THESIS

EFFECT OF ELEPHANT DIGESTIVE PRETREATMENT BY ANAEROBIC MIXED CULTURE WITH ELEPHANT DUNG ON SUGARCANE BAGASSE FOR CELLULOSE EXTRACTION

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GRADUATE SCHOOL, KASETSART UNIVERSITY
Academic Year 2021

THESIS APPROVAL
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THESIS

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CHOTIWIT SRIWONG

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Biotechnology)
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Chotiwit Sriwong : Effect of Elephant Digestive Pretreatment by Anaerobic Mixed Culture with Elephant Dung on Sugarcane Bagasse for Cellulose Extraction. Master of Science (Biotechnology), Major Field: Biotechnology, Department of Biotechnology.
Thesis Advisor: Associate Professor Prakit Sukyai, Dr.nat.techn
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Simulated elephant colon pretreatment (SEP) to reduce chemical use in cellulose extraction from sugarcane bagasse (SCB) was investigated using elephant dung as inoculum in the fermentation. The 16S rRNA gene sequences showed microorganisms in elephant dung that corresponded to metabolites during pretreatment. Organic acid production in the fermentation broth was confirmed by the accumulation of lactic, acetic, propionic and butyric acids. Reducing sugar indicated the degradation of SCB and its utilization by the microorganisms. Lignin peroxidase, manganese peroxidase and xylanase detected in the pretreatment enhanced the removal of lignin. The SEP fiber showed increased cellulose content while lignin content decreased, with reduced bleaching time from 7 to 5 h, and high whiteness and crystallinity indices. Successful removal of hemicellulose and lignin was confirmed by Fourier transform infrared spectroscopy and scanning electron microscopy.

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Student's signature Thesis Advisor's signature

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Chotiwit Sriwong

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B5-SEP-F	=	Sugarcane bagasse + Simulation of elephant colon with elephant dung pretreatment + Bleaching five times
B7-SEP-F	=	Sugarcane bagasse + Simulation of elephant colon with elephant dung pretreatment + Bleaching seven times
B7-SP-F	=	SCB + Simulation of elephant colon without elephant dung pretreatment + Bleaching seven times
CH_3COOH	=	Glacial acetic acid
Cm	=	Centimeter
CrI	=	Crystallinity index
˚C	=	Degree Celsius
DNS	=	3,5-Dinitrosalicylic acid
EDPM	=	Elephant dung pretreatment medium
FTIR	=	Fourier transform infrared spectroscopy
g	=	Gram
h	=	Hour
µm	=	Micrometer
M	=	Molar
mg	=	Milligram

mL	=	Milliter
mmol	=	Millimole
min	=	Minute
$NaClO_2$	=	Sodium chlorite
nm	=	Nanometer
PC	=	Plant cellulose
KBr	=	Potassium bromide
rpm	=	Round per minute
SCB	=	Sugarcane bagasse
SEM	=	Scanning electron microscopy
SEP	=	The simulated elephant colon pretreatment with elephant dung
SEP-B	=	Fermentation broth from simulation of elephant colon with elephant dung pretreatment
SEP-F	=	Sugarcane bagasse + Simulation of elephant colon with elephant dung pretreatment
SEP-S	=	Supernatant from simulation of elephant colon with elephant dung pretreatment
SP	=	The simulated elephant colon pretreatment without elephant dung
SP-B	=	Fermentation broth from simulation of elephant colon without elephant dung pretreatment

SP-F	=	Sugarcane bagasse + Simulation of elephant colon without elephant dung pretreatment
SP-S	=	Supernatant from simulation of elephant colon without elephant dung pretreatment
TAPPI	=	Technical association of pulp and paper industry
U	=	Unit of enzymes
WI	=	Whiteness index
XRD	=	X-ray diffraction

INTRODUCTION

Sugarcane bagasse (SCB) is a solid residue obtained after extraction of juice from sugarcane. It is composed of cellulose, hemicellulose and lignin, with more than one-third to half being cellulose (Lam *et al.*, 2017). Normally, SCB is burnt by sugar mills to supply thermal, mechanical and electrical power to industrial units (Salinas *et al.*, 2020). One way to increase the value of SCB for more efficient utilization is by producing cellulose. Cellulose extraction from lignocellulosic biomass has recently attracted intensive research and offers tremendous advantages (Khairnar *et al.*, 2021). However, SCB requires pretreatment to break up the molecular structure before obtaining cellulose fibers (Mahmud and Anannya, 2021).

A two-step process combining pretreatment and bleaching has been used to extract cellulose from SCB. This pretreatment process disrupts the lignocellulosic matrix and removes some lignin and hemicellulose as an essential step before cellulose extraction. Several other pretreatment processes have also been used for cellulose extraction, either as single or two-stage pretreatments to improve efficiency and increase cellulose purity (Vanitjinda *et al.*, 2019a). Pretreatments for the effective conversion of lignocellulosic biomass include physical methods as mechanical techniques (Tian *et al.*, 2019) and irradiation (Kassim *et al.*, 2016), chemical methods as ozonolysis (Perrone *et al.*, 2021) and acid hydrolysis (Zhang *et al.*, 2020), biological methods as fungal (Gu *et al.*, 2021), microbial consortium (Raut *et al.*, 2021), enzymatic (Vanitjinda *et al.*, 2019a) and combinatorial pretreatment methods (Meesupthong *et al.*, 2021). Physical, chemical and combinatorial pretreatments are the most expensive processes in the conversion of lignocellulosic biomass because of the high energy and chemical requirements (Sankaran *et al.*, 2020). Therefore, this study focused on biological pretreatment because it is selective with no chemical addition, low severity and no

release of hazardous or toxic compounds to the environment (Sindhu *et al.*, 2016; Ummalyma *et al.*, 2019). Biological pretreatment, especially enzymatic pretreatment, has some drawbacks. Enzymatic pretreatment is specific, with less unwanted by-products and operates under mild conditions but it is limited by the high cost associated with enzyme production (Kumar *et al.*, 2020). To overcome this problem, elephant dung was used as a source of enzymes and other microbial metabolites to pretreat SCB. Previous studies have examined the effects of elephant dung on SCB for hydrogen production (Fangkum and Reungsang, 2011a, 2011b; Saripan and Reungsang, 2014); however, no research has considered the effect of elephant dung on fiber properties. Thus, here, microorganisms in elephant dung were assessed for cellulose extraction from SCB. Abundant microorganisms in elephant dung were hypothesized to show efficiency in cellulose extraction from SCB by reducing the use of chemicals in the bleaching process, and an elephant colon model was simulated for use in SCB pretreatment.

This study reported an enhanced cellulose extraction process from SCB as a biological pretreatment method utilizing microorganisms in elephant dung. The effects of pretreatment on fiber with and without addition of elephant dung were studied at different processing stages and reported in terms of whiteness index, chemical composition, functional change, crystallinity structure and morphological properties. Liquid portions after fermentation were also studied and assessed for microbial community composition, organic acid content, reducing sugar concentration and enzymatic activities.

OBJECTIVES

1. To utilize elephant colon simulation system as pretreatment combined with elephant dung for cellulose extraction from sugarcane bagasse.

2. To study the effect of elephant colon pretreatment on fiber properties.

LITERATURE REVIEW

Cellulose

1. Source

Cellulose is one of the biopolymers, which is most abundantly found in nature from several sources such as plants, animals, algae, as well as microbes. The properties and structure of cellulose depend on sources (Siqueira *et al.*, 2010). Generally, cellulose can be found in many forms and the most cellulose form in nature is in plant cell wall, which has cellulose as main components (Zuluaga *et al.*, 2007). Figure 1 showed that cellulose fiber obtained from various sources, which exist naturally in two native forms. The first is called complex cellulose, the most abundant form of the cellulose present in nature and as component of the plant cell wall. The second is called pure cellulose, which is present in algae, some animals, and it can be synthesized by some microorganisms (Liu and Sun, 2010; Pecoraro *et al.*, 2007).

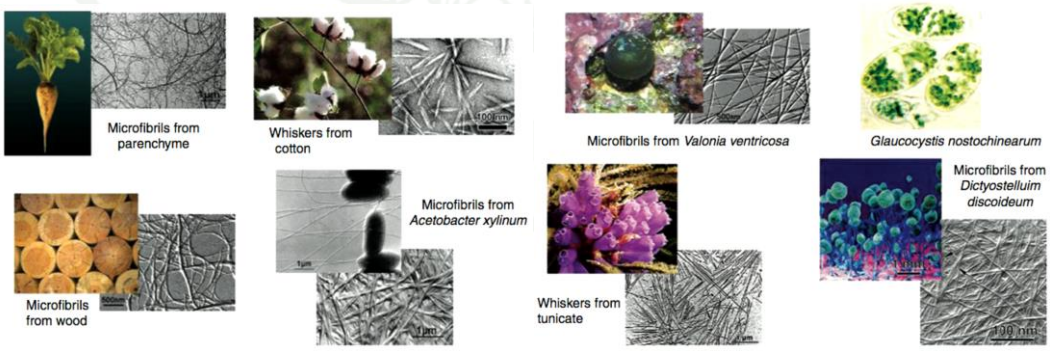

Figure 1. Cellulose microfibrils from various sources in nature.

Source: Perez and Samain (2010)

In nature, cellulose exists naturally in two native forms. The first is called complex cellulose that most of the celluloses present in nature and as component of the plant cell wall. The second is called pure cellulose, which presented in algae, some animals, cotton fibers and synthesize by some microorganisms (Belgacem and Gandini, 2008), table 1 showed comparison properties of plant cellulose and bacterial cellulose.

Table 1. Properties of plant cellulose (PC) and bacterial cellulose (BC)

Properties	**PC**	**BC**
Diameter of fibers	1.4 – 4.0 x 10^{-2} mm	70-80 nm
Crystallinity	56 – 65%	65 – 79%
Degree of polymerization	13,000 – 14,000	2,000 – 6,000
Young's modulus	5.5 – 12.6 GPa	15 – 30 GPa
Water content	60%	98.5%

1.1 Plant cell wall

Plant cell wall is complex structure which consist of cellulose, hemicellulose and lignin, mostly referred to as lignocellulosic compound (Yu *et al.*, 2006), and may be composed of other polysaccharides such as chitin, mannan and pectin, which varies in different kind of plant species. The main components of plant cell wall is cellulose, which is approximately 20-30% on dry weight basis (Perez and Samain, 2010). A major function of cell wall is to maintain and support cell shape, provide mechanical strength, physical barrier and act as barrier to potential pathogens

(Liu and Sun, 2010). The chemical composition of natural fibers varies according to their origin as summarized in Table 2.

1.2 Bacterial cellulose (BC)

Bacterial cellulose (BC) is synthesized by many genera. For insteace, Gluconacetobacter sp., A. xylinum, A. pasteurianus, A. hansenii, A. sucrofermantans, A. acetigenum and Acetobacter sp. The biosynthesis of bacterial cellulose splendid properties such as free other polymeric components, highly crystalline and greater surface area. Bacterial cellulose is used in many medical and industrial applications such as medical implants, coating, adhesives and composites (Cherian *et al.*, 2013).

Table 2. Chemical composition of some typical cellulose-containing materials.

Source	**Composition (%)**			
	Cellulose	**Hemicellulose**	**Lignin**	**Extract**
Hardwood	43-47	25-35	16-24	2-8
Softwood	40-44	25-29	25-31	1-5

Coir	32-33	10-20	43-49	4
Corn cobs	45	35	15	5
Corn stalks	35	25	35	5
Cotton	95	2	1	0.4
Hemp	70	22	6	2
Kenaf	36	21	18	2
Ramie	76	17	1	6
Sisal	73	14	11	2
Wheat straw	30	50	15	5

Source: Adapted from Khalil *et al.* (2012)

2. Structural and properties

Cellulose can be described as a homopolymer, consists of a linear polysaccharide composed of β-D-glucopyranose units linked by β-1,4-linkages. The basic structure of cellulose is shown in Figure 2. The non-etherified anomeric C1-OH is potential aldehyde and is known as the reducing end-group. The terminal glucopyranose residue with a free C4-OH is termed the non-reducing end group. Each structural unit has one primary OH group and two secondaries OH groups, which act in chemical reactions (e.g. esterification). The length of cellulose chain varies according to the sources of cellulose or species of plants (Siqueira *et al.*, 2010).

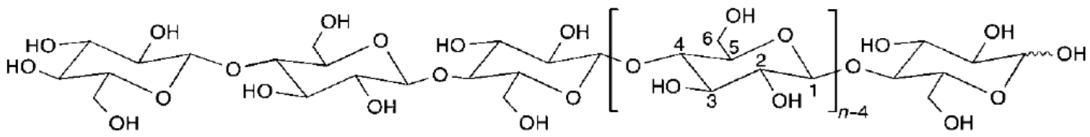

Figure 2. Basic chemical structure of cellulose showing the cellulosic repeat unit (n = DP, degree of polymerization).

