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Fermentation of Rice Straw by Vermiwash for Bioethanol Production

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A B S T R A C T

Rice straw is abundant in the world and the waste management became a global issue. In this paper, vermiwash is used for rice straw hydrolysis and then followed fermentation to produce bioethanol. This study involves the optimization of factors involved in bioethanol production and also microbiological study of the vermiwash. The hydrolysis of rice straw by vermiwash presents the highest reducing sugar yield of 18.70mg mL^{-1} and highest enzymatic activity of 0.151 IU mL^{-1}. Formation of clear zones indicates presence of cellulolytic microorganism. The highest bioethanol yield obtained was 0.0896mg mL^{-1}. The obtained result from ANOVA indicated that the significant factors were biomass loading and vermiwash ratio with P-value <0.05. In conclusion, it was found that the microbial consortiums in the vermiwashwere able to degrade rice straw and to convert the fermentable sugars to bioethanol.

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INTRODUCTION

Rapid development, increasing energy demand and concerns about greenhouse gas emissions has prompt the search for renewable fuels. The use of biofuels has received much attention in recent years due to the shortage and negative impact of fossil fuels towards the environment. The advantages of using renewable energy sources are reduction in global warming and climate changes due to fossil fuels burning [1]. One of the renewable energy sources is ethanol. Bioethanol can be use as substitute for current fossil fuels. Besides being readily biodegradable, bioethanol can also reduce greenhouse gases level due to the utilization of atmospheric carbon dioxide from the assimilation of biomass by feedstock crops [2]. The fundamental benefits from shifting to bioethanol are renewable resources that can serve as a sustainable fuel supply for a long period of time [3-6].

Abundant availability of lignocellulosic waste materials from agricultural residues such as rice straw shows promise for use as feedstock for bioethanol production. Lignin, a natural polymer can be found in herbaceous and woody plants [7]. Rice is the third most important grain crop in the world behind wheat and corn, in terms of total production[8]. In this context, rice straw would be a potential candidate for our future energy needs.

Production of bioethanol from lignocellulosic biomass involves two steps (1) hydrolysis of biomass and (2) ethanol fermentation. Prior to enzymatic hydrolysis, pretreatment is needed to enable the lignocellulosic materials to open up due to the cellulose and hemicelluloses that are bound together by lignin [9]. Several pretreatment methods such as dilute acid, alkaline, steam explosion have been used for hydrolysis of lignocellulosic biomass [10]. Equipment corrosion problems, acid recovery are some of the shortcoming from the use of acid pretreatment [11]. Recently biological pretreatment using microorganism has become a preference in lignin and hemicelluloses degradation. Biological pretreatment offer advantages such as low capital cost, low energy, no chemical requirements and mild environmental conditions [7]. After pretreatment, enzymatic hydrolysis by microorganism plays a role in breaking down cellulose and hemicelluloses into monomeric sugar. Fermentation using various microorganisms converts carbohydrate

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monomers to ethanol [12, 13].Current fermentation uses yeast, however for this work microbe from vermiwash was adopted. Vermiwash is a liquid extract of vermicomposting process whereby waster is release by microorganism during organic matter decomposition and it contains soluble nutrients and microbial consortium [14]. Beneficial microbial communities from vermiwash have been demonstrated to improve plant health, yield and nutritive quality [15].

Response surface methodology (RSM) was use to assess the effect of experimental parameters on bioethanol production. Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems. Response surface methodology (RSM) is a competent tool use for process optimization in which a response of interest is affected by combined effect of independent variables [16].The objective of RSM is to determine the optimum operating conditions [17].
The experiment was conducted using central composite design (CCD) in RSM by Design Expert Version 6.0.4. In the present work, the combined effect of biomass loading, vermiwash ratio and retention time on bioethanol yield was investigated. Therefore, the study was conducted in aiming the yield of bioethanol via fermentation of rice straw using vermiwash as a fermentation agent substrate.

MATERIALS AND METHOD

Preparation of biomass

Rice straw obtained from a paddy field located in Kedah was used as a biomass feedstock for fermentation process. Samples were collected after the harvesting season. The rice straw collected were washed and dried at 60^{o}C for 24 hours. Then the samples were grinded to the size of less than 1 mm. Vermiwash was obtained from a local agricultural company.

