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CRUDE CELLULASES FROM SUBTERRANEAN TERMITE EXTRACT (*Macrotermes gilvus* Hagen) FOR SACCHARIFICATION OF LEAF LITTER AND BIOSOFTENING PROCESS IN SKELETON LEAF CRAFTS MAKING

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ABSTRACT

Recently, leaf litter has been used as compost or piled up and burned openly in most regions of Indonesia. This litter has the potential for its lignocellulosic components as the source of sugars. Also, the leave skeleton structure is promising for creative industrial purposes. Besides, termites (*Macrotermes gilvus* Hagen) can digest lignocellulosic biomass containing high fiber. In this research, termite extract contained lignocellulases and was used in the leaf litter pretreatment process for sugar preparation and leaf litter biosoftening. This study aimed to extract termite enzymes, identify, evaluate the activity of crude enzymes, and characterize the hydrolytic ability of enzymes to degrade lignocellulosic materials in three different leaf litters, i.e., *Polyalthia longifolia* (PL), *Pterocarpus indicus* (PI), and *Filicium decipiens* (FD). The leaf litter biosoftening process was performed in pulp removal for *Annona muricata* (AM) skeleton leaf crafts making. The results showed that *M. gilvus* Hagen extract exhibited high activity of endo-β-D-1,4-glucanase (CMCase), moderate activity of avicelase, β-D-1,4-mannanase, β-D-1,4-xylanase, and low β-D-1,4-glucosidase. The optimum pH of CMCase, β-D-1,4-mannanase, and β-D-1,4-glucosidase were 6.5, 5.0, and 6.0, respectively, while the optimum temperatures were 40 °C, 50 °C, and 55 °C, respectively. Cellulases from termite extracts were suitable pretreatment of leaf litter biomass, as evidenced by saccharification activity, with the highest saccharification activity in FD leaf litter. Enzymes were also successfully employed to help remove AM leaf pulp at the volume of 100 mL for 14 days, with the removal efficiency at 98.7%.

Keywords: termite enzymes, lignocellulose, saccharification, leaf litter, biosoftening

INTRODUCTION

The need for clean energy is increasing constantly and simultaneously around the world. Along with the increase in population and industrialization, bioethanol demand is growing as an alternative to fuel oil. Commercially, bioethanol production from low-cost agricultural wastes rich in lignocellulosic materials is promising. Rice stalks, grain, leaf litter, corn husks, corn cobs, sugar cane stalks, cotton stalks, and bamboo waste are cellulose materials often exploited as feedstocks (**Ravindranath *et al*., 2011**). Every year, this cellulosic material residue increases in number. The amount is incomparable to the minimal utilization of animal feed and fertilizer. Then, it was piled up, and some people burned it openly. The leaf litter is rich in lignocellulosic materials, and this waste has the potential to be transformed into clean energy. Moreover, leaf litter could be transformed into a value-added product, such as skeleton leaf crafts, which is gaining popularity in some countries, including Indonesia. A chemical process is usually performed to produce this skeleton leaf. The leaf pulp is removed by adding sodium carbonate or washing soda. Even though Environmental Protection Agency (EPA) lists sodium bicarbonate as generally recognized as safe (GRAS), there are still some concerns and issues related to this compound, especially its toxicity to humans and animals as well as its mutagenic properties for a long time exposure (**Waple, 2017**). Therefore, a greener process than a chemical process is essential for this industry.

Lignocellulose or fibers are the main compounds of plant cell walls, consisting of celluloses, hemicelluloses, and lignins. The concentration of fibers and other components varies in each type of plant. The lignocellulosic degrading enzymes are lignocellulase enzymes produced commercially from microorganisms. Microbial cellulase enzymes have wide applications in the chemical, textile, food, and pharmaceutical industries. In the fuel and chemical industries, current applications are in producing ethanol and other commodity products derived from cellulosic biomass, whereas in the paper industry, cellulases are used in refining pulp and recycling used paper. In the textile industry, cellulase enzymes are used for surface modification of fiber and woven fabrics (**Kumar & Chandra, 2020**). However, the activity of these microbial enzymes has lower specific protein activity than amylase. For instance, the commercial cellulase produced by *Trichoderma viridae* (Sigma-Aldrich) shows activity at 6-10 units per mg of protein (product number C1794), whereas the α-amylase made from *Bacillus amyloliquefaciens* is 1000-1500 units per mg of protein (product number A0521). The low specific protein activity in commercial cellulases results in the high bulk of enzymes required in some applications, and consequently, it is expensive.

