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Bioethanol Production from Corncob Hydrolysed by Cellulase of *Aspergillus niger* Using *Zymomonas mobilis* and *Saccharomyces cerevisiae* Isolated from Palm Wine

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Abstract

Cellulase is an inducible enzyme complex involving synergistic action of endoglucanase, exoglucanase and cellubiase; and it is produced by a number of bacteria, actinomycetes and fungi including *Trichoderma* species and *Aspergillus* species. However, fungi are the most common producer of cellulose used for industrial processes. Corn cob which is composed mainly of cellulose is a major component of agricultural and domestic waste in many parts of the world. It can be converted to energy in form of bioethanol as an effective and efficient means of waste management. Production of cellulase was induced in the fungi *Aspergillus niger* by growing it in mineral salt medium containing alkali pre-treated corn cobs. The cellulases were partially purified. Alkali pre-treated corn cob was hydrolysed with the partially purified cellulases and the product of hydrolysis was fermented using *Saccharomyces cerevisiae* and *Zymomonas mobilis*. The cellulosic hydrolysate yielded 1.78 gl^{-1} sugars which produced 9.10 gl^{-1} ethanol fermentation using *Zymomonas mobilis*, compared to ethanol yield at 8.20 gl^{-1} using *Saccharomyces cerevisiae*. Alkali pre-treated corn cob, hydrolysed with cellulases of *A. niger* is a suitable feedstock for bioethanol production. Also *Z. mobilis* is the most relevant organism when compared to *Saccharomyces cerevisiae* for industrial bioethanol production. Agricultural and industrial wastes litter the environment and cause environmental pollution. But the conversion of these materials into useful products (especially by microbial activity including those mediated by cellulase-activity) is critical to assuaging or preventing the environmental problems that these wastes cause. Thus, our study has presumptively shown that corncob hydrolyzed with cellulase extracted from *A. niger* with the help of *Z. mobilis* and *S. cerevisiae* isolated from palm wine could be a putative source of bioethanol. Further study is therefore needed to scale up this protocol to a large scale industrial production for local bioethanol production in this region.

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Saccharomyces cerevisiae
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Introduction

The importance of ethanol as a clean and safe transportation fuel has increased with the anticipated shortage of fossil fuel reserves and increased air pollution caused by the usage of fossil fuels (Lin and Tanaka, 2006; Solomon et al., 2007). Fuels derived from cellulosic biomass offer an alternative to conventional energy sources that supports national economic growth, national energy security, and

environmental sustainability. However a dramatic increase in ethanol production using the current starch-based technology may not be practical in Nigeria because it will compete for the limited agricultural land needed for food and feed production, thus affecting food security. Lignocellulosic biomass is a cheap, renewable, abundantly available resource (Hansen et al., 2005), and its conversion to glucose and other fermentable sugars has been considered to be an attractive route for ethanol production (Cao et al., 1996; Cazetta et al., 2007). In Nigeria, corn is processed to a variety of diets including pap which is a major diet used for weaning, and the capacity for corn production in Nigeria is high. Corn cobs form about 30 % of maize agro-wastes of which application in bioethanol industry are the focus of many researches aimed at achieving an effective and efficient waste management scheme (Olsson and Hahn-Hägerdal, 1996).

Currently the corn cobs (which contain 32.3-45.6 % cellulose, 39.8 % hemicelluloses and 6.7-13.9 % lignin) are burnt as fuel in households of peasant rural farmers which leads to severe environmental constraints. But it can be converted to fermentable sugar for ethanol production via enzymatic and microbial action. Physicochemical pre-treatment, cellulose saccharification and glucose fermentation to ethanol are the three main stages involved in the production of bioethanol from corn cobs (Jeffries and Jin, 2004), and the hydrolysis of cellulose has been achieved with dilute acids under high temperature and pressure and with concentrated acids at low temperature and atmospheric pressure. Large-amounted, concentrated agricultural waste corn cob is generated annually in Nigeria, and often causes environmental pollution due to the lack of its effective utilization. Thus, this study determined the production of bioethanol from corncob through the action of cellulase of *Aspergillus niger* and combined activity of *Zymomonas mobilis* and *Saccharomyces cerevisiae* isolated from palm wine.

