Degradation Properties of Oil Palm Trunk Fiber by Novel Thermophilic Anaerobic Xylan Degrading Bacteria *Caldicoprobacter* sp. CL-2

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WIDYASTI Erma

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WIDYASTI Erma

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Acronyms and Abbreviations

OPT	*Oil Palm Trunk*
CPO	*Crude Palm Oil*
f-OPT	*fibrillated-Oil Palm Trunk*
SEM	*Scanning Electron Microscopy*
TLC	*Thin-Layer Chromatography*
SDS-PAGE	*Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis*
DNA	*Deoxyribonucleic Acid*
USDA	*United States Department of Agriculture*
Topt	*Optimum temperature*
WDM	*Wet Disk Milling*
HPLC	*High-Performance Liquid Chromatography*
RID	*Refractive Index Detector*
BM	*Basal Medium*
BM7CO	*Basal Medium pH 7 with CO2 bubbling*
rRNA	*ribosomal Ribonucleic Acid*
PCR	*Polymerase Chain Reaction*
BLAST	*Basic Local Alignment Search Tool*
CMC	*Carboxymethyl Cellulose*
X2	*Xylobiose*
X3	*Xylotriose*
X4	*Xylotetraose*
X5	*Xylopentaose*
X6	*Xylohexaose*
GH	*Glycoside Hydrolase*
kDa	*kilo Dalton*
CBM	*Carbohydrate Binding Module*

Chapter 1
Introduction

1.1. Background

There has been enormous research and study for more than 40 years to find respectable sources for sustainable energy to replace the fossil fuel. Fossil fuel (consist of oil, coal, and natural gas) consumption has reach approximately 80% of the global energy demand. This fossil fuels possess heaps of difficulties such as the fluctuation of Crude Oil Prices in the world that influence the economic sector, and moreover bring threat to the environment as it has produced air pollution and the accumulation of its "Green House Gas" has led to the Global Warming (Hosseini *et al.,* 2015).

The use of renewable and sustainable energy resources has been an interest to reduce these problems. One of these sustainable resources are plant biomass. It is the most abundant natural material on earth. It represents as an enormous source for food and energy in nature. This plant biomass consist of Cellulose (35-50 %), Hemicellulose (20-35 %), and Lignin (10-25 %) which the component composition varied depend on the species (Saha, B.C., 2003). To utilize this source, a lot of research has been conducted. And to convert this source, biological saccharification has been studied, involving fungi and bacteria as the converter agent, and also the enzyme cocktails that has been the major economic barriers technology to apply for its high cost.

It has been for several decades since lignocellulosic biomass acknowledged as a potential sustainable source for biofuels (from fermentation of its mixed sugars) and also for other biomaterials product. This abundant biomass are produced from the agricultural waste, forestry wastes and thinnings, waste paper, and energy crops (Himmel *et al.,* 2007). One of this agricultural waste was from Oil Palm plantation. A large sum of lignocellulosic biomass residue, which one of them are felled oil palm trunks (OPTs), are created along with CPO (Kunasundari *et al.,* 2017). Indonesia and Malaysia, has been noticed dominated the global palm oil production. Both country take the first and second place as the World Oil Palm Producer. This has contributed on the byproduct of palm oil production, especially when it comes to replanting the Oil Palm plantation. The felled Oil

Palm Trunk (OPT) which will mount and may cause many problems, give the challenge to utilize it into useful products in an effective way.

1.2. Objectives and Outline

In this dissertation, we study about bacteria capable of OPT degradation and the mechanisms to degrade it. The aim of this study are:

1. To established the OPT fiber pretreatment in order to lowering the recalcitrant of the fibers.
2. To isolate thermophilic anaerobic microorganism capable of degrade Oil Palm Trunk Fiber and to characterized enzymes property of this isolate responsible for the OPT degradation
3. To study the mechanism of degradation by the isolated strain and its enzymes for efficiency of OPT degradation in the future.

This study has driven by the efforts to utilize felled unused Oil Palm Trunk that annually would have a great amount and would also cause environmental problems. The first chapter would explained about the background of the biomass degradation as an effort to seek for sustainable energy replacing the use of fossil fuels. Next described about the source, the content, and the potential of these biomass especially in the two world biggest Palm Oil producer country. It also mention about the previous studies has been conducted to utilize biomass, and the potential microorganisms capable to conduct biomass degradation and also a respectable source to isolate it. This chapter also reference about efforts and studies that has been conducted to utilize different component of OPT.

The second chapter will discussed about appropriate pretreatment for OPT to lowering the recalcitrance, and the isolation steps to isolate potential microorganisms capable to degrade it. It will mentioned the OPT degradation ability analysis. It also gave explanation of how to characterize the isolated microbes. The identification of isolated cultures also would take credits on this chapter, along with the phylogenetic study and how it would lead to the novel strain propose suggestion. The degradation mechanism study of OPT by the isolated strain conducted by comparison with the noticed thermophilic anaerobic cellulose-degrading bacteria by observing the morphology of degraded OPT and weighing the biomass residue.

The third chapter would described about the characterization of crude enzyme of CL-2 produced using varied substrate. It characterized the optimum pH and temperature, measure the thermostability and pH stability of the crude enzyme, the best substrate for measuring activity, and also the purification by ultrafiltration, salting out, and SDS-PAGE and Zymogram, to see which enzyme responsible for OPT degradation. And also to isolate the enzyme protein that has hydrolytic activity against substrate to characterize it further with the next step that will described on the next chapter.

The last chapter would described about the effort to identified, purified, and characterized one of the major enzymes produced by isolated strain by molecular technique, with cloning, transformation, expression, and purification of the recombinant enzymes. And comparing with the crude enzymes to degrade OPT, to understand the degradation mechanism conducted by this isolate. Last was to study the synergistic characteristic of the recombinant enzyme in order to discover the efficient mechanism of OPT degradation.

1.3. Literature Review

1.3.1. Indonesian Biomass Potential

Indonesia has lead amongst the South East Asian country for its biomass potential which consist mainly from agricultural biomass and added with woody biomass. According to Singh and Setiawan (2013), the biomass potential in Indonesia estimated reach about 50 GW. This contributed to 1/3 of the total energy consumption or about 27 % in 2010. The Biomass consist of 15.45 million cubic meters of forest residues per year, 64 million tons of plantation residues per year, and also agricultural residue, and municipal waste. Unfortunately, these biomass potential were remain underestimated and underutilized. Especially in the rural areas, these biomass, usually from woody biomass or agricultural residues, only used for cooking and water heating, and this practice were inefficient and environment unfriendly.

1.3.2. Oil Palm Trunk

Palm Oil has been used in many kind of products such as foods, cosmetics, medicine and soap. And the demand in the world for Palm Oil is increasing every year. Indonesia

and Malaysia has been known led the global palm oil production. This country take the first and second place as Oil Palm Producer with Indonesia for 28.5 million tons and Malaysia for 18.5 million tons of the worldwide production (88 %) in 2012 and 2013 (USDA statistics: PS&D online https://apps.fas.usda.gov/psdonline/psdHome.aspx). The history of Indonesia Palm Oil were started to give significant changes was begin with the government action on policy during 1980's. In order to rise per capita income, Indonesia has promote a great oil palm plantation expansion on 1980's, and deforestation areas was continue unceasingly happening until present. Regardless of being an opportunity, this expansion fetch more negative possessions especially for the environment and biodiversity.

Because of the lower price compare to soybean oil or others oil, Palm Oil has been used broadly for many kinds of industrial utilities, in food such as margarine and frying oil, for detergents and cosmetics, and even for bioplastics and biodiesel (Kosugi *et al.,* 2010).

The felled palm trunks exemplify one of the most important biomass resources in two highest palm oil producing countries in the world, Indonesia and Malaysia. Oil palm tree (*Elaeis guinensis*), which derive from West Africa, need to be replanted after 20-25 years because its palm oil productivity declines as the tree grows older (Kosugi *et al.,* 2010, Kunasundari *et al.,* 2017). The felled trunks of the oil palm has been an environment harms if it just leaved unused on the field. There are already several work to utilize these felled trunk such as for handicraft and plywood manufacturing. But the inner part of the trunk is not resilient enough to use as craft and plywood material, and has been cast-off on the field. Several effort has been conducted to find the useful potential of the inner part of the trunk. such as the core lumber utilization for wood material (Khalil *et al.,* 2012), the OPT Sap utilization for bioethanol production (Yamada *et al.,* 2010; Kosugi *et al.,* 2010), the OPT Sap utilization for lactic acid production (Kosugi *et al.,* 2010; Kunasundari *et al.,* 2017), the OPT Nanocrystals isolation (Lamaming *et al.,* 2017), and the Parenchyma and vascular bundle of OPT fibers utilization for ethanol production (Prawitwong *et al.,* 2012). Characterization of the sugars in the sap of the felled trunks has been conducted and has found large amounts of sap with a high glucose content (Kosugi *et al.,* 2010). The estimated cellulosic residue remaining after pressing sap out

was approximately 30% (w/w) of the trunk without the bark (Lokesh *et al.,* 2012), indicating that the trunks are promising as feedstocks.

1.3.3. Thermophilic Anaerobic Bacteria

Thermophilic bacteria has been studied to use as a good candidates for biomass conversion into energy and other useful products. They produce an active and forceful carbohydrate degrading enzymes. They also survived under strict bioprocessing conditions, so no pretreatment necessary to convert plant biomass into fermentable sugars (Blumer-Schuette *et al.,* 2013). Another group are thermophilic anaerobic bacteria. Several member of this group has been found to have special enzymes system that can breakdown lignocellulose immediately and expeditiously. This bring the potential candidate as the bio degrader high expected from this group. Many member of this group have special enzymes system that can breakdown lignocellulose immediately and expeditiously (Waeonukul *et al.,* 2012). Using their multi-enzyme complexes, cellulosome producing bacteria can saccharify lignocellulose during cultivation, which would greatly reduce operating costs (Lynd *et al.,* 2002; Tamaru *et al.,* 2010; Artzi *et al.,* 2014). There are also results in higher concentrations of fermentation products as compared to simultaneous saccharification with fungal cellulase and yeast (Yee *et al.,* 2012). From the degradation study amongst cellulolytic and xylanolytic degrading bacteria, it is assumed that Biological saccharification of lignocellulosic biomass using combination of thermophilic anaerobic cellulolytic and xylanolytic bacteria is one of useful and cost effective ways.

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Chapter 2

Oil Palm Trunk Pretreatment, Screening, Isolation and Identification of Oil Palm Trunk Degrading Thermophilic Anaerobic Bacteria

2.1. Introduction

Biological saccharification of lignocellulosic biomass in nature is generally considered to be performed by a variety of microorganisms or microbial communities, including fungi and bacteria. Microbial deconstruction of lignocellulosic biomass for second-generation biofuels production (Himmel *et al.,* 2007) has gain interest in high-temperature ([Topt], 70 °C), carbohydrate-utilizing microorganisms from terrestrial niches. Numerous sources has been investigated to screening potential microorganism capable of degrading biomass. Some of these sources are from compost (Rainey *et al.,* 1994; Sizova *et al.,* 2011), animal excrement (Yokoyama *et al.,* 2010), terrestrial hot spring (Larsen *et al.,* 1997; Hammilton-Brehm *et al.,* 2009; Bouanane-Darenfed *et al.,* 2011 & 2013). From all these studies, it was propose that compost is a respectable source for screening potential microorganism for biodegradation.

Another remarks for the efficient biodegradation is to deal with the recalcitrance structure of OPT fiber. OPT fibers offer challenge to convert due to its recalcitrant. This was a blockade for degrader Microorganisms to do the saccharification. The degree of crystallinity also affects the efficiency of enzymatic hydrolysis toward lignocellulosic biomass (Li *et al.,* 2016). Consequently, OPT pretreatment is an important step due to the typically breaks down of macroscopic firmness of biomass and decreases the physical blockades to mass transport (Himmel *et al.,* 2007).

Biomass, can be made into liquid biofuels via sugars fermentation of its cellulose and hemicellulose, but it has to receive pretreatment to lower the recalcitrant of its structure in order for microorganisms and its enzymes able to degrade it efficiently, so the sugars needed for fermentation can be freed (Agbor *et al.,* 2011). This pretreatment of biomass using thermochemical has long been acknowledged as a serious technology to produce materials with tolerable enzymatic digestibility (Himmel *et al.,* 2007).

2.2. Material and Methods

2.2.1. OPT Pretreatment

OPT sample were collected from felled old Palm Oil Tree from local Palm Oil Plantation in Malaysia. Felled old Oil Palm Trunks estimated more than 20 years aged were selected and the 7 cm thick plate were cut from the middle part of the trunk. The plate then peeled to remove the bark, and cutted into 5-10 cm size cubes. This OPT cubes then compressed to separate the sap from the fibers using a -laboratory scale press at 80 MPa (Kosugi *et al.*, 2010). The squeezed OPT then split into smaller size by grinding it manually, resulting OPT fiber ready to put into Super Mass Colloider.