Termites (*Macrotermes gilvus* Hagen) can digest wood with a high fiber content due to the activity of enzymes produced by microbes such as flagellated protists, yeasts, and bacteria in termite nests and termite digestive tracts (**Ni & Tokuda, 2013**). Termites are responsible for causing damage to homes in Indonesia and around the world, and a termite colony may contain several hundred thousand termites. Cellulase gene sequences from termites differ from cellulase gene sequences from microbes. These findings suggest that termite cellulase is produced not only by microbes in the digestive tract but also by the termites themselves. However, the function of cellulase-producing genes has yet to be reported (**Watanabe *et al*., 1998**).

The three enzymes necessary for cellulose digestion are endo-β-D-1,4-glucanase, which randomly hydrolyzes cellulose into cellodextrin, cellobiose, and glucose; exo-β-D-1,4-glucanase, which removes cellobiose from the non-reducing end of the cellulose chain, and β-D-1,4-glucosidase, which splits cellobiose into two glucose molecules. The activity of enzymes for digesting cellulose is heavily influenced by the cellulose structure, which consists of crystalline and amorphous regions, in addition to the action of enzymes at various sites on the cellulose structure. Carboxymethyl cellulase (CMCase) and avicelase can detect amorphous and crystalline cellulase activity. Because hemicellulose contains xylan, galactan, mannan, glucomannan, galactomannan, arabinan, and glucoronoxylan, the enzymes involved in digestion have a more complex structure than cellulase. Endo-β-D-1,4-xylanase and endo-β-D-1,4-mannanase randomly break down xylan and mannan middle chains. Whereas glycosidases such as β-D-1,4-xylosidase, α-D-1,6-galactosidase, β-D-1,4-glucosidase, and β-D-1,4-mannosidase remove chain ends of xylan, galactomannan, glucomannan, and mannan (**Kumar & Chandra, 2020**).

Termite extracts containing lignocellulolytic enzymes, as it was found in the digestive tract of *Coptotermes formosanus,* there was a distribution and properties of β-D-1,4 endoglucanase (**Nakashima & Azuma, 2000**). Xylanase activity can be detected in the gut of termites, where the xylanolytic bacteria are isolated (**Ni & Tokuda, 2013**). In research with *Nasutitermes takasagoenis*, all bacterial mixtures found could degrade 28% of dealkali lignin, while one of the isolated bacteria, i.e., *Burkholderia cepacia* KK01 degraded 60-95% dimer lignin (**Kato *et al*., 1998**). The objectives of our study were to find alternative enzymes from termites for the pretreatment process of leaf litter rich in lignocellulosic compounds

to obtain sugar and for the biosoftening of leaf litter to support leaf pulp removal in the skeleton leaf crafts making.

MATERIAL AND METHODS

Leaf litter and chemicals

Leaf litter from three trees, i.e., *Polyalthia longifolia* (PL) or false Ashoka, *Pterocarpus indicus* (PI) or Angsana, and *Filicium decipiens* (FD) or Ferntree were obtained from the Universitas Negeri Semarang area. Substrates for enzyme activity assays were purchased from (Sigma-Aldrich®, USA) and (Carbohydrate Synthesis Limited, UK). In this study, the leaf litter was analyzed using proximate analysis techniques, and the leaf litter biomass extraction included hexane extraction, ethanol extraction, water extraction, cellulose, hemicellulose, and lignin **(Duke, 2018)**.