Morphological and biochemical test of vermiwash

The aim for morphology and a few biochemical tests is to isolate the different bacteria in the vermiwash and to confirm the ability of the bacteria in the vermiwash to produce enzyme for degradation of cellulose and ferment sugars.

Bacteria isolation and gram staining

Sample from vermiwash was obtained and used for morphological and biochemical test. Method for morphological and biochemical test were performed [18]. Five isolates were attained from the bacteria isolation, streaking and gram staining for further analysis as in the following subtopic.

Starch test/amylase

The isolates from 2.2.1 was further analyzed for starch test. Starch hydrolysis test was used to identify the production of the exoenzyme amylase which breaks down starch in the rice straw. The bacteria were inoculated on a starch agar plate using aseptic technique. The plate was then incubated at 37^{o}C for 48hours. After incubation, gram's iodine was added to the plate to make a blue precipitate. Formation of clear zone indicates the presence of amylase.

Carboxymethylcellulose (CMC) test

Isolates obtained were further analyzed for cellulose degrading activity through CMC test by Congo red assay. CMC test was done to test for cellulase producing activity of bacterial isolates. Method for CMC test is developed in the literature [19]. The isolates were grown on CMC agar and incubated at 37oC. The agar medium was flooded with 0.1% Congo red to visualize the hydrolysis zone.The cellulose hydrolyzing activity was measured by the appearance of clear zones around the culture batches.

Phenol red test

Phenol red broth test was use to identify the bacteria's ability to ferment sugars. The broth contains phenol red as pH indicator and a Durham tube which is use to identify the formation of carbon dioxide via the presence of a bubble. The broth were inoculated with the isolates using aseptic technique and incubated at 37oC for 18-24 hours. When using phenol red as pH indicator, a yellow color indicates that enough acid products have been produced by fermentation.

Pretreatment and enzymatic hydrolysis of rice straw biomass

Rice straw was transferred into a 250mL Erlenmeyer flask. Distilled water was added prior to autoclave. Using heat as pretreatment the rice straw were autoclave (TOMY, Japan) at 121^{o}C for 10 minutes under 15 psig pressure. The rice straws were further heated in hot water at 90^{o}C using water bath (MEMMERT). After the flasks were cooled to room temperature, vermiwash was added into the flasks. The samples were transferred to an orbital shaker (WISD, Korea) at ambient temperature for 24 hours in order to allow the vermiwash to react with the rice straw. The prehydrolyzed samples (rice straw and vermiwash) were measured for total reducing sugars after 24 hours. Total reducing sugar was determined [20]. The absorbance values of the total reducing sugars in the samples were measured using HACH spectrophotometer DR2800 at 550nm. The prehydrolyzed samples were incubated in incubator shaker (DAIKI) to enable enzymatic hydrolysis at 35^{o}C for 48 hours at 120rpm. This method was slightly modified found in literature [21] with addition of

vermiwash to replace the commonly use enzyme which is cellulase. Enzyme activities were determined [22].

TABLE 1. Range and levels of the independent variables for bioethanol yield

Factors	Level				
	-α	**-1**	**0**	**+1**	**α**
Biomass Loading (A)	3.42	5	7.5	10	11.58
Compost : H_2O Ratio (B) Vermiwash %	22.79	33	50	67	77.21
Heating Time During Pretreatment (C)	20.51	30	45	60	69.49

DNS method was use for reducing sugar estimation. Enzyme activity was expressed as international unit (IU) defined as the amount of enzyme that forms 1µmol glucose/min.

Design of experiment for rice straw hydrolysis

To determine the effects of independent variables on the response and factor interactions, all the experiments of the rice straw hydrolysis will follow the central composite design (CCD).

CCD with five coded levels and a total 20 runs were carried out to optimize the chosen variables which are biomass loading, vermiwash ratio and retention time. The design consists of a full 2 factorial design with six axial points and six replication of the center points. The range and levels used in the experiments are shown in Table 1. For response surface methodology (RSM), the most commonly used second-order polynomial equation developed to fit the experimental data and optimize the variables can be expressed as shown in Equation 1

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{1 \le i \le j}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where Y represents the predicted response; ethanol yield, $\beta_o$the constant coefficient, β_i the linear coefficients, β_{ii} the quadratic coefficients, β_{ij} the interaction coefficients and x_i, x_j are the coded values of the variables.

