COMPARATIVE KINETICS OF THE ETHANOL FERMENTATION OF Zymomonas mobilis WITH Saccharomyces cerevisiae USING SUGARCANE AND SWEET SORGHUM SYRUP

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ABSTRACT
Z. mobilis strains (PNCM 1005, 1121, 1832) were used to produce ethanol from diluted sugarcane and sweet sorghum syrup in comparison with the industrial strain S. cerevisiae PNCM 2030. Initial screening experiments using flask fermentation revealed that Z. mobilis had comparable fermenting abilities with the industrial yeast strain in sugarcane, but not in sweet sorghum syrup. A 5-L Sartorius BIOSTAT A-plus fermenter with a working volume of 2.5 L was used to determine the fermentation profiles and ethanol production kinetic parameters of the bacterial and yeast strains. Results showed that Z. mobilis PNCM 1005 gave the best performance using sugarcane syrup as substrate, with fermentation efficiency of 89%. On the other hand, the industrial yeast performed much better than the bacterial strains in sweet sorghum syrup, giving better fermentation efficiency after 48 hrs.

KEYWORDS: Bioethanol, fermentation, Zymomonas mobilis, Saccharomyces cerevisiae, yeast, sugarcane, sweet sorghum, green technology

1. INTRODUCTION
Bioethanol has been identified worldwide as an alternative or supplementary fuel to compensate for the dwindling supply of fossil fuels. In the Philippines, the Biofuels Act of 2006 mandated 5% and 10% addition of locally-produced bioethanol to gasoline in 2007 and 2011, respectively. However, local supply is still very minimal and bioethanol is still mostly imported. Saccharine crops, such as sugarcane and sweet sorghum, are still the feedstock materials of choice since the technology for lignocellulosic biomass conversion to bioethanol is still difficult and costly.

In the Philippines, according to its Department of Energy (DOE), sugarcane is the most readily available feedstock, with sweet sorghum and cassava as other potential substrate materials. But sugarcane is mainly for sugar production and large production of ethanol from sugarcane blackstrap molasses are mainly for alcoholic beverages unlike Brazil, where they largely produce ethanol (~79%) from sugarcane for biofuel [1]. Countries like India and United States also primarily use sugarcane as the main feedstock for the production of ethanol for transportation [2].

In response to this, the Philippines tries to cope up with the increasing demand for bioethanol for biofuel use without opting for importation by searching for other potential substrates. Sweet sorghum is considered to be one of convenient substrates for manufacturing alcohol, as it exhibits great potential since it has better characteristics than sugarcane, which leads to lower ethanol production costs [3]. Based on ICRISAT study in 2005, the cost of cultivation is three times lower than that of sugarcane.

The foremost concern for selecting the right substrate material is evaluating the appropriate biological material for each fermentable substrate. Even though there
are a vast number of known yeast strains that can accumulate high concentrations of ethanol, the baker’s yeast *Saccharomyces cerevisiae* is still the most employed yeast strain in utilizing different feedstock materials [4]. This organism is able to grow in concentrations of 8-12% (v/v), to survive exposure to concentrations of up to 15% (v/v), and to ferment glucose to produce ethanol up to concentrations of around 12% (v/v) for normal fermentations and up to 20% (v/v) during sake fermentations [5]. As with many microorganisms, *S. cerevisiae* metabolizes glucose by Embden-Meyerhof (EM) pathway. However, some bacteria are also able to ferment sugary materials with superior fermentation efficiencies. *Zymomonas mobilis* is an unusual Gram-negative microorganism that has several appealing properties as a biocatalyst for ethanol production. The microorganism has a homoethanol fermentation pathway and tolerates up to 120g/l ethanol. It has a higher ethanol yield (5-10% more ethanol per fermented glucose) and has a much higher specific ethanol productivity (2.5x) than *S. cerevisiae* sp. [6]. The high ethanol yield and productivity observed for *Zymomonas* are a consequence of its unique physiology [4]. *Zymomonas* is the only microorganism that metabolizes glucose anaerobically using the Entner-Doudoroff (ED) pathway [6,7]. The ED pathway yields only half as much ATP per mole of glucose as the EM pathway. As a consequence, *Zymomonas* produces less biomass than yeast, and more carbon is funnelled to fermentation products. Also a consequence of the low ATP yield, *Zymomonas* maintains a high glucose flux through ED pathway [6]. Despite its advantage as an ethanologen, *Z. mobilis* is not well suited for all the biomass resources conversion because it ferments only glucose, fructose or sucrose [4,8].

