Thesis for the Degree of Master of Science

Valorization of Animal Manure: A Case Study of Bioethanol Production from Horse Manure

by

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Department of Microbiology

The Graduate School

Pukyong National University

August 2020

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Advisor: Prof. Young Jae Jeon

by

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Contents

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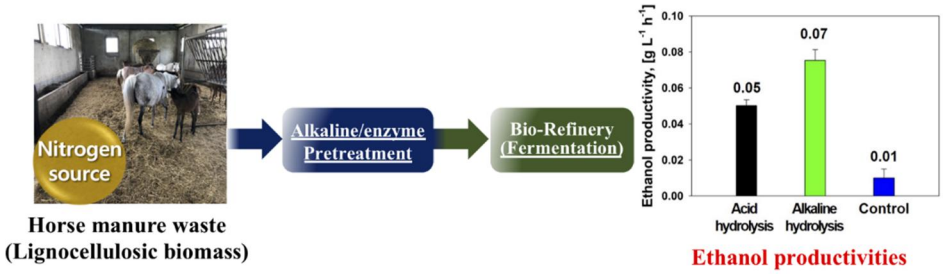
GRAPHICAL ABSTRACT



Abstract

Insecure supply chain of biomass has been regarded as one of the critical constraints for deteriorating the practical implementations of bioethanol (BE). To find a raw feedstock beyond lignocellulosic biomass, horse manure (HM) was converted into BE. To realize the grand premise, two pretreatment methods, acid-/alkaline-pretreatments using H_2SO_4/NaOH, were used for HM. To optimize acid-/alkaline-pretreatments, the surface methodology response with the Box-Behnken design was done. Under the optimized conditions, alkaline-pretreatment showed higher maximum sugar recovery yield (80%) than that from acid- pretreatment (71%), which offers that alkaline-pretreatment is suitable for BE synthesis from HM. The fermentability of acid/enzyme- and alkaline/enzyme-hydrolysates without a supplement of

nitrogen source were tested using GRAS strain of yeast, *Pichia stipitis*. The results indicated that alkaline/enzyme- hydrolysates showed higher BE productivities (0.075 g L^{-1} h^{-1}) than those of acid/enzyme-hydrolysates (0.050 g L^{-1} h^{-1}). To elucidate the possible reasons of such higher BE productivities from the alkaline/enzyme- hydrolysates, the potential fermentative inhibitory compounds for *Pichia stipitis* such as acetic acid, furfural, and hydroxymethylfurfural were quantitatively analyzed. The results indicated that the presence of less amounts of toxic compounds from the alkaline/enzyme- hydrolysates may lead to such higher BE productivities as compared those present in acid/enzyme-hydrolysates. Also, all experimental results suggested a potential for saving production cost of BE using HM as the C/N sources without an additional nitrogen source supplement.

Keywords: *Waste-to-energy; biomass valorization; livestock manure; saccharification; fermentation; bioethanol*

1. Introduction

Global energy consumption reached 14 billion tons of oil equivalent in 2018 [1, 2], and more than 80% of it were obtained from fossil fuels [3]. Heavy reliance on fossil fuels becomes a driving force to accelerate global warming [4, 5]. CO_2 emissions from fossil fuels to fulfill global energy demand are surpassing the Earth's full capacity to sequester carbons [6]. Note that CO_2 emissions from the anthropogenic activities (the use of fossil fuels) is equivalent to 32.5 billion tons in 2017, of which amount is 1.4 % more than that in 2016 (IEA, 2017). To mitigate catastrophic environmental consequences arising from global warming, a great deal of researches on carbon-free [7-9] and carbon-neutral energies [10, 11] has been conducted as a precautious CO_2 mitigation measure [12].

As compared to carbon-free energies, the practical implementations of biomass-derived carbon-neutral energies (*i.e.,* biofuels) has been readily realized because of their high compatibilities with the current infrastructures in the energy sector [13]. Indeed, biofuels share the distribution networks with the transportation fuels (gasoline and diesel) by simple blending [14]to be used in current internal combustion (IC) engines [10]. Their practical uses have been further expanded by the legislative enactments, renewable fuel

standard (RFS) [15]. DuPont (USA) announced establishment of the world largest cellulosic ethanol production process [16], which necessitated the process development for cost-effective and sustainable bioethanol (BE) production using lignocellulosic biomasses rather than crops [17, 18]. Nonetheless, insecure supply chain of lignocellulosic biomass from regional/seasonal uncertainties has been regarded as a critical constraint for retarding the commercialization of cellulosic-based BE production [19-21]. As such, it is desired to discover a new-class feedstock beyond a typical plant biomass.

Utilization of livestock manure as a raw material for BE may offer a breakthrough to circumvent insecure supply chain. Considering that livestock manure was composted or anaerobically digested (AD) [22-25], adopting livestock manure for BE production likely offers an alternative sustainable measure for valorizing a waste material. Also, the utilization of livestock manure to be used as a raw feedstock for BE offers a practical measure to abate the environmental burdens (such as greenhouse gas emissions, pathogen releases, and eutrophication) [26, 27] arising from the conventional manure treatments (such as composting and anaerobic digestion). Generally, the use of HM as a soil fertilizer using composting/vermicomposting is a beneficial

because livestock manure-derived amendments can enhance the soil quality, However, metallic contents and P/N sources could exceed the requirement for crop growth and lead to water contamination due to surface runoff. Also, it is available to produce large amount of biomethane, meaning the emissions of uncontrolled greenhouse gas into the atmosphere [28]. AD process leads to biogas (CH_4 and CO_2) production under the controlled process through hydrolysis, acidogenesis, acetogenesis, and methanogenesis of HM [29], while the maximum yield of biogas could be obtained after a few months of AD process due to the slow reaction kinetics of methanogenesis [30]. However, the intermediates of AD process such as fermentable sugars obtained from hydrolysis and acetogenesis can be used as useful feedstocks for BE production with a faster reaction kinetics and reduction of CO_2 production. The combustion of HM results in mass decrease of HM waste volume, but it also produces a huge amount of CO_2 without valorization of HM. Thus, BE production could be one of promising environmentally benign options to valorize and dispose of HM.

Also, BE conversion from manure may provide several advantageous in terms of less energy consumption to depolymerize the crystallinity of holocellulose. Given that manure contains the pre-depolymerized

holocellulose materials via acid-hydrolysis reaction provided by herbivores' digestion system for BE conversion [31], a considerable energy used in a pretreatment step could be saved. Moreover, the content of N-source in manure is beneficial to ethanologens during the fermentation process. N-source is indeed essential for the growth for ethanologens. As such, cost benefits as alternative N-source for BE production by using horse manure (HM) are referred to as 7.7-18.2 USD million per annum [32, 33]. Diverse BE processes from lignocellulosic biomass using separate enzyme hydrolysis and fermentation, simultaneous saccharification with fermentation, and consolidated bioprocessing have been developed [34, 35]. In parallel, several earlier works also reported BE production and other chemical productions, such as lactic acid, fumaric acid, and chitin [36-38]. Nonetheless, further extensive feasibility studies for BE production from the animal wastes still have not been carried out sufficiently.

