International Journal of Microbiology and Biotechnology
2016; 1(1): 33-39
http://www.sciencepublishinggroup.com/j/ijmb
doi: 10.11648/j.ijmb.20160101.15

Bioethanol Production from Rice Winery Cake Using Lactic Acid Bacteria and Yeasts by the Process of Simultaneous Saccharification and Fermentation

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To cite this article:
Abdulkadir Adedeji Shittu, Orukotan Abimbola Ayodeji, Mohammed Sani Sambo Datsugwai. Bioethanol Production from Rice Winery Cake Using Lactic Acid Bacteria and Yeasts by the Process of Simultaneous Saccharification and Fermentation. *International Journal of Microbiology and Biotechnology.* Vol. 1, No. 1, 2016, pp. 33-39. doi: 10.11648/j.ijmb.20160101.15

Received: October 28, 2016; **Accepted:** November 29, 2016; **Published:** December 27, 2016

Abstract: Bioethanol is a sustainable energy source which serves as an alternative to fossil fuel and contributes to a clean environment. Bioethanol was produced by individual activities of lactic acid bacteria and yeasts from rice cake waste using a simultaneous saccharification and fermentation process. The rice cake waste is a filtered solid waste of fermented rice wine mash and contains 78.04% of total carbohydrate, 10.88% of protein, 2.26% of ash, 8.12% of moisture and 0.7% of fat. The rice cake was mixed with raw starch digesting enzyme of *Aspergillus niger* and (*Lactobacillus fermentum*, *Rhodotorula minuta*, *Rhodotorula mucilagnosa*, *Candida krusei*, *Kodamara ohmeri*) respectively into different fermenting chambers. *Rhodotorula minuta* produced the highest efficiency of ethanol of 52.06% at the temperature of 30°C and pH of 2.58. Reducing sugar was observed to decrease with increase in bioethanol production and cell growth increased as the fermentation time increases. Bioethanol can also be produced from rice cake waste of a fermented rice mash, which can serve as a bio fuel and contributes to a healthy environment.

Keywords: Rice Cake, Simultaneous Saccharification Fermentation, *Rhodotorula minuta*, *Aspergillus niger*

1. Introduction

Bioethanol is an attractive, sustainable energy source for fuel that can contribute to a cleaner environment [1]. Bioethanol manufactured from renewable resources by microbial fermentation is an attractive alternative as it is carbon dioxide neutral, meaning that the amount of CO_2 released from the fermentation is 100% offset by the amount of CO_2 absorbed by the plants grown to make it [1]. The production of ethanol from the starch of wheat, barley, cassava, or maize by fermentation with the traditional yeast *Saccharomyces cerevisiae* is already a well-known process [1]. By altering the nutritional conditions, it is also possible to increase the ethanol yield and the survival of the yeast at high concentrations of ethanol [1]. Agriculture based industries generate a large amount of solid waste, such as peels from cassavas, plantains, bananas, and oranges [2]; however, instead of allowing this waste to become solid municipal waste, it is more beneficial to convert it into useful end products. Thus, it has now been recognized that such waste can be utilized as cheap raw materials for certain industries or as inexpensive substrates for microbiological processes [2]. The use of waste material is also economical, as it is more readily available and much cheaper. So far, agro-waste, such as cassava-peel hydrolysate [3], *Carica papaya* [4], and kitchen waste have already been used for ethanol production [5]. Henry Ford (1925) stated that, "The fuel of the future is going to come from apples, weeds, sawdust almost anything. There is fuel in every bit of vegetable matter that can be fermented." Today Henry Ford's futuristic vision significance can be easily understood. Bioethanol has emerged as the most suitable renewable alternatives to fossil fuel as their quality constituents match diesel and petrol, respectively. Bioethanol can be grouped in different generations: first generation bioethanol is made from carbohydrate based feedstock like corn, sugar beet, sugarcane, barley. Second generation bioethanol is produced from feedstock containing cellulosic biomass such as stalks, leaves and husk from corn plants, woodchips and saw dust. Third generation of bioethanol is produced by algae. In the