Termite species and feeding methods

The subterranean termites (*Macrotermes gilvus* Hagen) were obtained from the Laboratory of Biology Universitas Negeri Semarang Indonesia collection. Sugarcane bagasse was used as the primary source of their diet. Bagasse was mechanically pretreated by chopping with a knife and then milled to 0.5 mm particle size. The milled sugarcane bagasse was dried at 65 °C for two days. Then it was saturated with distilled water, compacted into cube-sized 1 cm^3, and dried at 65 °C for two days. Approximately 45 termites were released in a small Petri plate (35 mm) containing bagasse cubes and placed in a plastic sealable container at room temperature (30 ± 2 °C) in the dark. The relative humidity at 70% was maintained for 12 days. Some moist paper towels were placed in the container to prevent termites from desiccation **(Afzal *et al*., 2022).**

Crude enzyme extraction

Termites were washed and rinsed twice with sterilized cold distilled water. Then, it was added with McIlvaine buffer pH 6.2 with a composition of 1:5 (10 grams of termites in 50 ml of buffer) before being gently homogenized with a mortar and pestle. The extraction was carried out at 4 °C, and the mixture was then centrifuged at 10,000 ×*g* for 20 min at 4 °C. The supernatant was transferred to new sterilized 1.5 mL tubes and 0.2% sodium azide was added. Then, it was stored at -20 °C until further use.

Enzymes activity assays and characterization

Endoglucanase enzymes were tested by identifying reducing sugar as glucose from carboxymethylcellulose (CMC) and cellulose microcrystalline. Beechwood xylan and wheat arabinan were used as substrates to determine hemicellulase enzymes such as xylanase and mannanase. The sugar-reducing standards were xylose and mannose. As much as 90 µL substrate and 10 µL crude enzyme were mixed, and the reactions were conducted at room temperature (30 ± 2 °C) for 60 min, then 100 3.5 µL dinitro salicylic acid (DNS) was added to stop the reaction. Finally, the mixture was incubated at 100 °C for 15 min and cooled on ice for reducing sugar measurement at 546 nm. The quantity of enzyme needed to produce 1 mmol of glucose, xylose, and mannose per minute/mL under the assay conditions is considered one unit of the enzyme. The enzyme activity was calculated in the U/g dried weight of termites. β-glucosidase was assayed using 10 mM p-nitrophenyl β-D-1,4-glucoside as the substrate. The reaction was stopped by adding 0.6 M Na_2CO_3 at 1 mL. The absorbance was recorded at 420 nm, and 1 unit of the enzyme is defined as the amount of enzyme required to release 1 mmoL pNP for 1 min under test conditions. The specific activity of the enzyme is calculated in units per gram of soluble protein **(Afzal *et al*., 2022; Karl & Scharf, 2015)**. The effect of pH on the activity of the enzymes was determined at various pHs with 0.1 M acetate-acetic acid buffer (pH 3.0-6.0) and phosphate buffer (pH 6.0-8.0), whereas the effect of temperature was determined at various temperatures (from room temperature to 60 °C).

Application in saccharification of leaf litter

The leaf litter was chopped to 0.5 mm particle size. The feedstock was varied from 0.1 to 2% (w/v) and incubated at 40 °C. The mixing was carried out in 1.5 mL tubes containing 150 µL crude enzyme and 500 µL buffer (0.1 M HEPES pH 6.5). The reactions were performed at 45 °C, 200 rpm for different time intervals (1, 2, 4, 8, 14, 24, and 48 h) and stopped by adding 5 mM EDTA. A glucose and maltose detection kit (Thermo Scientific™, USA) was used to calculate the liberated glucose, and colorimetric assays were performed to estimate the cellulolytic and hemicellulolytic activity of the crude enzyme. All experiments were conducted in triplicates. The feedstocks without crude enzyme were used as blanks. Enzyme activity was determined in µmol of glucose produced per minute under test conditions **(Afzal *et al*., 2022; Batool *et al*., 2018)**.

