Bioethanol extraction from Coconut (*Cocos nucifera* Linnaeus) embryo using *Saccharomyces cerevisiae*

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ABSTRACT

The world oil crisis is looming as supply levels are under threat. This study is the first attempt to extract bioethanol from Coconut (*Cocos nucifera* Linnaeus) embryo using *Saccharomyces cerevisiae*. Samples of coconut embryos were collected, ground, and subjected to acid hydrolysis. These were then filtered, and the residues collected were treated with two different amounts of buffer solution. Cultured strains of *S. cerevisiae* were inoculated into the prepared Yeast Extract-Peptone-Dextrose (YPD) broth. Buffer-treated samples were then inoculated with 5 ml of the yeast solution and allowed to ferment at various times. After different fermentation times, samples were filtered, and the obtained filtrates were subjected to the distillation process for bioethanol concentration determination. Results showed that samples allowed to ferment at 72 h and 96 h yielded 7.11% and 12.22% bioethanol, respectively. The samples treated with 50 ml and 100 ml of buffer solution produced 9.02% and 10.31% bioethanol, respectively. The main effect of fermentation time on bioethanol concentration was statistically substantial such that samples allowed to ferment in 96 h yielded higher bioethanol concentration than samples permitted to ferment in 72 h. Based on the results, bioethanol could be extracted from a novel, cheap, and readily available coconut embryo using *S. cerevisiae*.

Keywords: acid hydrolysis, agri-food industry waste, bioconversion, fermentation

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INTRODUCTION

Due to the continuous annual oil consumption, the reserves are quickly being depleted, with scientists predicting that at the current rate of use, in just 40 years, the entire fossil fuel supply will run entirely out (Lazkano et al. 2017). While oil has numerous uses in the global community, burning it increases the trace gas concentration in the atmosphere and causes environmental problems. The global annual energy demand of over 12 GT of oil results in the emission of 39.5 GT of carbon dioxide, and the yearly emission of this gas would increase up to 75 GT when future energy demand will rise to 24–25 GT of oil (Abas et al. 2015). The produced carbon dioxide gas, a principal constituent of greenhouse gases, is accountable for global warming and other harmful effects to the ecosystem, such as the melting of ice caps and glaciers, reduction of dissolved oxygen in oceans, death of coral reefs and drought (Shakun et al. 2012).

The world community supported the grand energy transition from fossil fuels to renewable and more economically viable alternative fuel sources to decelerate climate change and develop sustainable energy resources for domestic and industrial uses. Previous research found out that hydroelectric power, fuel cells, solar energy wind power, and bioethanol were feasible as renewable and alternative energy sources (Bhatia 2014; Zou et al. 2016; Islam et al. 2018).

Bioethanol, a clear, colorless, and biodegradable liquid known to be a potential alternative to gasoline, is the best alternative fuel to replace the current fossil fuel. It is most commonly formulated with liquid gas in concentrations of 10% bioethanol to 90% gasoline, known as E10, and nicknamed gasohol. When used, it burns up to 75% cleaner than fossil fuels. The fermentation process produces this energy source from the crops that are rich in carbohydrates. Reports available suggest that previous natural substrates for bioethanol production have included wheat straw, corn, and sugarcane bagasse (Schwietzke et al. 2009; Talebnia et al. 2010; De Souza et al. 2014). However, these substrates yielded smaller amounts of bioethanol, and the processes of extraction were too expensive to sustain.

The Food and Agriculture Organization of the United Nations (FAO 2017) reported that the Philippines is the world's largest producer of coconuts, producing 14,049,131 T . However, there are lots of unused excess parts of it, notably the embryo, which is called "Buwa" in Philippine vernacular. These are wastes and sometimes, used as animal feed, and may contribute to the country's garbage problem. Furthermore, the country is experiencing an issue in waste management. According to Remo (2017), about 35,000 tons of municipal solid waste is generated by the Philippines daily. These wastes would accumulate, taking up valuable real estate and become a source of land

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pollution. As a solution to this problem, researchers came up with a study about the bioconversion of these waste products.

When a coconut fruit falls from the bush, the water inside after some time converts into a tissue-sponge. This tissue nourishes and helps in the germination of a new plant that will become another coconut tree. This sponge is called a coconut embryo. Coconut fruit, along with coconut embryo, composed of 60% carbohydrates (Decker 2018), making it a viable substrate for bioethanol.

Previous reports explored the bioethanol yield from coconut husk fiber (Goncalves et al. 2015; Cabral et al. 2016) and produce a comparable concentration. The potency of coconut waste water was also explored and estimated to produce ethanol at a rate of 50-60 kg day^{-1}; however, the study found that the material has higher potential for producing alcoholic beverage for profitability. Based on the review, there appears to be no published study that has assessed the potential of using coconut embryo as substrate for bioethanol production. For this reason, the coconut embryo, an unexploited material of coconut, is worthwhile to investigate.