Based on these reasons, this work highlighted the conversion of livestock manure into BE. For an in-depth study, converting HM into BE was made as a case study. Quantitative analysis with respect to the compositional matrix of HM and original horse feed was determined first. As a fundamental study for BE production from HM, we attempted to examine the feasibility of HM

as a potential raw material for BE production using separate enzyme hydrolysis and fermentation process. To evaluate the worth of HM as a raw feedstock for BE production, typical acid-/alkaline-pretreatments were performed to lower the degree of HM crystallinity followed by enzymatic saccharification, and their efficiencies were determined by response surface methodology. Lastly, saccharified products were fermented with/without a N-source supplement, and the total BE yield and productivity were evaluated under various fermentation conditions.

2. Materials and Methods

2.1. Feedstock

The horse manure (HM) was provided from mews located in National Institute of Animal Science (Jeju Island, Korea) during the winter season 2019. The collected sample was dried at 104 ˚C and then pulverized mechanically with a Hammer-Cutter Mill (Culatii AG, Switzerland). Powdered samples were further shift through 4 mm sieve repeatedly, and the prepared samples were stored at 20 ˚C in a sealed container before their use. Prior to analyses of sugar recovery and fermentability for BE conversion, the composition analysis and ultimate analysis of HM was quantitatively done [36, 39]. The overall methodology used in this study to produce BE from HM is summarized in Figure 1.

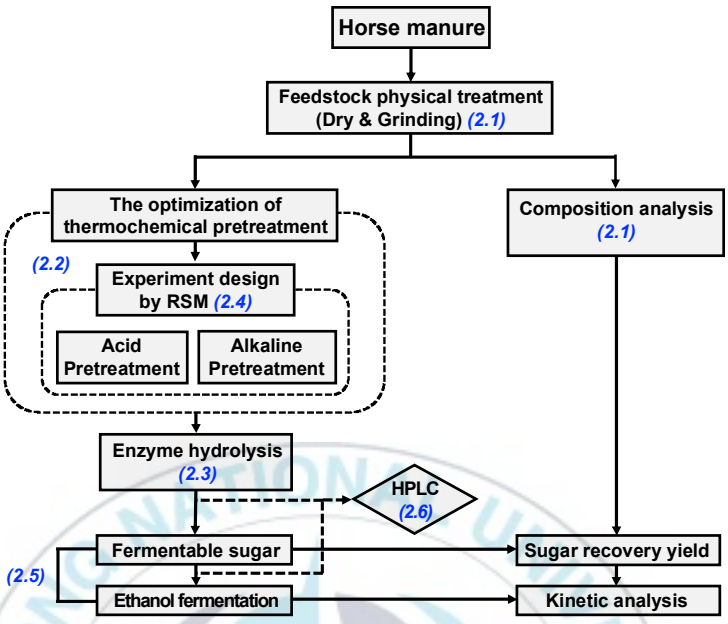


Figure 1. The overall methodologies used in this study for ethanol production from HM (blue font in brackets indicate corresponding experimental sections)

2.2. Acid-/alkaline-pretreatments for the effects of reaction conditions on the sugar recovery yields

Hydrothermal pretreatments using acid (H_2SO_4, Daejung, Korea) [40] and alkaline (NaOH, Daejung, Korea) catalysts [41] were evaluated using a single factor experimental analysis. These were carried out under three different concentrations of acid/alkaline catalysts (1, 2, and 3%), three different temperatures (100, 125, and 140 ˚C), five different reaction times (from 1 to 3 h), and three different substrate concentrations of raw materials (100, 150, and 200 g L^{-1}) to optimize the operational parameters for pretreatments. To this end, the predetermined concentrations of feedstocks and catalysts were loaded in 500 mL glass bottles, and then placed into an autoclave (SR Lab, Korea). Following the pretreatments, enzymatic hydrolysis was carried out to assess the recovery yield of sugary compounds from cellulose and hemicellulose parts in the HM sample. Overall experiments were carried out at least in duplicate.

2.3. Enzymatic hydrolysis and fermentation of HM

Prior to saccharification of the pretreated HM sample, acid-/alkaline-pretreated hydrolysates under the reported conditions was adjusted to pH 5.0 by the addition of 5M H_2SO_4 or $Ca(OH)_2$ (#239232, Sigma-Aldrich, USA) solution into the sample. To evaluate sugar recovery yields, enzymatic hydrolysis was conducted at 50 ˚C for 48 h and 200 rpm by adding 2 vol.% CTec2 cellulase (Novozymes, Bagsværd, Denmark) to acid-hydrolysates, or 2 vol.% CTec2 cellulase and HTec2 hemicellulase (Novozymes, Bagsværd, Denmark) to alkaline-hydrolysates. According to the manufacture's product information, the enzyme activities of cellulase mixture (CTec2) and hemicellulose (HTec2) contain 113 Filter Paper Unit (FPU) mL^{-1} and 1090 Fungal Xylanase Unit (FXU) mL^{-1} respectively. To acquire the liquid fractions in acid/enzyme- or alkaline/enzyme-hydrolysates, unsaccharified fibers were separated from the solution by centrifugation at 7000 *g* for 20 min. Only separated liquid fractions were used to analyze their sugar recovery and fermentability tests for ethanol production. The sugar recovery yield was calculated as following equation [36]:

$$\text{Sugar recovery yield (\%)} = \frac{\text{Glucose and xylose concentrations} \times 0.9 \times 100\ (\%)}{\text{Substrate concentrations} \times (\text{cellulose} + \text{hemicellulose}) \text{ percentage}}$$

2.4. Response surface method design for pretreatment

For the experimental design, a 3-level-3-factor response surface methodology (RSM) using Box-Behnken design based on the central point replicate was used using Design-Expert 12 software (Stat-Ease, Inc., USA) to optimize the pretreatment processes. The three experimental variables were acid/alkaline catalyst concentration (1, 2, and 3%), reaction time (from 1 to 3 h), temperature (100, 125, and 140 ˚C). The model equation was predicted by multiple regressions. The model accuracy was evaluated by the determination coefficients (R^2) and analysis of variance (AVOVA) [36].

2.5. Ethanol fermentation of acid or alkaline/enzyme-hydrolysates

For the test of fermentability, *Pichia stipitis* KCTC7222 was used as a

yeast for ethanol production. The yeast strain was cultured at 30 ˚C on YPD agar plates containing 5 g L^{-1} yeast extract (Duchefa Biochemie, Netherlands), 5 g L^{-1} peptone (Daejung, Korea), 20 g L^{-1} xylose (Junsei, Japan), 1 g L^{-1} $MgSO_4$ (Shinyo Chemical, Japan), 1 g L^{-1} KH_2PO_4 (Sigma, USA), and 16 g L^{-1} agar (Duchefa Biochemie, Netherlands). For the preparation of inoculum, a single colony of the yeast cells was transferred into a 250 mL Erlenmeyer flask containing 100 mL of the seed culture medium. The seed culture medium was consisted of yeast extract (5 g L^{-1}), peptone (5 g L^{-1}), glucose (5 g L^{-1}), xylose (20 g L^{-1}), $MgSO_4$ (1 g L^{-1}) and KH_2PO_4 (1 g L^{-1}). Then, the seed cultures were cultured at 30 ˚C until it reached to the optical density (3.0, OD_{600nm}). An aliquot of 10 mL inoculum was transferred 250 mL of Erlenmeyer flasks containing 90 mL of acid/enzyme- or alkaline/enzyme-hydrolysates with/without supplements of peptone (5 g L^{-1}) and yeast extract (5 g L^{-1}) to evaluate their fermentability for BE production. Fermentation tests of acid/enzyme- or alkaline/enzyme-hydrolysates were performed at least duplication using a shaking incubator (Vision, Korea) at 30 ˚C, 90 rpm. The fermentation samples were routinely taken out to analyze their fermentability by the measurement of the glucose, xylose and ethanol concentrations from acid/enzyme- or alkaline/enzyme-hydrolysates.

