Effect of Selected Fermentation Parameters on Bioethanol Production from Ripe Carabao Mango *(Mangifera indica)* Peelings

Asia Pacific Journal of Multidisciplinary Research
Vol. 3 No. 4, 29-35
November 2015 Part IV
P-ISSN 2350-7756
E-ISSN 2350-8442
www.apjmr.com

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Date Received: September 29, 2015; Date Revised: October 28, 2015

Abstract *–The study aimed to determine the effects of selected fermentation parameters on bioethanol production from ripe carabao mango (Mangifera indica)* peelings. *Based on the results of the study, untreated peelings has compositional analysis in w/w% of 41.51%, 32.10%, 25.94% and 20.05% of extractives, holocellulose, alpha cellulose and acid insoluble respectively. On the other hand, after dilute acid pretreatment, the compositional analysis of extractives, holocellulose, alpha cellulose, acid insoluble lignin, acid soluble lignin and hemicelluloses were 15.71%, 76.01, 73.21%, 48.88%, 5.57% and 2.81% respectively.Findings also showed that there is a significant difference in the aforementioned properties before and after undergoing dilute acid pre-treatment.*

Keywords *–carabao mango (Mangifera indica), dilute acid pretreatment, untreated peelings*

INTRODUCTION

Continuous search for alternative energy sources has been the focus of various researchers and scientists through the years due to the negative impacts of petroleum based products. Biofuels are now gaining increased public demand and scientific attention due to the depletion of fossil fuel resources and oil reserves and other environmental detriments brought about by the use of petroleum-based fuel. In fact, Philippines is now requiring a gasoline blend of 10% bioethanol in order to lessen its bad effects to the environment.

An alternative material for producing biofuels coming from non-food sources becomes more popular. This is now called the second generation biofuel sources which include the lignocellulose biomass which are either non-edible residues of food crop production or non-edible whole plant biomass. These materials also include wood wastes, agricultural residues and fruit peelings[1].

Mango fruit peeling which are being generated hugely by the mango processing industry contributes to the problem of waste disposal of most processing plants. Peelings contain high quality dietary fiber due to high starch content, high amount of cellulose and hemicellulose, high lignin content, high pectin content, low lipid content. Due to the mentioned properties of the mango peelings they are considered to be a viable source of bioethanol.

OBJECTIVES OF THE STUDY

This study aimed to determine the effects of selected fermentation parameters on bioethanol production from ripe carabao mango *(Mangifera indica* peelings.) Specifically, this study sought to determine if there is a significant difference on the properties of ripe carabao mango peelings before and after dilute acid pre-treatment; determine reducing sugar concentration in the filtrate after dilute acid pre-treatment; evaluate the effect of varying the fermentation time and yeast loading on the amount of bioethanol in the fermentation broth; and determine the percentage ethanol produced in the distillate using the best saccharification parameter.

MATERIALS AND METHODS

Preparation of Lignocellulosic Biomass and Dilute Acid Pre-treatment

Ripe carabao mangoes (RCM) were washed three to five times thoroughly to remove impurities and dirt. Then, washed mangoes were peeled off manually and the peelings were chopped for drying. Chipped peelings were rinsed using distilled water. The drying process was done by open sun drying for

an average of ten hours per day for seven days. After sun drying, the peelings were further dried at constant weight in the cabinet dryer at 48°C. The dried sample was milled using pulverizer and food grade blender.About 100 g of dried carabao mango peelings powder was mixed with one liter of 0.8 M H_2SO_4 solution. The resulting mixture was soaked for 48h at room temperature. The adherence of chemicals was then removed by washing using distilled water and was neutralized at pH 7.0. After filtration, solid residues was obtained and stored at 4^0C prior to Simultaneous Saccharifcation and Fermentation (SSF) [2].

Simultaneous Saccharification and Fermentation

The cellulase used in the experiment was obtained from BIOTECH laboratory in UP Los Baños. The enzyme had an activity of 1347 unit (1AGU, Amyloglucosidase Unit = amount of enzyme needed to hydrolyze one micrometer maltose/minute at 25°C, pH 5.5). *Saccharomyces cerevisiae* was also obtained from BIOTECH. Large production of the cultured enzyme was maintained on agar slants at 4˚C.

