

Full Length Research Paper

Evaluation of ethanol production from pito mash using *Zymomonas mobilis* and *Saccharomyces cerevisiae*

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Received 12 October, 2015, Accepted 30 June, 2016.

This study investigated the potential of pito mash (waste from sorghum brewing) as alternative and cost-effective feedstock for bioethanol production by means of fermentation using *Zymomonas mobilis* and *Saccharomyces cerevisiae* isolated from freshly tapped palm wine. Fermentation parameters such as pH, temperature and incubation period were studied. The fermentation microbes, *Z. mobilis* and *S. cerevisiae* were identified using API™ test kit and morphological characteristics, respectively. Analysis of reducing sugar residue was performed using dinitrosalicylic acid (DNS) method, while analysis of ethanol content was performed using gas chromatography. Pito mash recorded total starch content of 6.69%, reducing sugar content of 11.1 mg ml⁻¹ and cellulose content of 0.41 mg g⁻¹. Saccharification by malting increased reducing sugar content by 77.9% (19.75 mg ml⁻¹). The optimum fermentation conditions (pH, temperature and incubation period) for *Z. mobilis* and *S. cerevisiae* were 5.5, 35°C, 3 days and 6.0, 30°C, 4 day, respectively. The maximum ethanol yield of 3.03 g l⁻¹ and efficiency of 62% were obtained for *S. cerevisiae* while yield of 3.63 g l⁻¹ and efficiency of 74.2% were obtained for *Z. mobilis*. *Z. mobilis* conclusively may be better organism for ethanol production from pito mash.

Key words: Pito mash, agro-industrial wastes, *Zymomonas mobilis*, ethanol, reducing sugars.

INTRODUCTION

Energy consumption has increased steadily over the last century as the world's population increases, and more and more countries become industrialized. The traditional source of fuel, fossil fuel is continuously being depleted irrespective of the new geographical discoveries. There have also been concerns about the pollution and various

health risks associated with the use of petroleum as fuel. In view of these, the importance of alternative energy source has become even more necessary not only due to the continuous depletion of the limited fossil fuel stock but also for safe and better environment (Chandel et al., 2007). The interest in biomass as the alternative source

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of energy is gaining momentum more and more over the last century. Production of bioethanol from biomass is one of the alternative sources of fuel that has gained a lot of attention over the past years. Ethanol produced from renewable energy source is the most promising future biofuel (Marszalek and Kaminski, 2008). To be a viable substitute for a fossil fuel, Hill et al. (2006) contended that an alternative fuel should not only have superior environmental benefits over the fossil fuel it displaces, be economically competitive with it, and be producible in sufficient quantities to make a meaningful impact on energy demands, but it should also provide a net energy gain over the energy sources used to produce it. Bioethanol meets most of these criteria but the quantity of ethanol produced annually has not overtaken petroleum.

The use of starchy crops such as cereals and tubers as substrates for the production of ethanol has however, been reported to be much costly and non-sustainable accounting for about 70% of ethanol production (Ramesh et al., 2004). This drawback has caused an emergence of alternative substrates such as agro-industrial wastes that are relatively abundant in the environment and serve as renewable energy resources. Some of these agro-industrial wastes are rice straw, sugarcane bagasse, corn cob, pito mash, rice husk, municipal wastes to mention a few (Ramesh et al., 2004). The problem, however, is the technology for conversion of the lignocellulosic part of these materials to bioethanol. The choice of the best technology for the conversion of lignocelluloses to bioethanol should be decided on the basis of overall economics (lowest cost), environmental (pollutants), and energy (higher efficiencies). Many investigations have been performed on the appropriate technology for the conversion of the lignocellulosic to ethanol as well as substrate with little or no lignin such as molasses (Chandel et al., 2007), but research work on the utilization of pito mash has been very limited.

Even though several microorganisms, including *Clostridium* species, have been considered as ethanologenic microbes, the yeast *Saccharomyces cerevisiae* and facultative bacterium *Zymomonas mobilis* are better candidates for industrial alcohol production (Castro, 2013). Traditionally, *S. cerevisiae* has been used for the production of ethanol; however, it has been associated with low alcohol tolerance and low productivity which for efficient ethanol production requires improvement. *Z. mobilis*, a gram negative bacterium possesses advantages over *S. cerevisiae* with respect to ethanol productivity and tolerance. *Z. mobilis* strain grown under anaerobic conditions can produce about 1.5 to 1.9 mol of ethanol from each mol of glucose, which is much better than ethanol produced by *S. cerevisiae* (Gonzalez-Sanchez et al., 2011). *Zymomonas* grows and ferments glucose very fast; its preference for low pH prevents contamination and grows in high glucose and ethanol concentration (He et al., 2014). The present study

focuses on the potential use of pito mash as a substrate for both *Z. mobilis* and *S. cerevisiae* for bioethanol production under optimum fermentation conditions such as temperature, pH and incubation periods.