Source: Klemm *et al*. (2005)

The structure of cellulose chain has three hydroxyl groups located at C-2, C-3 and C-6, and each hydroxyl group is linked together by hydrogen bond. The hydrogen bonding occurs in cellulose chain (intra- and intermolecular) resulting in the chain forming microfibrils with two regions, that is crystalline and amorphous region (Liu and Sun, 2010). Many cellulose microfibrils are fused together by hydrogen bond to form cellulose fibrils. Cellulose can be used to produce a broad variety of products by chemical modification such as methylation, esterification, sulphonation, nitration and deoxyamination (Belgacem *et al*., 2008). Therefore, many researches have been conducted to improve the properties of cellulose for suitable applications. An X-ray patterns obtained from X-ray diffraction (XRD) and ^{13}C-nuclear magnetic resonance (^{13}C-NMR) showed the existence of more than one crystalline form referred to as polymorphism (Perez and Samain, 2010). There are four major polymorphs of cellulose been reported, cellulose I, II, III and IV (Mandal and Chakrabarty, 2011). The relationships between the different cellulose allomorphs and some prototypical fibers diffractograms are shown in Figure 3.

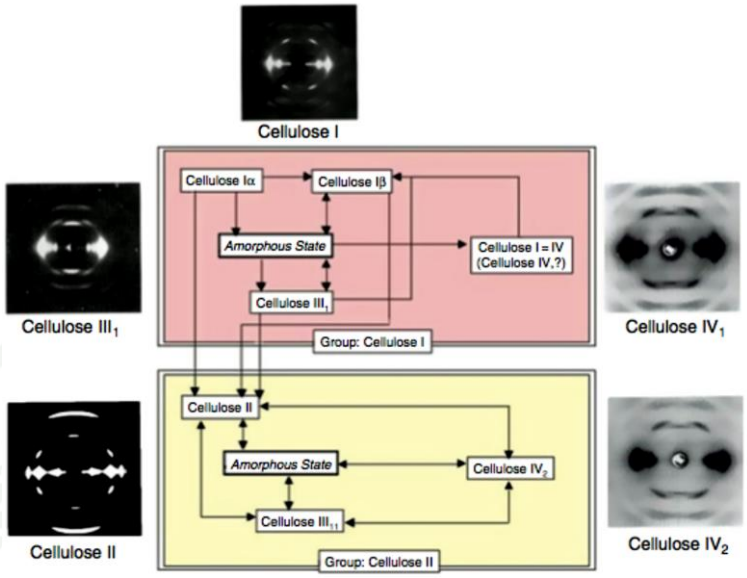

Figure 3. Relationships between the different cellulose allomorphs and some prototypical fibers diffractograms.

Source: Perez and Samain (2010)

Generally, cellulose I is the native cellulose and predominant in nature such as in bacteria, some animals and higher plants. Cellulose I or native cellulose can be converted into other polymorphs depending on the method of isolation. Moreover, cellulose I has two forms, cellulose I_α and cellulose I_β. The cellulose I_α is predominant in bacteria and algae, while cellulose I_β is predominant in higher plant (Liu and Sun, 2010). A result from ^{13}C-NMR spectra of cellulose I shown that at ~106 ppm I_α is singlet, while I_β is doublet, the conformation of the polysaccharides is similar with different hydrogen bonding (Siqueira *et al.*, 2010). Cellulose III could be obtained from both cellulose I and II, when treated with liquid ammonia at -80 °C, while cellulose IV is obtain by heating cellulose III in glycerol at 260 °C, depending on the starting material (Perez and Samain, 2010).

Cellulose extraction

Plant-based cellulose has been extracted from a variety of lignocellulosic materials which is well-known to consist of complex structure. The cellulose in plant cell wall is embedded with amorphous non-cellulosic materials including, lignin, hemicellulose, and other polysaccharides (Abraham et al., 2011). Hence it is necessary to eliminate non-cellulosic materials using chemical or physico-chemical treatments. Different approaches have been applied to isolate cellulose from different materials, the process differ depending on the cellulose sources (Klemm et al., 2005; Lu and Hsieh, 2010; Klemm et al., 2011).

1. Pretreatment

The properties of lignocellulosic biomass get it resistant to biodegradation. Due to the complexity and variability of biomass chemical structures, the optimal pretreatment method and conditions depend on the types of lignocellulose present. Several structural and compositional properties were found to have impacts on the biodegradability of lignocellulosic biomass, including cellulose crystallinity, accessible surface area, degree of cellulose polymerization, presence of lignin and hemicellulose, and degree of hemicellulose acetylation (Kim and Holtzapple, 2005, 2006). The goal of pretreatment is to alter such properties to improve biomass goodness to enzymes, microbes and chemicals. The effects of different pretreatment methods on the chemical composition and physical characteristics of lignocellulosic biomass are summarized in Table 3.

Table 3. Effect of pretreatment on the compositional and structural alteration of lignocellulosic biomass.

Pretreatment	**Increase of accessible surface area**	**Decrystallization of cellulose**	**Solubilization of hemicellulose**	**Solubilization of lignin**	**Alteration of lignin structure**	**Formation of furfural/ hydroxymethylfurfural (HMF)**
Mechanical	*	*				
Irradiation	*	+	+			+
Steam-explosion	*		*	+	*	*
Liquid hot water	*	ND	*	+	+	+
Catalyzed steam-explosion	*		*	*/+	*/+	*
Acid	*		*	+	*	*
Alkaline	*		+	*/+	*	+

Oxidative	*	ND		*/+	*	+
Ionic liquids	*	*	+			
Thermal acid	*	ND	*			*
Thermal alkaline	*	ND	+	*/+	*	+
Thermal oxidative	*	ND	+	*/+	*	+
Ammonia fiber explosion	*	*	+	*	*	+
Biological pretreatment	*	ND	*	*	*	

* = **major effect,** + = **minor effect, ND** = **not determined, and blank** = **no effect**.

Source: Adapted from (Zheng *et al.*, 2014)

In general, different pretreatment methods affected these properties to different degrees; however, all methods had a major effect on the accessible area of lignocellulosic biomass. Therefore, different function of pretreatment influence for step of substrate hydrolysis of lignocellulosic biomass, the selection and integration of

pretreatment methods, parameters, and strategies may differ depending on downstream processes. Pretreatment methods are reviewed under three categories: physical, chemical and biological. The technical performances of present pretreatment methods, including those not currently feasible at commercial or pilot scale, are summarized

1.1 Physical pretreatment

Physical pretreatment refers to methods that do not use chemicals or microorganisms during the pretreatment processes. Previously developed physical pretreatment techniques include comminution (e.g. milling and grinding), extrusion, and irradiation (ultrasound and microwave).

1.1.1 Comminution

Comminution of lignocellulosic biomass is utilized to reduce particle size and is typically applied to most biofuel processes prior to other pretreatment methods to improve feedstock handling and treatment. Biomass particle size reduction can alter the inherent ultrastructure of lignocellulosic biomass, increase the accessible surface area, reduce the degree of cellulose crystallinity, and decrease the degree of cellulose polymerization for improved digestibility (Kratky and Jirout, 2011). Comminution of biomass can be accomplished by using milling or grinding machines, including ball, vibro, hammer, knife, two-roll, colloid, and attrition mills, as well as extruders. The selection of a comminution machine depends on the moisture content of the feedstock. Two-roll, hammer, attrition, and knife mills are usually used for comminuting dry biomass with moisture contents of up to 10e15% (wet basis), while colloid mills and extruders are suitable only for comminuting wet materials with

moisture contents of more than 15-20% (wet basis); the ball and vibro mills can be used for either dry or wet material (Kratky and Jirout, 2011; Taherzadeh and Karimi, 2008).

1.1.2 Extrusion

Extrusion is a process that combines multiple operations in one unit. Raw materials are fed into one end of the extruder and then transported along the length of the barrel with a driving screw. As the material moves along the barrel, it is subjected to friction heat, mixing, and vigorous shearing upon pressure release at the finishing end (Camire, 1998). The design of the screw can affect the effectiveness of extrusion processing.

1.1.3 Irradiation

Irradiation is a physical pretreatment method that includes microwave, ultrasound, gamma-ray, and electron beam. Microwave processing is the most studied irradiation pretreatment method. Energy is generated by an electromagnetic field and delivered directly to the material to provide rapid heating throughout the entire material with reduced thermal gradients. The microwave field and the dielectric response of a material determine its ability to be heated with microwave energy (Thostenson and Chou, 1999). Microwave technology could be an effective alternative to conventional heating (Eskicioglu *et al.*, 2007). It can more rapidly heat a large volume, reducing the treatment time, which could lead to considerable energy savings.

1.1.4 Steam-explosion

Steam-explosion, also called autohydrolysis, differs from catalyzed steam-explosion as no chemicals are added in the process. In this method, biomass particles are heated with high-pressure saturated steam for a short period of time and the pressure is swiftly reduced to terminate the reactions, which causes the biomass to undergo an explosive decompression. Typical pretreatment temperature, pressure, and time fall within the ranges of 160-260 C, 0.69-4.83 MPa, and several seconds to a few minutes, respectively (Sun and Cheng, 2002). Under these conditions, hemicellulose is hydrolyzed into its component sugars and lignin is transformed to a certain degree, thus making pretreated biomass more degradable. The hydrolysis of hemicellulose is often facilitated by organic acids such as acetic acids and other acids formed from acetyl or other functional groups. In addition, water, which possesses certain acid properties at high temperature, further catalyzes hemicellulose hydrolysis (Weil *et al.*, 1997). Therefore, the degradation of reducing sugars might happen during steam-explosion due to acidic conditions. Steam-explosion is one of the most common pretreatment methods for lignocellulosic biomass (Zheng *et al.*, 2014).

1.1.5 Liquid hot water (hydrothermolysis)

Liquid hot water (LHW) is one of the hydrothermal pretreatment technologies that does not require addition of chemicals and is used in the pulp industry and for bioethanol production. In LHW pretreatment, pressure is utilized to maintain water in the liquid state at elevated temperatures (Brandon *et al.*, 2008; Dien *et al.*, 2006; Negro *et al.*, 2003; Rogalinski *et al.*, 2008). Biomass undergoes high temperature cooking in water at a high pressure. During pretreatment, water can penetrate into the biomass cell structure, hydrating cellulose, solubilizing

hemicellulose, and slightly removing lignin. LHW pretreatment is highly effective for enlarging the accessible and susceptible surface area of cellulose and improving cellulose degradability to microbes and enzymes. LHW treatment has the potential to enhance sugar extraction, pentose recovery, and cellulose degradability with the advantage of producing prehydrolyzates with much lower concentrations of inhibitors, compared with low pH pretreatment methods such as dilute acid and acid catalyzed steam-explosion (Zheng *et al.*, 2014).

1.2 Chemical pretreatment

Chemical pretreatment refers to the use of chemicals, such as acids, bases, and ionic liquids, to alter the physical and chemical characteristics of lignocellulosic biomass. Among the three pretreatment categories discussed, chemical pretreatment has received the most research interest.

1.2.1 Alkaline pretreatment

Alkaline pretreatment uses bases, such as NaOH, $Ca(OH)_2$, KOH, and $NH_3 \cdot H_2O$, to remove lignin, hemicellulose, and/or cellulose, rendering lignocellulosic biomass more degradable to microbes and enzymes. Alkaline treatment with NaOH has been extensively used in the pulp and paper industry. The function of alkaline pretreatment is believed to be the saponification and cleavage of lignin-carbohydrate linkages (Tarkow and Feist, 1969). By removal of crosslinks, alkaline pretreatment leads to an increase of porosity and internal surface area, structural swelling, a decrease in the degree of polymerization and crystallinity, disruption of lignin structure, and a breakdown of links between lignin and other polymers. The effectiveness of alkaline pretreatment is associated with the lignin content of biomass materials.

1.2.2 Acid pretreatment

Acid pretreatment can be conducted either under concentrated acid (e.g. 30-70%) and low temperature (e.g. 40 C) or under dilute acid (e.g. 0.1%) and high temperature (e.g. 230 C). Both inorganic and organic acids, including sulfuric acid (H_2SO_4), hydrochloric acid (HCl), nitric acid (HNO_3), phosphoric acid (H3PO4), acetic acid, and maleic acid, have been used for dilute acid pretreatment, with H_2SO_4 being the most commonly used. Although concentrated acid is highly effective on cellulose hydrolysis, it is extremely toxic, corrosive, and dangerous, and requires expensive materials, such as specialized nonmetallic materials or alloys, for reactor construction. In addition, the acid must be recovered after biomass treatment for economic reasons, as it is an energy- and cost-intensive process. Therefore, dilute acid is favored over concentrated acid for lignocellulosic biomass pretreatment, and extensive studies have been conducted resulting in it being one of the most commonly applied chemical pretreatment methods. Dilute acid pretreatment primarily hydrolyzes up to 100% of the hemicellulose into its component sugars (e.g. xylose, arabinose and galactose), depending on the pretreatment conditions. It can also disrupt lignin to a high degree, but is not effective in dissolving lignin in most cases.