Fermentation of rice straw biomass

After hydrolysis of rice straw, all the samples from each run were filtered. The filtrates (100mL) obtained were further fermented for 72 hours at room temperature (28±5^{o}C). Simple distillation was used to separate ethanol from water [23]. The distillate will be further analyzed for bioethanol determination.

Ethanol determination by acid chromic assay

The fermented rice straw biomass will be analyzed for bioethanol concentration [24]. Potassium dichromate reagent solution was prepared by dissolving 1g of potassium dichromate in 6N concentrated sulphuric acid to a volume of 100mL. The prepared solution was mixed to ensure homogeneity. Sample size of 0.5 mL of distillate was oxidized with 2.5mL of potassium dichromate reagent solution at 90^{o}C for 15minutes. After the samples were cooled to room temperature, the ethanol absorbance was determined by spectrophotometer at wavelength of 590nm using HACH spectrophotometer DR2800 (Camlab, UK).

RESULTS AND DISCUSSION

Morphological and biochemical test of vermiwash

Base on morphology of the microorganisms and colony, five isolates were determined. The morphological and characteristics of the isolates from vermiwash is shown in Table 2. The colonies of the isolate were yellow, white and creamy with flat, raised and convex margin. The colony surface was dry, smooth and shiny. Among the isolates some were found to contain bacteria with rounded and irregular shapes; convex and raised or a flat surface. Some colonies were opaque, transparent or translucent. All the isolated microorganisms were gram positive under gram staining analysis. The biochemical tests were performed on the isolates and are illustrated as in Table 2. Some of the isolates showed positive results for starch test, CMC test and phenol test. This explains the microbial consortium in vermiwash that are able to perform various activities. From the biochemical starch test shows that isolates CT-1, CT-3 and CT-5 shows a positive effect. Clear areas around colonies indicate the presence of starch-digesting enzymes [18]. Isolate CT-1, CT-2, CT-4, and CT-5 also display clear zones around colonies which imply positive results for CMC test. These results indicated the isolated bacterial species in vermiwash have moderate to significant ability to produce cellulase which is in agreement to Hao et al. [25] that mentioned cellulotic enzymes can be use to hydrolyze cellulose and use as useful end products. Phenol test was use to confirm the ability of bacteria to ferment sugar into ethanol. Positive reaction in the phenol test was obtained from isolate CT-1, CT-2, CT-4, and CT-5. This indicates the vermiwash contain bacteria that are able to conduct fermentation process. As stated by Manero and Blanch [26], carbohydrate fermentation was considered positive when the media turned yellow while a red color shows a negative result.

Reducing sugar and enzymatic activity determination

Figure 1 shows the reducing sugar concentration of the prehydrolyzed samples. The highest concentration of reducing sugar was from 18.70, 18.52 and 18.21 mg mL^{-1} for samples 11, 14 and 2, respectively. The lowest reducing sugar concentration was obtained in sample 4 with 3.39 mg mL^{-1}.

