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Potential of Mao-Luang (*Antidesma thwaitesianum* Müll. Arg.) Waste for Bioethanol Production

Kaewkanlaya Sotthisawad[1,*], Jutaporn Sawaengkaew[2], Polson Mahakhan[2]

[1]*Program of Biology, Faculty of Science and Technology, Sakon Nakhon Rajabhat University, Sakon Nakhon 47000, Thailand*
[2]*Department of Microbiology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand*

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ABSTRACT

Mao-Luang (*Antidesma thwaitesianum* Müll. Arg.) is a local fruit plant indigenous to northeastern Thailand. Mao-Luang fruit is widely processed into various food products such as wine, juice, and jam. This study aims to investigate the potential for using Mao-Luang waste (MLW) in bioethanol production. Pretreatment of MLW involved dilution with acid (1% (v/v) H_2SO_4) at 121°C for a period of 90 min before cellulase (Cellic®Ctec 2) hydrolysis at 50 ºC for 24 h, these were the optimum conditions that provided maximum reducing sugar yields of 11.48±0.20 g/L. Images obtained using a scanning electron microscope (SEM) were used to confirm structural changes in the morphological features and surface characteristics of MLW after the pretreatment. Bioethanol production using the MLW that had been pretreated with acid and cellulase hydrolysis was found to produce a maximum ethanol yield of 3.91 g/L or 0.49 g ethanol/g sugar, which corresponded to 95.84% of the theoretical yield by *Saccharomyces cerevisiae* TISTR 5339. This study has revealed that MLW displays significant potential as a cheap lignocellulose source for bioethanol production and adds to the value of MLW to be utilized for renewable energy production.

Keywords: Mao-Luang (*Antidsma thwaitesianum* Müll. Arg.); Pretreatment; Lignocellulosic biomass; Bioethanol

1. Introduction

In the 21st century, the growing demand for energy for the purposes of transportation, heating, and industrial processes has led to rapid decreases in available resources of fossil fuels [1]. Therefore, sources of renewable energy and alternative energy are actively being researched and developed. Bioethanol that can be produced from any natural material containing carbohydrates is one of the most promising sources of renewable energy.

***Corresponding author:** kaewkanlaya@snru.ac.th
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Notably, it has been determined that bioethanol can be used as an alternative to petroleum- derived products [2]. Currently, the technological development for ethanol production from lignocellulosic materials, or what are identified as agricultural waste (straw, corncobs, and bagasse), woody feedstock, perennial grass, and municipal solid waste has been proposed for the purposes of second-generation bioethanol production [3]. The process of producing bioethanol is performed by the saccharification of lignocellulosic biomass, followed by microbial fermentation and product recovery [1]. In general, the saccharification or pretreatment of lignocellulosic biomass and subsequent enzymatic hydrolysis are key processes for digestion to ethanol fermentation because it reduces the long structural carbohydrates chains of cell walls into monomeric sugars that can then be fermented into biofuels [4]. Acid and alkaline pretreatments are well studied, common methods used to decompose lignocellulose, which have both been shown to be cost efficient and applicable on a large scale [5]. The main components of lignocellulosic biomass include cellulose, hemicellulose, and lignin. Cellulose is surrounded by lignin and is a β-glucan linear polymer of D-glucose that is linked by β-1,4-glycosidic bonds. Hemicellulose is a heteropolymer comprised of short, branched chain sugars consisting of D-xylose and L-arabinose, D-mannose, D-galactose, and D-glucose [6, 7]. Lignin is a large complex polymer of unrepeated phenolic monomers. It is known to contribute significantly to the process of water conduction and to the defense systems in plants [8]. Lignocellulosic biomass is relatively inexpensive as well as readily and locally available in large quantities [9]. Many agro-food residues are comprised of the substrates that are most frequently used for bioethanol production through anaerobic fermentation [2].

Mao-Luang or Mak-Mao (*Antidsma thwaitesianum* Müll. Arg.) (Fig. 1) is a tropical fruit as well as a wild plant that has been classified as a member of the Euphorbiaceae family. It grows abundantly in northeastern Thailand [10, 11] such as in Sakon Nakhon, Kalasin, Mudahan, and Nakhon Phanom. The entire plant is of medicinal value and is known to possess antioxidant, anticancer, and antidiabetic properties. The study on the effectiveness of Mao- Luang as a medicinal plant is gaining a significant amount of attention. In previous studies, phytochemical analysis of different species of Mao-Luang has confirmed the presence of varying amounts of phenolic acids, flavonoids, and anthocyanins [12-14]. Mao-Luang is popularly used as a raw material to produce many food products and beverages, such as healthy types of juice, concentrated juices, wine, and jam. These processes can produce a good deal of Mao-Luang waste (MLW) in the various industrial communities where the production processes take place. The burning of MLW has raised serious socio-environmental concerns around the world. Therefore, the use of MLW as a raw material for bioethanol production could be an advantageous way to manage this vast amount of organic waste, and add value to MLW by allowing it to become a source of renewable energy. Moreover, the present study will be the first of its kind to investigate the application of MLW as a new form of lignocellulosic biomass that can be used for bioethanol production. The lignocellulosic components of MLW have been assessed. The pretreatment of MLW using diluted acid/ alkaline solutions and enzyme hydrolysis was also investigated for the purposes of producing bioethanol.