Bioethanol production from carbohydrate feedstocks such as coconut embryo, requires fermentation process. This process is carried out by various microorganisms such as fungi, yeasts, and bacteria. The yeast *S. cerevisiae* is the most widely used and studied organism for bioethanol production at both household and industry levels (Tesfaw and Assefa 2014). It is superior to fungi, bacteria, and other yeasts because of its ability to hasten the fermentation process (Goncalves et al. 2015; Azhar et al. 2017). In addition, it tolerates a wide range of temperature and pH, two most essential variables that affect yeast growth and bioethanol production (Benjaphokee et al. 2012). *Saccharomyces cerevisiae* is generally regarded as safe for human consumption which improves its advantageous application more than other yeasts and microorganisms. Several studies found that the use of *S. cerevisiae* in the fermentation of coconut products such as husk fiber and water, produced the greatest bioethanol concentration compared to other microorganisms (Goncalves et al. 2015; Azhar et al. 2017).

The present study aimed to analyze the potential of Coconut (*C. nucifera* L.) embryo extract as an alternative source of bioethanol using *S. cerevisiae*. It also sought to examine the effect of fermentation time and the amount of buffer on bioethanol production. In doing so, it also attempted to come up with an alternative bioethanol source that is cheap and readily available.

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METHODS

Collection and Acid Hydrolysis of Sample

Coconut embryos (Figure 1) were collected from an authorized local coconut shop at Davao City, Philippines. The shop grows non-genetically modified coconuts in an organic method. Before collecting the embryos, all coconut fruits were submitted to a Botanist for taxonomic identification and verification of botanical specimen (Certificate #2019-00124). After verification, the samples were washed with 5% potassium permanganate solution and distilled water to disinfect and remove any debris (Ma et al. 2015). These were then ground into pulp using a house blender and placed in a 1,000 ml glass beaker. The ground sample was then subjected to acid hydrolysis.

Figure 1. Coconut (*Cocos nucifera* Linnaeus) embryo.

A weight of 800 g of the ground sample was added to 1,000 ml of 5% sulfuric acid. The utilization of 5% sulfuric acid solution was based on the study of Kuhad et al. (2010). The sample was then filtered using Whatman 11 µm-filter paper. The residue was then set aside at room temperature while the filtrate was discarded.

A weight of 50 g of the residues was equally transferred to eight 1,000 ml Erlenmeyer flasks. The flasks were then grouped into two; the first group, labeled as A, contained 50 ml of the prepared 2% sodium hydroxide buffer with a pH of 9.25 while the second group, labeled as B, contained 100 ml of the same buffer.

Preparation of the Culture and Inoculation Procedure

The preparation of the Yeast Extract-Peptone-Dextrose (YPD) broth followed the procedures of Ho et al. (2006). The medium was prepared in a flask by mixing 50 g of YPD agar to 1,000 ml of distilled water. The medium was brought to boil for one minute using the hot plate and stirred

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continuously. The solution was allowed to cool, and the mouth of the flask was covered tightly with a cotton plug wrapped with aluminum foil, and finally sealed with indicator tape. The container was stored away from light at 2-8°C to avoid loss of moisture.

The inoculation procedure followed the steps of Guevara (2005). Cultured strains of *S. cerevisiae* or Baker's yeast were inoculated into the prepared YPD broth. This mixture was then shaken for 10 minutes before inoculation into the fermentation medium.

Fermentation

All of the eight flasks containing 50 g of the sample and different amounts of 2% sodium hydroxide buffer (50 ml and 100 ml) were inoculated with 5 ml suspension of *S. cerevisiae*. The containers that were previously divided into two were further divided into two subgroups, forming two replicates at each buffer-fermentation time combination. These were labeled depending on the amount of buffer used and fermentation time (Table 1). The fermentation times, 72 h and 96 h, are standard bioethanol production parameters used to determine the minimum perceptible effect of fermentation time on bioethanol concentration; hence, these values were previously used in several studies (Srimachai et al. 2015; Hossain and Jalil 2017).

Bioethanol evaporation was intercepted, and aerobic conditions were kept by placing plugs on all flasks (Figure 2). After their fermentation times, the fermented sample was filtered using cheesecloth, and the filtrate of each flask was subsequently submitted for chemical analysis.

Table 1. Preparation of sample under different fermentation parameters.