2.6. Analysis of biochemicals

The concentrations of glucose, xylose, furfural, hydroxymethylfurfural (HMF), acetate, and ethanol were quantified using HPLC (Thermo-Fisher Scientific, USA) equipped with HPX-87H (300×7.8) column (Bio-Rad, USA) and a refractive index (RI) detector. The operational conditions for the HPLC were set at 50 ˚C for column oven temperature. 5 mM H_2SO_4 was used as a mobile phase and flow rate was set at 0.6 mL min^{-1}. The yeast cell growth was turbidometrically monitored at 600 nm (1 cm light path) using a spectrophotometer (Biochrom Libra S22, UK). To the calculate the kinetic parameters, the ethanol yields and productivities were calculated as previously reported [40, 41].

2.7. Nitrogen contents quantification

The nitrogen quantity in pretreated hydrolysates and after fermentation were identified with standard Kjeldahl method [42, 43] employing a Vapodest 50s analyzer (Gerhardt, Germany). For that, 10.0 ± 0.1 g of each sample containing nitrogen components was loaded with 15 mL of sulfuric acid (≥ 98.0 %) and thermally digested using block heating system from 20 to 405 ˚C

at a constant heating rate (15 ˚C min^{-1}). Total nitrogen content was measured by titration with 0.1 N (HCl) after distillation.

2.8. Statistical analysis

The results of sugar recovery yields from acid/enzyme- or alkaline/enzyme-hydrolysates were expressed as mean and standard deviation from three replicates. Statistical significance of the samples from the fermentability test experiments using the nitrogen and non-nitrogen supplemented groups was analyzed by multiple *t*-test. The *t*-ratios values greater than 1.96 (in absolute value) suggesting the coefficient is statistically significantly different from 0 at 95% confidence level were used to determine statistical significance. The analysis of variation (ANOVA) was used to analyze the statistical significance of the group difference between the acid/enzyme-hydrolysate group and the alkaline/enzyme-hydrolysate group using the values of volumetric ethanol productivity, ethanol yield and final ethanol production ($p<0.005$).

3. Results and Discussion

3.1. Characterization of HM and haylage

The constitutional matrices of haylage (horse feed) and horse manure (HM) were summarized in Table 1. HM used in this work was composed of cellulose (42.4 wt.%), hemicellulose (9.2 wt.%), and lignin (7.2 wt.%). Table 1 indicates that 14.9 wt. % of carbon in HM were reduced as compared to that of haylage after horse digestion. The content of lignin in haylage was decreased to 7.2 wt.% after digestion.

Table 1. Chemical and elemental compositions of haylage and HM used in this study

Raw Materials	Chemical components (%)				
	Cellulose	Hemicellulose	Lignin	Ash	Moisture
Haylage	37.2 ± 0.3	28.1 ± 0.6	8.4 ± 0.2	8.7 ± 0.1	0.4 ± 0.1
Horse manure	42.4 ± 0.6	9.2 ± 0.3	7.2 ± 0.3	15.7 ± 0.3	0.7 ± 0.1
Raw Materials	Elemental compositions (wt.%) (not including ash)				
	C	H	N	S	O
Haylage	42.2 ± 0.2	5.9 ± 0.03	1.8 ± 0.03	1.0 ± 0.02	49.2 ± 0.02
Horse manure	43.0 ± 0.2	5.7 ± 0.02	1.0 ± 0.02	0.8 ± 0.01	49.5 ± 0.02

3.2. Effects of reaction parameters on the recovery yield of sugary compounds

To seek suitable pretreatments for HM, hydrothermal pretreatments using dilute acid (H_2SO_4) and dilute alkaline (NaOH) methods were performed. The effect of catalyst loading was evaluated first at 120 ˚C for 1.5 h with 100 g L^{-1} substrate concentration. Following pretreatments, the acid-/alkaline-pretreated hydrolysates were further hydrolyzed using 2 vol.% CTec2 cellulase and/or 2 vol.% HTec2 hemicellulase respectively for 48 h at 50 ˚C, 200 rpm and pH 5.0 to measure the sugar recovery yields.

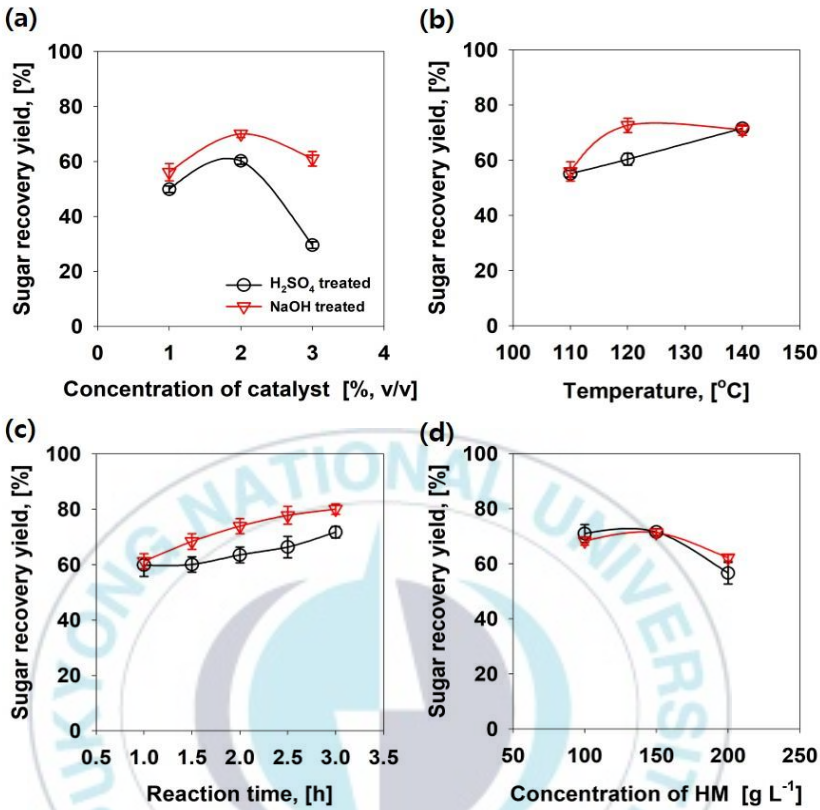


Figure 2. Comparison of sugar recovery yields from both dilute acid (H_2SO_4) and alkaline (NaOH) pretreated hydrolysates of HM following the enzyme hydrolysis. The pretreatments performed under various (a) catalyst concentrations; (b) temperatures; (c) reaction times; and (d) substrate concentrations.