last two decades, numerous microorganisms have been selectively engineered to produce bioethanol. Ethanol is high octane fuel that can replace lead as an octane enhancer in petrol by helping to oxygenate the fuel mixture so it burns more completely. The burning of ethanol closely represents carbon dioxide cycle because the released carbon dioxide is recycled back into plants using carbon dioxide to synthesize food during photosynthesis cycle [6]. Various bacteria like *Zymomonas mobilis*, *Klebsiella oxytoca* and fungi like *Trichoderma* and *Aspergillus* species can produce bioethanol by their action on different carbohydrate sources. *Zymomonas mobilis* is a unique bacterium offering a number of advantages over the current ethanol producing microorganisms and reported for maximum bioethanol production [7]. Rice wine cake (RWC) is the filtered solid waste from rice-wine fermentation. After completing the process of simultaneous saccharification and fermentation (SSF), the mash is filtered and the clear rice wine and RWC are separated. However, the possibility of using RWC for ethanol fermentation has not yet received much attention. The use of RWC in ethanol production can not only reduce the waste material created by wineries, but also lower the cost of ethanol production [1]. This research is aimed at comparing bio-ethanol produced from rice winery cake using lactic acid bacterium and different yeasts isolated from fermented foods.

2. Materials and Methods

2.1. Source of Organisms

Pure strains of *Lactobacillus fermentum*, *Candida krusei, Rhodotorula minuta*, *Rhodotorula mucilagnosa*, and *Kodamara ohmeri* were obtained from the culture collection of the department of microbiology, Kaduna state university, Kaduna, Nigeria. These organisms were maintained on Deman Rogosa and Sharpe agar (MRS) and potatoe dextrose agar (PDA) slants at 4°C respectively.

2.2. Proximate Analysis, Preparation, and Pre-treatment of Rice Winery Cake for Bioethanol Production

The rice winery cake was obtained from rice wine variety and proximate analysis was carried out. These as follows: (Solid, raw starch, protein, lipid, cellulose, ethanol, minerals, and vitamins,). The proximate analysis was carried out on the rice wine cake using the method of [29]. The rice winery cake was mixed with distilled water at different ratios (1:0.7 to 1:1.7) and homogenized using blender (KENWOOD BL 430 SERIES). There after the rice winery cake was mixed with distilled water based on a ratio of 1:1.3 with the resulting slurry contains 23.03% (W/V) raw starch. The slurry was treated with a raw starch digesting enzyme containing α-amylase. This gives a proportion of the raw starch digesting enzyme as 1.125 U/g which is the dry weight base [8]. The slurry was steamed and treated with a raw-starch-digesting enzyme (RSDE), amylase produced by *Aspergillus* sp, where the volume of the RSDE to substrate was 3ml. This proportion of RSDE to substrate was used in all the experiments. This procedure treated the slurry and created a favourable environment for lactic acid bacteria and yeasts isolates to produce a yield ethanol [1].

2.3. Starch Hydrolysis Test of Strains of Aspergillus niger

An inoculum from a pure culture of *Aspergillus niger* was streaked on a sterile plate of starch agar. The inoculated plate was incubated at 27°C for 5 to 7 days. Iodine reagent was then added to stain the growth. Presence of clear zone surrounding colonies confirmed the positive result and accounts for their ability to digest the starch and thus indicates presence of alpha-amylase 3 [9].

2.4. Preparation of Raw-Starch-Digesting Enzyme (RSDE) for Bioethanol Production

A liquid culture of *Aspergillus* specie (10% v/w) was inoculated onto solid wheat bran at 30°C for 3 days, the mouldy wheat bran was dried at 35°C for 12 hours. Distilled water was added to a ratio of 1:50 (w/v) and stirred for 1 hour at room temperature. The extracted liquid was then used as the crude RSDE [1].

2.5. Preparation of Inoculum for Bio-ethanol Production

One loopful of lactic acid bacteria and yeasts cells of a two (2) day old grown on Deman Rogosa and Sharpe (MRS) agar and potatoe dextrose agar respectively was inoculated into two separate testubes containing 9 ml of nutrient broth, and incubated anaerobically at 37°C and 30°C respectively overnight. 1 ml of both samples was drawn after the incubation period for appropriate serial dilution. This will provide specific number cells of inoculum of both samples at different concentrations [1]. In order to determine the specific number of inoculum cells which was used for fermentation, a McFarland standard was prepared and applied. This standard was be used as a reference to adjust the turbidity of bacterial suspensions, so that the number of bacteria was within a given range to standardise microbial testing. A 0.5 McFarland Standard is prepared by mixing 0.05 ml of 1.175% barium chloride dehydrate ($BaCl_2$+ $2H_2O$ with 9.95 ml of 1% sulphuric acid (H_2SO_4). A 0.5 McFarland standard concentration amounts to $1x10^8$ cells. The turbidity of this preparation will used as a reference source to the two different inoculum cells (lactic acid bacteria and yeast) of different dilutions in order to determine the specific cells which was used for fermentation.