Flask Code	Amount of Buffer (ml)	Fermentation Time (h)
XA1	50	72
YA1	50	72
XA2	50	96
YA2	50	96
XB1	100	72
YB1	100	72
XB2	100	96
YB2	100	96

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Bioethanol Determination by Distillation

The filtrates contained in the flasks of the two groups were sent to Science Resource Center–University of the Immaculate Conception, Davao City for bioethanol determination using the distillation method. Samples of each liquid filtrate in each of the flasks were loaded to the distillation equipment for chemical analysis, and the results of the test were recorded and analyzed.

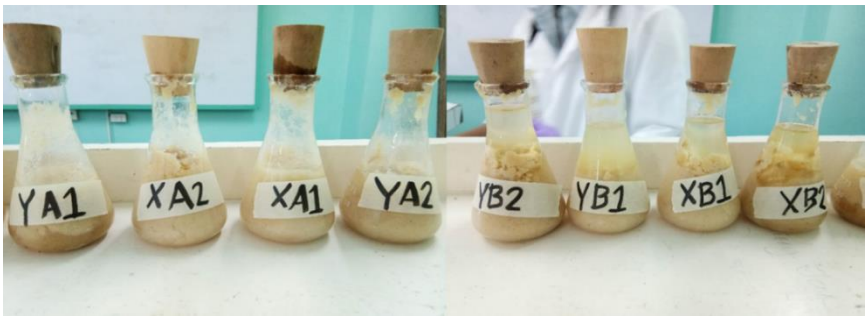

Figure 2. Flasks containing samples for fermentation.

Statistical Analysis

The experiment was conducted in three repeated trials to increase the validity of the results. The three trials (T1, T2 and T3) were conducted between 07 January and 02 February 2019, and each trial lasted 4 days. Mean ± standard deviation (SD) was used to express the average concentration of bioethanol produced per trial for three trials and the variability of the said concentrations. A two-way analysis of variance was employed to determine if there is a significant difference in the concentration of bioethanol produced when fermentation times and amounts of the buffer are varied. Furthermore, this was used to determine if there is a significant interaction between the varied fermentation times and the amounts of the buffer used. To confirm where the differences occurred between groups, pairwise comparisons using Bonferroni correction was employed. A *P*-value <0.05 was considered as statistically significant. The data were analyzed using the trial SPSS version 23.0 software package.

RESULTS

The samples that were treated with 100 ml buffer solution and allowed to ferment in 96 h yielded the highest bioethanol concentration at 16.05±0.01% compared to others (Table 2). On the other hand, the samples that were treated with 100 ml buffer solution and allowed to ferment at 72 h produced the lowest bioethanol concentration at 4.57±0.01%.

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Table 2. Concentration (% weight by volume) of bioethanol produced after 72 h and 96 h fermentation and using 50 ml and 100 ml of buffer solution in three trials (% w/v means % weight by volume).

Flask Code	Amount of Buffer (ml)	Fermentation Time (h)	Bioethanol Concentration (% w/v)						
			T1	Mean ± SD of T1	T2	Mean ± SD of T2	T3	Mean ± SD of T3	Overall Mean ± SD
XA1	50	72	9.68	9.65 ± 0.05	9.68	9.65 ± 0.05	9.68	9.66 ± 0.04	9.65 ± 0.01
YA1	50	72	9.61		9.61		9.63		
XA2	50	96	8.39	8.39 ± 0.01	8.42	8.39 ± 0.04	8.42	8.41 ± 0.02	8.39 ± 0.01
YA2	50	96	8.38		8.36		8.39		
XB1	100	72	4.60	4.57 ± 0.05	4.61	4.58 ± 0.05	4.60	4.56 ± 0.06	4.57 ± 0.01
YB1	100	72	4.53		4.54		4.52		
XB2	100	96	16.1	16.04 ± 0.08	16.07	16.04 ± 0.05	16.07	16.06 ± 0.01	16.05 ± 0.01
YB2	100	96	15.98		16.00		16.05		

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There was a statistically significant interaction between the effects of fermentation time and amount of buffer on bioethanol concentration, F=89.54, P<0.01 (Table 3). The table also presented the value of adjusted R squared which tells that 95.5% of the variance in bioethanol concentration is attributed to fermentation time and amount of buffer.

Table 3. Two-way ANOVA in the interaction effect of fermentation time and amount of buffer. *P<0.05. **P<0.01.

Source	***F*-statistic**
Fermentation time	57.73*
Amount of buffer	3.67
Fermentation time * Amount of Buffer	89.54**
R Squared = 0.974 (Adjusted R Squared = 0.955)	

At 72 h, the samples treated with 50 ml buffer solution yielded a statistically higher bioethanol concentration compared to those treated with 100 ml buffer solution (P<0.01) (Table 4). At 96 h, the samples treated with 100 ml buffer solution yielded a statistically higher bioethanol concentration compared to those treated with 100 ml buffer solution (P<0.01).