Application in biosoftening of leaf litter for skeleton leaf crafts making

The leaf litter of *Annona muricata* (soursop) trees was collected from a local park in Semarang, Indonesia, and used as the material for skeleton leaf crafts. The leaves were washed twice using distilled water to remove soil and dirt. Then, 25, 50, 75, and 100 mL of crude enzymes pH 6.5 were added to clean containers. Crude enzymes in each container were diluted with water until reaching 500 mL. Then, the leaves of similar size and approximately weighed 1.5 g of each leaf were soaked evenly in the mixture. The treatment was performed by soaking for 14 days at 40 °C. The removal of leaf pulp was performed by gentle brushing. The weight of leaves before and after soaking was measured, and the % removal of leaf pulp was calculated.

Data analysis

All experiments were done in triplicate, and Duncan's multiple range test was used to confirm statistical differences ($p < 0.05$).

RESULTS AND DISCUSSION

Leaf litter characteristics

By performing a physicochemical examination, leaf biomass potentials are first assessed. This study used proximate analysis to measure the biomass % moisture content, volatile components, charcoal content, and ash content. The leaf litter from the three tree species proximate analysis results are displayed in **(Table 1)**. These findings are important for further application evaluation.

Table 1 Results of leaf litter proximate analysis

Biomass	Proximate analysis (% by weight)				Calorific value (MJ/kg)
	Ash content	Volatile component	Charcoal content	Moisture content	
Polyalthia longifolia (PL)	8.67±0.65^b	75.34 ± 4.18^a	7.66± 0.71^b	7.34± 0.69^b	19.32± 0.73^a
Pterocarpus indicus (PI)	9.46±0.60^a	75.01 ± 4.77^a	7.71± 0.40^b	7.57± 0.47^b	18.37± 0.46^b
Filicium decipiens (FD)	3.70±0.28^c	76.05 ± 6.25^a	11.23± 0.78^a	8.32± 0.70^a	18.50± 0.45^b

Actual value = mean ± SD (*n* = 3)

Numerous cellulase enzyme applications include the chemical, textile, culinary, and pharmaceutical sectors. Cellulase enzymes are currently utilized in the fuel and chemical sectors to produce ethanol and other commodity products made from cellulosic biomass, while they are also used in the paper industry to refine pulp and recycle discarded paper. Cellulase enzymes are employed in the textile industry to modify the surface of woven fabrics and fibers **(Nanda *et al*., 2013)**. Additionally, biomass with high moisture content can be converted biochemically using lignocellulosic substrates, particularly when saccharification and simultaneous brewing are involved. Meanwhile, biomass with a reduced moisture content (40–50%) is suited for dry, congested environments. A higher ash concentration is thought to prevent the enzymatic saccharification of lignocellulosic biomass **(Bin & Hongzhang, 2010)**. As a result, biomass with a low ash concentration is preferred for bioethanol synthesis. When using biomass as the starting material for fermentation to create bioethanol, some extra processes are needed to reduce the amount of ash content. These actions include washing to remove water-soluble inorganic elements and correctly pretreating to reduce ash content.

As a result, biomass with a low ash concentration is preferred for bioethanol synthesis. When using biomass as the starting material for fermentation to create bioethanol, some extra processes are needed to reduce the amount of ash content. These actions include washing to remove inorganic elements that are water soluble and correctly pretreating to reduce ash content, as presented in **(Table 1)**; therefore, the leaves could be used as biomass for raw materials for ethanol production. Similar outcomes were observed with wheat straw, barley straw, flax straw, and timothy grass, which were reported to have moisture contents between 5.0 and 7.9 percent and ash contents between 1.1 and 8.8 percent **(Naik *et al*., 2010)**. Biomass from *Areca catechu, Ziziphus rugosa,* and *Albizia lucida* showed a moisture content of 6.6, 5.2, and 5.8% and ash content of 2.9, 1.3, and 1.5%, respectively **(Sasmal *et al*., 2012)**.

