Biofuel from Microorganisms

Glenn Chua Jia Hoe. Rick Wong Tuck Jun

Background and Purpose of Research Area

Fossil fuels such as petroleum and coal are widely used as the main source of energy needed for various applications, from running a car engine, to the production of electricity. However, fossil fuels are a non-renewable source of energy and are declining at an alarming rate. The combustion of fossil fuels has a detrimental effect on the environment as large volumes of greenhouse gases, such as carbon dioxide, are released.

Due to these reasons, microbial production of biofuel from food crops has gained much significance in recent years. Ethanol is a viable alternative fuel for the future (Gunasekaran and Raj, 1999), as it is a renewable source of energy. However, according to Banschbach and Letovsky (2010), there is a controversy in using food crops such as corn, sugarcane and soybean in producing biofuels. For example, when Brazilian rainforests were cleared for growing sugarcane, the carbon stored in the forests was released through cutting and burning the trees, which emitted about 50% more greenhouse gases than producing and burning gasoline (Tilman and Hill, 2007).

Given the food crop controversy, the production of ethanol from food wastes instead is worth exploring. According to the 2010 statistics of wastes generated and recycling from the National Environmental Agency, Singapore, food wastes are generated at a rate of 0.64 million tones / year. They are recycled at a rate of 0.10 million tonnes / year, thus representing only 16% of the total waste output. Wastes are recycled, incinerated or landfilled. By increasing the rate of recycling, there will be a lower need for incineration which releases toxic gases into the atmosphere, and landfills which are gradually reaching their maximum capacity.

The yeast *Saccharomyces cerevisiae* is commonly used in ethanolic fermentation from sugars. Subashini *et al*. (2011) studied the production of ethanol from sago waste using *S. cerevisiae*. Additionally, Dhabekar and Chandak (2010) utilized banana peels and beet waste for alcohol production. This project mainly utilises *Zymomonas mobilis*, a rod-shaped Gram-negative bacterium, in the production of ethanol. Its ability to utilize sucrose, glucose and fructose makes it a versatile organism in ethanol fermentation. Other benefits of *Z. mobilis* include a higher sugar uptake and a high tolerance to ethanol as compared to yeast (Gunasekaran and Raj, 1999).

Gunasekaran *et. al*. (1986) studied the fermentation pattern of *Z. mobilis* strains on different substrates such as cane juice and molasses. Ethanol yield was highest at pH 7 and using an initial sugar concentration of 15%. Doelle and Greenfield (1985) carried out single-batch ethanol fermentation from refined sucrose, sugarcane juice and syrup and obtained a high yield of ethanol

within 30 hours. Amin *et al*. (1987) immobilized *Z. mobilis* cells in polyurethane foam and investigated the production of ethanol from sucrose. A final ethanol concentration of 6.3% was obtained. More recently, Zhang and Feng (2010) attempted to produce ethanol from low-cost, non-grain feedstock such as raw sweet potato, and this offered an advantage over the use of food crops such as corn and wheat in producing ethanol, especially in developing countries.

Thus, in order to address the problems of using fossil fuels, the controversy of using food crops in the production of ethanol, and the increasing amount of food wastes generated over recent years, this project aims to investigate the feasibility and efficiency of producing ethanol from food wastes such as fruit peels and sugarcane bagasse.

Hypotheses of the Research

We hypothesise that fruit peels and sugarcane wastes contain varying concentrations of reducing sugars. Free and immobilized *Z. mobilis* produce different concentrations of ethanol when grown in different wastes. *S. cerevisiae* and *Z. mobilis* will give different ethanol yields from the same waste.

Research Methods and Materials

Preparation of extracts from wastes

30 g of fruit peels or sugarcane waste were blended in 300 ml deionised water using a blender. The residue was removed and the liquid containing sugars was collected. In order to increase the sugar content, 5 mg/ml cellulase enzyme was added to the extracts and incubated for 1 day at 30°C.

DNS test for reducing sugars in waste

To 0.5 ml of the extract, 0.5 ml of DNS (dinitrosalicylic acid) was added and placed in a boiling water bath for 5 min. 4 ml of water was then added. The absorbance was taken at 530 nm using a spectrophotometer. The concentration of reducing sugars was read from a maltose standard curve.

Growth of microorganisms

Z. mobilis ATCC 29191 were inoculated in 20 ml GY medium (2% glucose, 0.5% yeast extract). *S. cerevisiae* cells were inoculated in 20 ml PDB (potato dextrose broth). They were incubated at 30ºC for 2 days with shaking.

