EFFECTIVENESS OF FUNGAL TREATMENT AND ACCLIMATED MICROBIAL CONSORTIUM ON BIODEGRADATION AND BIOGAS YIELDS OF LIGNOCELLULOSIC GRASS

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A THESIS SUBMITTED AS A PART OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN ENVIRONMENTAL TECHNOLOGY

THE JOINT GRADUATE SCHOOL OF ENERGY AND ENVIRONMENT
AT KING MONGKUT'S UNIVERSITY OF TECHNOLOGY THONBURI

1ST SEMESTER 2014

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ABSTRACT

This study aims to improve the biodegradability of paragrass and its methane potential using (1) fungal pretreatment, and (2) acclimated microbial consortium (AMC) as the inoculum. The effectiveness of fungal treatment by *Coprinopsis cinerea* and *Polyporus tricholoma* on biodegradability and methane yield of paragrass was investigated. After 15 days fungal treatment, reduction in cellulose crystallinity was found in the grass treated with *C. cinerea* and *P. tricholoma*. Biogas production from the fungal treated grass and from the untreated grass with original sludge were compared. The maximum methane production rate of the treated grass occurred earlier than the untreated grass; i.e., at day 10 for the grass treated with *C. cinerea*, day 13 for the grass treated with *P. tricholoma*, and day 22 for the untreated grass. However, after 140 day anaerobic digestion, the methane yield of the grass treated by *C. cinerea* and by *P.tricholoma* was approximately 15% lower than that of the untreated grass, which was 368 mL STP/g VS added. Using the two-stage fungal treatment and anaerobic digestion, the recalcitrant cellulose in the fungal treated grass was significantly lower than that of the untreated grass ($P<0.05$), while the amounts of recalcitrant hemicellulose were approximately the same.

The specific methane yields of a wide variety of paragrass was investigated. The untreated grass was inoculated with two types of sludge: (1) a typical anaerobic sludge obtained from a domestic wastewater treatment plant, and (2) a sludge acclimated to fibrous substrates in raw palm oil mill effluent (POME). The acclimated microbial consortium could enhance the hydrolytic, acetogenic and methanogenic activities of the sludge significantly ($p < 0.05$). After 80 days of anaerobic digestion, the methane yield of the OS and the AMC were 277 and 316 mL STP/g VS added, respectively. The cellulose, hemicellulose and lignin were reduced by 40%, 48% and 37%, respectively, by the OS, while 51% cellulose, 59% hemicellulose and 40% lignin, respectively, by the AMC. The acclimatization of the

mesophilic microbial community in raw POME can significantly enhance the methanogenic activity, the biodegradation and the methane yield of the paragrass ($P < 0.05$).

Keywords : fungal pretreatment, acclimated microbial consortium, biodegradation, biogas yield, paragrass

ACKNOWLEDGEMENTS

The author is heartily thankful to her advisor Assoc. Prof. Dr. Chantarapron Phalakornkule, and co-advisor Assoc. Prof. Dr. Savitri Vatanyoopaisarn for the valuable guidance and advice. The author also thanks the other members of the thesis committee: Assoc. Prof. Dr. Anurak Petiraksakul, Assoc. Prof. Dr. Chalermraj Wantawin, Asst. Prof. Dr. Nipon Pisutpaisal, and Prof. Dr. Ajit P. Annachhatre for their helpful suggestions.

The author is grateful to the Science and Technology Postgraduate Education and Research Development Office (PERDO) and the Commission of Higher Education Thailand (Grant No. JGSEE/PROJECT/005-2011) for the financial support. The author is grateful to the Thailand Research Fund (The Royal Golden Jubilee Ph.D. Program, Grant no. PHD/0311/2551) and the Joint Graduate School of Energy and Environment (JGSEE) for the scholarship. The author also wishes to thank Ngaung-Khaem Water Quality Control Plant for providing sludge.

Special thanks to Dr. Elvin Moore for helping me to improve English. My teachers, family, friends, especially Pinanong Tanikkul, Maneerat Khemkhao and Nattaporn Chutichairattanaphum, for their helpful suggestions and valuable assistance throughout the entire research. The authors would like to acknowledge the Center of Technology Services of King Mongkut's University of Technology North Bangkok at Prachinburi Campus for providing technical assistance and the research facilities.

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NOMENCLATURE

a	Year
Å	Angstrom
C	Carbon
°C	Celsius
CH_4	Methane gas
cm	Centrimater
CO_2	Carbon dioxide
COD	Chemical oxygen demand
g	Gram
h	Hour
ha	Hectare
H_2	Hydrogen gas
k	Kilo-
L	Liter
m^3	Cubic mater
m	Milli-
M	Mole/l
min	Minute
MLVSS	Mixed liquor volatile suspended solid
MMPR	Maximum methane production rates
Nm^3	Volume at standard conditions of 0 °C, 101.325 kPa
%	Percentage
sCOD	Soluble chemical oxygen demand
t	Ton
T	Tera
TS	Total solids
µ	Micro
U	Unit
V	Volume
VFA	Volatile fatty acid

NOMENCLATURE (Cont')

VS	Volatile solid
W	Weight
Wh	Watt hour

CHAPTER 1
INTRODUCTION

1.1 Rationale

It has been estimated that biogas production would increase from 1.5 TWh/a to 15 TWh/a, if lignocellulosic materials could be utilized as the raw materials in addition to traditional substrates, such as wastewater from agro industry, animal manure, foodwaste and sewage sludge (Feng et al., 2013)[27]. Varieties of grass have been grown as dedicated energy crops. In Germany, it has been estimated that potential of methane (CH_4) production from grass is 4,060 Nm^3 CH_4/ha a (Weiland, 2003)[118], while in Sweden the estimate is 2,040 Nm^3 CH_4/ha a (Lantza et al., 2007)[55]. In Austria, clover grass yielded 3,000-4,500 Nm^3 CH_4/ha a on medium to good locations in 2004 (Amon et al., 2004)[4]. Methane yields of various grasses have been estimated to be as follows (Braun et al., 2009)[13]: clover grass 290-390 Nm^3/t VS; sudan grass 213-303 Nm^3/t VS; reed canary grass 340-430 Nm^3/t VS; ryegrass Nm^3/t VS. High biomass yield per hectare, good digestibility and regrowth ability after harvesting have been suggested to be important factors when choosing grass species for biogas production (Seppälä et al., 2009)[95]. Therefore, varieties of grass have been investigated more deeply in a number of recent studies to optimize their biogas outputs. For example, in Oleszek et al. (2014)[77], a wide variety of reed canary grass was found to have greater indigestible lignocellulosic content than cultivated grass. The higher content of the indigestible lignocellulosic content was found to be related to biogas quality and quantity, i.e., 406 Nm^3/t VS for the cultivated reed canary grass and 120 Nm^3/t VS for the wild variety. Seppälä et al. (2009)[95] reported that the specific methane yields of four grass species in Finland (cocksfoot, tall fescue, reed canary grass and timothy) and all harvests varied from 253 to 394 Nm^3/t VS and that the methane yields from different harvest years ranged from 1200 to 3600 Nm^3 CH_4/ha a. The methane yield per hectare of the 1st harvest was always higher than that of the 2nd harvest, and the higher methane yield per hectare was found to be related to higher dry matter yield per hectare and specific methane yield.

Paragrass (*Brachiaria mutica*), which is also known as buffalo grass, is a creeping perennial grass that grows only in warm weather. Information about the ecology, agronomy and production potential of paragrass is available in the literature (Troprical Forages, 2014)[107]. Paragrass can stand long-term flooding, and it can tolerate depths of water up

to 1.2 m in the tropics and up to 30 cm in the subtropics. Due to its ability to adapt to a wide range of soil types, paragrass is abundant in tropical countries, e.g. Thailand, and the Philippines as well as in subtropical countries, e.g. Australia. On fertilized land, dry matter yields were typically 5,000-12,000 kg/ha a, and up to 30,000 kg/ha a have been recorded. On unfertilized land, dry matter yields ranged between 2,000-4,000 kg/ha a. However, little research has been done to estimate its potential for biogas production.

Besides optimizing the habitat and the harvest method, another strategy to increase the biogas potential of grass is to increase its digestibility by pretreatment of recalcitrant lignocellulosic biomass before the anaerobic digestion stage. Over the past few decades, a number of different pretreatment techniques involving physical, chemical, and biological approaches have been investigated for the enhancement of biogas production from lignocellulosic biomass (Zheng et al., 2014)[129]. Compared with physical and chemical pretreatment methods, biological pretreatment requires far lower energy and chemical inputs. Biological pretreatment methods have mainly focused on fungal pretreatment, pretreatment by microbial consortium, and enzymatic pretreatment, but only enzymatic pretreatment has been studied on grass (jose tall wheatgrass) (Romano et al., 2009)[89]. The addition of enzyme products containing cellulase, hemicellulase, and β-glucosidase was found to have positive effects on the solubilization of the wheatgrass. However, no significant differences in biogas and methane yields and in volatile solids reduction was found when the enzyme products were tested in the anaerobic digestion systems. A recent study on napier grass reported the enhancement of the maximum methane yield of napier grass pretreated by microbial consortia constructed for the rapid degradation of lignocelluloses (Wen et al., 2015)[119]. The maximum methane yields of pretreated samples by the consortia MC1 (*Clostridium straminisolvens* as the most dominant microbial species), WSD-5 (*Coprinus cinereus* and *Ochrobactrum* sp.) and XDC-2 (mesophilic bacteria in the genera of *Clostridium*, *Bacteroides*, *Alcaligenes* and *Pseudomonas*) were 259, 279, 247 mL/ g VS, which were 1.39, 1.49 and 1.32 times greater than the values of the untreated controls.

The pretreatment of lignocelluloses by edible fungi is a relatively more environmentally friendly method that does not require toxic chemicals, harsh conditions, expensive specialized instruments and high energy input. Some edible fungi have been reported in the literature to disrupt the lignin-cellulose bindings in plant biomass and to increase the methane potentials of the biomass. Pretreatment of cotton stalk by solid-state fermentation using selected strains of *Pleurotus ostreatus* and *Phanerochaete*

chrysosporium improved the biodegradation of the biomass (Kerem et al., 1992)[52]. *P. chrysosporium* was found to have rapid, but nonselective degradation of the lignocellulose, while *P. ostreatus* grew more slowly with obvious selectivity for lignin degradation. Pretreatment of Japanese cedar wood with a selective white rot fungus, *Ceriporiopsis subvermispora*, in the presence of wheat bran was found to increase methane production from cedar wood (Amirta et al., 2006)[3]. Wheat bran has been shown to promote the growth and production of hemicellulolytic and ligninolytic enzymes of the fungus. Muthangya et al. (2009b)[72] reported that the methane yield of sisal leaf decortication residues (SLDR) can be enhanced by pretreatment with *Trichoderma reseei* in a solid-state fermentation for 8 days at 25% wet weight inoculum per SLDR. However, unpromising results have also been reported. For example, in the fungal pretreatment of straw by two edible strains of rot fungi *Pleurotus ostreatus* and *Pleurotus eryngii* by Feng et al. (2013)[27], the methane potential of the fungal pretreated straw was not significantly higher than that of the untreated straw. The authors suggested that some carbon in the straw had been lost during the aerobic treatment. In addition, Muthangya et al. (2009a)[71] found that the increase of the *T. reseei* inoculum concentration from 25% to 50% decreased the methane yield of the SLDR by 38%. A microbial consortium with the dominant fungi *C. cinerea* and the dominant bacteria *Ochrobactrum* sp. was successfully used in the biological pretreatment of napier grass (Wen et al., 2015)[119]. The maximum methane yield of napier grass was increased from 187 mL/ g VS of the untreated napier grass to 279 mL/ g VS of the pretreated samples. The results in the literature suggest that the success of edible fungal pretreatment on enhancing the methane yield depends on many factors such as the characteristics of biomass substrates, types of the fungal strains, inoculums concentrations and nutrition supplementation.

At present, there are few studies on enhancing the biomass digestibility of paragrass and its methane potential. This research aims to improve the biodegradability of the paragrass and its methane potential using (1) a fungal pretreatment and (2) an acclimated microbial consortium (AMC) as the inoculum.

Two fungal strains were selected for this study: (1) *Coprinopsis cinerea* and (2) *Polyporus tricholoma. C. cinerea* is a species of mushroom in the Psathyrellaceae family. Habitats of *C. cinerea* are in common surroundings, such as in mown fields, cow dung and grassplots after rain (Wang et al., 2011)[117]. *P. tricholoma* is a white-rot fungus, commonly found in the neotropics of Central America (Kruger et al., 2004)[54]. In chapter 3 of this thesis, the characteristics of the grass after aerobic treatment and after methane anaerobic

digestion were studied. Changes in the chemical compositions and crystallinity of the paragrass were investigated over a 45-day period. In addition to the digestibility, the rates and yields of methane production were compared between the naturally decayed grass and the fungal treated grass. Finally, the effectiveness of the fungal pretreatment on enhancing digestibility and biogas yields of the paragrass was analyzed.

In a previous study, microbial consortia, which effectively degrade palm fiber, can be developed from the sludge obtained from a domestic wastewater treatment plant by an acclimatization technique (Khemkhao et al., 2015)[53]. The microbial consortia are comprised of cellulolytic and hemicellulolytic bacteria enriched by raw palm oil mill effluent (POME). In chaper 4 of this thesis, the specific methane yields of a wide variety of paragrass was investigated. The untreated grass was inoculated with two types of sludge: (1) a typical anaerobic sludge obtained from a domestic wastewater treatment plant and (2) a sludge acclimated to fibrous substrates in POME. The results from this study not only demonstrate methods for enhancing methane yields from paragrass, but also suggest a biological approach for enhancing the methane yields of any grass.

1.2 Research Objectives

1.2.1 To enhance lignocellulose degradation of paragrass using isolated fungus from cow faeces and the white rot *P. tricholoma* (PT);

1.2.2 To investigate biogas production from fungal pretreated grass and to investigate its biodegradability;

1.2.3 To study the biodegradation and methane production of the paragrass using acclimated microbial consortium (AMC) as the inoculum;

1.2.4 To study the effects of the acclimatization of the mesophilic sludge in raw POME on the microorganism activities.

1.3 Scopes of Research Work

1.3.1 Paragrass samples were collected from Prachinburi, Thailand.

1.3.2 Fungus was isolated from cow faeces.

1.3.3 The grass was pretreated by the isolated fungus and *P. tricholoma* (PT) under aerobic conditions.

1.3.4 Microbial consortium was acclimated with POME for 2 weeks under a mesophilic condition.

1.3.4 The hydrolytic, acidogenic, acetogenic and acetoclastic activities of the AMC and the original sludge were observed;

1.3.5 Batch reactors were used for the Biochemical Methane Potential assays;

1.3.6 Investigated parameters included volatile solids (VS), mixed liquored dissolved solids (MLVSS), total solids (TS), cellulose, hemicelluloses, lignin, reducing sugar, volatile fatty acids (VFAs), biogas, cumulative biogas yields and biogas compositions.

CHAPTER 2
THEORY AND LITERATURE REVIEW

Grass is an important energy crop worldwide (Vogel, 2008)[114] with a great potential for biogas production via anaerobic digestion. However, the bioconversion of grass is limited by its aromatic constituents including both lignin and phenolic acid esters (Akin, 2007)[2]. A major limitation to biodegradation of nonlignified grass cell walls is due to the presence of esterified phenolic acids, i.e. ferulic and *p*-coumaric acid , especially in warm-season grass species (Akin, 2007)[2].

2.1 Components and Structures of Grass Lignocellulose

Grass typically consists of 25 - 40% cellulose, 25 - 50% hemicelluloses and 10 - 30% lignin (Malherbe and Cloete, 2002)[62]. The structure of the lignocellulose components are shown in Fig. 2.1a and 2.1b.

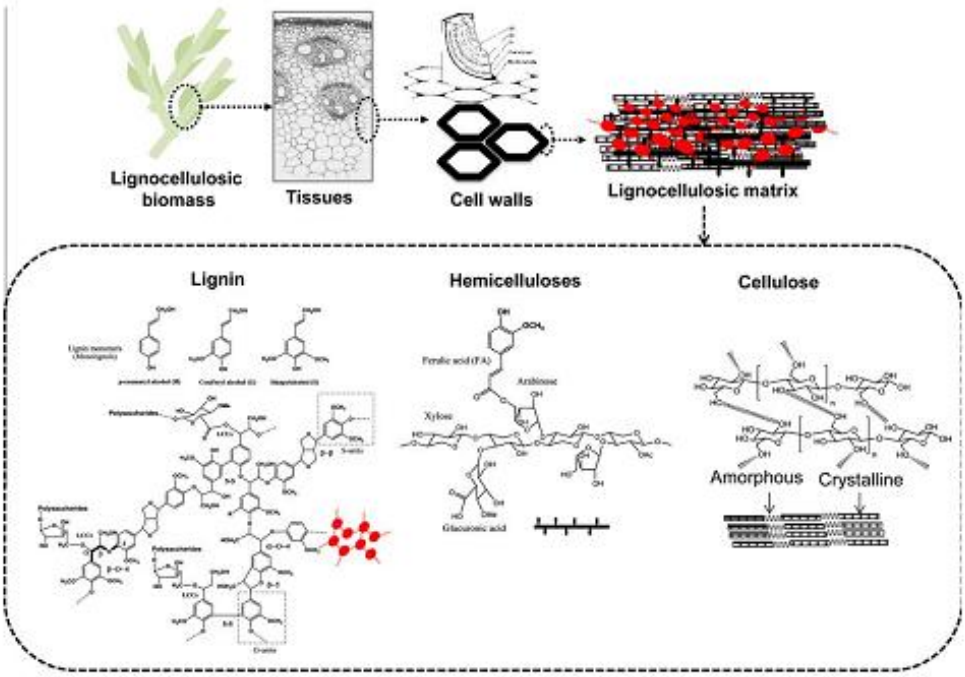


Fig. 2.1a Lignocellulosic biomass compositions (Barakat et al., 2013)[11].

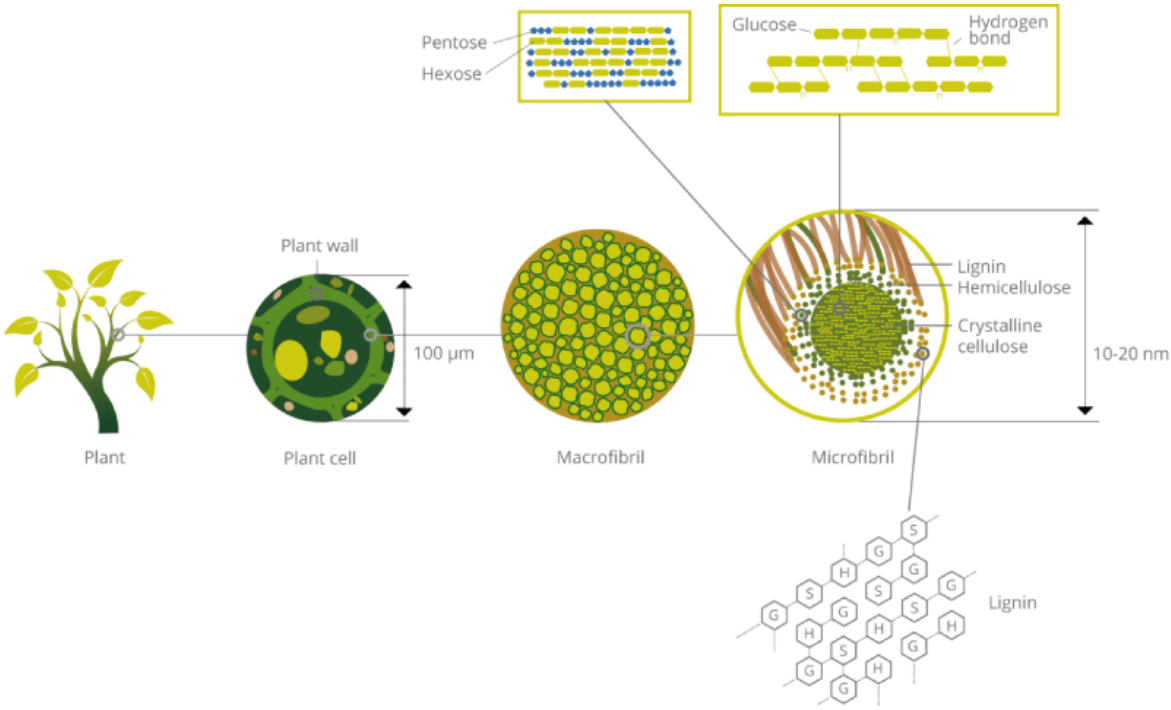


Fig. 2.1b Schematic structure of lignocellulose (Streffer, 2014)[100]. The hexagons denote the lignin subunits *p*-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (s).

Three main components of grass are cellulose, hemicellulose and lignin (Fig. 2.2).

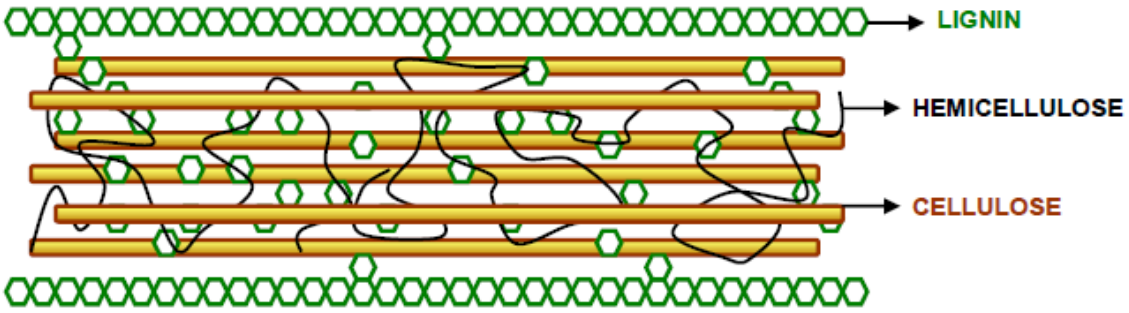


Fig. 2.2 Lignocellulose structure (Mussatto and Teixeira, 2010)[70].

Cellulose is a homo-polysaccharide that is composed of D-glucose subunits linked by β-1,4 glycosidic bonds forming the dimer cellobiose (Hatakka and Hammel, 2011)[42]. These form long chains (or elemental fibrils) linked together by hydrogen bonds and van der Walls forces. Cellulose may be presented in a crystalline form (crystalline cellulose) or non-organized cellulose chains (amorphous cellulose) (Sanchez, 2009)[93].

Hemicellulose is a heteropolysaccharide which is formed from D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methyl-glucuronic, D-galacturonic and D-glucuronic acids. Sugars are linked together by β-1,4- and all by β-1,3-glycosidic bonds. (Sanchez, 2009)[93]. The main hemicellulose component of grass is xylan (Malherbe and Cloete, 2002)[62] and glucoronoarabinoxylans (GAX) (Vogel, 2008)[114]. The GAX composes of β-1,4-linked xylose (Xyl) backbone with single arabinose (Ara) and glucuronic acid (GlcA). Fig. 2.3 shows the structure of glucoroarabinoxylans.

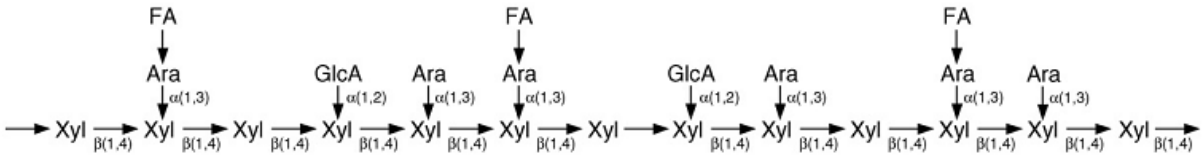


Fig. 2.3 Structure of glucoronoarabinoxylans (Vogel, 2008)[114].

Lignin is linked to both hemicellulose and cellulose. It is an amorphous hetero-polymer, non-water soluble and optically inactive. It is formed from phenylpropane units joined together by non-hydrolyzable linkages (Sanchez, 2009)[93]. The structure of lignin is shown in Fig. 2.4. Lignin consists of three phenylpropane units (*p*-coumaryl, coniferyl and sinapyl alcohol) with different kind of linkages (Fig. 2.5) (Hendriks and Zeeman, 2009)[44]. The total weight of grass consists of 5%-10% esterified *p*-coumaric acid. The *p*-coumaric acid molecules ester-linked with the side chain of lignin molecule. Fig. 2.6 shows a proposed structure of *p*-coumaric acid ester linkage in grass lignin (Jeffries, 1990)[51].

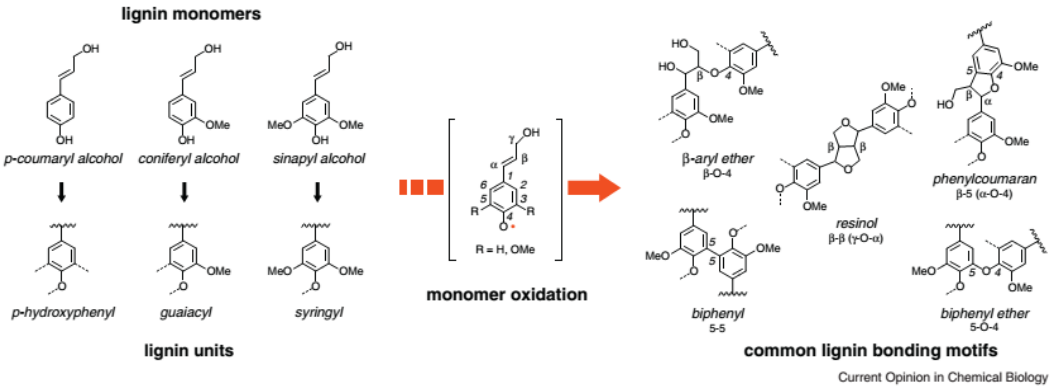


Fig. 2.4 Structure of lignin (Brown and Chang, 2014)[15].

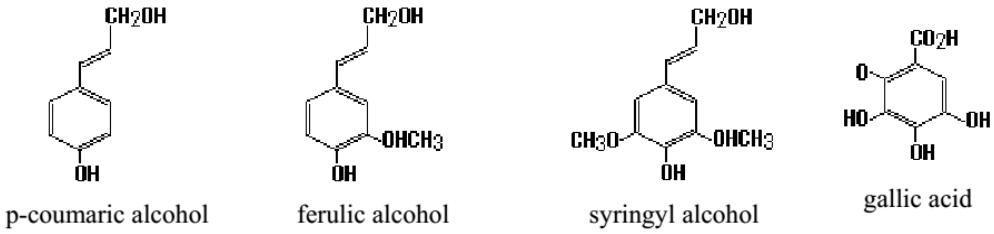


Fig. 2.5 Phenylpropane units of lignin (Samfira et al., 2013)[92].

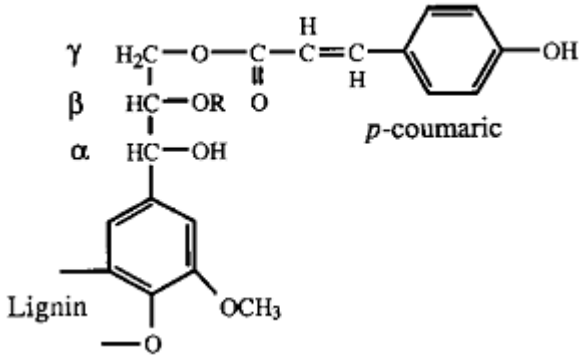


Fig. 2.6 Proposed *p*-coumaric acid ester linkage in grass lignin (Jeffries, 1990)[51].

In grass, ferulic acid and *p*-coumaric acid are esterified to hemicellulose and lignin. Xylan is a major interface between lignin and other carbohydrates. Ferulic acid anchors hydrophobic lignin to hydrophilic polysaccharide via alkali-sensitive ester bonds. Fig. 2.7 shows formation of diferulic acid in grass. Fig. 2.8 shows linkage of furulic acid ester and grass arabinoxylan. Intramolecular lignin bonds are usually of alkali-resistant ether type. This intricate association with lignin protects hemicellulose from direct enzymatic hydrolysis (Malherbe and Cloete, 2002)[62].

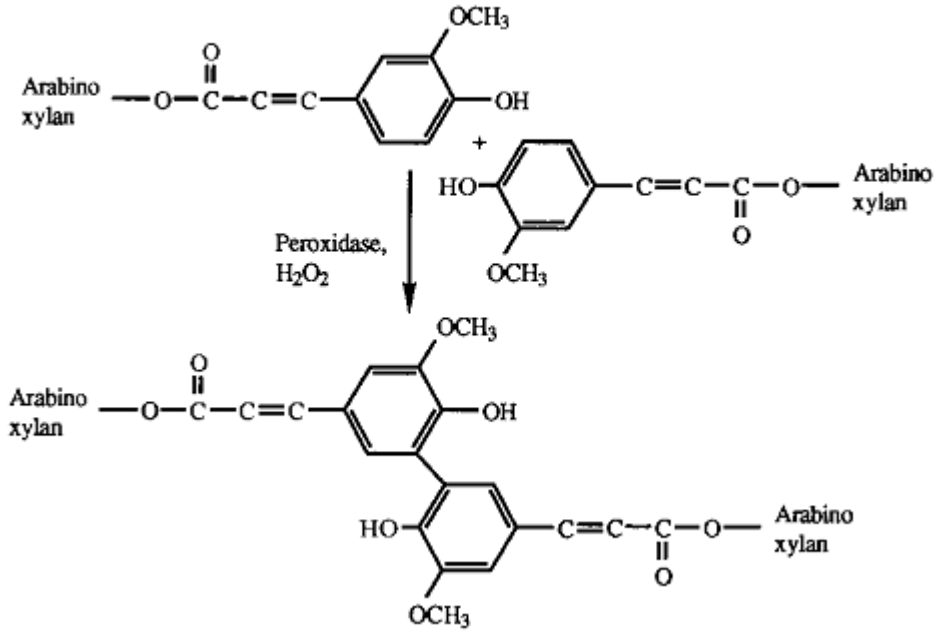


Fig. 2.7 Diferulic acid formation in grass (Jeffries, 1990)[51].

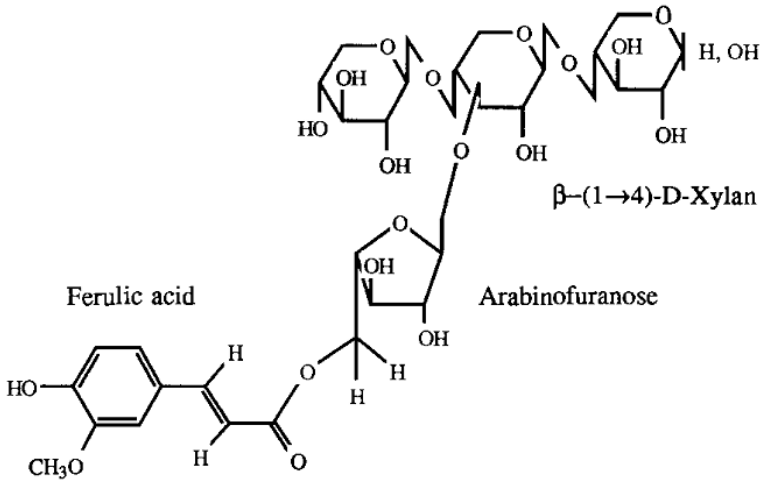


Fig. 2.8 Furulic acid ester linkage to grass arabinoxylan (Jeffries, 1990)[51].

2.2 Enzymatic Systems in Lignocellulosic Degradation

Feruloyl and *p*-coumaroyl esterases are relatively novel enzymes capable of releasing feruloyl and *p*-coumaroyl, and play an important role in the biodegradation of recalcitrant cell walls in grass. These enzymes act synergistically with xylanases to disrupt the hemicellulose-lignin association without mineralization of the lignin. Therefore,

hemicellulose degradation is required before efficient lignin removal can commence (Malherbe and Cloete, 2002)[62]. The enzymes degrading lignocellulosic substrate are shown in Fig. 2.9.