1.2.3 Wet oxidation

Wet oxidation is an oxidative pretreatment method that consists of the addition of water and an oxidizing agent (e.g. air, oxygen, and hydrogen peroxide [H2O2]) to feedstocks prior to pretreatment. The temperature, reaction time, oxygen pressure, and water content are the most critical parameters in wet oxidation. The process is usually performed under high temperature (125-300 C) and high pressure

(0.5e20 MPa). Reported treatment time varied from a couple of minutes to hours. The presence of oxygen can increase the reaction rates and production of free radicals. Although faster reaction rates can be achieved with high oxygen concentrations, using pure oxygen results in high operating costs. Therefore, air is usually used as an oxidizing agent in wet oxidation pretreatment. The wet oxidation process is exothermic, and therefore heat produced from reactions is, in most cases, sufficient to keep the temperature at a desired level once the pretreatment is initiated, hence eliminating or minimizing energy inputs. Thus, this process can be carried out at comparatively reduced temperature because of the generation of heat. Critical water content is needed for the process to occur. Additional water needs to be added for dry biomass such as wood and straw. During wet oxidation, the main reactions include oxidative cleavage of aromatic nuclei, electrophilic substitutions, displacement of side chains, and cleavage of alkyl aryl ether linkages (Hon and Shiraishi, 2000).

1.2.4 Ozonolysis pretreatment

Ozone is a powerful oxidant which can be used as a pretreatment (ozonolysis) for lignocellulosic biomass. Ozonolysis pretreatment results in a more degradable biomass primarily via lignin degradation, with a slight alteration of hemicellulose; however, it has very little effect on cellulose. Main pretreatment parameters are the water content in the reactor, particle size, and ozone concentration in the gas stream. Among these parameters, the water content is the most important factor affecting the solubilization of feedstocks (Taherzadeh and Karimi, 2008). When ozone is applied during pretreatment, ozone molecules disintegrate into hydroxyl radicals (OH) in water, resulting in a combination of oxidation by both ozone itself and the OH radicals. Therefore, the pH of the solution determines to a large degree the type of reactions that occur during ozonolysis pretreatment.

1.2.5 Ionic liquids pretreatment

Compared with other volatile organic cellulose solvents, ILs possess the advantages of low toxicity, low hydrophobicity, low viscosity, thermal stability, broad selection of anion and cation combinations, enhanced electrochemical stability, high reaction rates, low volatility with potentially minimal environmental impact, and non-flammable properties. The mechanism of cellulose dissolution in ILs involves the oxygen and hydrogen atoms of cellulose hydroxyl groups, which form electron donoreelectron acceptor complexes that interact with ILs (Feng and Chen, 2008). Upon interaction between cellulose's hydroxyl groups and ILs, hydrogen bonds are broken, leading to opening of the hydrogen bonds between molecular chains of the cellulose, resulting in cellulose dissolution. Solubilized cellulose can be rapidly precipitated with anti-solvents such as ethanol, methanol, acetone, or water. The recovered cellulose was found to have the same degree of polymerization and polydispersity as the initial cellulose, but significantly different macro- and micro-structures, especially decreased crystallinity and increased porosity (Zhu, 2008).

IL pretreatment is a relatively new technique for efficient utilization of lignocellulosic materials for biofuels and bio-based products, and there are still many challenges in putting these potential applications into practical use. These challenges include the high cost of ILs, regeneration requirements, lack of toxicological data and knowledge about basic physico-chemical characteristics, the effect of the action mode on hemicellulose/lignin contents, and inhibitor generation issues. Further research is required to address such challenges (Zheng *et al.*, 2014).

1.3 Biological pretreatment

Biological pretreatment involves minimal energy input and depends on incubation of the biomass with selected microorganism that produce extracellular enzymes to modify the biomass, and improving its ability to be used for either biological or thermochemical processing (Chen *et al.*, 2010). The largest use of biological pretreatment involves biopulping with indigenous microorganism. However, earlier work showed the advantage of adding fungal organisms to accelerate and control the process. Preferred inoculums are various fungal microorganisms, with white rot fungi having significant deligninification activities (Zeng *et al.*, 2012). However, the time for processing varies from 28 to 60 days, as reviewed by (Chen *et al.*, 2010). Therefore, biological pretreatments are viewed as too long to be economically viable for biofuel production.

1.3.1 Fungal pretreatment

Research on fungal pretreatment is mainly on evaluating fungi that selectively degrade lignin and hemicellulose, while utilizing little cellulose. Cellulose is more recalcitrant to fungal attack than other components. Degradation of lignin and hemicellulose result in increased digestibility of cellulose, which is preferred for AD processes. Several fungi classes, including brown-, white- and soft-rot fungi, have been used for pretreatment of lignocellulosic biomass for biogas production, with white-rot fungi being the most effective (Sun and Cheng, 2002).

1.3.2 Microbial consortium pretreatment

Microbial consortium pretreatment is conducted by microbes screened from natural environments in which rotten lignocellulosic biomass is the substrate. In contrast to fungal pretreatment, which mainly attacks lignin, a microbial consortium usually has high cellulose- and hemicellulose-degradation ability. For microbial consortium pretreatment, there are several ways of microbial sources for pretreating. For example, (Li *et al.*, 2017) used rumen fluid from cow to pretreat corn stover, which showed the removal of hemicellulose part from corn stover after the rumen fluid pretreatment, with the increase of crystallinity and specific surface area. The pretreatment efficiency was mainly attributed to the participation of the enzymes secreted by rumen microorganisms. There are also researches that used several kinds of animal dung to pretreat biomass. For example, the work of (Sukasem *et al.*, 2017), they utilized pig dung, elephant dung and bat dung with different alkali pretreatments to pretreat water hyacinth for methane production, which showed he suitable dung for the highest efficiency anaerobic digestion. Moreover, (Fangkum and Reungsang, 2011a, 2011b; Saripan and Reungsang, 2014) have examined the effects of elephant dung on sugarcane bagasse for hydrogen production, which indicated the potential of the natural microbial in the elephant dung to convert the sugarcane bagasse hydrolysate into hydrogen.

1.3.3 Enzymatic pretreatment

In anaerobic digestion of lignocellulosic biomass, hydrolysis of cellulose and hemicellulose is believed to be a rate-limiting step. The most commonly used enzymes included laccase, cellulase and hemicellulase. In most cases, the effect of enzymes in enhancing pretreatment was minimal, and the cost of enzymes was high; therefore, application of enzymatic pretreatment has been limited. In general, most biological pretreatments are not as efficient as chemical pretreatments and the retention time is relatively high. Before biological pretreatment is feasible for

application in commercial production of pretreatment, additional research is needed to address some key issues such as cost, selectivity, and efficiency. On the other hand, there is some successful research about, (Vanitjinda *et al.*, 2019b) used xylanase for pretreatment of sugarcane bagasse, then the pretreated fiber was used for film production. (Saelee *et al.*, 2016) used xylanase for pretreatment of sugarcane bagasse to achieve cellulose nanofibrils.

2. Bleaching

After pretreatment, the current lignin content left in cellulose fibers could impede the extraction of cellulose, but it is hardest chemical component to take away from lignocellulosic fibers (Acharya *et al.*, 2011; Jonoobi *et al.*, 2009). Hence, bleaching is an additional crucial step for further removing the residual cementing material mainly lignin in the alkali retted fibers, so this process also known as delignification (Panaitescu *et al.*, 2013; Shi *et al.*, 2011). According to literatures, the different performance of bleaching treatments have been summarized in Table 4 (Ng *et al.*, 2015),

Table 4. Various bleaching treatments of cellulose fibers

Sources	Chemicals/Equipment	Conditions	References
Coconut fibers	Sodium chlorite, glacial acetic acid	60~70 °C, 1 h, mechanical stirring, 1 and 4 times	(Rosa *et al.*, 2010)

Softwood wood flour	Sodium chlorite, glacial acetic acid	70~75 °C, 2 h intervals until the wood became white	(Hietala *et al.*, 2011)
Kenaf fibers	NaOH, Sodium chlorite, glacial acetic acid	80 °C, 4 h, mechanical stirring, three times	(Hietala *et al.*, 2011)
Kenaf fibers	Sodium chlorite (2%), Acetic acid (3%)	70 °C, 180mins,	(Jonoobi *et al.*, 2009)
	NaOH (1.5%), H_2O_2 (1%)	70 °C, 90mins,	
	Sodium chlorite (1.25%) Acetic acid (3%)	70 °C, 90mins,	
Kenaf fibers	Sodium chlorite (2%), Acetic acid (3%)	70 °C, 90mins,	(Shin *et al.*, 2012)
	NaOCl (1.2%)	room temperature, one hour.	
Rice husk	a buffer solution of acetic acid, aqueous chlorite (1.7 wt%) and distilled water at reflux	using a silicon oil bath at 100–130 °C, 4 h, four times.	(Johar *et al.*, 2012)

H. sabdariffa fibre	a mixture of NaOH, acetic acid, sodium hypochlorite solution	room temperature, one hour.	(Sonia *et al.*, 2013)
Wood fiber	acidified sodium chlorite solution	75°C, 1 h, 5 times	(Chen *et al.*, 2011)
Corncob	Equal parts (v:v) of 1) acetate buffer (27 g NaOH and 75 mL glacial acetic acid, diluted to 1 L of distilled water) 2) aqueous chlorite (1.7 wt.% $NaClO_2$ in water)	80 °C, 6 h	(Silvério *et al.*, 2013)

Source: Adapted from (Ng *et al.*, 2015)

Almost, the pretreated-fibers are bleached by boiling the fibers with sodium chlorite ($NaClO_2$) solution under acidic condition (Thiripura Sundari and Ramesh, 2012). The acidic condition is created by acetate buffer solution which is made up of NaOH and glacial acetic acid, diluted with distilled water (Acharya *et al.*, 2011; Savadekar and Mhaske, 2012). During the fibers are in an acidic buffer solution, sodium chlorite would break down into chlorine dioxide (ClO_2) under the presence of buffer salts that help to discharge the chlorine dioxide from sodium chlorite. The ClO_2 could oxidize with lignin which is left in the fibers by attacking the aromatic ring of the lignin, the loss of lignin

could result in further fibrillation of the alkalitreated fibers (Zainuddin *et al.*, 2013). As an oxidant, chlorine dioxide also has an oxidizing effect on the hemicelluloses and pectins found in cellulose fibers based on the FTIR indicates that the aromatic groups also existed in hemicelluloses and pectins (Karimi *et al.*, 2014; Shi *et al.*, 2011). It helps to solubilize them leading to further opening of fiber-bundles with making the fiber more even and workable (Zainuddin *et al.*, 2013)

By bleaching process, the high exodus of lignin and hemicellulose which hinder its pursuance with polymer matrices could produce relatively pure cellulosic fibrils which enhanced interfacial adhesion with the polymer matrix (Shi *et al.*, 2011; Zainuddin *et al.*, 2013). Generally, the bleaching process is necessarily repeated by increasing numbers of times or stages until the permanent white tissues/fabrics are achieved based on the ability of bleaching to destroy natural color matter of fiber (Thiripura Sundari and Ramesh, 2012). The resulting white color of bleached material indicates that a relative amount of lignin is significantly removed and decreased, confirming that bleaching is successful (Chen *et al.*, 2011). The increased stages of bleaching process are used to further increase the cellulose content and remove the lignin and hemicellulose, and, thus producing purer and separated cellulose fibers than the previous process (Shin *et al.*, 2012).

Elephant digestive system

Elephants are particularly non-selective feeders and their diet often contains more than 100 plant species. While considerable knowledge has been accumulated about the diet, there is a few about the microbial aspect of the digestion in the elephant. There is only the general notion that fermentation of structural plant carbohydrates may occur in the gastrointestinal tract and that especially the caecum and colon act as

fermentation chambers (Eltringham, 1976; Woodford, 1972). Throughout the study all intestinal samples were taken from 8 different locations. The numbers and locations of these sampling points are shown in Figure 4, the volume and mass of the ingesta in the eight different regions of the gut were determined in accordance with the divisions described in Table 5 and pH and bicarbonate concentration in different compartments of the alimentary tract of the African elephant was shown in Table 6.