2. Materials and Methods

2.1 Raw materials

MLW product (the remaining fruit mass after juice extraction) was collected from a local orchard in Sakon Nakhon,

Thailand and oven-dried overnight at 65ºC [12]. After that, the dried waste was mashed into small pieces and stored under low humidity conditions in a desiccator cabinet until further analysis was conducted.

2.2 Analysis of chemical composition of MLW

The lignocellulosic composition of MLW was analyzed, including cellulose, hemicellulose, lignin, ash, and moisture content using the modified method of Lima et al. [15].

2.3 Pretreatment of MLW

MLW pretreatment was performed in 100 mL Erlenmeyer flasks in which about 5 g of MLW was treated by adding 50 mL of pretreatment solution (ratio of 1:10) prior to autoclaving at 121 ºC for 90 min [16]. The pretreatment was performed using 6 different pretreatment solutions as follows:

(*I*)-Acid pretreatment with 1% (v/v) H_2SO_4 solution

(*II*)-Alkaline pretreatment with 1% (w/v) NaOH solution

(*III*)-Acid pretreatment with 1% (v/v) H_2SO_4 solution followed by hydrolysis by cellulase (Cellic®Ctec2) diluted 50-fold under a water bath at 50 ºC for 24 h

(*IV*)-Alkaline pretreatment with 1% (w/v) NaOH solution followed by hydrolysis by cellulase (Cellic®Ctec2) diluted 50-fold under a water bath at 50 ºC for 24 h

(*V*)-Alkaline-acid pretreatment with 1% (w/v) NaOH followed by 1% (v/v) H_2SO_4 solution

(*VI*)-Alkaline-acid pretreatment with 1% (w/v) NaOH followed by 1% (v/v) H_2SO_4 solution prior to hydrolysis using cellulase (Cellic®Ctec2) diluted 50-fold under a water bath at 50ºC for 24 h

The optimum pretreatment condition was determined by measure of the highest yield of reducing sugar (RS) that was released using the 3-5-Dinitrosalicylic acid (DNS) method analysis [17]. The commercial enzyme cellulase (Cellic®Ctec2, Novozyme, Denmark) was used in this study; this enzyme's activity had been previously assessed in terms of filter paper activity (FPase; 500 U/mL), carboxymethyl cellulase (CMCase; 1,085,926 U/mL), and Beta-glucosidase (23,837 U/mL).

2.4 Scanning electron microscopy

Scanning electron microscope (SEM) analysis was performed to characterize the structure of MLW after pretreatment, and the results were compared with unpretreated MLW. Before the analysis, samples were oven-dried at 60 ºC for 24 h [18]. The dried samples were then mounted on aluminum stubs and sputter-coated in a Cressington 108 auto sputter coater with a gold layer to improve the electrical conductivity of the sample surface. After that, all samples were imaged by SEM (LEO 1450 VP) at 12 kV by accelerating voltage with a scanning electron detector (SED) at a working distance (WD) of 14 mm.

2.5 Bioethanol fermentation

Saccharomyces cerevisiae TISTR 5339 was used for bioethanol fermentation of MLW [19]. Fermentation was carried out in 250 mL Erlenmeyer flasks under static conditions at 30°C for 96 h. The hydrolysates obtained from the enzymatic hydrolysis of pretreated MLW, and the liquid fraction obtained from the acid pretreatment of the MLW were used as a medium for the fermentation process; this medium was then supplemented with nutrients of yeast extract peptone dextrose (YPD) broth formula (without glucose). The YPD was comprised of 1.0 g/L of yeast extract and 2.0 g/L of peptone. The mixture was sterilized at 121 ºC for 15 min and allowed to cool under ambient conditions. Pre-cultures of the yeast grown to a stationary phase in YPD broth was used as an inoculum and added to the fermentation medium with 10% (v/v) of the yeast cell suspension yielding a cell density of 10^7

cell/mL. Each flask was sealed using a rubber stopper with an air lock. Samples were withdrawn at 0, 48, and 96 h after fermentation in order to determine the degree of RS concentration using the DNS method, and to measure ethanol production by gas chromatography (GC).