Ethanol fermentation by free cells

3 ml of *Z. mobilis* preculture was transferred to fermentation medium (1% yeast extract, 0.1% ammonium sulfate, 0.1% dipotassium phosphate, 0.05% magnesium sulfate in 50 ml waste extract) and incubated at 30ºC for 2 days for ethanol fermentation to take place. The cultures were centrifuged at 7000 rpm for 10 min to pellet the cells. The wet weight of cells was recorded. The supernatant was collected and sent for distillation to obtain the ethanol.

Ethanol fermentation by immobilised cells

The *Z. mobilis* or *S. cerevisiae* preculture was centrifuged at 7000 rpm for 10 min and the cell pellet was resuspended in 7.5 ml of GY medium. The absorbance of the culture was taken at 600 nm. 7.5 ml of 2% sodium alginate was added to the cell suspension and mixed well. The mixture was dropped into 0.1 mol dm^{-3} calcium chloride solution to form *Z. mobilis* or *S. cerevisiae* alginate beads. The beads were rinsed with 0.85% sodium chloride solution. 200 beads were added to 50 ml waste extract. The set-ups were incubated with shaking at 30ºC for 2 days for ethanol fermentation to occur. The beads were then removed and the extracts were distilled to obtain ethanol.

Determination of ethanol yield with the dichromate test

2.5 ml of acidified potassium dichromate solution was added to 0.5 ml of distillate in a ratio of 5:1 and placed in a boiling water bath for 15 min. The absorbance was measured at 590 nm using a spectrophotometer, and the concentration of ethanol was read from an ethanol standard curve.

Experimental Results

Ethanol fermentation using free *Z. mobilis* cells

Orange peel had the highest concentration of reducing sugars among the three samples tested (Figure 1). The ANOVA test had a p value of 0.0003, indicating a significant difference in the concentration of reducing sugars in the sugarcane waste, orange and watermelon peels. The yield of ethanol corresponded with the concentration of reducing sugars in the wastes. As such, orange peel resulted in the highest ethanol yield, followed by sugarcane waste and watermelon peel (Figure 2). The ANOVA test yielded a p value of 4.17×10^{-7}, showing significant differences in the concentration of ethanol obtained from the different wastes used.

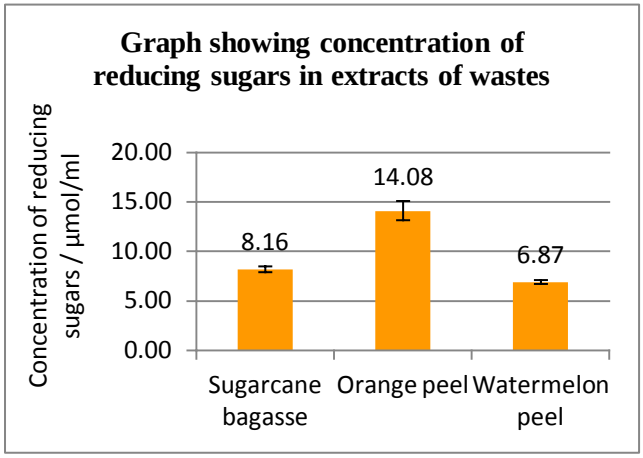

Figure 1: Concentration of reducing sugars in wastes used in ethanol fermentation by free *Z. mobilis* cells