The OPT fiber, 40 % solids were soaked into deionized water for 24 hour before processed in WDM. This WDM were conducted using SuperMassColloider model: MKZA6-2, disk model: MKGA6-80, with 150 mm diameter (Masuko Sangyo Co.Ltd. Japan) which equipped with two nonporous ceramic grinding which separated length arranged for 80 μm with the speed set at 1500 rpm. The milling performed for 1-2 cycle. The fibrillated OPT then filtered using non-woven fabric and washed with surfeit water. The wet f-OPT resulted used directly into the subculture medium for screening experiment. The whole process pictured in Fig. 2.1.

2.2.2. Fibrillated OPT (f-OPT) Analysis

Water content of f-OPT was analyzed by drying it at 105 °C for 48 hour. The starch content of dried f-OPT determined using Total Starch Assay Kit at standard condition following Manufacturer instruction (megazyme). Chemical composition of oven-dried f-OPT was analyzed base on Chemical Analysis Standard Procedure of National Renewable Energy Laboratory (US Department of Energy). Mono- and oligosaccharide content were determined using high performance liquid chromatography (Shimadzu Corp., Kyoto, Japan), with a Refractive Index Detector (Shimadzu RID-10A) inside Bio-Rad Aminex (HPX-87P) column which were operated at 80 °C using MiliQ-filtered water (Milipore). The Lignin acid-insoluble content measured as the weight of dried-oven filter cake (dried at at 70 °C until constant). The data were means from three experiment ± standard deviations.

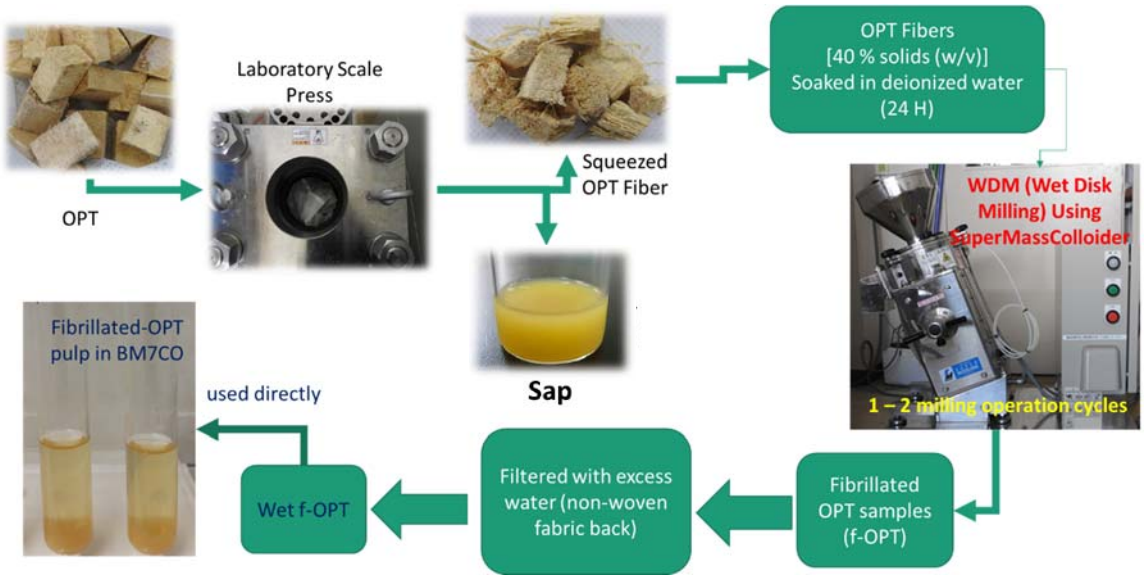


Figure 2.1. OPT pre-treatment (fibrillated OPT preparation)

The morphological characteristic of OPT and f-OPT investigated using Scanning Electron Microscopy (SEM). The sample prepared by coating with plasma osmium (OPC80T, Filgen, Nagoya, Japan).

2.2.3. Compost and Medium

The source for isolation of Bacteria potential as an OPT degrader was from compost sample from Ishigaki island compost facility (Ishigaki Taihi center, Ishigaki City, Japan). The sampling conducted in May 2015 at 20 cm depth and temperatures ranging from 60 to 75°C through sampling sites. The compost material contained two approximately equal parts: bovine excrement, and sugarcane leaves and stems. CO_2 gas flushing were conducted during primary sampling and subculturing to maintained strictly anaerobic conditions. Compost samples were put and saved in a Ziploc bag (Johnson Co., Ltd. Yokohama, Japan), designated ISI -1 to -20. Basal medium (BM) was prepared as described in Watthanalamloet *et al.* (2012) modified with following composition (l-1): 2.9 g K_2HPO_4, 1.5 g KH_2PO_4, 2.1 g urea, 3 g yeast extract, 0.01 g $CaCL_2{\cdot}2H_2O$, 4 g Na_2CO_3, 0.5 g L-Cystein, 0.001 g Resazurin and 200 µl mineral solution. This mineral solution (100 ml) are consisted of 25 g $MgCl_2{\cdot}6H_2O$, 3.75 g $CaCL_2.2H_2O$ and 0.0312 g $FeSO_4{\cdot}6H_2O$. The medium was conditioned anaerobically by degassing in boiling water and bubbled with high purity carbon dioxide gas.

2.2.4. Culturing and Isolation

Hungate tubes containing 15 ml of the BM7CO (Basal Medium adjusted to pH 7 and bubbling with CO_2 gas), pH 7.0-8.0, and 1 g of wet f-OPT pulp [moisture content 80 % ($^{w}/_{w}$)] was inoculated with around 2 g of compost sample. Each tube was immediately flushed with CO_2 and incubated at 70 °C on static culture. After 1 week of incubation, aliquot of the cultures were transferred to fresh medium containing f-OPT. Samples that showed effective degradation of the OPT fiber were selected and purified by continuously subcultured with gradually decreased preculture volume in BM7CO-f-OPT medium (2 x 500 µl, 2 x 100 µl, and 2 x 50 µl inoculated).

From serial dilution rows of anaerobic agar roll tubes technique (Hungate, 1969), the sample then isolated. The potential consortia were serially diluted into cellobiose-

agar medium that had been melted and cooled to 55°C and then shaped by roll tubes technique under strictly anaerobic conditions. After solidified, roll tubes were incubated at 65°C. After incubation for 4 to 5 days, the f-OPT-utilizing bacteria formed colonies. Single colonies formed then were picked with a sterile needle and inoculated into BM7CO-f-OPT medium. Five best isolate were selected base on the growth speed, turbidity, and gas formation on BM7CO-OPTF, and the efficient degradation of the OPT fiber was tested by measuring the dry weight of the OPT fiber (in percentage compare with control medium without inoculation) after 5 days incubation in 70 °C.

2.2.5. Biomass Degradation

To check the degradation of f-OPT, strain CL-2 was compared with that of *Clo. thermocellum* PAL 5. *Clo. thermocellum* PAL 5 were used as a comparation because it was an effective cellulose-fermenting thermophile. CL-2 strain and *Clo. thermocellum* PAL5 were grown on BM7CO medium contain 1% (w/v dry base) f-OPT at their optimum growth temperature, 70 °C for CL-2 and 60 °C for *Clo. thermocellum* PAL5, for 4 days.

2.2.6. DNA Extraction, PCR Amplification, and Phylogenetic Analysis

DNA extraction from 3-4 days culture cell was done using a NucleoBond® AXG column with NucleoBond® buffer set III (TaKaRa BIO), according to supplier's protocol. Afterwards was the amplification of 16S rRNA gene occupy the bacterial domain-specific primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1525R (5′-AAAGGAGGTGATCCAGCC-3′). The PCR amplification was conducted with commencing denaturation at 94 °C for 1 min, continued with 30 cycles of denaturation (94 °C, 30 seconds), annealing (55 °C, 30 seconds), and extension (72 °C, 1 minute). The 10 minutes final extension at 72 °C was applied. The PCR products then purified using a QIAquick PCR purification kit (Qiagen, Frederick, MD). The 16S rRNA gene sequences (GenBank accession number MF445145) were examined by the BLAST program and manually aligned compared to sequences in the GenBank database using CLUSTAL_X v1.81 (Thompson *et al.,* 1997). After that, the Phylogenetic tree was created by the neighbor-joining method using the programs BioEdit version 7.1.9 and

MEGA version 6.06 (Hall, T.A., 1999). And the Tree topologies and distances were estimated by bootstrap analysis build upon 1000 re-samplings (Tamura *et al.,* 2007).

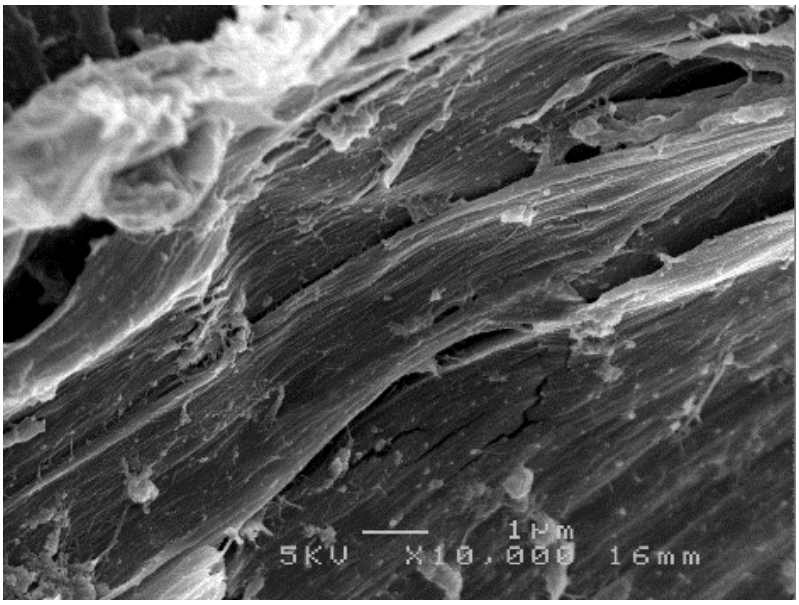
2.3. Results and Discussion

2.3.1. Fibrillated OPT (f-OPT)

Pretreatment technology has been proven to magnify the hydrolysis of carbohydrates into valuable sugars by enzymes and microorganisms by defeated the recalcitrance of its lignocellulosic structure. Without using chemicals, the particle size and crystallinity of lignocellulosic material can be decrease by mechanical pretreatment in order to rise the specific surface area and lessen the degree of polymerization. SEM images before (Fig. 2.2-A) and after fibrillation (Fig. 2.2-B) were captured from the systematic physical breakdown of the cell wall of squeezed OPT fiber by WDM. And it is clearly showed that the OPT fiber was mechanically fibrillated by the SuperMassColloider. The original OPT fiber contained of parenchyma and vascular bundle components which amounts almost equal, with an average width of 50–90 μm (Prawitwong *et al.,* 2012). Major of the fibers were highly fibrillated, to micro or nanofibrils, after treatment with the SuperMassColloider. This step called Wet Disk Milling has been used as an environmentally friendly pretreatment that appropriate for enzymatic saccharification. This WDM using SuperMass Colloider would increase the sugars yields. And with the energy consumption lower than other pretreatment methods, without the addition of chemicals such as strong acid or basic, this method is a good low cost pretreatment for enzymatic biological saccharification (Hideno *et al.,* 2009).

In order to authorize alterations in chemical composition caused by fibrillation, the glucan, hemicellulose, and Klason lignin contents were evaluated in fibrillated OPT (f-OPT) as shown in Table 2.1. The Klason lignin content of f-OPT (30.8% ± 2.2%) was slightly higher than that of untreated OPT (28.8% ± 2.2%) (w/w). These results point out that fibrillation of OPT by WDM may solubilize some parts of the cellulose From the enrichment, continuous subculture for 5-6 consecutive transfers, and isolation by Hungate roll tube of the compost sample, 5 positive culture was chosen base on the growth rate and the degradation ability toward f-OPT.

A



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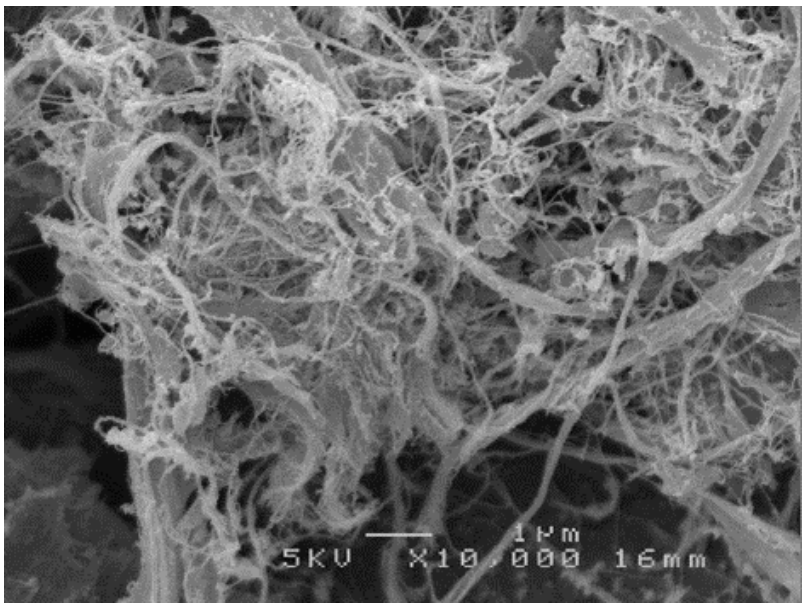


Figure 2.2. SEM images of (A) OPT before fibrillation and (B) micro-fibrillated OPT (f-OPT). The bar length are 1 μm.