PerkinElmer Clarus 680 GC with Elite-5 capillary column (length 30 m, diameter 320 μm) was used for ethanol concentration analysis using a flame ionization detector (FID). Helium gas was used as the carrier gas with a flow rate of 2.0 mL/minute. The temperature of the FID detector and the injection port was set at 250°C. Additionally, yeast growth was measured at 0, 24, 48, 72, and 96 h of fermentation by colony plate counting (colony forming unit; CFU/mL) in YPD agar after 24 h of incubation at 30°C.

Fig. 1. Mao-Luang (*A. thwaitesianum* Müll. Arg.).

3. Results and Discussion

3.1 Chemical composition of MLW

The lignocellulosic compositional analysis of MLW is shown in Table 1. It was revealed that MLW is rich in lignin and also contains significant proportions of cellulose, as well as hemicellulose. Cellulose is the main component of lignocellulose at 32.88%. It can be hydrolyzed to produce glucose and is easily fermented into ethanol. The hemicellulose content was estimated to be 13.27%. However, lignin, which acts as a preventive barrier against microbial or enzymatic hydrolysis, was recorded as having the highest cellulosic content in this study, at 40.48%.

The compositional analysis data revealed the presence of an abundance of cellulose in MLW. The cellulose content (32.88%) of the MLW employed in the current study is within the same range as was reported in palm empty fruit bunches (38.0%) [20] and vetiver grass (32.6%) [21].

Table 1. The compositions of MLW.

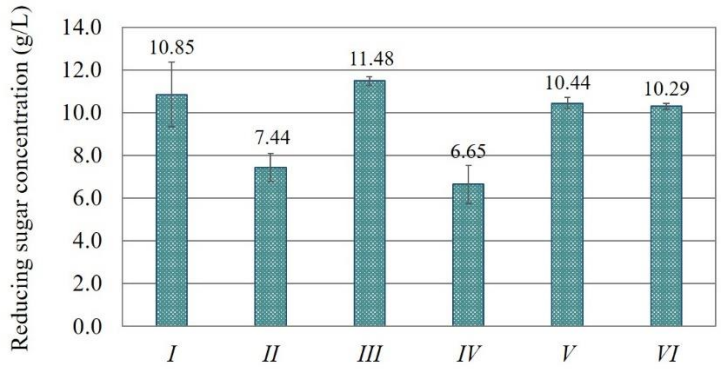
Component	Content (%w/w)
Cellulose	32.88 ± 4.99
Hemicellulose	13.27 ± 1.67
Lignin	40.48 ± 3.84
Ash	4.25 ± 0.61
Moisture	9.15 ± 0.18

However, the cellulose content was considerably higher than the amounts found in wheat bran (14.8%) [22] and in the leaves and stems of dry water hyacinth (25.64%) [23]. Meanwhile, the lignin content extracted from MLW (40.48%) was found to be quite high. This result is in concordance with the findings of previous studies that reported the potent antioxidant activities of Mao-Luang fruits, seeds, and marcs was due to their high quantities of phenolic compounds and flavonoids [11, 24]. Lignin is classified as a phenolic compound [1]. Both phenolic compounds and flavonoids are created via the lignin biosynthesis pathway in the plant [25]. However, previous studies have shown that lignin could be removed by delignification using an alkaline pretreatment process [18, 26].

3.2 Acid-alkaline pretreatment and enzyme hydrolysis

The yields of RS in the liquid fraction, using 5 g of MLW after acid/ alkaline pretreatments and enzyme hydrolysis under the 6 previously listed conditions, were analyzed and presented in Fig. 2. The findings reveal that the optimum conditions for the release of RS from MLW was pretreatment with 1% (v/v) H_2SO_4 followed

by cellulase (Cellic® Ctec2) hydrolysis (condition *III*), which produced RS yields of 11.48±0.20 g/L, followed next by pretreatment with 1% (v/v) H_2SO_4 (condition *I*) giving an RS yield of 10.85±1.51 g/L. It was clear that both the pretreatment and enzymatic hydrolysis were critical to improving the saccharification of MLW. This finding reveals another important step in the process of biofuel production that involves the need to yield the most important sugars (hexose and pentose). Similarly, Badal et al. [27] reported that the hydrolysate of wheat straw obtained from 0.5% (v/v) H_2SO_4 pretreatment at 140 ºC and enzymatic saccharification using cellulase (Celluclast), β-glucosidase (Novozyme 188), and xylanase (Viscostar 150 L) at 45 ºC at a pH of 5.0 for 72 h produced sugar yields of 313±8, 198±11, and 25±3 mg/g DNS of glucose, xylose, and arabinose, respectively. Recently, cellulase (Cellic®Ctec2) has also been used for the hydrolysis of oil palms from empty fruit bunchs by employing alkaline pretreatment for the purpose of reducing sugar production [28].