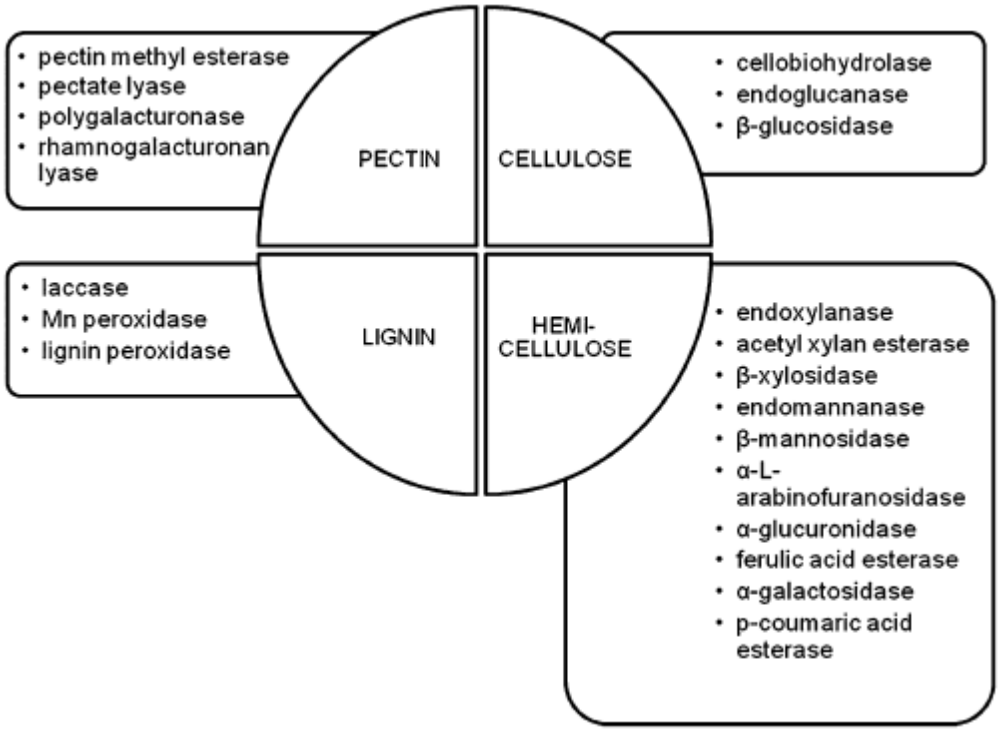


Fig. 2.9 Principal enzymes degrading lignocellulosic substrates (Cater et al., 2014)[16].

Cellulose hydrolysis

Cellulose is degraded by three enzymes, which are β-1,4-endoglucanase, exoglucanase or cellobiohydrolase and β-glucosidase, as shown in Fig. 2.10 (Mussatto and Teixeira, 2010)[70]. Endoglucanase attacks randomly at multiple internal sites in the amorphous regions of the cellulose fibre which opens-up sites for subsequent attack by the cellobiohydrolases. Cellobiohydrolase hydrolyzes highly crystalline cellulose. Cellobiohydrolase can remove monomer and dimers from the end of the glucanchain. Glucose dimers and cellulose oligosaccharides are hydrolyzed to glucose by β-glucosidase (Sanchez, 2009)[93].

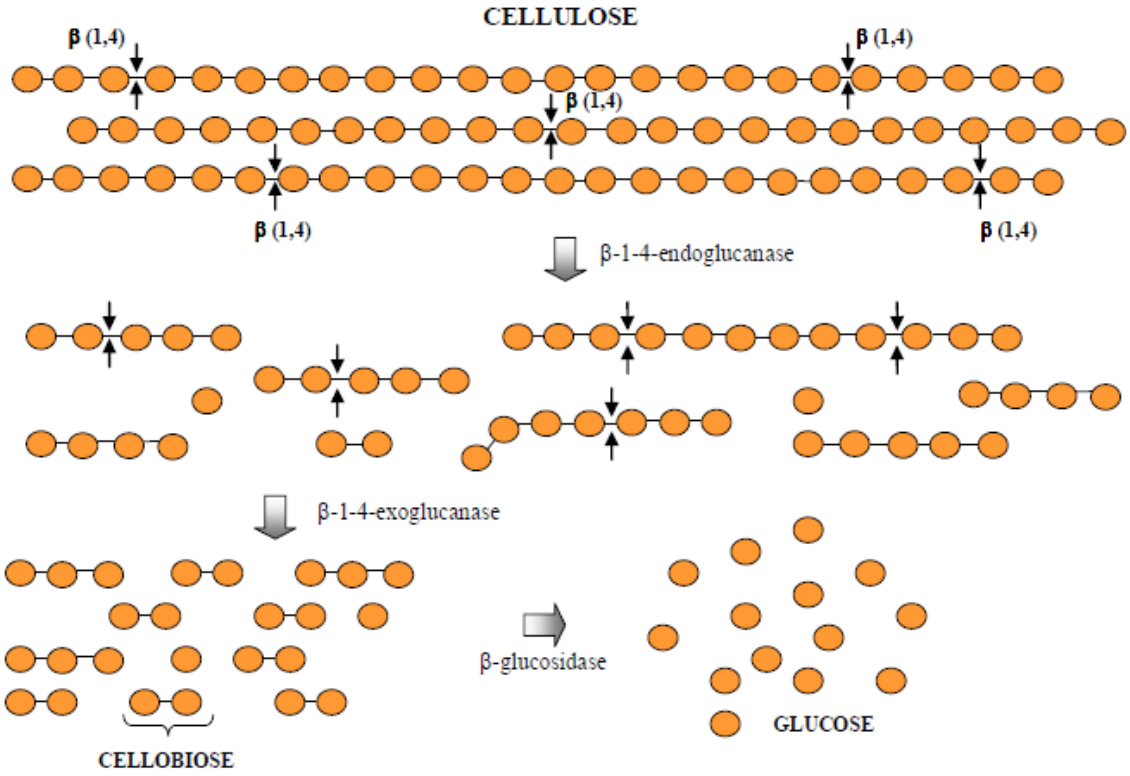


Fig. 2.10 Schematic structure of cellulose with cellulolytic enzymes (Mussatto and Teixeira, 2010)[70].

Hemicellulose hydrolysis

Hemicellulose is hydrolyzed by a specific set of dedicated carbohydrate active enzymes (Fig. 2.11): β-1,4-endoxylanase and β-1,4-xylosidase for xylan, xyloglucan active β-1,4-endoglucanase and β-1,4-glucosidase for xyloglucan, and β-1,4-endomannanase and β-1,4-mannosidase for (galacto-) mannan. Moreover, feruloyl/*p*-coumaroyl esterases can remove *p*-Coumaric acid and ferulic acid (Brink and Vries, 2011)[14]. O-acetyl-4-O-methylglucuronxylan is the most common hemicellulose, and it is degraded by four enzymes; β-1,4-endoxylanase, acetyl esterase, α-glucuronidase and β-xylosidase (Pérez et al., 2002)[80].

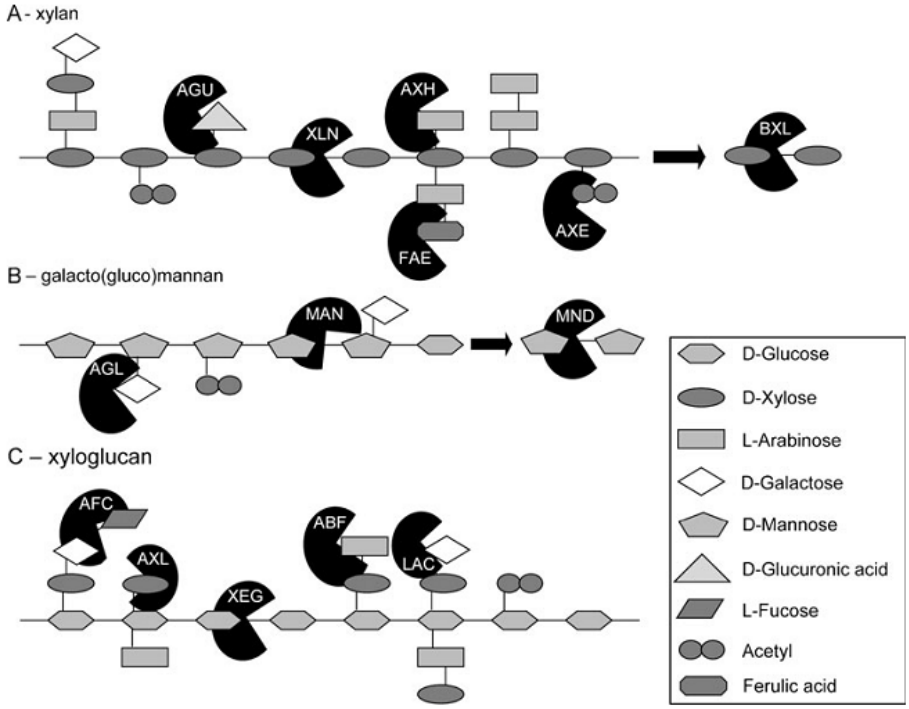


Fig. 2.11 Schematic structure of three hemicelluloses (xylan), galacto (gluco) mannan and xyloglucan) with hemicellulolytic enzymes; ABF: α-arabinofuranosidase, AFC: α-fucosidase, AGL: α-1,4-galactosidase, AGU: α-glucuronidase, AXE: acetyl (xylan) esterase, AXH: arabinoxylan-α-arabinofuranohydrolase, AXL: α-zylosidase, BXL: β-1,4-xylosidase, FAE: feruloyl esterase, LAC: β-1,4-galactosidase, MAN: β-1,4-endomannanase, MND: β-1,4-mannosidase, XEG: xyloglucan-active β-1,4-endoglucanase and XLN: β-1,4-endoxylanase (Brink and Vries, 2011)[14].

Lignin hydrolysis

Extracellular, oxidative and unspecific enzymes can liberate highly unstable products that further undergo many different oxidative reactions and catalyze the initial step of lignin depolymerization (Pérez et al., 2002)[80]. An oxidative process and phenol oxidases are the key enzymes of lignin biodegradation by white rot fungi. Non-phenolic lignin units are degraded by lignin peroxidase, while manganese peroxidase generates Mn^{3+} which acts as a diffusible oxidizer on phenolic or non-phenolic lignin units via lipid peroxidation reactions (Sanchez, 2009)[93]. Laccase is multi-copper enzymes and is defined as oxidoreductase which oxidizes diphenol and allied substances (Desai et al., 2011)[22]. Laccase oxidizes phenolic units in lignin to phenoxy radicals (Fukushima and Kirkk, 1995)[30] and also

oxidizes non phenolic lignin units (Moreno et al., 2014)[67]. Lignin peroxidase directly oxidizes both phenolic and non-phenolic compounds, while manganese peroxidase and laccase only act on phenolic compounds (Fig. 2.12). In the secondary pathway, these enzymes oxidize non-phenolic compounds indirectly by the action of a mediator. Finally, the catalytic mechanism of the versatile peroxidase can either be similar to lignin or manganese peroxidase (Moreno et al., 2014)[67].

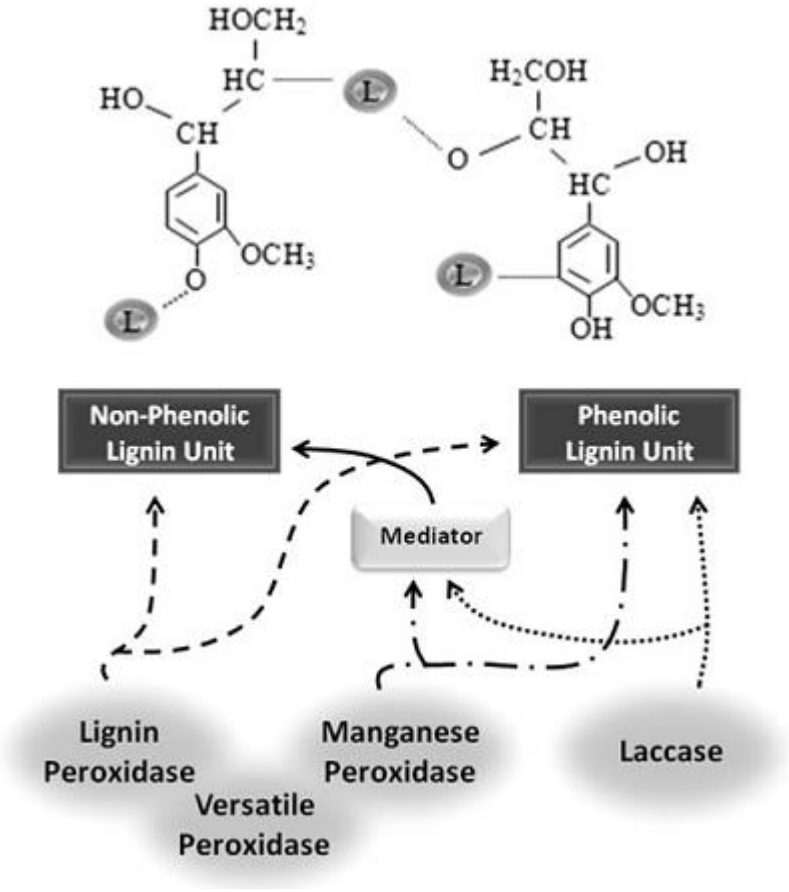


Fig. 2.12 Ligninolytic enzymes and their selective action on lignin components (Moreno et al., 2014)[67].

2.3 Fungal Solid-state Fermentation

This pretreatment uses microorganisms, such as fungi, which are able to degrade lignin. The lignin degradation requires oxygen and the pure lignin degradation process cannot serve as the sole energy and carbon source for microorganism (Streffer, 2014)[100].

Fungal pretreatment with lignin-degrading microorganisms has received renewed interest as an alternative to thermal or chemical pretreatments. The fungal pretreatment has feasibility for improving enzymatic digestibility of various biomasses such as corn stover, wheat straw, rice straw, cotton stalks and woody biomass. There are many advantages of the fungal pretreatment over thermal-chemical pretreatment including simple techniques, low energy requirements, reduced output of waste streams, reduced downstream processing costs (Wan and Li, 2012)[116].

Fungal pretreatment of lignocellulose is a method for digestibility improvement. White, brown and soft rot fungi are used for lignin and hemicellulose degradation. Brown rot attacks mainly cellulose. While white and soft rot attack both cellulose and lignin. White rot fungi are the most effective for biological pretreatment of lignocellulose materials (Mtui, 2009)[68]. White rot fungi involve powerful lignin degradation enzymes that enable them in nature to bridge the lignin barrier and overcome the rate limiting step in the carbon cycle (Malherbe and Cloete, 2002)[62].

White rot fungi are the most effective basidiomycetes for the biological pretreatment of lignocellulose (Mtui, 2009)[68] and the most active lignin degrading micro-organisms (Anderson and Akin, 2008)[5]. The fungi produce oxidative enzymes which degrade aromatic compounds. The oxidative enzymes include laccase, manganese peroxidase and lignin peroxidase (Anderson and Akin, 2008)[5].

Basidiomycetous white rot and some related litter-decomposing fungi are the only organisms which can capable of mineralizing lignin efficiently. More than 90% of all wood rotting basidiomycetes are the white rot type (Hatakka and Hammel, 2011)[42].

Usually syringl units of lignin are preferentially degraded while guaiacyl units are more resistant to degradation. Many white rot fungi colonize cell lamina and cause cell wall erosion (Fig. 2.13). Phenoloxidizing enzymes, such as laccase and peroxidase, are involved in lignin degradation by most species and are produced in quantity by white rot fungi (Garraway and Evans, 1984)[31]. However, white rot fungi also produce cellulases, xylanases and other hemicellulases (Isori et al., 2011)[49].

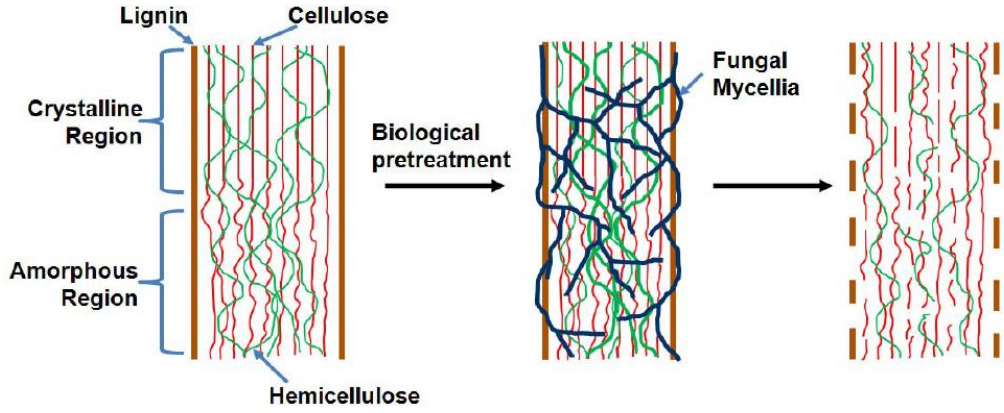


Fig. 2.13 Schematic diagram of lignocellulose degradation by white rot fungi (Isori et al., 2011)[49].

Lignin degradation by white rot fungi is described by Sanchez (2009)[93], shown in Fig. 2.14, the laccase or ligninolytic peroxidase oxidizes the lignin polymer and generates (a) aromatic radicals. Different non-enzymatic reactions are evolved including (b) C-4 ether breakdown, (c) aromatic ring cleavage, (d) Cα-Cβ breakdown and (e) demethoxylation. The substrates for H_2O_2 generation by AAO in cyclic redox reactions also involving AAD are the aromatic aldehydes released from Cα-Cβ breakdown of lignin or synthesized *de novo* by the fungus (f, g). Phenoxy radicals from C4-ether breakdown (b) can repolymerize on the lignin polymer (h) if oxidase is not first reduce the phenoxy radicals to phenolic compounds (i). Laccase or peroxidase can again reoxidize the phenolic compounds (j). Phenoxy radicals are subjected to Cα-Cβ breakdown (k), yielding *p*-quinones. Quinones from g and/or k contribute to oxygen activation in redox cycling reactions with QR, laccases, and peroxidase (l, m). This results in the reduction of the ferric iron present in wood (n), either by superoxide cation radicals or directly by the semiquinone radicals. Its reoxidation is concomitant with the reduction of H_2O_2 to a hydroxyl free radical (OH×)(o). The latter is a very mobile and very strong oxidizer that can initiate the attack on lignin (p) in the initial stage of wood decay, when the small size of pores in the still-intact cell wall prevents the penetration of ligninolytic enzymes. Then lignin degradation proceeds by oxidative attack of the enzymes. In the final steps, simple products from lignin degradation enter the fungal hyphae and are incorporated into intracellular catabolic routes.

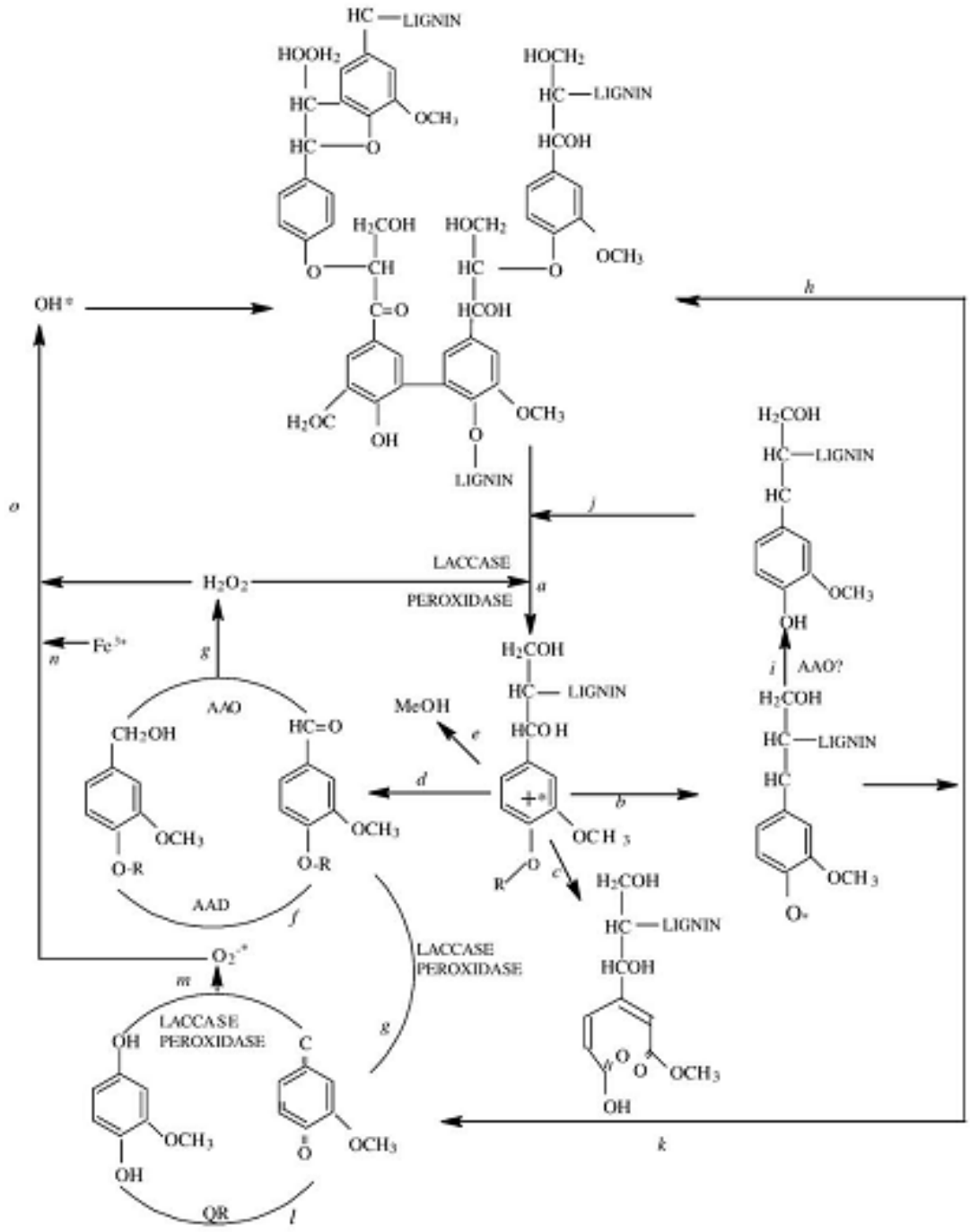


Fig. 2.14 Lignin biodegradation process by white rot fungi (Sanchez, 2009)[93].

Lignocellulose degradation needs the synergistic action of hydrolytic and oxidative enzymes. Xylanase and feruloyl esterase act synergistically with other hydrolytic enzymes to modify lignocellulose structure. The esterase cleaves covalent bonds between polysaccharide of hemicellulose and lignin. So, the combination between xylanase and

esterase is a key role in the matrix degradation of lignin-hemicellulose. However, esterase activity for white rot fungi grown on lignocellulose is limited (Dong et al., 2013)[24].

The degradation of lignin by fungi may be used to allow better access to the cellulose and hemicellulose components, and is considered to be an effective biological detoxification alternative (Mussatto and Teixeira, 2010)[70]. Fungi may also attacks cellulose and hemicellulose and hydrolysis rate in biological materials is very low (Mussatto and Teixeira, 2010)[70].

Coprinopsis cinerea (*Coprinus cinereus*) belongs to the genus *Coprinus*, family Coprinaceae in division Basidiomycota. Several *Coprinus* sp. are known and are used for food and medicine (Raymond et al., 2012)[87]. *C. cinerea* is usually found in fields and forest moreover, it can grow on forest humus soil (Guiraud et al., 1999)[40]. *C. cinerea* is defined as wood rotting fungi (Heinzkill et al., 1998)[43]. *C. cinerea* produces laccase (Hatakka and Hammel, 2011)[42] and cellulase including β-glucosidas, endocellulase, cellobiohydrolase (Zifcakova and Baldrian, 2012)[130]. Tuomela et al. (2000)[109] reports that lignin can be degraded by *C. cinerea*.

Polyporus tricholoma is a white rot fungi (Kruger et al., 2004)[54] that can produce laccase (Gnanasalomi and Gnanadoss, 2013)[37]. *P. tricholoma* belongs to the phylum Basidiomycota. It is an important source of medicinal substances, such as antibacterial metabolites (Vieira et al., 2008)[113].

Solid state fermentation (SSF) is defined as the fermentation process that involves a solid matrix (Singhania et al., 2009)[97] in the absence or near absence of free water (Pandey, 2003)[79]. In general, the solid state fermentation is the most suitable fungal and yeast cultures (Thomas et al., 2013)[106] especially the enzyme production by filamentous fungi (Couto and Sanroman, 2005)[19].

Solid state fermentation is combined with the capability of white rot fungi to make possible industrial scale application of lignocellulose-based biotechnologies (Fig. 2.15). There are many advantages such as outperforms conventional fermentation technologies with respect to simplicity, cost effectiveness and maintenance requirements (Isori et al., 2011)[49].

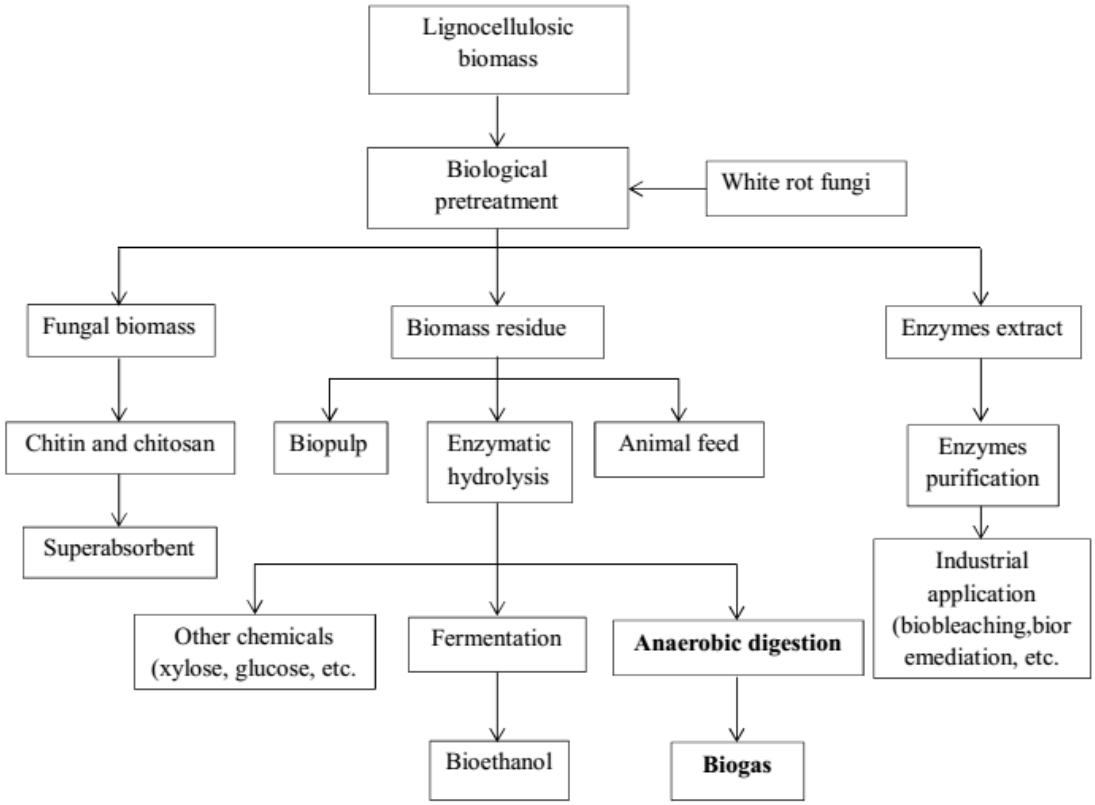


Fig. 2.15 Alternative application routes of lignocellulose pretreatment with white rot fungi (Isori et al., 2011)[49].

Fungi is the most organism that adapts to solid state fermentation because fungal hyphae can grow on particle surfaces and penetrate into the interparticle spaces, thereby colonizing solid substrates (Graminha et al., 2008)[38]. The fungi degradation process during solid state fermentation is descript by Holker and Lenz (2005)[46] and shown in Fig. 2.16.

The fungal hyphae develops into a mycelial mat (black) after sporulation and the hyphae spreads over the surface of the particles that contain the solid substrate (brown). From the mycerial mat, gaseous space is protruded by aerial hypha whereas liquid filled pores are penetrated by the other substrate growing. At normal moisture levels, gas fills the void spaces between the aerial hyphae (g), whereas liquid fill the void spaces within the mycelial mat and within the substrate (l). The metabolic activities show mainly occur near the substrate surface and within the pores; however, exposed regions of the mycelium (for instance the aerial hyphae) also show metabolism and there can be a transport of substances from the penetrative to the aerial hyphae. Hydrolytic enzymes (light blue), which are

produced by the mycelium, diffuse to the solid matrix and catalyse the degradation of macromolecules into smaller units (green). The latter are taken up by the fungus to serve as nutrients. O_2 is consumed and CO_2, H_20, heat and interesting biochemical products are produced during fermentation. Then, gradients develop within the biofilm that, for instance, force O_2 to diffuse from the gaseous phase into deeper regions of the biofilm (lilac) and CO_2 to diffuse from these regions to the gaseous phase (red). Heat development (Q; orange) leads to a fast increase in temperature (T), which is a serious problem during solid state fermentation. Heat is therefore removed from the substrate not only via conduction but also by evaporation, which is part of the complex balance of water in the system (dark blue). Beside evaporation, water balance includes water uptake by the mycelium in the course of growth, water consumption during hydrolysis reactions and water production through respiration. As another factor, local pH, might be changed owing to the release of carbon acids and the exchange of ammonia (grey). The biochemical products of interest (magenta) that are released into the solid matrix and the liquid-filled spaces during fermentation might absorb to the solid and might have to be extracted for further use at the end of the process. All these and many other phenomena can strongly influence the process performance during SSF.

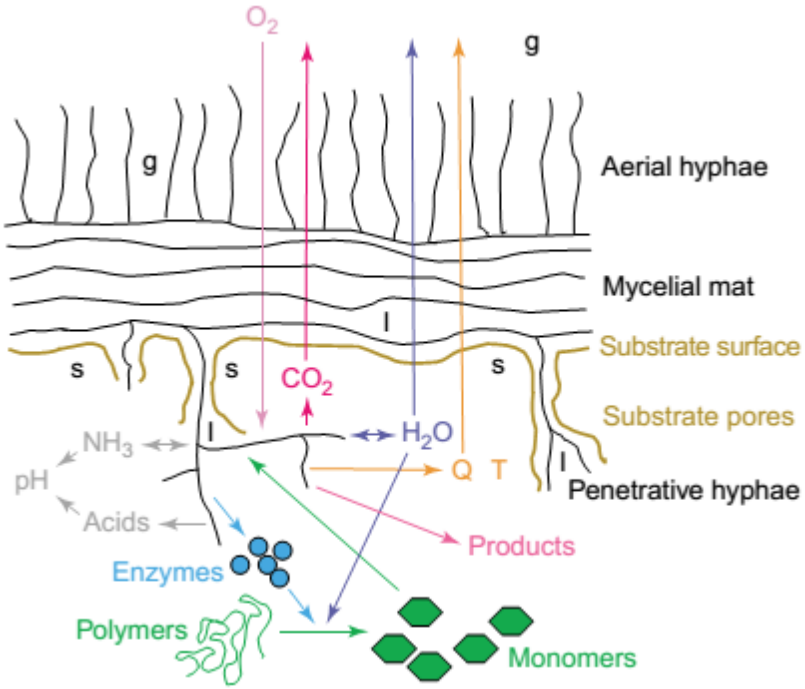


Fig. 2.16 Schematic of lignocellulose degradation by fungi during solid state fermentation on micro-scale (Holker and Lenz, 2005)[46].

Inoculum for solid state fungal pretreatment can be prepared by different methods, such as mycelium grown in liquid or agar medium, and spawn grown in cereal grains or fungal pre-colonized substrate (Wan and Li, 2012)[116]. The initial moisture content of the substrate is important to the fungal establishment and growth and affects secondary metabolism in fungal pretreatment. The initial moisture of the optimal level for the lignocellulose degradation and ligninolytic activities of most white rot fungi is range from 70 to 80% (Wan and Li, 2012)[116]. The low moisture content means that fermentation can only be carried out by a limited number of microorganisms (Couto and Sanroman, 2006)[20]. In general, high moisture content is favorable for formation of fungal mycelia but not necessarily for increased delignification (Wan and Li, 2012)[116]. Large particle size can hamper the penetration of fungi into cellulosic biomass and prevent the diffusion of air, water and metabolite intermediates into the particles. However, the reduced particle size with a decreased size of antiparticle channel may adversely affect interparticle gas circulation thus not necessarily giving an enhanced delignification rate (Wan and Li, 2012)[116]. Decontamination of feedstock can effectively kill or inhibit indigenous microorganisms in the feedstock and is generally required prior to fungal pretreatment. However, decontamination poses one of the major costs for fungal pretreatment (Wan and Li, 2012)[116]. Long pretreatment time, due to low delignification rates, is a major barrier to large-scale application of fungal pretreatment. Generally, several weeks to months are needed to obtain a high degree of lignin degradation (Wan and Li, 2012)[116].

White rot asscodiomycetes grow well around 39 °C, while white rot basidiomycetes grow between 15 and 35 °C. High lignification rate is obtained within an optimal temperature range 25 to 30 °C (Wan and Li, 2012)[116]. Lignin is degraded by an oxidative process therefore oxygen availability is important for ligninolytic enzyme activity of white rot fungi (Wan and Li, 2012)[116].