Materials and methods

Sample collection

A freshly tapped palm wine was purchased from a palm wine market at Ntezi, Ishielu Local Government Area, Ebonyi State with the use of a sterile container. The palm wine was used fresh after it was tapped. A sterile polythene bag was used to collect a decaying corncob at a dumpsite in Abakaliki metropolis. The corncob and palm wine was taken to the microbiology laboratory of Ebonyi State University, Abakaliki where they were further analyzed.

Isolation of microorganisms

The test organisms including *Aspergillus niger, Zymomonas mobilis* and *Saccharomyces cerevisiae* were isolated based on standard microbiology techniques (Cheesbrough, 2006). Briefly, Twenty (20) ml volume of the palm wine was centrifuged at 5000 rpm for 10 minutes, and a loopful of the palm wine sediment was aseptically collected using a wire loop, and this was inoculated on already prepared Sabouraud dextrose agar (SDA) and potato dextrose agar (PDA) growth media supplemented with 300 mg of Ketoconazole and 500 mg of chloramphenicol to inhibit the growth of saprophytic fungi and bacteria respectively.

After inoculation, the Petri dishes were packed in an anaerobic jar, and incubated at 37°C for 48 hrs for fungal growth and isolation. Recovered suspect colonies were subcultured onto freshly prepared culture media plates for the isolation of pure isolates. The identification and characterization of the pure bacterial isolates were based on morphological, physiological and biochemical tests as was previously described (Cheesbrough, 2006; Oyeleke et al., 2012).

Isolation of *Aspergillus niger* from dumpsite corncob

A sterile polythene bag was used to collect a decaying corncob at a dumpsite in Abakaliki. The corncob was taken to the laboratory after which 10 g of corn cob was immersed in 100 ml of distilled water. A four (4)-fold serial dilution of remnant of corncob water immersion was carried out and cultivation was done on potato dextrose agar (PDA). Pour-plate method was used to inoculate the growth medium prepared with 1 ml of the diluted sample; and were incubated for a period of 4 days at 37 ° C. Sub-culturing was carried out until pure cultures of *Aspergillus niger* was obtained and the pure *Aspergillus niger* colony was used to produce cellulase. After 4 days of cultivation, a small portion of the mycelia growth was carefully picked with the aid of a sterile wire loop and placed on a drop of lactophenol cotton blue on a microscope slide and covered with a cover slip. The slide was examined under the microscope with (40x) objective lens for morphological examination as described by Cheesbrough (2006). The isolates were characterized based on the colour of aerial, shape and kind of asexual spores, and the characteristics of spore head.

Screening of *Aspergillus niger* for cellulase activity

A loopful of growth culture of isolated colonies was inoculated on 3.9 g of Potato dextrose Agar (PDA) mixed with 0.2 g Carboxyl Methyl Cellulose (CMC), dissolved in 100ml distilled water. The PDA plates were incubated for 3 days at 37°C for *Aspergillus* isolate and observed for growth. To undertake ammonia steeping, 20 g of milled corn cobs of particle size of 2 mm was mixed with 100 ml 2.9 M NH_4OH solution in a 250 ml Erlenmeyer flask. The mixture was then incubated in a shaker for 24 hr at 30°C. The content was filtered using a 2 µm Whatman filter paper into 250 ml Erlenmeyer flask. It was further rinsed twice using distilled water. The corncobs were then dried at 30°C in an oven overnight.

Production of enzyme

Spores of *Aspergillus niger* were harvested by flooding 1 week old stock culture on agar slants with sterile distilled water (5 ml) as was previously described (Martin et al., 2002). The inoculums medium (100 ml) comprised of Mandels mineral salt, was inoculated with the entire spore suspension and incubated on a rotary shaker (150 rpm) at room temperature for 72 hrs. Each 250 ml flask containing 100 ml of Mandels mineral salt medium, into which was incorporated 10 g of cellulosic materials i.e. alkali pre-treated and untreated corn cob; and Carboxyl methyl cellulose (CMC). The medium was inoculated with inoculums culture (5 ml) containing approximately 4.6×10^6 spores/ml of the fungi and incubated at 28°C on a gyratory shaker (150 rpm) for 10 days. Enzyme filtrates were obtained, after which cultures were centrifuged and stored at -10°C until required.