MATERIALS AND METHODS

Sample collection

The substrate for the fermentation, pito mash was obtained from local pito brewers in Kumasi in the Ashanti Region of Ghana.

Standard solid media

Five hundred milliliters of standard media was prepared by dissolving 2.5 g of yeast extract and 10 g of glucose in 500 ml conical flask containing 150 ml distilled water. Exactly 10 g of agar was then added, made up to mark and autoclaved at 121°C for 15 min.

Synthetic media

Synthetic media containing g/L: K_2HPO_4 1.0 g, $(NH_4)_2SO_4$ 1.0 g, $MgSO_4$ 0.5 and 20 g glucose was prepared in 1 L volumetric flask with 750 ml of distilled water, made up to mark and autoclaved at 121°C for 15 min.

Malt yeast peptone glucose media

Five hundred milliliters of MYPG was prepared by dissolving 1.5 g of yeast extract, 2.5 g peptone, 1.5 g malt extract, and 10 g of glucose in 500 ml conical flask containing about 150 ml distilled water. Exactly 10 g of agar (melted) was added and made up to mark and autoclaved at 121°C for 15 min. The pH of each medium was adjusted to the appropriate pH values using 1 N NaOH and 1 N HCl.

Sorghum base medium (SBM)

The SBM was prepared substituting glucose and malt extract with pito mash in the MYPG medium

Isolation and identification of microorganisms

Z. mobilis was isolated from palm wine using the method of Cheesbrough (2003). The inoculated plates were incubated at 30°C for 3 days. Pure colonies were obtained from re-cultivation in MYPG agar. Purified isolates from fresh plates of MYPG medium were identified as *Z. mobilis* using API™ test kit. *S. cerevisiae* was serially diluted and the sediment inoculated in standard media supplemented with chloramphenicol (0.05 mg/L) (Nwachukwu, 2001) and incubated at 28°C for 24 h. Colonies suspected to be yeast were purified and morphological and fermentable sugars were determined.

Malting and mashing of sorghum grains

Sorghum cultivar grains were washed and steeped in 0.2% sodium hydroxide and water for 8 and 16 h, respectively at room

temperature. The steeped grains were allowed to germinate at room temperature for 4 days with daily sprinkling of water. The rootlets were broken and kept in oven for 24 h at 50°C. In the mashing process, a mixture of 20% malt and 80% mash was slurred and pre-heated to 45°C for 30 min after being raised to 100°C for 1 h. Complete saccharification was determined by iodine test.

Fermentation process

The procedures were adopted from Dowe and McMillan (2008). Fermentation was carried out in 500 ml Erlenmeyer flasks. The fermentation lock or bubble trap consisted of rubber stopper (with hole) through which a tube was inserted. A cotton plug was inserted in the tube and the tube was connected to silicone tubing. The other end was submerged in a test tube containing water. All mashes were cooled to a temperature between 27 and 30°C after liquefaction and saccharification and the pH adjusted with HCl. Saccharified mashes were then inoculated with 10 ml pre-culture *S. cerevisiae* and *Z. mobilis* in separate setups. Fermentation was performed in an incubator with intermittent shaking at optimized conditions. The fermentation process was monitored by measuring the sugar and ethanol contents.

Ethanol concentration was estimated by Gas Chromatography and sugar content was measured using 3,5-dinitrosalicylic acid (DNS) method. The expected ethanol amount was calculated after fermentation stoichiometry, assuming that 1.0 g of total sugars produced 0.511 g of ethanol. The efficiency of reducing sugar conversion into ethanol by both microorganisms (%) expresses the amount of produced ethanol relative to the theoretical quantity expected based on the sugar content of the malted sorghum, and it was calculated accordingly with the following equation:

$$\text{Efficiency (\%)} = \frac{\text{Ethanol produced (g/l)}}{[\text{TRS}_i - \text{TRS}_f]} \times 100$$

where TRS_i is the initial sugar content before fermentation and TRS_f is the final sugar content after fermentation (Carter, 2014)

Optimization of pH

Ten milliliters of the mashed sorghum was placed in different test tubes and pH adjusted to ranges of 4.0, 4.5, 5.0, 5.5 and 6.0 using 1 N HCl or 1 N NaOH.