2.4 Previous Studies on Degradation of Lignocellulose by Fungi

2.4.1 Effectiveness of Fungal Pretreatment on Biodegradation of Lignocellulose

Ejechi and Ogbimi (1996) [26] studied the biodegradation of wood (Oboche and mahogany, supplemented with potato dextrose broth) by *Gloeophyllum sepiarium, Gloeophyllum* sp. and *Pleurotus ostreatus*. The aerobic fungal pretreatment was carried at room temperature (30+2 °C) for 12 weeks. It was illustrated that *Gloeophyllum* sp. degraded

cellulose but not degraded lignin. *P. ostreatus* attacked lignin after substantially degrading of cellulose. Lignin peroxidase was detected only *P. ostreatus.*

Song and Deng (2004)[98] studied the biodegradation of straw (supplemented with media culture) by 13 edible fungi; *Trichotama mongolicum, Agaricus bisporus, Agarixus blazei, Coprinus comatus, Pleurotus ostreatus, P. cetrinipileatus, P. comucopiae, P. eryngfi* var. *nebrodensis* Inzenga., *Hericium erinaceus, Photiota nameko, Flammulina velutipes, F. velutipes,* and *Hohenbuehella serotina*. The aerobic fungal pretreatment was carried at 25 °C for 7 days. It was illustrated that all fungi except *T. mongolicum* could degrade the lignin and cellulose of the straw. *P. ostreatus* was the highest lignin degradation (17.86%) and the lowest cellulose degradation (2.24%).

Auer et al. (2005)[9] studied the nitrocellulose degradation (12% N content of alongside amino acids or as sole N source, and starch or carboxy-methyl cellulose as carbon source) by three lignocellulolytic fungi (*Trametes versicolor*, *Pleurotus ostreatus* and *Coprinus cinereus*) and two cellulolytic fungi (*Trichoderma reesei* and *Chaetomium elatum*). It was illustrated that *C. elatum* degraded nitrocellulose (43%) when the medium contained nitrocellulose as the only nitrogen source. *C. cinereus* decreased nitrocellulose (37%) when the amino acid and starch was the co-substrate. *T. versicolor*, *P. ostreatus* and *T. reesei* degraded only 10%-20% of nitrocellulose in all media. *C. cinereus* degraded nitrocellulose when starch was the carbon source and no organic N supplied. The white rot fungus *C. cinereus* could hydrolyse more nitro groups from the nitrocellulose polymer. Nitrate released could be absorbed by the fungus and used for metabolism, including that arising from autocatalytic hydrolysis or by active hydrolysis by the fungus.

Wu et al. (2005)[121] studied the lignin degradation of black liquor from a pulp and paper mill (Supplemented with KH_2PO_4, $MgSO_4.7H_2O$, glucose as the carbon source, and ammonium tartrate as the nitrogen source) by five fungi: *Phanerorochaeta chrysosporium, Pleurotus ostreatus, Lentinus edodes, Trametes versicolor* and S22. The aerobic fungal pretreatment was carried at 28 °C for 16 days. It was illustrated that three white fungi, P. *chrysosporium*, *P. ostreatus* and S22, degraded lignin at pH 9.0-11.0.

Isikhuemhen and Mikiashvilli (2009)[48] studied the biodegradation of solid waste (containing 70-80% wheat straw, 10-20% solid waste and 10-20% millet) by *Pleurotus ostreatus* strain MBFBL400. The aerobic fungal pretreatment was carried at 25 ± 2 °C. It was illustrated that *P. ostreatus* selectively used hemicellulose over cellulose in biomass and the organic matter loss was 45.8-56.2%.

Dong et al. (2013)[24] studied the biodegradation of sugarcane bagasses (supplemented with liquid Czapek culture medium) by three lignin degrading fungi: *Phanerochaete chrysosporium* PC2, *Lentinula edode* LE16 and *Pleurotus ostreatus* PO45. The aerobic fungal pretreatment was carried out at 25 °C for 13 weeks. It was illustrated that these fungi degraded lignin (85 – 93%), hemicellulose (64 - 88%) and cellulose (15 - 64%) in 12 weeks. The lignocellulose enzymes polyphenol oxidase (PPO) and manganese peroxidase (MnP) were produced first and the cellulolytic enzyme (CMCase) was produced subsequently. *P. ostreatus* PO45 degraded the syringyl units over guaiacyl units of SCB and this fungus primarily degraded the aromatic rings to aliphatic hydrocarbons by laccase. *P. ostreatus* PO45 destroyed the major ester linkages between lignin and hemicellulose by esterase.

2.4.2 Effectiveness of fungal pretreatment on enhancing biogas production

Ghosh and Bhattacharyya (1999)[32] studied the enhancement of biogas production from rice straw using the fungal pretreatment of the lignocellulose by white rot fungus, *Phanerochaete chrysosporium*, and brown rot fungus *Polyporus ostreiformis*. It was illustrated that the fungal pretreatment helped enhance the lignin degradation of 47.51% by *Phanerochaete chrysosporium* and 19.87% by *Polyporus ostreiformis*. The anaerobic digestion was carried out in a 5 L continuous stirred tank reactor (CSTR) at pH 7-8, temperature of 30 °C for 63 days and using inocular slurry as the inoculum. The biogas and methane production was increased about 34.73% and 46.19% in *Polyporus ostreiformis* -treated straw and 21.12% and 31.94% in *Phanerochaete chrysosporium* pretreated straw, respectively. VFA production also increased in *Phanerochaete chrysosporium* and *Polyporus ostreiformis* treated straw compared to control straw which were 76.73% and 30.69%, respectively. The rate of reduction of COD during the initial period of digestion after 21 operating days was 59.01%, 55.55% and 26.00% in *Phanerochaete chrysosporium* -treated straw, *Polyporus ostreiformis* -treated straw and control straw, respectively.

Amirta et al. (2006)[3] studied the enhancement of methane production from Japanese cedar wood (adding wheat bran) using fungal pretreatment of *Ceriporiopsis subvermispora* ATCC 90467, CZ-3, CBS 347.63 and *Pleurocybella porrigens* K-2855. It was illustrated that pretreatments with *C. subvermispora* ATCC 90467, CZ-3 and CBS 347.63 in the presence of wheat bran for 8 weeks decreased 74–76% of β-O-4 aryl ether linkages in the lignin to accelerate the production of methane. The anaerobic digestion was carried out in 500 ml Erlenmeyer flask at 35 °C for 60 days and using digested sludge from

the Noshiro sewage treatment plant (Noshiro, Akita Pref, Japan) as the inoculum. The methane yield of the pretreated wood with *C. subvermispora* ATCC90467 in the presence of wheat bran reached 35% and 25% of the theoretical yield based on the holocellulose contents of the decayed and original wood, respectively. The pretreated wood with *P. porrigens* was a lower ability to linin decomposition. Therefore, the pretreated wood with *C. subvermispora* promoted methane fermentation of soft wood in the presence of wheat bran.

Muthangya et al. (2009)[72] studied the enhancement of biogas production from sisal leaf decortication residues by fungal pretreatment of the lignocellulose by a ligninolytic CCHT-1 strain and *Trichoderma reseei*. It was illustrated that the fungal pretreatment helped enhance the degradation of neutral detergent fiber by 45.5 ± 1.8 % to 38.2 ± 1.1 % at 50% inoculum concentration. The anaerobic digestion was carried out in a 200 mL bioreactor at 28±2°C using anaerobic sludge as the inoculum. The methane production of the pretreated sisal leaf decortication residues with CCHT-1 for 4 days was 0.203 ± 0.019 m^3 CH_4/ kg VS_{added}, while the methane yield of the pretreated sisal leaf decortication residues with *T. reseei* for 8 days was 0.192 ± 0.024 m^3 CH_4/kg VS_{added}. The methane yield from the untreated sisal leaf decortication residues was 0.145 ± 0.015 m^3 CH_4/kg $VS_{added.}$ Moreover, increasing the *T. reseei* inoculum concentration led to a decrease in methane yield. The increasing of fungal inoculum concentration resulted to the methane yield decreasing because more polysaccharide was removed than lignin and starch. The methane yield decreasing with increasing of fungal inoculum concentration led to a decrease in readily available nutrient for biogas production. White rot fungi metabolize sugar and starch in preference to lignin and cellulose in cultures so white rotted material did not contain much nutrient.

Phutela et al. (2011)[82] studied the enhancement of biogas production from chopped and moist paddy straw (3-4 cm) using the fungal pretreatment of the lignocellulose by *Trichoderma reesei* MTCC 164 and *Coriolus versicolor* MTCC 138. It was illustrated that the *T. reesei* pretreatment had helped enhance the degradation of cellulose, hemicellulose and lignin by 28.9 %, 24.8 % and 11.6 %, respectively. The *C. versicolor* pretreatment helped enhance the degradation of cellulose, hemicellulose and lignin by 26.0 %, 23.6 % and 9.1%, respectively. The anaerobic digestion was carried out in a 2 L digester for 25 days using digested cattle dung slurry and cattle dung as the inoculum. The biogas was increased about 20.8% in the *T. reesei* pretreated paddy straw. The biogas was increased about 26.2% in the *C. versicolor* pretreated paddy straw.

Mackulak et al. (2012)[61] studied the enhancement of biogas production from the sweet chestnut (*Castanea sativa*) leaves and hay using fungal pretreatment of the lignocellulose by *Auricularia auricular-judae*. The anaerobic digestion was carried out in a continuously stirred tank reactor (CSTR) at 37 – 38 °C, using sludge as the inoculum. The biogas production was increased by 15%. The utilization of the pretreated leaves and hay leaded to a gradual increase of the concentration of formic, acetic and volatile fatty acids as well as to the formation of some aldehydes, ketones and alcohols.

Phutela et al. (2012)[81] studied the enhancement of biogas production from paddy straw using the fungal pretreatment of the lignocellulose by *Pleurotus florida*. It was illustrated that the fungal pretreatment had helped enhance the degradation of cellulose and lignin by 19.3% and 55.1%, respectively. The anaerobic digestion was carried out in a 2 L digester at 37 °C for 45 days using the digested cattle dung slurry as the inoculum. The biogas of the pretreated paddy straw was increased about 15.4%

Feng et al. (2013)[27] studied the enhancement of biogas production from straw using the fungal pretreatment of the lignocellulose by 12 fungal strains. The anaerobic digestion was carried out in 250 mL bottle at 25 °C, for 88 days. It was illustrated that the fungi could grow well on the straw, but the methane potential of fungal pretreated straw gave no significantly higher biogas potential than that of untreated straw. Some carbon from straw was lost during the growth of fungi under pretreating times.

Jasko et al. (2013)[50] studied the enhancement of biogas production from sawdust using the fungal pretreatment of the lignocellulose by *Pleurotus ostreatus*. The anaerobic digestion was carried out in two continuously stirred tank reactors at 37 °C. The methane yield of the pretreated sawdust was 610 ± 23 L/ kg VS, while that of the untreated substrate was 252 ± 9 L/ kg VS.

Liu et al. (2014)[58] studied the enhancement of biogas production from corn stover and corn stover silage using the fungal pretreatment of the lignocellulose by *Phanerochaete chrysosporium*. It was illustrated that the fungal pretreatment had helped enhance the degradation of cellulose, hemicellulose and lignin by 54.7%, 64.0% and 61.1%, respectively. The anaerobic digestion was carried out in a 250-mL glass serum vials at 37 ± 1 °C for 60 days. The methane yield of the pretreated corn stover was increased about 10.5% because pretreatment degreased dry mass loss about 14.2% and increase substrate biodegradability (19.9% cellulose, 32.4% hemicellulose and 22.6% lignin). In contrast, the higher dry mass loss in corn stover about 55.3% after microbial pretreatment was accompanied by 54.7%

cellulose, 64.0% hemicellulose and 61.1% lignin degradation but did not significantly improve biogas production.

Zhao et al. (2014a)[127] studied the enhancement of biogas production from yard trimmings using the fungal pretreatment of the lignocellulose by *Ceriporiopsis subvermispora*. It was illustrated that the fungal pretreatment had helped enhance the degradation of lignin, cellulose, hemicellulose and dry weight for 30 days by 14.8 - 20.2%, 8.1 - 15.4%, 20.7 - 27.8% and 9.8 - 16.2%, respectively. The anaerobic digestion was carried out in a 1 L glass bottle reactor at 37 ± 1 °C for 28 days. The methane yields of the pretreated yard trimmings was 34.9 - 44.6 L/kg VS, while that of the untreated substrate was 20 L/kg VS.

Zhao et al. (2014b)[128] also studied the enhancement of biogas production from yard trimmings using the fungal pretreatment of the lignocellulose by *Ceriporiopsis subvermispora*. It was illustrated that the fungal pretreatment had helped enhance the degradation of lignin by 20.9% but the fungal pretreatment limited cellulose degradation by 7.4%. The anaerobic digestion was carried out in a 1-L glass reactor at 37 ± 1 °C for 40 days. The methane yield of the pretreated yard trimmings was 44.6 L/kg VS, while that of the untreated substrate was 17.6 L/kg VS. The increasing of methane production was probably caused by the lignin digestion during the fungal pretreatment.

2.5 Hydrolysis of Lignocellulose by Anaerobic Bacteria

Organic material digestion of methane and carbon dioxide is a complex system of biochemical anaerobic reactions (Fig. 2.17). The reactions can be divided into four groups: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Each step has specific microorganisms groups (Lidholm and Ossianaaon, 2008)[56].

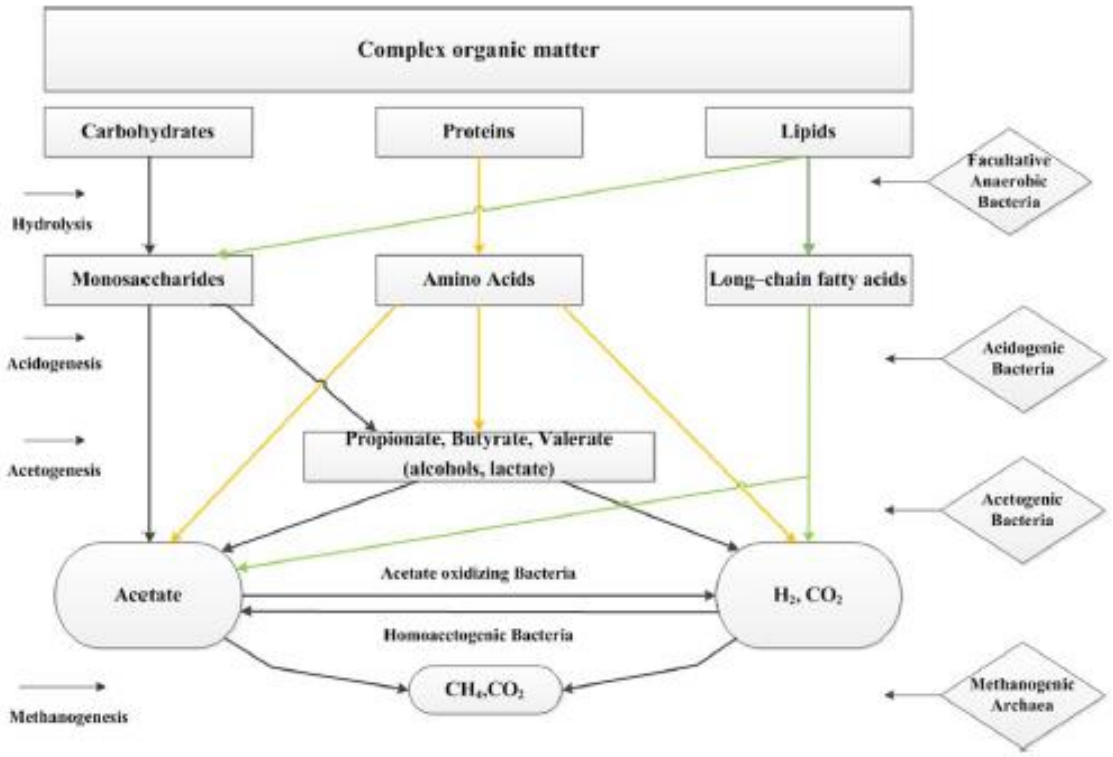


Fig. 2.17 Anaerobic model (Christy et al., 2014)[17].

In the first state, complex organic materials are broken down into their constituent parts in a process by extracellular enzymes. The result is soluble monomers: proteins are broken down into amino acid, fats into long chain fatty acids, and carbohydrates into simple sugar, while the liquefaction of complex biological polymers, especially cellulose, to simple, soluble substrate is often the rate-limiting step in digestion (Lidholm and Ossianaaon, 2008)[56]. Methanogenesis passes through extracellular enzymes from the group of hydrolases such as amylases, proteses and lipases produced by hydrolytic bacteria (Shah et al., 2014)[96].

Hydrolysis is often the rate limiting step when the particulate matter is not readily degradable or in systems with high loading rates. Even though the dynamics of hydrolysis of some individual substrates are known, the process is often described as a simple first order process due to extensive variations in substrate composition (Batstone, 2006)[12]. The rate of hydrolysis is governed by the nature and availability of the substrate, bacterial population, temperature, and pH.

In terms of lignocellulose degradation, a microbial consortium has high cellulose and hemicellulose degradation ability (Zheng et al., 2014)[129], but the digestion rate decreases in the presence of lignin (Barakat et al., 2014)[10]. Many factors limit the hydrolysis of lignocellulose such as lignin content, crystallinity of cellulose, particle size (Hendriks and Zeeman, 2009)[44], protection of cellulose by lignin and by hemicellulose (Taherzadeh and Karimi, 2008)[103], accessible surface area, degree of cellulose polymerization and degree of hemicellulose acetylation (Zheng et al., 2014)[129]. Therefore, pretreatment is usually performed to improve the digestibility of lignocellulose (Hendriks and Zeeman, 2009)[44].

Cellulose fibers are tightly linked to other polymers, such as hemicellulose and lignin, so cellulose-containing materials are difficult to degrade. Bacteria usually hydrolyze cellulose slowly (Tsavkelova and Netrusov, 2012)[108]. Aerobic bacteria able to degrade cellulose including *Acidothermus*, *Bacillus*, *Caldibacillu*, *Cellulomonas*, *Cellvibrio*, *Cytophaga*, *Dyella*, *Erwinia*, *Microbacterium*, *Micromonospora*, *Pseudomonas*, *Pseudoxanthomanas*, *Sporocytophaga*, *Rhodothermus*, *Streptomyces* and *Thermobifida* (Tsavkelova and Netrusov, 2012)[108]. Cellulolytic anaerobic bacteria include *Acetivibrio*, *Anaerocellum*, *Bacteroides*, *Butyrivibrio*, *Caldicellulosiruptor*, , *Cellobacterium*, *Clostridium*, *Desulfurococcus*, *Enterococcus*, *Eubacterium*, *Fibreobacter*, *Halocella*, *Ruminococcus*, *Spirochaeta* and *Thermotoga* (Tsavkelova and Netrusov, 2012)[108].

Anaerobic thermophilic cellulose degraders are mainly specialized. Many species cannot grow on mono-, oligo- or polysaccharides consisting of monomers other than glucose (Tsavkelova and Netrusov, 2012)[108]. Unlike aerobes, which degrade cellulose with an extracellular enzyme complex, anaerobes do it with multienzyme cellulose complexes, known as cellulosomes, as shown in Fig. 2.18 (Tsavkelova and Netrusov, 2012)[108]. In aerobic conditions, cellulose is completely degraded to carbon dioxide and water but cellulose degradation under anaerobic conditions releases carbon dioxide, methane and water (Pérez et al., 2002)[80]. *Clostridium* sp. is the most complex and the best investigated cellulosome especially, thermophilic bacterium *Clostridium thermocellum* (Schwarz, 2001)[94], gram positive, sporulated bacterium (Pérez et al., 2002)[80]. Anaerobic bacteria can hydrolyze crystalline cellulose, as shown in Table 2.1.

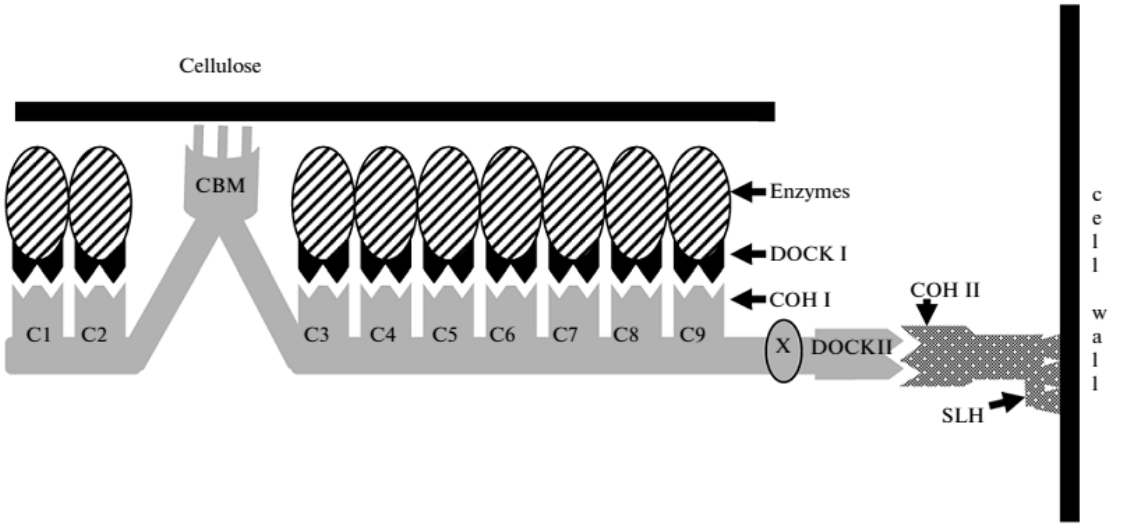


Fig. 2.18 Cellulosome structure of *C. thermocellum*. The module structure of scaffoldin is shown in gray, enzyme components in dark gray, CBM: carbohydrate binding module, x: X module, C1-C9: cohesins, COH I, II: type I and TT cohesion domains, DOCK I, II: type I and II dockerin domains; SLH: the surface layer homologous module which binds a complex to a bacterial cell wall (Tsavkelova and Netrusov, 2012)[108].

Bacterial xylanases have been found in several aerobic species and some ruminal genera (Pérez et al., 2002)[80]. Thermophilic xylanases have been described in actinobacteria (formerly actinomycetes), such as *Thermomonospora and Actinomadura* (Pérez et al., 2002)[80]. Moreover, a very thermostable xylanase has been isolated from the hyperthermophilic primitive bacterium *Thermotoga* (Pérez et al., 2002)[80]. However, *Bacillus* sp. has been found in xylanases active in alkalines (Pérez et al., 2002)[80]. Xylan can be hydrolyzed by *Bacteroides, Butyrivibrio, Prevotella, Ruminococcus, Clostridium* and *Lacnospira* (Tsavkelova and Netrusov, 2012)[108]. β-Xylosidases have been found in *B. stearothermophilus* and the ruminal bacterium *Butyrivibrio fibrisolvens* (Pérez et al., 2002)[80].

Table 2.1 Anaerobic bacteria actively hydrolyzing crystalline cellulose (Schwarz, 2001)[94]. Column T- m: mesophilic, h: thermophilic above 50 °C

Phylogeny	Genus	Species	T	Source
Family	*Caldocellulosiruptor*	*saccharolyticus*	h	Hot spring
Syntrophomonodaceae	*Caldocellulosiruptor*	*lactoaceticus*	h	
	Caldocellulosiruptor	*kristjanssonii*	h	Hot spring
	Anaerocellum	*thermophilum*	h	
Family	*Butyrivibrio*	*fibrisolvens*	m	Rumen
Lachnospiraceae	*Ruminococcus*	*flavefaciens*	m	Rumen
	Ruminococcus	*succinogenes*	m	Rumen
	Ruminococcus	*albus*	m	Rumen
Family Eubacteriaceae	*Eubacterium*	*cellulolyticum*	m	Rumen
Family Clostridiaceae	*Clostridium*	*acetobutylicum*	m	Soil
	Clostridium	*chartatabidum*	m	Rumen
	Clostridium	*cellulovorans*	m	Wood fermenter
	Clostridium	*herbivorans*	m	Pig intestine
	Clostridium	*cellulosi*	m	Manure
	Clostridium	*cellobioparum*	m	Rumen
	Clostridium	*papyrosolvens*	m	Paper mill
	Clostridium	*josui*	h	Compos
	Clostridium	*cellulolyticum*	m	Compost
	Clostridium	*aldrichii*	m	Wood fermenter
	Clostridium	*stercorarium*	h	Compost
	Clostridium	*thermocellum*	h	Sewage soil
	Clostridium	*cellulofermentans*	m	Manure
	Clostridium	*celerescens*	m	Manure
	Clostridium	*thermopapyrolyticum*	h	Mud
	Clostridium	*thermocopriae*	h	Hot spring
	Clostridium	*sp. C7*	m	Mud
	Bacteroides	*sp. P-1*	h	Rotting biomass
	Bacteroides	*cellulosolvens*	m	Sewage
	Acetivibrio	*cellulolyticus*	m	Sewage
	Acetivibrio	*cellulosolvens*	m	Sewage

Celulosomes are large extracellular enzyme complexes that can degrade cellulose, hemicellulose and pectin, and are produced by anaerobic bacteria (Table 2.2). Cellulosomes degrade crystalline cellulose hemicellulose, chitin and pectin, depending on the source of cellulosomes (Doi et al., 2003)[23]. Table 2.3 shows the cellulosomal hemicellulases including xylanase and mannanase which occur frequently in the cellulosome.

Table 2.2 Cellulosome-producing anaerobic bacteria (Doi et al., 2003)[23]; m: mesophilic, h: thermophilic above 50 °C

Species	Optimal growth temperature	Source
Acetivibrio cellulolyticus	m	Sewage
Bacteroides cellulosolvens	m	Sewage
Butyrivibrio fibrisolvens	m	Rumen
Clostridium acetobutylicum	m	Soil
Clostridium cellulovorans	m	Wood fermenter
Clostridium cellobioparum	m	Rumen
Clostridium cellulolyticum	m	Compost
Clostridium josui	m	Compost
Clostridium papyrosolvens	m	Paper mill
Clostridium thermocellum	h	Sewage soil
Ruminococcus albus	m	Rumen
Ruminococcus flavefaciens	m	Rumen
Ruminococcus succinogenes	m	Rumen

Lignin degradation and lignin degrading enzymes have been detected in actinobacteria from *Streptomyces* genus (Pérez et al., 2002)[80]. However, lignin biodegradation is accepted as an aerobic process but some authors have reported that anaerobic microorganisms in the rumen may later, if not partially degrade, portions of lignified plant cell (Pérez et al., 2002; Tuomela et al., 2000)[80,109]. Both fungi and bacteria can metabolize lignin but their differential reactivity with this substrate indicates that they may utilize different chemical strategies for its breakdown (Brown and Chang, 2014)[15].

In conclusion, the bacteria of the genus *Clostridium* are common among anaerobic organisms hydrolyzing cellulose, as well as the genera *Acetivibrio, Bacteroides, Ruminococcus, Butyrivibrio, Fibrobacter,* and *Cellobacterium*. Starch is degraded by *Ruminobacter, Bacteroides, Prevotella, Clostridium, Succinimonas, Butyrivibrio, Streptococcus*, and *Thermoanaerobacterium* xylan and pectin, by *Bacteroides, Butyrivibrio, Prevotella, Ruminococcus, Clostridium*, and *Lachnospira*; and proteins and amino acids, by *Bacteroides, Clostridium, Acidaminococcus, Peptostreptococcus, Selenomonas, Syntrophomanas, Fusobacterium*, etc. (Tsavkelova and Netrusov, 2012)[108].

Table 2.3 Cellulosomal subunits of mesophilic *Clostridia* (Doi et al., 2003)[23]

Cellulosomal enzyme of mesophilic *Clostridium*	Function	Mol mass (kDa)	Modular structure[a]
C. cellulovorans			
ExgS	Exoglucanase	80	GH48-DS1
EngH	Endoglucanase	79	GH9-CBM3-DS1
EngK	Endoglucanase	97	CBM4-Ig-GH9-DS1
EngL	Endoglucanase	58	GH9-DS1
ManA	Mannanase	47	DS1-GH5
EngM	Endoglucanase	96	CBM4-Ig-GH9-DS1
EngE	Endoglucanase	112	(SLH)3-GH5-X-DS1
EngY	Endoglucanase	80	CBM2-GH9-DS1
EngB	Endoglucanase	49	GH5-DS1
PelA	Pectate lyase	94	X-CBD2-GPL9-DS1
XynA	Xylanase	57	GH11-DS1-CE4
C. acetobutylicum			
CelF	Exoglucanase	81	GH48-DS1
CelA	Endoglucanase	54	GH5-DS1
CelH	Endoglucanase	80	GH9-CBM3-DS1
EngA	Endoglucanase	67	GH44-DS1
CelG	Endoglucanase	77	GH9-CBM3-DS1
CelL	Endoglucanase	60	GH9-DS1
ManA	Mannanase	47	GH5-DS1
CAC0919	Sialidase	91	GH74-DS1
CelE	Endoglucanase	96	CBM3-Ig-GH9-DS1
CAC3469	Endoglucanase	110	(SLH)3-GH5-X-DS1
C. cellulolyticum			
CelF	Exoglucanase	78	GH48-DS1
CelC	Endoglucanase	51	GH8-DS1
CelG	Endoglucanase	80	GH9-CBM3-DS1
CelE	Endoglucanase	97	CBM4-Ig-GH9-DS1
CelH	Endoglucanase	83	GH9-CBM3-DS1
CelJ	Endoglucanase	85	GH9-CBM3-DS1
ManK	Mannanase	48	DS1-GH5
CelD	Endoglucanase	63	GH5-DS1
CelA	Endoglucanase	50	GH5-DS1
CelM	Endoglucanase	58	GH9-DS1
C. josui			
CelD	Exoglucanase	80	GH48-DS1
CelB	Endoglucanase	51	GH8-DS1
CelE	Endoglucanase	81	GH9-CBM3-DS1
AgaA	α-Galactosidase	52	GH27-DS1

2.6 Acidogenesis, Acetogenesis and Methanogenesis

Acidogenesis

The monomers, which are released from hydrolysis, are converted in short-chain organic acids, mainly volatile fatty acid, alcohols, hydrogen, and carbon dioxide by fermentative bacteria (Teghammar, 2013)[104]. Acetic, propionic, butyric, and valeric acids are referred as VFA. Acidogenesis can be two-directional due to the effects of various populations of microorganisms. This stage may be divided into two types including hydrogenation and dehydrogenation. The basic transformation pathway passes through acetic acid, carbon dioxide (CO_2) and hydrogen (H_2) while other acidogegenesis products play an insignificant role. Electrons accumulation by compounds such as lactic acid, ethanol, propionic acid, butyric acid and higher volatile fatty acids is the bacterial response to hydrogen concentration increasing in the solution (Shah et al., 2014)[96]. *Enterobacteriaceae* (*Escherichia*, *Enterobacter*, *Salmonella*, *Klebsiella* and *Shigella*) ferment glucose to a mixture of acetate, formate, lactate, succinate and ethanol under anaerobic condition or in the absence of alternate electron acceptors (Moat et al., 2003)[66]. Moreover, butyrate, butanol, acetone, isopropanol or 2,3-butanediol, H_2, CO_2, acetate and ethanol are produced by *Clostridium*, *Butyrivibrio* and *Bacillus* (Moat et al., 2003)[66]. Propionate, acetate and CO_2 are the major products of the glucose, glycerol and lactate by *Propionibacterium*, *Veillonella*, *Bacteroides* and some species of clostridia (Moat et al., 2003)[66].

Acetogenesis

Acetogenic bacteria are strict anaerobes and have optimum pH of about 6. The bacteria require long lag phase periods for adjust to new environmental condition (Christy et al., 2014)[17]. Hydrogen-producting acetogenic bacteria oxidize alcohols and VFAs into acetate, hydrogen and carbon dioxide. Acetic acid is formed from hydrogen and carbon dioxide by hydrogen-oxidizing acetogenic bacteria (Surendra et al., 2014)[102]. This stage is a phase which depicts the efficiency of biogas production because about 70% of methane arises in acetic acid reduction process. The wastes degradation produces approximately 25% acetic acid and 11% hydrogen (Shah et al., 2014)[96]. Acetogens make syntrophi associations with hydrogen consuming methanogens because they depend on low hydrogen partial pressure for their degradation (Christy et al., 2014)[17].

$$H_3CH_2COO^- + 3H_2O \longleftrightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2 \quad (2\text{-}1)$$

$$C_6H_{12}O_6 + 2H_2O \longleftrightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (2\text{-}2)$$

$$CH_3CH_2OH + 2H_2O \longleftrightarrow CH_3COO^- + 2H_2 + H^+ \quad (2\text{-}3)$$

The propionate converts to acetate are only achievable at low hydrogen pressure in Eq. 2-1. Glucose is converted into acetate in Eq. 2-2. The ethanol is transformed to acetate in Eq. 2-3. The acetogens cannot convert ethanol to methane and carbon dioxide directly, it must convert the ethanol to acetic and consequent release of molecular hydrogen (Christy et al., 2014)[17]. Acetogenic bacteria and the sources of isolate are listed in Table 2.4. However, the acetate can be produced by two genera aerobes including *Acetobacter* and *Gluconobacter*. (Moat et al., 2003)[66].