Assay for Cellulase activities

Endoglucanase activity – Carboxyl methyl cellulose: Reaction mixture comprising of 0.5ml carboxyl methyl cellulose (2 % w/v) in 0.05M citrate buffer, pH 4.8 and 0.5ml culture filtrate in test tubes. Mixture was incubated at 50°C for 1 hr. After incubation 1ml of di-nitrosalicyclic acid reagent was added to stop the reaction. The reactants in test tubes were boiled for 5 mins in boiling water bath and transferred to cold water bath. 10 ml of distilled water was added and absorbance was measured at 540 nm in spectrophotometer. Amount of reducing sugar was read from a curve obtained by plotting value of absorbance against concentration. One unit of enzyme activity is defined as the amount of enzyme that released 1 µm of reducing sugar.

Bioethanol production

Bioethanol production from the corncob was microbiologically analyzed using previous methodology; and the methods employed in this assay included enzyme hydrolysis, fermentation and distillation process as was previously described (Yanase et al., 2005; Oyeleke et al., 2012).

Hydrolysis of corn cob with the partially purified cellulose of isolated fungi

The method of Oyeleke et al. (2012) was employed. Citrate buffer (100 ml) containing alkali treated corn cob (10 % w/v) in 250 ml Erlenmeyer flask was inoculated with 10 ml of the partially purified filtrate and incubated at 50°C for 7 hrs. Samples (1 ml) was withdrawn aseptically from flask at 1 hr interval and analysed for reducing sugar by the DNSA method to determine the optimum time in hours for cellulose digestion of corn cob. The hydrolysate was then used for fermentation to produce ethanol.

Fermentation of the products of corncob digestion to ethanol

The fermentation of the products of corncob digestion to ethanol was carried out by a previously described methodology (Oyeleke et al., 2012). Yeast peptone dextrose broth (100 ml) in 250 ml Erlenmeyer flask was inoculated with pure colonies of yeast from agar slant with the aid of an inoculating loop. This was incubated at 28°C on gyratory shaker at 150 rpm for 48 hrs. 0.3 g malt extract, 0.3 g yeast extract, 2 g glucose, 0.5 g peptone and 0.002 g of actidone dissolved in water was inoculated with pure colonies of *Zymomonas mobilis* from agar slant with the aid of inoculating loop. The fermentation broth (100 ml) comprising of (%w/v), Peptone, 8 g; yeast extract, 8 g; and the product of hydrolysis of corn cob as the fermenting sugar. The broth (80 ml) was filled into a 100 ml sealable bottle, sterilized in an autoclave and inoculated with 10 ml of each of the inoculums. The bottles were sealed with the aid of an adhesive tape and incubated at 28°C for a period of 8 – 48 hrs. Bottles were removed at 8 hrs intervals to determine the amount of ethanol produced and the residual sugar in the medium.

Determination of ethanol concentration by the acidified dichromate/thiosulphate titration method

The fermented broth was assayed for ethanol using the acidified dichromate/thiosulphate titration method. Ten

ml acid dichromate solution (0.01 M in 5.0 M sulphuric acid) was placed in 250 ml Erlenmeyer flask. This was connected to 10 ml of fermented broth in another 250 ml Erlenmeyer flask which was placed in water bath set at 80°C. The set up was allowed to stand for 3 hrs, during which ethanol produced by the fermentation of the broth evaporated into the acid dichromate solution. All the flasks were fixed with rubber stopper and sealed with wax to avoid leakages. After the incubation period, the set up was dismantled, 100 ml distilled water and 1.0 ml Potassium iodide (1.2 M) was added to the dichromate solution. This was then titrated with Sodium thiosulphate (0.03 M) until the brown colour turned yellow, at which point 1% starch solution (1 ml) was added as indicator of iodine, and further titrated until the blue colour fades. Three flasks consisting of 10 ml acid dichromate was set up as blanks and titrated first so as to monitor the volume of thiosulphate required. The difference between thiosulphate used in the titration of the blank and that of the sample was used in calculating the amount of ethanol.

Determination of residual sugar in the fermentation medium

The amount of sugar in the fermentation medium after each period of fermentation was determined following the DNSA method of Miller (1959). Dinitrosalicylic acid (DNSA) reagent (1 ml) was added to an aliquot (1 ml) of the fermentation medium in a test tube and properly mixed. The mixture was boiled for 5 mins and cooled under running tap water. 5 ml of 40 % Rochelle salt solution was added to the mixture and absorbance read in spectrophotometer at 540 nm. Amount of reducing sugar was read from a standard glucose curve and expressed as mg ml^{-1}.