The National Institutes of Molecular Biology and Biotechnology at the University of the Philippines Los Baños (BIOTECH, UPLB) has embarked on research activities aimed to provide support to the country’s Bioethanol Program, and to give the Philippines a competitive edge in ethanol production. In this study, the fermentation profile of *Z. mobilis* strains using sugarcane and sweet sorghum syrup was evaluated and compared to that of *S. cerevisiae* 2030.

2. MATERIALS AND METHODS

**Yeast and Bacterial Isolates, and Culture Media.** Three strains of *Z. mobilis* (PNCM 1005, 1121, 1832) with high ethanol production in flask fermentation screening were used to compare with *S. cerevisiae* 2030 in a bioreactor system. Three *Z. mobilis* strains were microaerophilically grown and maintained at 4°C on Roger’s Medium (RM) (Yeast extract, 10g; Glucose, 20g; Potassium phosphate (KH2PO4), 2g; Agar, 20g per liter). *S. cerevisiae* was grown and maintained at 4°C on Yeast Extract Peptone Dextrose or YEPD (Yeast extract, 10g; Bactopeptone, 20g; Glucose, 20g; Agar, 20 per liter water) slants. All organisms were obtained from the Philippine National Culture Collection (PNCM), BIOTECH, UP Los Baños.

Sugarcane and sweet sorghum syrup were obtained from Central Azucarera de Tarlac (a sugar factory) and Mariano Marcos Memorial University, respectively.

**Successive Inoculum Build-up for Batch Bioreactor Fermentation.** Seed inoculum with increasing sugar content (5%, 10% and 15% w/v total sugar) were prepared, supplemented with the following nutrients per 1 L of water: 1.4g KH2PO4, 0.25 MgSO4, and 1.0 (NH4)2SO4. pH was adjusted to 5.0 and 4.5 (for bacteria and yeast, respectively). Inoculum build-up was started with an 18 hrs old culture in RM broths (bacteria) and YEPD slant (yeast). Successive inoculations from 5% to 15% sugarcane and sweet sorghum were maintained at 10% v/v and were all
incubated at 30°C for 18-20hrs with (yeast) and without shaking (bacteria).

**Sterile Bioreactor Runs.** 5 L Biostat Sartorius A Plus was used for all the bioreactor runs, both for bacterial and yeast strains. The 2.25 L fermentation media was prepared by diluting sugarcane and sweet sorghum syrup to an initial sugar content of 20% w/v with the usual nutrients. The pH of the media was adjusted to 5.0 and 4.5 (bacteria and yeast, respectively). Corn oil (5% of total volume) was then added as antifoam for bioreactor runs using yeast and then sterilized for 15 min at 15 psi.

Fermentation broths were inoculated with approximately 10^8 cells/mL seed inocula from 15% w/v total sugar. Cells were concentrated by centrifugation. Seed inoculum was then transferred to the fermentor at 20% v/v inoculation level. Fermentation parameters were maintained as follows: temperature of 30°C, pH of 5.0 (bacteria) and 4.5 (yeast), initial agitation rate of 50rpm (bacteria) and 200rpm (yeast). Fermentation runs were monitored for 48-hrs and samples were drawn every 2-hrs for the first 24-hrs and every 4-hrs thereafter. Samples were collected for the analysis of ethanol content, residual sugar content, and viable cell count. Time profiles for each variable was then constructed to calculate for the fermentation kinetic parameters.

**Analytical Methods.** The percent ethanol concentration was measured using Shimadzu GC-8A gas chromatograph with the following settings: nitrogen gas as carrier at 60mL/min; air pressure at 0.5 kg/cm²; H2 pressure at 0.7 kg/cm²; column temperature set at 180°C; detector temperature set at 200°C and Porapac Q as column material. Sugar concentration (initial and residual) was measured by phenol-sulfuric acid assay using Shimadzu UV-VIS spectrophotometer. Cell counts were obtained by plating samples at appropriate media.

