aerobic growth conditions. The mean values for the triple experiments are presented. Error bars show ± standard deviation.

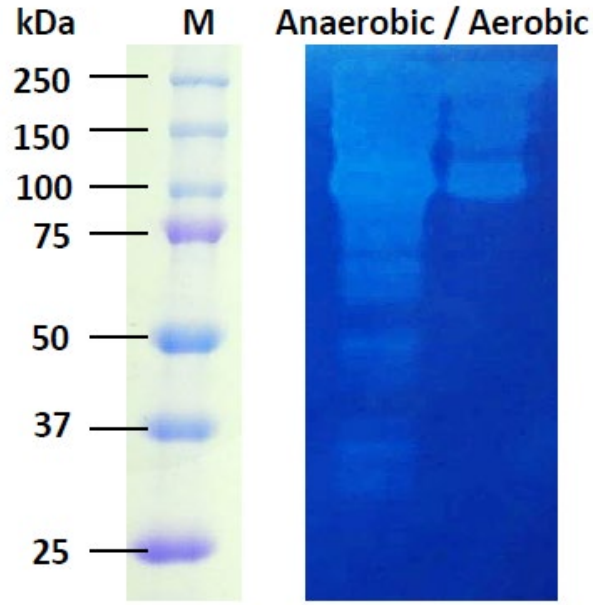


Figure 4.4. Extracellular enzymes activity of strain DA-C8 on zymogram containing 2% of beechwood xylan. SDS-PAGE and zymogram using extracellular enzymes (10 µg protein) from DA-C8 bacterium cultured in an enrichment medium containing 2% of beechwood xylan as a carbon source under anaerobic and aerobic condition. Lane M is a standard protein molecular mass marker.

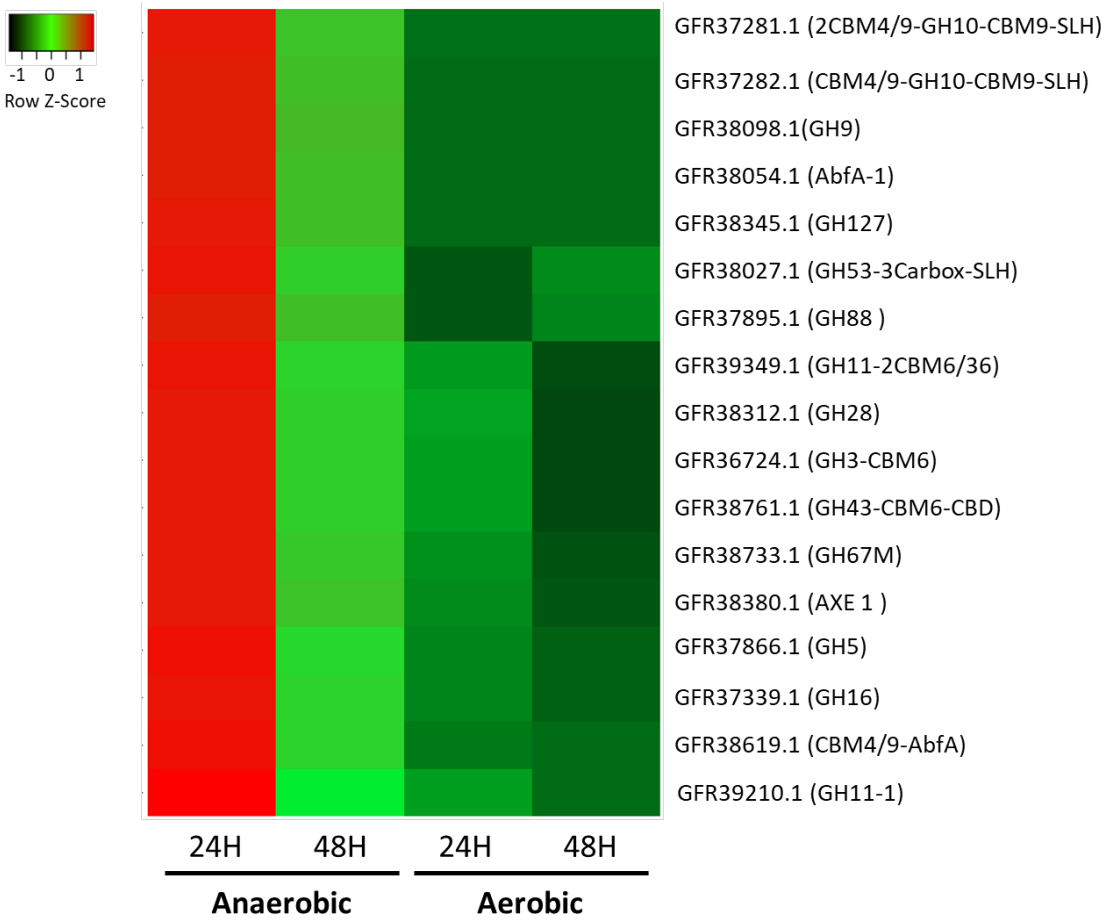


Figure 4.5. The expression levels of genes from strain DA-C8 grown under anaerobic and aerobic condition (Heat mapper)