Table 2.4 Acetogenic bacteria (Daniel et al., 2008)[21]; m: mesophilic, h: thermophilic above 50 °C, p: psychrotrophic and nr: not reported

Species	Optimal growth temperature	Source
Acetitimaculum ruminis	m	Rumen fluid, steer
Acetoanaerobium noterae	m	Sediment
Acetoanaerobium romashkovii	m	Oil field
Acetobacterium bakii	p	Wastewater sediment
Acetobacterium carbinolicum	m	Freshwater sediment
Acetobacterium dehalogenans	m	Sewage digester sludge
Acetobacterium fimetarium	p	Digested cattle manure
Acetobacterium malicum	m	Freashwater sediment
Acetobacterium paludosum	p	Fen sediment
Acetobacterium	m	Subsurface sandstone
psammolithicum	p	Tundra soil
Acetobacterium tundra	m	Sewage digester
Acetobacterium wieringae	m	Marine sediment
Acetobacrterium woodii	m	Freshwater sediment
Acetobacterium sp. AmMan1	m	Wastewater pond
Acetobacterium sp.B10	m	Sewage sludge
Acetobacterium sp. HA1	m	Sewage sludge
Acetobacterium sp. LuPhet1		

Table 2.4 Acetogenic bacteria (cont.)

Species	Optimal growth temperature	Source
Acetobacterium sp. MrTac1	m	Marine sediment
Acetobacterium sp. OyTac1	m	Freshwater sediment
Acetobactium sp. RMMac1	m	Marine sediment
Acetobacterium sp.69	m	Sea sediment
Acetobacterium sp.	p	Tundra wetland soil
Acetohalobium arabaticum	m	Saline lagoon
Acetonema longum	m	Wood-eating termite, gut
Bryantella formatexigens	m	Human feces
Butyribacterium	m	Sewage digeater
methylotrophicum	h	Hot spring
Caloramateor fervidus	m	Soil
Clostridium aceticum	m	Rabbit feces
Clostridium	m	Lagoon sediment
autoethanogenum	nr	Mice feces, human feces
Clostridium carboxidivorans	m	Rumen, newborn lamb
Clostridium coccoides	m	Coal mine pond sediment
Clostridium difficile AA1	m	Sewage
Clostridium drakei	m	Sewage
Clostridium formicaceticum	m	Sea-grass roots
Clostridium glycolicum 22	m	Chicken waste
Clostridium glycolicum	m	Freshwater sediment
Clostridium ljungdahlii	m	Soil-feeding termite, gut
Clostridium magnum	m	Olive oil mill wastewater
Clostridium mayombei	m	Soil, coal mine pond sediment
Clostridium	m	Swine manure digester
methoxybenzovorans	m	
Clostridium scatologenes		
Clostridium ultunense		
Clostridium sp. CV-AA1		

Table 2.4 Acetogenic bacteria (cont.)

Species	Optimal growth temperature	Source
Clostridium sp. Ag4f2	nr	Human feces
Clostridium sp. TLN2	nr	Human feces
Eubacterium aggregans	m	Olive oil mill waster
Eubacterium limosum	m	Rumen fluid, sheep
Holophaga foetida	m	Freshwater ditchmud
Moorella glycerini	h	Hot spring sediment
Moorella mulderi	h	Bioreactor
Moorella thermoacetica	h	Hourse manure, soil
Moorella	h	Hot spring
thermoautotrophica	h	Soil
Moorella sp. F21	h	Mud
Moorella sp. HUC22-1	m	Soda lake deposits
Natroniella acetigena	m	Soda lake deposits
Natronincola histidinovorans	m	Rumen fluid, steer
Oxobacter pfennigii	m	Human feces
Ruminococcus	m	Sewage digester
hydrogentrophicus	m	Sewage digester
Ruminococcus productus	m	Rumen, 3 day old lamb
Ruminococcus productus	nr	Human feces
Marburg	m	Distillation waste
Ruminococcus schinkii	m	Soil-eating termite gut
Ruminococcus sp. TLF1	m	Freshwater sediment
Sporomusa acidovorans	m	Silage
Sporomusa aerivorans	m	Lake sediment
Sporomusa malonica	m	Beech forest soil
Sporomusa ovate	m	River mud
Sporomusa paucivorans	m	Wood-eating termites, gut
Sporomusa silvacetica		
Sporomusa sphaeroides		
Sporomusa termitide		

Table 2.4 Acetogenic bacteria (cont.)

Species	Optimal growth temperature	Source
Sporomusa sp. DR6	nr	Rice field soil
Sporomusa sp. DR1/8	nr	Rice field soil
Syntrophococcus	m	Rumen fluid, steer
sucromutans	h	Pulp wastewater reactor
Thermoacetogenium phaeum	h	Lake sediment
Thermoanaerobacter kivui	m	Alkaline lake sediment
Tindallia californiensis	m	Termite, hindgut
Treponema azotonutricium	m	Termite, hindgut
Treponema primitia		

The hydrogenotrophic methanogens, acetogens, sulfate reducers, and/or sulfur reducers are syntrophic microorganisms, such as *Synthrophomonas* and *Synthrophobacter* (Tsavkelova and Netrusov, 2012)[108]. However, hydrogenotrophic methanogens can use hydrogen for CO_2 reduction to methane. Syntrophic metabolism based on hydrogen transport has been proved by *Syntrophobotulus glycolicus* and *Syntrophococcus sucromutans*. In addition to hydrogen, formate is an electron transporter in a methanogenic community. For example, propanoate is consumed by *Syntrophobacter fumaroxidans* and butyrate by *Syntrophomonas bryantii*, these reactions are only occurred with a methanogen that equally consumed both hydrogen and formate, but not with a methanogen consuming only hydrogen (Tsavkelova and Netrusov, 2012)[108].

Methanogenesis

Methane is produced as a metabolic byproduct in anoxic conditions by methanogenic microorganisms belonging to Archaea (Christy et al., 2014)[17]. The main methanogenesis substrate are carbon dioxide and hydrogen (hydrogenotrophic methanogenesis), acetic acid (acetoclastic methanogenesis), formate, methanol and methyl amines (Tsavkelova and Netrusov, 2012)[108]. Methane producing bacteria (Methanogens) can be divided into two group including acetotrophic methanogens, and hydrogenotrophic methanogens. Acetotrophic methanogens produce methane and carbon dioxide from acetic acid in Eq. 2-4, while hydrogenotrophic methanogens produces methane from hydrogen, and carbon dioxide in Eq. 2-5 (Myint et al., 2007)[73]. *Methanosarcina* species can consume acetic acid, H_2/CO_2, methanol and methyl amines, whereas *Methanosaeta* (*Methanothrix*) consume

acetic acid as an energy source (Tsavkelova and Netrusov, 2012)[108]. The hydrogen-consuming methanogens (*Methannospirillum hungatei*, *Methanoculles receptacli*) are faster growing than the aceticlastic methanogens (*Methanosarcina thermophile*). The maximum doubling time for hydrogenotrophic methanogens has been estimated to be six hours compared with slowing growing aceticlastic methanogens which takes 2.6 days (Christy et al., 2014)[17].

$$CH_3COOH \longrightarrow CH_4+CO_2 \qquad (2\text{-}4)$$

$$CO_2+4H_2 \longrightarrow CH_4+2H_2O \qquad (2\text{-}5)$$

Methanogens have very slow growth rates. Their metabolisms are usually considered as a rate-limiting step in the anaerobic digestion. Waste stabilization is accomplished when methane and carbon dioxide are produced. The methane formation is very important in anaerobic digestion, because it can produce methane gas and regulates pH by converting VFA into bicarbonate. The bacteria utilizing propionic and acetic acids are the most important among the methanogens (Lien, 2004)[57]. Hill et al. (1987)[45] proposed that an anaerobic system failure occurs when the propionic acid to acetic acid ratio is greater than 1.4.

In general, biogas consists of 50-75% CH_4 and 25-50% CO_2 with other trace components, such as water vapor, hydrogen sulfide (H_2S) and ammonia (NH_3). However, the biogas composition varies with type of feedstock (Surendra et al., 2014)[102]. The biogas can be used in the electric power production and in combustion. The biogas has advantages over other alternative fuels for examples; the bio-methane produces less noxious emissions than gasoline or diesel fuel, methane stores about three times as much energy as hydrogen fuel and the biogas production from biomaterials does not require growing energetic plants in contrast to biodiesel fuel or bioethanol (Tsavkelova and Netrusov, 2012)[108].

2.7 Microbial Activity Tests

The anaerobic microorganism activity test is generally measured as the specific rate of substrate consumption which refers to the biomass (e.g. volatile suspended solids) and the targeted microbial population. The activity considers with slope of curve of substrate utilization and products in unit of mass of substrate or product per unit mass per unit time (Rozzi and Remigi, 2004)[90]. The substrate used for acidogenic activity determination is

usually glucose, which is considered as the main intermediate in the pathway of anaerobic digestion of carbohydrate complex organic. Volatile fatty acids were used as the substrate for methanogenic activity determination, especially acetate (Effebi et al., 2011)[25].

2.8 Previous Studies on Degradation of Lignocellulose by Anaerobic Bacteria

Neo et al. (2012)[74] studied biogas production from agricultural wastes (wheat straw and corn stalks) using cattle manure are compared it with activated sludge used as inoculums. The biomass of their study was wheat straw and corn stalks. Those biomass were milled using a kitchen mixer after that the biomass were subjected to a combination of thermal and chemical pretreatment (2% NaOH, autoclaving for 30 min at 121 °C, 2 bar). The anaerobic batch was operated at constant temperature (37 °C). The results shown that corn stalks added no extra biogas production potential comparing with cattle manure and the biogas production was low using this agricultural waste as substrate even inoculated with activated sludge. However, wheat straw increased 39.2% biogas production potential by using cattle manure as the inoculum. Moreover, the biogas production of wheat straw inoculated with cattle manure (59%) was higher than that of wheat straw inoculated with activated sludge (47%). Therefore, cow manure can be used as inoculum of anaerobic fermentation especially wheat straw.

Xia et al. (2012)[122] studied thermophilic anaerobic digestion (55 °C) of microcrystalline cellulose in batch experiments using microcrystalline as the sole carbon source and anaerobic digestion sludge (ADS) as the seed sludge. The original culture produced 566 mL/L methane with 14.7% substrate degradation in 380 hr. The ADS was enriched at 55 °C for 18 days. The enriched consortium was able to degrade 100% cellulose in 140 hr with 6,770 mg/L acetate, 2,674 mg/L methanol as the major products. 16S rDNA result shown that the dominant of the cellulose-degrading consortium was the genus of *Thermoanaerobacterium* (4 clones out of total 9 clones), *Bacill*us (2 clones), *Tepidiphilus* (2 clones) and unknown strain.

Yan et al. (2012)[124] studied a mesophilic lignocellulolytic microbial consortium BYND-5 for rice straw degradation to enhance the biogas production. The degradation efficiency of BYND-5 for rice straw was more than 49.0 ± 1.8% after 7 days of cultivation at 30 °C. The BYND-5 diversity was analyzed by ARDRA (Amplified ribosomal DNA restriction analysis) of the 16S rDNA clone library. The results show that bacterial groups represented in the clone library were 5.96% *Firmicutes*, 40.0% *Bacteroidetes*, 8.94%

*Deferribacter*es, 16.17% *Protrobacteria*, 2.13% *Lentisphaerae,* 1.7% *Fibrobacteraceae* and 25.1% uncultured bacterium. The microbial community was a potential candidate consortium for the degradation of lignocellulose and enhancement of biogas production under mesophilic temperature conditions.

Zainudin et al. (2013)[126] studied the composting of lignocellulosic oil palm empty fruit bunch (OPEFB) with the continuous addition of palm oil mill (POME) anaerobic sludge, which contained nutrients and indigenous microbes. Through the continuous addition of POME anaerobic sludge, which contained indigenous microbes and nutrients, rapid composting of lignocellulosic OPEFB could be completed in 40 days. Twenty-seven cellulolytic bacterial strains of which 23 strains were related to *Bacillus subtillis*, *Bacillus firmus*, *Thermobifida fusca*, *Thermomonospora* spp. *Cellulomonas* sp., *Ureibacillus thermospharicus*, *Paenibacillus barengoltzii*, *Paenibacillus campinasensis*, *Geobacillus thermodenitrificans*, *Pseudoxanthomonas byssovorax*, which were known as lignocellulose degrading bacteria and commonly involved in lignocellulose degradation. Four isolated strains related to Exiguobacterium acetylicum and Rhizobium sp. with cellulolytic and hemicellulolytic activities.

CHAPTER 3

EFFECTIVENESS OF FUNGAL TREATMENT BY *Coprinopsis cinerea* AND *Polyporus tricholoma* ON DEGRADATION AND METHANE YIELDS OF LIGNOCELLULOSIC GRASS

3.1 Materials and Methods

3.1.1 Materials

A wide variety of paragrass (*Brachiaria mutica*) collected from Prachinburi Province in Thailand during the summer season was used as the carbon fiber substrate. The grass was dried at 60 °C until the moisture content was less than 10% (Wan and Li, 2011)[115]. The grass was chopped into 20 mm pieces and then ground into 1 mm powder by a blender. The grass was stored in a black bag at room temperatures between 30-35 °C prior to use. Fiber compositions of the paragrass were 32.06 ± 0.44% cellulose, 31.92 ± 3.91 % hemicellulose and 8.63 ± 1.35 % lignin. The paragrass samples were autoclaved before being stored for the experiments to avoid growth of airborne microorganisms on the samples (Amirta et al., 2006)[3]. Fiber compositions of the paragrass after the sterilization process were 31.35 ± 0.40% cellulose, 30.48 ± 0.76% hemicellulose and 10.06 ± 0.33% lignin.

Mesophilic anaerobic sludge was used as the inoculum for anaerobic biogas production. This sludge was obtained from the Ngaung-Khaem Water Quality Control Plant, a domestic wastewater treatment plant in Bangkok, Thailand. The sludge had 83.43 ± 0.16 g TS/L and 40.35 ± 0.58 g VS/L, and contained 0.27 ± 0.02 g cellulose, 0.11 ± 0.01 g hemicellulose and 0.04 ± 0.01 g lignin per gram of TS sludge. Throughout this chapter, this sludge will be referred to as original sludge (OS).

3.1.2 Isolation of the Fungal Strain

Faeces of cattle were collected directly from adult dairy cattle in the eastern region of Thailand. The faeces samples were collected in plastic bags and stored at 4 °C for 12 h. One gram of faeces was added to 10 mL of the liquid defined medium containing antibiotics (Lowe et al., 1985)[60] and 1% (w/v) glucose. The bottles were agitated for 1 min and were incubated at 39 °C for 7 days and were inverted to disperse their content. After the incubation, 0.5 mL of the culture supernatant was transferred to a petri dish containing the medium B agar with antibiotics and 1% (w/v) cellulose. The dish was incubated at 39 °C for

4 days. The observed individual colonies were transferred with a sterile mounting needle into a fresh petri dish containing the same medium. This procedure was repeated until the cultures were free of bacteria (Lowe et al., 1987)[59].

3.1.3 Identification of the Fungal Strain

The identification of the isolated strain was performed using the 18s rDNA sequences database by the National Center for Genetic Engineering and Biotechnology (Thailand) (Sri-indrasutdhi et al., 2010)[99].

The identification showed that the white mycelia fungus was *Coprinopsis cinerea* (99% similarity). The nucleotide sequence was as follows.

GCCCGTCACCTTTATTTCTCCACCTGTGCACACACTGTAGGCCTGGATACCTCTCGT
CGCAAGGCGGATGCGTGGCTTGCTGTCGCTTTCGAAAGAAGGCCGGCTTGCCATGAA
TTTCCAGGTCTATGATTTCTTACACACCCCAAACTGAATGTTATGGAATGTCATCTC
AAGGCCTTGGTGCCTATAAACCTATACAACTTTCAGCAACGGATCTCTTGGCTCTCG
CATCGATGAAAAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAAAATTCAGTGA
ATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCAAGGAGCATGCCTGT
TTGAGTGTCATTAAATTCTCAACCTCACCAACTTTGTTGTGTGCAGG

3.1.4 Fungal Pretreatment of the Paragrass

The fungal strain isolated from cattle rumen was used as the inoculum in the aerobic treatment of the paragrass. A commercially available strain of *P. tricholoma* (BCC22851) obtained from the National Center for Genetic Engineering and Biotechnology (Thailand) was also used as the inoculum in some experiments.

Five grams dry weight of the paragrass powder were transferred into a 250 mL Erlenmeyer flask. The grass was moisturized with 15 mL of tap water to obtain 75% moisture (Wan and Li, 2011)[115]. Then the grass was inoculated with 10 disks of a fungal colony, each of which was 1 cm in diameter. The grass samples in the flask were inoculated with a monoculture of either *C. cinerea* to obtain samples (CC-grass) or *P. tricholoma* (PT-grass). The grass cultures were then incubated at 28 °C for 5, 10, 15, 30 or 45 days, depending on the trial.

After each specified treatment period, the pretreated grass was washed with 15 mL of distilled water and filtered under a vacuum (Valaskova and Baldrian, 2006)[110]. The liquid fractions were analyzed for pH and chemical content.

The solid fractions were dried at 100 °C and then analyzed for dry weight and cellulose, hemicellulose and lignin content. The surface structure and the degree of

cystallinity of grass samples after the aerobic pretreatment and of grass samples that had naturally decayed for the same period were then investigated. Scanning electron microscopy (SEM) was used to investigate surface structure and X-ray diffraction (XRD) to determine degree of crystallinity.

3.1.5 Anaerobic Digestion

The grass samples were treated for 30 days according to the method described in Section 3.1.4. The treated grass was then transferred to 100-mL serum bottles. The total working volume was 60 mL. The bottles were inoculated with the OS at a ratio of 1 g VS/g VS and then flushed for 1 min with 99.995% argon. The bottles were incubated under mesophilic temperatures between 28-30 °C for 140 days. Each serum bottle was closed with a rubber stopper. Anaerobic digestion of the naturally decayed grass (the untreated grass or UT-grass) was also carried out using the sludge, and a control experiment with only the sludge was run in parallel. The volume of the gas produced from each sample was measured periodically and the gas composition was analyzed by gas chromatography. After each specified incubation period, the samples were collected for physical and chemical analysis.

3.1.6 Enzyme Assays

Total cellulase activity: Fifty mg of Whatman no.1 filter paper strip (6 cm x 1 cm) was used as the substrate. The filter paper was coiled in the bottom of a test tube. One mL of 0.05 M citrate buffer at pH 4.8, and 1 mL of sample was then added to the test tube, and the tube was incubated at 50 °C for 60 min (Ghose, 1987)[34]. After that, the sample was centrifuged, and the supernatant was analyzed for the content of reducing sugar by the dinitrosalicylic acid (DNS) method.

β-glucosidase activity: The reaction mixture consisted of 1 mL of 15 mM cellobiose in 0.05 M citrate buffer at pH 4.8, 1 mL of 0.05 M citrate buffer at pH 4.8 and 1 mL of sample. The mixture was incubated at 50 °C for 30 min and then boiled for 5 min (Ghose, 1987)[34]. The mixture was analyzed for glucose content by the DNS method.

Xylanase activity: The reaction mixture consisted of 1 mL of 0.05 M citrate buffer at pH 4.8, 1 mL of 100% xylan and 1 mL of sample. The mixture was incubated at 50 °C for 30 min, boiled for 5 min (Ghose and Bisaria, 1987)[35], and then analyzed for reducing sugar content by the DNS method.

One unit of enzyme activity is defined as 1 μmol of glucose or xylose equivalents released per minute under the given conditions (Isikhuemhen and Mikiashvilli, 2009)[48].

3.1.7 Analysis

Measurements of cellulose, hemicellulose and lignin content were performed by the detergent method (Van Soest and Wine, 1967)[112]. The hemicellulose content was calculated from the difference between neutral detergent fibre (NDF) and acid detergent fibre (ADF). The lignin content was the difference between ADF and permanganate lignin (PML). After the PML analysis, the cellulose content was estimated from the weight loss of the sample when held at 550 °C for 3 h. Carbon and nitrogen content was analyzed by a CHNS/O analyzer (Perkin Elmer PE2400 Series II).

The crystalline structure of the cellulose was analyzed by X-ray diffraction (XRD, Bruker AXS D8 Discover, Germany) using Cu K α radiation (λ1 = 1.54 Å) generated at a voltage of 40 kV and a current of 40 mA. The scanning was performed from 5° to 50° at a speed of 3°/min. The degree of crystallinity ($\boldsymbol{x_c}$) was defined as the area of the crystal region divided by the total area of the crystal and noncrystalline regions. The degree of crystallinity ($\boldsymbol{x_c}$) was calculated by Sun et al. (2009)[101].

$$x_{\mathrm{C}} = \frac{F_c}{(F_c + F_a)} \qquad \text{Eq.3-1}$$

Where $\boldsymbol{F_C}$and $\boldsymbol{F_a}$ are the area of the crystal and non-crystalline regions, respectively.

The grass after the fungal pretreatment for 5, 15 or 30 days was examined for changes in its surface structure using a SEM (Jeol JSM 5410LV, Japan).

Reducing sugar was measured by the DNS method (Miller, 1959)[65]. The COD was determined using the closed-tube method (Finnish Standard Association, 1988)[28]. The concentrations of total solids (TS) and volatile solids (VS) were determined by the Standard Methods (APHA, 1998)[8]. The pH was measured by a pH meter (Schott Lab 850, Germany).

The biogas generated was collected using either 25 mL- or 50 mL-hospital needle syringes (Owen et al., 1979)[78]. The biogas generated was collected at standard temperature and pressure (STP: 0 °C and 1 atmosphere). Biogas composition was determined by a gas chromatograph equipped with a thermal conductivity detector (SHIMADZU GC-2014, Japan) and a unibeads C column under an argon flow rate of 25 mL/min. Maximum methane production rate (MMPR) was estimated from the slope of the initial linear part of each methane production curve versus time. Methane yield was determined from the maximum plateau achieved in the methane production curves (Neves et al., 2004)[75].

In this study, the measurements were repeated three times. The standard errors were all within 10% of the mean value. A test of significant difference based on the paired t-statistic was performed using the Excel Solver Add-in. The difference was regarded as not significant if the paired t-statistic showed Probability: $P > 0.05$ and significant if $P < 0$ .05.

3.2 Results and Discussion

3.2.1 Detection of Cellulolytic and Hemicellulolytic Enzyme Activities

Enzyme activities were investigated in the culture of *C. cinerea* on paragrass. As shown in Table 3.1, cellulase, β-glucosidase and xylanase activities were all detected, but at different levels. Cellulase activity varied from 0.029-0.175 U/mL during 30 days of the treatment by *C. cinerea.* The cellulase activity was highest on day 5, but much lower on other days. β-glucosidase activity varied in a narrow range of 0.117-0.261 U/mL, and xylanase activity in the range of 0.900-1.112 U/mL. The data suggested that among the cellulase, β-glucosidase and xylanase activities of *C. cinerea*, the xylanase activity was found to be the highest. The level of xylanase activity in this study was comparable with that obtained from *Pleurotus ostreatus* grown on wheat straw and/or solid waste before their fruiting periods (Isikhuemhen and Mikiashvilli, 2009)[48].

Table 3.1 Cellulolytic and hemicellulolytic enzyme activities of *C. cinerea* at different periods

Enzyme activity (U/mL)	5 days	10 days	15 days	30 days	45 days
Cellulase	0.175 ± 0.024	0.054 ± 0.010	0.029 ± 0.004	0.087 ± 0.032	0.099 ± 0.010
β-glucosidase	0.260 ± 0.046	0.234 ± 0.005	0.117 ± 0.013	0.219 ± 0.040	0.261 ± 0.003
Xylanase	0.900 ± 0.049	1.069 ± 0.019	0.994 ± 0.112	1.028 ± 0.092	1.112 ± 0.012

3.2.2 Changes in Compositions and Structures of the Paragrass by the Fungal Treatment

The growth and colonization of the grass by the fungi was clearly seen, and the treated grass appeared to have more moisture than the untreated grass. Table 3.2 compares the chemical compositions of the insoluble parts and the soluble parts of the untreated grass and the grass treated with either *C. cinerea* or *P. tricholoma* BCC22851. It was evident that the reductions of solid, cellulose and hemicellulose in the grass were enhanced by the treatment with the two fungi. On day 30, the dry weight, cellulose and hemicellulose were reduced by 27%, 16% and 27%, respectively, by *C. cinerea*, and 29%, 32% and 20%, respectively, by *P. tricholoma*. In contrast, the dry weight, and cellulose and hemicelluloses content of the untreated grass were hardly reduced. Even though reductions of cellulose and

hemicellulose can be achieved by the aerobic fungal treatment, the reduction of lignin was not found to be significantly different from the natural decay (P>0.05). The effect of the fungal treatment was different from that of alkaline treatment. In the alkaline treatment, NaOH can ionize the carboxylic and phenolic groups, causing the lignin to be dissolved from a hemicelluloses-lignin matrix (Gierer, 1985)[36]. However, the alkaline treatment hardly reduces cellulose content in grass silage (Xie et al., 2011)[123].

Table 3.2 Comparative components in the grass samples after the fungal pretreatment

		5 days			15 days			30 days			45 days	
Parameters	0 day	UT-grass	CC-grass	PT-grass	UT-grass	CC-grass	PT-grass	UT-grass	CC-grass	PT-grass	UT-grass	CC-grass
Insoluble part												
Dry weight (g)	0.96	0.96	0.86	0.88	0.96	0.80	0.77	0.97	0.70	0.68	0.98	0.65
Cellulose (g)	0.31	0.31	0.28	0.32	0.30	0.26	0.30	0.31	0.26	0.21	0.31	0.24
Hemicellulose (g)	0.30	0.30	0.26	0.31	0.30	0.24	0.22	0.31	0.22	0.24	0.30	0.18
Lignin (g)	0.10	0.10	0.09	0.11	0.10	0.09	0.09	0.10	0.09	0.08	0.10	0.09
Carbon (g)	0.38	-	-	-	-	-	-	-	0.29	-	-	-
Nitrogen (g)	0.013	-	-	-	-	-	-	-	0.007	-	-	-
Soluble part												
sCOD (mg)	144	139	94.69	146	161	109	147	154	106	125	169	101
Reducing sugar (mg)	48.75	43.79	12.15	45.77	42.78	8.28	26.14	43.88	10.28	19.09	31.68	11.86
pH	5.98	5.92	5.51	5.87	5.71	7.28	5.44	5.50	7.16	5.49	5.39	6.78

Rot fungi are known to secrete cellulase from their hyphae (Popescu et al., 2011)[84]. This leads to the formation of microscopic cavities inside cellulosic materials (Hamed, 2013)[41]. Fig. 3.1 shows the SEM images of the untreated grass and the grass treated by fungus under aerobic conditions for 30 days. The structure of the treated grass was cracked, while that of the untreated grass had hardly changed from its original form. The hyphae can be observed in the cell wall of the grass treated with *C. cinerea*. The cellulase released from fungal hyphae has been reported to diffuse freely within grass substrates, making the grass lose its strength and shrink (Popescu et al., 2011)[84].

For the soluble parts, the contents of reducing sugar and soluble COD (sCOD) in the fungal treated grass were much lower than those of the untreated grass. The nitrogen content in the grass treated with *C. cinerea* was reduced to approximately half of the initial value on

day 30. The data suggested that both fungi had utilized the reducing sugar and nitrogen compounds during their growth. Muthangya et al. (2009b)[72] reported that rot fungi metabolize sugar and starch in preference to lignin and cellulose.

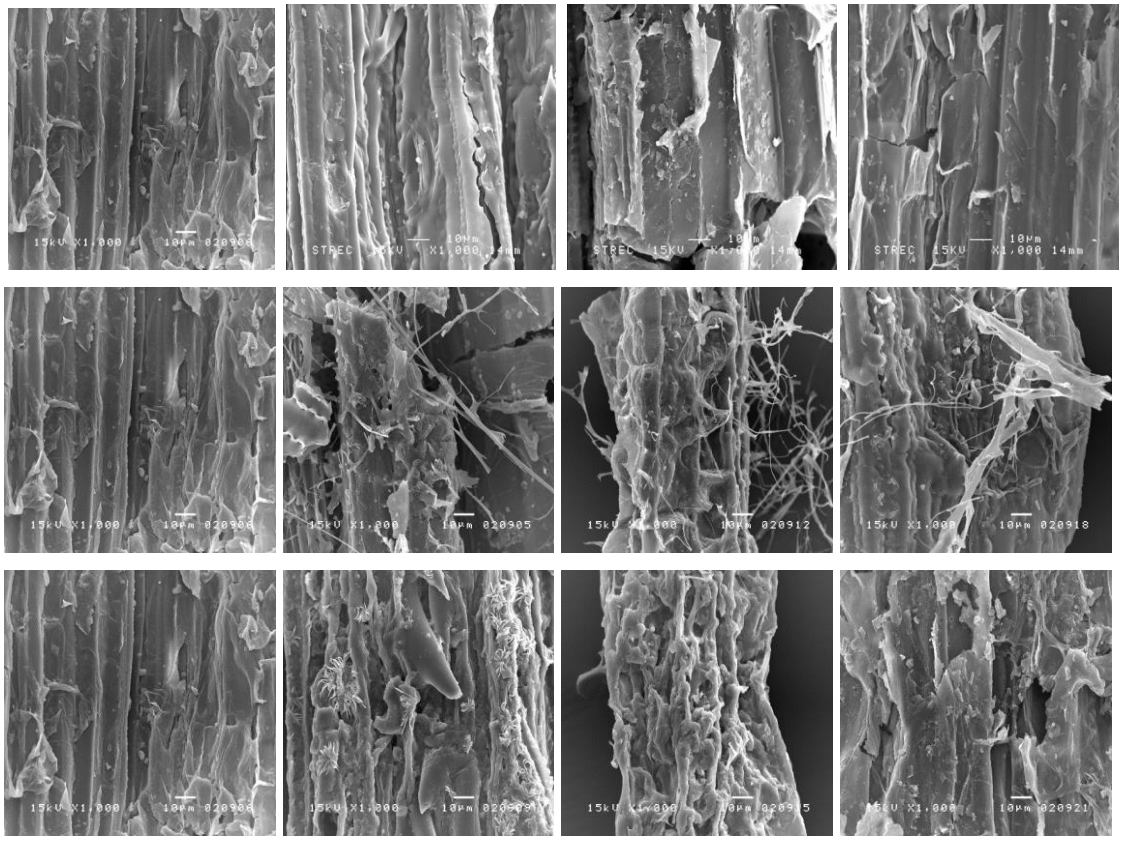


Fig. 3.1 Scanning electron micrographs of the grass samples after 0, 5, 15 and 30 days; untreated grass (top), grass treated by *C. cinerea* (middle) and grass treated by *P.tricholoma* (bottom).

Cellulose digestion is a common property of many fungi. However, some fungi can degrade amorphous cellulose, but not crystalline cellulose. The biodegradation of crystalline cellulose requires cellobiohydrolases, the exo-acting enzymes that are required for the operation of a complete, synergistic cellulase system (Hatakka and Hammel, 2011)[42]. Alternatively, the biodegradation of crystalline cellulose can occur with synergistic activities of non-processive cellulases and some low-molecular weight oxidants (Cohen et al., 2005; Yoon et al., 2008)[18,125]. Fig. 3.2 shows the XRD profiles, and Fig. 3.3 shows the degree of crystallinity of the untreated and the fungal treated grass. The reduction in the crystallinity in the grass treated with *C. cinerea* can be observed to occur beginning at day 5 and

continuing to day 30. The reduction in crystallinity in the grass treated with *P. tricholoma* was first observed on day 15. On day 30, the degree of crystallinity of the grass treated with *C. cinerea* had decreased to 23% and that treated with *P. tricholoma* had decreased to 24% from the original degree of crystallinity of 28%.