2.6. Ethanol Tolerance Test

The yeast isolate was tested for ethanol tolerance. The yeast strain was inoculated in 10ml of YPG broth containing different concentrations of ethanol (0, 2, 4, 6, 8, 10, 12, 14, and 16%). The tubes were incubated at 30°C for 48 hrs. After incubation, the viability of yeast cells were checked by serially dilution with sterile distilled water and plated on YPG agar medium. After incubation, the results were tabulated in CFU/ml [10].

2.7. Inoculation of Substrate for Simultaneous Saccharification and Fermentation

The substrate was inoculated with starter cultures (Lactic acid bacteria and different yeasts isolates) using McFarland Standard. This standard was used as a reference to adjust the turbidity of bacterial suspensions, so that the number of bacteria was within a given range to standardise microbial testing. A 0.5 McFarland Standard is prepared by mixing 0.05 ml of 1.175% barium chloride dehydrate ($BaCl_2$+ $2H_2O$ with 9.95 ml of 1% sulphuric acid (H_2SO_4). At this stage, ethanol production by simultaneous saccharification and fermentation was conducted at 30°C in a 3-l flask containing two (2) litres of the rice winery cake slurry, pH 4.5, treated with the raw starch digesting enzymes. These isolates convert sugar to ethanol. Fermentation was done at 30°C for 48 hours at 120 rpm on an orbital shaker for two days. The hydrolysate was treated at optimal conditions for liquefaction and saccharification with an initial biomass loading of 10 and 20 weight percentage [11, 12].

2.8. Determination of Cell Density and Quantitative Analysis of Reducing Sugar Present in Bioethanol

At 24 hour intervals, samples were taken aseptically from the fermentation media to determine growth, residual sugar and ethanol concentrations. The growth was determined by measuring the cell density (optical density) at 650 nm using spectrophotometer, the residual sugar was determined using Dinitrosalicylic acid (DNS) method described by [13]. The quantitative analysis was carried out using 3, 5 dinitrosalicylic acid. The concentration of the reducing sugar present in the samples was determined by adding 1cm of 3, 5-dinitrosalicylic acid to 1 cm^3 of each of the samples and boiled for 5 minutes while 10 cm^3 distilled water was added. The absorbance of each of sample was determined at 540nm using JENWAY 6400 spectrophotometer. Thus, the concentration values were extrapolated from the glucose standard curve [14].

2.9. Physicochemical Analysis of Bioethanol

i. Temperature

The temperature of the fermenting rice was taken every 24 hours until the end of fermentation using a laboratory thermometer (glass, alcohol filled, -0°C to 10°C, graduation 1°C with a card board/plastic cover and cotton on both ends). The pH during liquid fermentation was also measured. On the other hand, the alcohol content and total titratable acidity were determined at the end of fermentation period. At the final stage of rice liquor production, the total amount of liquor, alcohol content (%), purified alcohol and percentage yield were determined [15].

ii. pH

pH meter was calibrated using pH buffers 4 and 7. Enough amount of sample was placed in a beaker with volume 100ml and the pH was recorded [15].

iii. Alcohol (%)

One hundred mls (100 ml) of rice liquor was poured into a 100 mL capacity graduated cylinder. This was refrigerated for 15 minutes until the temperature of the liquor reached 15°C. The alcohol meter was allowed to float freely on the sample and then the alcohol content was recorded. The reading was expressed as% alcohol (v/v) (Alan, 2011). The purified alcohol was calculated using the formula [15]:

$$\text{Purified alcohol (L)} = \frac{\text{Volume of Alcohol (L) x Alcohol percentage (\%)}}{100}$$

Yield= It is defined as the quantity of final product per kilogram of raw material used. Yield was calculated using the formula:

$$\text{Yield (L/Kg)} = \frac{\text{Purified alcohol (L)}}{\text{Weight of raw rice (Kg)}}$$