Optimization of temperature

Ten milliliters of the mashed sorghum was placed in different test tubes at varying initial temperature values of 30, 35, 40 and 45°C.

Optimization of incubation period

Ten milliliters of the mashed sorghum was placed in different test tubes and incubated at 30°C, pH 4.5 and periods of 24, 48, 72, 96 and 120 h.

Chemical analysis

The amount of reducing sugars was estimated by dinitrosalicylic acid (DNSA) using methods described by Negulescu et al. (2012). Ethanol concentration was determined using a Perkin Elmer,

Autosystem XL, Gas Chromatograph (USA) equipped with a flame ionization detector (FID), coupled to a Yokogawa 3021 Pen recorder.

RESULTS AND DISCUSSION

Ethanol produced from spent sorghum using *S. cerevisiae* and *Z. mobilis* separately

S. cerevisiae and *Z. mobilis* were employed in fermenting pito mash hydrolysate containing 19.75 g ml⁻¹ reducing sugar. In both organisms, a continuous increase in ethanol yield was accompanied with decreased reducing sugar concentration during the whole period of fermentation (Figures 1 and 2). The fermentation with *Z. mobilis* proceeded very rapidly and was essentially completed in three days with maximum yield of 3.63 g l⁻¹. Fermentation with *S. cerevisiae* required three days to complete with a yield of 3.03 g l⁻¹. In all cases, the sugar utilization was faster in *Z. mobilis* than in *S. cerevisiae*. T-test analysis showed significant difference between the amounts of sugar utilized by *Z. mobilis* and *S. cerevisiae* on each day at 95% confidence interval. This indicates that the utilization of reducing sugar on each day is dependent on the microorganism used. In both cases, the percentage yield of ethanol produced at each fermentation time examined using *Z. mobilis* was higher compared to that using *S. cerevisiae*. Bacteria are known to multiply faster than yeast thus *Z. mobilis* might reached the lag phase faster than *S. cerevisiae* and therefore utilized its substrate faster. The ethanol yield for *Z. mobilis* was higher than that of *S. cerevisiae* at all fermentation periods. *S. cerevisiae* is known to employ the EMP pathway to metabolize glucose producing 2 moles of ATP from 1 mole of glucose whereas *Z. mobilis* employing the E-D pathway produces 1 mole of ATP from 1 mole of glucose (Wang et al., 2014). Ming et al. (2014) reported that a significant amount of the carbon source is converted into biomass as a result of the E-D pathway used by this microorganism. All the enzymes involved in fermentation are expressed constitutively, and fermentation enzymes comprise as much as 50% of the cells' total protein (Ming et al., 2014). *Z. mobilis* maintains a high level of glucose flux through the pathways to compensate for its low yield (Rutkis et al., 2013). Clark et al. (2013) reported that there is linearity between maximum cell growth) and ethanol production with *S. cerevisiae* strains. However, *Z. mobilis* perform less biomass formation and efficient production of ethanol compared to *S. cerevisiae* (Sootsuwan et al., 2013). The low ethanol conversion efficiency by *S. cerevisiae* might therefore be due to the fact that a portion of the substrate was converted to cell mass and other products. Although liquefaction and saccharification might probably kill some microorganism that might cause contamination, both organisms were able to metabolize their substrate faster