Under the predefined condition at three different catalyst loadings (1, 2, and 3%), the sugar yields are plotted in Figure 2(a). Based on the results, the effect of different alkaline catalyst concentrations showed relatively higher influence in sugar yields (56-70 %) as compared to those from the respective acid catalyst concentrations (30-60%). In particular, 2% H_2SO_4 treated

hydrolysates showed the highest sugar recovery yield at 60%, while 2% of alkaline-pretreated HM showed the highest at 70%. However, the further increase in the acid/alkaline catalyst loading to 3% resulted in a negative effect on the sugar recovery yields. This indicates that 2% of the acid/alkaline catalyst concentration is sufficient for both the pretreatments.

The effect of temperature was also tested with the range from 110 to 140 ˚C under the fixed conditions at 2% both catalysts for 1.5 h with 100 g L^{-1} feedstock concentration. When the reaction temperature increased from 110 to 120 ˚C, the sugar recovery yields in particular from the alkaline/enzyme hydrolysates showed a 17% increase from 56 to 73 %, wherease the acid/enzyme hydrolysates showed only small increase in sugar yields from 55% to 60 % (Figure 2(b)). With the further increased temperature at 140ºC, in particular, the acid pretreatment provided the highest sugar recovery yield at 72%, whereas the alkaline pretreatment performed under the same temperature showed began to decrease in the sugar recovery yield from 73 to 71%. This result indicates that 120 ˚C was sufficient temperature for the alkaline pretreatments, but the acid pretreatments required higher temperature at 140ºC.

The effect of the reaction time on the sugar yields was also investigated under 5 different reaction time ranging from 1 to 3 h under the fixed conditions at 2% of both catalyst loadings, 120 ˚C, and 10% substrate concentrations followed by the enzyme hydrolysis (Figure 2(c)). Within the range of 1 to 2 h reaction time, the acid pretreated/enzyme hydrolysates showed only small increase in the sugar recovery yields from 60 to 63%. Under the same reaction time, however the sugar yield from alkaline-pretreated hydrolysates were substantially increased from 61 to 74%. In particular, as the further reaction time increased up to 3h, only minor increase in sugar yields from both acid/alkaline pretreatments was noticeable. The alkaline enzyme hydrolysates showed the yields increase from 74 to 80%. Also, a small sugar yield increase (from 63 to 72%) from the acid pretreatment was observed under the same conditions. This result suggests that the reaction time at the range of 1 to 2 h were sufficient for both pretreatments.

Finally, the effect of substrate concentration was also investigated on the range from 100 to 200 g L^{-1} under the fixed condition at 2% sulfuric acid, 120 ˚C and 2 h reaction time. The results are shown in Figure 2 (d). As the concentration of HM increased from 10 to 15%, the sugar recovery yields

from the acid pretreatment were maintained at 71% whereas the alkaline-pretreatment showed only a small increase from 68 to 71%. In particular, the further increase of substrate concentrations to 20% of HM showed that the sugar yields from both acid/alkaline pretreatments were rather decreased to 57% for acid and 62% for alkaline. Moreover, high concentrations of substrate (>150 g L^{-1}) found to lead to a lack of solid-liquid mixing issue because of the hygroscopicity of dried HM. Therefore, the concentrations of substrates were not considered as a single factor for the process optimization using these feedstocks. Similar trends were also reported by Yang et al [36].

In sum, all experimental results in Figure 2 offered that the major fermentable sugars recovered from HM were glucose and xylose. Similar results were previously reported using daily manure as a feedstock materials for ethanol production [36]. After several single factor analysis experiments, we concluded that four parameters including temperature, reaction time, catalyst concentrations and substrate concentrations affected to the recovery yields of sugary compounds from HM. Nonetheless, high concentrations (>150 g L^{-1}) of substrate led to a deficient solid-liquid mixing issue. Therefore, the high concentrations of the substrate HM, were not used as only a single factor during the optimization of the process.

3.3. Scanning electron microscopy (SEM)

Before and after acid-/alkaline-pretreatment at HM each of them was photographed by SEM to observe the change in the shape of the surface area. (Figure 3), it was confirmed that the surface area was wider when the alkaline-pretreatment was performed than the acid treatment.

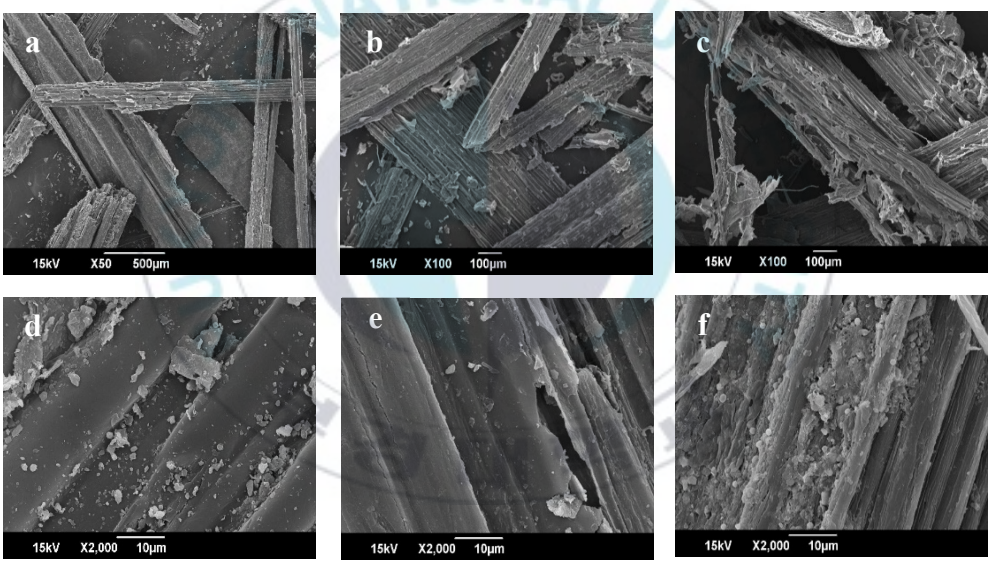


Figure 3. Horse manure(HM) before pretreatment and after acid-/alkaline-pretreatment: SEM images under different magnification (a) 50X HM before pretreatment ; (b) 50X HM after acid-pretreatment; (c) 50X HM after alkaline-pretreatment; (d) 2000X HM before pretreatment; (e) 2000X HM after acid-pretreatment and (f) 2000X HM after alkaline-pretreatment

3.4. Optimization using Response surface analysis (RSM)

Based on single factor experimental analysis, a central composite design (CCD) was carried out using 3-factors-3-levels to optimize the effects of parameters on sugar recovery yields. Table 2 shows the design and actual values of sugar recovery yields obtained from combinations used for the response surface methodology. The prediction models for the recovery yields of sugary compounds from both acid/enzyme- and alkaline/enzyme-hydrolysates were fitted to the second order polynomial equations, having R^2 values between 0.876 and 0.883 (Table 3). Both predicted and experimental results have a good agreement, thereby suggesting that the quadratic prediction models could provide a reasonable prediction of the sugar recovery yields from HM. Table 4 shows the results from ANOVA for the quadratic models of sugar yields from each pretreatment and enzyme hydrolysis. In all instances, the *F*-values present the significance of the model equations. The value of "Prob > F" less than 0.05 indicates that the model terms are also significant. In the sugar yield model for the acid pretreatment, Temperature (˚C), Catalysis concentration (%) and Time (h) are represented as X_1, X_2 and X_3, respectively. In such sugar recovery model, one linear coefficients (X_1) and two quadratic coefficients (X_1^2, X_2^2) were significant model terms.

