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Forage crops as substrate for animal feed and ethanol production in Thailand

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Five forage crops, namely ruzi (*Brachiaria ruziziensis*), purple guinea (*Panicum maximum*), atratum (*Paspalum atratum*), plicatulum (*Paspalum plicatulum*), and rhodes grass (*Chloris gayana*), were experimented for their possibility of ethanol and animal feed utilization. All tested forage crops were harvested 45 and 75 days after being planted. The results indicate the effect of harvesting time on their composition, including the contents of cellulose, lignin, and crude protein, thus affecting the ethanol yield and quality of animal feed. Ruzi grass, harvested 45 days after being planted, was shown to be the most suitable substrate for animal feed due to its highest crude protein content (12.49%), whereas purple guinea and atratum grasses provided highest expected yield of ethanol (2,688.40 and 2,613.20 L/ha/year, respectively).

Key words: Ethanol, animal feed, forage crops.

INTRODUCTION

The high price of fossil fuels in the world's markets has led to the search for other energy sources to alleviate the problem. Second-generation energy crops, such as forage crops, are considered to be part of the future of the bioenergy industry. Compared with first-generation energy crops that are based on human food stocks such as sugarcane and cassava, lignocellulosic forage crops produce the same amount of energy, reduce greenhouse gas emissions, and do not affect the human food supply. Bioenergy crops can be used in two major ways: for direct combustion or for conversion to alcohol. For direct combustion, the most important factors affecting energy levels are ash and element concentrations in crops (Lewandowski and Kicherer, 1997). For ethanol conversion, grass cell walls (cellulose and hemicellulose) are important as they are abundant renewable materials (Pauly and Keegstra, 2008).

Among many species of forage crops available, a few have been extensively examined with respect to the conversion processes required to produce energy. These include miscanthus (*Miscanthus giganteus*) (Clifton-Brown and Lewandowski, 2002), switchgrass (*Panicum*

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virgatum L.), reed canarygrass (*Phalaris arundinacea* L.), alfalfa (*Medicago sativa* L.) (Sanderson and Adler, 2008), bermuda grass (*Cynodon dactylon*) (Xu et al., 2011), forage sorghum (*Sorghum bicolour* L. Moench) (Griffith et al., 2014), and elephant grass (*Pennisetum purpureum*) (Eliana et al., 2014; Menegol et al., 2014). Miscanthus is recognized as a model herbaceous biomass feedstock in Europe. It is primarily used as a feedstock in combustion steam-generating electrical plants (Clifton-Brown and Lewandowski, 2002). Switchgrass is a perennial plant being investigated as a source for biofuels in USA since 1992. Until now the plant breeders have already increased its biomass yield by 30-40% and hope to double those yields by 2020 (Casler, 2012). Reed canarygrass is a highly productive perennial grass found in northern Europe (Lewandowski et al., 2003). Bermuda grass is a warm-season perennial grass used extensively as a ruminant feed and can be an ideal feedstock for ethanol production due to its high biomass production (Xu et al., 2011). Alfalfa is one of the oldest and highest-value forage crops in North America and is envisioned as a dual-use crop for both biomass feedstock and high-quality animal feed (Delong et al., 1995). Forage sorghum has been proposed as an annual energy crop due to its broad genetic diversity, thus providing the opportunity to develop varieties adapted to diverse climates (Griffith et al., 2014). Elephant grass is a tropical species, considered as an alternative energy crop due to its high production yield approximately 40-50 tons/ha/year (Eliana et al., 2014; Menegol et al., 2014). These crops offer additional advantages in management because they can be used for either biomass or forage. Converting the forage plantation to large-scale second-generation cellulosic bioenergy farming could push the traditional forage-livestock industry to more marginal lands.