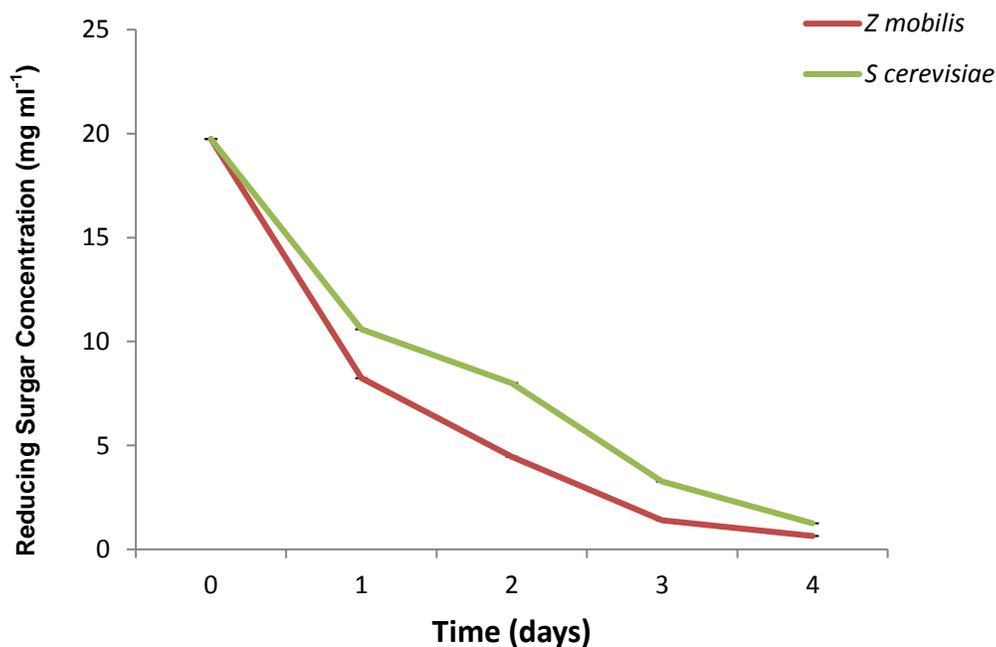


Figure 1. Sugar utilization by *Z. mobilis* and *S. cerevisiae*.

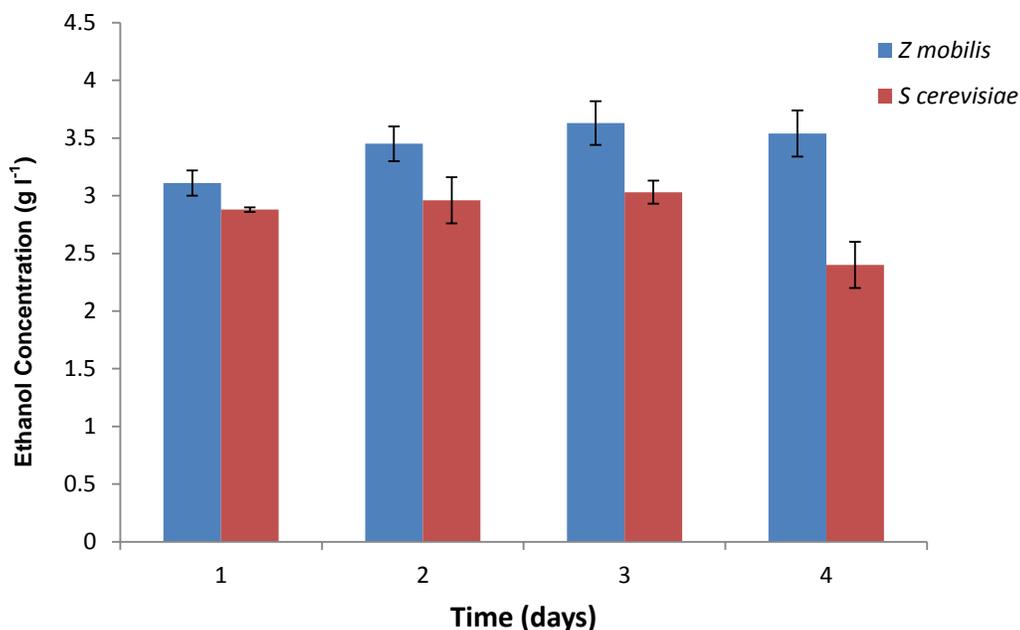


Figure 2. Ethanol produced from pito mash using *S. cerevisiae* and *Z. mobilis* separately.

thus competitively inhibiting the growth of other microorganisms. They can therefore be used to produce ethanol using non-sterile substrate. This could reduce energy cost involved in sterilizing the substrate. According to Tao et al. (2005) and Aggarwal et al. (2001),

cheap raw material, low processing cost and high productivity are the main considerations for most ethanol production. This work therefore shows that under appropriate conditions pito mash can be used as alternative and cost-effective feed stock for the