2.10. Separation, Purification and Qualitative Estimation of Bioethanol

In the simultaneous saccharification and fermentation process, the saccharification step and fermentation process was carried out simultaneously for 48 hours. After fermentation, the product mixture was separated into liquids and solid phases using a fabric filter. Thereafter the liquid phase was centrifuged at 5000 rpm for 5 minutes before it was forced through a syringe filter to obtain a clear liquid product [16]. The fermented product was purified using an evaporator to remove residual water and impurities. Purification conditions were studied using a rotary vaccum evaporator, which was operated at a tempreture of 65°C for 45 minutes [16]. Bioethanol production was examined with jones reagent ($K_2Cr_2O_7$+H_2SO_4) 1 ml K_2CrO_7 (2%), 5 ml H_2SO_4 and 3 ml sample was added after incubation. It was observed that ethanol will oxidize to acetic acid with an excess of potassium dichromate in the presence of sulphuric acid, giving off a green colour. The presence of green colour indicates that the used carbon source was able to produce bioethanol after confirmation [7].

3. Results

Table 1 showed the proximate analysis of rice winery cake used for bioethanol production. This analysis was carried out based on six (6) parameters which are moisture, fat, total carbohydrate, protein, energy and ash contents. The proximate values of the total carbohydrate and Energy had high values of 78.04 and 361.98 kcal/g.

Thirteen yeasts isolates were analysed based on the level of ethanol tolerance. Five yeasts isolates were able to thrive on ethanol concentration ranging from (2%-14%), while the rest of the yeasts isolates tolerated ethanol concentration between the range of (2%-8%), but sparingly tolerated the concentration of ethanol from (10%-14%) as shown in table 2.

In table 3 it was observed that there was no significant difference at ($P<0.05$) between the pH of bioethanol produced from different fermenting organisms between day 1 and day 5.

Table 4 illustrates the tempreture analysis during

bioethanol production with no increase in the level of significant difference at (P<0.05) within the different time intervals from the 24th hour to 120th hour. At the 24th hour, *Candida krusei* and *Rhodotorula mucilagnosa* had the highest tempreture of 30°C compared to *Rhodotorula minuta* that had the least tempreture value of 28°C.

Cell density analysis during bioethanol production showed the increase in the microbial load of bioethanol produced. This implies as fermentation increases, the turbidity of the bioethanol increases as a result of the increase in the microbial population involved during fermentation. The level of significant difference at (P<0.05) increases at the various time intervals. At the 24th hour, *Rhodotorula mucilagnosa* had the highest value of cell density of 0.294 compared to *Rhodotorula minuta* that had the least value of 0.199, and there was increase in significant difference at (P<0.05) within this columns as shown in table 5.

Table 1. *Proximate Analysis of Rice Winery Cake after Rice Wine Production.*

Rice winery cake	
Parameters	**Value%**
Moisture	8.12
Ash	2.26
Protein	10.88
Fat	0.7
Total carbohydrate	78.04
Energy	361.98 kcal/g

Table 2. *Ethanol Tolerance Test of Fermenting Isolates.*

Fermentation Isolates	Ethanol concentration						
	2%	**4%**	**6%**	**8%**	**10%**	**12%**	**14%**
R. minuta	++	++	++	++	++	++	++
R. mucilagnosa	++	++	++	++	++	++	++
K. ohmeri	++	++	++	++	++	++	++
C. krusei	++	++	++	++	++	++	++
C. humicola	++	++	++	++	+	+	+
T. mucoides	++	++	++	++	+	+	+
P. ohmeri	++	++	++	++	+	+	+
C. laurentii	++	++	++	++	+	+	+
C. ciferri	++	++	++	++	+	+	+
C. colliculosa	++	++	++	++	+	+	+
C. magnolia	++	++	++	++	+	+	+
C. terrus	++	++	++	++	+	+	+
c. utilis	++	++	++	++	+	+	+

Key:
++ - positive growth
+ - sparingly positive growth
Cryptococcus humicola, *Trichosporon mucoide*, *Pichia ohmeri*, *Cryptococcus laurentii*, *Candida ciferri*, *Candida krusei*, *Rhodotorula minuta*, *Candida pelliculosa*, *Candida magnolia*, *Candida magnolia*, *Rhodotorula mucilagnosa*, *Cryptococcus terrus*, *Rhodotorula minuta*, *Candida utilis*

Table 3. *Effect of Submerged Fermentation on the pH of Bioethanol Produced from Rice Winery Cake.*