Considering all the information, forage crops are potential biomass feedstock for bioenergy utilization. Two forage species widely studied as bioenergy feedstock in Thailand are napiergrass (*Pennisetum purpureum* Schumach) (Rengsirikul et al., 2011) and vetiver grass (*Vetiveria zizanioides* Nash) (Wongwatanapaiboon et al., 2012). Rengsirikul et al. (2011) showed that both cellulose and lignin concentrations in napiergrass increased with an increase in inter-cutting interval. High levels of neutral detergent fiber in forage led to low digestibility for livestock, however, the amount of ethanol that can be produced from plant material is positively affected by the cellulose content and negatively affected by the lignin content.

This research thus shows the possibility of using forage crops as the source for ethanol production. It is however important to identify when the harvesting would provide the optimal combination of lignin and cellulose concentrations for maximum ethanol production. Wongwatanapaiboon et al. (2012) compared 18 types of napiergrass and vetiver grass for ethanol production.

Alkaline peroxide was applied as the pretreatment step, followed by enzymatic hydrolysis and simultaneous saccharification and cofermentation for ethanol production. It was shown that the highest yield of ethanol was obtained from Sri Lanka vetiver grass (1.14 g L^{-1} or 0.14 g g^{-1} substrate).

The purpose of this research was to investigate an appropriate harvesting time for a variety of forage crops in Thailand, namely ruzi (*Brachiaria ruziziensis*), purple guinea (*Panicum maximum*), atratum (*Paspalum atratum*), plicatulum (*Paspalum plicatulum*), and rhodes grass (*Chloris gayana*), to obtain the maximum cellulose yield for the conversion into ethanol. The chemical compositions of each crop, harvested at 45 and 75 days, were analyzed and compared to show their potential as substrates for ethanol conversion. The amount of NDF was also quantitatively measured to evaluate its potential as animal feed.

MATERIALS AND METHODS

Five forage crops, namely ruzi (*B. ruziziensis*), purple guinea (*P. maximum*), atratum (*P. atratum*), plicatulum (*P. plicatulum*), and rhodes grass (*C. gayana*), were selected for this study. These crops were selected because they are well-known forage crops in Thailand and farmers are familiar with their management and already have the capacity to grow, harvest, and store them. The field experiment was conducted at the Faculty of Science and Technology, Thammasat University, Pathumthani Province, Thailand. The trial commenced during rainy season (August-October) in 2011. The design of the experiment was a randomized complete block. The treatments consisted of five cultivars (ruzi, purple guinea, atratum, plicatulum, and rhodes grass), while the blocks were harvesting times (45 and 75 days). Forty-five and seventy-five days after being planted, the five forage crops were harvested by cutting at 10 cm above the ground. They were then dried at 60°C and kept in sealed plastic bags until use. The dry samples were ground with a Wiley mill (Kinematica AG Co. Ltd.; Tokyo, Japan), then passed through a 420-μm sieve prior to composition analysis. Their chemical composition was determined according to TAPPI standards as described below.

Preparation of pretreated substrates from forage crops

The fractionation of cellulose, hemicellulose, and lignin was conducted under high temperature and pressure. First, 20 g of dry forage crop in 200 mL of 3% (w/v) sulfuric acid solution was steamed at 121°C and 15 pounds/inch^2 for 60 min. After that, the pulp and hydrolyzate were separated with a sheet cloth. The remaining hemicellulose and lignin were removed from the cellulose pulp by washing with hot distilled water (80°C) until pH 7 was achieved. The pulp was then dried at 60°C and kept in sealed plastic bags until use (Punsuvon et al., 2008).

Ethanol fermentation

The ethanol fermenting yeast, *Saccharomyces cerevisiae* TISTR 5339, was purchased from the yeast collection of Thailand Institute of Scientific and Technological Research (TISTR; Pathum Thani Province). It was initiated in a YPD slant (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} dextrose, and 15 g L^{-1} agar) at room temperature (25 to 30°C) for 24 h. To prepare the inoculum, yeast

Table 1. Composition of forage crops after harvesting at 45 and 75 days.