Reflecting the individual factor, the sequence of effects on the recovery yields of sugary compounds for the acid pretreatment were $X_1 > X_2 > X_3$. The effect of interaction sequencing was $X_2X_3 > X_1X_2 > X_1X_3$. However, in the alkaline-pretreatment, two linear coefficients (X_2, X_3) and one quadratic coefficients (X_2^2) were significant model terms. The sequence of the single factor effects on the sugar yields for the alkaline-pretreatment were $X_2 > X_3 > X_1$. However, the effect of interaction sequencing had different order: $X_1X_2 > X_2X_3 > X_1X_3$. Other study [39] established a sugar recovery yield model through alkaline-pretreatment (2% NaOH) using cow manure as a feedstock. They found that reaction time was the most influencing factor followed by temperature and concentration of catalyst as $X_3 > X_1 > X_2$. However, this study found that the catalyst concentration was the most influencing factor for sugar recovery from HM followed by time, and temperature. These results are likely derived from different feed ingredient used for the herbivore diet.

Table 2. 3-level-3-factor response surface analysis in Box-Behnken design

X_1, Temperature (℃)	X_2, Catalyst conc. (%)	X_3 Time (h)	Sugar recovery yields (%)	
			H_2SO_4/enzyme hydrolysis	NaOH/enzyme hydrolysis
110	3	2	46.40	64.30
125	2	2	67.80	72.90
110	2	1	53.40	59.60
140	2	1	61.70	62.30
110	1	2	40.00	51.60
140	2	3	70.60	64.80
125	3	3	60.51	77.30
125	2	2	66.50	72.80
125	2	2	62.50	75.01
125	3	1	65.60	59.80
110	2	3	63.70	71.00
125	1	3	68.80	74.00
125	1	1	58.80	35.80
125	2	2	67.80	77.30
140	1	2	45.10	39.60
140	3	2	66.30	76.80

Table 3. Final model equations for sugar recovery yield responses resulting from experimental design

Response (%)	Final equation in terms of actual factors	Model	R^2
H_2SO_4/enzyme hydrolysis	Sugar yield = -573.48167 + 9.72658 X_1 + 11.07542 X_2 - 6.33000 X_3 + 0.248167 X_1X_2 - 0.025000 X_1X_3 - 3.765000 X_2X_3 - 0.039350 X_1^2 - 7.82375 X_2^2 + 5.07875 X_3^2	Quadratic	0.876
NaOH/enzyme hydrolysis	Sugar yield = -441.51361 + 7.06703 X_1 + 7.31583 X_2 + 50.28708 X_3 + 0.407833X_1X_2 - 0.146667 X_1X_3 – 5.18250 X_2X_3 - 0.039350 X_1^2 - 9.57125 X_2^2 – 3.22375 X_3^2	Quadratic	0.883

X_1: temperature (˚C), X_2: catalyst concentration (%), and X_3: time (h)

Table 4. Analysis of variance (ANOVA) for sugar recovery yield models

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	Prob > F[a,b]
H_2SO_4/enzyme hydrolysis					
Model	1135.83	9	126.20	4.69	0.0368
Residual	161.43	6	26.91		
Lack of fit	142.86	3	47.62	7.69	0.0639
Pure error	18.58	3	6.19		
Cor total	1297.26	15			
NaOH/enzyme hydrolysis					
Model	2222.46	9	246.94	5.01	0.0316
Residual	295.79	6	49.30		
Lack of fit	282.53	3	94.18	21.30	0.0159
Pure error	13.26	3	4.42		
Cor total	2518.25	15			

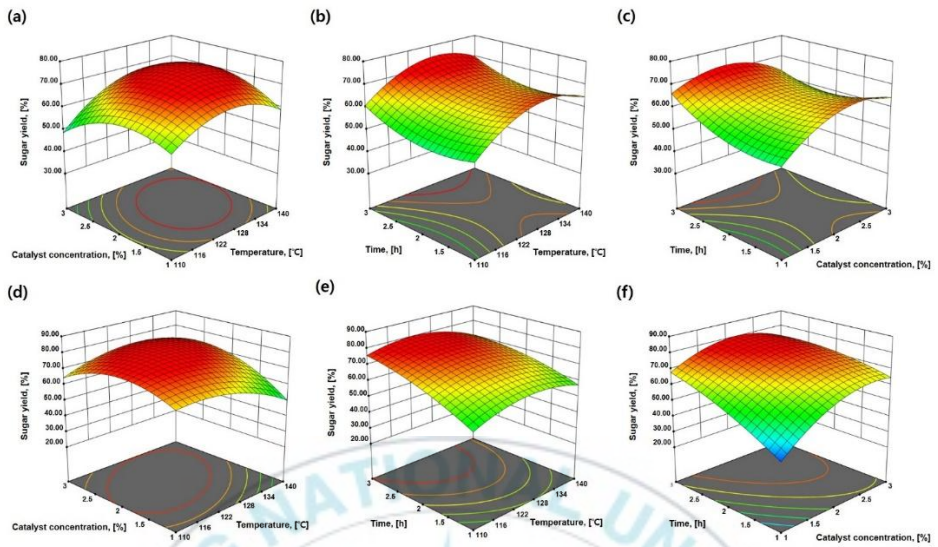


Figure 4. 3D surface plot analysis for the combined effects of different treatment conditions on the yields of sugary products. (a-c) dilute acid-pretreatment, (d-f) dilute alkaline-pretreatment; (a) temperature and H_2SO_4 concentration, (b) temperature and time, (c) H_2SO_4 concentration and time; (d) temperature and NaOH concentration, (e) temperature and time, (f) H_2SO_4 concentration and time.

The interaction effects on the reducing sugar yield are shown in Figure 4. Based on the CCD results, the optimal pretreatment conditions were determined: 3 h of reaction time at 120 °C with 2% concentration of catalyst when the substrate concentration was 100 g L^{-1}. When the optimal condition was used, the dilute acid pretreatments were predicted to be 72%, whereas the dilute alkaline-pretreatments showed 80% of sugar recovery yields from HM. Verification tests showed similar recovery yields with 71% for acid pretreatment and 80% for alkaline-pretreatment.

3.5. Fermentability test

The yeast strain, *Pichia stipitis* capable of co-metabolizing glucose and xylose as carbon sources, was used to fermentability test of acid/enzyme-pretreatment and alkaline/enzyme-pretreatment hydrolysates with/without a supplement of N-sources including 5 g L^{-1} of yeast extract and peptone. The control fermentation experiments using synthetic medium containing 25 g L^{-1} glucose and 25 g L^{-1} xylose with the same N-source supplemented group (CAN) and the same synthetic medium without the supplement of the same N-source group (CNN) were simultaneously performed under the same conditions. The fermentation profiles are plotted in Figure 5, and analysis of the fermentation kinetics are summarized in Table 5. As the sugar consumption and ethanol production profiles of the acid/enzyme-hydrolysate with non-additional nitrogen source group (AHNN) were compared with those from the group of acid/enzyme-hydrolysate with the additional supplement of nitrogen source group (AHAN) by *t*-test analysis using the Holm-Sidak methods, AHNN and AHAN are significantly not different (*t*-ratio< 1.96). As similar manner to AHNN and AHAN, the sugar consumption and ethanol production profile of the alkaline/enzyme-hydrolysate with non-additional nitrogen source group (AKNN) were also compared with those

from the group of alkaline/enzyme-hydrolysate with the additional supplement of nitrogen source group (AKAN). The results indicated that AKNN and AKAN also showed similar sugar consumption and ethanol production profile each other (t-rato<1.96). This indicated that AHNN and AKNN hydrolysates contain enough nitrogen source capable of efficiently converting the glucose and xylose in the hydrolysates into ethanol.