### 3. RESULTS AND DISCUSSION

**Sugarcane Syrup as Substrate.** Figure 1 shows the ethanol concentration, residual sugar and viable cell count profiles of bacterial isolates along with that of the yeast. In Figure 1A, strain 2030 produced ethanol concentrations of 9.83% and 12.10% (v/v) after 24 and 48-hrs. Compared to the yeast strain 2030, *Z. mobilis* 1005 exceeded ethanol product with ethanol concentrations of 11.71% (v/v) and 12.64% (v/v) after 24- and 48-hr, respectively. Fermentation can be terminated as early as 16th or 18th hour for 1005 attaining high ethanol concentrations between 9-10% (w/v).

The ethanol produced by *Z. mobilis* strains 1832 and 1121 after 24-hrs, were comparable to that of *S. cerevisiae* strain 2030. The average ethanol production rates for the first 24-hr of strains 1832 and 1121 were 2.90 and 3.05 mg ethanol/ml-hr, respectively. The residual sugar concentration profiles for the 4 strains are shown in Figure 1B. A significant difference between the residual sugar concentration of the bacterial strains and the yeast strain was observed throughout the course. The average sugar consumption rate from 0-24 hours of strain 1005 was the highest at 8.57 mg total sugar/ml-hr compared to strains 1832 (7.99), 1121 (7.98) and 2030 (7.90).

Different growth phases of the bacterial strains and the yeast strain 2030 were observed using sugarcane medium as the substrate (Figure 1C). Increase in cell concentration was most profound for bacterial strain 1005. This was not expected because *Z. mobilis* strains usually produce less biomass compared to *S. cerevisiae*. However, strain 1005 maintained high rate of ethanol production in the 48-hr fermentation run.

All *Z. mobilis* strains had a very low cell count at the end of the 48 hour bioreactor runs, which may be due to the depleting
nutrient components of the substrate or due to non optimal growth of the bacterial strains in high ethanol concentration. Meanwhile, the yeast strain 2030 showed a constant number of viable cells until the end of the run indicating that this yeast isolate is very robust, withstanding low nutrient levels and high ethanol concentration.

![Ethanol concentration (A), residual sugar content (B), and viable cell count (C) over time (hours) using sugarcane medium.](image)

**Figure 1.** Ethanol concentration (A), residual sugar content (B), and viable cell count (C) over time (hours) using sugarcane medium (2030, 2032, 1121, 1005).

**Sweet Sorghum Syrup as Substrate.** Ethanol, residual sugar and viable cell count profiles of *Z. mobilis* 1832, 1121 and 1005 along with that of *S. cerevisiae* 2030 using sweet sorghum are shown in **Figure 2**. Trends for ethanol and sugar concentrations were the same as for the sugarcane medium. Cell concentration did not show any trend but were erratic for yeast strain 2030 and two *Z. mobilis* strains 1121 and 1005. Strain 1832 was the only strain that exhibited a negative slope meaning there was no growth and cell death progressed from the start up to the end of the bioreactor run.

The rate of ethanol production by 2030 was way better than the bacterial strains as can be seen in **Figure 2A**. It attained 9.93 % and 10.40% v/v of ethanol concentration after 24- and 48-hrs of the fermentation run.

![Ethanol concentration (A), residual sugar content (B), and viable cell count (C) over time (hours) using sweet sorghum.](image)
Table 1. Summary of kinetic parameters for S. cerevisiae 2030, Z. mobilis 1832, 1121, and 1005 using sugarcane and sweet sorghum medium.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sugarcane Medium</th>
<th>Sweet Sorghum Medium</th>
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<tbody>
<tr>
<td></td>
<td>2030</td>
<td>1832</td>
</tr>
<tr>
<td>Ethanol Yield Coefficient with respect to Cell Concentration at 1st exponential phase, $Y_{E/X}$ (g EtOH/cell)</td>
<td>1.00E-09</td>
<td>4.00E-10</td>
</tr>
<tr>
<td>Specific ethanol productivity at 1st exponential phase, $Q_E$ (g EtOH/cell-hr)</td>
<td>1.45E-10</td>
<td>5.16E-11</td>
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<td>Ethanol Yield Coefficient with respect to Substrate consumed, $Y_{E/S}$ (g EtOH/g TS consumed)</td>
<td>0.421</td>
<td>0.463</td>
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<tr>
<td>Ethanol Concentration after 24 hours (%v/v)</td>
<td>9.83</td>
<td>9.21</td>
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<tr>
<td>Ethanol Concentration after 48 hours (%v/v)</td>
<td>12.10</td>
<td>12.50</td>
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<tr>
<td>Overall Fermentation Efficiency (%)</td>
<td>77.94</td>
<td>85.69</td>
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<tr>
<td>Volumetric Ethanol Productivity after 24 hours (g EtOH/L-hr)</td>
<td>3.19</td>
<td>2.90</td>
</tr>
<tr>
<td>Overall Volumetric Ethanol Productivity (g EtOH/L-hr)</td>
<td>1.97</td>
<td>1.99</td>
</tr>
</tbody>
</table>