Forage crops	% Content (on dry basis)			
	Neutral detergent fiber (NDF)	Crude protein	Cellulose	Total lignin
Cultivar				
Atratum	72.81^{d}	9.22^{a}	44.31^{ab}	18.23^{a}
Purple guinea	76.44^{b}	7.04^{b}	46.16^{a}	15.88^{b}
Ruzi	68.97^{e}	9.02^{a}	41.24^{cd}	17.19^{ab}
Rhodes	78.33^{a}	7.41^{b}	40.19^{d}	15.98^{b}
Plicatulum	74.56^{c}	6.97^{b}	43.41^{bc}	18.21^{a}
SE	0.33	0.32	0.77	0.51
Harvesting time				
45 days	73.17^{b}	10.17^{a}	40.82^{b}	16.59^{b}
75 days	75.23^{a}	5.69^{b}	45.31^{a}	17.60^{a}
SE	0.21	0.20	0.49	0.32

Values within each column not followed by the same letter are significantly different at $P < 0.05$; SE = standard error.

was subcultured into 100 mL of fresh media (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, and 50 g L^{-1} dextrose) in a 250-mL flask for 48 h at room temperature with 200 rpm shaking. The yeast cells were harvested by centrifugation at × 8000 *g* for 15 min and then washed with sterile water before being used for ethanol fermentation.

Each pretreated pulp from forage crops was simultaneously hydrolyzed and fermented using the simultaneous saccharification and fermentation (SSF) process in a total volume of 100 mL. First, 5 g of pulp was sterilized in a 125 mL flask. Then, 100 mL of 0.1 M acetate buffer with a pH of 5.5 containing 0.5 g L^{-1} $(NH_4)_2HPO_4$, 0.025 g L^{-1} $MgSO_4.7H_2O$, 13.8 g L^{-1} $NaH_2PO_4.H_2O$, and 1 g L^{-1} yeast extract was added. Then, 15 Filter Paper Unit (FPU) of Celluclast® 1.5L (Sigma-Aldrich, USA), 15 Unit of Novozyme® 188 (Novozymes A/S, Denmark) *per* g of pulp, and 10 mL of inoculum (0.05 g (dry matter) mL^{-1} suspension of *S. cerevisiae* TISTR5339) were added. The SSF process was performed at room temperature for 168 h with 100 rpm shaking. The samples were collected at regular time intervals for the analysis of ethanol yield.

$$\% \text{ Ethanol yield} = \frac{[EtOH]_f - [EtOH]_0}{0.51(f\,[\text{Biomass}]\,1.111)} \times 100$$

Where $[EtOH]_f$ is ethanol concentration at the end of fermentation (g/L); $[EtOH]_0$ is ethanol concentration at the beginning of fermentation (g/L); [Biomass] is dry biomass concentration at the beginning of the fermentation (g/L); *f* is cellulose fraction of dry biomass (g/g); 0.51 is conversion factor from glucose to ethanol; 1.111 is conversion factor from cellulose to equivalent glucose

Analysis

The contents of cellulose and lignin were determined using TAPPI methods [TAPPI T203 om-88 (1992) and T222 om-88 (1988), respectively]. Crude protein contents was determined according to AOAC (2000). Analysis of neutral detergent fiber (NDF) was adapted from Goering and Van Soest (1970). A general linear statistical model was applied and the difference in mean data was tested for significance at $P < 0.05$. The amount of ethanol was measured by gas chromatography (GC; Chromosorb-103, GC4000, GL Sciences, Japan). The GC was performed with an HP5 capillary column (30 m x 0.32 mm x 0.25 μm, J&W Scientific, USA) and a FID detector under the following conditions: splitless ratio 50:1; split flow 25.1 mL min^{-1}; air flow 400 mL min^{-1}; H_2 flow 40 mL min^{-1}; 40°C (hold 5 min) ramp to 250°C at 15°C min^{-1} (hold 15 min); injection volume 1 μL). Reducing sugar was quantitatively measured using the Somogyi-Nelson method (Somogyi, 1926).