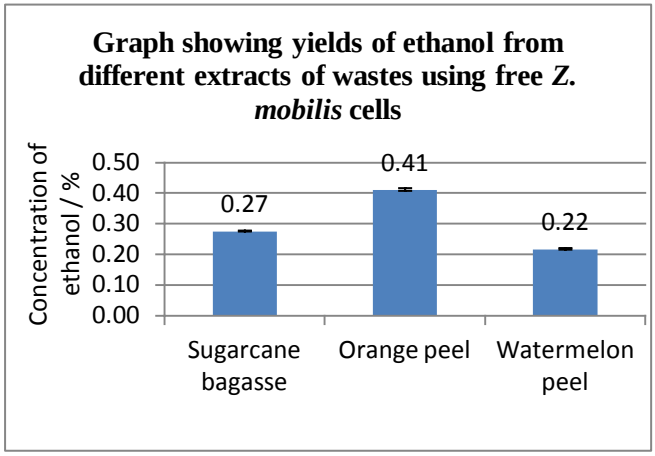

Figure 2: Yield of ethanol from free *Z. mobilis* cells

Ethanol fermentation using immobilized *Z. mobilis* cells

A different batch of wastes was used in ethanol fermentation using *Z. mobilis* cells immobilised in calcium alginate. The concentration of reducing sugars in wastes showed a different trend as compared to ethanol fermentation using free *Z. mobilis* cells. Watermelon peels had the highest concentration of reducing sugars, followed by orange peels and sugarcane waste (Figure 3). The ANOVA test revealed a significant difference in the concentration of reducing sugars (p value of 1.42×10^{-6}). However, the ethanol yield did not correlate well with the reducing sugar concentration for sugarcane extract. Sugarcane extract contained the lowest reducing sugar concentration, yet it resulted in the highest ethanol yield, as compared to orange and watermelon peels (Figure 4). There could be the presence of non-reducing sugars in sugarcane wastes such as sucrose that were utilized by *Z. mobilis* for ethanol fermentation. The ANOVA test yielded a value of 0.0588, showing insignificant differences in the concentration of ethanol obtained from the different wastes used. No ethanol was detected for the control, consisting of beads without *Z. mobilis* cells.

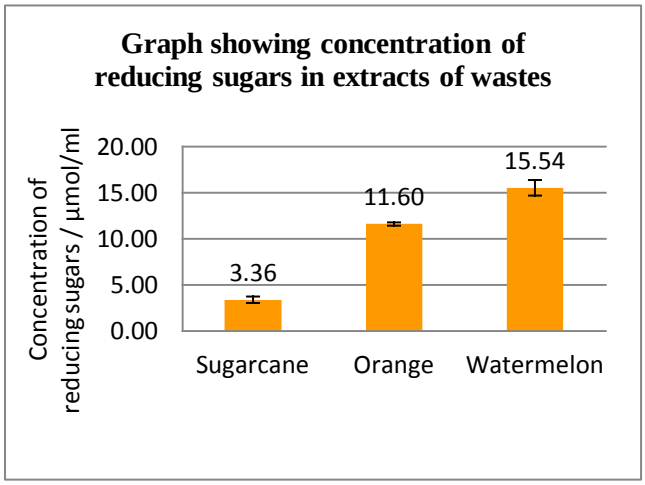

Figure 3: Concentration of reducing sugars in wastes used in ethanol fermentation by immobilised *Z. mobilis* cells

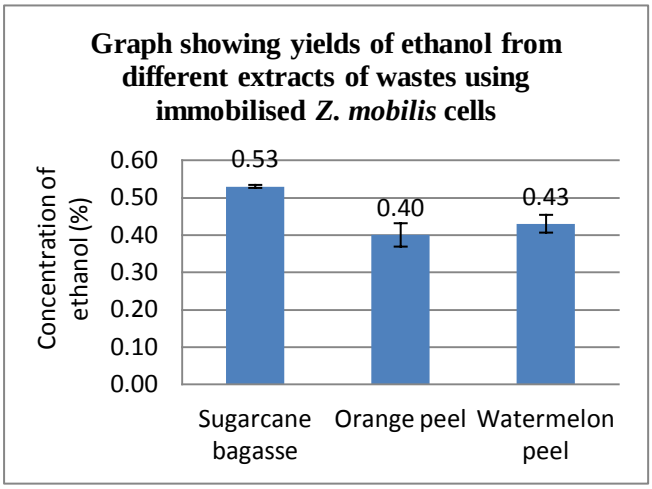

Figure 4: Ethanol yield from immobilised *Z. mobilis* cells

Addition of cellulase to increase reducing sugar concentration

Sugarcane waste extract was prepared from a different batch of waste, to which 5 mg/ml cellulase was added. There was a significant increase in the reducing sugar concentration after cellulase treatment, with t-test p value of 0.0002 (Figure 5). There was also a corresponding increase in the ethanol yield with immobilized *Z. mobilis* cells, with t-test p value of 0.001 (Figure 6). These

results suggest that there is a good potential of increasing ethanol yield by increasing the reducing sugar concentration by treating the wastes with cellulase enzyme.