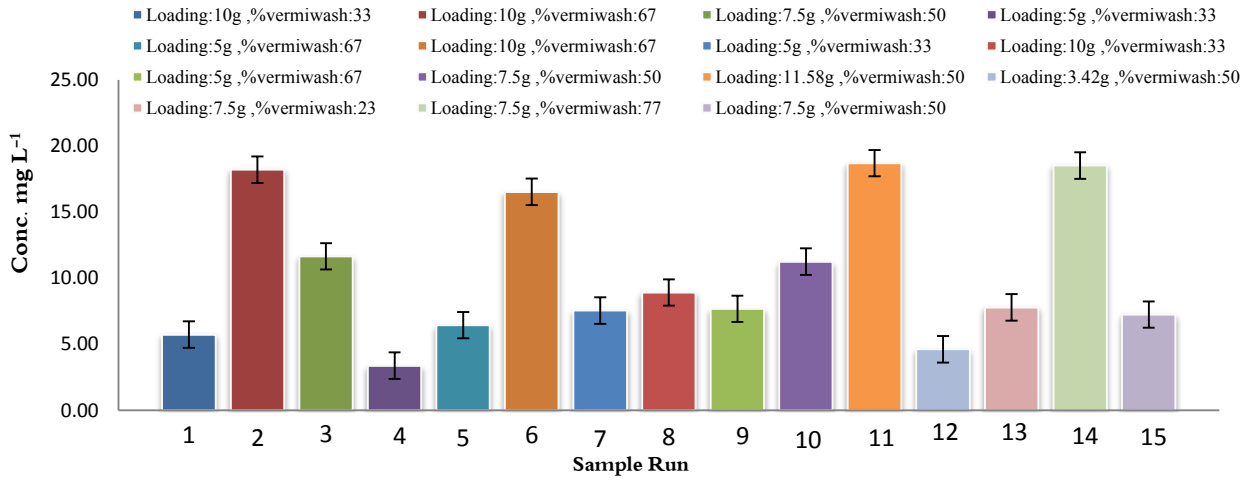

Figure 1. Reducing sugar concentration for 20 runs.

TABLE 2. Morphological and biochemical test of the different isolates from vermiwash

Isolates	Morphological					Biochemical test		
	Colony surface	Colony color	Opacity	Shape of colony	Elevation	Starch	CMC	Phenol
CT-1	Dry	Yellow	Opaque	Irregular	Raised	+	+	+
CT-2	Smooth	Yellow	Translucent	Rounded	Convex	-	+	+
CT-3	Shiny	Creamy	Translucent	Rhizoid	Slightly raised	+	-	-
CT-4	Smooth	White	Transparent	Circular	Convex	-	+	+
CT-5	Dry	Creamy	Transparent	Lobate	Flat	+	+	+

TABLE 3. Enzyme activity of 20 runs

Runs	Reducing sugar concentration (mg mL^{-1}) (y=0.4608x) R2=0.9871	Enzyme activity (IU mL^{-1})
1	0.167	0.062
2	0.206	0.151
3	0.176	0.065
4	0.256	0.043
5	0.2	0.074
6	0.178	0.066
7	0.204	0.075
8	0.143	0.053
9	0.115	0.095
10	0.126	0.047
11	0.145	0.054
12	0.202	0.075
13	0.143	0.053
14	0.148	0.108
15	0.408	0.076
16	0.263	0.097
17	0.269	0.100
18	0.297	0.110
19	0.291	0.055
20	0.254	0.094

CMC = mg reducing sugar released × 0.37
1.0mg glucose =1.0/0.18 × 0.5 × 30 µmol min^{-1} mL^{-1} substrate cleavage = 0.37 units mL^{-1}

High reducing sugar concentration is probably due to the high biomass loading and vermiwash concentration which permit more access of the bacteria in the vermiwash to attach to the biomass in order to produce fermentable sugars. Li et al. [27] reported by increasing the raw material a high sugar concentration can be attained, therefore leading to a high ethanol concentration.

The enzymatic activity for 20 runs was obtained in the range of 0.043 to 0.151 IU mL^{-1}. Maximum and minimum amount of enzyme release was insamples 2 and 4 with 0.151 and 0.043 IU mL^{-1}, respectively (see Table 3). Besides endoenzymes, many bacteria produce exoenzymes and release them through the cell or plasma membrane [18]. The results suggest the ability of the enzyme in vermiwash to yield reducing sugar. Hsu et al. [21] evaluated cellulase activity produced by *Streptomyces* sp. has the potential to improve hydrolysis efficiency of cellulosic materials.

Optimization of rice straw hydrolysis by using central composite design (CCD)

In this study, the effect of three main variables (ratio, loading and time) during rice straw pretreatment and hydrolysis for bioethanol production was investigated. The experimental design matrix by CCD generated

using the Design –Expert 6.0.4 was tabulated in Table 3. The resulting CCD model for ethanol production could be expressed using Equation 2 in term of coded factors:

$$Y = 0.071 + 0.020A + 0.013B - (1.932E - 003)C - (8.567E\text{-}003)A^2 - 0.013B^2 - (8.605E - 003)C^2 + (6.637E - 003)AB - (6.187E - 003)AC \quad (2)$$

where Y is the predicted response and A, B and C are the coded values for loading, ratio and time, respectively. Positive sign in front of the terms indicates synergistic affect whereas negative sign indicates antagonistic effect.