Fermentation Isolates	24th h	48th h	72th h	96th h	120th h
Lactobacillus fermentum	2.72±0.40^{a}	2.62±0.60^{a}	2.61±0.20^{a}	2.62±0.20^{a}	2.69±0.50^{a}
Candida krusei	2.71±0.40^{a}	2.53±0.30^{a}	2.60±0.50^{a}	2.65±0.50^{a}	2.65±0.50^{a}
Rhodotorula minuta	2.78±0.40^{a}	2.58±0.10^{a}	2.41±0.30^{a}	2.45±0.30^{a}	2.58±0.30^{a}
Rhodotorula mucilagnosa	2.71±0.06^{a}	2.57±0.24^{a}	2.70±0.27^{a}	2.51±0.27^{a}	2.63±0.25^{a}
Kodamara ohmeri	2.75±0.23^{a}	2.58±0.28^{a}	2.68±0.30^{a}	2.67±0.30^{a}	2.68±0.24^{a}

Values are Mean ± SD: Values with different superscript within the columns are statistically different (P<0.05)

Table 4. *Effect of Submerged Fermentation on the Temperature of Bioethanol Produced from Rice Winery Cake.*

Fermentation Isolates	24th h	48th h	72th h	96th h	120th h
Lactobacillus fermentum	29.00±2.00^{a}	30.00±1.00^{a}	30.00±2.00^{a}	30.00±3.00^{a}	30.00±4.00^{a}
Candida krusei	30.00±1.00^{a}	30.00±1.00^{a}	30.00±2.00^{a}	30.00±2.00^{a}	30.00±1.00^{a}
Rhodotorula minuta	28.00±2.00^{a}	29.00±4.00^{a}	30.00±3.00^{a}	30.00±4.00^{a}	30.00±1.00^{a}
Rhodotorula mucilagnosa	30.00±3.00^{a}	30.00±6.00^{a}	30.00±6.00^{a}	30.00±6.00^{a}	30.00±6.00^{a}
Kodamara ohmeri	29.00±1.00^{a}	29.00±1.00^{a}	30.00±2.00^{a}	30.00±4.00^{a}	29.00±2.00^{a}

Values are Mean ± SD: Values with different superscript within the columns are statistically different (P<0.05)

Table 5. *Effect of Submerged Fermentation on the Cell Density of Bioethanol Produced from Rice Winery Cake.*

Fermentation Isolates	24th h	48th h	72th h	96th h	120th h
Lactobacillus fermentum	0.253±0.026^{ab}	0.373±0.029^{ab}	0.398±0.028^{a}	0.463±0.019^{b}	0.562±0.092^{b}
Candida krusei	0.272±0.047^{b}	0.305±0.072^{a}	0.329±0.019^{a}	0.369±0.060^{a}	0.369±0.009^{a}
Rhodotorula minuta	0.199±0.023^{a}	0.398±0.027^{b}	0.524±0.035^{b}	0.576±0.054^{c}	0.657±0.045^{bc}
Rhodotorula mucilagnosa	0.294±0.034^{b}	0.428±0.062^{b}	0.506±0.080^{b}	0.534±0.026^{bc}	0.557±0.089^{b}
Kodamara ohmeri	0.248±0.039^{ab}	0.411±0.020^{b}	0.682±0.028^{c}	0.732±0.038^{d}	0.732±0.085^{c}

Values are Mean ± SD: Values with different superscript within the columns are statistically different (P<0.05)

At the end of this analysis, it was observed that there was increase with significant difference of the cell density (turbidity). At the 24th hour, *Rhodotorula minuta* had highest value of reducing sugar compared to *Candida krusei* which had the least with values of 0.826 and 0.381 respectively, as shown in table 6.

The specific gravity of a bioethanol decreases which makes alcoholic content of bioethanol to increase. Table 7 and 8 reveals the increase in significant difference at (P<0.05) within the columns of both tables. At the 24th hour, bioethanol produced with *Lactobacillus fermentum* produced more alcohol compared to bioethanol produced with *Candida krusei* with values of 0.57 and 0.13 respectively.