The most crucial component of lignocellulosic raw materials is their gross calorific value (GCV), which quantifies the energy or quantity of heat emitted after combustion with extra air. The three different leaf litter forms were found to have biomass with a calorific value of 18–19 MJ/kg **(Table 1)**. These results showed almost the same results as previous studies on Timothy grass, wheat straw, and pine wood, with calorific values of 15.9, 15.6, and 18.1 MJ/kg **(Nanda *et al.*, 2013)**. The calorific value of wheat straw (20.3 MJ/kg), pine wood (19.6 MJ/kg) **(Naik *et al.*, 2010)**, Bonbogori biomass (21.24 MJ/kg), and Moj (20.3 MJ/ kg) **(Sasmal *et al.*, 2012)**, which turns biomass more efficient for bioenergy production.
The outcomes of extracting leaf litter biomass samples with hexane, alcohol, and water are shown in **(Table 2)**. Before processing lignocellulosic biomass for the production of ethanol, polar and non-polar molecules must be removed since they enhance the value of other chemicals. Compounds, including terpenoids, hydrocarbons, and non-polar lipids, were separated using the extraction process. Hexane and ethanol were used to extract polar substances such as chlorophyll, polar waxes, sterols, and other minor components. Water extraction was done to separate non-structural sugars from inorganic materials. The carbohydrate components cellulose and hemicellulose can be fermented to create biofuels like pentoses and hexoses. Lignin might obstruct the conversion process due to its complicated structure for degradation.

Table 2 The results of the extraction and lignocellulosic components of leaf litter biomass

Extraction component (g/100 g)	% Biomass		
	Polyalthia longifolia (PL)	*Pterocarpus indicus* (PI)	*Filicium decipiens* (FD)
Hexane	13.24±0.19[a]	10.65±1.14[b]	9.95 ± 0.66[c]
Ethanol	14.88±0.78[a]	12.77±0.91[b]	10.47 ± 0.41[c]
Water	19.42±0.80[a]	14.34±0.22[b]	14.39 ± 0.78[b]
Cellulose	54.30±0.23[a]	52.71±1.11[a]	26.06 ± 1.64[b]
Hemicellulose	57.60±0.13[a]	41.25±0.71[b]	23.81 ± 1.12[c]
Lignin	42.40±0.09[a]	37.22±0.29[b]	14.45 ± 0.78[c]

Actual value = mean ± SD (n = 3)

Enzyme activity and protein concentration

Fresh extracts of termites showed all enzyme activities (CMCase, avicelase, β-D-1,4-mananase, β-D-1,4-xylanase, and β-D-1,4-glucosidase) as shown in **(Table 3)**. The CMCase (endo-D-1,4-glucanase), which breaks down amorphous cellulose, had the highest activity. The extract was unsuitable for digesting natural fiber because natural fiber has a large amount of the enzyme (avicelase) responsible for breaking down crystalline cellulose. Microbial activity in the nest or ligninase in the digestive system are both factors that affect termites' capacity to break down wood. Similar findings were also found in *C. formosanus*, where five different forms of CMCase—EG-A, B, C, D, and E—were discovered in digestive tract extracts from the termites. While protozoa most likely produced EG-C and D in the hindgut, salivary glands provided EG-A, B, and E for isolation. EG-E was comparable to CMCase and demonstrated activity on amorphous cellulose **(Nakashima & Azuma, 2000)**. According to absorbance measurements, the concentration of soluble protein in the crude extract of the enzyme was 186.31 ± 12.41 mg/mL. At the same time, the total protein from the crude extract of the enzyme was 46,578.71 mg. The analysis results of the enzyme-specific activity are presented in **(Table 3)**.