In this process, the amount of carabao mango peelings used was 3g in wet basis. The media was composed of (g/L): 25 glucose, 10 peptone, 2 KH_2PO4, 1 MgSO4 and 4 $(NH_4)_2SO_4$ and pre-treated biomass was used in 250mL Erlenmeyer flasks. The flasks contained culture medium with the prepared sodium citrate buffer mixed within the flask. 20 mL cellulase with an enzyme activity of 2000 U/mL was supplemented to the culture medium. The fermentation started with the addition of yeast. Loading of *Saccharomyces cerevisiae* was varied at 10 mL, 15 mL, and 20 mL. Samples were withdrawn at the fermentation time of 36 h, 72 h, and 108 h.

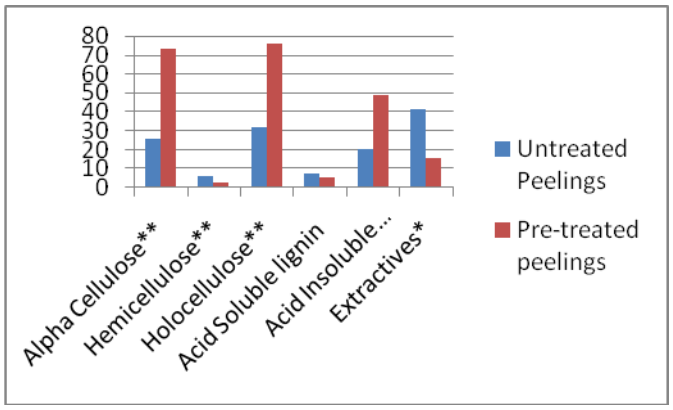
Distillation

The reaction broth of the samples prepared utilizing the best SSF parameter was filtered using Whatman No. 1 filter paper to remove unfermented hydrolyzates and yeast biomass. 100 mL of the fermented hydrolyzate at 200^0C and was placed into the distillation flask with 50 mL of water. The sample was distilled slowly into the same 100 mL volumetric flask at temperatures between 78.3 - 80°C. Then distillate of about 15 mL of distillate was collected and was analyzed for percent ethanol concentration by Gas Chromatography [3].

Determination of reducing sugar concentration was done using Dinitro Salicylic Acid (DNS) method. The percentage (%) ethanol in the sample was determined using Gas Chromatography (GC)

RESULTS AND DISCUSSION

Properties of Ripe Carabao Mango (RCM) Peelings Before and After Pre-treatment



*Note: * - Moisture free basis; ** - Moisture free and extractive free basis*

Figure 1.Properties of untreated and treated ripe carabao mango peelings.

Based on Figure 1, it can be seen that the untreated peelings has compositional analysis in w/w% of 41.51 percent, 32.10 percent, 25.94 percent and 20.05 percent of extractives, holocellulose, alpha cellulose and acid insoluble respectively. After dilute acid pre-treatment, the compositional analysis of extractives, holocellulose, alpha cellulose, acid insoluble lignin, acid soluble lignin and hemicelluloses were 15.71 percent, 76.01 percent, 73.21 percent, 48.88 percent, 5.57 percent and 2.81 percent respectively.

It is explicitly confirmed in this study that ripe carabao mango peelings have the potential of providing high amount of fermentable sugars due to the presence of 25.94 percent alpha cellulose and 6.16 percent hemicellulose. Being a lignocellulosic biomass, ripe carabao mango peelings are naturally resistant to degradation, hydrolytically stable and structurally robust mainly because of cross linking between lignin and polysaccharides like cellulose and hemicellulose by means of ester and ether linkages [4].

This characteristic was reflected in the lignin content of the peelings amounting to 7.79 percent acid soluble lignin and 20.05 percent acid insoluble. Besides, the peelings also contained non-structural materials such as non-structural sugars, nitrogenous material, chlorophyll, and waxes called extractives amounting to 41.51 percent.

A high increase from 25.94 percent to 73.21 percent in the alpha cellulose composition connotes disruption of the crystalline structure of the cellulose, swelling of the accessible area for cellulase to do further cellulose degradation in the hydrolysis step following pre-treatment, and separation of hemicellulose and lignin from the cellulose itself.