Table 6. *Effect of Submerged Fermentation on the Reducing Sugar of Bioethanol.*

Fermentation Isolates	24th h	48th h	72th h	96th h	120th h
Lactobacillus fermentum	0.592±0.047^{b}	0.180±0.023^{b}	0.179±0.004^{c}	0.127±0.014^{c}	0.112±0.010^{c}
Candida krusei	0.381±0.006^{a}	0.261±0.050^{b}	0.084±0.001^{b}	0.040±0.010^{a}	0.021±0.005^{a}
Rhodotorula minuta	0.826±0.025^{d}	0.725±0.080^{d}	0.077±0.002^{b}	0.053±0.004^{b}	0.053±0.001^{b}
Rhodotorula mucilagnosa	0.699±0.036^{c}	0.596±0.029^{c}	0.046±0.004^{b}	0.041±0.006^{a}	0.012±0.003^{a}
Kodamara ohmeri	0.703±0.046^{c}	0.037±0.002^{a}	0.026±0.002^{a}	0.016±0.003^{a}	0.011±0.001^{a}

Values are Mean ± SD: Values with different superscript within the columns are statistically different (P<0.05)

Table 7. *Effect of Submerged Fermentation on the Alcoholic Content of Bioethanol.*

Fermentation Isolates	24th h	48th h	72th h	96th h	120th h
Lactobacillus fermentum	0.57±0.08^{c}	0.78±0.02^{b}	1.20±0.20^{b}	2.32±0.03^{b}	10.52±1.20^{b}
Candida krusei	0.13±0.04^{a}	0.25±0.03^{a}	0.37±0.02^{a}	2.32±0.01^{b}	6.74±0.80^{a}
Rhodotorula minuta	0.17±0.05^{a}	0.28±0.03^{a}	0.46±0.01^{a}	4.31±0.20^{c}	52.06±1.40^{d}
Rhodotorula mucilagnosa	0.13±0.07^{a}	0.25±0.02^{a}	0.90±0.10^{b}	4.66±0.03^{d}	28.09±1.32^{c}
Kodamara ohmeri	0.37±0.08^{b}	0.78±0.08^{b}	1.20±0.30^{b}	2.14±0.01^{a}	5.32±0.84^{a}

Values are Mean ± SD: Values with different superscript within the columns are statistically different (P<0.05)

Table 8. *Effect of Submerged Fermentation on the Specific Gravity of Bioethanol.*

Fermentation Isolates	24th h	48th h	72th h	96th h	120th h
Lactobacillus fermentum	0.9995±0.0001^{a}	0.9988±0.0002^{a}	0.9982±0.0003^{a}	0.9951±0.0003^{c}	0.9856±0.0011^{c}
Candida krusei	0.9997±0.0001^{ab}	0.9994±0.0002^{b}	0.9987±0.0001^{ab}	0.9951±0.0003^{c}	0.9904±0.0010^{d}
Rhodotorula minuta	0.9999±0.0001^{b}	0.9996±0.0001^{b}	0.9993±0.0004^{c}	0.9937±0.0002^{b}	0.9247±0.0010^{a}
Rhodotorula mucilagnosa	0.9997±0.0001^{ab}	0.9994±0.0002^{b}	0.9988±0.0001^{b}	0.9932±0.0001^{a}	0.9647±0.0010^{b}
Kodamara ohmeri	0.9995±0.0002^{a}	0.9988±0.0002^{a}	0.9982±0.0001^{a}	0.9968±0.0001^{d}	0.9923±0.0010^{e}

Values are Mean ± SD: Values with different superscript within the columns are statistically different (P<0.05)

4. Discussion

The proximate analysis of rice winery cake (RWC) was based on moisture, ash, protein, fat, energy and total carbohydrate, revealed that RWC was low in moisture, fat and ash with the values of 8.12%, 0.7% and 2.26% respectively, but was high in total carbohydrate and protein with values of 78.04% and 10.88% respectively. This findings agrees with the report of [1] who also carried out proximate analysis on RWC for bioethanol production and revealed that the RWC sample was high in starch, protein, and lipids with values of 53%, 5.84%, and 0.62% respectively. The RWC used in this research work was very much high in carbohydrate which can still be utilized by starter microbes and microbial enzymes for bioethanol production.

The fermentation periods had effect on ethanol production. Fermentation time increases with increase in percentage of ethanol contents. This agrees with the findings of [1] who reported that fermentation time had significant effect on ethanol production where the ethanol yield continue to increase from day one (1) to day four (4). [1] further reported that a longer fermentation time beyond four days produces no further increase of ethanol production. It is also in conformity with the findings of [7] who also observed the increase in ethanol concentration during the fermentation time produced from different carbohydrate sources, from day one (1) to day five (5).