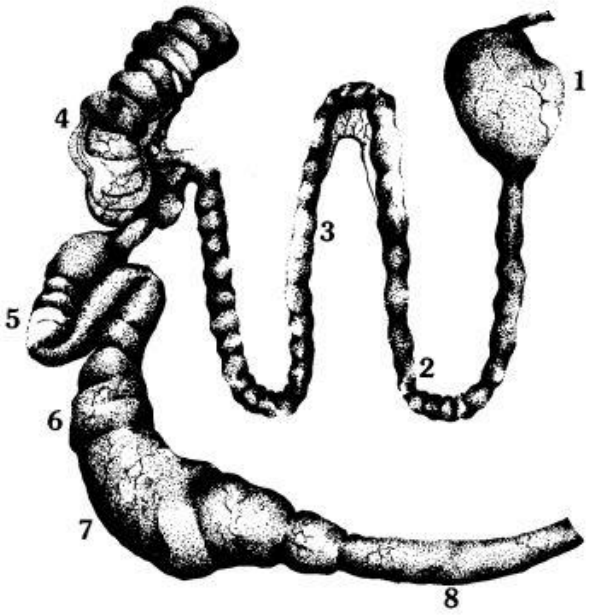

Figure 4. 1 Gut of adult elephant. 1 - Stomach. 2 - Small intestine (duodenal). 3 - Small intestine (ileal). 4 - Caecum. 5 - Anterior colon. 6 -Middle colon. 7 - Posterior colon. 8 - Mesocolon.

Source : (Van Hoven *et al.*, 1981)

Table 5. Names, numbers, location and content of intestinal areas investigated. The figures are based on the average of 10 adult African elephants.

Intestinal area number	Name	Location	Volume	Content kg (wet)	Protozoa number per mL x 10^4
1	Stomach	Anterior	60 ± 5	51 ± 4	0
2	Duodenum	First 8 m from stomach	21 ± 3	20 ± 3	1,0 ± 0,4
3	Ileum	Second 8 m from stomach	19 ± 4	18 ± 4	3,2
4	Caecum	At small intestine and colon junction	90 ± 10	86 ± 9	4,1
5	Anterior colon	First 2 m behind caecum	180 ± 16	157 ± 14	4,6
6	Middle colon	Second 2 m behind caecum	109 ± 7	96 ± 6	4,4
7	Posterior colon	Third 2 m behind caecum	40 ± 6	36 ± 7	3,8
8	Mesocolon	Hind 2 m	36 ± 9	23 ± 5	1,1
Total		Length: 27 m	555 ± 60	487 ± 52	

Source : Adapted from (Van Hoven *et al.*, 1981)

Table 6. pH and bicarbonate concentration in different compartments of the alimentary tract of the African elephant.

Compartment	Sampling site	pH	mmol/ l bicarbonate	pH
stomach	1A	<2,0	0	
stomach	1B	2,30 ± 0,37	0	
duodenum	2	6,52 ± 0,08	44,6 ± 5,6	
ileum	3	6,94 ± 0,38	41,6 ± 20,2	
caecum	4	5,88 ± 0,11	21,8 ± 3,8	
anterior colon	5	6,00 ± 0,33	25,4 ± 6,5	
posterior colon	7	6,42 ± 0,31	15,6 ± 8,1	
mesocolon	8	6,78 ± 0,29	10,8 ± 6,4	

Source : Adapted from (Hoven *et al.*, 1981)

1. Stomach

About 100 larvae were seen in each stomach investigated. The mean content of the stomach in ten adults was 60 ± 5 L and this was the only site in the whole digestive tract that contained no protozoa. No fermentation could be recorded here, very little

hydrolysis of cellulose takes place and no DL-Iactate hydrolysis or any bicarbonate was recorded. The highest rate of starch hydrolysis was found in the stomach but here hydrolysis is probably caused by high acidity.

2. Small intestine

The total length of this section between the stomach and the caecum is 16 m. It contained on average 40 I of which about 90070 was fluid. In the duodenum 10 000 protozoa per ml were found which increased to 30 200 distally in the ileum. No fermentation rate through gas production could be recorded in the small intestine. The rate of soluble starch hydrolysis was about half that found in the stomach and the lowest rate of cellulose hydrolysis took place here. An increase in the rate of both starch and cellulose digestion was found in the ileum compared to the duodenum. No DL-Iactate digestion was found in the whole small intestine.

The pH values found in duodenal and ileal contents ranged from 6,4 to 7,4. High concentrations of $HC0_3^-$ were present and these probably originate from pancreatic secretions. Somewhat higher lactic acid concentrations were found of which most were in the L-form. In concentration the VFA was not different from that found in the stomachs, however the 30% propionic acid to 55% acetic acid ratio in the oesophageal part of the stomach was unique in the gut.

3. Caecum

This is the first important site of fermentation in the digestive tract. In 10 adults it was found to contain 90 ± 10 I of which only 8,1% represented solids. A well-

established protozoa population was present and together with the bacteria, fermented the available substrates at an average rate of about 160 /lmol gas/g DM/h. The caecal gas consisted of 44,2% methane, 2,2% hydrogen with the balance being CO_2, an in vitro estimation of the rate of VFA formation in caecal contents of animal L7 gave a rate of 1,46 mmol per 100 g wet contents per hour. The VFA had a high concentration of acetate. Little or no lactic acid was found and that which was present was mostly of the L-configuration.

4. Colon

The colon can be regarded as the main area of digestive and absorptive activity. In the average adult elephant, it contains 329 ± 30 I of which the solid matter content increases from the front to the rear due to reabsorption of water. The highest number of protozoa per ml was found in the colon and the rate of fermentation was the highest in the anterior colon decreasing linearly to the rear. The methane content of the gas produced in the colon increased from 520/0 anterior to 65% in the posterior colon with CO_2 exhibiting just the opposite tendency. The VFA and lactic acid concentrations in the anterior and posterior colon are comparable to those found in caecal contents. Slightly higher pH values are found and soluble sugar concentrations remain low. Results of the in vitro fermentation rate measurements showed a rate of 1,66 mmol of VFA per 100 g of wet contents per hour. Proportions of acetic propionic and butyric acid in the colon were quite similar to those in the caecum, with a preponderance of acetic acid. However, increased concentrations of iso-butyrate, 2-methyl-butyrate, 3-methylbutyrate and also valerate were found especially in the posterior colon. The presence of increased amounts of branched- chain VFA is indicative of protein fermentation.

4. Mesocolon

The posterior 2 m of the digestive tract are referred to as the mesocolon and the sampling site 8 was in the middle of this section. On average this whole section contained 36 ± 9 litres of which 86,4% was fluid. From colon to mesocolon a reduction of about 75% occurred in the protozoa numbers which could well also be the case with the bacteria since a considerable reduction in rate of fermentation is evident here. The highest percentage of methane, an average of 68,7070was present in the mesocolon gas phase. The pH values near neutrality, a lowered bicarbonate concentration and somewhat reduced concentration of VFA were found. Most of the VFA in the mesocolon probably originates in the colon with its much higher fermentative activity. The capacity to ferment lactic acid has been lost in this caudal section which is probably the reason for a slight accumulation of the acid.

Materials and Equipment

1. Materials and chemicals

Sugarcane bagasse (SCB) was supplied by Mitrphol Sugar Factory (Suphanburi, Thailand) and used as raw material for the experiment. Elephant dung was collected from Ayutthaya Elephant Palace & Royal Kraal (Thailand). Sodium chlorite ($NaClO_2$) was purchased from Ajax Finechem (New Zealand). Glacial acetic acid (CH_3COOH) was purchased from QRec Chemical Co., Ltd. (New Zealand). All other chemicals were of analytical grade and used as received.

2. Methods

2.1. Preparation of inoculum medium

After collection, the elephant dung was transferred into a freeze box at -12°C with oxygen absorber (Pack- Anaero) to maintain activities of the contained microorganisms. The elephant dung was compressed through a Stomacher (Model 80C, Seward) and mixed with phosphate buffered saline at ratio 1:4 (w/v) to adjust the pH to 6.0 as elephant colon condition. Finally, the dung was mixed with basal medium (Table 7) at ratio 1:18 (v/v) as nutrients for elephant dung microorganisms to obtain elephant dung pretreatment medium (EDPM).

Table 7 Composition of basal medium.

Nutrient	Usage (g/L)
(A) $MgSO_4$	0.01
(B) $CaCl_2$	0.016

(C) KH_2PO_4, K_2HPO_4	0.04
(D) Tryptone, Yeast extract, NaCl	0.1
(E) Peptone, $NaHCO_3$	2
(F) Tween	2.2 (2 mL/L)
(G) Distilled water	998

2.2. Pretreatment of SCB using simulated elephant colon model

SCB was triturated by a high-speed universal smashing machine into small particles and screened at 35 mesh to conduct batch experiments. SCB was mixed with EDPM at a ratio of 1:19 (w/v) as the simulated elephant colon pretreatment (SEP). Simulation of elephant colon pretreatment without elephant dung (SP) was used as the control. The mixture was stirred using a magnetic stirrer at 120 rpm and the pH was adjusted to 6.0 using phosphate buffered saline. The temperature of the fermenter was kept constant at 39°C under nitrogen atmosphere to maintain an anaerobic environment. Pretreatment time lasted 24 h in a two-liter (2 L) fermenter. After fermentation, the solid portions were washed with water and dried at 80°C in an oven to obtain SEP fibers (SEP-F) and without using elephant dung condition (SP-F). SEP-F and SP-F were studied for their fiber properties. Fermentation broths at 0, 6, 12 and 24 h were filtered and centrifuged at 8,000 rpm for 10 min to obtain SEP broth (SEP-B) and without using elephant dung condition (SP-B). The SEP-B and SP-B were sterilized through a 0.45 µm filter membrane to obtain supernatant as SEP-S and SP-S, respectively.

2.3. Bleaching process

SCB, SEP-F and SP-F were bleached using acid chlorite seven times as listed in Table 8. The fibers were placed in 250 mL Erlenmeyer flasks using fiber to initial liquid

ratio of 1:10, bleached with 1.4% (w/v) sodium chlorite ($NaClO_2$) and maintained at pH 4 by adjusting the solution with acetic acid. The flasks were incubated at 70°C in a water bath. Fresh sodium chlorite and acetic acid were added to the reaction every hour at the same loading ratio until the fibers turned white. The samples were then washed with distilled water until the pH became neutral and dried at 55°C for 24 h.

A schematic representation of the processes used to obtain cellulose from SCB, fermentation broth, fermentation supernatant and a list of abbreviations are presented in Fig. 5 and Table 8, respectively.

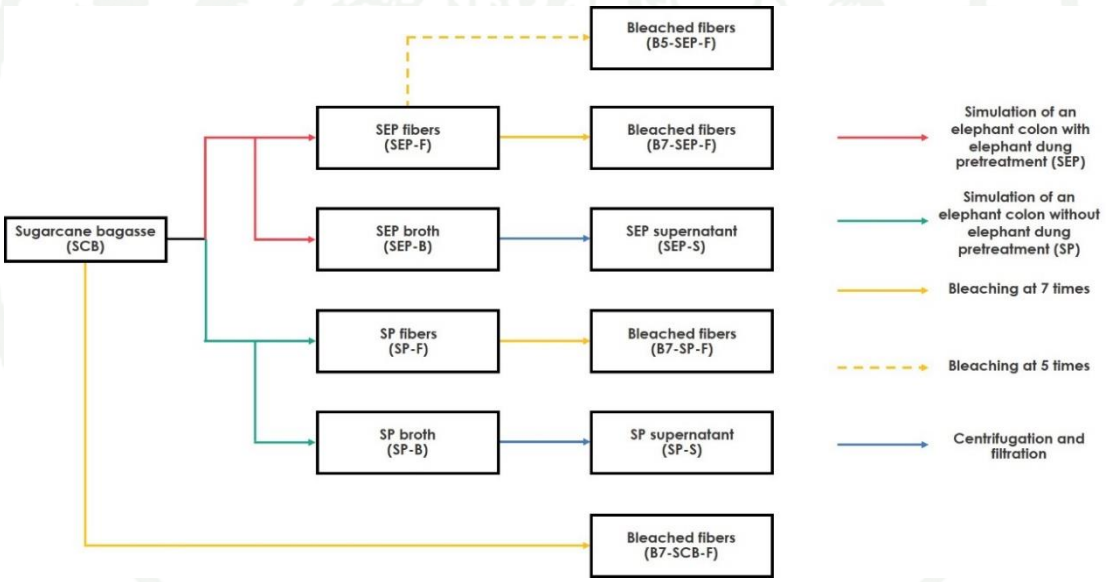

Figure 5 Schematic representation of the processes used to obtain cellulose fibers from SCB and liquid portions after pretreatment in fermenters.

Table 8 Fibers and liquid portions obtained at each stage of pretreatment and bleaching processes.