Table 2.1. Chemical Composition of OPT before and after fibrillation

Carbohydrates	**OPT before fibrillation***	**OPT after fibrillation (f-OPT)***
Glucan	36.2 ± 0.8	24.2 ± 0.8
Xylan	25.7 ± 0.7	23.8 ± 3.0
Arabinan	4.8 ± 0.1	5.1 ± 0.1
Galactan	2.0 ± 0.1	2.7 ± 0.1
Mannan	1.9 ± 0.1	2.0 ± 0.1
Klason Lignin	28.8 ± 2.2	30.8± 2.2

* Percentage (%) on a dry weight basis (w

For further, these 5 best culture were identified by 16S rDNA (Table 2.2) to check the culture purity. Only 2 of it shown purity as *Thermoanaerobacter* sp. and *Acetomicrobium faecale* (now *Caldicoprobacter faecalis*). The pure cultures then characterized further for its properties and potential and hemicellulose, and it slightly increased the lignin ratio in f-OPT. WDM also extended the physiologically-available surface area of the material.

Table 2.1 indicated that fibrillation of OPT fiber by WDM has effects that may dissolved some part of cellulose and hemicellulose, and slightly increased lignin ratio of f-OPT. On the other hand, OPT fiber could achieve to extend physiologically the surface area by the fibrillated process using WDM as shown on the SEM pictures on Fig. 2.2.

2.3.2. Best Culture for OPT Degradation

2.3.3. Ethanol and Organic Acid Production

Lactate and ethanol were the main metabolic products of CL-2, alongside with lower amounts of acetic acid as seen in Fig. 2.3; furthermore, butyrate and butanol were not observed.

2.3.4. Substrate Utilization

The two identified cultures, named CL-1 and CL-2, then grown on different artificial and Biomass substrates to check their ability to utilize those carbon sources. The data were collected on Table 2.3. CL-2 utilized a varied range of carbon sources such as sucrose, glucose, xylose, fructose, cellobiose, maltose, xylan, arabinoxylan, starch, arabinogalactan, pectin, guar gum, but not cellulosic polysaccharides such as microcrystalline cellulose, filter paper, and carboxymethyl cellulose (CMC). This strain characterization was conducted to select which identified strain has more positive properties to use as OPT degrader. And CL-2 was chosen for characterized further. And PAL 5, respectively. The f-OPT fiber surface from each bacterial growth in overall were smoother compare to the f-OPT without bacterial growth (Fig. 2.4). This may cause by the spreading of the extracellular enzyme on f-OPT surface. Cl-2 can use xylan, arabinoxylan and arabinogalactan, but cannot use cellulosic materials, whilst *Clo. thermocellum* degradation using its high cellulose degrading activity (Lynd *et al.,* 2002) that suggest the degradation of xylanolytic polymers hold an important role in the efficiency degradation of OPT fibers.

Table 2.2. Identification result of 5 best selected cultures.

No	Isolate Code	Identification
1	OPT-1	Mixed culture
2	OPT-2	Mixed culture
3	OPT-3	Mixed culture
4	CL-1	*Thermoanaerobacter* sp. (99 %)
5	CL-2	*Caldicoprobacter faecalis* strain DSM ATCC 20678^{T}(98.6 %)

Mixed culture: more than one species of bacteria were identified.

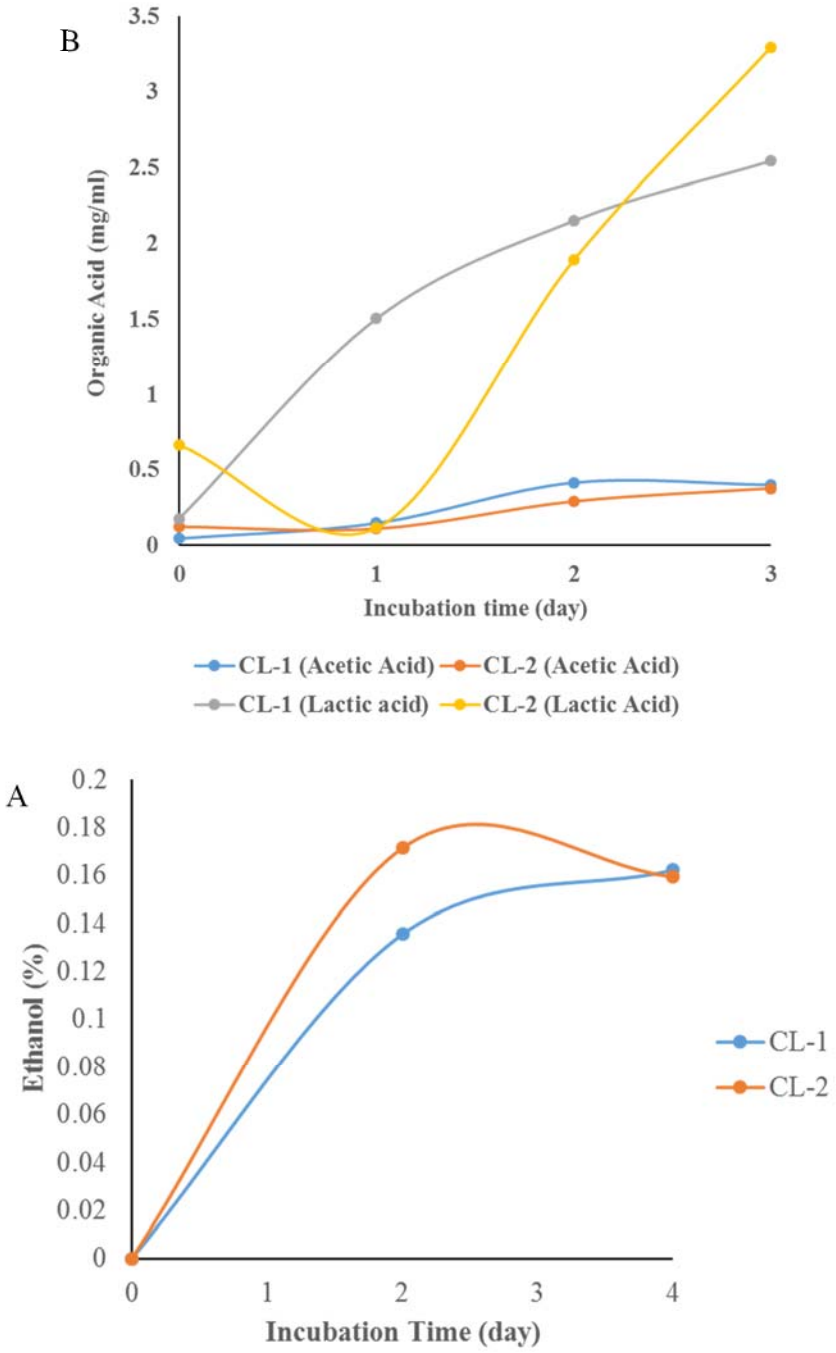


Figure 2.3. Ethanol production (A) and Organic acid (B) production of CL-1 and CL-2 cultures

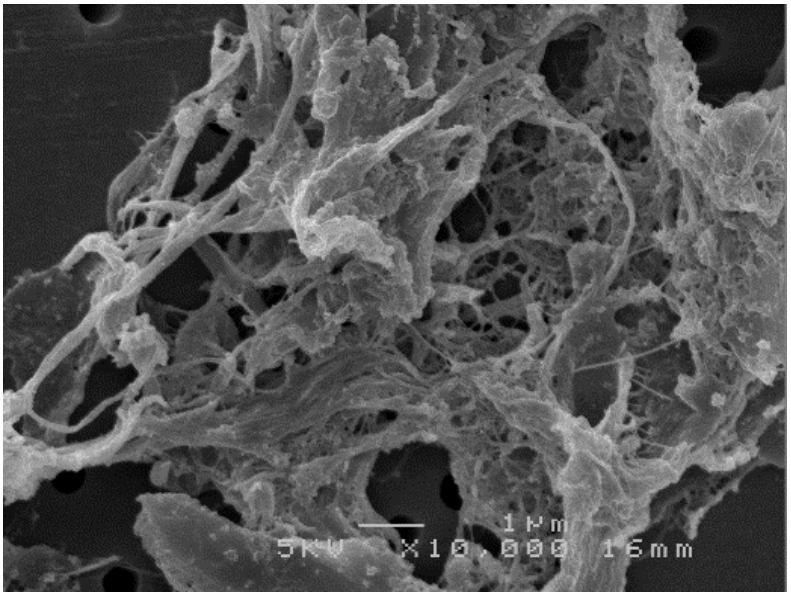
Table 2.3. Artificial and biomass substrates utilization of 2 identified isolates.

No.	Substrate	CL-1	CL-2
1	Cellobiose	+	+
2	Avicell	+	-
3	Filter Paper	-	-
4	Lactose	+	+
5	D-Galactose	+	+
6	Sucrose	+	+
7	D-Glucose	+	+
8	Trehalose	+	+
9	L-Arabinose	+	+
10	D-Rhamnose	+	+
11	D-Mannose	+	+
12	Maltose	+	+
13	D-Xylose	+	+
14	D-Fructose	+	+
15	Raffinose	+	+
16	Corn Hull	+	+
17	Rice Straw	+	+
18	Erianthus	+	+
19	Chitosan	-	-
20	Guar Gum	+	+
21	Corn Starch	+	+
22	Soluble Starch (Wako)	+	+
23	Soluble Starch - Sigma	+	+
24	Oatspelt Xylan	+	+
25	Beechwood Xylan	+	+
26	Arabinogalactan from Larchwood	+	+
27	Sorbitol	+	+
28	Mannitol	+	+
29	Glycerol	-	-
30	Chitin	-	-
31	Pectin from Apple	+	+
32	Gelatin	-	-
33	Salicin	+	-

+ : growth were detected on the inoculated medium contained substrates by turbidity or color changing

-: no changing on the inoculated medium contained substrates (either turbidity or color).

A



B

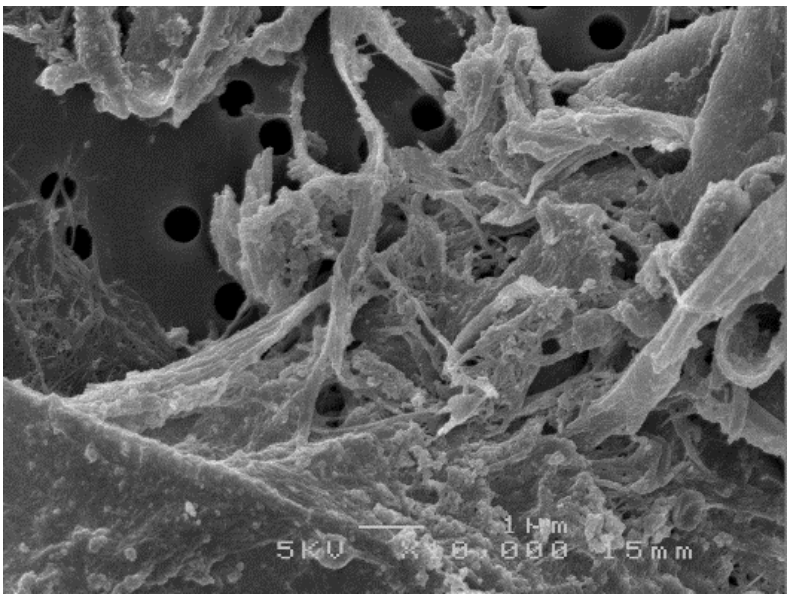


Figure 2.4. SEM images of biodegraded f-OPTs by *C. thermocellum* PAL5 (A) and *Caldicoprobacter* strain CL-2 (B)

2.3.5. Biomass Degradation by CL-2 Extracellular Enzyme

CL-2 f-OPT degradation were compare with the degradation by *Clostridium* (*Clo*) *thermocellum* PAL5. Both strain revealed the darting growth in f-OPT medium. After fermentation, the f-OPT residue were dried for overnight, and the dried weight used to subtracts the initial weight, resulting weight decreasing of 32.7 % (w/v) for CL-2 and 39.0 % (w/v) compared with the total initial dried weight of f-OPT after inoculated by CL-2