Pretreatment conditions:	
I	1% (v/v) H_2SO_4
II	1% (w/v) NaOH
III	1% (v/v) H_2SO_4 + Cellulase
IV	1% (w/v) NaOH + Cellulase
V	1% (w/v) NaOH + 1% (v/v) H_2SO_4
VI	1% (w/v) NaOH + 1% (v/v) H_2SO_4 + Cellulase

Fig. 2. Concentration of RS released after MLW pretreatments under various conditions.

It is interesting to note that the RS yield resulting from cellulase hydrolysis after MLW pretreatment with 1% (w/v) NaOH at 121 ºC for 90 min was lowest yield. This may be due to the large amount of lignin obtained from alkaline pretreatment inhibiting cellulase activity [29].

Although enzymatic hydrolysis has been referred to as a form of biological pretreatment, it has the potential to biodegrade and provide high RS yields. However, the cost of using commercial cellulase is notably expensive [30]. Therefore, the application of cellulolytic enzymes that are produced from potent fungi by solid state fermentation using MLW as a cheap substrate may require researchers to improve this process in further studies.

3.3 Scanning electron microscopy

Investigation by SEM can give a different level of detail of the biomass surface. This investigation involved a comparison of the untreated and pretreated samples with the optimum methods, which may lead to valuable insight into the use of this biomass [30]. The surface area and morphological features of the untreated MLW, and the MLW pretreated with acid prior to cellulase hydrolysis were imaged by SEM, as is depicted in Fig. 3.

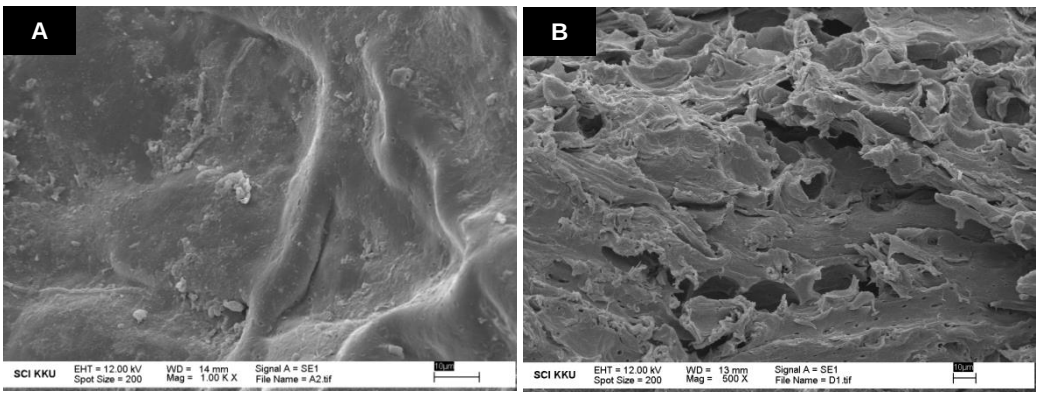


Fig. 3. Scanning electron microscopic (SEM) image of untreated MLW (A) and hydrolyzed MLW by cellulase, after pretreatment with 1% (v/v) H_2SO_4 at 121°C for 90 min (B).

For the untreated MCWM, the SEM images revealed the thick fibrous structure of cellulose and a smooth and continuous surface (Fig. 3A). Notably, after acid pretreatment of MLW followed by enzymatic hydrolysis, the surface, fibers, and internal structure of MLW were obviously deformed and broken down (Fig. 3B). The images indicate that the pretreatment process resulted in the destruction of external fibers and surface area.

The SEM images suggest that acid pretreatment and enzymatic hydrolysis could promote the breakdown of MLW structure, as well as lead to the liberation of many soluble sugars. The results are similar to the findings of previous research conducted by Sotthisawad et al. [16], which found that acid pretreatment and enzymatic hydrolysis brought about the disintegration of the structure of mushroom cultivation waste material and the release of fermentable sugars. Yücel and Aksu [31] also suggested that the effect of diluted acid hydrolysis treatment caused a disruption in the polymeric structure of sugar beet pulp, based on SEM analysis.