RESULTS AND DISCUSSION

The compositions of the forage crops harvested after 45 and 75 days are presented in Table 1. Neutral detergent fiber (NDF) and crude protein are the most common measures used for animal feed analysis. The NDF measures most of the structural components in plant cells (that is, lignin, hemicellulose, and cellulose). The level of NDF in the animal ration influences the intake of dry matter and the time of rumination, although the concentration of NDF in feeds is negatively correlated with energy concentration (Van Soest et al., 1991). Protein is the basic structure used to make all tissue. It is important not only for growth and milk production, but also is needed daily because the body is constantly repairing itself and replacing lost cells and tissue. Protein is made up of amino acids, which are used by animals to build and replace tissue. From Table 1, slightly higher NDF was observed in all forage crops harvested 75 days after being planted, implying there is no significant effect of harvesting time on the NDF amount of the studied forage crops. These values, however, are far higher than those reported in forage crops planted in the USA or Europe (~40 to 50% NDF). High NDF content indicates the unsuitability of crops as animal feed, as it reduces digestibility and intake potential (Linn and Martin, 1999). The level of crude protein in all forage crops was shown to be affected by harvesting time. The results in Table 1 indicate the significantly ($p < 0.05$) lower level of crude protein in all tested crops harvested 75 days after being

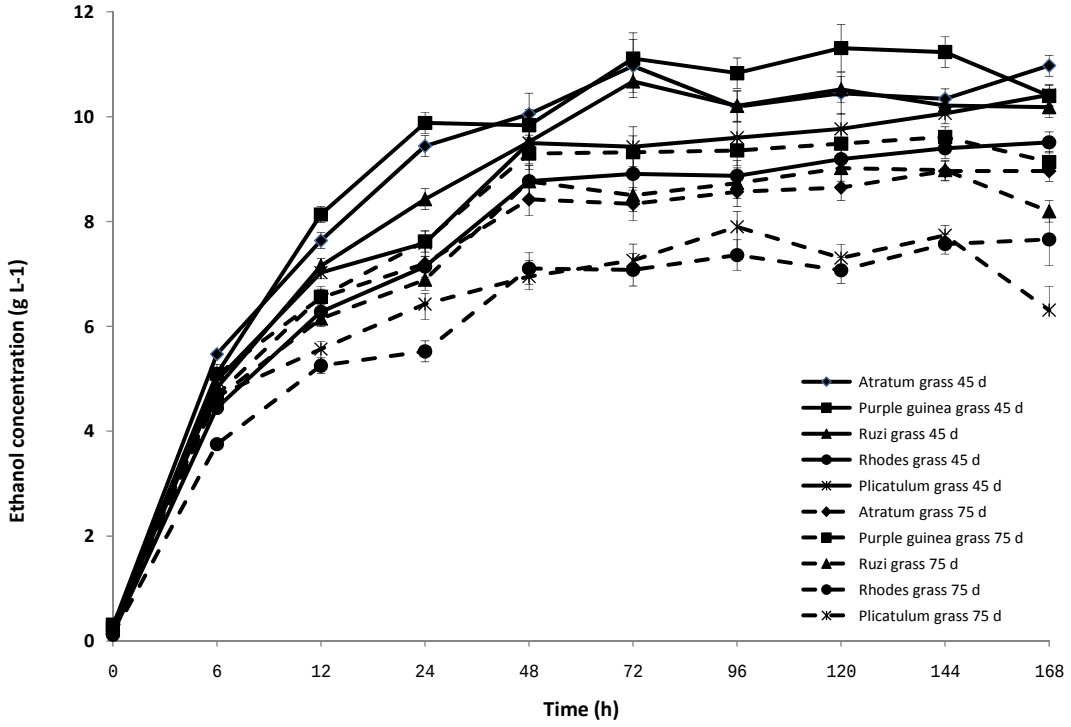

Figure 1. Ethanol yield from simultaneous saccharification and fermentation (SSF) of pretreated forage crops (after 45 and 75 days of growth) with *S. cerevisiae* TISTR 5339 at room temperature for 168 h.