Table 3 Results of crude enzymes activity assay at pH 6.2 and room temperature (30 ± 2 °C)

Enzymes	Enzyme activity (U/g dry weight*)	Specific activity (U/g protein)
CMCase	526.67±3.37[a]	4906.22[a]
Avicelase	0.95±0.02[e]	8.85[e]
β-D-1,4-xylanase	3.50±0.05[d]	32.61[d]
β-D-1,4-mannanase	11.15±0.12[b]	103.87[b]
β-D-1,4-glucosidase	6.98±0.34[c]	65.05[c]

*Dry weight was derived from the extracted termites to obtain crude enzyme

Nakashima and Azuma (2000) reported that CMCase activity in the salivary glands, foregut, midgut, and hindgut of termites varied from 326 to 8,240 U/mg protein. The enzymes extracted from termite bodies were still mixed with other protein components, resulting in low enzyme activity in this study. The low activity of avicelase was then explained as a result of the digestive tract microbial population not producing enough enzymes to digest crystalline cellulose. Some avicelase activities are found in many fungal and mold species that inhabit termite nests. For example, *Penicillium nalgiovense* S11 isolated from Termitidae hives had high crystalline cellulase activity **(Nurbayti, 2002)**. The activity of β-mannanase in the extract had higher activity than β-xylanase. Even so, its activity was still lower when compared to CMCase activity. The activity of β-mannanase proved the presence of mannan in termite food substrates. In addition to the protozoa that digest xylan, several bacteria produce xylanase in termites' digestive tract. In this study, CMCase activity as cellulase, β-D-1,4-mannanase as hemicellulose, and β-D-1,4-glucosidase as glycosidase were selected for further characterization, including determination of optimum temperature and pH. Avicelase and β-D-1,4-xylanase investigations were discontinued because of their low activities.

Determination of optimum temperature and optimum pH

The termite enzyme activity was tested at room temperature (30 ± 2 °C), and a pH range of 4.0–7.5, whereas the optimum temperature determination was performed by testing the enzyme activity at each optimum pH and at temperatures between 27 °C and 60 °C. The three enzymes varied in their properties, as revealed by pH and temperature measurements. CMCase, β-D-1,4-mannanase, and β-D-1,4-glucosidase had optimum activity at pH values of 6.5, 5.0, and 6.0, respectively **(Figure 1)**.

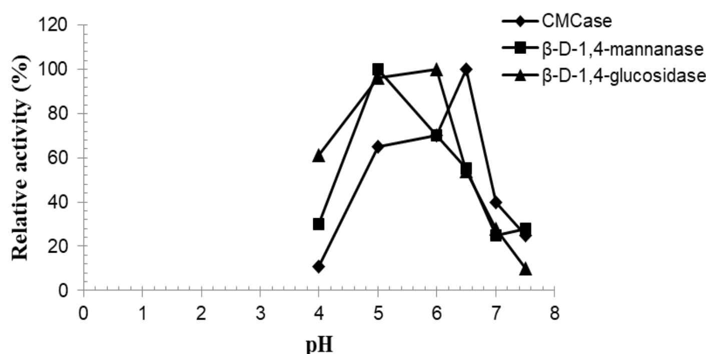

Figure 1 The optimum pH of CMCase, β-D-1,4-mannanase, and β-D-1,4-glucosidase extracted from termites. Enzyme activity was tested at room temperature (30 ± 2 °C)

Furthermore, as shown in **(Figure 2)**, each enzyme, CMCase, β-D-1,4-mannanase, and β-D-1,4-glucosidase, had an optimal temperature of 40 °C, 50 °C, and 55 °C, respectively. CMCase was the only enzyme to exhibit up to 10% reduction in activity at 60 °C. The other enzymes, such as β-D-1,4-mannanase and β-D-1,4-glucosidase, have a high retention rate at comparatively high temperatures. Even if a certain microorganism produces an enzyme or exhibits activity on the same substrate, it has a distinct optimal pH and temperature (isoenzyme). The pH in the foregut and hindgut of *Thoracotermes macrothorax* is within the same pH range as the optimal pH of the three different enzymes **(Brune, 1998)**. The optimum pH was affected by the abundance of CMCase and β-D-1,4-glucosidase in the salivary glands near the foregut. The optimum temperature is the most suitable environment for microorganisms that live in the digestive tract of termites to produce enzymes.