2.3.6. Isolation and identification of Oil Palm Trunk Degrading Thermophilic Anaerobic Bacteria

A single culture that has been isolated, exhibit a noteworthy degradation toward f-OPT. Study of the 16S rDNA gene sequence (GenBank accession number MF445145) found that it shared 98.6% sequence identity with the 16S rDNA gene of *Acetomicrobium faecale* strain DSM 20678^T, now reclassified as *Caldicoprobacter faecalis* DSM 20678^T (Winter *et al.,* 1987; and Bouanane-Darenfed *et al.,* 2015). A phylogenetic tree were build using the 16S rRNA gene sequences and the neighbor-joining method appointed that CL-2 should be appropriate to the family *Caldicoprobacteraceae*. This species found has formed a slightly separate clade from its closest relatives (Winter *et al.,* 1987; Yokoyama *et al.,* 2010, and Bouanane-Darenfed *et al.,* 2011, 2013, 2015) (Fig. 2.5). The experiment on physiology, morphology, and biochemistry revealed that CL-2 has oval shape with size: 0.3–0.4×1.7–2.8 μm, and it was a Gram-positive bacterium (Table 2.4; Fig. 2.6). CL-2 can utilize a broad range carbon source such as glucose, fructose, xylose, sucrose, maltose, cellobiose, soluble starch, xylan, arabinoxylan, pectin, guar gum and arabinogalactan, but cannot use cellulosic polysaccharides such as microcrystalline cellulose, filter paper and CMC. The main metabolic product of CL-2 are ethanol and lactate with a low amount of organic acid, while butanol and butyrate were not observed (Fig. 2.3). The closest relatives microbes, *Caldicoprobacter faecalis* DSM 20678T [isolated from sewage sludge (Winter *et al.,* 1987)] and *Caldicoprobacter algeriensis* TH7C1 (DSM 22661T) [isolated from a hydrothermal hot spring (Bouanane-Darenfed *et al.,* 2011)]. Although they can use varied hexose and pentose, they cannot utilize polysaccharides such as starch, cellulose and xylan as energy sources (Winter *et al.,* 1987;

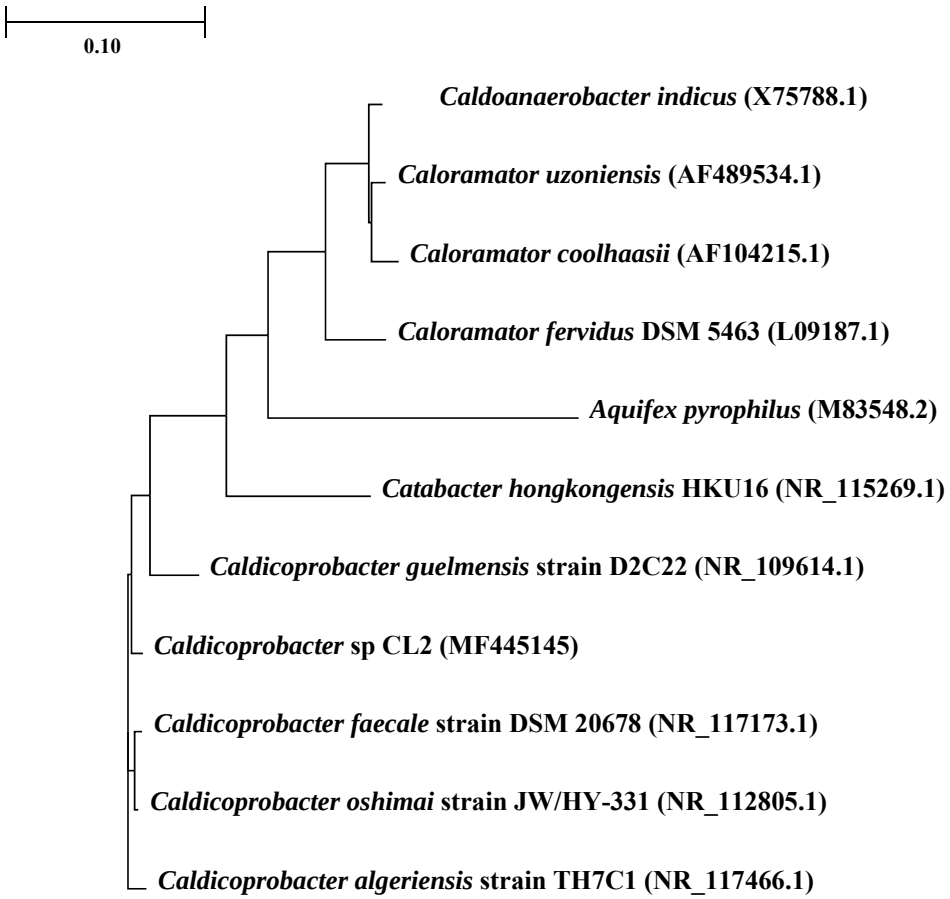


Fig. 2.5. Neighbor-joining phylogenetic tree of strain CL-2 created using 16S rRNA gene sequences. The tree shows the relationships between strain CL-2 and representatives of the family Caldicoprobacteraceae. Numbers at nodes represent bootstrap percentages (above 50%) from 1000 replicates. Bar, 0.1 substitutions per nucleotide position. GenBank accession numbers are shown in parentheses.

Table 2.4. Phenotypic characteristics differences between strain CL-2 and it's close phylogenetic neighbors

Characterization	**1**	**2**	**3**	**4**	**5**
Isolation source	Compost	Sludge samples	Hot spring	Hot spring	Sheep faces
Cell morphology	Straight rods	Rod	Rod	Rods	Spore-forming rod
Cell Length/Width (μm)	1.7–2.8/0.3–0.4	3.0–7.0/0.6	0.7–1.0/2.0–6.0	8.0–9.0/0.3–0.4	4.0–14.0/0.4–0.5
Gram stain	+	–	+	+	+
Motility	–	+	–	–	–
Temperature range/ optimum (°C)	37–60/55	ND/70–73	55–75/65	45–85/65	44–77/70
pH range/optimum	5.0–9.0/6.8	5.5–9.0/6.5	6.2–8.3/6.9	5.0–9.0/6.8	5.9–8.6/7.2
Polysaccharide utilization					
Xylan	+	–	+	+	+
Starch	+	ND	–	ND	–
Cellulose	–	–	–	–	–
CMC	+	ND	–	–	–
Growth substrates					
Xylose	+	ND	+	+	+
Arabinose	+	+	+	+	ND
Cellobise	+	ND	+	+	+
Fermentation products	A, E, L, CO_2, H_2	A, E, L, CO_2, H_2	A, E, L, CO_2, H_2	A, E, L, CO_2, H_2	A, E, L, CO_2, H_2
G+C (mol%)	ND	45.0	44.7	41.6	45.4
Reference	This study	Winter *et al.* (1967)	Bouanane-Darenfed *et al.* (2005)	Bouanane-Darenfed *et al.* (2013)	Yokoyama *et al.* (2010)

Strains: 1, *Caldicoprobacter* sp.CL-2; 2, *Caldicoprobacter faecale* DSM 20678^T; 3, *Caldicoprobacter algeriensis* TH7C1 (DSM 22661^T); 4, *Caldicoprobacter guelmensis* sp. nov.; 5, *Caldicoprobacter oshimai* JW/HY-331 (DSM 21659^T). CMC, carboxymethyl cellulose; End products, A, acetate; E, ethanol; L, lactate. +, Positive; –, negative; ND, no data available.

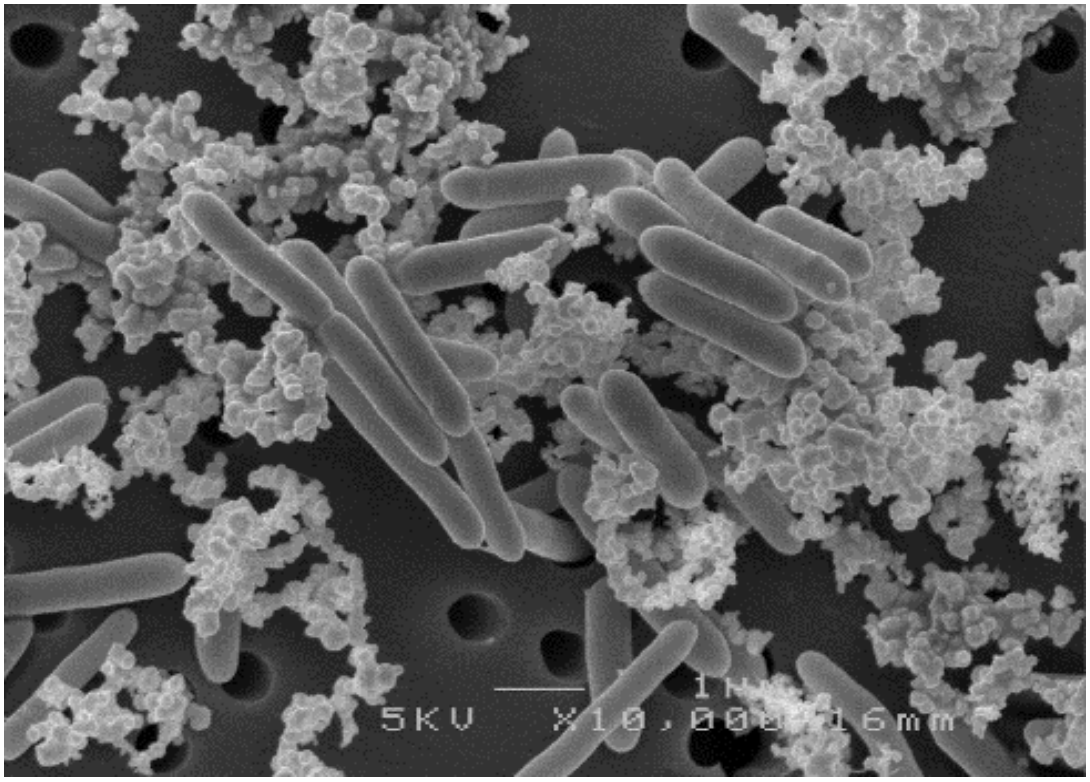


Fig. 2.6. SEM image of Strain CL-2 cell grown at 70 ºC on basal medium containing 0.5 % of beechwood xylan as carbon source. Bar indicate scales length are 1 μm.

and Bouanane-Darenfed et al., 2011). Caldicoprobacter oshimai (isolated from sheep faeces) and Caldicoprobacter guelmensis (isolated from hydrothermal hot spring) notwithstanding able to utilize xylose, glucose, and xylan as carbon and energy sources, but they cannot use dextran, soluble potato starch, CMC, or cellulose powder (Yokoyama et al., 2010, and Bouanane-Darenfed et al., 2013) (Table 2.4). Build upon previous physiological, morphological and biochemical analyses, we propose that CL-2 should be classified as a novel species of the genus Caldicoprobacter.

2.4. Conclusions.

In order to conduct anaerobic biodegradation in an effective way, with only simple pretreatment, thermophilic anaerobic microorganism were isolated from cow manure and sugarcane agricultural waste compost, using OPT that has been fibrillated which were prepared by Wet Disk Milling (WDM). Majority of OPT become micro fibrils after WDM treatment using SuperMassColloider. The Klason Lignin content of f-OPT has slightly increased compare to untreated OPT, indicated that some of cellulose and hemicellulose might be dissolved on WDM process. An anaerobic, thermophilic bacterium, *Caldicoprobacter* sp. CL-2, which 16S rDNA gene has 98.6 % sequence identity with that of *Caldicoprobacter faecalis* DSM 20678, display high degradation activity toward f-OPT -32.7 % decreasing from total dried solid at 70 °C. CL-2 strain able to utilize several polysaccharide such as xylan, arabinoxylan, starch, pectin and CMC as carbon sources , but not cellulose fiber. Base on the characteristic of polysaccharide utilization, morphology, and phylogenetic analysis based on 16S rRNA sequences, strain CL-2 might be a new species from Caldicoprobacter.

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Chapter 3

Characterization of Extracellular Enzymes and The OPT Degradation Mechanism

3.1. Introduction

Because no microorganism thus far able to convert biomass directly into biofuels (Blumer-Schuette *et al.,* 2014), degradation of biomass has generate efforts to developing various technologies. The production of biofuels has four successive phases of technologies such as the pre-treatment of biomass material, the formulation of enzyme cocktails (hydrolysis), fermentation by microorganisms consortia, and product separation (Barcelos *et al.,* 2015). These technologies were still scrutinized in order to create a cost-effective way to convert this lignocellulosic biomass into renewable source of energy and other useful product. One of this technology involve enzyme that produce mostly from microorganisms.

Lignocellulosic biomass mainly consist of cellulose, hemicellulose, and lignin with minor quantity of other components such as acetyl groups, minerals and phenolic substituents (Iskigor and Becer, 2015). Microorganism that can produce enzymes capable to degrade all of this component were in pursuit. Thermostable enzymes that has been isolated from different sources, especially from thermophilic anaerobic bacteria, are a potential genetic reservoir of biomass degrader agent.

This thermophilic plant biomass degrader were divided into two groups, cellulosomal (multienzyme complexes) and noncellulosomal (free enzyme systems). Three major enzymes that mostly involved in Biological Saccharification are cellulase, hemicellulose, and accessories enzyme. Accessories enzymes which comprise hemicellulose debranching, phenolic acid esterase, and possibly lignin degrading and modifying enzymes (Himmel *et al.,* 2007). These enzymes degrade biomass that resulting fermentable oligosaccharides, allowed other microbes to convert into final product as biofuels and other valued material.

In order to learn the degradation mechanism of OPT by CL-2, crude enzyme of CL-2 were produced, concentrated and characterized for its properties. What kind of enzymes produced by the selected culture, how the properties are, and which substrate that gives optimum activity, optimum pH and temperature of the enzyme and the hydrolysis product

was also investigated in order to design an effective biomass degradation using this isolate or its enzymes.