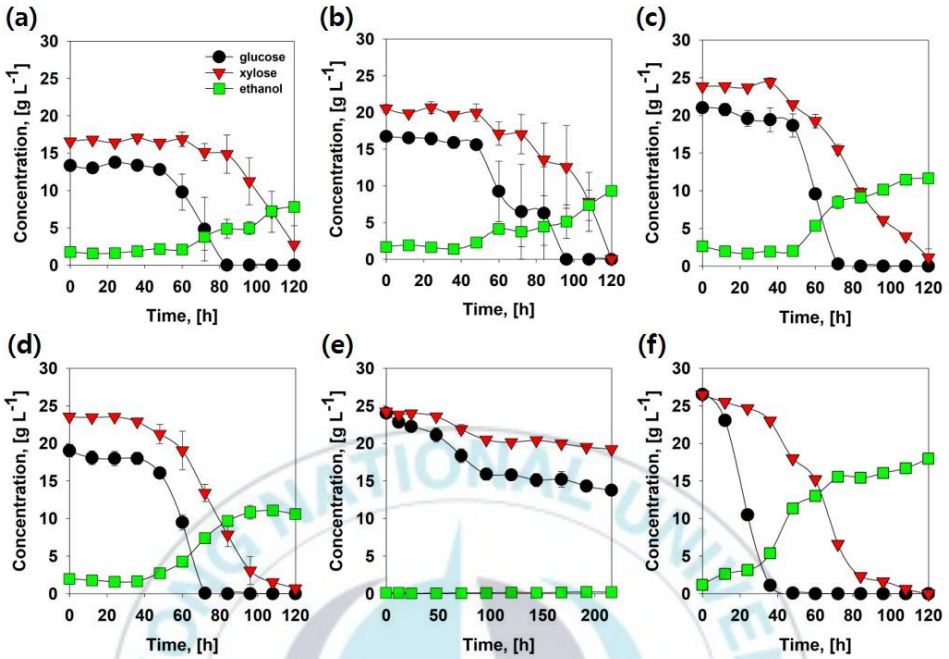


Figure 5. Fermentation profile of *Pichia stipitis* using acid/enzyme- or alkaline/enzyme-hydrolysates for ethanol production. (a) acid/enzyme-hydrolysates of HM in the absence of nitrogen source (AHNN), (b) acid/enzyme-hydrolysates in the presence of nitrogen source (AHAN), (c) alkaline/enzyme-hydrolysates in the absence of nitrogen source (AKNN), (d) alkaline/enzyme-hydrolysates in the presence of nitrogen source (AKAN), (e) synthetic medium containing glucose 25 g L^{-1} and xylose 25 g L^{-1} in the absence of nitrogen source (CNN), (f) synthetic medium glucose 25 g L^{1} and xylose 25 g L^{-1} in the presence of nitrogen source (CAN). The results were expressed as mean values from duplicates

Kinetic analysis of ethanol yields from AHNN and AHAN showed the same ethanol yields each other at 0.22 g g^{-1}, while AKNN and AKAN showed 0.21 and 0.23 g g^{-1} respectively (Table 5). These results further support that acid- and alkaline/enzyme hydrolysates without the nitrogen supplement (AHNN and AKNN) have sufficient nitrogen source for ethanol production. However, as the ethanol productivities were compared between AHNN and AKNN, AKNN (0.075 g L^{-1} h^{-1}) showed higher volumetric productivity than that from AHNN (0.05 g L^{-1} h^{-1}) ($p<0.05$) indicating the alkaline-pretreatment method for HM is better for ethanol production (Figure 6). As the productivities of AHNN and AKNN were also compared to the additional N-source providing groups (AHAN and AKAN), both the absence group of N-source (AHNN and AKNN) showed less productivities than those from the nitrogen supplemented group (Table 5, Figure 6). The higher productivities from the nitrogen source containing groups (AHAN and AKAN) could probably explain by the effects of other nutritional benefits derived from yeast extracts and peptone used in this studies which can also supply vitamins and other trace elements to support various enzyme catalytic activities involved in microbial cell growth and carbon metabolisms [44-46]. However, the results of ethanol yield and productivity from HM were comparable to the

results reported other studies [41, 47] (Table 6). Kim et al. [41] evaluated the ethanol production from H_2SO_4/enzyme- and NaOH/enzyme-hydrolysates of oak tree using the same strain of *Pichia stipitis* KTCC7222. They reported that 0.29 g g^{-1} ethanol yield and 0.084 g L^{-1} h^{-1} ethanol productivity from the NaOH/enzyme-hydrolysates of oak tree with the supplement of N-source including 5 g L^{-1} yeast extract and 5 g L^{-1} peptone. Canilha et al. [47] reported comparable ethanol yield (0.20 g g^{-1}) and ethanol productivity (0.04 g L^{-1} h^{-1}) from NaOH/enzyme-hydrolysates of sugar cane bagasse with the same amounts N-source supplement. Note that AKNN and AHNN without a supplement of N-source showed similar or higher ethanol yields and productivities. However, it is difficult to compare the ethanol production parameters of the same microorganism grown in different kind of lignocellulose hydrolysates which contain different sugar contents and fermentation inhibitory compounds leading to the change in cultivation conditions and consumption of carbon source in the hydrolysates.

Table 5. Kinetic analysis of *Pichia stipitis* in various hydrolysates of HM (10% substrate; chemical pretreatment using 2% H_2SO_4 or 2% NaOH at 120 ˚C for 2h; enzyme hydrolysis using 2% CTec2 cellulase for the acid hydrolysates or using 2% CTec2 cellulase and 2% HTec2 hemicellulase for the alkaline- hydrolysates at 50°C for 24h)

	Synthetic media		2% H_2SO_4 (v/v)		2% (w/v) NaOH (w/v)	
Nitrogen source	Absence (CNN)	Presence (CAN)	Absence (AHNN)	Presence (AHAN)	Absence (AKNN)	Presence (AKAN)
Ethanol production (g L^{-1})	0.10±0.01	16.80±0.02	6.30±0.40	7.60±0.90	9.60±0.40	9.50±0.05
Ethanol yield (g g^{-1})	0.01±0.01	0.32±0.03	0.22±0.01	0.22±0.01	0.2±0.01	0.23±0.01