Sample	Condition
SCB	Untreated sugarcane bagasse
SEP-F	SCB + Simulation of elephant colon with elephant dung pretreatment
SP-F	SCB + Simulation of elephant colon without elephant dung pretreatment

SEP-B	Fermentation broth from simulation of elephant colon with elephant dung pretreatment
SP-B	Fermentation broth from simulation of elephant colon without elephant dung pretreatment
SEP-S	Supernatant from simulation of elephant colon with elephant dung pretreatment
SP-S	Supernatant from simulation of elephant colon without elephant dung pretreatment
B7-SCB-F	SCB + Bleaching seven times
B5-SEP-F	SCB + Simulation of elephant colon with elephant dung pretreatment + Bleaching five times
B7-SEP-F	SCB + Simulation of elephant colon with elephant dung pretreatment + Bleaching 7 times
B7-SP-F	SCB + Simulation of elephant colon without elephant dung pretreatment + Bleaching seven times

2.4. DNA extraction

Total DNA of SEP-B and SP-B were extracted via the DNeasy PowerSoil Kit for microbial genetic DNA isolation from liquid samples (QIAGEN, Denmark). The samples were added to a bead beating tube for rapid and thorough homogenization. Cell lysis was performed by mechanical and chemical methods and total genomic DNA was captured on a silica membrane in a spin column format. The DNA was washed and eluted from the membrane. The isolated DNA was used for PCR analysis and 16S metagenomic sequencing.

2.5. Characterization

2.5.1 Characterization of liquid portions

2.5.1.1. Microbial community composition

SEP-B and SP-B at 0 and 24 h were used for molecular analysis. The V3-V4 region of 16S rRNA was amplified using a forward primer (TCGTCGGCAGCGTCA GATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and reverse primer (GTCTC GTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). The 20 µL PCR reaction mixture was composed of 2.5 µL of microbial DNA (5 ng/µL), 5 µL of amplicon PCR forward primer 1 µM, 5 µL of amplicon PCR reverse primer 1 µM and 12.5 µL of 2x KAPA HiFi HotStart ready mix. The PCR program consisted of initial denaturing at 95°C for 3 min followed by 25 cycles of denaturing at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extending at 72°C for 30 seconds and completed with a final extension at 72°C for 5 min. The expected PCR product size of 460 bp was purified with the AMPure XP beads and PCR Clean-up (Machery-Nagel, Neumann-Neander-Str, Germany).

The PCR product was processed using the Illumina sequencing platform (MiSeq, San Diego, CA, USA). The MiSeq sequencing data were demultiplexed and quality-filtered using Quantitative Insight Into Microbial Ecology 2 platform (QIIME 2, Version 2018.4; https://docs.qiime2.org/2018.4/), using a pre-trained Naïve Bayes classifier based on the Greengenes database (http://greengenes.lbl.gov/). An operational taxonomic unit (OTU) database was computed from a sequence depth of 210 reads per sample and applied to pair-end sequence reads to generate a taxonomic table.

2.5.1.2. Organic acid

SEP-S and SP-S at 0, 6, 12 and 24 h were measured using high performance liquid chromatography (HPLC) with an ultraviolet detector (Waters; Mildford, MA, USA). An Aminex HPX-87H (Bio-Rad Labs; Hercules, CA, USA) operating at 60°C with 5 mM H_2SO_4 as a mobile phase and flow rate of 0.6 mL/min was used for separation. Detection was performed at 280 nm. Lactic, acetic, propionic and butyric acids were analyzed.

2.5.1.3. pH

The pH values of SEP-S and SP-S at 0, 6, 12 and 24 h were measured with a glass electrode pH meter (FiveEasy Plus pH meter FP20-Std-Kit, Mettler-Toledo International Inc., Switzerland) for each sample.

2.5.1.4. Reducing sugar concentration

The dinitrosalicylic acid (DNS) assay method was used to analyze reducing sugar concentration (Miller, 1959) in SEP-S and SP-S at 0, 6, 12 and 24 h. A GENESYS™ 10S Vis Spectrophotometer (Thermo Scientific™) was used to measure absorbance at 540 nm. All assays were performed in triplicate to obtain reproducible data.

2.5.1.5. Lignin peroxidase activity

Lignin peroxidase activity of SEP-S and SP-S at 0, 6, 12 and 24 h was determined spectrophotometrically (Levin *et al.*, 2004) with veratryl alcohol as the substrate. A 500

µl aliquot of the reaction mixture containing enzyme was diluted to 750 µL of H_2O, 250 µL of 4 mM $MnSO_4$, 500 µL of 0.25 M d-tartaric acid (pH 2.5) and 500 µL of 10 mM veratryl alcohol. The reaction was monitored by measuring the change in absorbance at 310 for 0 and 60 seconds. One activity unit was defined as the quality of enzyme required to liberate 1 micromole of veratryl alcohol per minute. The activities were expressed in U L^{-1} and calculated using Eq. 1.

2.5.1.6. Manganese peroxidase activity

Manganese peroxidase activity of SEP-S and SP-S at 0, 6, 12 and 24 h was determined spectrophotometrically following the method of (Heinfling *et al.*, 1998) with 2, 6-dimetoxyphenol as the substrate. A 500 µl aliquot of the reaction mixture containing enzyme was diluted to 750 µL of H_2O, 250 µL of buffer (20 mM sodium acetate buffer, pH 5), 250 µL of 0.1 mM H_2O_2, 250 µL of 1mM $MnSO_4$ and 500 µL of 1 mM 2, 6-dimetoxyphenol. The reaction was monitored by measuring the change in absorbance at 469 for 0 and 60 s. One activity unit was defined as the quality of enzyme required to liberate 1 micromole of 2, 6-dimetoxyphenol per minute. The activities were expressed in U L^{-1} and calculated using Eq. 1.

Enzyme activities = ((ΔAbs/min) x total volume x 10^6)/ ε x sample volume (1)

Where; ΔAbs/min = Increase of absorbance within 60 s

ε = Extinction coefficient

-Lignin peroxidase = 9,300 $M^{-1}cm^{-1}$

-Manganese peroxidase = 27,500 $M^{-1}cm^{-1}$

2.5.1.7. Xylanase activity

Xylanase activity of SEP-S and SP-S at 0, 6, 12 and 24 h was measured following the dinitrosalicylic acid (DNS) method (Miller, 1959). A 0.3 mL aliquot of 0.1 M sodium acetate buffer with 7.0 pH and 0.2 mL of 5% birchwood xylan solution was prepared in the same buffer and added to 0.5 mL enzyme solution (SEP-S and SP-S). The reaction solution was incubated for 15 min at 37°C. For the blank, the reaction mixture, to which 0.1 M pH 7.0 sodium acetate buffer solution was added, was used instead of enzyme solution. After incubation, 1 mL DNS solution was added to the reaction mixture to stop the reaction. The mixture was kept at 90°C for 5 min and absorbance was measured at 540 nm by a GENESYS™ 10S Vis Spectrophotometer (Thermo Scientific™). One xylanase activity unit was defined as the quality of enzyme required to liberate 1 micromole of xylose equivalent per minute at 50°C.

2.5.2. Characterization of solid portions

2.5.2.1. Whiteness index

Color changes in the fibers at each stage of treatment were measured using a colorimeter (Konica Minolta D/8 UV). For the whiteness index, a 360 to 740 nm SCI/SCE CM- 3600A Spectrophotometer (China) was used for color reflectance transmission. A white (lightness) values ranging from 0 (black) to 100 (white), a* (red and green) values ranging from 80 (greenness) to 100 (redness), and b* (blue and yellow) values ranging from 80 (blueness) to 70 (yellowness). All measurements were performed in triplicate. The whiteness index (WI) was calculated using Eq. 2 (Ghanbarzadeh *et al.*, 2010):

$$WI = 100-[(100-L)^2 + a^2 + b^2]^{0.5} \quad (2)$$

2.5.2.2. Chemical composition

Chemical composition of the fibers at each stage of treatment was analyzed according to the standard TAPPI methods. Lignin content was determined by reaction with 72% (w/v) sulfuric acid according to T222 om-98. Holocellulose (α-cellulose + hemicellulose) content was determined using the acid chlorite method and the α-cellulose content was estimated according to TAPPI T202 om-88. Hemicellulose content was calculated as holocellulose minus the α-cellulose content (Meesupthong *et al.*, 2021; Vanitjinda *et al.*, 2019a). All determinations were conducted with three replications.

2.5.2.3. Fourier transform infrared (FTIR) spectroscopy

FTIR spectra of the samples were recorded using a Fourier transform infrared spectrometer (Bruker model Tensor 27, USA). The fibers were ground to powder, then dried and mixed with KBr and compressed to form a disk. The spectrum for each sample was analyzed in the region 4000-400 cm^{-1} at a resolution of 4 cm^{-1} (Kawee *et al.*, 2018).

2.5.2.4. X-ray diffraction (XRD)

The crystallinity of the fibers was measured using an X-ray Diffractometer (Bruker D8 Advance, Germany) with Cu-Kα radiation (λ = 1.54 Å) at 40 kV. The samples were scanned at 2θ angles from 5° to 50° at a rate of 5°/min. The peaks were

deconvoluted into crystalline and amorphous contributions and the crystallinity index (CrI) was calculated according to Eq. 3 (Ruan *et al.*, 2016).

$$\text{Crystallinity index (CrI)} = \frac{\sum A_{cryl}}{\sum A_{cryl} + \sum A_{amph}} \times 100 \quad (3)$$

where $\sum A_{cryl}$ is the integrated area of all crystalline peaks and $\sum A_{amph}$ is the integrated area of all amorphous peaks.

2.5.2.5. Scanning electron microscopy (SEM)

Fiber morphology was characterized using a JEOL JSM5600LV (Japan) Instrument. The sample was coated with gold and analyzed using a scanning electron microscope operated at an accelerating voltage of 10 kV.

standard plate was used to calibrate the instrument with color values of L = 100, a = 0 and b = 0. The L*, a* and b* color values were measured as L*

RESULTS AND DISCUSSION

The pretreatment is an important tool for disrupting lignocellulosic matrix and removing some lignin and hemicellulose, which is an essential step for the preparation of cellulosic materials from sugarcane bagasse. Whereas, the physical, chemical and combinatorial pretreatment are the most expensive process in the conversion of lignocellulosic biomass because of high energy and chemical requirement. In this work, we addressed those issues by using green technique involving simulated elephant colon with elephant dung to pretreat sugarcane bagasse for cellulose extraction. This is the first study establishing the use of elephant dung to pretreat sugarcane bagasse as a bioresource for cellulose extraction.

1. Microbial community and metabolites during pretreatment

1.1. Microbial community composition

SEP-B and SP-B were analyzed using 16S metagenomic sequencing for the Illumina MiSeq system. Illumina high-throughput sequencing was applied to characterize bacterial community composition and their changes during pretreatments at the family level (Fig. 6). Relative abundance of microorganisms in SEP-B showed higher diversity than SP-B due to microorganisms from the elephant dung. Relative abundances of *Bacillaceae* were proportion at the initial stage of pretreatment but dramatically decreased from 45.1% to 3.7% at 0 and 24 h in SEP-B and from 99.3% to 27.3% at 0 and 24 h in SP-B. The *Bacillaceae* family contains rhizospheric microorganisms that have the ability to produce endospores, which can be dormant and resistant to environmental and chemical stress. Bacillaceae also perform fundamental roles in soil ecology, such as the cycling of organic matter in plant health and growth stimulation via suppression of plant pathogens and phosphate solubilization (Han *et al.*, 2020). The *Bacillaceae* originated from the soil along with the SCB and were abundant

during the initial stage of pretreatments. Relative abundance of *Bacillaceae* in SEP-B was lower than in SP-B because of increased microorganism diversity from the added elephant dung. Relative abundances of *Clostridiaceae* were 3.3% and 20.8% at 0 and 24 h in SEP-B and 0% and 17.2% at 0 and 24 h in SP-B. Compositions of *Paenibacillaceae* were recorded as 0.1% and 1.4% at 0 and 24 h in SEP-B and 0.2% and 35.7% at 0 and 24 h in SP-B. *Paenibacillaceae* showed remarkable characteristics significantly higher in SP-B than SEP-B at 24 h because of the lower diversity of microorganisms. Relative abundance of *Enterobacteriaceae* in SEP-B was higher than SP-B as 0.1% and 40.5% at 0 and 24 h in SEP-B and 0.2% and 17.2% at 0 and 24 h in SP-B. Some species of *Enterobacteriaceae* as well as *Leuconostocaceae, Streptococcaceae, Veillonellaceae, Lactobacillaceae, Synergistaceae, Ruminococcaceae, Lachnospiraceae, RFP12, Paraprevotellaceae, Spirochaetaceae and Prevotellaceae* presented in SEP-B were previously reported in gastrointestinal animal tracts (Ishiguro *et al.*, 2018; Jumas-Bilak *et al.*, 2007; Maier *et al.*, 2014; Nagao-Kitamoto *et al.*, 2016; Neubauer *et al.*, 2020; Papizadeh *et al.*, 2017; Tran *et al.*, 2019). *Leuconostocaceae* increased from 0.1% to 19.4%, *Streptococcaceae* increased from 0.0% to 6.8%, *Veillonellaceae* increased from 1.2% to 3.7% and *Lactobacillaceae* increased from 0.0% to 0.1% at 0 and 24 h, respectively. By contrast, *Synergistaceae* decreased from 3.0% to 0.4%, *Ruminococcaceae* decreased from 4.1% to 0.4%, *Lachnospiraceae* decreased from 5.6% to 0.1%, *RFP12* decreased from 4.6% to 0.3%, *Paraprevotellaceae* decreased from 4.0% to 0.0%, *Spirochaetaceae* decreased from 1.2% to 0.0% and *Prevotellaceae* decreased from 1.0% to 0.0% at 0 and 24 h, respectively. An unknown bacterial family decreased from 26.6% to 1.5% at 0 and 24 h in SEP-B but increased from 0.1% to 1.1% at 0 and 24 h in SP-B.