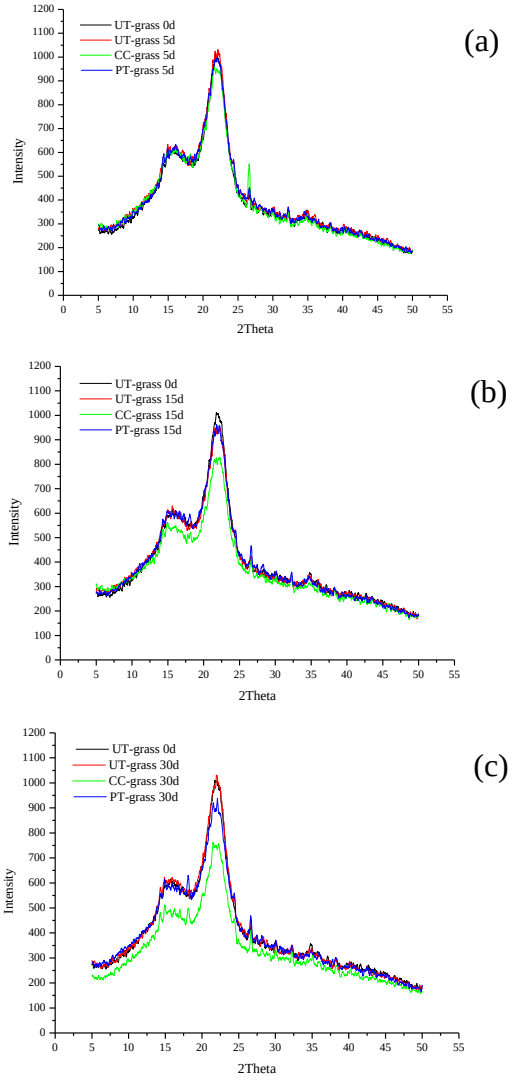


Fig. 3.2 XRD profiles of the untreated grass and the fungal treated grass (a) after 5 days, (b) 15 days and (c) 30 days; untreated grass (UT-grass), grass treated by *C. cinerea* (CC-grass) and grass treated by *P.tricholoma* (PT-grass).

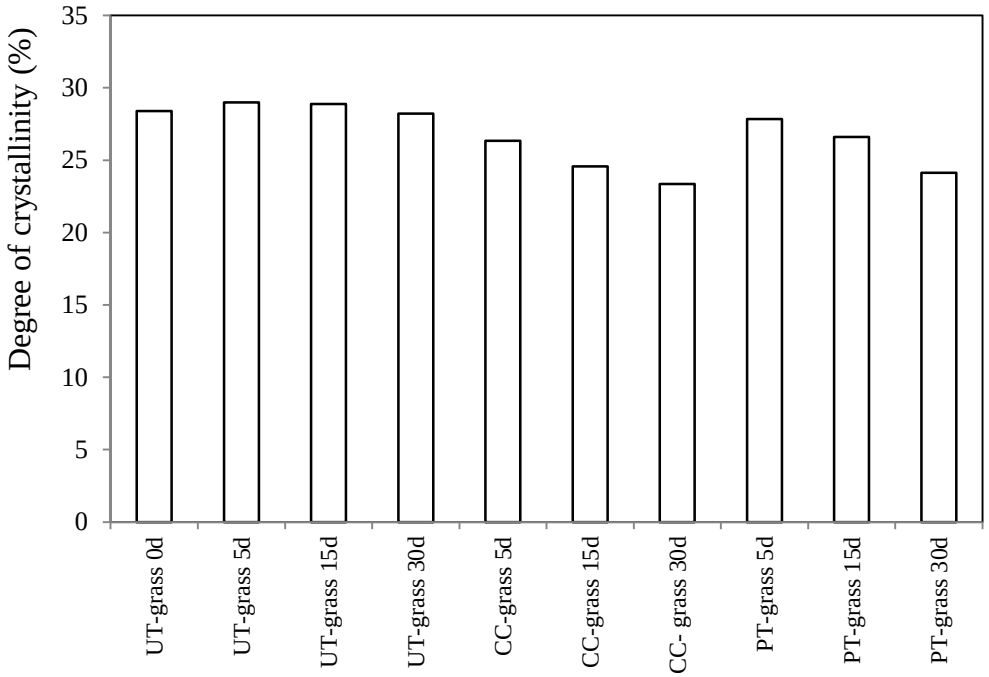


Fig. 3.3 Degree of crystallinity of the untreated and fungal treated grasses after 5 days, 15 days and 30 days; untreated grass at the initial stage (UT-grass 0d), untreated grass after 5 days (UT-grass 5d), 15 days (UT-grass 15d) and 30 days (UT-grass 30d), grass treated by *C. cinerea* for 5 days (CC-grass 5d), 15 days (CC-grass 15d) and 30 days (CC-grass 30d), and grass treated by *P.tricholoma* for 5 days (PT-grass 5d), 15 days (PT-grass 15d) and 30 days (PT-grass 30d).

3.2.3 Biogas Production Rates and Yields

Fig. 3.4 shows the methane content in the biogas produced from the untreated and the fungal treated grass. The methane content in the biogas produced from the fungal treated grass reached a maximum of 60% within 4 days, while that of the untreated grass increased slowly and reached a maximum of 55% on day 10. The methane content in the biogas produced from the untreated grass did not reach the level of that from the treated grass until approximately day 30, and then the methane contents of the samples remained approximately the same and fluctuated between 50-64% during the remainder of the fermentation period of 140 days.

Fig. 3.5 compares the cumulative methane production from the untreated and the fungal treated grass. The methane production rate associated with the digestion of the fungal treated grass was constant at 11.2 mL/g·d during the first fourteen days. In contrast, the

methane production rates associated with the digestion of the untreated grass can be divided into two phases. During the first seven days, the methane production rate was 4.1 mL/g·d, which was lower than half of that of the fungal treated grass. During the second seven days, the methane production rates became comparable. The results indicated that the fungal treatment helped accelerate the anaerobic digestion at the initial stage. However, after day 14, the methane production from the fungal treated grass continued to increase, but at a lower rate. In contrast, the methane production from the untreated grass continued at the same rate of 11.2 mL/g·d until day 36. As a consequence, the cumulative methane produced from the untreated grass reached those produced from the fungal treated grass on day 22, at which the cumulative methane was 178 mL/g VS. The yield was 57% and 48% of the methane yield on day 140 produced from the fungal treated grass and from the untreated grass, respectively. After day 140, the untreated grass produced a higher amount of methane.

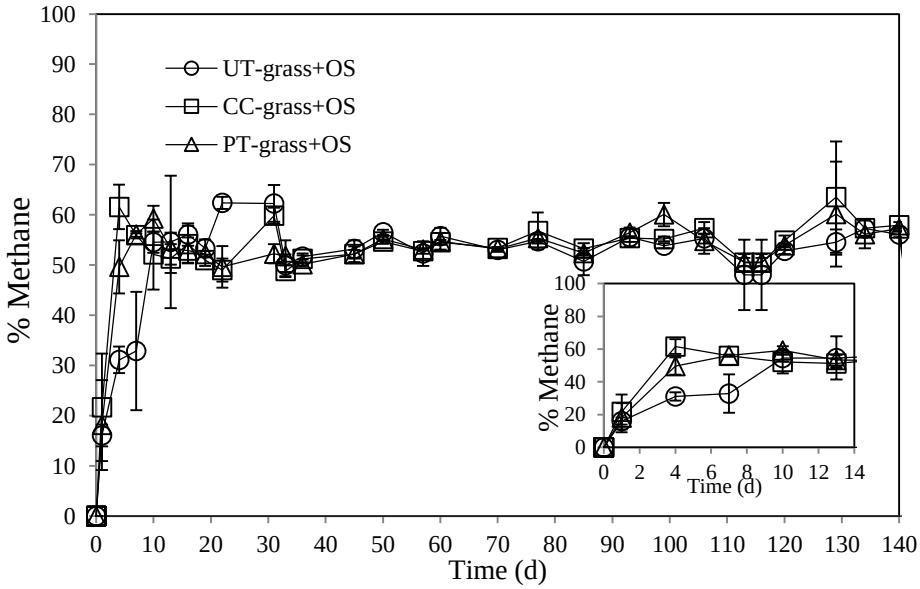


Fig. 3.4 Methane content from the anaerobic digestion of the untreated grass (UT-grass+OS), the grass treated by *C. cinerea* (CC-grass+OS), and the grass treated by *P.tricholoma* (PT-grass+OS).

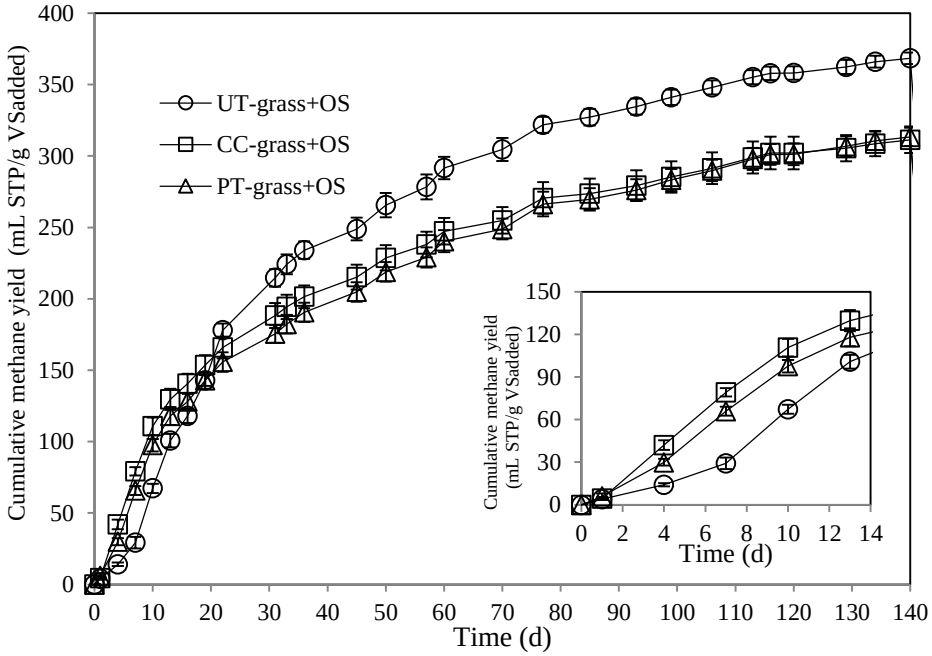


Fig. 3.5 Cumulative methane from the anaerobic digestion of (a) the untreated grass (UT-grass+OS), the grass treated by *C. cinerea* (CC-grass+OS), and the grass treated by *P.tricholoma* (PT-grass+OS).

Table 3.3 summarizes the methane yields and the MMPRs of the untreated grass and the fungal treated grass in 140 days, which were estimated from the initial slope of each methane production curve. The MMPR of the grass treated by *C. cinerea* and by *P.tricholoma* were comparable with that of the untreated grass, but the MMPR of the treated grass occurred earlier; i.e. at day 10 for CC-grass, day 13 for PT-grass and day 22 for the untreated grass. The methane yield of the grass treated by *C. cinerea* and by *P.tricholoma* was approximately 15% lower than that of the untreated grass. Feng et al. (2013)[27] reported similar results that the methane yields of the grass treated with various strains of *Pleurotus* spp. were 11% lower than that of the naturally decayed grass. It is known that under aerobic conditions, complete degradation of cellulose leads to the loss of carbon as carbon dioxide. Therefore, a possible explanation for the lower methane yields of the fungal treated grass was that some carbon in the grass substrate treated with the fungi was lost during the aerobic treatment.

Table 3.3 The methane yields after 140 days of anaerobic digestion and the maximum methane production rates (MMPR) of untreated grass and fungal treated grass

Sample	Methane yield (mL STP/g VS added)	MMPR (R^2) (mL STP/g VS·day)
UT-grass+OS	368	9.41 (0.99)
CC-grass+OS	311	11.44 (1.00)
PT-grass+OS	313	9.51 (0.99)

3.2.4 Digestibility of the Paragrass in the Two-stage Aerobic Treatment and Anaerobic Digestion

Table 3.4 shows the normalized weight and the weight loss of cellulose, hemicellulose and total solids in the untreated grass and the fungal treated grass after the two-stage aerobic treatment and anaerobic fermentation. During the fungal aerobic treatment, the fungal treated grasses lost around 18-32% of their cellulose, 20-28% of hemicellulose and around 27-29% of total solid, while the untreated grass lost the compounds by less than 4% by a natural process. During the following anaerobic digestion, the grass treated by *C. cinerea* lost 53% of its cellulose, while the untreated grass lost 32%. The data indicated that the fungal treatment helped enhance the cellulose digestion by the anaerobic bacteria. The combined degradation of cellulose in the grass treated by *C. cinerea* followed by the anaerobic digestion was 61%, while that in the untreated grass followed by the anaerobic digestion was 48%. The recalcitrant cellulose in the fungal treated grass was significantly lower than that of the untreated grass ($P<0.05$).

During the anaerobic digestion, the grass treated by *C. cinerea* lost 50% of its hemicellulose, while the untreated grass lost 53%. The combined hemicellulose degradation in the grass treated by *C. cinerea* followed by anaerobic digestion was 64%, while that in the untreated grass followed by anaerobic digestion was 59%. The amounts of recalcitrant hemicellulose in the untreated grass and in the fungal treated grass were approximately the same. The data indicated that the fungal treatment did not enhance hemicellulose degradation. An explanation is that the remaining hemicellulose forms chemical bonds with lignin, while *C. cinerea* has been known not to degrade lignin even though it contains several

laccase-encoding genes (Hatakka and Hammel, 2011)[42]. Since parts of the hemicellulose were consumed by the fungus during the aerobic treatment, the grass treated by *C. cinerea* had lower amounts of the hemicellulose available for the anaerobic digestion.

Table 3.4 Normalized weight and percentage loss of cellulose and hemicellulose after the two-stage aerobic treatment (30 days) and anaerobic digestion (140 days)

Sample	Component	After the aerobic treatment[1]		After the anaerobic fermentation		Cumulative loss
		Normalized weight	% loss[2]	Normalized weight	% loss[2]	
UT-grass+OS	TS	0.962	3.8	0.650	32.4	35.0
	Cellulose	0.851	14.9	0.524	38.3	47.6
	Hemicellulose	0.869	13.1	0.413	52.5	58.7
	Lignin	0.879	12.1	0.687	21.8	31.3
CC-grass+OS	TS	0.723	27.7	0.488	32.5	51.2
	Cellulose	0.821	17.9	0.387	52.9	61.3
	Hemicellulose	0.717	28.3	0.362	49.5	63.8
	Lignin	0.889	11.1	0.530	40.5	47.0
PT-grass+OS	TS	0.709	29.1	0.489	31.0	51.1
	Cellulose	0.681	31.9	0.399	41.7	60.1
	Hemicellulose	0.800	20.0	0.369	54.0	63.1
	Lignin	0.818	18.2	0.457	43.9	54.3

Notes [1.] The weight loss of each component in the untreated grass was due to a natural decay.
[2.] The percentage loss during each treatment.

The fungal treatment of *P.tricholoma* had similar effects to the fungal treatment by *C. cinerea* on the degradation of cellulose and hemicellulose. That is, the recalcitrant cellulose in the fungal treated grass was significantly lower than that of the untreated grass ($P<0.05$), but the amounts of recalcitrant hemicellulose in the untreated grass and in the fungal treated grass were not significantly different ($P>0.05$). The recalcitrant cellulose in the fungal treated grass was significantly lower than that of the untreated grass because both

C.cinerea and *P.tricholoma* can reduce the crystallinity in the grass substrate. In contrast, the degradation of hemicellulose was restricted, possibly because under aerobic conditions, neither *C. cinerea* nor *P.tricholoma* produced ligninolytic enzymes that can degrade lignin. The lignin binds the hemicellulose and makes it difficult for the microbial degradation.

After the two-stage aerobic treatment and anaerobic digestion, the lignin removal in the untreated grass, the CC-grass and the PT-grass was 31%, 47% and 54%, respectively. Even though the lignin degradation during the fungal treatment was not evident, the anaerobic digestion of lignin in the fungal treated grass was significantly higher than that of lignin in the untreated grass ($P<0.05$). The results may seem to contrast with most data in the literature about lignin biodegradation that were obtained by aerobic processes (Pérez et al., 2002)[80]. However, facultative microorganisms may alter or partially degrade portions of lignified plant cells by either anaerobic or aerobic processes (Akin, 1980)[1]. The data in this study suggests that the fungal pretreatment had helped the attack on lignified cell walls by facultative bacteria through an anaerobic process.

CHAPTER 4
EFFECTS OF ACCLIMATED MICROBIAL CONSORTIUM ON GRASS LIGNOCELLULOSIC DIGESTION AND BIOGAS PRODUCTION

4.1 Materials and Methods

4.1.1 Sludge

Mesophilic anaerobic sludge was used as the inoculum for anaerobic biogas production. This sludge was obtained from Ngaung-Khaem Water Quality Control Plant, a domestic wastewater treatment plant in Bangkok, Thailand. The sludge had 63.95 ± 0.01 g TS/L, 21.33 ± 0.44 g VS/L and 20.49 ± 0.09 g MLVSS/L. The total solids in the sludge contained 0.141 ± 0.003 g cellulose, 0.098 ± 0.006 g hemicelluloses and 0.041 ± 0.004 g lignin. This sludge will be referred to as original sludge (OS) in this chapter.

A portion of the OS was acclimated in a continuously stirred tank reactor (CSTR). The CSTR was fed with 52.8 g COD/L of POME at a flow rate of 300 mL/d for 2 weeks. The feed POME consisted of 12.64 ± 0.40% (w/w) cellulose, 4.31 ± 0.61% (w/w) hemicelluloses and 4.03 ± 0.20% (w/w) lignin. This sludge will be referred to as acclimated microbial consortium (AMC) in this study. The AMC had 50.32 ± 0.20 g TS/L, 25.08 ± 0.12 g VS/L and 23.55 ± 0.11 g MLVSS/L. The TS sludge in the AMC contained 0.194 ± 0.005 g cellulose, 0.076 ± 0.002 g hemicelluloses and 0.079 ± 0.004 g lignin.

4.1.2 Grass Samples

A wide variety of paragrass (*Brachiaria mutica*) collected from Prachinburi Province, Thailand during the summer season was used as the carbon fiber substrate. The grass sample was dried at 60 °C until the moisture content was less than 10%. The grass was chopped into 20 mm pieces and was ground into 1 mm powder by a blender. The grass was stored in a black bag at room temperatures between 30-35 °C prior to use. Characteristics of the grass are shown in Table 4.1. This report will refer to these grass samples as untreated grass.

Table 4.1 Properties of paragrass and other grass biomass

		Grass type			
Parameter	**Unit**	Paragrass *Brachiaria mutica*	Switchgrass *Panicum vergatum* (Fresh summer harvested)	Wild canary grass *Phalaris arundinacea*	Napier grass *Pennisetum purpureum*
pH		6.67	n.a.	5.57	n.a.
TS	% wet weight	29.37 ± 0.27	42.7 ± 6.7	35.72 ± 1.02	19.99[a]
VS	% wet weight	25.80 ± 0.22	39.6 ± 6.4	35.17 ± 0.13	18.20[a]
Cellulose	% TS	34.50 ± 0.36	24.6	29.76 ± 0.30	45.66[b]
Hemicellulose	% TS	28.34 ± 1.46	37.4	25.80 ± 0.84	33.67[b]
Lignin	% TS	10.07 ± 0.96	28.1	8.04 ± 0.02	20.60[b]
Carbohydrate	% TS	87.99 ± 0.36	n.a.	n.a.	n.a.
Crude protein	% TS	4.62±0.27	n.a.	9.14 ± 0.26	7.20[c]
Crude lipid	% TS	1.06±0.05	n.a.	2.05 ± 0.63	n.a.
Carbon (C)	% TS	44.87 ± 0.22	n.a.	n.a.	n.a.
Nitrogen (N)	% TS	1.52 ± 0.05	0.61	n.a.	n.a.
Ammonia nitrogen	mg/g TS	1.84 ± 0.03	n.a.	n.a.	n.a.
C/N ratio		29.5 ± 0.81	92	n.a.	43.6[a]
Hydrogen	% TS	6.39 ± 0.13	n.a.	n.a.	n.a.
Phosphorous (P)	% TS	n.a.	0.09	n.a.	0.19[c]
moisture	% wet weight	70.39 ± 0.45	n.a.	n.a.	73.03[a]
Reference		This study	Frigon et al. (2012) [29]	Oleszek et al. (2014) [77]	[a]Wilawana et al. (2014)[120] [b]Reddy et al. (2012)[88] [c]Tessema and Baars (2004)[105]

n.a. = not available

An alkaline pretreatment was used to prepare chemically treated paragrass samples. The alkaline pretreatment applied the method of Salvachua et al. (2011)[91]. Five grams of the paragrass samples were soaked with 15 mL of NaOH solution 5% (w/v). After that, the paragrass samples were autoclaved for 15 min at 121 °C.

In the biochemical methane potential tests described below, the alkaline treated grass (AKL-grass) and the native untreated paragrass (UT-grass) were used as the substrates.

4.1.3 Biochemical Methane Potential Tests

One hundred milliliter serum bottles, each with a rubber stopper, were used as batch reactors. Each bottle contained a native untreated grass sample, and either the OS or the AMC anaerobic sludge at a ratio of 1 g VS/g VS. The total working volume was 60 mL. The reactors were flushed for 1 min with 99.995% argon to ensure anaerobic conditions. The bottles were incubated under mesophilic temperatures between 32 - 35 °C for 80 days. The volume of the gas produced was measured periodically and the gas composition was analyzed by gas chromatography. After each specified incubation period, the samples were collected for physical and chemical analysis. For comparison, the biochemical methane potential of the alkaline treated grass was tested with the OS as the inoculum. The methane potential of the alkaline treated grass with AMC as the inoculum was not studied in these experiments.

4.1.4 Microbial Activity Tests

The inoculum activities were tested in 100 mL serum bottles at 30 - 35 °C. Each serum bottle contained 70 mL of a mineral solution mixed with the substrate specific for each activity test (Table 4.2) (Angelidaki et al., 2009)[6]. The mineral stock solution contained per liter; 2.5 g KH_2PO_4, 1 g K_2HPO_4, 1 g NH_4Cl, 0.213 g $MgCl_2 \cdot 6H_2O$, 0.118 g $Na_2S \cdot 9H_2O$, 3.0 g $NaHCO_3$ and 0.2 g yeast extract (Valcke and Verstraete, 1983)[110]. The OS and the AMC were centrifuged to remove the dissolved organic. Thirty mL of the centrifuged sludge sample (OS and AMC) was inoculated into the bottle, corresponding to 6 g VSS sludge per liter of reactor. The pH was adjusted to 7.0 with 3 N NaOH or 1 N HCl. Anaerobic conditions were initiated by flushing the headspace of each serum bottle for 1 min with argon.

Table 4.2 The substrates for activity determination of different anaerobic microorganism groups

Microorganism group	Initial substrate concentration	Data collection
Hydrolytic	3 g amorphous cellulose per liter	Glucose concentration
	3 g xylan per liter	Xylose concentration
Acidogenic	3 g glucose per liter	VFA and glucose concentrations
Acetogenic	3 g propionate per liter	VFA concentration
	3 g butyrate per liter	VFA concentration
Acetoclastic	3 g acetate per liter	VFA concentration and methane production

In the hydrolytic activity test, the reducing sugar concentration was monitored every 2 days. In the acidogenic activity test, the reducing sugar concentration was monitored every 2 h, and the volatile fatty acids (VFA) concentrations, the amount of gas and gas composition were monitored every 4 h. In the acetogenic activity test, VFA concentrations were measured every 2 days. In the acetoclastic activity test, VFA concentrations, the amount of gas and gas composition were monitored every 2 days. For the liquid samples, 1.5 mL of liquid was sampled without opening the serum bottles. The substrate utilization rate (or the product production rate) was determined from the slope of the initial linear part of each plot between substrate (or product) concentration versus time.

4.1.5 Analysis

The amount of biogas generated was collected using either 25 mL or 50 mL hospital needle syringes (Owen et al., 1979)[78]. The amount of biogas generated was collected at standard temperature and pressure (STP: 0 °C and 1 atmosphere). Biogas composition was determined by a gas chromatograph equipped with a thermal conductivity detector (SHIMADZU GC-2014, Japan) and a unibeads C column under an argon flow rate of 25 mL/min. Maximum methane production rate (MMPR) was estimated from the slope of the initial linear part of each methane production curve versus time. Biodegradability was the maximum plateau achieved in the methane production curves (Neves et al., 2004)[75].

Measurements of cellulose, hemicelluloses and lignin contents were performed by the detergent method (Van Soest and Wine, 1967)[112]. The hemicelluloses content was calculated from the difference between neutral detergent fiber (NDF) and the acid detergent

fiber (ADF). The lignin content was the difference between ADF and permanganate lignin (PML). After the PML analysis, the cellulose content was estimated from the weight loss of the sample when held at 550 °C for 3 h.

VFA concentration was determined by a gas chromatograph (SHIMADA GC-2010, Japan) equipped with a flame ionization detector. The initial column temperature of 60 °C was increased at the rate of 10 °C/min to a temperature of 230 °C. The detector temperature was set at 250 °C. The samples were centrifuged, filtered through a 0.45 μm glass fiber filter and acidified with 17% (v/v) H_3PO_4 in order to lower the pH value below 3, and to ensure that the acid was un-ionized.

Reducing sugar content was measured by the DNS method (Miller, 1959)[65]. The COD was determined using the closed-tube method (Finnish Standard Association, 1988)[28]. The concentrations of total solids (TS), volatile solids (VS), mixed liquored suspended solid (MLVSS) and ammonia nitrogen were determined by the Standard Methods (APHA, 1998)[8]. Carbohydrate, crude protein and fat were analyzed by the methods of the Association of Official Analytical Chemists (AOAC, 1995)[7]. The pH was measured by a pH meter (Schott Lab 850, Germany).

In this study, the measurements were repeated three times. The standard errors were all within 10% of the mean value. A test of significant difference based on the paired *t*-statistic was performed using the Microsoft Excel Solver Add-in. The difference was regarded as not significant if the paired *t*-statistic showed probability; $P > 0.05$ and significant if $P < 0.05$.

4.2 Results and Discussion

4.2.1 Comparative Microbial Activities of the OS and the AMC

During the anaerobic digestion of cellulosic biomass, the first step, and often the limiting step that takes place, is the hydrolysis of the complex substrate to organic acids, alcohols, sugars, hydrogen and carbon dioxide (CO_2) by the hydrolytic fermentative (acidogenic) bacteria. Subsequently, the fermentation products are converted to acetate and CO_2 by hydrogen-producing and acetogenic organisms. Acetate is further degraded by acetoclastic methanogens into CO_2 and methane (CH_4).

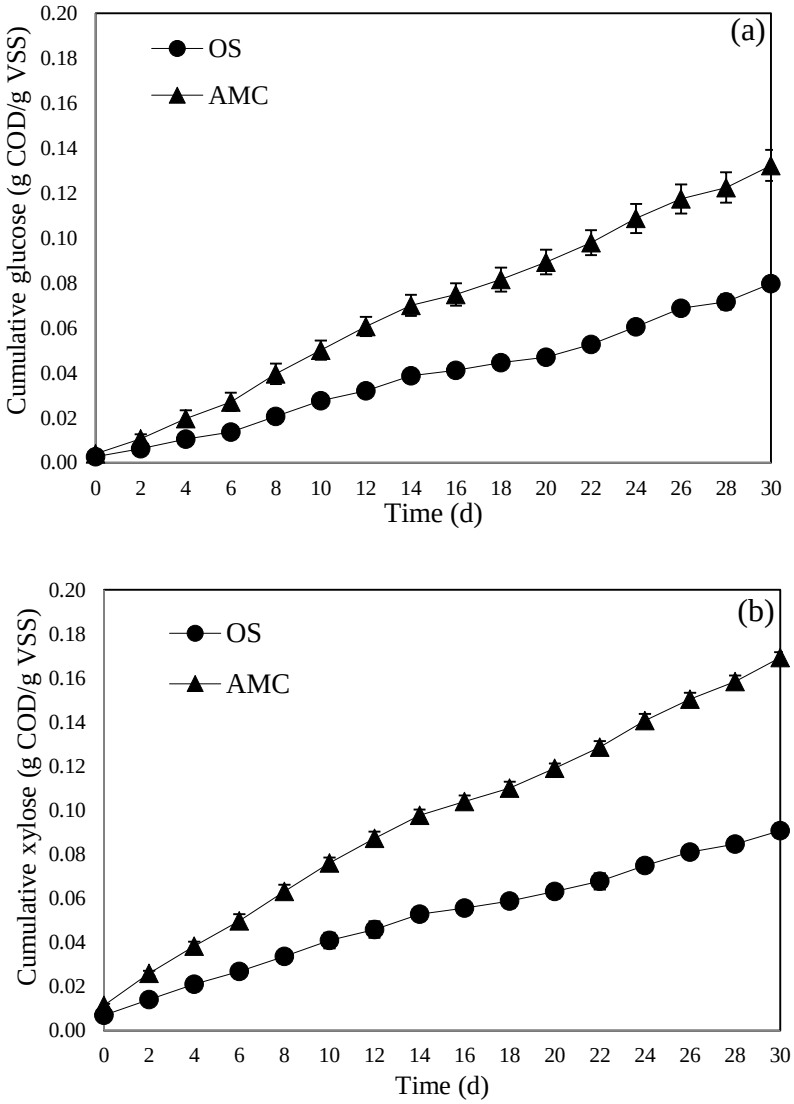
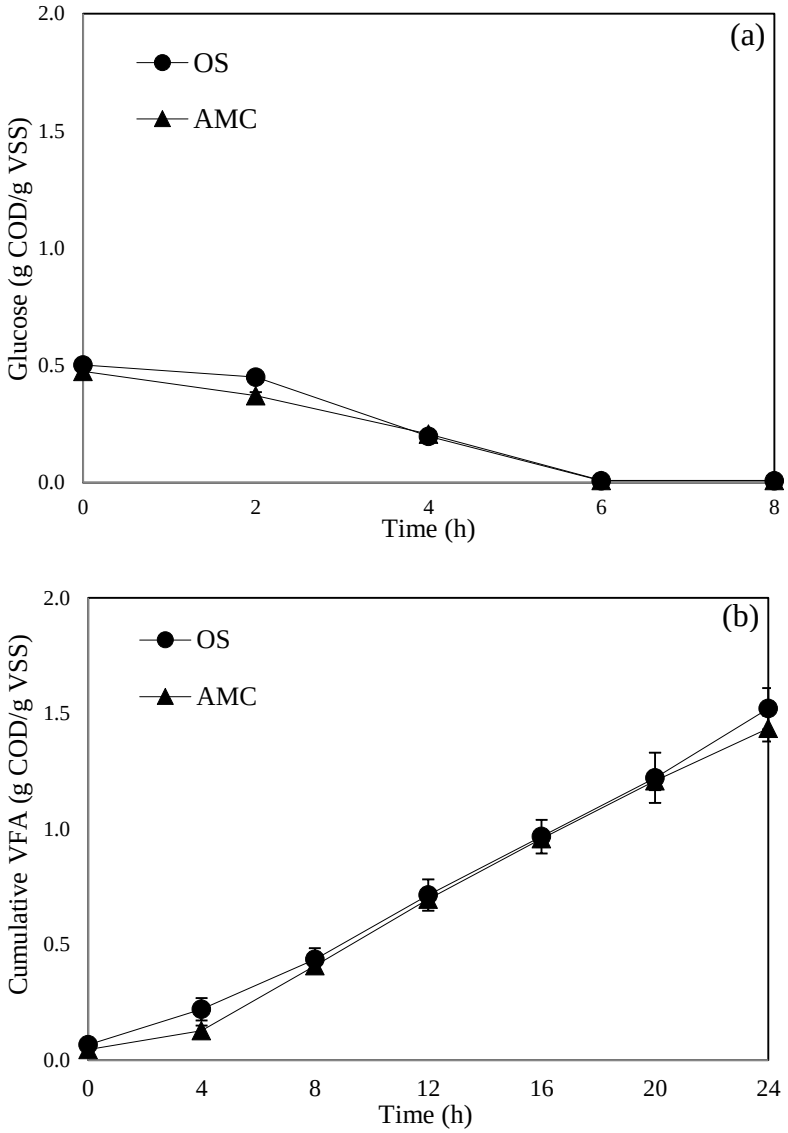


Fig. 4.1 Comparative hydrolytic activities of the original sludge (OS) and the acclimated microbial consortium (AMC): (a) glucose production; (b) xylose production.

Fig. 4.1 illustrates the cumulative hydrolytic fermentative products, glucose and xylose, from the anaerobic digestion of cellulose and xylan, respectively. The rates of glucose and xylose production by the AMC were twice as fast as those by the OS (Table 4.3). The results indicated that the acclimatization of the mesophilic microbial community in raw POME which contained fibrous substrates can enhance their capacity for degrading cellulose and xylan significantly ($P < 0.05$). These results are expected because previous studies have shown the presence of cellulolytic and hemicellulolytic bacteria which can effectively degrade palm fiber in POME anaerobic sludge (Khemkhao et al., 2015; Zainudin et al., 2013)[53,126].