3.2. Material and Methods

3.2.1. Enzyme Assay

The amount of reducing sugar released from birchwood xylan, oat-spelt xylan and arabinoxylan (Sigma-Aldrich) were measured for the determination of xylanase activity using Nelson-Somogyi method. About 100 µl (50-100 µg) of enzyme were added into 900 µl of substrate solution consist of 0.5% (w/v) xylan substrate in 0.1M sodium acetate buffer at pH 6.0. The mixture then incubated for 10 minute at optimum temperature, the mixture was reaction were stopped by adding Somogyi reagent and boiling in water. The boiled mixture then cooled down, and added with Nelson reagent. Lastly the mixture were centrifuged to remove the solid part, and the supernatant were measured by Spectrophotometer using xylose as standard (Sermsathanaswadi *et al.,* 2014).

The definition of Xylanase activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar in 1 min at the previous conditions. Carboxymethylcellulose (CMC), barley glucan, carob galactomannan, arabinan and polygalacturonic acid (all from Sigma-Aldrich) at a 0.5% (w/v) final concentration were used to measure the activity of endoglucanase, mannanase and pectinase. Activities were measured based on the amount of reducing sugar liberated from the substrates. And for β-Glucosidase, β-xylosidase and α-L-arabinofuranosidase activities, were determined by measured the concentration of p-nitrophenol release from p-nitrophenyl β-D-glucoside, p-nitrophenyl β-D-xyloside and p-nitrophenyl β-D-arabinopyranoside (all from Sigma-Aldrich), respectively.

3.2.2. pH Optimization

For the determination of optimum pH, beech wood xylan was used as buffer at pH range 4.0 to 10.0. Sodium acetate buffer were used for acidic range pH (4.0 – 6.0), phosphate buffer used for neutral range (6.0 – 8.0), and for basic range (8.0 – 10.0) Tris-HCL buffer were used at 0.1 M concentration. And for pH stability, the prepared enzyme were pre-incubated without substrate in different pH buffers for 1 hour at 70 °C. Xylanolytic activity then assayed under the determined optimum pH condition, at pH 6.0.

3.2.3. Temperature Optimization and Thermal Stability

The temperature optimization was conducted by incubated the prepared enzyme at temperature range 40 – 80 °C with 10 degree interval at optimum pH condition. And the thermostability observed by pre-incubating the prepared enzyme without any substrate at 40 – 100 °C for 30 minutes. Residual enzyme activity on each temperature then assayed under standard assay conditions.

3.2.4. Degradation on Biomass Substrate

To determine the degradation ability of CL-2 extracellular enzymes, Beechwood xylan, OPT, and Cassava Pulp were prepared as a substrate in pH 6 acetate buffer solution, and the enzymes were added. The mixture then shaked and incubated at 80 ºC and the reducing sugar resulted from the enzymes activity were measured every 30 minutes.

3.2.5. Protein Assays, SDS-PAGE and Zymogram

The protein concentration of the prepared enzyme were determined by Coomassie (Bradford) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) using BSA (bovine serum albumin) as the standard. A 5-20 % gradient polyacrylamide gels (ATTO, Tokyo, Japan) of SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used to analyze the homogeneity of purified enzyme. The gels then was stained using Coomassie Brilliant blue R-250 (Bio-Rad Laboratories). The molecular mass standards were from Bio-Rad Laboratoris. Beechwood xylan was added into acrylamide gels to perform zymogram. The gel were incubate in acetate buffer at 80 °C to expose which enzyme responsible for the xylan degradation.

3.2.6. TLC Analysis of Hydrolysis Product

For the determination of hydrolysis product, the prepared crude enzyme was incubated in sodium acetate buffer (pH 6.0) containing 0.5% (w/v) of substrates as: xylooligosaccharides (X2-X6; xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose) (Megazyme International, Wicklow, Ireland) and beechwood xylan, separately, for overnight at 70ºC. The reactions then stopped by boiling the mixture, and then centrifugated at 12,100 × g for 10 min to separate and removed the solid. Boiled

crude enzyme was used as a negative control. TLC on silica gel 60 F254 plates (20 × 20 cm; catalog No. 105554, Merck KGaA) with a mixture of n-butanol/acetic acid/water (2:1:1) as the mobile phase were used to determine the hydrolysis products. Spraying the sample plate with a mixture of 4 g of α-diphenylamine, 4 ml of aniline, 200 ml of acetone, and 30 ml of 80% phosphoric acid were conducted in order to display the sugar spots by heating the plates to 100°C after.

3.3. Results and Discussion

3.3.1. Characterization of extracellular enzymes from *Caldicoprobacter* sp. CL-2

In order to apprehend the glycoside hydrolase (GH) properties of *Caldicoprobacter* sp CL-2, extracellular enzymes were prepared from 5 days culture at 70 ºC in 300 ml of BM7CO medium supplemented with 1% (w/v) beechwood xylan and f-OPT as the sole carbon source, respectively. The bacterial culture were centrifuged at 8500 g, for 10 min, at 4 °C to separate between the supernatant and the solids part [the substrate (beechwood xylan or f-OPT) and *Caldicoprobacter* sp. CL-2 cells]. The supernatant then were added by Ammonium sulfate (Wako Pure Chemical) gradually to the cell free supernatant (maintained at 4 °C) to obtain 80% saturation, and stirred for approximately 15 h. After centrifugation (30 min at 15,000 g, 4 °C), the pellet was re suspended in 50 mM sodium phosphate buffer, pH 7.0, and loaded onto an Econo-Pac 10DG column (Bio-Rad) equilibrated with 50mM phosphate buffer, pH 7.0 for desalting. The total proteins were eluted from the column with distilled water and concentrated using Amicon Ultra centrifugal filters (Merck Millipore Corp., Darmstadt, Germany). The proteins were used as the extracellular enzymes of *Caldicoprobacter* sp. CL-2.

Activities toward β-glucosidase and β-xylosidase was observed, and also activities toward CMC at a low level. In accordance with the degradation ability of extracellular enzymes, since all enzyme activities were much higher when cells were grown on this substrate, beechwood xylan performed to be a better carbon source compare to f-OPT (Table 3.1).

The lignocellulose degradation ability of CL-2 is mainly dependent on xylanases and α-amylases due to the inability of extracellular enzymes to degrade microcrystalline cellulose or amorphous cellulose. This hemicellulose degradation properties of CL-2 extracellular enzyme possess considerable potential to saccharifying various pretreated

Table 3.1. CL-2 extracellular enzymes and recombinant enzymes *CalXyn11A* activity against soluble and insoluble substrates

Substrates	Specific activity (U/mg protein)[a]		
	Beechwood[b]	f-OPT[b]	*Cal*Xyn11A[c]
Beechwood xylan	1887.9 ± 15.4	771.7 ± 17.6	218.4 ± 6.7
Oat-spelt xylan	1957.5 ± 14.2	530.7 ± 22.0	35.4 ± 5.9
Arabinoxylan	355.1 ± 6.4	254.1 ± 4.1	85.4 ± 6.4
Soluble starch	10.1 ± 0.27	1.91 ± 0.02	ND
Carboxymethylcellulose	0.2 ± 0.01	0.09 ± 0.001	ND
Amorphous cellulose	ND	ND	ND
Microcrystalline cellulose	ND	ND	ND
p-nitrophenyl β-D-glucoside	0.17 ± 0.01	0.09 ± 0.001	ND
p-nitrophenyl β-D-xyloside	0.19 ± 0.02	0.1±0.02	ND

Notes:

[a] Enzyme activity [1 unit (U)] was defined as the total of enzyme that liberated 1 μmol of reducing sugar or *p*-nitrophenol in 1 min.

[b] Beechwood and f-OPT were used as sole carbon sources for enzyme production from *Caldicoprobacter* sp.CL-2 culture, separately.

[c] Recombinant *Cal*Xyn11A was assayed for the degradation activity. The substrate concentration used for specific activity determination was 10 mg/ml for all polysaccharides. The released reducing sugars were determined by the Somogyi-Nelson method. Results are means ± standard deviation (n = 3).

ND; not detected.

agricultural and forestry residues, and other biopulping wood, coffee processing, fruit and vegetable maceration and also high-fiber baked goods. And without activity against cellulose, this enzyme also potential to applied at the pulp and paper industry. Without harming the cellulose fiber, CL-2 enzyme can facilitate the lignin removal of paper pulp regarding of their ability to boost pulp bleaching (biobleaching) that would significantly reduce the consumption of chlorine, an environmentally unfriendly waste of pulp bleaching (Saha, B.C., 2003).

3.3.2. Optimum pH and Temperature, and Thermal Stability

For optimum condition of CL-2 extracellular enzymes activity, it was observed that xylan degradation was maximal (100% relative activity) at pH 6.0 in the range 60 to 80°C (Figure 3.1). Around 80% of the activity was retained after incubation at 70°C for 6 h in the pH range 5.0–7.0.

3.3.3. Degradation on Biomass Substrate

From the degradation activity measurement of CL-2 extracellular enzymes, it was found that Beechwood xylan gave the highest result (reducing sugar released), whilst OPT was the second for degradation, higher compare to degradation toward CP as shown on Fig. 3.2. Both sets of extracellular enzymes showed relatively high degradation activity toward oat-spelt and beechwood xylan, and starch degradation activity (Table 3.1). We also observed activities toward β-glucosidase and β-xylosidase, and, at a low level, CMC . In terms of the degradation ability of extracellular enzymes, beechwood xylan appeared to be a better carbon source than f-OPT, since all enzyme activities were much higher when cells were grown on the former substrate (Table 3.1). The extracellular enzymes could not degrade microcrystalline cellulose or amorphous cellulose, indicating that the lignocellulose degradation ability of CL-2 is mainly dependent on xylanases and α-amylases

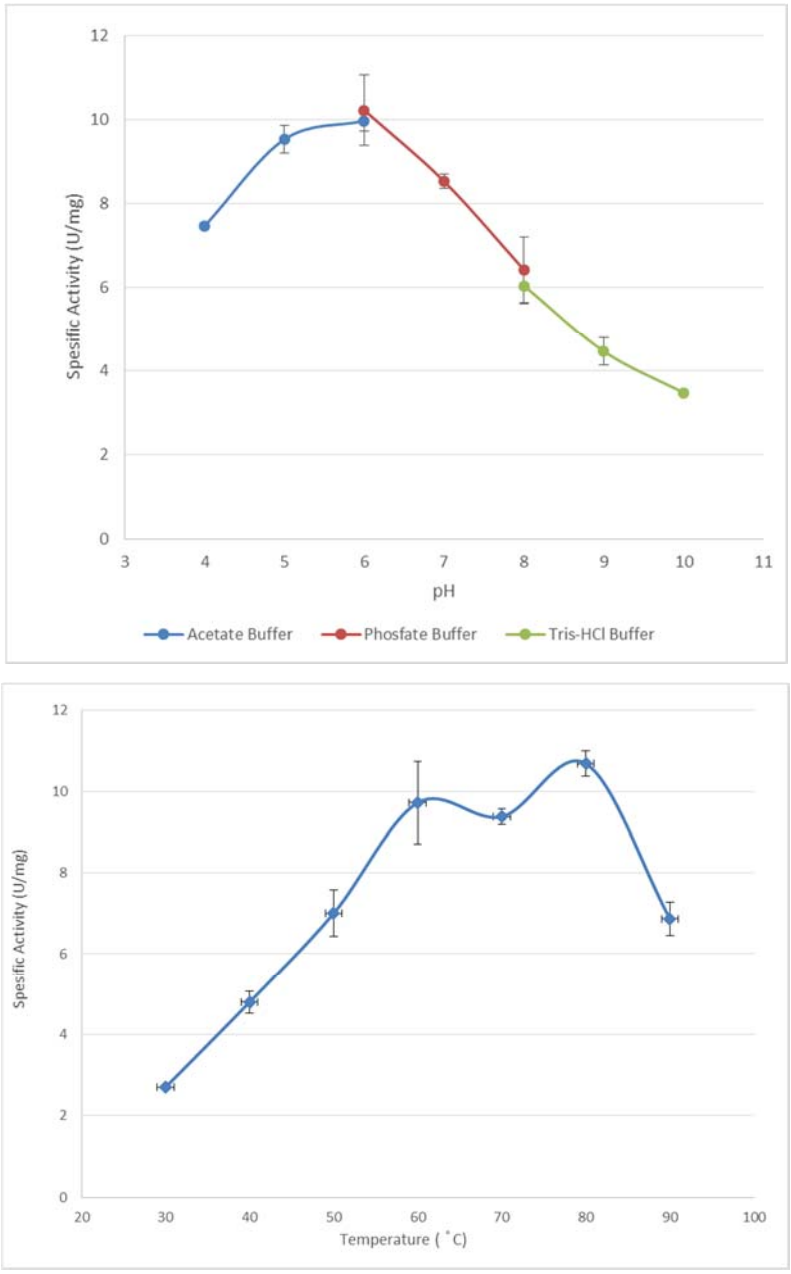

Figure 3.1. Optimum pH (top) and temperature (bottom) of CL-2 xylanase activity of Extracellular enzyme. The data were mean value of three independent experiment with error bars shown the SD (standard deviation) value.