3.4 Bioethanol fermentation

The feasibility of bioethanol production from MLW using *S. cerevisiae* TISTR 5339 was carried out under anaerobic conditions at room temperature for 96 h, and the results are shown in Fig. 4. The growth of yeast was determined by colony plate counting on YPD agar.

It was found that yeast grew rapidly during the first 24 h and then cell growth decreased slowly. The concentrations of the RS and ethanol yields were determined and illustrated changes in the levels of the sugars and ethanol between fermentation. It was noted that sugars were consumed rapidly during the first 48 h of fermentation and then the sugars gradually decreased until the end of the fermentation process. After 96 h of fermentation, the maximum ethanol concentration was recorded at 3.91 g/L and the ethanol yield reached a level of 0.49 g ethanol/g sugar. The theoretical efficiency

yield of ethanol conversion from fermentable sugar was recorded at 95.84%.

Similar findings were obtained in a study conducted by Lima et al. [15] who investigated ethanol production by *S. cerevisiae* from acid pretreated sisal (3% (v/v) H_2SO_4) and found that the maximum amount of ethanol produced was 0. 47 g ethanol/ g sugar. This result corresponded to 92% of the theoretical yield. Meanwhile, Maeda et al. [32] examined the second generation of ethanol production from sugar cane bagasse under appropriate pretreatment conditions with 1% (v/v) H_2SO_4 at 121ºC, resulting in high ethanol concentrations at the end of the fermentation process, corresponding to a fermentation efficiency of 78%. Additionally, the efficiency of ethanol production, using sugar obtained from a variety of lignocellulosic biomasses, by *S. cerevisiae* TISTR 5339 was reported to be in the range of 30-82% of the theoretical efficiency yield as reported by [16, 33] and [34]. These results indicate that acid pretreatment followed by cellulase hydrolysis are well- suited for the improvement of RS yields obtained from MLW. Additionally, it was determined that the use of lignocellulosic biomass as a source material for bioethanol production could be highly appropriate.

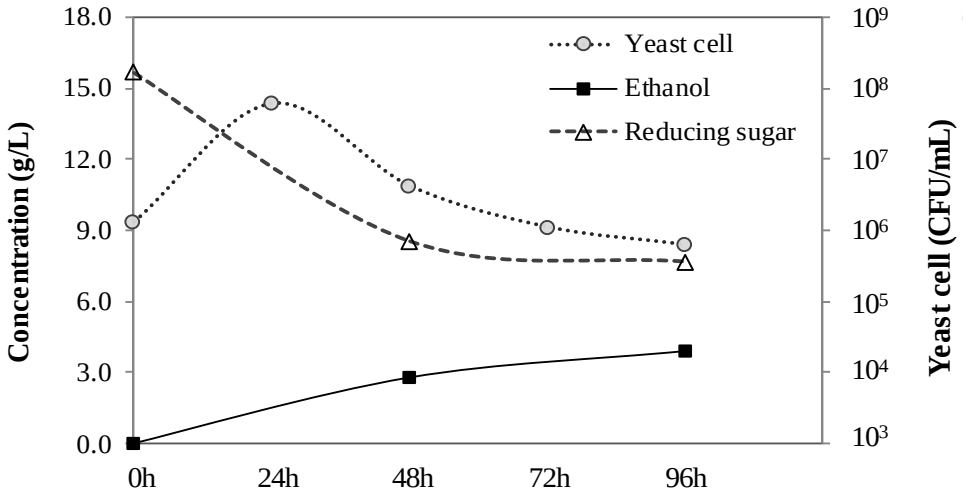


Fig. 4. Ethanol production, reducing sugar consumption and growth of *S. cerevisiae* TISTR 5339 during 96 h fermentation process.

4. Conclusion

Based on the compositional data, MLW containing cellulose at 32.88% (w/w) is considered an appropriate biomass source for bioethanol production. Acid pretreatment and enzymatic hydrolysis were found to be effective in producing a high yield of fermentable sugars. SEM analysis was carried out in order to compare the differences in morphology and surface characteristics between untreated and pretreated MLW. In this study, the maximum yield of bioethanol produced from MLW by *S. cerevisiae* TISTR 5339 was 0.49 g ethanol/g sugar, which corresponds to 95. 84% of the theoretical efficiency yield. This indicates that MLW could be considered a low cost and appropriate source of lignocellulosic biomass for bioethanol production or another biofuel i.e. biogas. However, it was found to be more beneficial to use the cellulolytic enzymes extracted from microorganisms as opposed to using the commercially produced forms of cellulase, in

order to reduce production costs. This conclusion should be further considered.

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