planted. Rengsirikul et al. (2011) also reported that N concentration declined from 2.0 to 1.2% as inter-cutting interval increased from 1 to 12 months. These results thus indicate that ruzi grass harvested 45 days after being planted is the most suitable crop to be used as animal feed due to its lowest NDF and highest crude protein contents.

The contents of cellulose and lignin are the most common indicators of the suitability of a substrate for ethanol production. Cellulose is one of the primary components of lignocellulose. It consists of long chains of glucose molecules that are similar to starch molecules, but with a different structural configuration. In theory, approximately 51% of the cellulose in lignocellulosic biomass can be converted into ethanol (Badger, 2002). As shown in Table 1, the highest cellulose content (49.11%) was obtained from purple guinea grass harvested 75 days after being planted; purple guinea grass thus has the potential to produce the highest amount of ethanol. The lignin content in the raw material is also a factor in ethanol conversion because lignin has been reported to encapsulate cellulose and hemi-cellulose, thus making cellulosic material more difficult to hydrolyze than starchy material (Linn and Martin, 1999). The results show higher lignin content in forage crops harvested 75 days after being planted, implying that it is more difficult to reach and hydrolyze cellulose in these crops.

To confirm the ethanol yield obtained from selected forage crops, ethanol conversion was carried out through the simultaneous saccharification and fermentation (SSF) process. Figure 1 shows ethanol production, as expressed by produced ethanol concentration, obtained through the SSF process from pretreated forage crops (harvested 45 and 75 days after being planted) by *Saccharomyces cerevisiae* TISTR 5339 at room temperature after 168 h. Maximum ethanol production was observed after 72 h of incubation in all tested crops. Figure 2 shows the effect of harvesting time on ethanol yield from SSF of pretreated forage crops. The crops harvested after 75 days contained higher cellulose content, however, resulted in lower ethanol yield. The highest ethanol yield (97.98% of the theoretical yield) was produced from ruzi grass harvested 45 days after being planted, not, as expected, from purple guinea grass harvested 75 days after being planted. This may be explained by the fact that even though purple guinea grass harvested 75 days after being planted contained highest cellulose content, it also contained high content of lignin, which can hinder the enzymatic cellulose hydrolysis and ethanol production process, resulting in low ethanol yield (Martin et al., 2007). This explanation is also shown in Figure 2, in which forage crops harvested 75 days after being planted provided lower ethanol yield, even though they contained more cellulose. This study unfortunately did not analyze the composition of pretreated