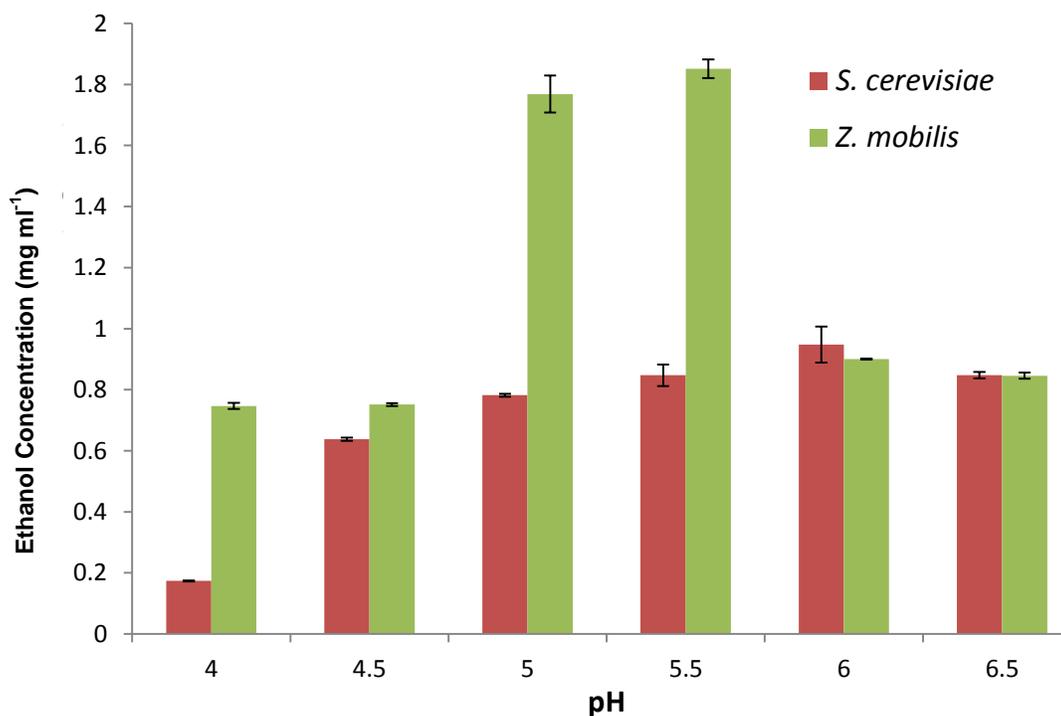


Figure 3. Effect of pH on ethanol production by *S. cerevisiae* and *Z. mobilis* using pito mash as substrate.

production of bioethanol without supplementing the fermentation broth with other nutrients. The substrate was able to support the growth of both organisms without the addition of nutrients indicating that pito mash has high carbohydrate (starch) and protein content. The products of saccharification of the pito mash also did not inhibit ethanol production by *Z. mobilis* and *S. cerevisiae* as indicated by the high fermentation efficiencies. This is advantageous compared to lignocellulosic materials which require pretreatment that sometimes leads to production of inhibitory compounds that, in turn, decrease ethanol production by the organisms (Chandel et al., 2007).

Optimization of pH

Generally, ethanol concentration increased with increased pH in both *S. cerevisiae* and *Z. mobilis*. However, the increase was more pronounced in *S. cerevisiae* than *Z. mobilis*. For *S. cerevisiae*, ethanol concentration began to increase with increased pH till it reached maximum at pH of 6, and then decreased at pH 6.5 (Figure 3). In the case of *Z. mobilis*, fermentation took place at pH of 4 and gave higher ethanol concentration compared to ethanol concentration produced by *S. cerevisiae* at the same temperature. Ethanol concentration reached maximum at pH 5.5, beyond which

it began to decrease. In the case of *S. cerevisiae*, there was significant ($p < 0.05$) difference between the ethanol produced at all pH values. Optimum pH for ethanol was between 6.0 and 6.5 with pH of 6.0 producing the maximum ethanol volume of 0.948 mg ml^{-1} for *S. cerevisiae*. For *Z. mobilis*, there was significant difference in ethanol produced all pH values. The optimum pH was between 5.0 and 5.5 with 5.5 producing the highest ethanol of 1.85 mg ml^{-1} . In all cases, ethanol produced by *Z. mobilis* was higher compared to *S. cerevisiae*. The result agrees with observation by Hwang et al. (2004) who reported that the activities of ethanol producers are slightly suppressed at pH below 4.5.

Optimization of temperature

In *Z. mobilis*, there was initial increase in ethanol concentration with temperature increase from 30 to 35°C; however, beyond 35°C increasing temperature became inhibitory to ethanol production (Figure 4). The decrease was more pronounced at 45°C. *S. cerevisiae* produced maximum amount of ethanol at 30°C and further increase in temperature (35 to 45°C) was inhibitory to its ethanol production ability. Analysis of variance indicated that for *S. cerevisiae*, there was significant ($p < 0.05$) difference in the ethanol produced at each temperature. However, there was no significant ($p < 0.05$) difference in ethanol