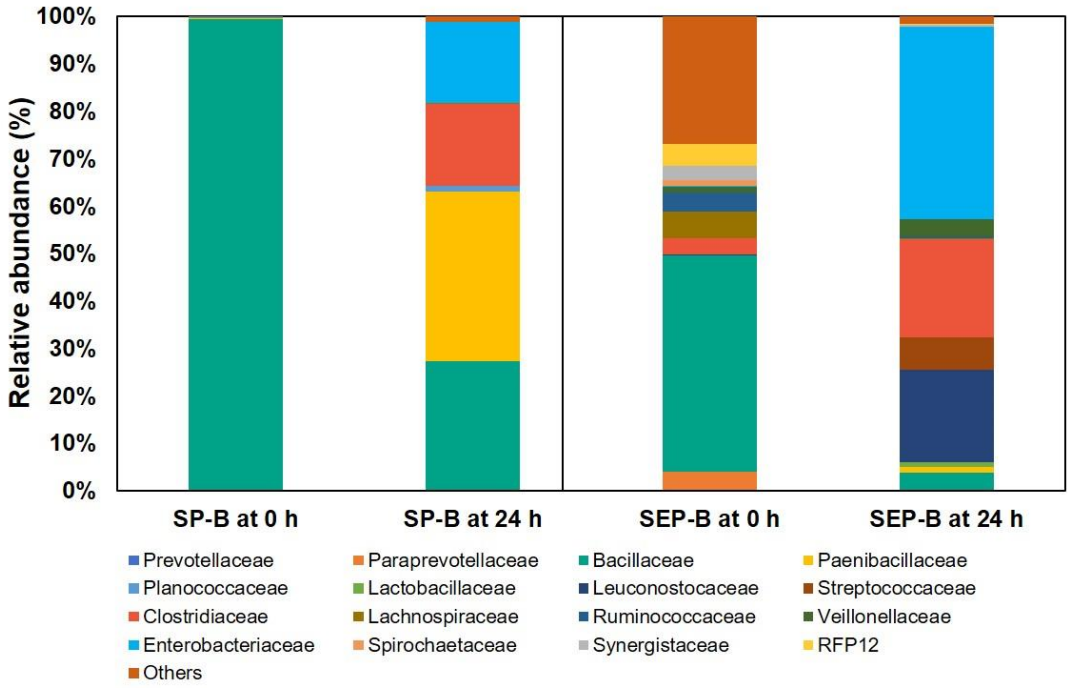

Figure 6 Relative abundance of fermentation broth from the fermenter at different times.

1.2. Organic acid production

Organic acid concentration of SEP-S and SP-S was investigated. Results showed the production of four compounds as lactic, acetic, propionic and butyric acids. At the initial hours of pretreatments, the microorganisms produced similar amounts of organic acids in SEP-S and SP-S as shown in Table 9. This occurred because *Bacillaceae* as the main proportion at 0 h were able to produce a variety of organic acids (Mumtaz *et al.*, 2019; Yan *et al.*, 2013). Furthermore, *Leuconostocaceae* in SEP-B increased significantly from 0.1% to 19.4% at 0 and 24 h, respectively. *Leuconostocaceae* ferment glucose heterofermentatively yielding lactic acid, carbon dioxide, ethanol and/or acetate (Nieminen *et al.*, 2014) as the reason for the presence of lactic acid in SEP-S at the initial hours. However, SEP-S at 12 h showed only acetic and butyric acids at 22 and 20.6 mmol/mL, respectively. The acetic and butyric acid concentrations in SEP-S

accumulated continuously from 9.9 to 30.5 mmol/mL and from 13.5 to 23.4 mmol/mL from 0 to 24 h, respectively while SEP-S at 24 h showed no lactic acid and propionic acid. Depletion of lactic acid in SEP-S at 12 and 24 h was caused by *Veillonellaceae* in SEP-B that require lactate for growth but are unable to metabolize normal dietary carbohydrates. Thus, they used lactate produced by other microorganisms and converted it into a range of weaker and less cariogenic organic acids such as propionic acid (Actor, 2012; Salliss *et al.*, 2021). Furthermore, the disappearance of propionic acid in SEP-S at 12 and 24 h was due to the influence of acetic acid concentration on propionic acid degradation to methane (Fukuzaki *et al.*, 1990; Liu *et al.*, 2012). The disappearance of lactic acid and propionic acid in SEP-S was caused by their conversion into other compounds by microorganisms and the influence of other organic acid concentrations leading to the degradation of these compounds. Conversely, SP-S showed four organic acids including lactic acid, acetic acid, propionic acid and butyric acid, with an increasing trend from the initial stage of pretreatment, except for acetic acid concentration of SP-S, which sharply decreased from 26.8 to 1.9 mmol/mL from 12 to 24 h, respectively. Acetic acid depletion in SP-S at 24 h was caused by the relative abundance of the methanogenic bacteria *Paenibacillaceae* that oxidized acetate to methane (Manyi-Loh *et al.*, 2019). Moreover, some species in *Paenibacillaceae* produce antimicrobial substances that affect the production of acidogenic bacteria in the SP system (Grady *et al.*, 2016). The appearance of butyric acid in SEP-S and SP-S *throughout the pretreatments was attributed to* the presence of *Clostridiaceae*. Some species of *Clostridiaceae* showed important characteristics as butyric acid fermenters (Ciani *et al.*, 2008; Esquivel-Elizondo *et al.*, 2017), while others were also reported to produce acetate and other products such as butyrate, ethanol or butanol (Wiegel *et al.*, 2006). This result concurred with a previous report which showed that organic acids could be produced by microbial processes as mostly natural or intermediate products in major metabolic microbial pathways (Nuryana *et al.*, 2019).

The pH values for both conditions slightly decreased due to the accumulation of organic acids, causing a decrease in the initial pH from 6.0 to 5.43 in SEP-S and 6.0 to 5.53 in SP-S at 0 and 24 h, respectively (Table 9). The pH changes reflected the organic acid production as explained earlier. The pH value did not change significantly because of the high buffering capacity from the phosphate buffered saline used for the initial pH adjustment before pretreatment.

Table 9 Dynamics of pH and organic acid contents during pretreatment.

Item	**Supernatant**	**Pretreatment times (h)**			
		0	6	12	24
pH	SP-S	6	5.9	5.55	5.53
	SEP-S	6	5.56	5.55	5.43
Lactic acid (mmol/mL)	SP-S	1.7	2	2.3	6
	SEP-S	1.8	2.3	0	0
Acetic acid (mmol/mL)	SP-S	9.6	18.3	26.8	1.9
	SEP-S	9.9	19.2	22	30.5
Propionic acid (mmol/mL)	SP-S	6	7.2	10	11.9
	SEP-S	5.5	9.7	0	0
Butyric acid (mmol/mL)	SP-S	12.8	15.9	20.2	20.9
	SEP-S	13.5	20.5	20.6	23.4

1.3. Reducing sugar concentration

Reducing sugar concentrations at different pretreatment times are presented in Fig. 7. In the initial stage of pretreatment, the reducing sugar concentration at 0 h in SEP-S showed a higher value than SP-S at 328.33 and 196.87 mg/L, respectively. The higher value in SEP-S at 0 h was attributed to the reducing sugar in elephant dung and SCB. Peaks of reducing sugar concentration in both SEP-S and SP-S were highest at 6 h as 382.84 mg/L and 209.70 mg/L, respectively. This was due to the lignocellulosic degradation of SCB by microorganisms in the elephant dung. However, reducing sugar of SEP-S at 12 h decreased sharply to 182.12 mg/L from utilization by microorganisms,

while SP-S at 12 h also decreased to 154.55 mg/L. Reducing sugar concentrations of SEP-S and SP-S slightly decreased to 169.93 mg/L and 138.51 mg/L at 24 h, respectively. Changes in reducing sugar concentration reflected the conversion of SCB to reducing sugar, while depletion of reducing sugar illustrated the production of organic acids by acidogenic bacteria.

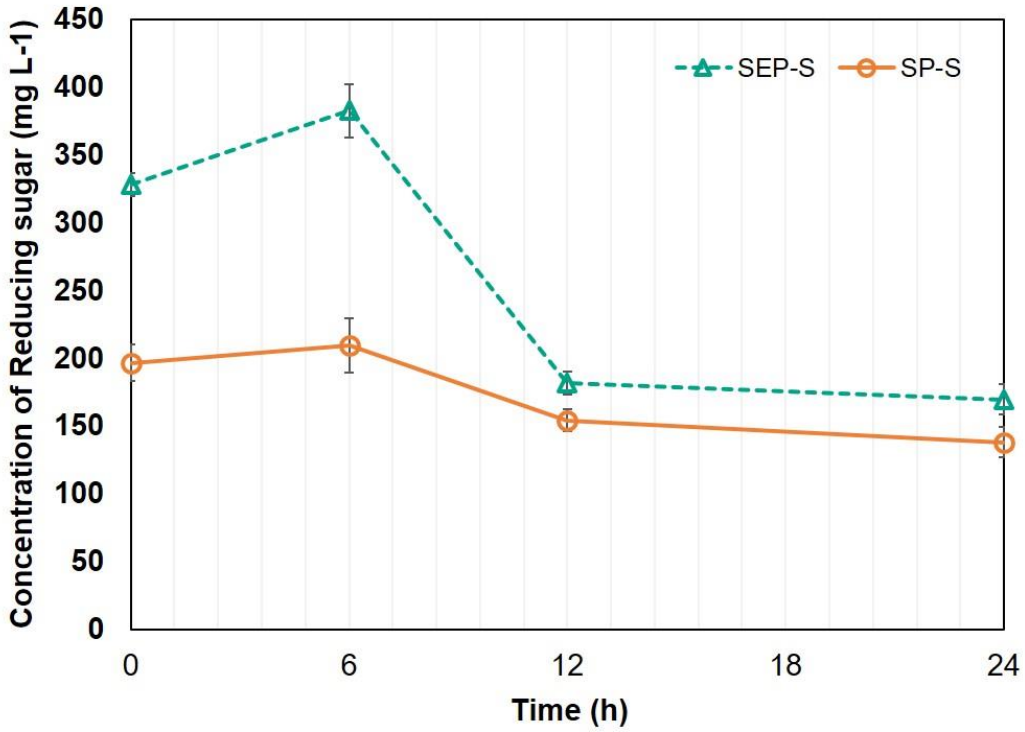

Figure 7 Relationship between reducing sugar concentrations at different pretreatment times.

1.4. Change of enzymatic activities

Lignin peroxidase, manganese peroxidase and xylanase activities in SEP-S and SP-S were investigated. Enzymatic activities during pretreatment are presented in Table 10. All enzymatic activities were detected in SEP-S, whereas enzymatic activity in SP-S was only represented by xylanase, which clearly explained the importance of

microorganisms in elephant dung as major sources of enzymes. Lignin peroxidase, manganese peroxidase and xylanase activities in SEP-S and xylanase activity in SP-S all showed a downward trend. Lignin peroxidase activity in SEP-S decreased continuously from 1.703 to 1.120, 0.896 and 0.851 U/mL at 0, 6, 12 and 24 h, respectively, while manganese peroxidase activity in SEP-S decreased from 0.848 to 0.167 U/mL at 0 and 6 h and was undetected at 12 and 24 h. Xylanase activity in SEP-S and SP-S slightly decreased. SEP-S decreased from 1.072 to 1.023, 0.943 and 0.642 U/mL while SP-S decreased from 0.454 to 0.414, 0.408 and 0.407 U/mL at 0, 6, 12 and 24 h, respectively. Decrease in enzymatic activities from the beginning of the pretreatments was caused by the adsorption of SCB. The enzymes generated by microorganisms were absorbed by SCB. (Li *et al.*, 2017) studied the adsorption of enzymes during pretreatment of corn stover. This explained the continuous reduction in enzymatic activities in SEP-S and SP-S. Throughout the 24 h of pretreatment, lignin peroxidase, manganese peroxidase and xylanase activities in SEP-S were higher than SP-S as a result of the enzymes secreted by microorganisms in the elephant dung. Makhuvele, R. et al. revealed that about 58% of molds in herbivore dung and most molds existing in elephant dung belonged to the genus *Aspergillus* (Makhuvele *et al.*, 2017). Copious reports have documented the ability of *Aspergillus* species to produce lignocellulolytic enzymes and delignify lignocellulosic biomass (Conesa *et al.*, 2000; Janusz *et al.*, 2017; Kuhad *et al.*, 2013; Kumar and Goswami, 2006; Milstein *et al.*, 1984). Xylanase activities found in SEP-S and SP-S samples were related to bacteria found in animal gastrointestinal tracts that contaminated SCB. Lignin peroxidase and manganese peroxidase are well documented for lignin degradation by their redox potentials (Biko *et al.*, 2020). Xylanase depolymerized xylan precipitated on the surface of the fibers, followed by opening the pulp structure to enable access by bleaching chemicals, and also released chromophores with smaller residual lignin molecules as a result of glycosidic bond cleavage in the carbohydrate portion of the lignin-carbohydrate complex (Saelee *et al.*, 2016). After adsorption, the enzymes participated in hydrolysis of SCB to produce reducing sugar.