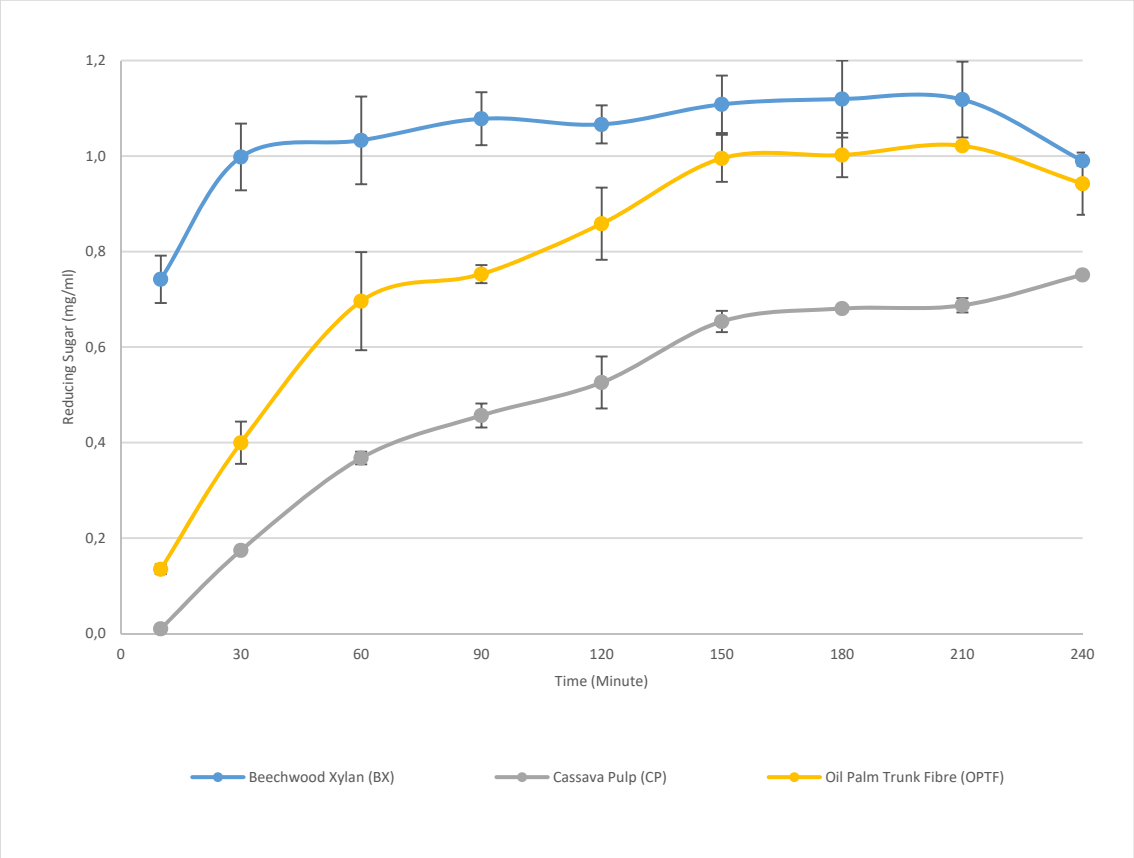

Fig. 3.2. CL-2 extracellular enzymes degradation profiles towards beechwood xylan, f-OPT and Cassava Pulp. The results are means of three independent experiments. Error bars exemplify ± standard deviation (n=3).

3.3.4. SDS-PAGE and Zymogram Analysis

To obtain clear evidence for the xylanase activity of CL2, zymogram analysis was used on the extracellular fraction prepared from the culture with beechwood xylan as carbon source Major active xylanase bands were observed at 50 and 40 kDa, and minor activity was observed at 35 kDa (Fig. 3.3). The 50 kDa- and 40 kDa-xylanases may be responsible for f-OPT degradation by CL2.

3.3.5. TLC Analysis of Hydrolysis Product

From the Hydrolysis result of CL-2 extracellular enzymes against Xylan and xylooligosaccharide substrates, it was evidenced that this enzymes was able to convert xylotriose into xylose and xylobiose, also for higher sugar, but it was inactive against xylobiose as shown in Fig. 3.4. Although CL-2 crude enzyme shown slight activity against p-nitrophenyl β-D-xyloside, but CL-2 seems could not able to degrade xylobiose as shown on figure 3.4. This might cause the majority of CL-2 crude enzyme are an endo-xylanase that working by attacks the main chains of xylans. And for β-xylosidase, it hydrolyzes xylooligosaccharides to xylose (Saha, 2003). And also this might due to the strong competitive type of inhibition by xylose (van den Brink *et al.,* 2013), although this phenomenon are more common on fungal xylanase.

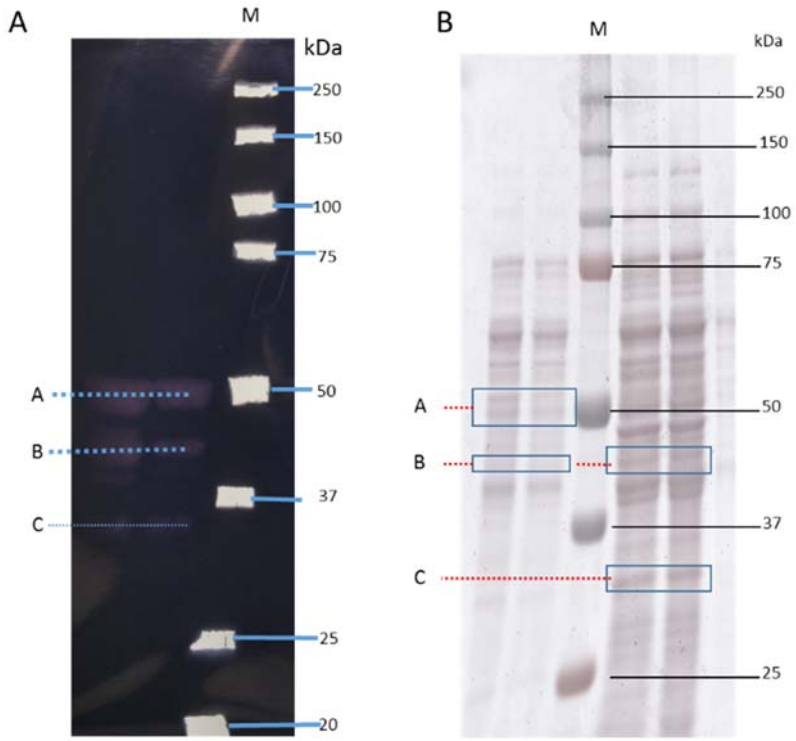

Fig. 3.3. Zymogram analysis (A) and SDS-PAGE (B) of *Caldicoprobacter* sp. CL-2 extracellular enzymes grown on beechwood xylan. Lane M, standard protein molecular mass markers, protein band A and B: major xylanase, protein band C: minor xylanase.

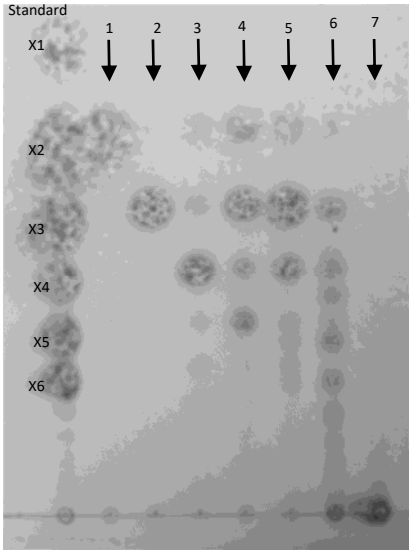

Figure 3.4. Thin-layer chromatography of hydrolysis products of xylooligosaccharides obtained using extracellular enzymes from strain CL-2

Note:

5 µg enzyme, 2 hours incubation. Authentic xylooligosaccharides were used as standards (at the left-hand side of the image): X1, xylose; X2 to X6, xylobiose to xylohexaose. Lane 1: xylobiose hydrolyze with enzyme; lane 2: xylotriose hydrolyze with enzyme; lane 3: xylotetraose hydrolyze with enzyme; lane 4: xylopentaose hydrolyze with enzyme; lane 5: xylohexaose hydrolyze with enzyme; lane 6: beechwood xylan hydrolyze with enzyme; and lane 7: beechwood xylan without enzyme (unhydrolyzed).

3.4. Conclusions

CL-2 crude enzymes optimum activity was at pH 6 and between 60 - 80 °C. Around 80% of the activity was retained after incubation at 70°C for 6 h in the pH range 5.0–7.0. CL-2 crude enzyme shown to release sugar of f-OPT as the second place after beechwood xylan. The 50 kDa- and 40 kDa-xylanases may be responsible for f-OPT degradation by CL2. And this enzyme was inactive against xylobiose. The next step was to purified this crude enzyme to understand better the degradation mechanism of OPT by major pure enzyme in crude enzymes mixture.

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Chapter 4

Cloning, Expression, Production and Characterization of Recombinant Enzyme CalXyn11A, and the Synergistic Study with Crude Enzyme from CL-2.

4.1. Introduction

Lignocellulosic biomass degradation in nature, were conducted by many enzymes which produce by various cellulolytic microorganisms. These microorganisms produce enzymes that act synergistically and subordinate with the microorganism such as the cellulosome or perform independently (as most fungal and lots of bacterial cellulases) (Himmel *et al.,* 2007).

As the second most abundant polysaccharide in nature, hemicellulose, contain xylan as the major component. This component found in an enormous amounts at wood and plant material. The backbone of xylan contain of D-xylose residues, and habitually contain also glucuronic acid and α-L-arabinofuranose as the side chains, and most of this xylans are also acetylated. Numerous dissimilar enzymes are essential to perform the complete hydrolysis of xylan. Endo-1,4-β-xylanase (EC 3.2.1.8) were one among this xylanase that pledges the hydrolysis of xylan into xylo-oligosaccharides (Luthi *et al.,* 1990).

From the previous experiment, the extracellular enzymes produced by CL- 2 strain has xylanase activity which are the major enzyme from the mixture, shown by the highest activity of xylan hydrolysis compare with other substrate hydrolysis. Base on the Carbohydrate-Active enZYmes (CAZy) database, there are four GH families which xylanase mainly classified into it, which is GH5, GH10, GH11, and GH43. Most of endo-β-1,4-xylanases which are classified within GH11 group, has shown high xylan-degradation activity.

The next step of the degradation mechanism was to study the pure enzyme involved in the degradation with cloning and expressed it and characterize the recombinant enzyme properties, and also applying this enzymes into OPT degradation. It is an important to study the enzymes produces by CL-2 at the molecular level of its chemical linkages and how this enzyme able to distinguish their substrates and catalyze their reactions in order to produce optimized enzyme cocktail especially for xylan deconstruction (Dodd and

Cann, 2009). This step was necessary in order to better understand the degradation mechanism of OPT. And also to design future work to degrade OPT, and Lignocellulosic Biomass in general with a more efficient way.

4.2. Material and Methods

4.2.1. Genomic Walking, Cloning, and Sequencing of *Caldicoprobacter* sp. CL-2 Xylanase Gene

Once comparing xylanases from *Caldicellulosiruptor* species amino acid sequences of GH11 (more than five), we designed a pair of degenerate primers (des-xynA11F, 5′-TAY GAR YTN TGG AAR GAY TAY GGN AAY- 3′ and des-xynA11R, 5′-TTN GCR TAN CCN SWR TTY TGR TAN CCY TCN AC-3′) based on two highly-conserved amino acid sequences (YELWKDYGN and VEGYQNSGYAN). And to amplify the corresponding region from *Caldicoprobacter* sp. CL-2 genomic DNA, PCR was performed using ExTaq polymerase (Takara Bio) in standard conditions following the manufacturer's guidelines. Ligation of purified PCR products conducted using with pTAC-1 (Biodynamics Laboratory Inc., Tokyo, Japan). Both strands were sequenced using M13 primers. Homology analysis by BLAST confirmed that the amplified region was a fragment of a xylanase gene. To perform genome-walking PCR, two gene-specific primers (5′- ATTGACAACTAAACCTTC-CTCCTGTGTCAA-3′ and 5′-AAAGGT-CTGAACTTGGGTACAATTGACCAA-3′) were designed using the Universal Genome Walker 2.0 kit (Takara Bio) following the manufacturer's instructions. pTAC-1 were used to cloned and sequenced the purified products of the nested PCR. GENETYX v. 12 software (GENETYX Corporation, Tokyo, Japan) was used to accomplish the Sequence assembly. BLASTn and BLASTp programs, respectively used to analyze the nucleotide and amino acid sequences (https://blast.ncbi.nlm.nih.gov/Blast). And the *Caldicoprobacter* sp. CL-2 GH11 xylanese gene were then called as *calxyn11A*.

4.2.2. Plasmid Construction

To amplify the open reading frame of the *calxyn11A* gene and to afford restraint sites for producing fragments for ligation and cloning, specific primers namely *xyn11A*-F and *xyn11A*-R, with *Bam*HI and *Bpu*1102I restriction enzyme sites, were designed as

shown in table 4.1. PCR using PrimeSTAR® HS DNA Polymerase (Takara Bio) was used to produced A 1144-bp fragment of the *calxyn11A* gene. The PCR condition was set as followed. First: 98 °C – 1 minute – 1 cycle. Second: 30 cycle of 98 °C – 10 second , 60 °C – 1 minute, and 68 °C – 2 minute for extention. Third: additional extention at 72 °C for 10 minutes. Agarose gel electrophoresis then were employed to refine the PCR products. And then digested by *Bam*HI plus *Bpu*1102I restriction enzyme. The digested fragment then cloned into pET22b vector which already digested before using the same restriction enzymes. The producers' protocols (Qiagen) were followed to prepared plasmid DNA. And this constructed plasmids then were transformed into *Escherichia coli* competent cells. Positive clones then verified by DNA sequencing.