Results

Cellulase production by *Aspergillus niger*

The result of this investigation showed that there was progressive increase in enzyme activity from 24 to 144th hr after incubation. The highest cellulase activity was obtained on the 5th day (2.30 mg /ml/sec) and the least on the first day (1.3 mg /ml/ sec) this corresponds to the day with the highest and lowest biomass activities (Fig. 1).

Effect of cellulase dosage on hydrolysis of corn cob powder

Fig. 2 shows the results of hydrolysis experiments performed with 100gl^{-1} substrate and different dosage of the crude cellulose at pH 4.8 and 50°C. The reducing sugar concentration increased sharply with an increase in the cellulase dosage.

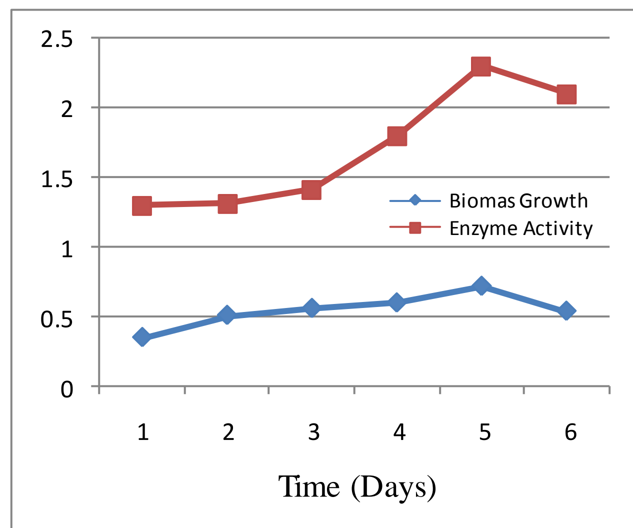

Fig. 1: Production of cellulase by *Aspergillus niger.*

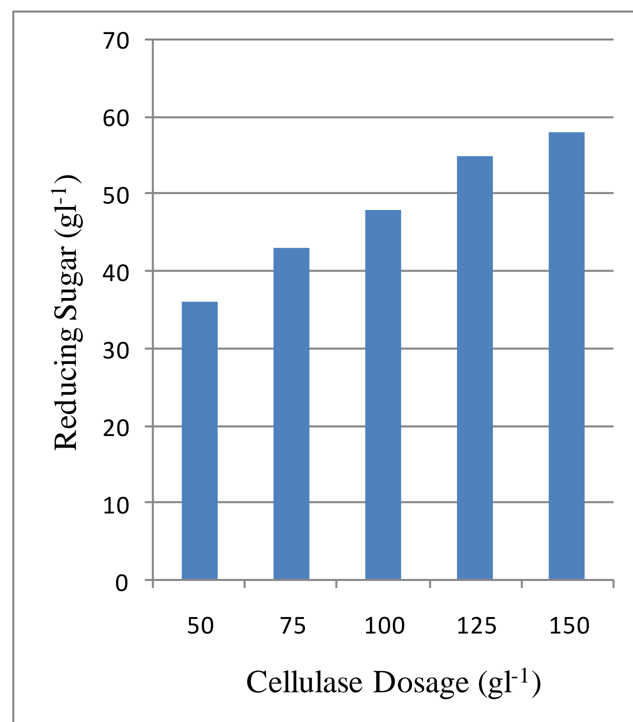

Fig. 2: Effect of cellulase dosage on hydrolysis of corncob.

Time course for saccharification of corn cob powder

The result of this experiment showed that there was an increase in saccharification of corn cob from 0 to 7th hrs. The increase was steeper up to 5th hr than from 5 to 7th hrs (Fig. 3).

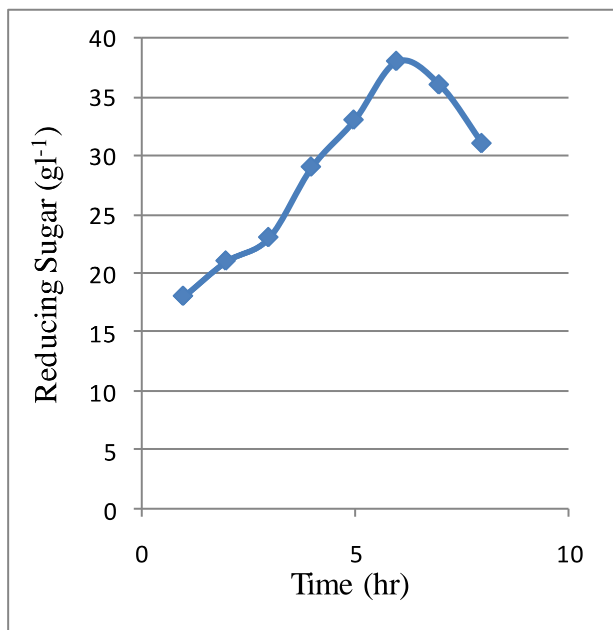

Fig. 3: Time course for Saccharification of corn cob powder.