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Chapter 5

General Conclusions

Plant cell walls are the significant repository of fixed carbon in nature. The major constituent of the plant cell wall is lignocellulose containing lignin, hemicellulose, and cellulose. Xylan is a major component of hemicelluloses in the plant cell wall and natural polymer included agricultural residues such as corn hull/straw, corn cop, rice straw that remains from other consumers and has been discarded in landfill improperly ways which caused environmental issues. The energy of the plant polymers can be conserved by using microbes in fermentation under anaerobic or aerobic conditions. Using enzymes from microorganisms to degrade those wastes is one of the environmentally friendly methods. They convert the polysaccharides of the plant cell wall to soluble sugars. Somehow, the hydrolysis of xylan depends on the source and ability of the isolated microbe to produce the enzyme production needed for completed degradation. So, this thesis aimed to isolate the bacterium with strong xylan degrading ability under anaerobic thermophilic conditions and study its properties and function.

A pure culture of strain DA-C8 was isolated from bovine compost having strong xylan degradation under anaerobic thermophilic conditions. The strain has gram-negative, straight or slightly curved rods. It could use mono- and disaccharide but could not use amide derivatives of glucose, N-acetyl glucosamine, and sugar alcohol such as adonitol, mannitol, and sorbitol. Base on 16S rRNA sequences analysis, strain DA-C8 was closely related to *P. cisolokensis* (93.7% identity; accession no. NR_151901) and *X. composti* K-13 (92.8% identity; NR_159899). Accordingly, ANI value of whole genomic sequences between strain DA-C8 and its closest relative strains were revealed that strain DA-C8 closely related to *P. cisolokenesis* (68.17%, accession number: BOVJ00000000.1), and following by *P. composti* K-13 (67.98%, accession no: BOVK00000000.1).

Strain DA-C8 should be presented as a new genus *Paenibacillus,* rather than genus *Xylanibacillus* because of sugar utilization properties. In terms of the basics of genome analysis, strain DA-C8 and *X.composti* K-13 were found that many predicted genes regarding β-1,4-xylanases (EC.3.2.1.8) and endo-β-1,4-glucanases (EC 3.2.1.89) belonging to GH family-5, 9, 10 and 11 were found in the genome sequence of both strains. Meanwhile, genes of β-glucosidases (EC.3.2.1.185) and β-xylosidases (EC.3.2.1.21) belonging to GH family-3 and 16 were abundantly confirmed in the genome sequence of stain DA-C8 than *X. composti*.

The high activities of β-glucosidase and β-xylosidase in extracellular fractions prepared from

strain may reflect the existence of these genes. In addition, genes corded the accessory enzymes such as α-L-arabinofuranosidase, β-galactosidase, α-glucosidase, and polygalacturonase were also confirmed to the genome of strain DA-C8. Additionally, the catalyzes the hydrolytic release of unsaturated glucuronic acids from oligosaccharides produced through the reactions of polysaccharide lyases such as gellan, xanthan, hyaluronate, and chondroitin lyases, only found in strain DA-C8. It is well known that multiple hydrolase activities such as not only β-1,4-xylanases and β-1,4-glucanases but also β-xylosidase, α-L-arabinofuranosidase, β-galactosidase, and α-glucosidase, are necessary to achieve complete hydrolysis of lignocellulosic biomass. Strain DA-C8 was revealed to equip various genes regarding lignocellulose degradation more than *X. composti* K-13. Strain DA-C8 is a unique regulation system depending on growth conditions might be the first report among facultative microbes belonging to *Paenibacillus* because of few reports on metabolic changes of anaerobic and aerobic cultures in genus *Paenibacillus*.

Acknowledgments

CHHE CHINDA acknowledges the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan to provide the scholarship to conduct the current research under the MEXT Special Scholarship Program on Trans-world Professional Human Resources Development Program on Food Security & Natural Resources Management (TPHRD) for Doctoral Course.

I would like to sincerely express my gratitude to Prof. Dr. Akihiko Kosugi (Graduate School of Life and Environmental Sciences, University of Tsukuba and Japan International Research Center for Agricultural Sciences; JIRCAS) for guiding and supporting me during my study and life in Japan.

In the meantime, I would like to thank sincerely the Biological Resources and Post-Harvest Division, Japan International Research Center for Agricultural Sciences (JIRCAS), where I conducted my experiments and provided necessary resources and facilities for my research.

I am grateful to all my lab mates for their busy time assisting me in lab work and giving advice in my research. Additionally, I appreciated your kindness in taking good care of me during my hard time.

I would like to express my sincere to the Faculty of Agro-industry, Royal University of Agriculture, Cambodia, which gave me an excellent opportunity to proceed with this doctoral degree.

Finally, I would like to thank my beloved family, who handles and settles all issues during my absence especially; their love and motivation inspired me to keep moving on and get through a hard time. Also, my beloved friends and juniors who spent their tightly time taking good care of me as well as their encouragement and motivation during my hard time