4.2.3. Production and Purification of Recombinant *Cal*Xyn11A

Vector pET22b with *calxyn11A* gene inserted from the first cloning into *E. coli* DH5α then were transformed into *E. coli* BL21 in order to produce the recombinant enzyme, and to characterize the enzyme properties. The positif transformants were selected using ampicillin (100 μg/ml) contained LB plates. And the expression of the *calxyn11A* gene were conducted by growing *E. coli* BL21 containing the *calxyn11A* expression vector in 300 ml of LB medium containing 100 μg/ml of ampicillin. The growth were observed until the absorbance at 600 nm reached 0.6–0.8, and the cell then were harvested. The cell wall then lyzed by adding lysing mixture and aplly sonication. The lysed cell solution then was purified by the Profinia Affinity Chromatography Protein Purification System using a Bio-Scale Mini Profinity IMAC cartridge and a Bio-Gel P6 desalting cartridge following the producer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). Coomassie (Bradford) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin as the standard were used to determine the concentration of proteins. And to observed the homogeneity of the purified recombinant enzyme, the sample was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

TABLE 4.1. Oligonucleotide primers used for *xynA* expression plasmid

Primer	Sequence (5′→3′)
xyn11A-F	CGCGGATCCG ATGGGGGTTTACAAAATGAAAGGG
xyn11A-R	ATTGCTCAGCTCATTGTATTAACAAATAATCTGCATA

*BamH*I and *Bpu*1102I restriction sites are underline

The recombinant enzymes then were studied for its properties, such as optimum stability on pH and temperature as described on the previous chapter. The Lineweaver-Burk method were applied to analyze the Km and Vmax values of the purified recombinant enzyme. Enzyme activity was analyzed for 30 min at 60 °C in sodium acetate buffer (pH 6.0) containing 1 mg/ml to 10 mg/ml beechwood xylan.

4.2.4. Biodegradation of f-OPT by *Caldicoprobacter* sp. CL-2 Extracellular Enzymes and Recombinant *Cal*Xyn11A

To witness the synergistic relation between recombinant enzyme *Cal*Xyn11A with its extracellular enzymes against f-OPT and beechwood xylan, both of these enzymes are prepared and combined, by incubating 100 µl of extracellular enzymes and recombinant *Cal*Xyn11A separately and a mixture of the two enzymes in 20 mM sodium acetate buffer, pH 6.0 with 5 ml of substrate (either f-OPT or beechwood xylan) with concentration are 1 g/l in 50 mM phosphate buffer, pH 6.0 at 70 °C and shaking at 150 rpm. The final total protein concentration was arranged up to 50 µg. Recombinant *Cal*Xyn11A was added to the extracellular enzymes with concentrations of 5, 10, or 15 µg in the mixture of synergistic experiments. The mixture were taken samples by aliquots 500 µl of the reaction solutions at 24 h, respectively, centrifuged, and examined for soluble reducing sugars using xylose as the standard. Somogyi-Nelson method were used to measure the released sugars in supernatants.

4.3. Results and Discussion

4.3.1. Recombinant enzyme *Cal*Xyn11A

Xylanases are mostly classified into four GH families, which amongst are GH5, GH10, GH11 and GH43 base on the Carbohydrate-Active enZYmes (CAZy) database. And amongst these member, GH11, are the group with mostly endo-β-1,4-xylanases that show high xylan-degradation activity. Members of this group are also commonly considered as the real xylanases due to their strict substrate specificity against xylan (Paes *et al.,* 2012). One member of this GH11 gene were cloned from CL-2, with 526 bp gene fragment that amplified by PCR using degenerate primers based on two highly-conserved amino acid sequences (YELWKDYGN and VEGYQNSGYAN). PCR-based genome walking were used to amplify the 5′- and 3′- flanking regions which then draw together

with the recognized partial sequence. The result, a new xylanase gene called *calxyn11A* that later were cloned, with the functional annotation was performed using the Conserved Domains Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). This novel xylanase gene consist of 1143 bp which is encode a 380 amino acids proteins with 41.7 kDa molecular mass calculated. The GenBank accession number for *calxyn11A* is MF463700. In *calXyn11A,* amino acid residues 48-57 and 213-223 were the YELWKDYGN and VEGYQNSGYAN conserved amino acid sequences. The putative GH11 xylanases from *Cal. bescii* (with 81% identity, WP_015906728), *Cal. owensensis* (with 78% identity, WP_013411078.1), *Dictyoglomus thermophilum* (with 78% identity, WP_012547705.1), and *Cal. kronotskyensis* (with 71% identity, WP_013429154.1) showed high homology with the inferred amino acid sequence. The protein comprises a putative N-terminal signal peptide, as forecast by the SignalP 3.0 server. It was specified that *Cal*Xyn11A is a modular enzyme, consisting of a signal peptide, a GH11 catalytic domain and a family 36 carbohydrate-binding (CBM36) module, in that order from the N-terminus, from the analysis using the Pfam database (http://pfam.sanger.ac.uk/). CBM36, a member of 'glycan-chain-binding' type B CBMs (based on structural and functional similarities) is well known to be a calcium-dependent xylan binding module that can form direct interactions with the substrate through a single atom of the bivalent metal (Boraston *et al.,* 2004).

4.3.2. Expression, Characterization and Biomass Degradation of *Cal*Xyn11A

The cloned xylanase gene *calxyn11A* was expressed in *E. coli* BL21, purified to electrophoretic homogeneity using immobilized-metal affinity chromatography (Fig. 4.1; lane 2), and assayed for activity toward xylan and other substrates to characterize the enzymatic properties of *Cal*Xyn11A.

Once analyzed at 70 °C and pH 5.0, *Cal*Xyn11A presented high specific activity against beechwood xylan and arabinoxylan, but low activity was witnessed toward oat-spelt xylan and arabinan, (Table 3.1). And from the determination of *Cal*Xyn11A hydrolysis product, it is evidenced that this recombinant enzyme was also able to convert xylotriose to xylobiose and xylose but was inactive toward xylobiose (Fig. 4.2; lanes 1 and 2).

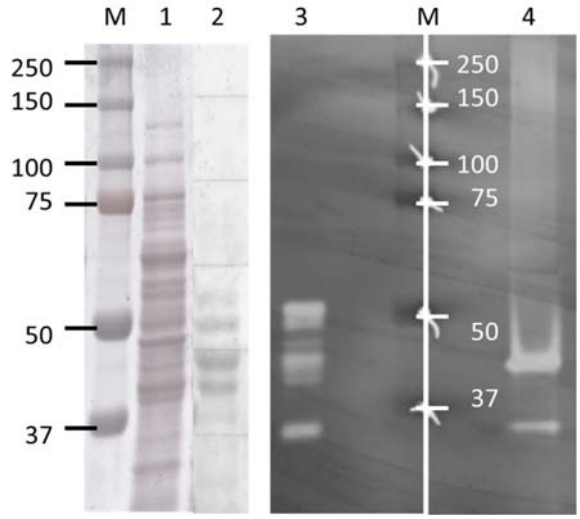

Figure 4.1. SDS-PAGE and Zymogram analysis of extracellular enzymes and *Cal*Xyn11A from CL-2.

Notes: SDS-PAGE (lanes M, 1, and 2) and zymogram analysis (lanes 3 and 4) of the extracellular enzymes prepared from CL-2 culture grown on beechwood xylan and of recombinant *Cal*Xyn11A. Lane M, standard protein molecular mass markers; lane 1, extracellular enzymes from CL-2 (10 µg of protein); lane 2, partially purified, recombinant *Cal*Xyn11A (3.5 µg of protein); lane 3, zymogram analysis of the extracellular enzymes (10 µg of protein); lane 4, zymogram analysis of partially-purified recombinant *Cal*Xyn11A (3.5 µg of protein

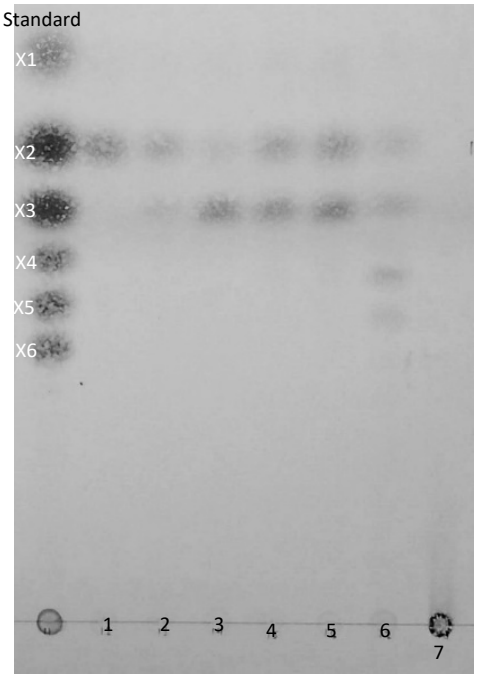

Figure 4.2. Thin-layer chromatography (TLC) of hydrolysis products of xylobiose and xylotriose obtained by recombinant *CalXyn11A*. Veritable xylooligosaccharides were used as standards (at the far left side of the image): X1, xylose; X2 to X6, xylobiose to xylohexaose. Lane 1, xylobiose with *CalXyn11A*; lane 2, xylotriose with *CalXyn11A*, up to lane 5, xylohexaose, lane 6 beechwood xylan with *CalXyn11A*, and lane 7, beechwood xylan unhydrolyzed as control

Kinetic parameters were ascertained by Michaelis-Menten assay to calculate the substrate affinity and catalytic efficiency of *Cal*Xyn11A toward xylan. The Km and Vmax values for *Cal*Xyn11A were appraised to be 20 ± 4.5 mg/ml and 500 ± 5.5 μmol/min/mg protein, respectively, corresponding to a kcat of 124,069/s.

The optimal pH for xylanase activity of *Cal*Xyn11*A* was 5.0, lower then the extracellular one. And the enzyme was stable over the pH range of 5.0–6.0. The temperature for maximum activity was at 80 °C with 70 - 90 °C range as shown on Fig. 4.3. These enzymatic properties and substrate specificity are similar to those members of GH11 β-1,4-xylanases. Nevertheless, when compared with the activity determined for the extracellular enzymes preparation, the degradation of f-OPT and beechwood by recombinant *Cal*Xyn11A were remarkably low (Fig. 4.4). These results might cause by the *Cal*Xyn11A role on the degradation. That *Cal*Xyn11A may not play a major role in f-OPT degradation, but may act synergistically with other GH hydrolases.

In zymogram analysis, we compare the recombinant enzyme with extracellular enzyme of CL-2 to see the similarity and the differences and last to find the role of this recombinant enzymes in the whole crude enzymes of CL-2. Xylanase activities were observed by the clear zone formed at 40 kDa and 35 kDa, comparing to the calculated molecular mass of 41.7 kDa, this indicated *CalXyn11A* may have suffered proteolysis (Fig. 4.1. lane 4).

Compare to its extracellular enzymes, CL-2 *Cal*Xyn11A could not efficiently degrade f-OPT, even though xylanase activity is important for f-OPT degradation as shown on Fig 4.4.