The experimental data were analyzed by fitting to a second order polynomial model, which was statistically validated by performing Analysis of Variance (ANOVA) and lack-of-fit test to evaluate the significance of the model. The results of ANOVA with the estimated effects and coefficients for the model are shown in Table 4. Model terms were evaluated by the P-value with 95% confidence level. The P-values were used to estimate whether F was large enough to indicate statistical significance of each coefficient. Values of "Prob>F" less than 0.05 indicate model terms are significant. It was observed from Table 5 that the coefficients for loading (A), ratio (B), and the interaction terms (AB and AC) including the square terms (A^2, B^2 and C^2) were significant to the response at 95% confidence level. However factor C shows P-value of 0.3804 which is insignificant to the response. The Model F-Value of 25.53 implies the model is significant. The lack of fit F-value of 1.54 is not significant as the p-value is >0.05. Non significant lack of fit is good as it shows the model is valid.

A normal probability plot in Figure 2 shows the relationship between the actual and predicted values of Y. It can be seen the data points are well distributed close to a straight line which suggests an excellent relationship between the experimental and predicted values of response. R^2 value close to 1 is desirable to establish an adequate adjustment of the quadratic model to the experimental data. This includes a reasonable agreement with the adjusted R^2. From the ANOVA results, the predicted R^2 of 0.7248 is in reasonable agreement with the adjusted R^2 of 0.9203.

Figures 3 and 4 illustrate the mutual interactive effects of the combination of independent variables on ethanol yield in the manner of 3D surface plots. These plots were represented as a function of two factors by holding other factors at a fixed level. Figure 3 depicts the effect of both ratio and loading with time kept as constant at centre point value; while Figure 4 illustrates the effect of both time and loading was studied with ratio kept as constant. From Figure 3 it shows that increase of loading rate, the production of ethanol increases, furthermore it can be seen that ratio show a higher ethanol yield at increasing ratio value and the maximum ethanol yield was obtained when the ratio is 93.86mL. Han et. al. [28] mentioned as the biomass loading increased, the ethanol concentration accordingly increased. For Figure 4, with the prolongation of retention time and the increase of loading, the ethanol concentration underwent a process of first increasing then decreasing. This can be seen by the curvature form at approximately 45minutes. Kuhad et al. [29] state that as a result of treatment period beyond 45 min, there is a decrease in the final sugar concentration that is a result of sugar degradation at severe condition.

TABLE 4. Central composite design experiments and experimental results for response

Run	Blocks	X_1:BiomassLoading (g)	X_2 : Vermiwash Ratio (mL)	X_3 : Retention Time (min)	Y_{EXP}RS
1	1	1	-1	1	0.0292
2	1	1	1	-1	0.0896
3	1	0	0	0	0.0677
4	1	-1	-1	-1	0.0132
5	1	-1	1	1	0.0247
6	1	0	0	0	0.0796
7	2	1	1	1	0.0665
8	2	0	0	0	0.0741
9	2	-1	-1	1	0.0153
10	2	0	0	0	0.0692
11	2	1	-1	-1	0.0532
12	2	-1	1	-1	0.0244
13	3	0	0	α	0.0553
14	3	α	0	0	0.0815
15	3	-α	0	0	0.0177
16	3	0	-α	0	0.0132
17	3	0	0	0	0.071
18	3	0	α	0	0.0632
19	3	0	0	-α	0.0437
20	3	0	0	0	0.0614

TABLE 5. ANOVA results for the quadratic model of the responses

ANOVA						
Source	**Sum of Squares**	**DF**	**Mean Square**	**F Value**	**Prob>F**	
Block	6.222E-007	2	3.111E-007			
Model	0.021	8	1.493E-003	25.53	<0.0001	significant
A	5.270E-003	1	5.270E-003	90.12	<0.0001	
B	2.322E-003	1	2.322E-003	39.70	0.0001	
C	4.976E-005	1	4.976E-005	0.85	0.3804	
A^2	9.693E-004	1	9.693E-004	16.57	0.0028	
B^2	2.178E-003	1	2.178E-003	37.24	0.0002	
C^2	9.778E-004	1	9.778E-004	16.72	0.0027	
AB	3.525E-004	1	3.525E-004	6.03	0.0365	
AC	3.063E-004	1	3.063E-004	5.24	0.0479	
Residual	5.262E-004	9	5.848E-005			
Lack of Fit	3.974E-004	6	6.624E-005	1.54	0.3880	Not Significant
Pure Error	1.289E-004	3	4.296E-005			
Cor Total	0.012	19				