Enzymatic activities corresponded with reducing sugar and organic acid yields. When reducing sugar and organic acid yields increased after the initial stage of pretreatments, the enzymes were gradually adsorbed.

Table 10 Dynamics of enzymatic activities during pretreatment.

Enzyme	Supernatant	Pretreatment times (h)			
		0	6	12	24
Lignin peroxidase (U/mL)	SP-S	-	-	-	-
	SEP-S	1.703	1.120	0.896	0.851
Manganese peroxidase (U/mL)	SP-S	-	-	-	-
	SEP-S	0.848	0.167	-	-
Xylanase (U/mL)	SP-S	0.454	0.414	0.408	0.407
	SEP-S	1.072	1.023	0.943	0.642

2. Changes in fibers during the pretreatment and bleaching processes

2.1. Whiteness index (WI)

The main purpose of the bleaching process is to obtain white cellulose fibers. Whiteness was achieved by removing the lignin contributing to the color by destroying the coloring matter with the help of bleaching agents. The WI was used to estimate the color of fibers after the bleaching process, as presented in Fig. 8 and Fig. 9. After pretreatments, SEP-F (Fig. 9c) was whiter than SCB (Fig. 9a) and SP-F (Fig. 9b) with 63.53%, 61.89% and 61.59% WI, respectively. Furthermore, after bleaching, all fiber samples indicated a noticeable increasing trend of WI from the initial stage. However, B5-SEP-F (Fig. 9f) exhibited WI value of 84.67%, while B7-SCB-F (Fig. 9d) and B7-SP-F (Fig. 9e) showed similar whiteness index of 83.60% and 82.43%, respectively. Pretreatment with elephant dung before the bleaching process reduced the number of

required bleaching times from seven to five, resulting in a 28.57% reduction in chlorine consumption. Changes in the color of samples were due to the removal of lignin and hemicellulose during the pretreatment process (Meesupthong *et al.*, 2021). Increased fiber whiteness was achieved due to the degradability of lignin by acetic acid, lignin peroxidase, manganese peroxidase and xylanase that precipitated on the fiber surface, opening the fiber structure to chlorine dioxide (ClO_2), which then oxidized and extracted the lignin-derived chromophores by cleaving the glycosidic bonds in the carbohydrate portion of the lignin-carbohydrate complex (Biko *et al.*, 2020; Ko *et al.*, 2010; Muurinen, 2000; Rahimi *et al.*, 2014). This resulted in higher delignification efficiency in SEP-F than SCB and SP-F.

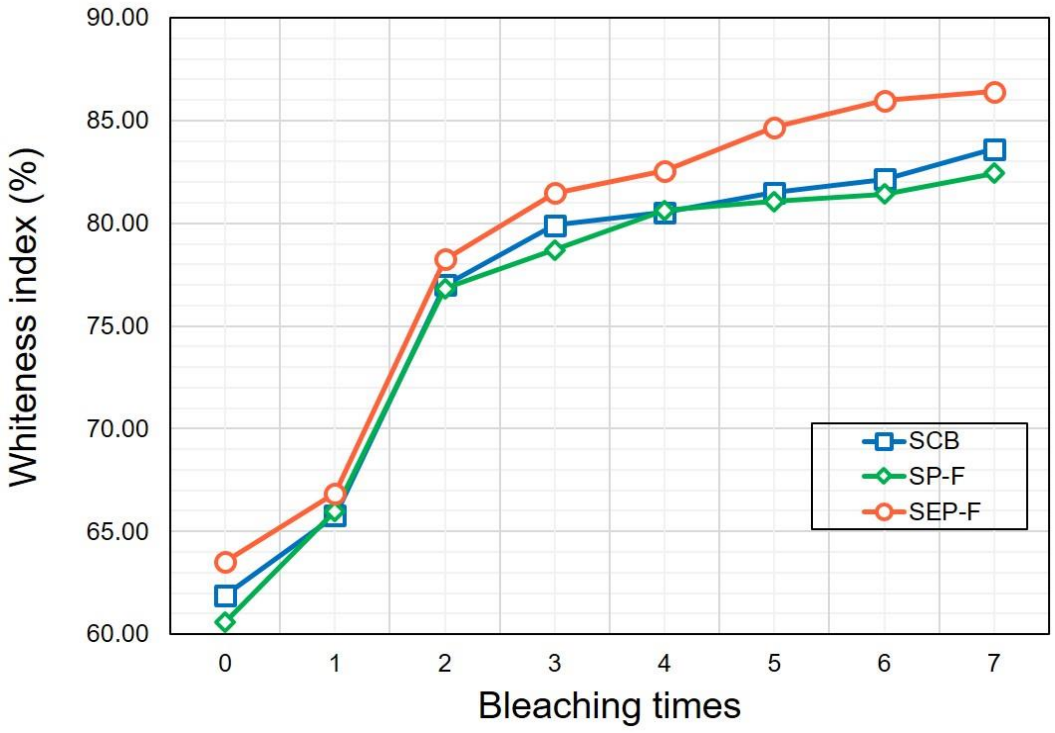

Figure 8 Relationship between whiteness index and bleaching times.

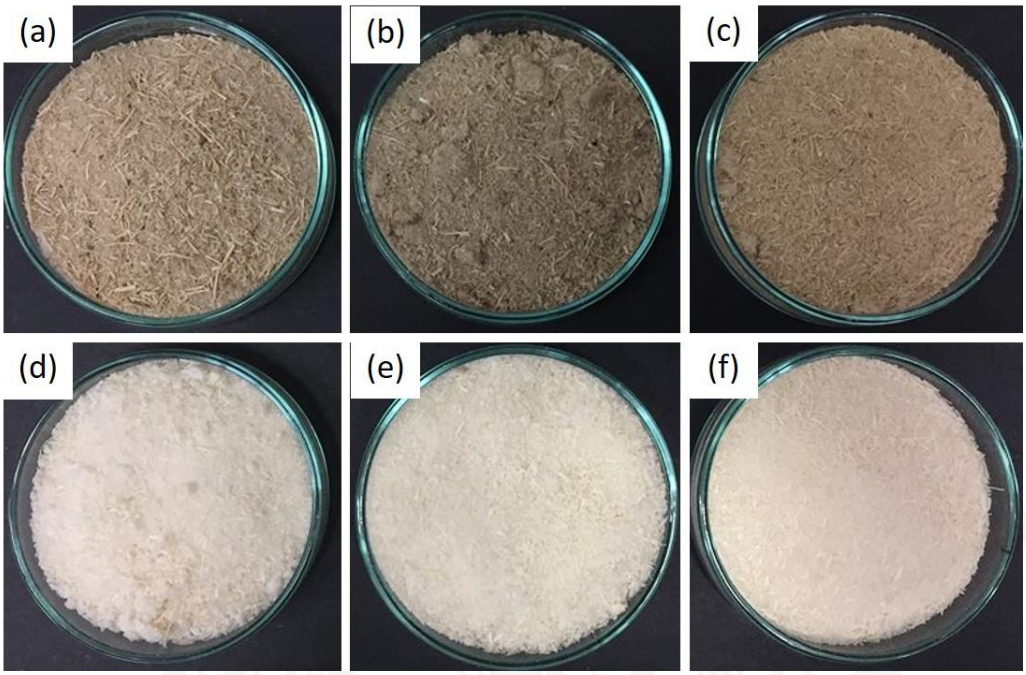

Figure 9 Photographs of (a) SCB, (b) SP-F, (c) SEP-F, (d) B7-SCB-F, (e) B7-SP-F and (f) B5-SEP-F.

2.2. Chemical composition

Changes in chemical composition of the samples after pretreatment and bleaching processes are presented in Table 11. The SCB fibers had cellulose content of 49.95%, with hemicellulose and lignin of 23.58% and 21.75% w/w, respectively. After pretreatment with elephant dung (SEP-F), the cellulose content increased to 56.83% w/w, while hemicellulose content decreased to 22.32% w/w and lignin content decreased to 18.78% w/w. This occurred because of the efficiency of substances secreted by the microorganisms in elephant dung that participated in delignification. By contrast, the chemical composition of SP-F was similar to SCB fibers because the phosphate buffered saline combined with basal medium pretreatment did not allow significant substances produced from the microorganism within the elephant dung to promote efficient hydrolysis. Furthermore, after the bleaching process, hemicellulose and lignin clearly

decreased in all bleached samples because the chemicals involved in the bleaching process broke the bonds of lignin and hemicellulose carbohydrate complexes, resulting in production of purified cellulose (Saelee *et al.*, 2016). After bleaching five times, B5-SEP-F still showed similar cellulose content with B7-SCB-F and B7-SP-F as 59.82%, 60.86% and 61.59% w/w, respectively. However, the seven times bleached fibers (B7-SEP-F) demonstrated the highest cellulose content at 64.13% w/w from participation of microorganisms after elephant dung pretreatment.

Change in yield of SEP-F was lowest compared to SCB and SP-F (90.31%, 100% and 97.93%, respectively). The yield of B7-SEP-F also showed the lowest value compared to B7-SCB-F, B7-SP-F and B5-SEP-F (39.16%, 40.37%, 41.84% and 44.65%, respectively) due to the removal of non-cellulosic fractions. These results confirmed the removal of some compounds (especially, hemicellulose and lignin) from SCB during the different processing stages.

Table 11 Chemical composition and yield of each fiber at different processing stages.

Sample	**Chemical composition** (% w/w)			**Yield** (%)
	α-Cellulose	**Hemicellulose**	**Lignin**	
SCB	49.95 ± 0.15	23.58 ± 1.46	21.75 ± 0.19	100.0
SP-F	50.60 ± 0.03	23.44 ± 0.78	21.67 ± 0.50	97.93
SEP-F	56.83 ± 0.06	22.32 ± 0.86	18.78 ± 0.95	90.31
B7-SCB-F	60.86 ± 0.40	20.31 ± 1.59	3.84 ± 1.05	40.37
B7-SP-F	61.59 ± 0.06	19.65 ± 0.58	3.82 ± 0.45	41.84
B5-SEP-F	59.82 ± 0.16	20.33 ± 0.49	5.07 ± 0.26	44.65
B7-SEP-F	64.13 ± 0.03	17.63 ± 0.17	2.19 ±0.83	39.16

* ± mean standard derivative

2.3. Functional changes of fibers

FTIR analysis was applied to determine changes in chemical composition of the fibers during pretreatment, as presented in Fig. 10. The vibration was attributed to the CO stretching mode where non-cellulosic vibration bands were located (Saelee *et al.*, 2016). All the samples presented wide bands in the wavenumber range between 3100 and 3700 cm^{-1}, representing the O-H stretching vibrations of the hydroxyl groups in cellulose molecules as well as intermolecular and intramolecular hydrogen bonds (Penjumras *et al.*, 2014). All the samples had a band around 2920 cm^{-1}, which was attributed to the stretching of the C-H aliphatic bonds of cellulose, hemicellulose and lignin (Mondragon *et al.*, 2014). The O-H bending vibration band of absorbed water was observed around 1631 cm^{-1} in all samples (Lu *et al.*, 2016). Furthermore, the band at 1427 cm^{-1} in the spectrum of all samples was associated with the bending vibration of CH_2 symmetry in cellulose (Abidi *et al.*, 2014), while the band at 897 cm^{-1} observed in all samples referred to C–H deformation and C–O–C glycosidic stretching (*β*-glycosidic bonds) for cellulose (Fadele *et al.*, 2019).