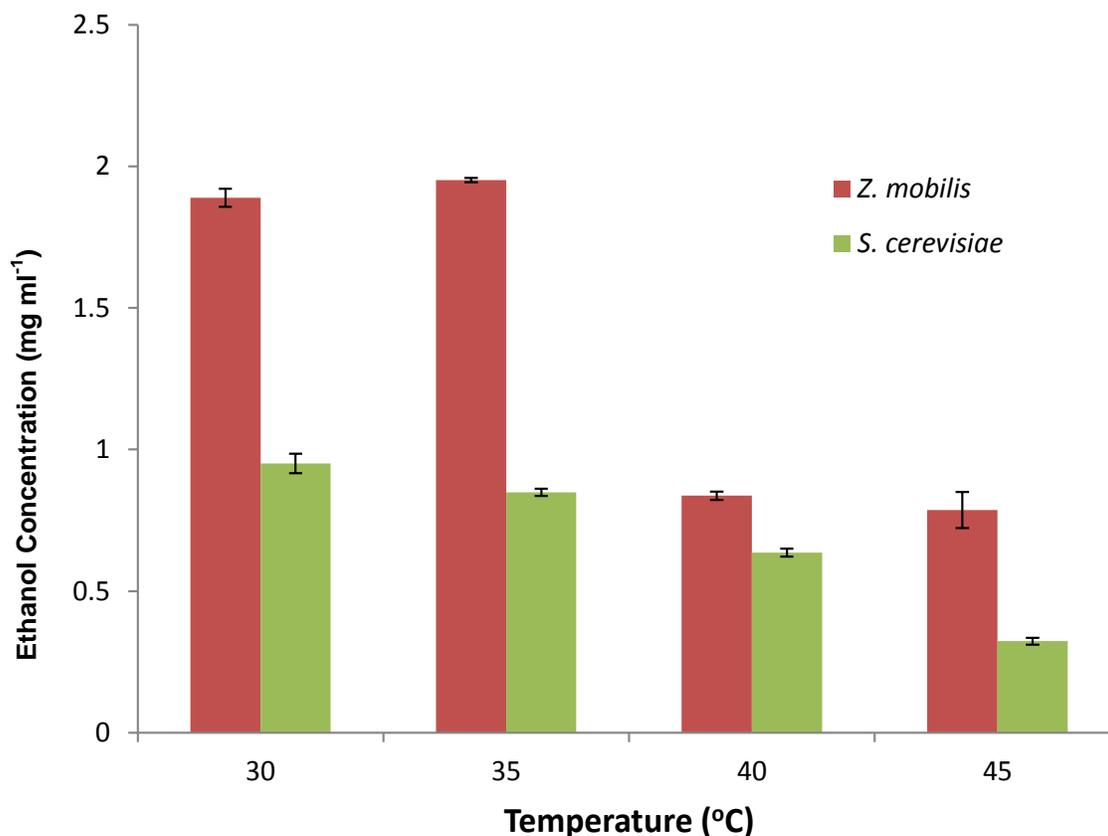


Figure 4. Effect of temperature on ethanol production by *S. cerevisiae* and *Z. mobilis* using pito mash as substrate.

produced at the temperature of 35 to 45°C. The highest concentration (0.951 mg l⁻¹) was produced at temperature of 30°C for *S. cerevisiae*, followed by 0.849 mg l⁻¹ at 35°C. The lowest volume (0.323 mg l⁻¹) was produced at 45°C. In the case of *Z. mobilis*, there was significant ($p < 0.05$) difference between ethanol produced at all temperatures. However, there was no significant ($p < 0.05$) difference between ethanol produced at 30 to 35 and 40 to 45°C. The highest concentration of 1.951 mg l⁻¹ was produced at temperature of 35°C followed by 1.889 mg l⁻¹ at the temperature of 30°C. At all temperature values, the concentration of ethanol produced at each fermentation examined using *Z. mobilis* was significantly different from using *S. cerevisiae*. Similar observations were made by Panesar et al. (2007). It was also indicated that, decrease in the membrane phospholipids content may be responsible for the unique thermal sensitivity of *Z. mobilis* cells grown at higher temperature (Panesar et al., 2007).

Optimization of fermentation period

As shown in Table 1, maximum ethanol production of

0.855 mg ml⁻¹ for *S. cerevisiae* was observed on the fourth day of fermentation whereas maximum ethanol production of 1.269 mg ml⁻¹ was observed for *Z. mobilis* on the third day of fermentation. In both organisms, there was a sharp increase in ethanol concentration within the first two days of fermentation period (Figure 5). Generally, there was a decline of ethanol production after the optimum for both organisms (Figure 5) which could be attributed to the build-up of inhibitory toxins produced in the fermentation medium as reported previously by Zakpaa et al. (2009).