Temperature was very much effective during the production of bioethanol produced from RWC using different isolates of lactic acid bacteria and yeasts. The temperature of fermentation was between 28°C to 30°C, and the optimum temperature for bioethanol production was observed to be at 30°C and the percentage of ethanol yield recovered was 52.06%. This agrees with the reports of [1] and [17] that fermentation temperature was found to have significant effect on the ethanol production from RWC where the optimum temperature range for ethanol production was 25°C to 30°C and low ethanol was produce between the ranges of 37°C to 45°C. [17] also reported that 30°C is suitable for saccharification of raw starch by raw starch digesting enzyme of *Rhizopus* sp and ethanol fermentation by starter microbes. This is also in conformity with the findings of [9] who produced ethanol from banana peels using co-cultures of *Saccharomyces cerevisiae* and *Aspergillus niger* and the optimum temperature for ethanol production was 30°C. It also agrees with the report of [18], who observed that 30°C is the optimum temperature for ethanol production from rambutan fruits using *Saccharomyces cerevisiae*. It also agrees with the findings of [19] who observed that ethanol production using *Aspergillus niger* and non-starch digesting and sugar fermenting saccharomyces cerevisiae was

produced at 30°C. It is also in conformity with the findings of [20] who observed that 30°C was the optimum temperature for ethanol production using agricultural waste. It also agrees with the findings of [21] who reported that optimum temperature for ethanol production using banana waste by *Saccharomyces cerevisiae* was 30°C. But it does not agree with the findings of [22] who produced ethanol from cashew apple juice and the optimum temperature was 32°C, this could be as a result of difference in substrates used during the production of bioethanol.

The pH of fermentation during bioethanol production from the use of RWC decreased as the level of ethanol concentration increased. At the initial stage of fermentation, the pH of all RWC samples during the bioethanol production were all at the range of 2.7. At the end of the bioethanol production, one of the RWC samples which was inoculated with pure culture of *Rhodotorula minuta* and used for bioethanol production had a pH of 2.5 with a percentage bioethanol yield of 52.06%. This does not agree with the report of [1] who also produced ethanol using RWC. Their reports revealed that the maximum ethanol produced was 16.1% at pH of 4.5 after 114th hour. [23] reported that pH below 3.0 or above 9.0 produce a sharp decrease in ethanol production. [1] also reported that the optimum pH also varies depending on the substrate used for fermentation stating that pH 4 to 4.5 for sucrose, and pH 2.8 to 3.4 is for sugar cane. The variation of values obtained from these findings could be as result of differences in rice species due to the fact that several species of rice are grown at different geographical regions. The findings in this present research also agrees with [1] who further explained that a low pH is known to prevent microbial contamination while promoting relatively high ethanol productivity.

The cell density during bioethanol production from RWC increased throughout fermentation time resulting to mass yield of ethanol. This could be as a result of the presence of the organisms utilizing the nutrients present in the substrate for growth and ethanol production. This agrees with the findings of [1] who reported that the initial yeast cell number had a marked effect on the ethanol production, more ethanol was produced as the cell density increased. He further reported that a low cell density causes a lower ethanol yield and requires a larger time to complete fermentation whereas if the cell density is high, fermentation would easily be completed. It is also in agreement with the findings of [13], who produced bioethanol from corn cobs, and observed that cell density of the test microorganisms was responsible for the increase of a steady cell density during the fermentation period.

The Analysis of reducing sugar during bioethanol production implies the amount of sugars, microorganisms make use of during fermentation. As the fermentation time increases, sugar present decreases making the environment more acidic and alcoholic. The reducing sugar in the fermentation of the rice winery cake was observed to decrease with increase in fermentation time. This could be as a result of the sugar which serves as a carbon source to these microorganisms, for growth and utilization of sugar resulting to the production of ethanol. This findings agrees with the reports of [13] who produced ethanol from corn cobs and reported that the residual sugar in the fermentation media observed to decrease with increase in fermentation which could be as a result of utilization of sugar by starter microbes present in the substrate.

5. Conclusion

In conclusion, this research work has yield results in the production of bioethanol using RWC which is a waste from rice wine production, as a fermentation substrate and several yeasts and lactic acid bacterium. This indicates that these organisms exhibited good fermentation attributes and were successfully used for bioethanol production using the simultaneous saccharification and fermentation process.

Acknowledgements

The authors of this research would like to thank the staff and students of the department of microbiology (food and industrial research unit) for their scientific contributions towards the success of this work.

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