The residual sugar concentration profiles for the 4 strains are shown in Figure 2B. A significant difference between the residual sugar concentration of the bacterial strains and the yeast strain was observed throughout the course of the fermentation run, though all exhibit a downward slope for each curve. The average sugar consumption rate from 0 to 24-hrs of yeast strain 2030 was highest at 3.56 mg total sugar/ml-hr compared to bacterial strains.

In Figure 2C, initial cell count of 1832 continued to drop after the start of the inoculation up to 14 hours then levelled off with cell counts of 6.50E+05 to 4.50E+05 (14 to 28 hours). After 28 hours, the number of viable cells continued to drop until it reached 2.50E+04 cells/mL. The higher biomass production of 2030 resulted from the sequential utilization of the sugars present in the sweet sorghum syrup resulting in many exponential growth phases in the fermentation run.

All bacterial strains had a less than one percent (1% v/v) ethanol concentration after 10-hrs of fermentation compared to 2030 with 4.33% v/v. Strain 1832 only reached 1% v/v ethanol concentration after 24-hrs and only doubled after 48-hrs with 2% v/v. Using sweet sorghum media, 2030 is a superior fermentative microorganism than the bacterial strains.
Fermentation Kinetic Parameters.
Kinetic parameters were computed for the bacterial strains and yeast strain, obtained from the gathered raw data from the bioreactor profile of each strain using sugarcane and sweet sorghum (Table 1).

All the values for ethanol yield coefficient with respect to cell concentration ($Y_{E/X}$) of all bacterial strains for both sugarcane and sweet sorghum revealed that they have lower ethanol production, relatively due to their smaller physiological size compared to that of yeast cells. However, in sugarcane medium, the product yield with respect to substrate consumed ($Y_{E/S}$) were generally higher for all bacterial strains, ranging from 0.451 to 0.487, than 2030 (0.421 g EtOH/ g TS consumed), suggesting that bacterial strains have more efficient fermentative utilization.

The fermentation efficiency of strain 1005 was 90.17%, compared to 2030 which only had a value of 77.94% using the sugarcane medium. All bacterial strains had higher fermentation efficiencies than the yeast strain ranging from 85 to 90%.

Strain 2030 exhibited the highest ethanol produced (9.93% v/v), $Y_{E/X}$ (1.00E-09), and $Y_{E/S}$ (.548) values using sweet sorghum medium. This shows that strain 2030 is more efficient fermentative microorganism for sweet sorghum.

Fermentation efficiencies of the bacterial strains after 24-hrs did not even reach 50% and volumetric ethanol productivity values of the three bacterial strains barely reached the value of 1 g EtOH/L-h after 48-hrs. These obtained values suggest that bacterial strains of *Z. mobilis* are not better fermentative microorganisms for sweet sorghum as substrate for bioethanol production.

4. CONCLUSION

Some of these tested *Z. mobilis* strains can produce more ethanol than the industrial yeast strain 2030 using sugarcane syrup as substrate. Highest maximum ethanol produced was obtained from strain 1005 followed by strains 1832, 2030 and 1121 using sugarcane medium. This suggests that bacterial strains can be an option as a good ethanologenic microorganism for sugarcane medium as a substrate. Ethanol concentration of 11.71% v/v produced by 1005 after 24 hrs was significantly higher than that of 2030 (9.83% v/v). This is a desirable characteristic of an industrial microorganism with high ethanol production at a shorter period of time, maximizing the productivity potential in a large scale fermentation system. However, industrial yeast strain 2030 is still more suitable ethanologenic microorganism for fermentation using sweet sorghum syrup.

5. ACKNOWLEDGMENT

The authors wish to thank the Philippine Council for Industry, Energy and Emerging Technology Research and Development (PCIEERD), Department of Science and Technology (DOST) for the research funds.

6. REFERENCES