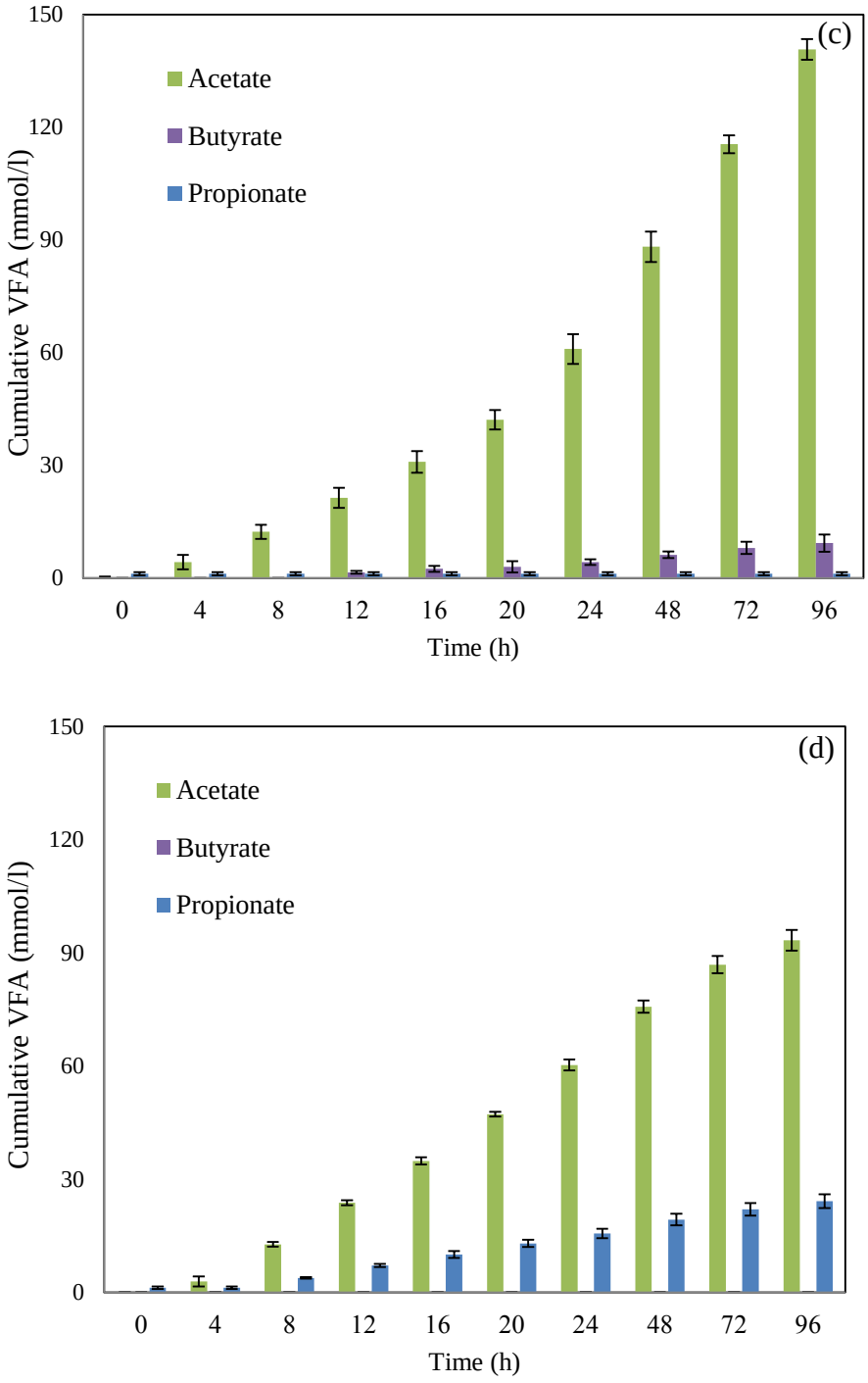


Fig. 4.2 Comparative acidogenic activities of the original sludge (OS) and the acclimated microbial consortium (AMC): (a) glucose utilization; (b) cumulative VFA production; (c) cumulative individual VFA production by the OS and (d) cumulative individual VFA production by the AMC.

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Table 4.3 Comparative substrate utilization rates and production rates of each group of microorganisms in the original sludge (OS) and the acclimated microbial consortium (AMC)

Microorganism group	Substrate	Substrate utilization rate (g COD/g VSS d)		Product	Production rate (g COD/g VSS d)	
		OS	AMC		OS	AMC
Hydrolytic	Cellulose	-	-	Glucose	0.0026	0.0044
	Xylan	-	-	Xylose	0.0030	0.0056
Acidogenic	Glucose	2.650	2.170	Total VFA	1.562	1.578
Acetogenic	Propionate	0.044	0.110	Acetate	0.016	0.037
	Butyrate	0.134	0.223	Acetate	0.084	0.093
Acetoclastic	Acetate	0.033	0.078	Methane	0.058	0.062

In contrast to the hydrolytic activity, the acidogenic activity of the sludge had been hardly enhanced at all by the acclimatization (Table 4.3). The curves of glucose uptake and VFA production of the OS and the AMC almost coincide (Fig. 4.2). However, the fact that the acclimatization did not enhance the acidogenic activity of the sludge should not affect the effectiveness of the technique because hydrolysis is often the rate-limiting step of anaerobic digestion of fibrous materials (Huntňan et al., 1999)[47]. The results from this study also showed that the rates of VFA production in the acidogenesis step were approximately 500 times faster than the rates of glucose production in the hydrolytic fermentation.

The acetogenic assay illustrated the difference in the acetogenic activity of the OS and the AMC. When propionate was used as the substrate, the substrate utilization rate (Fig. 4.3a) and the acetate production rate (Fig. 4.3b) of the AMC were approximately double those of the OS. The accumulative acetate in the assay with the AMC inoculum was markedly lower than that in the assay with the OS inoculum. The lower accumulative acetate corresponded to the earlier biogas production in the assay with the AMC (Fig. 4.3c). The biogas production in the assay with the AMC started almost immediately with the accumulation of acetate. The pH of the culture was around 6.5, which is known to be suitable

for methane production. In contrast, when the OS was used as the inoculum, the incubation period before the biogas production increased to approximately 10 days.

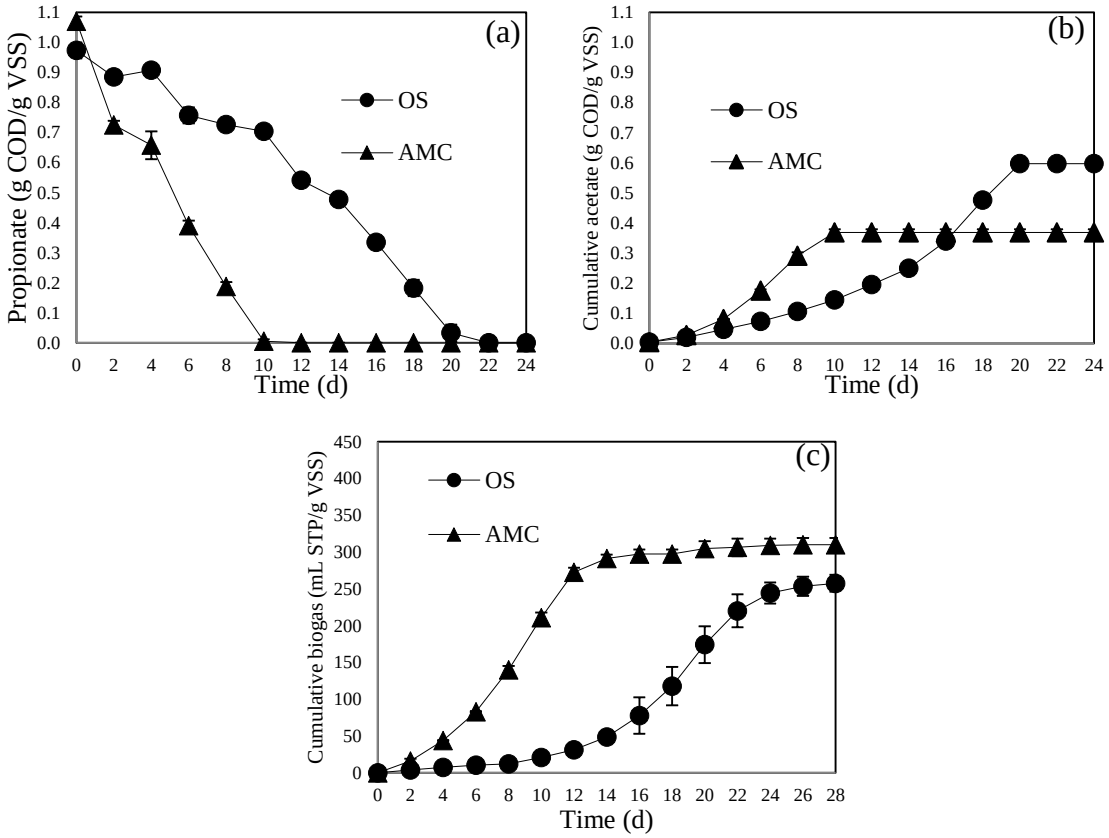

Fig. 4.3 Comparative acetogenic activities of the original sludge (OS) and the acclimated microbial consortium (AMC) when propionate was the substrate: (a) propionate utilization; (b) cumulative acetate production; (c) cumulative biogas production.

Similarly, when butyrate was used as the substrate, the substrate utilization rate of the AMC was approximately double that of the OS (Fig. 4.4a). The acetate production by the AMC started without a lag period and continued until day 8. In contrast, there was a lag phase of 2 days in acetate production by the OS (Fig. 4.4b). After that, the acetate production rate of OS was comparable with that of the AMC. The accumulative acetate in the assay with the AMC inoculum was markedly lower than that in the assay with the OS inoculum. The lower accumulative acetate corresponded to the earlier biogas production in the assay with the AMC (Fig. 4.4c).

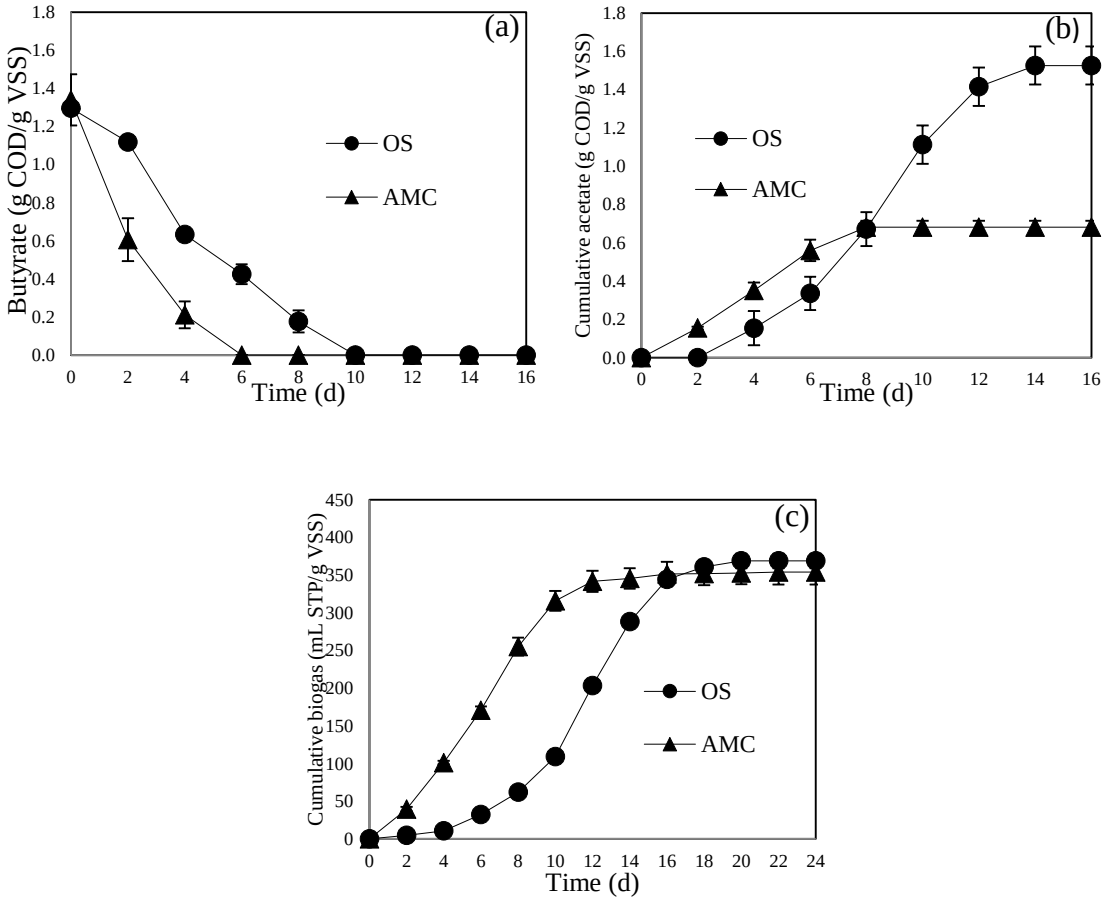

Fig. 4.4 Comparative acetogenic activities of the original sludge (OS) and the acclimated microbial consortium (AMC) when butyrate was the substrate: (a) butyrate utilization; (b) cumulative acetate production; (c) cumulative biogas production.

The methanogenic assay illustrated the difference in the methanogenic activity of the OS and the AMC. The consumption of acetate of the OS can be divided into two phases (Fig. 4.5a). In the first phase from day 0 to day 8, the acetate consumption occurred at a lower rate of 0.033 g acetate COD/g VSS d. In the second phase from day 8 to day 14, the acetate consumption was faster at a rate of 0.076 g acetate COD/g VSS d. In contrast, the consumption of acetate by the AMC occurred in one phase, and the acetate was completely depleted within 8 days. Considering the CH_4 product, the CH_4 production by the AMC took place without a lag phase. In contrast, when the OS was used as the inoculum, the incubation period before the rate of CH_4 production increased was about 8 days (Fig. 4.5b). The results indicated that the acclimatization of the mesophilic microbial community in raw POME had helped to enhance the methanogenic activity significantly ($P < 0.05$).

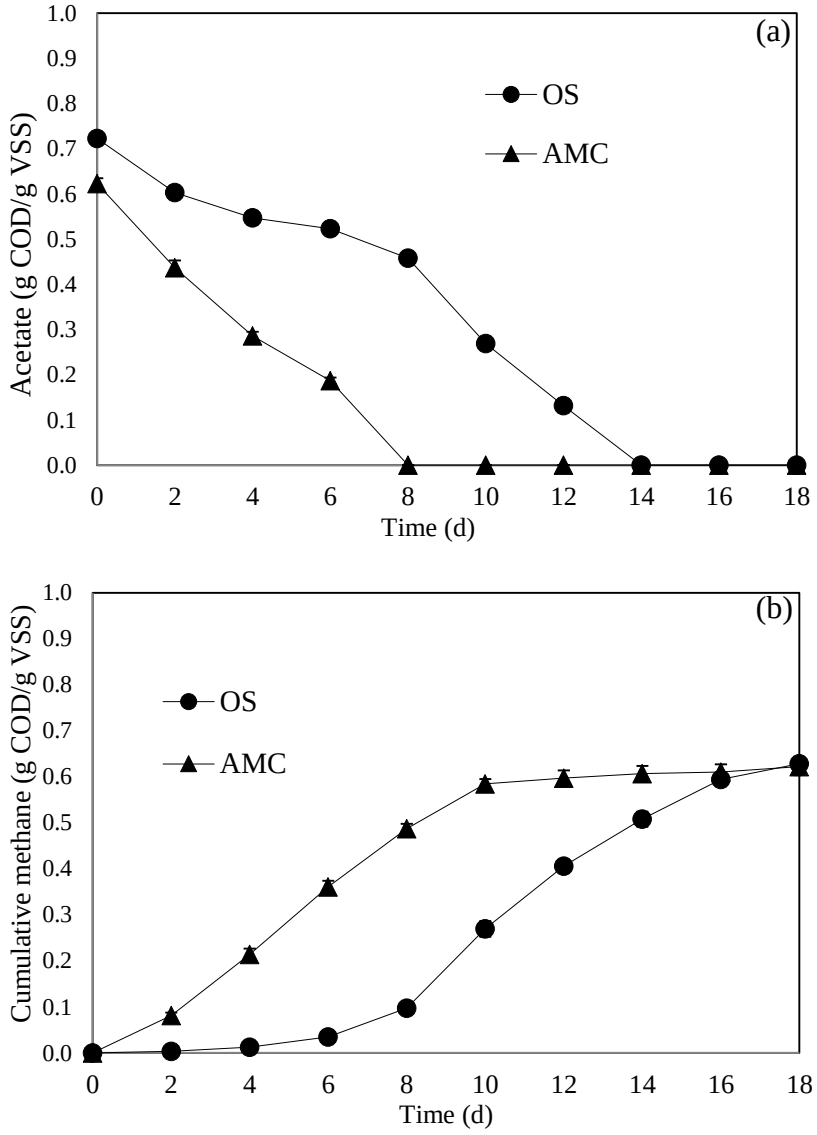

Fig. 4.5 Comparative acetoclastic activities of the original sludge (OS) and the acclimated microbial consortium (AMC): (a) acetate utilization; (b) cumulative methane production.

4.2.2 Production of Biogas from Paragrass using the OS and the AMC

Fig. 4.6 shows the biogas production from paragrass using the OS and the AMC as the inoculums. It can be seen that when the OS was used as the inoculum, there was an incubation period of 8 days before the amount of CH_4 had increased, while the CH_4 production by the AMC occurred without a lag phase. These behaviors are in good agreement with those observed in the methanogenic assays. The maximum methane production rates of both inoculums were comparable; i.e., 12.5 ± 0.5 mL STP/ g VS d. The methane yields of

paragrass after 80 days anaerobic digestion inoculated with the OS and the AMC were 277 and 316 mL STP/g VS added, respectively. Using the AMC as the inoculums increased the methane yield by approximately 15%. The acclimatization of the mesophilic microbial community in raw POME results in a better adaptability of the inoculums and helps enhance the methane yield of the paragrass. The importance of inoculum sources has been previously reported for the anaerobic digestion of rice straw (Gu et al., 2014)[39]. The better adaptability of inoculum sources to cellulose and hemicelluloses substrates was found to relate to the higher cellulose and hemicelluloses degradation rates and the higher biogas production from rice straw. In this study, the increase in the methane yield by the biological method (303 mL STP/g VS added at day 60) was comparable with the increased methane yield obtained by the alkaline hydrolysis using sodium hydroxide (306 mL STP/g VS added at day 60). However, the acclimatization technique is a more environmentally friendly and cheaper method as it does not require extra energy and chemical input.

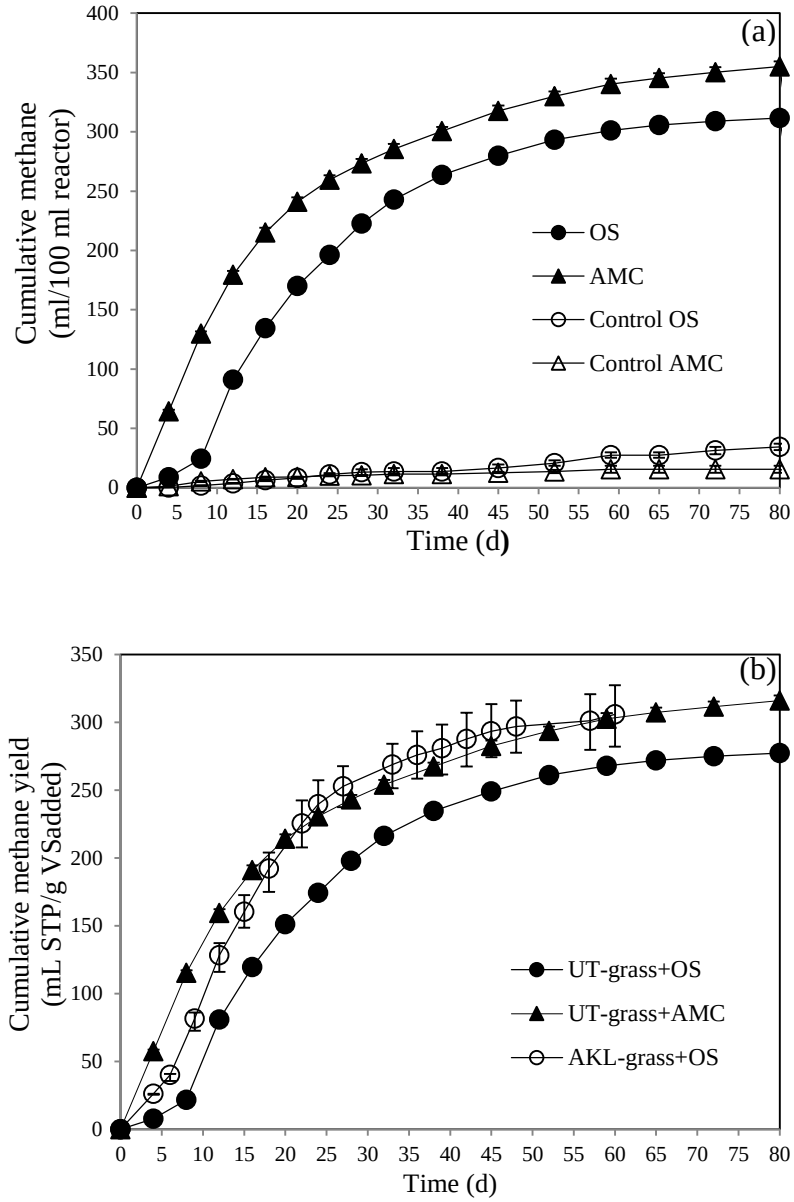

Fig. 4.6 Comparative (a) cumulative methane and (b) cumulative methane yield from the anaerobic digestion of the paragrass by the original sludge (UT-grass+OS), the paragrass by the acclimated microbial consortium (UT-grass+AMC) and the alkaline pretreated paragrass by the OS (ALK-grass+OS).

Table 4.4 reports the normalized weight and the percentage loss of TS, cellulose, hemicelluloses and lignin after 80 days anaerobic digestion. During the anaerobic digestion, the grass inoculated with the AMC lost 51% of its cellulose, while the grass inoculated with the OS lost only 40%. The results were in good agreement with those from the hydrolytic assays, which illustrated that the AMC can degrade cellulose more effectively.

Table 4.4 Normalized weight and percentage loss of cellulose and hemicellulose after anaerobic digestion for 80 days

Substrate/ Inoculums	Component	Before	After the anaerobic fermentation	
		Normalized weight	Weight	% Loss
Paragrass/OS	Cellulose	0.345	0.206	40.24 ± 0.66
	Hemicellulose	0.283	0.146	48.35 ± 0.55
	Lignin	0.100	0.063	37.17 ± 2.71
Paragrass/AMC	Cellulose	0.345	0.169	51.16 ± 1.40
	Hemicellulose	0.283	0.116	59.14 ± 2.54
	Lignin	0.100	0.060	40.47 ± 1.33

The hemicellulose degradation in the grass inoculated with the OS was 48%, while that in the grass inoculated with the AMC was 59%. The amounts of recalcitrant hemicelluloses in the grass inoculated with the AMC were significantly lower than those in the grass inoculated with the OS ($P < 0.05$). The data indicated that the acclimatization helped enhance degradation of hemicelluloses. Biodegradation of lignin in the paragrass was also observed. Even though lignin biodegradation is more often obtained by aerobic processes (Pérez et al., 2002)[80], it has been reported that facultative microorganisms may alter or partially degrade portions of lignified plant cells by either anaerobic or aerobic processes (Akin, 1980)[1]. This may explain the lignin degradation during the anaerobic digestion. Furthermore, the lignin binds the hemicelluloses and makes it difficult for the microbial degradation. As some facultative microorganisms present in the inoculums can alter or partially degrade portions of lignified plant cells, the degradation of hemicelluloses was possible. The higher hemicelluloses degradation capacity of the AMC is a main factor that leads to the higher methane potential of the paragrass.

The conversion of hemicelluloses in the paragrass was found to be 8% higher than the conversion of cellulose, regardless of different types of the inoculums. The data was consistent with those reported early by Ghosh et al. (1985)[33] that the conversion of hemicelluloses in various grass substrates via anaerobic digestion was higher than those of cellulose and protein under mesophilic conditions. However, cellulose was utilized in

preference to hemicellulose during mesophilic fermentation of Bermuda grass in the presence of supplemented nitrogen.

4.2.3 Potential of Paragrass as an Energy Crop

The potential of methane production from paragrass ($PMP_{paragrass}$) can be estimated from its methane yield and its dry matter yield. Using the dry matter yield ranging between 5,000 – 12,000 kg/ha a, the VS/TS ratio of the paragrass 0.88, and the methane yield of 316 mL STP/g VS added using the AMC inoculums, the $PMP_{paragrass}$ was estimated to be between 1,390 – 3,337 Nm^3 CH_4/ha a. The $PMP_{paragrass}$ drops to 1,218 – 2,925 Nm^3 CH_4/ha a, when calculated from the methane yield of 277 mL STP/g VS added using the OS inoculums. As for a biogas plant, a few hundred hectares of land are typically dedicated for growth of energy crops (Braun et al., 2009)[13], the enhanced methane yield of 15% has an impact on the amount of methane production.

The maximum $PMP_{paragrass}$ estimated from the maximum dry matter yield of 12,000 kg/ha a, i.e. 3,337 Nm^3 CH_4/ha a, is comparable to that of clover grass of 3,000 – 4,500 Nm^3 CH_4/ha a (Amon et al., 2004)[4]. The maximum $PMP_{paragrass}$ is highly comparable with those of cocksfoot, tall fescue, reed canary grass and timothy of 1,200 – 3,600 Nm^3 CH_4/ha a (Seppälä et al., 2009)[95]. The maximum $PMP_{paragrass}$ is also comparable with the methane production of 3,450 Nm^3 CH_4/ha a of an existing biogas plant, using solely solid enegy crop substrates, i.e. maize silage and grass (Braun et al., 2009)[13]. Therefore, based on the maximum $PMP_{paragrass}$ which is comparable with other existing energy crops, paragrass was found to be an economically feasible energy crop for biogas production.

4.2.4 Energy analysis

The energy flow in a biogas plant was analyzed by the potential of methane production from paragrass ($PMP_{paragrass}$) using the dry matter yield of 5,000 kg/ha a and VS/TS ratio of 0.88. The methane yield of the grass inoculated with the AMC was 316 mL STP/g VS added and the methane yield of the grass inoculated with the OS was 277 mL STP/g VS added. The energy content in methane gas is 55.7 kJ/g CH_4. Heat production from the combined heat and power (CHP) was 30% of total energy from methane production. Electrical production from CHP was 47.5% of the total energy from methane production. Heat and electrical usage in the biogas plant were 543 kWh/ha a and 4,783 kWh/ha a, respectively. Fig. 4.7 and Fig. 4.8 show the energy analysis of paragrass using the OS and the AMC as the inoculums, respectively.

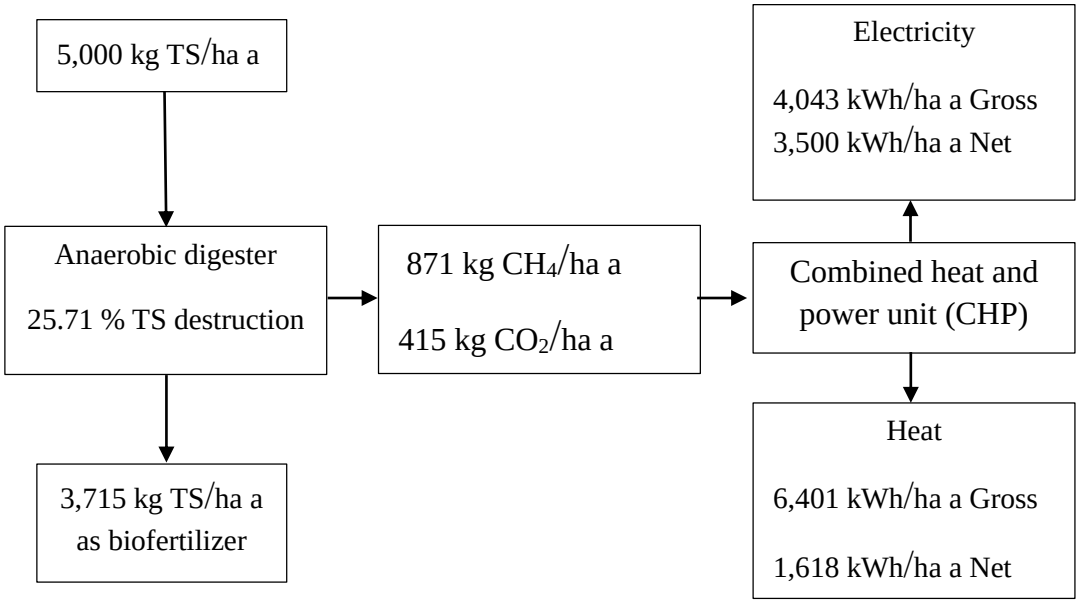

Fig. 4.7 The energy analysis of paragrass using the OS as the inoculums.

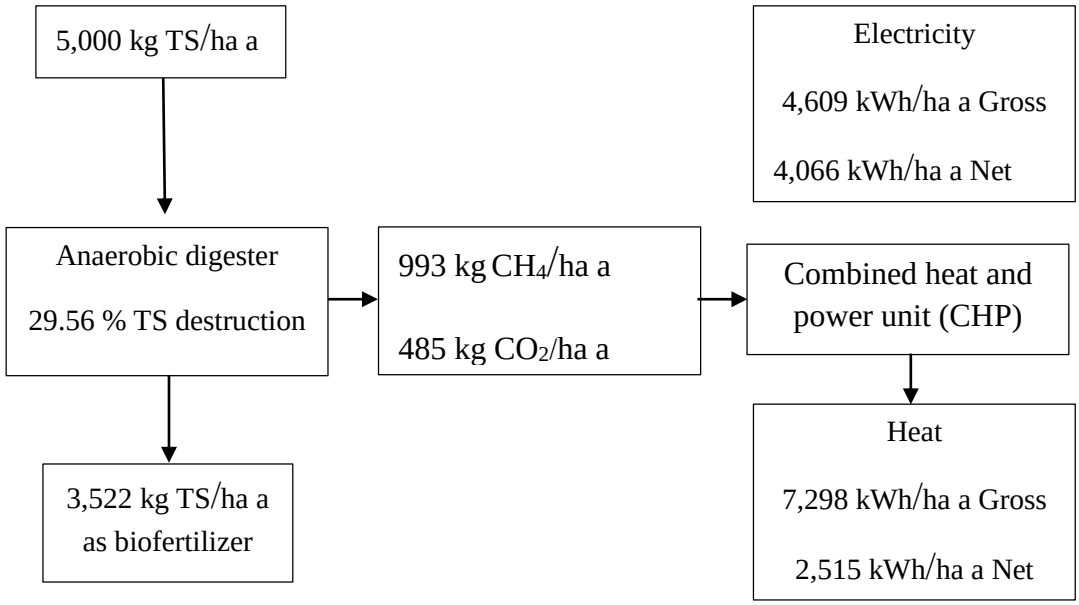

Fig. 4.8 The energy analysis of paragrass using the AMC as the inoculums.

4.2.5 Comparative energy potential of anaerobic technique and other techniques

Anaerobic digestion is the conversion of organic material directly to gas, termed biogas, a mixture of mainly methane and carbon dioxide with small quantities of other gases, such as hydrogen sulphide (McKendry, 2002)[63].

Combustion is used over a wide range of outputs to convert the chemical energy stored in biomass into heat, mechanical power, or electricity. Combustion of biomass produces hot gases at temperatures around 800 ˚C to 1,000 ˚C. It is possible to burn any type of biomass, but in practice combustion is feasible only for biomass with a moisture content less than 50%, unless the biomass is pre-dried. High moisture content biomass is better suited to biological conversion processes (McKendry, 2002)[63].

Fermentation is used to produce ethanol from sugar crops and starch crops. The biomass is ground down and the starch converted by enzymes to sugars, then converting the sugars to ethanol with yeast (McKendry, 2002)[63].

Table 4.5 reports the energy potential of anaerobic digestion and other techniques using the dry matter yield of 5,000 kg/ha a and the VS/TS ratio of the paragrass 0.88. The gross energy potential of the grass from anaerobic digestion was 10.4 to 11.9 MWh/ha a, While the gross energy potential of the grass from combustion was 22.6 to 25.0 MWh/ha a, using heating value of 16.3 to 18.0 MJ/kg TS (Prochnow et al., 2009)[85]. The gross energy potential of the grass via ethanol production was 11.9 0 MWh/ha a, based on the reported that 1 L of ethanol was produced from 2.5 kg of switchgrass and 5.13 million kcal was achieved from 1,000 L of ethanol (Pimentel and Patzek, 2005)[83]. The energy potential of the grass from anaerobic digestion could also compare with that of combustion and ethanol production. However, in ethanol production process, the conversion of grass is more complex, due to the presence of long-chain polysaccharide molecules and requires acid or enzymatic hydrolysis before the resulting sugars can be fermented to ethanol. Such hydrolysis techniques are currently at the pre-pilot stage (McKendry, 2002)[63]. Grass combustion is possible as stand-alone biomass-firing or with other fuels, but the grass harvest usually involves drying in the field and clearing with conventional farm machinery. Moreover, pelleting or briquetting of grass may be required to improve the biofuel quality from combustion (Prochnow et al., 2009)[85].