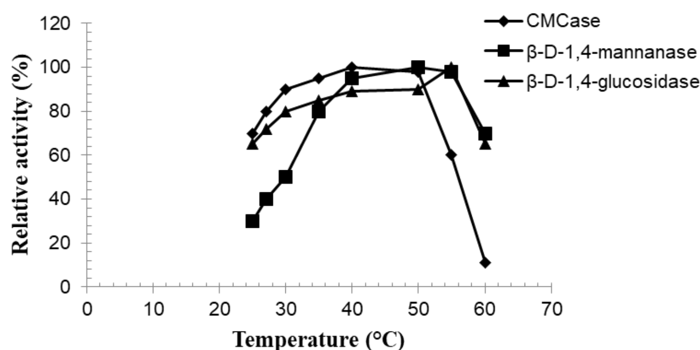

Figure 2 Optimum temperatures of CMCase, β-D-1,4 mannanase, and β-D-1,4-glucosidase extracted from termites. Enzyme activity was tested at each optimum pH

Leaf litter saccharification test

Saccharification activity was determined by testing avicelase activity using leaf litter as a substrate. The effectiveness of enzyme application as a leaf litter hydrolysis agent for ethanol production is influenced by the incubation time for hydrolytic activity. This activity is closely related to the fiber structure, amount of litter, and enzyme properties. Termite extracts can digest fiber (carbohydrates) from the three types of leaf litter into reducing sugars (**Figure 3**). The formation of reducing sugars increased throughout the incubation time. For all leaf litter substrates, the formation of reducing sugars started from 1 h and increased to 60 h. It can be concluded that enzymes from termite extracts took part in the hydrolysis process of leaf litter components. The amount of reducing sugar is affected by the type of substrate. The determination of reducing sugar production during the incubation and saccharification process is shown in (**Table 4**).

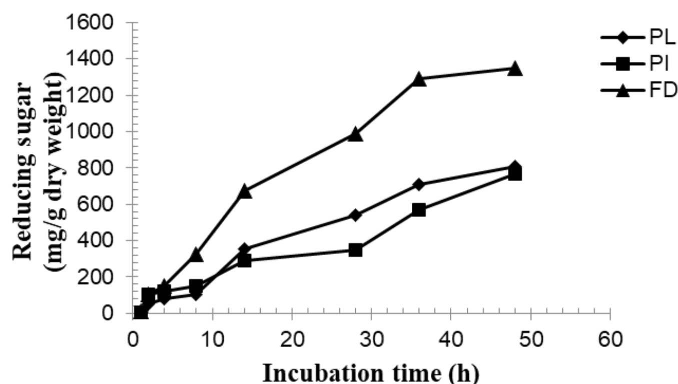

Figure 3 Reducing sugar produced from the saccharification process of leaf litter by crude extracts of termite enzymes. All reactions were carried out by adding enzymes with the same concentration. Dry weight is the dry weight of termites

The most suitable substrate for termite enzymes was the leaf litter of FD, followed by the leaf litter of PI and PL. Although the activity of avicelase was deficient, high saccharification activity was obtained in the FD leaf litter substrate. These results indicate that termite enzymes have lignocellulase components (CMCase and ligninase) compared to amylase. The saccharification activity in dissolved materials is also influenced by the repressive effect of reducing sugars on soluble carbohydrates.

Table 4 Saccharification activity of crude enzymes on leaf litter at pH 6.5 and 40 °C

Biomass	Saccharification activity (µmol/g dry weight*)
Polyalthia longifolia (PL)	1.21±0.71[c]
Pterocarpus indicus (PI)	7.29±0.11[b]
Filicium decipiens (FD)	18.40±0.29[a]

*Dry weight was derived from the extracted termites to obtain crude enzyme

Biosoftening of leaf litter for skeleton leaf crafts making

The leaf pulp removal (%) after soaking in crude enzymes is presented in (**Table 5**), whereas the appearance of skeleton leaves is shown in (**Figure 4**). The best volume for the biosoftening process was 100 mL, as the leaf pulp removal reached 98.7%. In this enzyme level, the biosoftening was effective, and the gentle brushing on leaf pulp removal was easier than at other levels. The skeleton was also cleaner and more manageable for further processing compared to the three treatments performed.