Since lignin is considered as the most recalcitrant component of a certain biomass, it has been concluded that reducing the lignin content usually leads to higher bioavailability of the substrate for bioethanol production. As to this study, a drop in the acid soluble content of the peelings from 7.79% to 5.57% denotes that part of the structure which could hinder the enzymatic activity of the cellulase was removed upon undergoing the pre-treatment consequently making it possible for the substrate to undergo further degradation. Aside from lignin content, other factors like lignin composition, its chemical structures, and lignin-carbohydrate complex linkages present in biomass have important impact on biomass digestibility and such was confirmed in this study due to the increase in the acid insoluble lignin composition of the peelings from 20.05 percent to 48.88 percent [5]. Even though some of the lignin was solubilised, it underwent re-condensation resulting to the formation of pseudo-lignin which yields higher percentage of acid insoluble insoluble lignin [6]. Lignin depolymerization and repolymerization are due to the formation of a common carbocation intermediate during acid pre-treatment [7]. Utilizing the lignin`s melting temperature changes the lignin into larger molten bodies which migrate within and out of the cell wall and then re-deposit as droplets on the surface of biomass cell walls [8].

As extractives also hinder cellulose accessibility during enzymatic hydrolysis, mango peelings possessed high amount of extractives, they must be extracted prior to sugar degradation through their solubility in various solvents. This was achieved in this study as indicated by the decrease in extractives from 41.51 percent to 15.71 percent. Also, the process of removing extractives contributed to the high precision of the analysis of the peelings. However, the removal was accomplished primarily because of the continuous washing of the pre-treated peelings using water until pH 7 was reached.

In order to determine the statistical significance of the gathered data set regarding the chemical properties of RCM peelings, one tailed paired t-test with arbitrary assigned p- value of 0.05 was used in this part of the study.

Table 1: Statistical results of the chemical properties of Carabao mango peelings before and after pre-treatment

Properties	P-Value	Decision Ho	Verbal Interpretation
Alpha Cellulose	0.00001	Reject	Significant
Hemi-cellulose	0.00074	Reject	Significant
Holo-cellulose	0.00006	Reject	Significant
Acid Soluble Lignin	0.00101	Reject	Significant
Acid Insoluble Lignin	0.00130	Reject	Significant
Extractives	0.00013	Reject	Significant

α=0.05

The computed p-values for the chemical properties of ripe carabao mango peelings in terms of alpha cellulose, hemicellulose, holocellulose, acid soluble lignin, acid insoluble lignin, and extractives are 0.00001, 0.00074, 0.00006, 0.00101, 0.00130, and 0.00013 respectively. These values are less than the 0.05 level of significance thus resulting to the rejection of the null hypothesis. Accordingly, it can be said that there is a significant difference in the aforementioned properties before and after undergoing dilute acid pre-treatment.

A significant increase in the acid insoluble lignin was mainly because of the severity of the pre-treatment which made way to the formation of artificial lignin. Despite of this, the recalcitrance of the substrate was dominantly overcome due to the significant increase in the alpha cellulose and decrease in the other components. Cellulose accessible surface area still increased even if the lignin was re-distributed since such weakened the carbohydrate - lignin matrix [9], [10]. Some of the benefits in employing dilute acid pre-treatment: hydrolysis of the hemicellulose producing syrup of monomeric sugars such as pentoses, exposure of cellulose for enzymatic digestion by removal of hemicellulose and part of lignin, and solubilization of heavy metals in the feed

[11]. Such process has the ability to give high reaction rates and improve cellulose hydrolysis [12].

Reducing Sugar Concentration in the Filtrate after Dilute Acid Pre-treatment

Table 2: Reducing sugar concentration in the filtrate

Sample No.	*Concentration (mg/mL)*
1A	0.254
1B	0.261
2A	0.268
2B	0.264
Ave	**0.2618**

As shown in Table 2 given below, minimal amount of reducing sugar was lost in the pre-treatment of peelings with 0.8M H_2SO_4 and 48h soaking time. This only means that more sugar remained in the substrate which is essential in attaining high yields of ethanol. More than 95 percent of the hemicellulosic sugars of a lignocellulosic biomass could be recovered by dilute acid pre-treatment depending on the substrate and the conditions of the process.

Effect of Fermentation Time and Yeast Loading on the Amount of Ethanol in the Fermentation Broth

Table 3: Ethanol Yield in the Fermentation Broth at Varying SSF Parameters

Trial	*Fermentation Time (hr)*	*Yeast Loading (mL)*	*Percentage Ethano l(v/v)*
1	36	10	0.22
2	36	10	0.24
1	36	15	0.27
2	36	15	0.22
1	36	20	0.33
2	36	20	0.33
1	72	10	0.08
2	72	10	0.16
1	72	15	0.16
2	72	15	0.09
1	72	20	ND
2	72	20	ND
1	108	10	0.3
2	108	10	ND
1	108	15	ND
2	108	15	0.09
1	108	20	0.16
2	108	20	0.27

**ND – Not Detected*

Table 3shows the percentage ethanol in the fermentation broth when SSF was carried out at varying fermentation time and yeast loading.