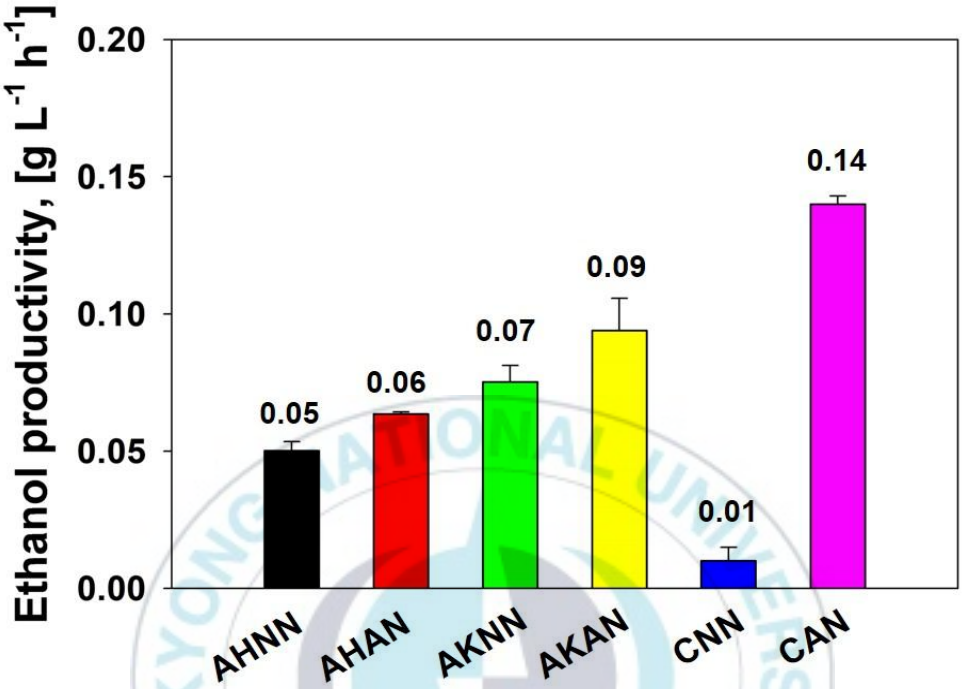


Figure 6. Comparison of ethanol productivities from various hydrolysates of HM. The control groups were used to synthetic media containing 25 g L^{-1}glucose and 25 g L^{-1} xylose. Nitrogen source used in these studies were 5 g L^{-1} yeast extract and 5 g L^{-1} peptone

Table 6. The comparison of sugar yield, ethanol production yield and ethanol productivity from various raw materials and pretreatment methods.

Raw materials	Pretreatment conditions	Microorganisms	Sugar yield (%)	Ethanol yield ($g\ g^{-1}$)	Ethanol Productivity ($g\ L^{-1}\ h^{-1}$)	References
Horse manure	2% H_2SO_4, 120°C, 2h	*Pichia stipitis*	72.10	0.22	0.050	This study
	2% NaOH, 120°C, 2h	*P. stipitis*	78.70	0.21	0.075	This study
Sugar cane bagasse	2% H_2SO_4 at 150°C for 30min	*P. stipitis* DSM3651	-	0.20	0.040	[47]
Oak	2% H_2SO_4, 121°C, 2.5h	*P. stipitis*	68.10	0.01	0	[41]
	2% NaOH, 121°C, 2.5h	*P. stipitis*	49.80	0.29	0.084	[41]

3.6. Fermentation inhibitory compounds in pretreated/saccharified hydrolysates.

During pretreatment of lignocellulosic feedstocks, several byproducts such as furfural, hydroxymethyl furfural (HMF), and acetic acid are formed, and they negatively influence the enzyme hydrolysis and microbial fermentation steps [40, 41]. The byproducts such as furfural, HMF and acetic acid were also identified in the acid/enzyme- and alkaline/enzyme-hydrolysates of HM and the results are summarized in Table 7. The results indicated that the acid/enzyme- and alkaline/enzyme-hydrolysates contained different amounts of inhibitory compounds. However, the acid/enzyme-hydrolysates harbor higher content of acetic acid, furfural, HMF than those from the alkaline/enzyme-hydrolysates. The presence of these inhibitory compounds in the acid/enzyme-hydrolysates possibly caused the combined negative effects of ethanol production which may have led to lower ethanol productivities than those from the alkaline/enzyme-hydrolysates. Similar results also reported by other studies [40, 41]. To overcome such issues, many studies to improve the ethanol productivities and yields have been reported for the strain developments [48-50] as well as detoxification process against such compounds [51, 52].

Table 7. Reducing sugar content and inhibitory compounds from various hydrolysates of HM derived from the pretreatment conditions (10% substrate; catalysts concentrations using 2% H_2SO_4 or 2% NaOH at 120°C for 2h; enzyme hydrolysis using 2% CTec2 cellulase for the acid hydrolysates or using 2% CTec2 cellulase and 2% HTec2 hemicellulase for the alkaline-hydrolysates at 50°C for 48h)

	Synthetic media		2% H_2SO_4/enzyme hydrolysates		2% NaOH/enzyme hydrolysates	
Nitrogen source	Absence (CNN)	Presence (CAN)	Absence (AHNN)	Presence (AHAN)	Absence (AKNN)	Presence (AKAN)
Glucose (g l^{-1})	24.05±0.50	25.75±0.70	13.57±0.18	16.73±0.11	21.01±0.07	19.03±0.82
Xylose (g l^{-1})	24.25±0.20	25.25±0.50	17.14±0.44	20.53±0.54	23.83±0.12	23.53±0.16
Reducing sugars (g l^{-1})	48.25±0.30	51.00±0.50	30.71±0.26	37.27±0.65	44.84±0.04	42.57±0.66
Acetate (g l^{-1})	N/A	N/A	2.17±0.40	2.46±0.62	1.25±0.50	0.73±0.40
Furfural (g l^{-1})	N/A	N/A	4.44±0.55	4.20±0.01	0.02±0.01	0.01±0.01
HMF (g l^{-1})	N/A	N/A	4.16±0.10	4.03±0.20	0.15±0.08	0.13±0.11

3.7. Evaluation of removal capacity of nitrogen eutrophication through the ethanol fermentation using HM as initial feedstocks.

Following ethanol fermentation of the hydrolysates of HM, the removal capacity of total N-source in the animal manure was determined using the standard Kjeldahl method [43] since the ethanologens consume N-source for the cell growth and metabolisms. As shown in Figure 7, the total N contents after the fermentation of the acid/enzyme-hydrolysates of HM were reduced from 0.4 to 0.07%. The alkaline/enzyme-hydrolysates was also decreased from 0.8 to 0.13 %. Interestingly, ~83% total nitrogen in the acid/enzyme- and alkaline/enzyme-hydrolysates were utilized by *Pichia stipitis* for ethanol production. Similar results have been reported by You et al. [36]. The authors reported that 45% of ammonia in daily manure was utilized for ethanol production by *Zymomonas mobilis*. Although the higher nitrogen removal capacity found in this study could plausibly come from different analytic methods used for nitrogen contents, HM has sufficient carbon and nitrogen source for ethanol production allowing a potential to improve economics of ethanol production industry. These results also indicated that the ethanol production process using HM can offer potential economic benefits in wastewater treatment process.