Conclusion

Pito mash (waste from sorghum brewing) was a suitable substrate for bioethanol production by *Z. mobilis* and *S. cerevisiae* since high yield of ethanol was produced. However, *Z. mobilis* (3.63 g l⁻¹) demonstrated higher biomass conversion efficiency, hence higher ethanol concentration compared to *S. cerevisiae* (3.03 g l⁻¹). Pito mash could therefore be used for large scale bioethanol production, hence reducing its threat to the environment.

Table 1. Effect of fermentation period on ethanol production.

Fermentation period (days)	Ethanol concentration/ mg ml ⁻¹	
	<i>Saccharomyces cerevisiae</i>	<i>Zymomonas mobilis</i>
1	0.199 ± 0.10 ^a	0.196 ± 0.030 ^a
2	0.748 ± 0.04 ^b	0.760 ± 0.033 ^b
3	0.838 ± 0.01 ^b	1.269 ± 0.063 ^{b,c,d}
4	0.855 ± 0.007 ^b	0.844 ± 0.047 ^{b,e}
5	0.747 ± 0.06 ^b	0.686 ± 0.046 ^{b,f}

Numbers in column followed by different superscript are significantly different at P<0.05.

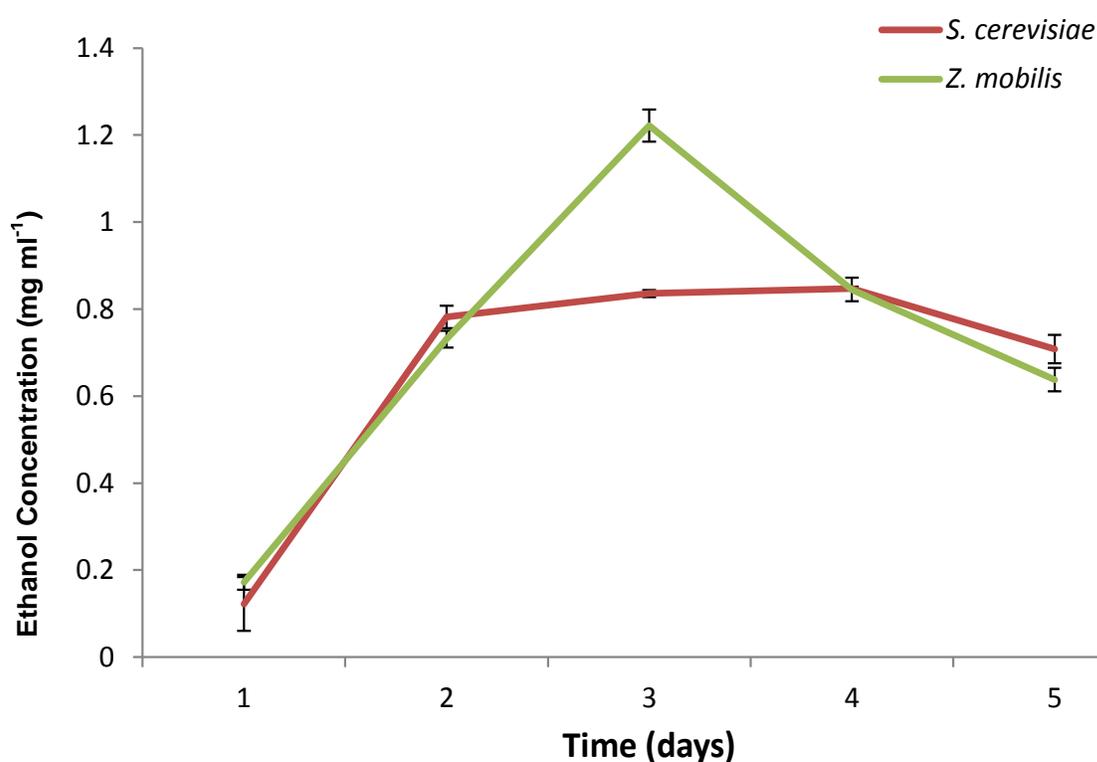


Figure 5. Effect of time duration on ethanol production by *S. cerevisiae* and *Z. mobilis* using pito mash as substrate