Fig. 10(a-g) shows FTIR spectra of the fibers at different processing stages. The absorption bands of SEP-F (Fig. 10c), were similar with untreated SCB (Fig. 10a) and SP-F (Fig. 10b), suggesting no significant changes in chemical composition of the raw material. Peaks at 1512 and 1606 cm^{-1} were assigned to C=C stretching of the aromatic ring of lignin (Emmanuel *et al.*, 2014; Moosavi Nejad *et al.*, 2016; Pucetaite, 2012), while the absorption peak at 1460 cm^{-1} was assigned to asymmetric bending of CH_3 in the methoxyl groups of lignin (Lionetto *et al.*, 2012; Moosavi Nejad *et al.*, 2016). The peaks at 1512 and 1606 cm^{-1} disappeared, while 1460 cm^{-1} decreased in spectra of B7-SCB-F, B7-SP-F, B5-SEP-F and B7-SEP-F due to the removal of some fractions of lignin from their structure by the bleaching process (Penjumras *et al.*, 2014). The FTIR results

supported the chemical composition analysis, indicating that most of the lignin was removed from the SCB structure after the cellulose extraction process.

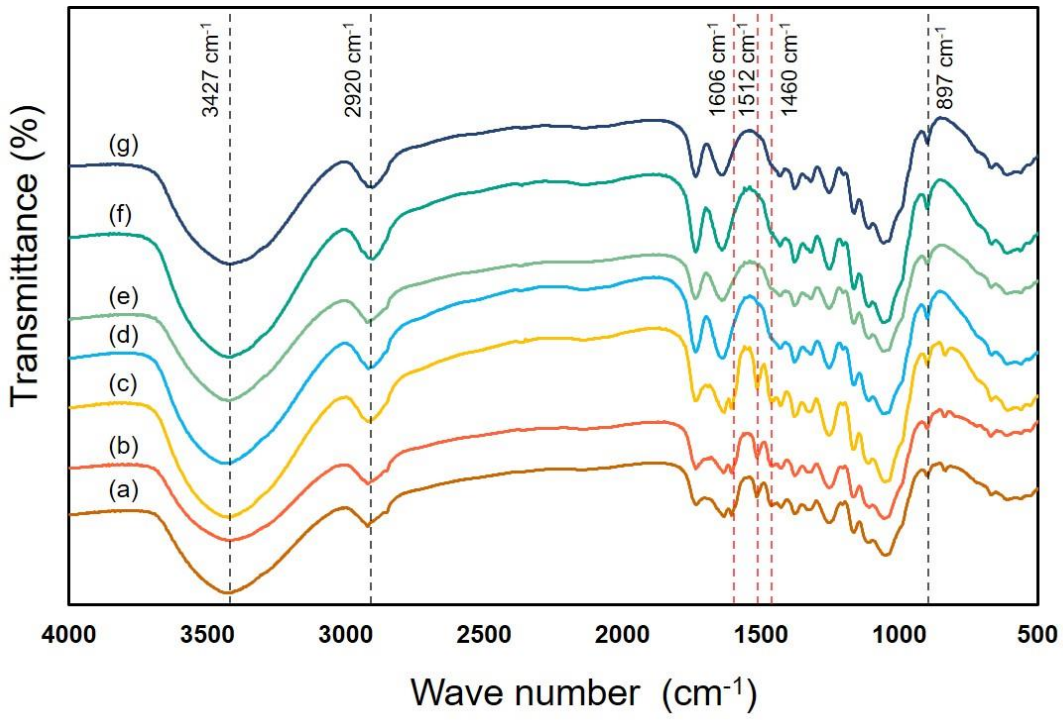

Figure 10 Fourier transform infrared spectroscopy of (a) SCB, (b) SP-F, (c) SEP-F, (d) B7-SCB-F, (e) B7-SP-F, (f) B5-SEP-F and (g) B7-SEP-F.

2.4. Crystallinity of fibers

The XRD patterns of the cellulose fibers in Fig. 11 illustrated the crystalline phase of the samples with crystallinity index (CrI) values presented in Table 12. The XRD profiles of cellulose fibers showed typical diffraction of cellulose I at 2θ = 16°, 22.5° and 34°, which were assigned to (110), (002) and (004) planes, respectively (Senapitakkul *et al.*, 2020). The calculated CrI values of SCB, SP-F and SEP-F were 51.58%, 47.17% and 56.43%, respectively. The CrI of SEP-F fibers increased compared to

SCB fibers and SP-F, due to the degradation of non-cellulosic constituents by the secreted substances of elephant microorganisms. After the bleaching process, the CrI values of B7-SCB-F, B7-SP-F, B5-SEP-F and B7-SEP-F increased to 66.41%, 56.01%, 62.13% and 66.77%, respectively. The increase in crystallinity was due to the removal of lignin and hemicellulose as the amorphous portion in the fiber structure (Meesupthong *et al.*, 2021; Vanitjinda *et al.*, 2019a). The CrI of B7-SEP-F was higher than B7-SCB-F and B7-SP-F, due to their original crystalline structure and the efficient removal of both hemicellulose and lignin compounds from fibers by pretreatment with elephant dung. The CrI values of SP-F and B7-SP-F were also significantly lower than the other fibers caused by cellulase production from *Bacillaceae* and *Paenibacillaceae* in SP-B at 0 and 24 h. These families formed the main microbial composition in SP-B and have been reported as potent cellulose degraders (Liu *et al.*, 2021).

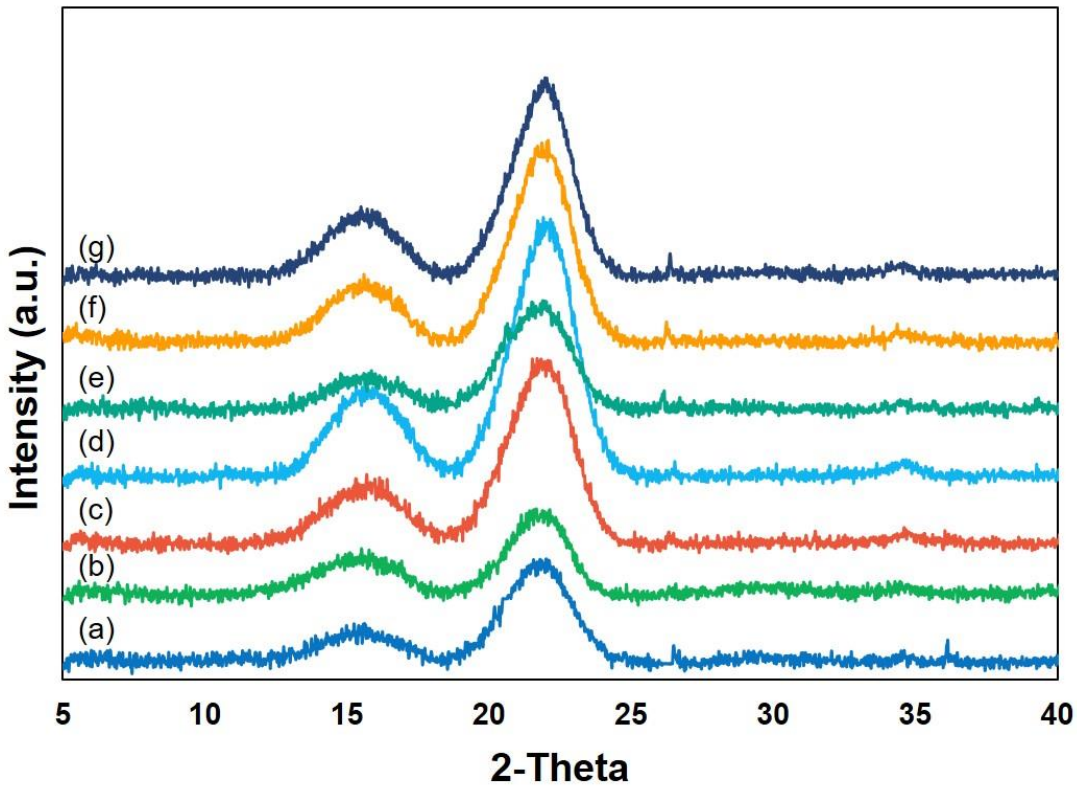

Figure 11 X-ray diffraction patterns of fibers at each processing stage (a) SCB, (b) SP-F, (c) SEP-F, (d) B7-SCB-F, (e) B7-SP-F, (f) B5-SEP-F and (g) B7-SEP-F.

Table 12 Crystallinity indices of fibers after different stages of treatment.

Sample	Crystallinity index (%)
(a) SCB	51.58
(b) SP-F	47.17
(c) SEP-F	56.43
(d) B7-SCB-F	66.41
(e) B7-SP-F	56.01
(f) B5-SEP-F	62.13
(g) B7-SEP-F	66.77

2.5. Morphological analysis

Fibers obtained from each of the processing stages were observed using SEM to investigate the effects of pretreatment by elephant dung on morphological structure (Fig. 12a-f). The SCB surface (Fig. 12a) had non-fibrous components and was slightly smooth similar to the surface of SP-F (Fig. 12b) due to the presence of extractives such as pectin, waxes and oil (Vanitjinda *et al.*, 2019a). After pretreatment with elephant dung, the surface of SEP-F became rougher, with the appearance of parallel sheaths (Fig. 12c) due to removal of lignin and some extractives, resulting in increased internal surface area and opening up the fiber structure. This provided a higher surface area for subsequent enzymatic and acidic reactions (Sun and Cheng, 2002). Surfaces of the fibers after pretreatment with substances secreted from microorganisms in the elephant dung became rough and heterogeneous, with small filaments due to the partial hydrolysis of lignin on the fiber surface that acted as a physical barrier to chemical bleaching agents.

Following delignification by the bleaching process of the fibers, B7-SCB-F, B7-SP-F and B5-SEP-F (Fig. 12d, 12e and 12f, respectively) were dispersed into individual smooth fibers with average diameter of 20-30 µm. However, B5-SEP-F showed higher individual fibers compared to B7-SCB-F and B7-SP-F. The diameter of the fibers reduced as the non-cellulosic constituents were removed.

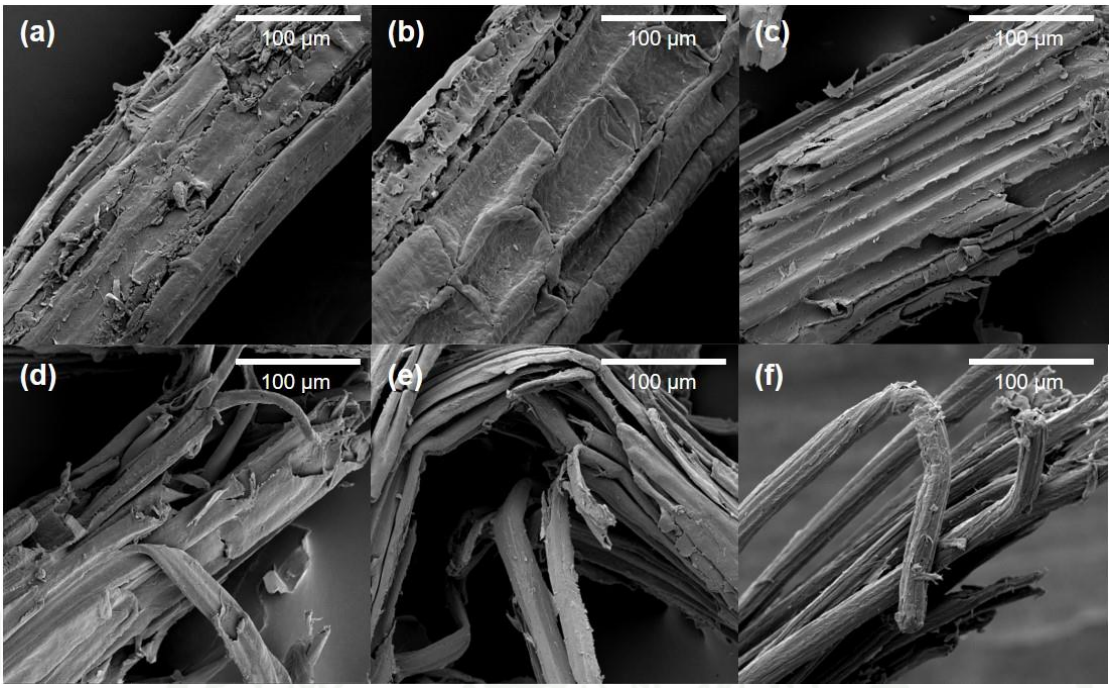

Figure 12 SEM images of fibers at different processing stages (a) SCB, (b) SP-F, (c) SEP-F, (d) B7-SCB-F, (e) B7-SP-F and (f) B5-SEP-F.

CONCLUSION

Simulation of an elephant colon using elephant dung as inoculum for extracting cellulose from sugarcane bagasse was achieved. The efficiency of the method was attributed to the microbial community of the elephant dung, which produced organic acids and enzymes. These products from the microorganisms synergistically facilitated cellulose extraction by removing non-cellulosic constituents and resulted in increased cellulose content, whiteness index, crystallinity and internal surface area. Pretreatment significantly reduced bleaching time and chemical agents. This simulated elephant colon using elephant dung pretreatment is eco-friendly and sustainable as a cost-effective alternative to chemical and physical methods for cellulose extraction.

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