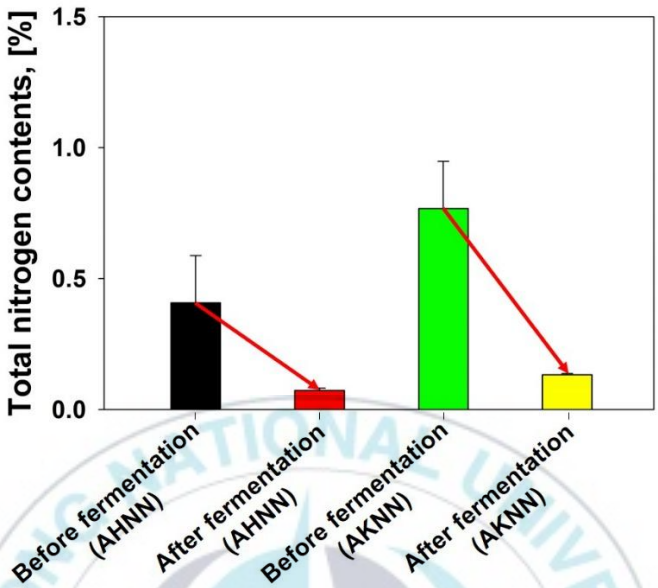


Figure 7. Total nitrogen contents after the fermentation of AHNN and AKNN hydrolysates.

The daily production of HM is 0.051 kg per 1 kg of horse, and the mean weight of horse among 27,819 heads of horse is 450 kg [53] in Korea. Considering the fraction of solid horse feces in HM is approximately 24 % [54], the estimated production of solid feces to be used a feedstock for BE production would be 56.3×10^6 kg in Korea. As shown in the Table 6, the sugar recovery yield (78.7 wt.%) from holocellulose part (51.6 wt.%) of HM, and the BE production yield from fermentable sugars was 21 wt.% in this

study. Thus, entire BE yield from HM was 8.5 wt.%. Based on our experimental result, estimated annual production of BE from HM would be approximately 4.8×10^6 kg (6.1×10^6 liter). Considering that the gasoline price in Korea is around $1.5 per liter, this gasoline alternative from HM would worth to approximately $9.2M per year.

Table 8. Population, HM production, and estimated BE production from HM in Korea

Population (ea)	27,819 [55]
Mean weight of Horse (kg)	450 [53]
Daily production of HM per weight of horse, wet basis (kg day^{-1} kg^{-1} horse)	0.051 [53]
Annual production of HM per weight of horse, wet basis (kg $year^{-1}$ kg^{-1} horse)	18.6
Annual production of HM per weight of horse, dry basis (kg $year^{-1}$ kg^{-1} horse)[a]	4.5
Annual production of HM in Korea (kg)	56.3×10^6
BE production yields in this study (per dry basis kg of HM)	8.5 wt.%
Estimated annual production of BE production	4.8×10^6 kg (6.1×10^6 liter)

[a] Assuming 24% solid contents in HM [54]

Nonetheless, further cost analysis and process developments are required to make this process more attractive. Note that BE production yield could be further improved when more relevant enzymes and process conditions are established because the current study adapted these experimental conditions

from BE process of cellulosic biomass. For example, further process development is required to focus on high gravity (HG) ethanol fermentation process for brewing industries [56]. The exact statistics of annual HM-based fertilizer production was not established, because individual livestock farms use the HM-based fertilizer. However, it is believed that the main application of livestock manure (about 90%) is for farming in Korea. Therefore, the further study for economic viability between BE and fertilizer production from HM would be meaningful study in line with the investigation of environmental impacts.

4. Conclusions

In this study, we found that a N-rich lignocellulosic material such as HM is a feasible raw feedstock as carbon and nitrogen sources for BE production. Two typical acid-/alkaline-pretreatments using H_2SO_4 and NaOH were evaluated for HM as an initial raw material for BE production. In comparison of sugar recovery yields from two different pretreatment process, the alkaline-pretreatment showed higher sugar recovery yield (80%) than that from the acid pretreatment (71%) indicating the alkaline-pretreatment method is more suitable for BE production using HM. The fermentability of acid/enzyme- and alkaline/enzyme-hydrolysates without a supplement of N-source were evaluated using GRAS strain of yeast, *Pichia stipitis*. The results indicated that the alkaline/enzyme-hydrolysates showed higher ethanol productivities (0.075 g L^{-1} h^{-1}) than that of the acid/enzyme-hydrolysates (0.050 g L^{-1} h^{-1}) supporting that the alkaline-pretreatment method is more suitable for the conversion of HM into BE. Moreover, this result depicts that HM can be used as feasible feedstocks for carbon and nitrogen source which possibly integrated into lignocellulosic ethanol production process providing potential saving cost of N-source supplement. During the ethanol fermentation of both acid/enzyme- and alkaline/enzyme-hydrolysates, *Pichia stipitis* are able to

remove about 83% total nitrogen indicating this process also can mitigate the nitrogen eutrophication in effluent water from animal farms.

5. Acknowledgement

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6. Nomenclature

BE: bioethanol

HM: horse manure

IC: internal combustion

RFS: renewable fuel standard

RSM: response surface analysis

ANOVA: analysis of variance

HMF: hydroxymethylfurfural

RI: refractive index

CCD: central composite design

CAN: control fermentation experiments with additional nitrogen source

CNN: control fermentation experiments without additional nitrogen source

AHNN: acid/enzyme-hydrolysate with non-additional nitrogen source

AHAN: acid/enzyme-hydrolysate with the additional nitrogen source

AKNN: alkaline/enzyme-hydrolysate with non-additional nitrogen source

AKAN: alkaline /enzyme-hydrolysate with the additional nitrogen source

7. 국문 초록

Biomass의 안전하지 않은 공급망은 Bio-Ethanol (BE)의 산업의 중요한 요인 중 하나로 여겨져 왔다. Lignocellulosic-biomass의 원료 공급 원료를 찾기 위해, 말 분뇨를 BE로 전환시켰다. 발효를 하기 위해 H2SO4/NaOH를 사용한 산/알칼리 전처리의 두 가지 전처리 방법을 사용했습니다. 산/알칼리 전처리를 최적화하기 위해 Box-Behnken 설계를 사용한 표면 방법론을 사용하였습니다. 최적화된 조건 하에서, 알칼리 전처리(80 %)는 산 전처리 (71 %)보다 높은 최대 당 회수수율을 보였으며, 이는 알칼리 전처리가 마분으로부터의 BE 합성에 적합하다는 것을 제공한다. 질소원을 보충하지 않은 산/효소-및 알칼리/효소-가수 분해물의 발효 성은 효모인 *Pichia stipitis*를 사용하였습니다. 결과는 알칼리/효소 가수 분해물이(0.075 g L-1 h-1) 산/효소 가수 분해물 (0.050 g L-1 h-1)보다 높은 BE 생산

성을 나타냈다. 알칼리/효소 가수 분해물로부터 이러한 높은 BE 생산성의 가능한 이유를 설명하기 위해, acetic acid, furfural, and hydroxy-methyl-furfural(HMF) 같은 발효균주에 대한 잠재적 발효 억제 화합물을 정량적으로 분석하였다. 결과는 알칼리/효소 가수 분해물이 산/효소 가수 분해물보다 더 적은 양의 발효 저해 물질이 존재하는 것을 통해 알칼리 전처리가 BE 생산에 더 적합한 것을 확인하였다. 또한 모든 실험 결과는 추가 질소원 보충없이 HM을 탄소원과 질소원으로 사용하여 BE의 생산 비용을 절감할 수 있는 가능성을 시사했습니다.

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