Table 4.5 Energy potential of anaerobic digestion and other techniques from the grass

Technique	**Anaerobic digestion**	**Combustion**	**Bioethanol**
Gross energy potential (MWh/ha a)	10.4 – 11.9 (This study)	22.6 – 25.0 (Prochnow et al., 2009) [85]	11.9 (Pimentel and Patzek, 2005) [83]
Utilises entire organic biomass (Milledge et al., 2014)[64]	Yes	Yes	No
Requires biomass drying after harvesting (Milledge et al., 2014)[64]	No	Yes	No
Primary energy product (Milledge et al., 2014)[64]	Gas	Heat	Liquid

CHAPTER 5
CONCLUSIONS

5.1 Effects of Fungal Pretreatment on Lignocellulose Degradation and Biogas Production of Paragrass

Based on the results in this study and those reported in the literature, it is clear that the pretreatment by edible fungi helps increase the biodegradability of lignocellulolytic substrates. This is because the most edible fungi can produce cellulase and hemicellulase, and some can produce lignin-degrading enzymes. However, the effectiveness of the fungal pretreatment on enhancing the methane potential of lignocellulosic substrates is uncertain because a portion of carbon source in the substrate is utilized by the fungi for their own metabolism. The net gain or loss in the methane yield will depend on whether the amounts of pretreated recalcitrant carbon compounds which become available for anaerobic bacteria are greater or less than the amounts of carbon consumed by the fungi for their metabolism. In the case of the paragrass treated with monoculture of *C. cinerea* or *P. tricholoma*, the amounts of recalcitrant carbon compounds which become available for anaerobic bacteria after pretreatment (crystalline cellulose) are less than the amounts of carbon consumed by the fungi for their metabolisms (a portion of hemicellulose). Therefore, the methane yields of the treated grass were lower than that of the untreated grass. However, the results show that the methane yields of the treated grasses occur at much shorter times than the yield from the untreated grass and therefore the pretreatment could decrease the cost of the methane production. However, the fungal pretreatment results in the earlier methane production approximately 20 days, and approximately a 1.5-fold increase in the degraded biomass.

5.2 Effects of the Unacclimated and the Acclimated Microbial Consortium as the Inoculum on Lignocellulose Degradation and Biogas Production of Paragrass

The acclimatization of sludge to fibrous substrates in raw palm oil mill effluent can enhance the hydrolytic, acetogenic and methanogenic activities of the sludge significantly. The production rates of glucose and xylose from cellulose and xylan by the acclimated microbium consortium were two-fold faster than those by the original sludge. The acclimated microbium consortium could increase the methane yield of paragrass by

approximately 15%. The potential of methane production from paragrass estimated from its methane and dry matter yields was 3,337 Nm^3 CH_4/ha a. The paragrass grown on unfertilized land in tropical regions can be a potential for biogas production.

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APPENDIX A

EXPERIMENTAL DATA OF THE FUNGAL TREATED GRASS

A.1 Culture Media for Fungal Isolation

Medium contained per liter: basal solution 764.15 mL, 68 g/L KH_2PO_4 solution 9.43 mL, 50 g/L yeast extract solution 9.43 mL, 37.5 g/L glucose 94.34 mL, 80 g/L Na_2CO_3 solution 4.72 mL, vitamin solution 9.43 mL, reducing agent solution 9.43 mL, antibiotic solution 4.72 mL and lysozyme solution 9.43 mL (Lowe et al., 1987)[59].

Basal contained KCl 0.6 g, NaCl 0.6 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCl_2 \cdot 2H_2O$ 0.2 g, NH_4Cl 0.54 g, trypticase peptone 1 g, PIPES buffer 1.5 g, coenzyme M solution 10 ml, fatty acid solution 10 mL, trace elements solution 10 mL, haemin solution 10 mL, 1 g/L resazurin solution 1 mL. The pH of the solution was adjusted to 6.8 with 1 M KOH and the volume was made up to 810 mL with 10 g/L cellulose solution. For solid media, oxoid bacteriological agar no.1 was added to the basal solution to a final medium concentration of 18 g/L.

The vitamin solution was prepared in 5 mM HEPES buffer containing (g/L): 1,4-naphthoquinone 0.25, calcium D-pantothenate 0.2, nicotinamide 0.2, riboflavin 0.2, thiamin HCl 0.2, pyridoxine HCl 0.2, biotin 0.025, folic acid 0.025, cyanocobalamin 0.025, and *p*-aminobenzoic acid 0.025.

The reducing agent solution contained $Na_2S \cdot 9H_2O$ 2.5 g and L-cysteine·HCl 2.5 g in water 100 ml.

The antibiotic solution contained (g/L): streptomycin sulphate 2, penicillin G 8, chloramphenicol 6, oxytetracycline 5 and neomycin sulphate 6.

The lysozyme solution contained (g/L): lyzozyme 4 and EDTA (disodium salt) 3.

The coenzyme M solution was prepared by dissolving the sodium salt of 2-mercaptoethane sulphonic acid in water to give a concentration of 4 g/L.

The fatty acid solution was prepared by mixing 6.85 mL 95% acetic acid, 3.0 mL 95% propionic acid, 1.84 mL 98% butyric acid, 0.55 mL 85% 2-methylbutyric acid, 0.47 mL 95% isobutyric acid, 0.55 mL 95% valeric acid and 0.55 mL 95% isovaleric acid with 700 mL of 0.2 M NaOH. The pH of the fatty acid mixture was adjusted to 7.5 with 1 M NaOH and its volume was adjusted to 1 L with water.

The trace element solution was prepared in 0.2 M HCl and contained (g/L): $MnCl_2 \cdot 4H_2O$ 0.25, $NiCl_2 \cdot 6H_2O$ 0.25, $NaMoO_4 \cdot 2H_2O$ 0.25, H_3BO_3 0.25, $FeSO_4 \cdot 7H_2O$ 0.20, $CoCl_2 \cdot 6H_2O$ 0.05, SeO_2 0.05, $NaVO_3 \cdot 4H_2O$ 0.05, $ZnCl_2$ 0.025 and $CuCl_2 \cdot 2H_2O$ 0.025.

The haemin solution was prepared by dissolving 0.1 g haemin in 10 ml ethanol and the volume to 1 L with 0.05 M NaOH.

The KH_2PO_4 and yeast extract solutions were prepared right before use.

The antibiotic, lysozyme and vitamin solution were sterilized by membrane filtration (0.22 μm pore diameter). The glucose solutions were autoclaved at 115 °C for 10 min, and all other solutions and agar media were autoclaved at 121 °C for 15 min.

Table A.1 Total solid, cellulose, hemicellulose and lignin of the untreated grass and treated grass before and after pretreatment

Sample	parameter	initial weight (g)	weight after pretreatment (g)
UT-grass	Total solid	5.0004	4.8035
		5.0001	4.8045
		5.0005	4.8291
	Cellulose	1.7648	1.5805
		1.8963	1.5780
		1.8723	1.5445
	Hemicellulose	1.7603	1.5680
		1.7682	1.4997
		1.7320	1.5044
	Lignin	0.5418	0.4894
		0.5896	0.4981
		0.5873	0.5214
CC-grass	Total solid	4.8035	3.4938
		4.8045	3.6416
		4.8291	3.2999
	Cellulose	1.5805	1.2942
		1.5780	1.2764
		1.5445	1.2893
	Hemicellulose	1.5680	1.0831
		1.4997	1.1099
		1.5044	1.0826
	Lignin	0.4894	0.4286
		0.4981	0.4483
		0.5214	0.4652
PT-grass	Total solid	4.8035	3.3151
		4.8045	3.4797
		4.8291	3.4449
	Cellulose	1.5805	1.0336
		1.5780	1.0732
		1.5445	1.0938
	Hemicellulose	1.5680	1.1961
		1.4997	1.2349
		1.5044	1.2222
	Lignin	0.4894	0.3792
		0.4981	0.4199
		0.5214	0.4367
AKL-grass	Total solid	4.8035	5.5078
		4.8045	5.5251
		4.8291	5.5408
	Cellulose	1.5805	1.4699
		1.578	1.4522
		1.5445	1.4559
	Hemicellulose	1.568	0.5315
		1.4997	0.5415
		1.5044	0.5983
	Lignin	0.4894	0.1709
		0.4981	0.1838
		0.5214	0.2237

Table A.2 Biogas production and biogas composition of the untreated grass anaerobic digestion (UT-grass+OS) for 140 days

Operating day (d)	Temperature (°C)	Biogas production (mL)			Biogas composition (%)								
					1			2			3		
		1	2	3	H_2	CH_4	CO_2	H_2	CH_4	CO_2	H_2	CH_4	CO_2
0	35.0	0.0	0.0	0.0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
1	34.0	28.3	32.2	25.5	0.12904	14.01402	41.74334	0.00000	15.83262	45.85076	0.00000	58.20036	27.30889
4	35.5	38.0	35.0	38.0	0.02983	29.54011	53.92345	0.02941	29.69472	53.91452	0.12201	78.54413	8.66883
7	34.0	52.0	52.0	54.0	0.00000	40.35743	51.79338	0.00000	38.94891	54.51577	0.00000	0.07832	0.00000
10	34.0	77.0	80.0	80.0	0.00000	52.13148	44.54844	0.00000	55.54049	42.24905	0.00000	44.52832	24.06291
13	32.0	71.0	70.0	67.5	0.00000	45.61074	51.66373	0.19530	48.46509	45.90473	0.03210	49.59934	33.14794
16	34.0	36.0	34.0	35.5	0.05576	53.84198	44.72689	0.00000	58.27993	40.93215	0.00000	63.70856	6.47299
19	32.0	52.5	54.0	51.0	0.00000	55.31885	42.52565	0.00000	52.85592	46.79728	0.00000	49.94982	31.83447
22	32.5	60.5	66.0	65.0	0.00000	63.67578	36.32422	0.00000	61.30425	38.29757	0.00000	53.82672	30.31956
31	31.0	66.0	68.5	64.0	0.00000	59.10139	40.38392	0.00000	61.45871	38.20119	0.02927	62.84285	33.19857
33	31.0	20.0	23.0	21.0	0.00000	47.50193	45.81375	0.00000	50.61785	48.93117	0.01980	45.37622	30.15384
36	31.0	24.0	20.0	21.0	0.00000	51.44191	48.21369	0.00000	51.28769	47.84037	0.00000	60.42882	30.38813
45	31.0	27.5	30.0	36.0	0.00000	54.11653	45.50846	0.00000	51.14411	48.48624	0.00000	69.15365	28.91213
50	30.5	33.5	30.0	36.0	0.00000	56.89203	42.85427	0.00000	56.69145	43.09636	0.00000	63.99187	30.14649
57	32.0	29.0	26.0	28.0	0.00000	49.54502	50.21190	0.00000	54.17964	45.60876	0.00000	65.58857	32.31897
60	31.0	28.0	26.5	26.0	0.00000	57.62212	42.04995	0.00000	55.12781	44.65285	0.00000	44.47318	17.12053
70	31.0	27.0	27.5	27.5	0.00000	53.04918	46.58411	0.00000	52.63000	47.07041	0.00000	64.38230	30.53451
77	32.0	40.0	37.0	30.0	0.00000	54.24852	45.37424	0.00000	54.99961	44.86623	0.00000	66.55719	31.53959
85	31.0	12.6	11.2	12.6	0.00000	51.14756	48.18759	0.00000	47.75914	51.84363	0.00000	49.46402	26.69746
93	30.0	16.4	13.4	14.8	0.00000	55.64505	43.64398	0.00000	55.68092	43.79549	0.00000	49.77690	33.37834
99	31.0	14.2	13.5	13.2	0.00000	54.30878	45.02328	0.00000	53.97982	45.68150	0.00000	51.16853	28.60805
106	33.0	15.0	14.0	13.0	0.00000	56.41380	43.15289	0.00000	54.75470	44.85928	0.00000	57.05103	28.06785
113	31.0	18.2	16.4	16.0	0.00000	40.17580	34.67088	0.00000	53.53804	45.95321	0.00000	51.68468	27.80003
116	30.0	6.0	8.4	5.0	0.00000	40.17580	34.67088	0.00000	53.53804	45.95321	0.00000	52.59193	28.86985
120	30.0	0.0	0.8	0.0	0.07050	53.62402	41.68486	0.02795	52.58374	44.29394	0.00000	57.59196	21.23576
129	30.0	7.6	10.1	8.7	0.00000	56.00710	41.56328	0.02784	51.68243	45.23237	0.02498	51.65872	26.96717
134	30.0	8.6	7.2	5.8	0.00000	56.47925	42.16055	0.06641	59.29752	39.72177	0.00000	54.60378	27.62388
140	30.0	4.8	5.4	4.2	0.00000	56.27892	42.38891	0.00000	56.56135	42.55553	0.00000	57.06003	28.53170

Table A.3 Biogas production and biogas composition of the *C. cinerea* treated grass (CC-grass+OS) for 140 days

Operating day (d)	Temperature (°C)	Biogas production (mL)			Biogas composition (%)								
					1			2			3		
		1	2	3	H_2	CH_4	CO_2	H_2	CH_4	CO_2	H_2	CH_4	CO_2
0	35.0	0.0	0.0	0.0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
1	34.0	18.5	24.1	35.5	0.00000	32.21974	34.51076	0.18218	21.90457	66.85494	0.00000	10.84549	44.10845
4	35.5	70.0	70.0	66.5	0.03293	65.24500	24.66990	0.03866	56.64006	40.36379	0.09470	62.84115	33.22003
7	34.0	74.0	74.5	75.5	0.00000	55.65155	41.99890	0.00000	56.34468	43.24290	0.00000	55.90790	43.50622
10	34.0	68.0	67.0	68.0	0.05250	54.73306	43.85985	0.00000	44.19644	40.98435	0.00000	57.38311	42.19549
13	32.0	43.5	40.0	40.0	0.02818	54.11749	45.49862	0.18093	51.44543	46.95131	0.00000	48.38807	50.71276
16	34.0	20.5	23.5	23.5	0.00000	58.35305	41.35925	0.04203	52.71593	44.24090	0.04372	51.37595	47.35162
19	32.0	28.0	29.0	29.5	0.00000	51.06169	48.38079	0.00000	51.99051	47.81786	0.00000	49.79247	49.84138
22	32.5	29.0	28.0	26.5	0.00000	50.12180	49.35754	0.00000	46.38355	53.36793	0.00000	50.55294	49.10859
31	31.0	45.0	41.0	39.0	0.00000	60.40247	39.04361	0.00000	61.10492	38.28217	0.00000	58.03114	41.83568
33	31.0	13.0	13.0	15.0	0.00000	47.48727	48.58631	0.01612	49.79773	49.93035	0.02013	49.08948	48.66448
36	31.0	13.5	16.0	17.0	0.00000	52.00289	47.53914	0.01291	50.61721	48.75609	0.00000	51.15068	48.31221
45	31.0	29.5	27.0	31.0	0.00000	52.71534	46.80165	0.00000	50.41072	47.19778	0.00000	53.36519	46.27878
50	30.5	28.0	27.5	27.0	0.00000	55.00591	44.63091	0.00000	54.90605	44.90350	0.00000	54.17805	45.52240
57	32.0	20.0	20.0	19.5	0.00000	52.90401	46.75353	0.00000	52.16612	46.40315	0.00000	53.16889	46.43928
60	31.0	20.0	18.0	18.5	0.00000	54.15816	45.53309	0.00000	55.02155	44.72179	0.00000	54.51313	45.13518
70	31.0	16.0	16.0	15.5	0.00000	53.30804	46.30460	0.00000	53.33171	46.17417	0.00000	53.61296	45.94672
77	32.0	32.0	29.0	31.0	0.00000	61.02063	38.64946	0.00000	54.91832	44.18792	0.00000	54.42833	45.02463
85	31.0	5.4	7.0	6.6	0.00000	52.85236	46.35755	0.00000	52.42783	45.78304	0.00000	54.52105	44.22874
93	30.0	11.0	11.6	11.6	0.00000	57.24849	41.89311	0.00000	53.75604	44.29256	0.00000	55.19556	43.02948
99	31.0	12.6	11.8	11.8	0.00000	55.77726	43.61713	0.00000	54.98275	43.65489	0.00000	54.86350	44.06959
106	33.0	12.0	12.0	12.0	0.00000	58.75881	40.31345	0.00000	56.42729	42.25601	0.00000	56.85513	42.12163
113	31.0	16.0	16.4	17.6	0.00000	51.64880	41.10433	0.00000	48.48353	44.56140	0.00000	51.25710	41.19889
116	30.0	7.0	6.6	6.5	0.00000	51.64880	41.10433	0.00000	48.48353	44.56140	0.00000	51.25710	41.19889
120	30.0	0.0	0.0	0.0	0.04202	55.69774	41.11776	0.03516	53.76474	41.30797	0.01669	55.11806	40.10313
129	30.0	1.4	8.0	9.0	0.00000	67.56398	29.88833	0.10084	72.02412	25.74959	0.04567	50.97905	46.68513
134	30.0	5.8	6.9	6.0	0.00000	55.71987	37.85784	0.00000	58.71809	39.44540	0.00000	57.58839	40.46243
140	30.0	6.0	4.0	5.4	0.00000	58.60457	40.35034	0.00000	57.71430	40.94389	0.00000	57.42968	41.09219

Table A.4 Biogas production and biogas composition of the *P. tricholoma* treated grass (PT-grass+OS) for 140 days

Operating day (d)	Temperature (°C)	Biogas production (mL)			Biogas composition (%)								
					1			2			3		
		1	2	3	H_2	CH_4	CO_2	H_2	CH_4	CO_2	H_2	CH_4	CO_2
0	35.0	0.0	0.0	0.0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
1	34.0	32.0	44.2	40.0	0.00000	28.26935	24.58553	0.00000	11.21509	60.39876	0.14813	14.98789	39.29924
4	35.5	57.5	53.0	52.2	0.04080	46.07268	45.39254	0.03567	55.68779	34.08554	0.04960	47.08893	48.00770
7	34.0	72.0	71.2	71.0	0.00000	56.58148	42.52352	0.00000	56.35435	42.20378	0.00000	55.23541	42.81849
10	34.0	59.0	60.0	60.0	0.00000	61.38177	38.16856	0.00000	59.99505	39.19877	0.00000	56.37870	42.83080
13	32.0	40.0	47.0	40.0	0.00000	56.32909	43.11692	0.00000	53.46912	45.63253	0.03928	50.02105	49.22454
16	34.0	21.0	21.5	22.0	0.09828	50.05084	48.88725	0.01506	55.02578	44.37085	0.02079	53.64812	44.98093
19	32.0	29.0	32.0	32.0	0.00000	52.88544	46.73115	0.00000	52.02664	47.49126	0.00000	51.71799	47.71390
22	32.5	28.0	30.0	29.0	0.00000	49.00603	50.51278	0.00000	54.05182	45.38497	0.00000	45.85684	44.48349
31	31.0	39.0	42.0	44.0	0.01833	50.11131	49.41378	0.00000	52.95238	46.63152	0.00000	53.70514	45.68409
33	31.0	15.5	14.5	13.5	0.00000	55.41194	44.32679	0.00000	50.28911	49.25355	0.00000	49.68169	49.73818
36	31.0	18.0	19.0	17.5	0.00000	50.52587	48.92067	0.00000	50.06767	49.43808	0.00000	49.90150	49.50038
45	31.0	29.5	31.0	30.0	0.00000	52.80457	46.89545	0.00000	51.20112	48.14129	0.00000	52.54388	47.15039
50	30.5	27.5	28.5	28.0	0.00000	55.20253	44.48574	0.00000	54.77986	45.00090	0.00000	55.64703	44.06957
57	32.0	21.0	22.0	21.0	0.00000	53.31981	46.31718	0.00000	52.84553	46.89697	0.00000	53.12149	46.61181
60	31.0	21.5	24.0	23.0	0.00000	54.23923	45.47122	0.00000	56.57530	43.23948	0.00000	53.35201	41.91647
70	31.0	16.0	17.5	19.5	0.00000	53.60612	45.99189	0.00000	52.99031	46.56005	0.00000	52.93557	46.82583
77	32.0	32.0	39.0	33.5	0.00000	55.54287	43.92993	0.00000	54.60409	44.90052	0.00000	55.97890	43.60883
85	31.0	8.4	4.8	7.8	0.00000	52.84834	45.96347	0.00000	51.82015	47.51105	0.00000	52.40236	46.85735
93	30.0	12.8	12.8	12.8	0.00000	56.06263	42.80669	0.00000	55.67535	43.59376	0.00000	57.62111	41.71112
99	31.0	13.4	12.6	12.8	0.00000	58.04777	40.98082	0.00000	62.60272	36.84196	0.00000	59.52917	39.64167
106	33.0	12.5	13.0	15.0	0.00000	51.87660	47.01570	0.00000	56.35915	42.97567	0.00000	56.20363	43.05259
113	31.0	16.8	18.4	17.6	0.00000	51.48046	41.61948	0.00000	50.56013	42.12292	0.00000	49.82767	41.39117
116	30.0	7.6	5.6	7.4	0.00000	51.48046	41.61948	0.00000	50.56013	42.12292	0.00000	49.82767	41.39117
120	30.0	0.0	1.0	0.0	0.00000	54.22113	39.78529	0.02859	53.88131	42.64814	0.00000	53.60067	42.15430
129	30.0	8.8	10.4	12.1	0.01870	71.84836	26.77487	0.02702	51.75909	41.19651	0.00000	56.80314	42.22835
134	30.0	7.0	7.0	8.0	0.00000	57.59709	40.96218	0.00000	52.89936	38.77813	0.00000	57.92198	40.64808
140	30.0	4.8	5.2	6.0	0.00000	57.34259	41.52368	0.00000	56.85856	42.08566	0.00000	57.57377	41.49429

Table A.5 Total weight, cellulose, hemicellulose and lignin of the untreated grass and fungal treated grass before and after anaerobic digestion

Sample	Operating time (d)	Parameter	Initial weight (g)	Weight after anaerobic digestion (g)
UT-grass+OS	140	Total solid	3.4278	2.2997
			3.4323	2.3304
			3.4322	2.3231
		Cellulose	0.9182	0.5331
			0.9182	0.5774
			0.9182	0.5894
		Hemicellulose	0.5292	0.2557
			0.5292	0.2448
			0.5292	0.2539
		Lignin	0.1596	0.1143
			0.1600	0.1197
			0.1600	0.1410
CC-grass+OS	140	Total solid	3.4438	2.3171
			3.4435	2.3533
			3.4528	2.3081
		Cellulose	0.8195	0.3944
			0.8195	0.4074
			0.8195	0.3573
		Hemicellulose	0.6761	0.3708
			0.6761	0.3202
			0.6761	0.3339
		Lignin	0.2213	0.1172
			0.2213	0.1426
			0.2213	0.1354
PT-grass+OS	140	Total solid	3.4327	2.4033
			3.4379	2.3793
			3.4384	2.3322
		Cellulose	0.5081	0.2492
			0.5097	0.3067
			0.5099	0.3353
		Hemicellulose	0.5771	0.2439
			0.5786	0.2739
			0.5788	0.2807
		Lignin	0.2130	0.1367
			0.2137	0.1247
			0.2137	0.0977

APPENDIX B

EXPERIMENTAL DATA OF THE ACCLIMATED MICROBIAL CONSORTIUM

Table B.1 Glucose production from cellulose in the hydrolytic activity test

Sample	Sludge volume (gVSS/L)	Operating time (d)	Glucose production (mg/L)		
			1	2	3
OS	5.96	0	11.72	19.17	15.98
		2	15.98	20.24	21.30
		4	22.37	28.76	21.30
		6	15.98	15.98	20.24
		8	39.41	39.41	38.34
		10	37.28	40.47	38.34
		12	22.37	22.37	30.89
		14	39.41	39.41	33.02
		16	17.04	11.72	11.72
		18	19.17	18.11	19.17
		20	14.91	12.78	12.78
		22	34.08	26.63	35.15
		24	39.41	48.99	42.60
		26	71.36	27.69	39.41
		28	20.24	12.78	13.85
		30	41.54	48.99	46.86
AMC	6.09	0	18.11	27.69	23.43
		2	30.89	38.34	45.80
		4	41.54	50.06	61.78
		6	38.34	44.73	43.67
		8	71.36	63.91	77.75
		10	61.78	59.65	58.58
		12	59.65	61.78	59.65
		14	51.12	54.32	55.39
		16	23.43	33.02	25.56
		18	37.28	34.08	42.60
		20	41.54	48.99	42.60
		22	47.93	53.26	46.86
		24	56.45	60.71	67.10
		26	50.06	46.86	51.12
		28	27.69	27.69	31.95
		30	56.45	54.32	57.52

Table B.2 Xylose production from xylan in the hydrolytic activity test

Sample	Sludge volume (gVSS/L)	Operating time (d)	Xylose production (mg/L)		
			1	2	3
OS	5.96	0	35.53	40.33	39.37
		2	38.41	46.09	36.49
		4	32.65	38.41	46.09
		6	27.85	32.65	35.53
		8	39.37	33.61	41.29
		10	34.57	38.41	48.97
		12	26.89	27.85	29.77
		14	44.17	37.45	35.53
		16	16.32	15.36	14.40
		18	16.32	16.32	19.21
		20	24.97	24.01	24.97
		22	22.09	26.89	28.81
		24	46.09	39.37	34.57
		26	32.65	36.49	32.65
		28	18.25	21.13	22.09
		30	32.65	37.45	32.65
AMC	6.09	0	61.46	63.38	70.10
		2	80.66	83.55	85.47
		4	62.42	77.78	70.10
		6	64.34	78.74	53.78
		8	72.02	72.02	84.51
		10	78.74	72.02	71.06
		12	57.62	62.42	71.06
		14	62.42	56.66	60.50
		16	37.45	40.33	30.73
		18	32.65	32.65	39.37
		20	55.70	50.90	46.09
		22	50.90	56.66	56.66
		24	67.22	68.18	72.02
		26	56.66	55.70	54.74
		28	47.05	42.25	48.01
		30	62.42	58.58	63.38

Table B.3 Glucose utilization and total VFA production in the acidogenic activity test

Sample	Sludge volume (gVSS/L)	Operating time (h)	Glucose utilitzation (mg/L)		
			1	2	3
OS	5.96	0	2,822.52	2,779.91	2,821.45
		2	2,473.16	2,468.90	2,602.04
		4	1,144.98	1,163.09	1,003.32
		6	38.34	47.93	53.26
		8	51.12	45.80	40.47
		10	58.58	48.99	43.67
		12	35.15	40.47	4.08
		16	47.93	48.99	52.19
AMC	6.09	0	2,710.68	2,691.51	2,738.37
		2	2,128.07	2,027.95	2,195.17
		4	1,099.18	1,281.32	1,170.54
		6	53.26	55.39	53.26
		8	61.78	44.73	58.58
		10	60.71	61.78	64.97
		12	60.71	56.45	66.04
		16	53.26	47.93	57.52

Table B.4 VFA production in the acidogenic activity test

Sample	Sludge volume (gVSS/L)	Operating time (h)	VFA production (mmol/L)														
			Acetate			Butyrate			Ethanol			Propanoate			Valerate		
			1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
OS	5.96	0	0.00	0.47	0.00	0.00	0.00	0.00	1.78	1.92	1.51	0.85	1.02	1.65	1.13	0.15	0.00
		4	6.20	1.90	4.20	0.00	0.00	0.00	7.49	5.90	7.03	0.00	0.00	0.00	0.00	0.00	0.00
		8	8.09	8.18	7.84	0.00	0.00	0.00	8.21	8.30	7.70	0.00	0.00	0.00	0.00	0.00	0.00
		12	9.80	8.17	9.18	1.83	1.15	1.56	8.94	8.74	8.46	0.00	0.00	0.00	0.00	0.00	0.00
		16	9.73	9.35	9.62	1.07	1.72	0.00	8.01	7.79	7.44	0.00	0.00	0.00	0.00	0.00	0.00
		20	11.18	11.95	10.59	1.61	0.00	0.00	8.22	6.54	7.37	0.00	0.00	0.00	0.00	0.00	0.00
		24	20.53	18.44	17.50	0.00	1.89	1.84	3.39	4.04	4.33	0.00	0.00	0.00	0.00	0.00	0.00
		48	27.36	27.34	27.00	1.46	2.39	2.03	1.21	0.97	0.88	0.00	0.00	0.00	0.00	0.00	0.00
		72	25.36	28.06	28.42	0.42	2.47	2.56	1.34	1.25	1.10	0.00	0.00	0.00	0.00	0.00	0.00
		96	25.70	25.23	24.91	0.53	1.88	1.47	0.63	1.02	0.77	0.00	0.00	0.00	0.00	0.00	0.00
AMC	6.09	0	0.00	0.00	0.00	0.00	0.00	0.00	1.20	1.77	1.44	1.45	1.37	0.87	0.00	0.00	0.00
		4	4.29	2.89	1.62	0.00	0.00	0.00	3.61	3.00	3.04	0.00	0.00	0.00	0.00	0.00	0.00
		8	9.03	9.17	11.24	0.00	0.00	0.00	7.60	7.92	9.09	2.60	2.22	3.00	0.00	0.00	0.00
		12	11.17	11.06	10.82	0.00	0.00	0.00	7.68	6.41	6.99	3.60	3.21	3.14	0.00	0.00	0.00
		16	11.51	11.09	10.68	0.00	0.00	0.00	6.22	5.64	5.55	3.45	2.98	2.25	0.00	0.00	0.00
		20	11.93	12.57	12.58	0.00	0.00	0.00	4.96	4.06	3.73	2.93	2.96	2.93	0.00	0.00	0.00
		24	13.77	13.51	11.90	0.00	0.00	0.00	2.76	2.78	2.01	2.84	2.87	2.23	0.00	0.00	0.00
		48	15.88	14.88	15.61	0.00	0.00	0.00	1.46	1.39	1.49	4.06	3.46	3.52	0.00	0.00	0.00
		72	11.85	10.91	10.63	0.00	0.00	0.00	1.18	1.00	0.90	2.77	2.93	2.45	0.00	0.00	0.00
		96	6.98	6.19	6.15	0.00	0.00	0.00	0.40	1.20	1.00	2.29	2.25	1.91	0.00	0.00	0.00

Table B.5 Propionate utilization, acetate and biogas production in the acetogenic activity test

Sample	Sludge volume (gVSS/L)	Operating time (d)	Temperature (°C)	Propionate utilization (mmol/L)			Acetate production (mmol/L)			Biogas production (ml)		
				1	2	3	1	2	3	1	2	3
OS	5.96	0	37	50.36	53.22	52.02	0.42	0.42	0.43	0.0	0.0	0.0
		2	34	47.41	47.65	46.42	1.49	1.46	1.44	3.0	3.0	2.0
		4	34	48.33	48.93	47.79	2.43	2.58	2.36	2.0	2.0	3.0
		6	33	39.91	39.17	41.91	2.42	2.46	2.43	1.0	3.0	2.0
		8	35	38.14	38.76	39.17	2.97	3.28	3.15	0.0	4.0	0.0
		10	35	37.30	37.46	37.82	3.65	3.28	3.67	4.6	6.6	6.0
		12	33	29.06	27.63	29.85	4.96	4.47	4.78	8.0	6.0	7.0
		14	33	25.38	25.61	25.34	4.61	4.96	5.29	12.0	12.0	11.0
		16	34	17.58	17.99	17.86	7.95	8.59	8.78	27.0	29.0	2.6
		18	34	8.09	10.65	10.47	12.29	12.56	12.87	26.0	28.0	26.0
		20	33	2.42	0.00	2.82	11.62	11.23	10.87	36.0	38.0	39.0
		22	32	0.00	0.00	0.00	0.00	0.00	0.00	32.0	28.0	32.0
		24	32	0.00	0.00	0.00	0.00	0.00	0.00	20.0	6.0	22.0
		26	32	0.00	0.00	0.00	0.00	0.00	0.00	6.2	4.4	7.8
		28	32	0.00	0.00	0.00	0.00	0.00	0.00	2.0	2.0	4.0
AMC	6.09	0	37	59.06	57.27	58.29	0.47	0.42	0.43	0.0	0.0	0.0
		2	34	39.97	38.50	39.81	2.17	2.27	2.10	13.0	9.0	11.0
		4	34	36.04	33.17	38.17	5.07	4.89	5.09	17.0	21.0	19.0
		6	33	21.19	20.30	22.13	8.48	9.09	9.32	26.0	27.0	27.0
		8	35	9.91	9.65	11.13	10.70	10.28	11.94	37.0	43.0	38.0
		10	35	0.23	0.67	0.00	7.45	7.34	7.19	50.0	50.0	46.0
		12	33	0.00	0.00	0.00	0.00	0.00	0.00	43.0	41.0	42.0
		14	33	0.00	0.00	0.00	0.00	0.00	0.00	15.0	11.0	12.0
		16	34	0.00	0.00	0.00	0.00	0.00	0.00	5.0	4.0	4.0
		18	34	0.00	0.00	0.00	0.00	0.00	0.00	0.0	0.0	0.0
		20	33	0.00	0.00	0.00	0.00	0.00	0.00	8.0	5.0	2.0
		22	32	0.00	0.00	0.00	0.00	0.00	0.00	3.0	0.0	0.0
		24	32	0.00	0.00	0.00	0.00	0.00	0.00	0.0	2.0	4.0
		26	32	0.00	0.00	0.00	0.00	0.00	0.00	0.0	1.4	0.0
		28	32	0.00	0.00	0.00	0.00	0.00	0.00	0.0	0.0	0.0