4.3.3. Synergistic Study between Crude and Purified CL-2 Xylanase

Synergistic relation between *Cal*Xyn11A and CL-2 extracellular enzymes (prepared with beechwood xylan as substrate) were conducted to evaluate the role of *Cal*Xyn11A in f-OPT degradation. The quantity of reducing sugars released when the extracellular enzyme preparation alone incubated with f-OPT was much higher compare to the amount of reducing sugars released when *Cal*Xyn11A alone incubated with the substrate (Fig. 4.5-A). Nevertheless, when *Cal*Xyn11A was added incrementally to the extracellular enzyme preparation, the sugar concentration released was synergistically increased.released sugar

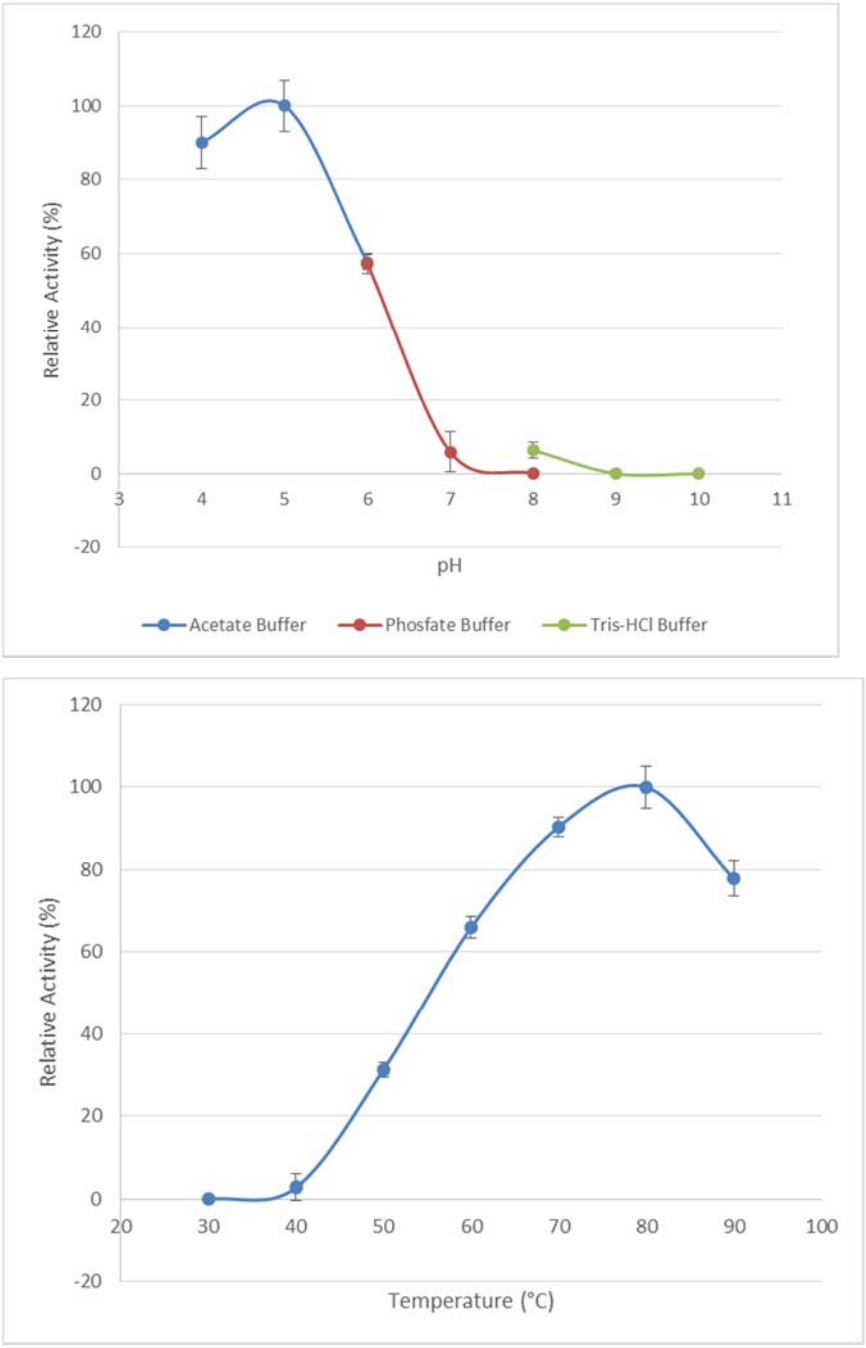

Figure 4.3. Optimum pH (above) and optimum temperature (bottom) of *Cal*Xyn11A.
Note: The data were mean value of three independent experiment with error bars shown the SD (standard deviation) value.

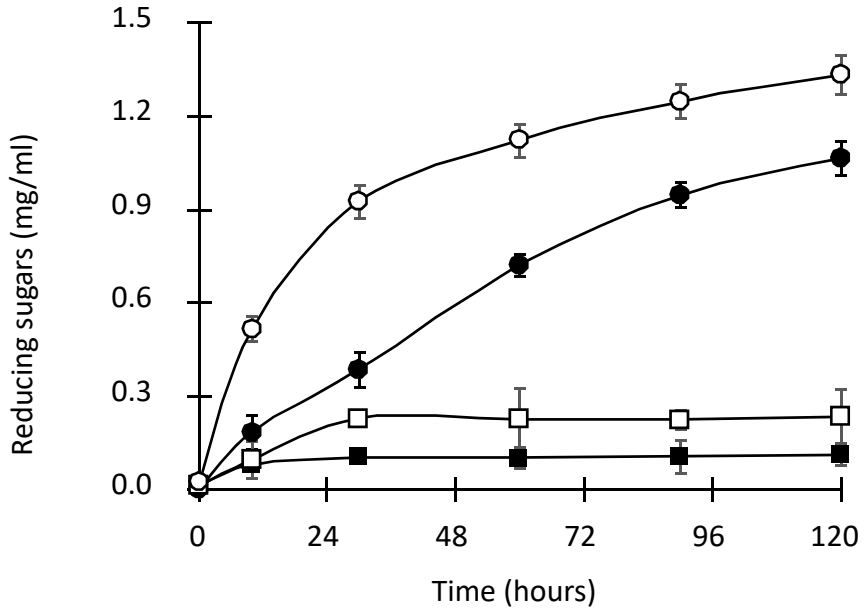

Fig. 4.4. Degradation profiles of f-OPT and beechwood xylan using extracellular enzymes from strain CL-2 (30 µg) and recombinant CalXyn11A (15 µg). The data are means of three independent experiments. Error bars represent ± standard deviation (n=3)

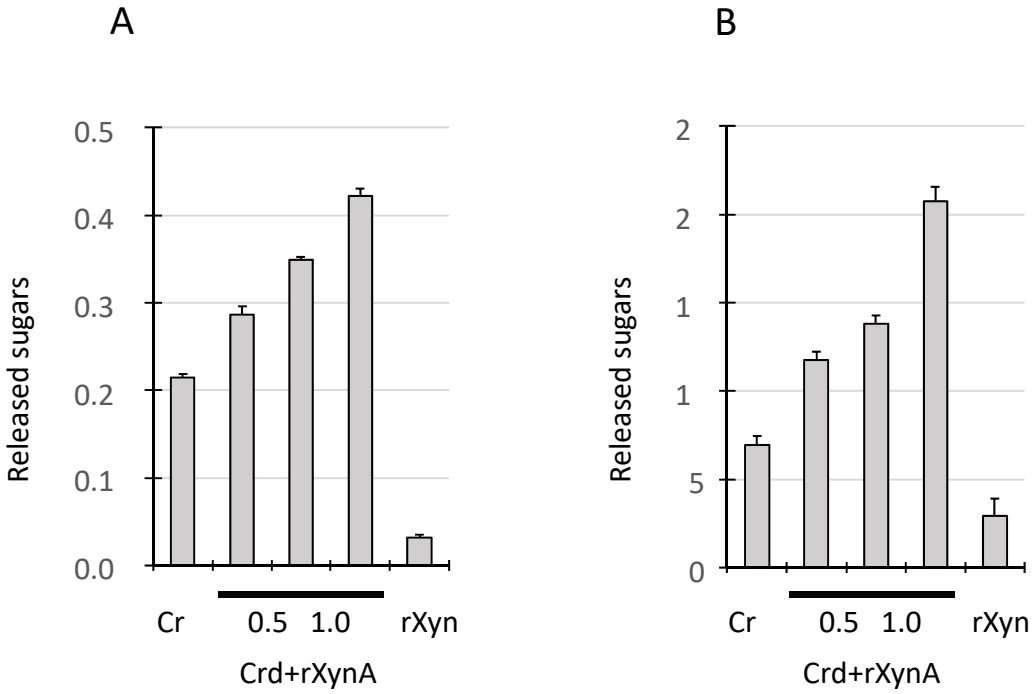

Fig. 4.5. Synergistic effects of extracellular enzymes produced with beechwood as substrate and recombinant CalXyn11A on hydrolysis of (A) f-OPT and (B) beechwood xylan.

Note: Recombinant CalXyn11A (rXynA; 5, 10, or 15 μg) was respectively added to the reaction mixture including the extracellular enzymes (Crd). Enzymatic activity was defined as the concentration (μM) of total released reducing sugars for 24 h. The data are means of three independent experiments. Error bars represent ± standard deviation (n=3).a.

The highest concentration of sugar released reach 1.7 times compare to the from extracellular enzymes action alone (Fig. 4.5-A). The confirmation of synergistic effect of *Cal*Xyn11A to the extracelluar enzyme was conducted by addition of *Cal*Xyn11A to the extracellular enzyme using beechwood xylan as substrate (Fig. 4.5-B), which similar synergistic effects were observed also. It has been noticed that exceedingly synergistic effects could resulted from the combination of xylan hydrolysis enzymes belong to GH10 and GH11 [Sermsathanaswadi *et al.* (2017) and Valls *et al.* (2016)]. Members of GH11 are generally regarded as true xylanases owing to their stringent substrate specificity toward xylan (Paës *et al.,* 2012). Some GH10 xylanases can hydrolyze other polysaccharides, such as barley β- glucan (Shi *et al.,* 2010). *Cal*Xyn11A able to subsidize to the speeding up of f-OPT degradation by cooperation with other xylanases such as GH10 members.

4.4. Conclusions

According to Saha (2003), xylan are the most profuse hemicellulose. As a result, its degradation to the constituents sugars which majority are xylose and arabinose that will continued to subsequent fermentation for efficient biofuel production from plant biomass is precarious. One of the main xylanases produced by CL-2 was cloned and characterized in order to comprehend the degradation system on f-OPT. The recombinant enzyme resulted were an endo-β-1,4-xylanase (*Cal*Xyn11A) which has a modular structure consisting of a GH family 11 catalytic domain and a family 36 carbohydrate-binding module. From the zymogram analysis, *Cal*Xyn11A was found to be one of the two major xylanases in CL-2 extracellular enzyme. Even though CalXyn11A displayed small degradation activity against f-OPT, strong synergistic degradation activity could be observed for f-OPT as well as xylan when CalXyn11A was added to the extracellular enzymes prepared from CL-2. These results specify that CalXyn11A may have xylan degradation activity, and cooperate with other extracellular glycosyl hydrolases of CL-2 synergistically for efficient biodegradation of f-OPT.

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Chapter 5

General Conclusions.

The utilization of felled OPT has been conducted in many studies from several aspect. The huge amount of this biomass waste of Palm Oil plantation resulted annually was one of the trigger. The research such as using the plywood that only a small part of the whole trunk, and also the efforts to strengthening the inside soft-part of the trunk as a material for woodcraft. The other studies were try to utilize the trunk on biological saccharification by using the trunk inner part, which is mostly unused, through the separation process into sap and the fiber, to produce valuable product, fermentable sugar, up to biofuels.

OPT fiber gave the challenge to utilize especially for microbial degradation. The recalcitrance of the structure make it hard to degrade, resulting the screening efforts to search for potential degrader candidate using this biomass substrate are difficult to practice. One of the pretreatment that chosen was WDM which is environmentally friendly leaving the chemicals use with low cost to conduct. From the pretreatment study of OPT fiber WDM using SuperMass Colloider, it was notice that this method are a suitable pretreatment that success to lowering the recalcitrant of OPT fiber and make it informal to degrade by microbes and hydrolyze by enzymes.

From overall of the OPT degrader microbes screening, it was noticed that a novel thermophilic anaerobic bacteria has been found from Bovine manure and sugarcane residue compost of Ishigaki island. This strain, proposed as *Caldicoprobacter* sp. CL-2, capable to degrade OPT and exhibited high degradation ability (32.7 % reduction) against total dry solids of f-OPT at 70 °C. This bacteria has 98.6 % sequence identity with the 16S rDNA gene of *Caldicoprobacter faecale* DSM 20678. This strain also could utilize xylan, arabinoxylan, starch, pectin and CMC except of cellulose fiber as carbon sources. From the utilization properties of polysaccharides, morphology and phylogenetic tree based on 16S rRNA sequence, strain CL-2 suggested to be classified as a novel species of Caldicoprobacter.

CL-2 strain produces crude enzymes with the highest activity measured against xylan substrate. The optimum xylanase activity was at pH 6 and between 60 - 80 °C. Around 80% of the activity was retained after incubation at 70°C for 6 hours in the pH

range 5.0–7.0. The crude enzyme of CL-2 shown able to release reducing sugar from f-OPT as the second place after beechwood xylan. From the zymogram analysis, the 50 kDa- and 40 kDa-xylanases may be responsible for f-OPT degradation by CL-2.

From the f-OPT degradation comparation between CL-2 and *Clo. thermocellum* PAL5, it is measured that the residue weights of CL-2 decreased by 32.7% (w/v) while the residue weights of *Clo. thermocellum* PAL5 decreased by 39.0%. *Clo. thermocellum* PAL5 efficiently and directly degrade lignocellulose using the high cellulose-degrading activity of multienzyme complexes, whilst CL-2 can use xylan, arabinoxylan and arabinogalactan, but not cellulosic materials; however, CL-2 showed 32.7% degradation of f-OPT, this mean that degradation of xylanolytic polymers may hold an important role in the efficient degradation of OPT fibers.

One of the major xylanase detected on CL-2 crude enzyme was cloned and express to produce the pure recombinant enzyme. The cloned endo-β-1,4-xylanase (*Cal*Xyn11A) has a modular structure consisting of a GH family 11 catalytic domain and a family 36 carbohydrate-binding module. *Cal*Xyn11A was found to be one of two major xylanases in culture supernatants of CL-2. The characterization study of the crude enzymes and recombinant enzyme *Cal*Xyn11A reveal the importance role of xylanase on biomass degradation, particularly OPT. The study between recombinant enzyme *calxyn11A* with its crude enzymes show synergistic relation, whilst *Cal*Xyn11A has low degrading activity towards OPT, but it gave significant rise to the whole degrading activity when added to the crude enzyme. This gave a feature to a more effective OPT degradation mechanism specifically and for the consolidated lignocellulosic biomass degradation in general.

Chapter 6

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