Table 5 Removal of leaf pulp (%) after soaking in crude enzymes at 25 mL, 50 mL, 75 mL, and 100 mL at pH 6.5 and 40 °C for 14 days

The volume of crude enzymes (mL)	Leaf pulp removal (%)
25	83.7%
50	89.1%
75	95.2%
100	98.7%

Generally, the leaf pulp removal process employs natural biodegradation by direct soaking in water, which takes approximately 1-2 months to get >90% of leaf pulp removal. This process is quite long and always leads to a problem, including the unpleasant smell from the leaf. Then, recently, most of the skeleton leaf crafters have been starting to use the boiling method and adding washing soda to the boiled leaf to speed up the softening process. However, even though this method is faster than the natural biodegradation process, the long exposure period to this chemical can lead to some health issues, such as cancer. Also, environmental problems are raised when these compounds reach the main water body without proper handling. The boiling method also needs more energy to generate the heat, and the demand for electricity, fuel, or gas is increased. Therefore, the biosoftening process using crude termite enzymes could be an alternative to this method as an eco-friendly approach. Three eco-friendly enzymes (hemicellulase, pectinase, and cellulase) were also used to biosoftening the banana fiber by removing pectins, waxes, lignin, and hemicellulose. It was shown that treating fiber with a mixture of enzymes produced better outcomes than treating fiber with a single enzyme. When a combination of enzymes was used to treat the fiber, the non-cellulosic contaminants were removed from the fiber more uniformly because the enzymes work synergistically (**Shroff *et al.*, 2015**). An enzymatic biosoftening was also performed in *Agave americana* L. fiber. The enzyme application increased the efficacy of softening process in textile raw material with an environmentally friendly process (**Mafaesa, 2020**).

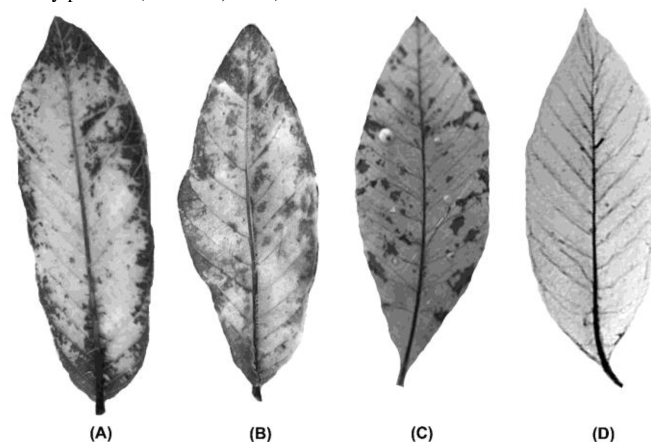

Figure 4 *Annona muricata* leaves after soaking in crude enzymes: 25 mL (A), 50 mL (B), 75 mL (C), and 100 mL (D) at pH 6.5 and 40 °C for 14 days

CONCLUSION

Termite extract contained high activity of endo-β-D-1,4-glucanase (CMCase), moderate activity of avicelase, β-D-1,4-mannanase, β-D-1,4-xylanase, and low activity of β -D-1,4-glucosidase. The cellulases from termite extracts were suitable for leaf litter pretreatment by saccharification to obtain simple sugar for further application in bioethanol production. Moreover, the enzymes showed unique characteristics and were applicable to the biosoftening process of skeleton leaf crafts making. It can be suggested that further studies regarding the optimization of leaf litter saccharification, further enzymes purification and characterization are required to obtain more complex information for advanced products and applications. The immobilization of the enzymes to anticipate a limited number of termites and massive termites killing and increasing enzymes reusability is necessary.

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