At 36 hrs of fermentation with 10, 15, and 20 mL of yeast, the broth had 0.22%, 0.27%, and 0.33% of ethanol for sample A respectively. For sample B, at 36 hrs of fermentation with 10, 15, and 20 mL of yeast, the broth had 0.24%, 0.22%, and 0.33% of ethanol respectively. When the fermentation was performed in sample 1 for a period of 72 hrs, the amount of ethanol in the crude were calculated to be 0.08% for 10 mL yeast loading and 0.16% for 15 mL yeast loading however, no ethanol was detected in the broth when 20 mL of yeast was consumed at SSF time of 72 hrs. At the fermentation time of 72 hrs for sample 2 the broth had 0.16% for 10 mL yeast loading, 0.09% for 15 mL yeast loading and not detected amounts for 20 mL yeast loading.

The results for the fermentation of sample 1 for 108 hrs were 0.30% for 10 mL yeast loading, 0.16 for 20 mL of yeast loading and undetected amounts for 15 mL of yeast loading. For sample 2 with 108 hrs of fermentation time, the amount of ethanol in the crude were 0.09 for 15 hrs, 0.27 for 20 hrs and for 10 hrs, no ethanol was detected. The results revealed that the highest percentage of ethanol was attained at the lowest time and highest yeast loading.

The amount of ethanol in 108 hrs fluctuated and did not conform to the general trend established by 36 hrs and 72 hrs of fermentation time. This could be due to the prolonged fermentation of the biomass. As the fermentation time increases, the risk of contamination greatly increases. Aside from this, unpredictable reactions occurring inside the system can alter the ethanol yield. Several studies had also concluded that after reaching the optimum condition in fermentation, ethanol production is supposed to decrease with time. Thus, the results obtained for 108 hrs of fermentation time is not reliable for the prediction of the trend of the fermentation reaction.

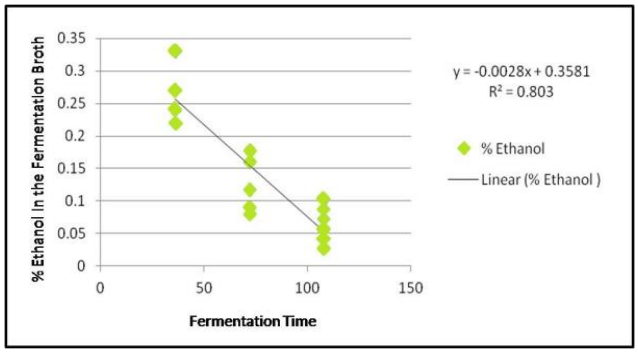


Figure 2.Effect of fermentation time in the amount of ethanol in the fermentation broth.

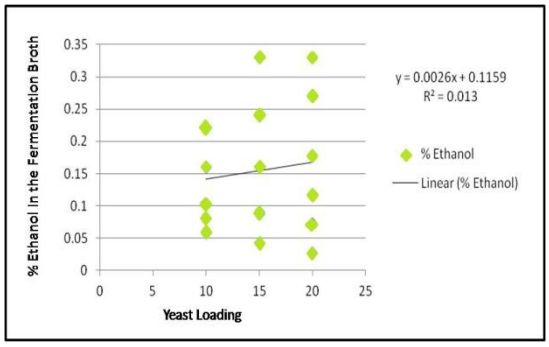
Figure 2 depicts the amount of ethanol produced as a function of time. It is evident in the graph that ethanol yield is inversely proportional to fermentation time at constant yeast loading.

The maximum amount of ethanol was obtained at 36 hrs. Prolonging the incubation period up to some extent caused a decrease in the ethanol concentration due to the fact that yeast suffers various stresses of which some stresses are synergistic, affecting the yeast cells more severely than any single one, leading to reduced yeast viability and vigour as well as lower ethanol yield.

Upon exposure to ethanol, microbial cells correspondingly adjust their intracellular metabolism thus resulting to yeast cell growth and ethanol production inhibitions [13]. Ethanol tolerance and thermotolerance are closely linked and related to the structure of the cell membrane, specifically the lipid content [14]. Moreover, unsaturated lipids enhance alcohol tolerance and membrane fluidity while saturated lipids diminish ethanol tolerance and make membrane more rigid. In anaerobic fermentation, a small amount of oxygen is necessary in order for the yeast cells to synthesized these fatty acids such as palmitoleic acid and oleic acid [15].