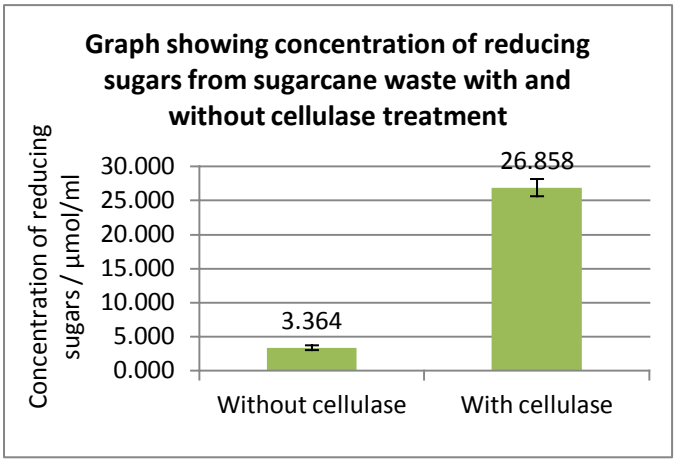

Figure 5: A comparison of reducing sugars in sugarcane waste with and without cellulase treatment

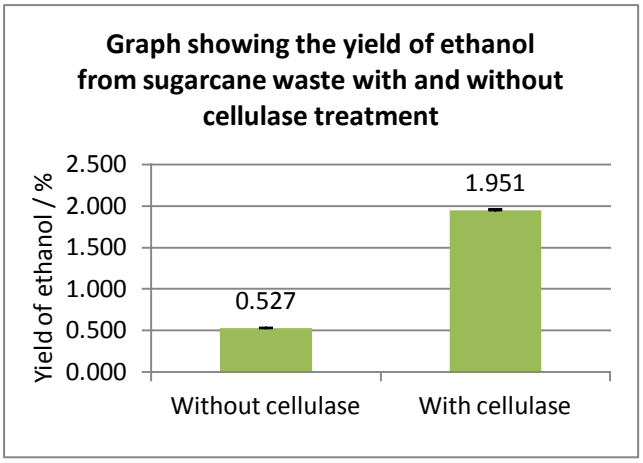

Figure 6: A comparison of ethanol yield from sugarcane waste with and without cellulase treatment

Ethanol yields of *S. cerevisiae* and *Z. mobilis*

A different batch of sugarcane waste was used to prepare the extract, with a reducing sugar concentration of 6.608 µmol/ml, was used for ethanol fermentation by immobilized *S. cerevisiae* and *Z. mobilis* cells. The mass of cells for both *S. cerevisiae* and *Z. mobilis* was standardised. There were three set-ups, the first with 200 *Z. mobilis* beads; the second with 200 *S. cerevisiae* beads, while the third contained a mixture of 100 *S. cerevisiae* and 100 *Z. mobilis* beads. The set-up with *S. cerevisiae* resulted in the highest ethanol yield (Figure 7). However, the mean difference in the ethanol yield was insignificant with the ANOVA test (p value of 0.41). There was a lower ethanol for immobilied *Z. mobilis* cells, compared with the previous experiment (Figure 4) as the density of cells per bead may be lower.

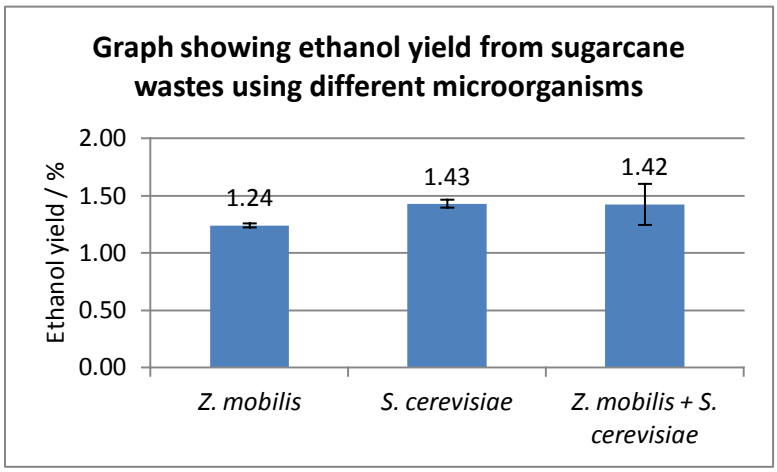

Figure 7: A comparison of ethanol yield from sugarcane waste by *Z. mobilis* and *S. cerevisiae*

In an attempt to increase the yield of ethanol, the number of *Z. mobilis* beads was varied. 200 or 300 beads were placed in orange peel extract for fermentation to take place. Figure 8 shows that the increase in ethanol concentration was insignificant (p value of 0.345) when the beads were increased from 200 to 300. Nevertheless, there is a potential to increase the ethanol yield by increasing the number of *Z. mobilis* immobilised cells.