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

- Aggarwal NK, Nilga P, Singh D, and Yadav BS (2001). Process optimization for the production of sugar for the bioethanol industry from sorghum, a non-conventional source of starch. *World J. Microb. Biotechnol.* 17:411-415.
- Carter MK (2014). Direct catalytic conversion of sugars to ethanol. Official Gazette of the United States Patent and Trademark Office Patents.
- Castro E (2013). Other ethanologenic microorganisms. *Lignocellulose Conversion: Enzymatic and Microbial Tools for Bioethanol Production*; DOI: 10.1007/978-3-642-37861-4_8; pp. 151-168.
- Chandel KA, Chan ES, Rudravaram R, Narasu LM, Rao VL, Ravindra Poqaku (2007). Economic and environmental impact of bioethanol production technologies: an appraisal. *Biotech. Mol. Biol. Rev.* 2(1):014-032.
- Cheesbrough M (2003). *Medical laboratory manual. Tropical health technology, low price edition.* Dordington, Cambridgeshire. England pp. 20-35
- Clark MH, Michelle L-C, Vladimir J, Marjorie LL, David EB. (2013). Ethanol Production and Maximum Cell Growth Are Highly Correlated with Membrane Lipid Composition during Fermentation as Determined by Lipidomic Analysis of 22 *Saccharomyces cerevisiae* Strains. *Appl. Environ. Microbiol.* 79(1):91-104.

- Dowe N, McMillan J (2008). SSF experimental protocols- lignocellulosic biomass hydrolysis and fermentation. Laboratory analytical procedure. NREL Technical Report. *NREL/TP-510-42630*
- He MX, Wu B, Qin H, Ruan ZY, Tan FR, Wang JL, Shui ZX, Dai LC, Zhu QL, Pan K, Tang XY, Wang WG and Hu QC (2014) *Zymomonas mobilis*: a novel platform for future biorefineries. *Biotechnology for Biofuels*, 7:101.
- Hill J, Nelson E, Tilman D, Polasky S, Tiffany D (2006). Environmental, economic and energetic costs and benefits of biodiesel and ethanol biofuels. *Proc Natl Acad Sci*. 103(30).
- Hwang MJ, Jang NJ, Hyun SH, and Kim IS (2004). Anaerobic biohydrogen production from ethanol fermentation: the role of pH. *J. Biotechnol*. 111:297-309.
- Marszałek J, Kamiński W (2008). Environmental impact of bioethanol production. *Proc. ECO pole*. 2(1):66-70.
- Ming XH, Bo W, Han Q, Zhi YR, Fu RT, Jing LW et al. (2014) *Zymomonas mobilis*: a novel platform for future biorefineries. *Biotechnol. Biofuels* 7:101.
- Negulescu A, Patrula V, Mincea MM, Ionascu C, Vlad-Oros BA, Ostafe V. (2012) Adapting the Reducing Sugars Method with Dinitrosalicylic Acid to Microtiter plates and Microwave Heating. *J. Brazilian Chem. Soc.* 23(12):2176-2182.
- Nwachukwu I (2001). Studies on the effects of *Ceiba pentandra*, *Lorantus bengwelensis*, *Cymbopogon citrate* on species of dermatophytes. M.Sc Thesis, University of Jos, Nigeria.
- Panesar PS, Marwaha SS, Kennedy J (2007). Comparison of ethanol and temperature tolerance of *Zymomonas mobilis* strain in glucose and molasses medium. *Indian J. Biotechnol*. 6:74-77.
- Ramesh B, Reddy PRM, Seenayya G, Reddy G (2004). Production of ethanol from cellulosic biomass by *Clostridium thermocellum* SS19 in submerged fermentation: screening of nutrients using Plack-Burman design. *J. Biochem. Biotechnol*. 113:133-141.
- Rutkis R, Kalnenieks U, Stalidzans E, Fell DA (2013) Kinetic modelling of the *Zymomonas mobilis* Entner-Doudoroff pathway: insights into control and functionality. *Microbiology* 159(12):2674-2689.
- Sootsuwan K, Thanonkeo P, Keeratirakha N, Thanonkeo S, Jaisil P, Yamada M. (2013) Sorbitol required for cell growth and ethanol production by *Zymomonas mobilis* under heat, ethanol and osmotic stresses. *Biotechnol for Biofuels*; 6 (1):180.
- Tao F, Miao JY, Shi GY, and Zhang KC (2005). Ethanol fermentation by an acid-tolerant *Zymomonas mobilis* under non-sterilized condition. *Process Biochem*. 40:183-187.
- Wang C, Zhang C, Chan YS (2014) Protection effect of pyrocatechol on the antioxidant defense system of *Zymomonas mobilis*. *J. Chinese Inst. Food Technol*. 14 (8):21-26.
- Zakpaa HD, Mak-Mensah EE, Johnson FS (2009). Production of bioethanol from corncobs using *Aspergillus niger* and *Saccharomyces cerevisiae* in simultaneous saccharification and fermentation. *Afr. J. Biotechnol*. 8(13):3018-3022.