Table B.6 Butyrate utilization, acetate and biogas production in the acetogenic activity test

Sample	Sludge volume (gVSS/L)	Operating time (d)	Temperature (°C)	Butyrate utilization (mmol/L)			Acetate production (mmol/L)			Biogas production (ml)		
				1	2	3	1	2	3	1	2	3
OS	5.96	0	37	49.07	47.88	47.63	0.00	0.00	0.00	0.0	0.0	0.0
		2	34	41.21	41.57	41.83	0.00	0.00	0.00	3.0	4.0	3.0
		4	34	22.81	24.64	23.18	7.08	23.37	12.31	5.0	3.0	4.0
		6	33	13.53	16.78	16.96	16.77	16.45	17.47	15.0	14.0	14.0
		8	35	4.88	5.85	8.96	29.97	30.38	33.09	20.0	21.0	19.0
		10	35	0.00	0.00	0.00	39.53	41.53	41.72	32.0	33.0	30.0
		12	33	0.00	0.00	0.00	27.51	26.97	29.87	64.0	63.0	62.0
		14	33	0.00	0.00	0.00	10.30	9.81	10.66	57.0	56.0	57.0
		16	34	0.00	0.00	0.00	0.00	0.00	0.00	39.0	32.0	42.0
		18	34	0.00	0.00	0.00	0.00	0.00	0.00	12.0	10.0	11.0
		20	33	0.00	0.00	0.00	0.00	0.00	0.00	4.0	3.6	8.2
		22	32	0.00	0.00	0.00	0.00	0.00	0.00	0.0	0.0	0.0
		24	32	0.00	0.00	0.00	0.00	0.00	0.00	0.0	0.0	0.0
AMC	6.09	0	37	48.63	56.69	47.26	0.00	0.00	0.00	0.0	0.0	0.0
		2	34	20.49	27.90	20.52	13.85	15.20	14.84	25.0	27.0	29.0
		4	34	9.93	4.92	9.13	14.69	20.26	20.78	44.0	41.0	42.0
		6	33	0.00	0.00	0.00	18.54	19.53	21.52	44.0	49.0	49.0
		8	35	0.00	0.00	0.00	14.15	10.07	10.42	53.0	59.0	62.0
		10	35	0.00	0.00	0.00	0.00	0.00	0.00	40.0	45.0	40.0
		12	33	0.00	0.00	0.00	0.00	0.00	0.00	17.0	19.0	17.0
		14	33	0.00	0.00	0.00	0.00	0.00	0.00	3.0	2.0	3.0
		16	34	0.00	0.00	0.00	0.00	0.00	0.00	2.0	6.0	4.0
		18	34	0.00	0.00	0.00	0.00	0.00	0.00	0.8	0.0	0.5
		20	33	0.00	0.00	0.00	0.00	0.00	0.00	1.0	0.2	0.0
		22	32	0.00	0.00	0.00	0.00	0.00	0.00	0.0	3.0	0.0
		24	32	0.00	0.00	0.00	0.00	0.00	0.00	0.0	0.0	0.0

Table B.7 Acetate utilization and biogas production in the acetoclastic activity test

Sample	Sludge volume (gVSS/L)	Operating time (d)	Temperature (°C)	Acetate utilization (mmol/L)			Biogas production (mL)		
				1	2	3	1	2	3
OS	5.96	0	37	67.87	66.80	66.48	0.0	0.0	0.0
		2	34	56.82	55.90	55.10	2.6	2.6	3.8
		4	34	52.19	49.61	50.57	3.4	4.6	3.2
		6	33	48.80	48.53	48.34	7.2	7.2	7.0
		8	35	43.43	42.08	41.94	16.0	18.0	18.0
		10	35	25.58	24.57	24.67	45.0	45.0	43.0
		12	33	12.16	10.89	13.56	35.0	32.0	35.0
		14	33	0.00	0.00	0.00	23.0	26.0	24.0
		16	34	0.00	0.00	0.00	20.0	20.0	22.0
		18	34	0.00	0.00	0.00	11.0	7.0	8.0
		20	33	0.00	0.00	0.00	0.0	0.0	0.0
		22	32	0.00	0.00	0.00	0.0	0.0	0.0
		24	32	0.00	0.00	0.00	0.4	0.4	0.4
AMC	6.09	0	37	59.42	59.90	57.76	0.0	0.0	0.0
		2	34	39.60	42.66	41.79	22.0	19.0	20.0
		4	34	26.08	27.82	27.35	33.0	32.0	34.0
		6	33	17.62	17.08	18.44	37.0	36.0	37.0
		8	35	0.00	0.00	0.00	30.0	31.0	32.0
		10	35	0.00	0.00	0.00	24.0	24.0	24.0
		12	33	0.00	0.00	0.00	5.0	2.0	2.4
		14	33	0.00	0.00	0.00	2.8	2.6	2.6
		16	34	0.00	0.00	0.00	1.0	1.2	1.0
		18	34	0.00	0.00	0.00	5.0	3.0	3.0
		20	33	0.00	0.00	0.00	0.0	0.0	0.0
		22	32	0.00	0.00	0.00	2.3	0.0	3.2
		24	32	0.00	0.00	0.00	1.6	0.0	5.8

Table B.8 Biogas compositions in the acetoclastic activity test

Sample	Operating time (d)	Temperature (°C)	Biogas composition (%)								
			1			2			3		
			H_2	CH_4	CO_2	H_2	CH_4	CO_2	H_2	CH_4	CO_2
OS	0	37	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
	2	34	0.00000	24.82274	25.60089	0.00000	19.38078	0.00000	0.00000	29.90820	29.90820
	4	34	0.00000	53.40772	20.12864	0.00000	62.62934	0.00000	0.00000	45.88412	45.88412
	6	33	0.00000	83.33742	0.00000	0.00000	77.01851	0.00000	0.00000	62.20577	62.20577
	8	35	0.00000	92.15503	0.00000	0.00000	89.33398	0.28178	0.00000	74.86327	74.86327
	10	35	0.00000	93.86595	0.00000	0.00000	91.06796	0.68913	0.00000	89.17561	89.17561
	12	33	0.00000	88.79063	2.80631	0.00000	97.72581	0.73431	0.00000	95.00814	95.00814
	14	33	0.00000	97.55430	0.00000	0.00000	99.40611	0.00000	0.00000	97.52354	97.52354
	16	34	0.00000	98.20693	0.00000	0.00000	99.59651	0.00000	0.00000	97.75485	97.75485
	18	34	0.00000	91.49600	7.20457	0.00000	99.54925	0.00000	0.00000	81.97627	81.97627
	20	33	0.00000	92.33228	0.00000	0.00000	77.34256	11.91410	0.00000	76.77177	76.77177
	22	32	0.00000	86.19313	9.77647	0.00000	85.07398	11.83742	0.00000	80.53992	80.53992
	24	32	0.00000	94.48387	0.63921	0.00000	85.57972	11.08394	0.00000	80.46021	80.46021
AMC	0	37	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
	2	34	0.00000	95.94809	0.00000	0.00000	95.98662	0.00000	0.00000	95.41966	95.41966
	4	34	0.00000	98.41165	0.00000	0.00000	92.76080	5.88442	0.00000	98.13392	98.13392
	6	33	0.00000	97.68118	1.39264	0.00000	99.33791	0.00000	0.00000	90.02274	90.02274
	8	35	0.00000	97.80876	1.19310	0.00000	98.14795	0.84863	0.00000	98.21068	98.21068
	10	35	0.00000	98.66470	0.44238	0.00000	98.40978	1.59022	0.00000	94.65704	94.65704
	12	33	0.00000	93.48911	4.19975	0.00000	94.00352	5.31962	0.00000	91.60706	91.60706
	14	33	0.00000	87.00167	11.86716	0.00000	87.64828	11.56148	0.00000	86.20923	86.20923
	16	34	0.00000	83.44117	14.60212	0.00000	87.64054	10.83618	0.00000	84.96367	84.96367
	18	34	0.00000	70.09291	15.62120	0.00000	86.52598	11.51747	0.00000	82.88953	82.88953
	20	33	0.00000	59.69543	0.00000	0.00000	80.84555	11.64070	0.00000	90.49188	90.49188
	22	32	0.00000	44.62907	11.76323	0.00000	86.02814	12.15997	0.00000	16.06845	16.06845
	24	32	0.00000	21.65084	11.70044	0.00000	82.74215	11.21742	0.00000	16.01366	16.01366

Table B.9 Biogas production and biogas composition from anaerobic digestion of the untreated grass with the original sludge (UT-grass+OS) for 80 days

Operating day (d)	Temperature (°C)	Biogas production (mL)			Biogas composition (%)								
					1			2			3		
		1	2	3	H_2	CH_4	CO_2	H_2	CH_4	CO_2	H_2	CH_4	CO_2
0	35	0.0	0.0	0.0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
4	33	28.0	34.0	28.0	0.12109	30.66141	58.87072	0.15925	29.86261	61.38230	0.16953	27.01146	58.48976
8	35	43.0	37.0	39.0	0.00000	41.50351	54.34744	0.00000	39.44660	54.91281	0.00000	37.46633	55.27572
12	35	105.0	105.0	108.0	0.00000	66.13826	32.63583	0.00000	61.63383	37.27525	0.00000	61.23728	36.42789
16	34	75.0	73.0	73.0	0.00000	54.47190	44.01019	0.00000	61.85016	38.14984	0.00000	60.39878	38.81868
20	34	60.0	63.0	60.0	0.00000	60.24488	39.22789	0.00000	55.71194	43.21554	0.00000	59.09509	40.35910
24	33	48.0	50.0	45.0	0.00000	54.86948	43.74704	0.00000	54.99660	44.44025	0.00000	54.05657	43.61862
28	31	49.0	49.0	49.0	0.00000	51.73831	46.94195	0.00000	54.92969	44.65073	0.00000	54.65260	43.86516
32	32	39.0	40.0	40.0	0.00000	48.45295	47.95999	0.00000	52.35475	46.58566	0.00000	53.70776	44.94759
38	33	39.0	42.0	40.0	0.00000	49.78257	43.39248	0.00000	51.46879	45.58575	0.00000	53.11723	45.18576
45	33	29.0	30.0	31.0	0.00000	51.51317	44.61799	0.00000	54.48750	45.27419	0.00000	54.03942	45.96058
52	34	27.0	27.0	26.0	0.00000	49.83073	46.86392	0.00000	51.14387	48.85613	0.00000	51.81799	47.82577
59	33	12.0	15.0	14.0	0.00000	52.83034	44.41294	0.00000	61.43599	37.83663	0.00000	52.41310	46.90324
65	33	8.0	8.4	9.4	0.00000	53.66301	43.28029	0.00000	52.30576	45.85720	0.00000	53.30250	45.92062
72	32	6.2	6.4	6.2	0.00000	52.17001	45.38314	0.00000	53.10111	45.63590	0.00000	54.83570	42.68131
80	32	5.0	5.0	4.8	0.00000	55.95909	41.12509	0.00000	49.79678	41.03568	0.00000	55.13694	42.20319

Table B.10 Biogas production and biogas composition from anaerobic digestion of the untreated grass with the acclimated microbial consortium (UT-grass+AMC) for 80 days

Operating day (d)	Temperature (°C)	Biogas production (mL)			Biogas composition (%)								
					1			2			3		
		1	2	3	H_2	CH_4	CO_2	H_2	CH_4	CO_2	H_2	CH_4	CO_2
0	35	0.0	0.0	0.0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
4	33	100.0	95.0	95.0	0.00000	65.88901	32.67034	0.00000	67.18183	31.54902	0.00000	66.76159	31.10045
8	35	103.0	105.0	112.0	0.00000	61.96546	38.03454	0.00000	60.91308	38.29380	0.00000	61.15620	38.38150
12	35	92.0	90.0	95.0	0.00000	53.00065	46.49649	0.00000	54.63538	45.36462	0.00000	53.92416	45.43321
16	34	70.0	70.0	70.0	0.00000	48.94202	50.82226	0.00000	50.79966	49.20034	0.00000	52.17120	47.82880
20	34	51.0	52.0	51.0	0.00000	51.29006	48.70994	0.00000	50.09742	49.90258	0.00000	50.41595	49.58405
24	33	37.0	38.0	37.0	0.00000	50.13409	49.86591	0.00000	49.62005	50.22662	0.00000	50.23622	49.76378
28	31	27.0	27.0	27.0	0.00000	51.65179	48.34821	0.00000	50.15540	49.50432	0.00000	50.67255	49.32745
32	32	23.0	23.0	24.0	0.00000	52.62521	47.37479	0.00000	51.23792	48.36059	0.00000	52.54502	47.19371
38	33	29.0	28.0	27.0	0.00000	54.84566	44.76700	0.00000	54.24317	45.60016	0.00000	52.35314	47.06882
45	33	28.0	29.0	32.0	0.00000	59.82723	39.59715	0.00000	55.54550	43.99329	0.00000	57.24581	42.34574
52	34	23.0	24.0	22.0	0.00000	54.96642	44.37071	0.00000	54.07101	43.03948	0.00000	52.88071	46.55568
59	33	17.0	16.0	19.0	0.00000	61.29554	37.55231	0.00000	57.22444	41.58012	0.00000	57.11331	42.30165
65	33	9.4	9.6	8.0	0.00000	59.73124	39.06436	0.00000	55.93203	42.97115	0.00000	55.88158	42.87984
72	32	8.4	8.6	8.6	0.00000	55.70281	42.59721	0.00000	55.61118	42.99739	0.00000	57.91793	40.93593
80	32	7.6	9.0	8.8	0.00000	54.77512	43.79224	0.00000	58.52702	40.22175	0.00000	58.31181	40.45645

Table B.11 Biogas production and biogas composition from anaerobic digestion of distilled water with the original sludge (DW+OS) for 80 days

Operating day (d)	temperature (°C)	Biogas production (mL)			Biogas composition (%)								
					1			2			3		
		1	2	3	H_2	CH_4	CO_2	H_2	CH_4	CO_2	H_2	CH_4	CO_2
0	35.0	0.0	0.0	0.0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
4	33.0	1.0	0.0	0.0	0.00000	6.34049	0.00000	0.00000	5.81062	0.00000	0.00000	5.81062	0.00000
8	35.0	2.0	2.3	2.7	0.00000	13.17139	0.00000	0.00000	13.26296	0.00000	0.00000	13.53398	0.00000
12	35.0	2.2	2.8	2.2	0.00000	17.12904	0.00000	0.00000	16.61192	0.00000	0.00000	17.14639	0.00000
16	34.0	3.0	3.0	4.0	0.00000	21.15071	0.00000	0.00000	20.28837	0.00000	0.00000	20.10750	0.00000
20	34.0	2.2	2.6	2.6	0.00000	22.92878	0.87594	0.00000	22.95565	0.00000	0.00000	22.02555	0.20025
24	33.0	3.6	3.1	3.8	0.00000	27.51677	0.00000	0.00000	24.97742	0.00000	0.00000	25.46495	0.00000
28	31.0	0.0	2.9	3.4	0.00000	25.84790	0.00000	0.00000	28.99513	0.00000	0.00000	28.35942	0.00000
32	32.0	0.0	0.0	1.4	0.00000	30.72793	0.00000	0.00000	31.73564	0.00000	0.00000	31.13359	0.00000
38	33.0	0.0	0.0	0.0	0.00000	36.03755	0.00000	0.00000	35.26880	0.00000	0.00000	32.76092	5.27148
45	33.0	3.0	4.0	3.0	0.00000	36.35033	0.00000	0.00000	37.85003	0.00000	0.00000	34.74662	0.00000
52	34.0	5.8	4.4	4.0	0.00000	40.85624	0.00000	0.00000	39.79855	0.00000	0.00000	38.33120	0.00000
59	33.0	9.0	9.2	8.6	0.00000	42.97064	9.84752	0.00000	41.68504	8.94082	0.00000	40.29354	7.84189
65	33.0	0.0	0.0	0.0	0.00000	44.25884	0.00000	0.00000	41.68227	0.00000	0.00000	41.24323	8.51713
72	32.0	4.4	4.4	5.4	0.00000	47.91415	11.46388	0.00000	45.68006	9.86867	0.00000	45.39908	0.00000
80	32.0	4.6	4.0	3.4	0.00000	48.97394	11.46173	0.00000	47.34197	9.38546	0.00000	46.76834	9.93179

Table B.12 Biogas production and biogas composition from anaerobic digestion of distilled water with the acclimated microbial consortium (DW+AMC) for 80 days

Operating day (d)	temperature (°C)	Biogas production (mL)			Biogas composition (%)								
					1			2			3		
		1	2	3	H_2	CH_4	CO_2	H_2	CH_4	CO_2	H_2	CH_4	CO_2
16	34.0	0.0	0.0	0.0	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.0000	0.0000	0.0000
20	34.0	0.0	3.8	0.0	0.00000	10.04166	0.00000	0.00000	9.86104	0.00000	0.0000	9.7658	0.0000
24	33.0	5.9	5.6	0.0	0.00000	14.67245	0.00000	0.00000	15.59902	0.00000	0.0000	13.6352	0.0000
28	31.0	2.8	2.8	0.0	0.00000	20.29486	0.00000	0.00000	19.75885	0.00000	0.0000	17.4372	0.0000
32	32.0	2.0	2.0	0.0	0.00000	20.60103	0.00000	0.00000	20.86648	0.00000	0.0000	19.5488	0.0000
38	33.0	0.4	0.4	0.0	0.00000	20.82422	0.00000	0.00000	20.61971	0.00000	0.0000	19.7040	1.2309
45	33.0	2.0	1.6	0.0	0.00000	24.18797	0.00000	0.00000	22.65597	0.00000	0.0000	21.7881	0.0000
52	34.0	0.0	0.4	0.0	0.00000	23.22715	0.00000	0.00000	22.97710	0.00000	0.0000	21.4317	0.0000
59	33.0	1.8	2.3	0.0	0.00000	24.45170	0.00000	0.00000	25.16033	0.00000	0.0000	23.9395	0.0000
65	33.0	0.0	0.0	0.0	0.00000	25.29606	0.00000	0.00000	25.14799	0.00000	0.0000	25.4666	0.4004
72	32.0	0.0	1.0	0.0	0.00000	22.64337	1.14615	0.00000	25.08862	0.00000	0.0000	25.2407	0.0000
80	32.0	0.0	1.8	0.0	0.00000	25.12847	0.00000	0.00000	25.52110	0.00000	0.0000	28.0847	0.0000

Table B.13 Total weight, cellulose, hemicellulose and lignin of the untreated grass and fungal treated grass before and after anaerobic digestion

Sample	Operating time (d)	Parameter	Initial weight (g)	Weight after anaerobic digestion (g)
UT-grass+OS	80	Total solid	4.1176	3.0772
			4.1176	3.0565
			4.1176	3.0431
		Cellulose	0.7591	0.4484
			0.7591	0.4584
			0.7591	0.4542
		Hemicellulose	0.5994	0.3126
			0.5994	0.3101
			0.5994	0.3061
		Lignin	0.2321	0.149
			0.2321	0.1499
			0.2321	0.1386
UT-grass+AMC	80	Total solid	3.1254	2.2014
			3.1254	2.1944
			3.1254	2.2086
		Cellulose	0.793	0.3914
			0.793	0.3957
			0.793	0.3747
		Hemicellulose	0.4562	0.1987
			0.4562	0.1757
			0.4562	0.1848
		Lignin	0.2675	0.1568
			0.2675	0.1576
			0.2675	0.1633

APPENDIX C

CALCULATIONS

C.1 Enzyme activity

One unit of enzyme activity is defined as 1 μmol of glucose or xylose equivalents released per minute under the given conditions (Isikhuemhen and Mikiashvilli, 2009)[48].

$$\frac{\text{Reducing sugar (mg)}}{\text{(mL)}} \left| \frac{\text{Total solution volume (mL)}}{\text{Sample volume (mL)}} \right| \frac{1}{\text{Molecular weight of sugar (mg/mmol)}} \left| \frac{1}{\text{Reaction time (min)}} \right.$$

Example: a) Cellulase activity of *C. cinerea* at 5 days

$$= \frac{689\ \text{mg}}{1{,}000\ \text{mL}} \left| \frac{1.5\ \text{mL}}{0.5\ \text{mL}} \right| \frac{1\ \text{mmol}}{180\ \text{mg}} \left| \frac{1}{60\ \text{min}} \right.$$

$$= 0.191 \times 10^{-3}\ \text{mmol/mL·min}$$

$$= 0.191\ \text{U/mL}$$

Cellulase activity (U/mL)				
Data 1	Data 2	Data 3	AVG	SD
0.191	0.186	0.148	0.175	0.024

b) β-glucosidase activity of *C. cinerea* at 5 days

$$= \frac{371\ \text{mg}}{1{,}000\ \text{mL}} \left| \frac{3\ \text{mL}}{1\ \text{mL}} \right| \frac{1\ \text{mmol}}{180\ \text{mg}} \left| \frac{1}{30\ \text{min}} \right.$$

$$= 0.206 \times 10^{-3}\ \text{mmol/mL·min}$$

$$= 0.206\ \text{U/mL}$$

β-glucosidase activity (U/mL)				
Data 1	Data 2	Data 3	AVG	SD
0.206	0.287	0.286	0.260	0.046

c) Xylanase activity of *C. cinerea* at 5 days

$$= \frac{1{,}272\text{mg}}{1{,}000\text{ mL}} \left| \frac{3\text{ mL}}{1\text{ mL}} \right| \frac{1\text{ mmol}}{150.13\text{ mg}} \left| \frac{1}{30\text{ min}} \right.$$

$$= 0.847 \times 10^{-3}\ \text{mmol/mL·min}$$

$$= 0.847\ \text{U/mL}$$

Xylanase activity (U/mL)				
Data 1	Data 2	Data 3	AVG	SD
0.847	0.910	0.943	0.900	0.049

C.2 Degree of crystallinity

The degree of crystallinity (X_c) was calculated by Sun et al. (2009)[101].

$$X_c = F_c/(F_c + F_{a)} \times 100$$

Where F_c and F_a are the area of the crystal and nincrystalline regions, respectively.

Example: The degree of crystallinity of untreated grass at the initial stage (UT-grass 0 d)

$$= (F_{c,101} + F_{c,002} + F_{c,040})/(F_{c,101} + F_{c,002} + F_{c,040} + F_a) \times 100$$

$$= (417.07+1{,}680.70+250.18)/8{,}271.84 \times 100$$

$$= 28.38\%$$

C.3 Grass components

$$\frac{\text{Cellulose / Hemicellulose / Lignin content (g)}}{\text{Total solid after fungal pretreatment (g TS)}} \left| \frac{\text{Total solid after fungal pretreatment (g TS)}}{\text{Initial dry weight (g TS)}} \right.$$

Example: Components of the treated grass with *C. cinerea* at 5 days

$$\text{Cellulose content} = \frac{0.3262\text{ g Cellulose}}{1\text{ g TS}} \left| \frac{0.8659\text{ g TS}}{1\text{ g TS}} \right.$$

$$= 0.2825\ \text{g cellulose/g initial dry weight}$$

$$\text{Hemicellulose content} = \frac{0.2904 \text{ g Hemicellulose}}{1 \text{ g TS}} \Big| \frac{0.8659 \text{ g TS}}{1 \text{ g TS}}$$

$$= 0.2515 \text{ g hemicellulose/g initial dry weight}$$

$$\text{Lignin} = \frac{0.1062 \text{ g Hemicellulose}}{1 \text{ g TS}} \Big| \frac{0.8659 \text{ g TS}}{1 \text{ g TS}}$$

$$= 0.0920 \text{ g lignin/g initial dry weight}$$

Sample	Component	Weight (g/g initial dry weight)				
		Data 1	Data 2	Data 3	AVG	SD
CC-grass (5 days)	Cellulose	0.28	0.29	0.27	0.28	0.01
	Hemicellulose	0.25	0.27	0.27	0.26	0.01
	Lignin	0.09	0.09	0.09	0.09	0.00

C.4 Percentage loss and cumulative loss of the grass components

Example:

Sample	Component	After the aerobic treatment		After the anaerobic fermentation		Cumulative loss
		Normalized weight	% loss[2]	Normalized weight	% loss[2]	
CC-grass+OS	TS	0.723	27.7	0.488	32.5	51.2
	Cellulose	**0.821**	**17.9**	0.387	52.9	61.3
	Hemicellulose	0.717	28.3	0.362	49.5	63.8
	Lignin	0.889	11.1	0.530	40.5	47.0

Normalized weight of cellulose = Cellulose after aerobic treatment (g) /Initial weight cellulose (g)

= 1.2942 g / 1.5805 g

= 0.8189

Normalized weight of cellulose				
Data 1	Data 2	Data 3	AVG	SD
0.819	0.809	0.835	0.821	0.013

% loss = (Initial weight – Weight after treatment)/Initial weight × 100%

= (1.5805- 1.2942) g/1.5805 g × 100

= 18.11%

% Loss of cellulose after the aerobic treatment				
Data 1	Data 2	Data 3	AVG	SD
18.1	19.1	16.5	17.9	1.3

C.5 Percentage of methane, methane production and methane yield

The grass sample was transferred to 100-mL serum bottles. The total working volume was 60 mL. The bottles were inoculated with the OS at a ratio of 1 g VS/g VS and then flushed for 1 min with 99.995% argon. The bottles were incubated under mesophilic temperatures between 28-30 °C for 140 days. The biogas composition was determined by a gas chromatograph equipped with a thermal conductivity detector (SHIMADZU GC-2014, Japan) and a unibeads C column under an argon flow rate of 25 mL/min.

Example: The biogas compositions of the grass treated with *C. cinerea* in day 1 from a gas chromatograph were 0.0758% H_2, 25.91318% air, 8.23211% CH_4 and 24.52085% CO_2. The biogas production was 28.3 ml. The batch reactor was operated at 34 ˚C.

Percentage of methane = 8.232112 / (0.0758+25.91318+8.23211+24.52085) × 100

= 14.014%

Methane production = 28.3 mL/100 mL reactor × 14.014 / 100

= 3.96 mL/100 mL reactor

Methane yield = 3.96 mL/100 mL reactor ×273 K / ((34+273)K × 1 g VS $_{added}$/100 mL reactor)

= 3.52 mL $_{STP}$/g VS $_{added}$

C.6 Maximum methane production rate (MMRP)

Maximum methane production rate (MMPR) was the slope of the initial linear part of each curve of methane production versus time (Neves et al., 2004)[75].

Example:

Maximum methane production rate (MMPR) = $\Delta y/\Delta x$

$$= \frac{(159.48 - 57.43) \text{ mL STP/g VS added}}{(12 - 4) \text{ d}}$$

$$= 12.76 \text{ mL STP/g VS added·d}$$

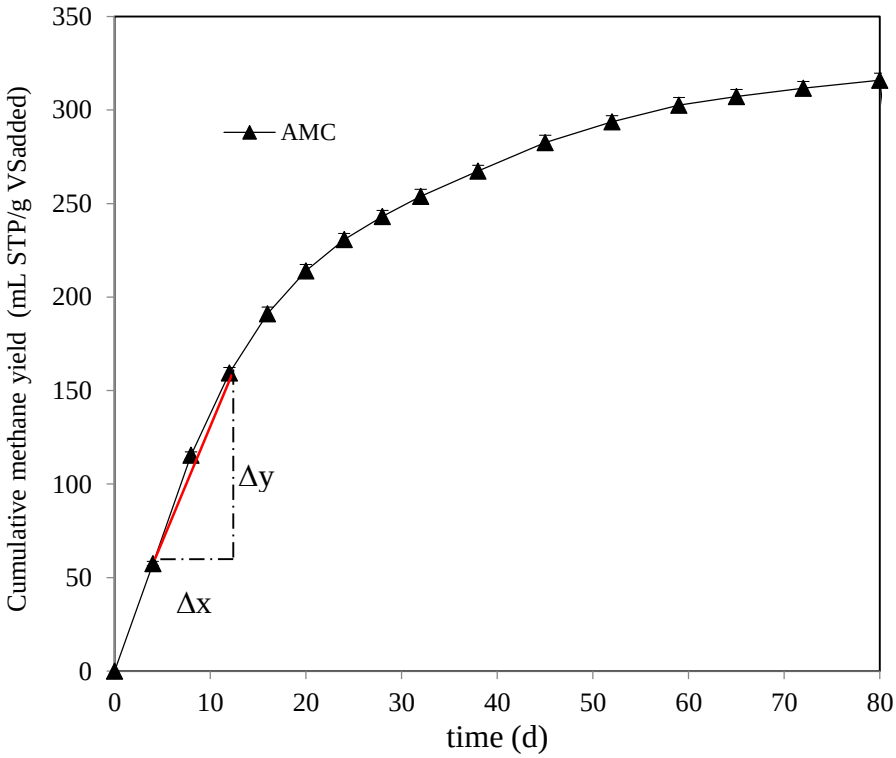

Fig.C.1 Cumulative methane yield from the anaerobic digestion of the paragrass by the original sludge (UT-grass+OS), the paragrass by the acclimated microbial consortium (UT-grass+AMC) and the alkaline pretreated paragrass by the OS (ALK-grass+OS).

C.7 Microbial activity

Glucose utilization rate = $\Delta \mathbf{y}/\Delta \mathbf{x}$

$$= \frac{(0.373\text{-}0.009)\ \text{g COD / g VSS} \mid 24\ \text{h}}{(6 - 2)\ \text{h} \mid 1\ \text{d}}$$

$$= 2.1816\ \text{g COD/g VSS·d}$$

Glucose utilization rate (g COD / g VSS ·d)				
Data 1	Data 2	Data 3	AVG	SD
2.182	2.074	2.254	2.170	0.091

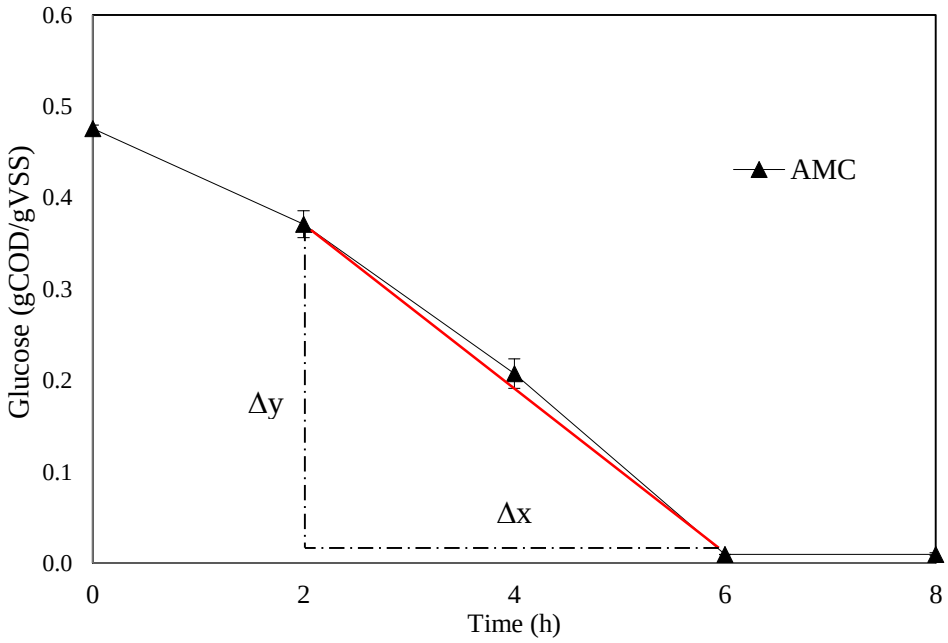

Fig. C.2 Glucose utilization of acidogenic activities of the acclimated microbial consortium (AMC).

C.8 Methane yield per hectare of the grass sample

- Dry matter yield of grass was 5,000 – 12,000 kg/ha a (Braun et al., 2009)[13].
- VS/TS ratio of the paragrass was 0.88.

Sample	Methane yield (mL/g VS added)	Estimated methane yield (Nm^3 CH_4/ha a)
Paragrass/OS	277	1,218 – 2,925
Paragrass/AMC	316	1,390 – 3,337

Example:

$$\text{Estimated methane yield of Paragrass/AMC} = \frac{316 \times 10^{-3}\ L_{STP}}{\text{g VS}} \cdot \frac{0.88\ \text{g VS}}{\text{g TS}} \cdot \frac{5{,}000 \times 10^{3}\ \text{g TS}}{\text{ha.a}} \cdot \frac{1\ m^3}{1000\ \text{L}}$$

$$= 1{,}390\ Nm^3\ CH_4/\text{ha a}$$