$R^2 = 0.9578$ Adj $R^2 = 0.9203$

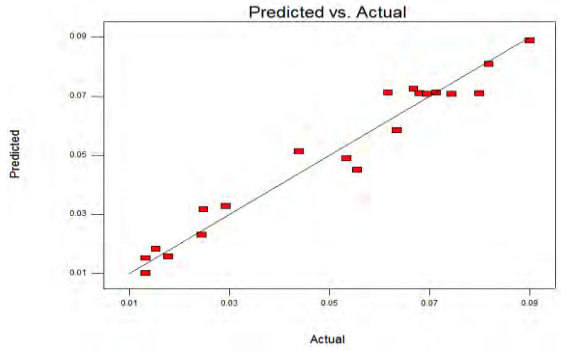

Figure 2. The actual and predicted value for bioethanol yield

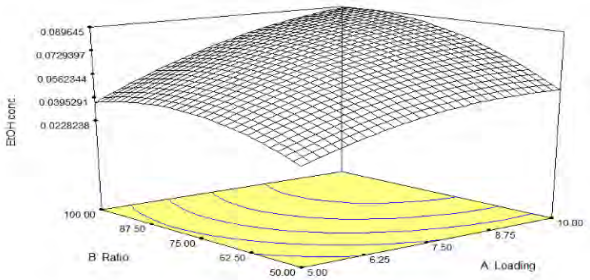

Figure 3. Response surface and contour plot for interaction between ratio and loading.

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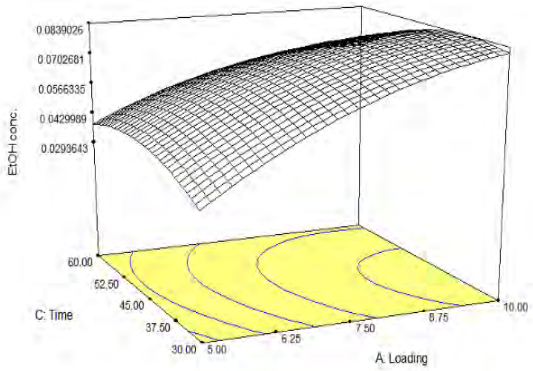

Figure 4. Response surface and contour plot for interaction between time and loading

CONCLUSION

Production of bioethanol from rice straw will give an advantage in terms of waste management and reducing environmental pollution. Rice straw can be use as feedstock to produce bioethanol with the assistance of vermiwash. This study demonstrates the potential of vermiwash in production of bioethanol from ricestraw base on the data from reducing sugar and enzymatic activity. The positive results from the biochemical test suggest vermiwash contains microbes that are able to carry out different activity such as fermentation and enzyme activity. Base on the central composite design, interaction between ratio and loading; time and loading show these factors influence the production of bioethanol.

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چکیده

کاه برنج در دنیا بسیار فراوان است و مدیریت پسماند تبدیل به یک مسئله جهانی شده است. در این مقاله ابتدا از ورمی واش برای هیدرولیز کاه استفاده شد و سپس تخمیر بر روی آن انجام شد تا بیواتانول حاصل شود. این پژوهش شامل بهینه سازی عوامل موثر در تولید بیواتانول و مطالعه میکروبیولوژیکی ورمی واش می باشد. هیدرولیز کاه برنج با ورمی واش مقدار ماکزیمم غلظت $mg\ ml^{-1}$ ۱۸/۷۰ برای قندهای احیا و ماکزیمم اکتیویته آنزیمی $IU\ Ml^{-1}$ ۰/۱۵۱ را نشان داد. تشکیل نواحی شفاف (بر روی کاه) نشان دهنده حضور میکروارگانیسم ها تجزیه کننده سلولز بود. بالاترین بازده تولید بیواتانول بدست آمده $mg\ ml^{-1}$ ۰/۰۸۹ می باشد. نتایج بدست آمده از ANOVA نشان داد که فاکتورهی موثر حجم بایومس و نسبت ورمی واش با P-value کوچکتر از ۰/۰۵ می باشد. در خاتمه، این نتیجه حاصل شد که کنسوسیوم میکروب ها در ورمی واش توانایی تجزیه کاه برنج و تبدیل آن به قندهای قابل تخمیر برای تولید بیواتانول را دارا بودند.