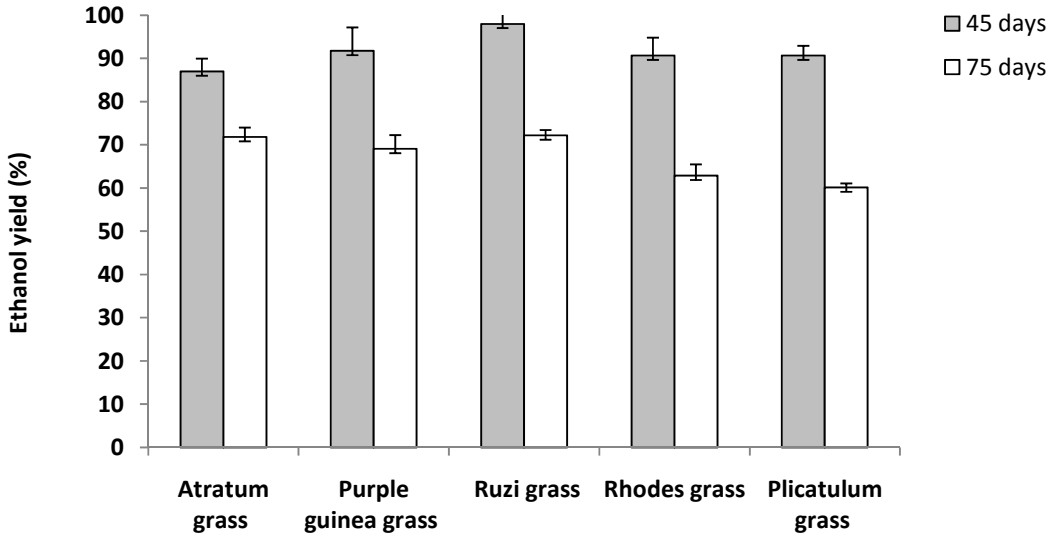

Figure 2. Effect of harvesting time on ethanol yield from SSF of pretreated forage crops (after 45 and 75 days of growth) with *S. cerevisiae* TISTR 5339 at room temperature for 168 h.

Table 2. Comparison of expected ethanol yield from SSF process of tested crops.

Tested crops	Dry biomass yield (ton/ha/year)	Max ethanol production by SSF		Expected ethanol yield (L/ha/year)
		g L^{-1}	g g substrate^{-1}	
Atratum	18.8*	10.97 ± 0.51	0.11 ± 0.04	2,613.20
Purple guinea	18.8*	11.31 ± 0.45	0.11 ± 0.04	2,688.40
Ruzi	14.1*	10.67 ± 0.31	0.11 ± 0.03	1,906.81
Rhodes	10.9**	9.51 ± 0.20	0.10 ± 0.01	1,313.80
Plicatulum	13.8***	10.41 ± 0.20	0.10 ± 0.01	1,820.76

Tested crops harvesting at 45 days (10 g) were pretreated. Saccharification and fermentation process was performed in 100 mL total volume for 72 h. *According to Wongwatanapaiboon et al. (2012), **Adapted from Mulualem et al. (2012), ***According to Buncharoen and Intisaeng (2010).

materials, thus the actual negative effect of lignin residual on enzymatic cellulose hydrolysis was not confirmed. However, it was reported that around 50% of lignin still remained on the pretreated pulp even though the fractionation pretreatment was carried out to remove part of lignin and hemicellulose from raw material such as sugar cane bagasse (Punsuvon et al., 2008). As a result, to take advantage of the high cellulose content of forage crops harvested after 75 days, a pretreatment process prior to ethanol fermentation should be further investigated. Alternatively, crops harvested 45 days after being planted might be suitable for ethanol production.

In order to determine the potential of suitable grass for cultivation as energy crop, the dry biomass yield of each tested crop was also included in the calculation as shown in Table 2. The maximum ethanol production was obtained from the SSF process of pretreated tested crops experimented in this study. Obviously, purple guinea and atratum grasses provided highest expected yield of ethanol (2,688.40 and 2,613.20 L/ha/year, respectively) compared with other tested crops, thus, they might have been appropriate to be applied as the energy crops for ethanol production in Thailand. Considering all the data, there is a high possibility that forage crops can be an alternative second-generation biomass source in Thailand. Principal management factors influencing crop productivity and quality should be further studied, especially those that are dedicated to the production of crop feedstock for ethanol.

Conclusions

Ethanol production from forage crops planted in Thailand was shown to be promising. Even though the highest ethanol yield (97.98% of the theoretical ethanol yield) was obtained from ruzi grass harvested 45 days after being planted, purple guinea and atratum grasses provided

provided highest expected ethanol yield when total dry biomass yield were included in the calculation. Ruzi grass, harvested at 45 days, was the most suitable substrate for animal feed due to its highest crude protein content (12.49%).

Overall, obtaining ethanol from forage crops holds great potential due to their abundant availability, short time rotation, and low cost. However, optimization of the process at each stage should be further studied to develop the most feasible conversion technology.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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