Table 4. Pairwise comparisons of bioethanol concentration by amount of buffer using Bonferroni correction. **P<0.01.

Fermentation Time (h)	**Amount of Buffer (ml)**	**Bioethanol Concentration (% w/v)**	**Mean Difference**
72	50	9.65	-5.08**
	100	4.57	
96	50	8.39	7.66**
	100	16.05	

Using 50 ml buffer solution, the samples fermented at 72 h yielded a comparable bioethanol concentration compared to those fermented at 96 h (P>0.05) (Table 5). Using 100 ml buffer solution, the samples fermented at 96 h produced a statistically higher bioethanol concentration compared to those fermented at 72 h (P<0.01).

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Table 5. Pairwise comparisons of bioethanol concentration (% weight by volume) by fermentation time using Bonferroni correction. $^{**}P<0.01$.

Amount of Buffer (ml)	Fermentation Time (h)	Bioethanol Concentration (% w/v)	Mean Difference
50	72	9.65	-1.26
	96	8.39	
100	72	4.57	11.48**
	96	16.05	

DISCUSSION

In this study, bioethanol was extracted from coconut embryos using *S. cerevisiae* and the interaction effect of fermentation time and amount of buffer on bioethanol yield was analyzed. The study produced bioethanol at 7.11-12.11% w/v which is higher compared to that of pineapple waste at 0.09% w/v, sweet potato waste at 0.079% w/v, Indian water chestnut waste at 0.045% w/v, and jackfruit waste at 0.045% w/v (Gosavi et al. 2017). The bioethanol yield was also higher compared to that of pretreated seagrass at 0.047% w/v (Ravikumar et al. 2011). Meanwhile, the standard deviations of bioethanol concentration across all samples ranged from 0.01-0.08. This is highly indicative that the measurements conducted in two replicates for three repeated trials were homogenous and means across the samples did not vary, thereby, increasing the validity of the study results (Hopkins 2017).

The amount of sugars, such as sucrose, fructose, and glucose, present in a material determines the bioethanol produced during fermentation. In the biochemical process, the sugar is converted into ethanol and carbon dioxide. Hence, the materials with higher amount of sugar would yield higher bioethanol compared to those with lower amount of sugar. In this study, the total sugar content of coconut embryos was not examined; however, the sample used may have higher sugar content compared to the samples used in the previous studies based on the previous theoretical assumption. To date, there is no existing study which analyzed the sugar content of coconut embryos; hence, preliminary test of total sugar estimation may be conducted in the future.

Simple main effect analyses were conducted using Bonferroni correction. The fermentation time of 96 h produced higher bioethanol concentrations than 72 h fermentation time. This finding is similar to previous reports of Fahrizal et al. (2013) and Sheikh et al. (2016). Based on their experiments, bioethanol yield increases from 24 h to 96 h fermentation time. However, bioethanol concentration decreases after 96 h. The decrease

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of bioethanol concentration after 96 h may be attributed to the decline in the total sugar content of the sample as most of its sugar were already converted to ethanol in the previous hours of fermentation (Fahrizal et al. 2013). Fermentation times beyond 96 h may be noteworthy to investigate in the future. While previous studies corroborate the findings of the present study, several scholarly works revealed that decreasing fermentation time results to improved bioethanol yield. El-Mekkawi et al. (2019) reported that bioethanol yield increases to 94% as the fermentation time is decreased from 72 h to 48 h. In their study, Gutierrez et al. (2015) demonstrated that the bioethanol yield decreases due to the formation of acetic acid, pyruvic acids, and lactic acids. These acids increase linearly with time and could significantly decrease the pH of the fermented sample and the production of ATP, which is critical to the survival of *S. cerevisiae*.

The bioethanol concentration did not significantly differ in samples treated with varied amounts of buffer. This finding is contradictory to the study of Kundiyana et al. (2011). Their report showed that samples treated without morpholinoethanesulfonic (MES) acid produced higher ethanol concentration. Future studies may be conducted to test for the effect of different types of buffer on bioethanol concentration.

This study successfully extracted bioethanol from coconut embryos; however, several limitations may be looked in the future. The distillation method may underestimate the ethanol content of the sample (Togarepi et al. 2012); hence, an accurate ethanol determination method such as gas chromatography may be used in the future. Other parameters such as pH may be considered to optimize the bioethanol yield. Although the present study did not extract pure ethanol for application in vehicles, this study is the first to successfully determine the ethanol content of processed coconut embryos. Nevertheless, the study came up with a novel bioethanol source that is cheap and readily available.

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