Another factor is the formation of acids like acetic acid, lactic acid, and pyruvic acid. Accumulation of such weak acids results to significant drop in intracellular pH and not enough production of ATP which is needed by the yeast to survive. The total acid generation increases linearly with time [16].

Phenolic compounds derived from lignin like vanillin, syringaldehyde, and ferulate also disrupt the integrity of the cell membrane of the organisms. Low molecular weight phenolic acids behave the same way as weak acids when it comes to ethanol production inhibition.



Figre3. Effect of yeast loading in the amount of ethanol in the fermentation broth

Higher concentrations of furfural and 5-hydroxymethyl furfural (HMF), generated due to the severity of the pre-treatment, are found to bring induce build up of reaction oxygen species in the cells thus causing cellular damage more specifically in mitochondria and vacuole membranes, actin cytoskeleton, and nuclear chromatin. This prevents yeast from growing and negative effect to ethanol production[17].

On the other hand, at constant time, ethanol yield increases with the increase in the amount of yeast used as shown in Figure 3. The yeast concentration, the rates increased rapidly with the increase in the amount of yeast added up to the yeast concentration of 8g/20g fruit pulp. However, beyond that point the formation of ethanol insignificantly increased since the substrate became the limiting and increasing the yeast did not increase the rate of reaction [18].

Table 4: Comparison of the effects of selected parameters in the ethanol yield

Source of Variation	Df	F-Value	p - value	*Decision on Ho*	Verbal Interpretation
Fermentation Time (hr)	2	7.988	0.0101	Reject	Significant
Yeast Loading (mL)	2	0.42	0.669	Accept	Not significant
Interaction	4	2	0.1782		
Within	9				
Total	17				

α=0.05

Using Analysis of Variance (ANOVA) Two Factors with Replication statistical method, it was found out that the difference in the mean values among the different levels of time is greater than would be expected by chance after allowing for effects of differences in yeast loading. Also, with 4 degrees of freedom, the calculated p-value of 0.0101 is less than the 0.05 level of significance. Thus, there is a significant difference in the ethanol yields as time is varied. In addition, F-value of 7.9879 is greater than F-critical of 4.2565 which lead to the rejection of null hypothesis.

On the other hand, since the computed value of p is greater than the 0.05 level of significance, no significant difference is exhibited by the percentage ethanol values obtained after varying the loading of yeast to the fermentation media. Besides, the F-value for this parameter is less than the F-critical as revealed by Table 5. Subsequently, the null hypothesis was accepted. This is because the difference in the mean values among the different levels of yeast loading is

not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in time.

The effect of different levels of time does not depend on what level of yeast loading is present. There is no significant interaction between the two parameters as the determined p value of 0.1782 is greater than the assigned value of level of significance.

The computed F-value for fermentation time (7.988) is higher as compared to the F-value for yeast loading (0.420). This suggests that fermentation time has greater effect in the ethanol yield than yeast loading when it comes to the simultaneous saccharification and fermentation of dilute acid pre-treated ripe carabao mango peelings.

Percentage Ethanol Produced in the Distillate Using the Best SSF Parameter

In ethanol production, distilling the desired product leads to higher yield due to the removal of more volatile components in the broth [19]. In accordance with this statement, the ethanol produced under the optimum condition of 36 hrs and 20 mL yeast loading were distilled and analyzed by gas chromatography. The distillate was found to contain 4.595 percent of ethanol

Aside from the previously discussed factors affecting fermentation, low ethanol concentration could also be attributed to the azeotropic property of ethanol water mixture which makes it hard to separate the two components at ordinary laboratory scale distillation.

CONCLUSION AND RECOMMENDATION

The alpha cellulose, holocellulose, hemicellulose, acid soluble lignin, acid insoluble lignin and extractives content of the peelings differ significantly after dilute acid pre-treatment; The sugar lost in the dilute acid pre-treatment as manifested in the filtrate is 0.2618 %; As fermentation time decreases and yeast loading increases, the percentage ethanol in the broth increases. **;** The percentage ethanol produced in the distillate under the optimum SSF condition of 36 hrs and 20 mL yeast loading is 4.595%.

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