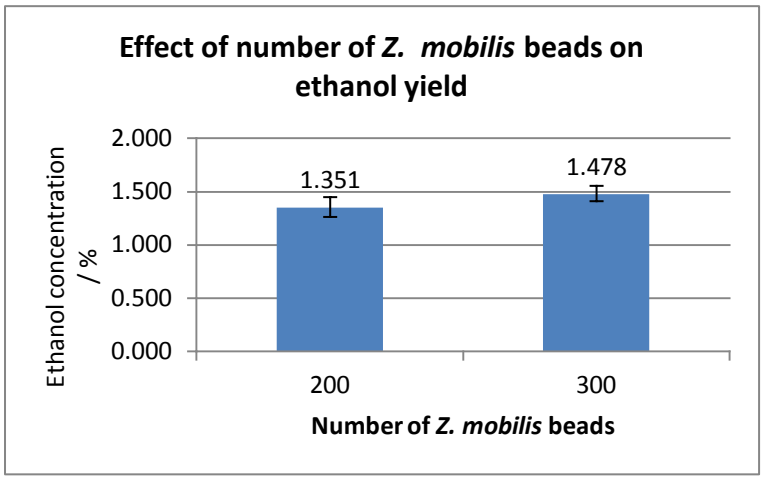

Figure 8: Graph showing the effect of number of *Z. mobilis* beads on the ethanol yield from orange peel extract

Conclusions and Recommendations

Orange peels contained the highest concentration of reducing sugars, and gave the greatest yield of ethanol when used as a substrate for fermentation to ethanol by *Z. mobilis* using free cells. There was a correlation between reducing sugar content and yield of ethanol, due a higher rate of utilization of these sugars by *Z. mobilis* in ethanol fermentation. Also, a higher reducing sugar content supported a higher growth rate of free *Z. mobilis* cells, thus resulting in a higher biomass and yield of ethanol.

On the other hand, for ethanol fermentation using immobilized *Z. mobilis* cells, watermelon peels had the highest concentration of reducing sugars, but gave the second greatest yield of ethanol when used as a substrate. Although sugarcane waste had the lowest concentration of reducing sugars, it gave the highest yield of ethanol. This could be due to the fact that sugarcane contained sucrose, a non-reducing sugar, which could be utilized by *Z. mobilis* cells.

The use of immobilised cells has more advantages compared to the use of free cells. According to Amin *et al*. (1987), cell immobilisation reduces the problems of washout of cells in continuous cultures, which limit productivity. Moreover, product inhibition of cells in batch cultures can be reduced by using immobilised cells. Any impurities in the waste extracts that could slow down growth of cells and the ethanol fermentation process could be overcome by

immobilising the cells in calcium alginate. The findings from this study have shown that significant yields of ethanol were obtained using immobilised cells, comparable to free cells.

Although *Z. mobilis* possesses advantages over *S. cerevisiae* with respect to ethanol productivity and tolerance, our results showed that ethanol yield from *S. cerevisiae* was slightly higher than that from *Z. mobilis*. This could be due to the fact that, under anaerobic conditions, *Z. mobilis* produces byproducts such as acetone, glycerol and acetate, which results in reduced production of ethanol from glucose (Gunasekaran and Raj, 1999). Co-cultures of *Z. mobilis* and *S. cerevisiae* did not result in a significant increase in ethanol yield, as sugars in the extracts could be utilised by both cultures. *S. cerevisiae* produces the enzyme invertase that catalyses the hydrolysis of sucrose to glucose and fructose (Phowchinda and Strehaiano, 1999). In contrast, research done by Abouzeid and Reddy (1986) showed that cocultures of the amylolytic fungus, *Aspergillus niger*, and *S. cerevisiae* resulted in a higher ethanol yield from potato starch.

There are a few benefits of using food wastes as substrates for producing ethanol. The wastes are recycled, thus saving costs in the production of ethanol. The production of ethanol from agricultural feedstocks is based on renewable resources. There have been debates over the use of large areas of agricultural lands for planting food crops such as corn and rice for ethanol production, instead of being consumed as food, especially in developing countries. Moreover, the large-scale planting and harvesting machinery of corn are powered by fossil fuels, resulting in the emission of considerable amounts of carbon dioxide, to the extent that driving a car fueled by corn-based ethanol only reduces the emission of greenhouse gases by a small margin, compared to a similar car powered by gasoline (Tilman and Hill, 2007). However, with the use of agricultural wastes instead of crops to produce ethanol, these problems can be alleviated. The cost feasibility and its impact on truly reducing the reliance on fossil fuels and waste management will definitely make further development into this area worthwhile.

Since initial findings indeed show a high potential of using wastes in ethanol fermentation, the next step would be to scale up the production of ethanol by increasing the wastes used as the starting substrate, so as to increase the concentration of sugars. The number of *Z. mobilis* beads can also be increased in order to achieve higher yield of ethanol.

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