Production of bioethanol with the use of *Saccharomyces cerevisiae*

The result obtained during production of ethanol with the use of *S. cerevisiae* is shown in Figure 4. The result showed that the value of the microbial cell density increased drastically from 8th hr up to the 32nd hr, at which point a lag phase of its growth was observed. Also the result showed an increase in ethanol concentration from 0.7 gl^{-1} at the 8th hr to 8.3 gl^{-1} at the 40th hr, after which there was decrease in its concentration. The result also showed that the sugar concentration inversely decrease with an increase in ethanol concentration observed at 8-16 hrs and 24-48 hrs intervals is the result of glucose utilization by *S. cerevisiae* either for biomass or ethanol production.

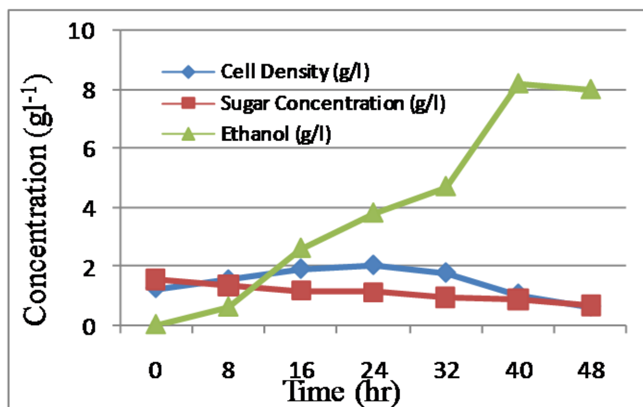

Fig. 4: Production of ethanol with the use of *S. cerevisiae*.

Bioethanol production from corn cob using *Zymomonas mobilis*

Fig. 5 shows the results obtained during ethanol production from corncob using *Z. mobilis*. The result shows a drastic increase in ethanol concentration with a corresponding decrease in sugar concentration. It also shows a simultaneous increase in the microbial cell density with increase in ethanol concentration. Also the decrease in sugar concentration observed at 0-24 hr is the result of glucose utilization by *Z. mobilis* either for biomass or ethanol production.

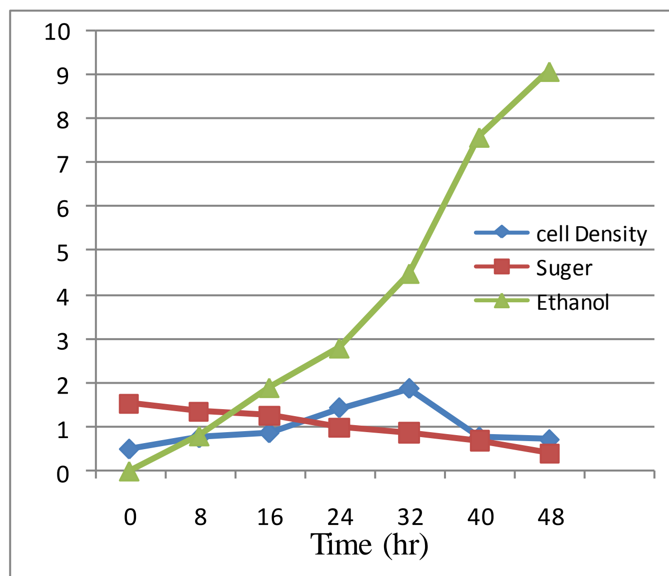

Fig. 5: Bioethanol production from Corncob using *Z. Mobilis*.

Discussion

This present day study evaluated the production of bioethanol from corncob hydrolysed by the cellulase activity of *A. Niger*; and using *Z. mobilis* and *S. cerevisiae* isolated from palm wine as an alternative source of feed stock for the local production of biofuels in this region. The increasing phenomenon of climate change and global warming (which are directly or indirectly linked to the use of fossil fuels) is affecting several human activities including agriculture. However, the development and use of alternative sources of fuels from natural sources such as corncob wastes (as investigated in this study) hold sway to the local production of cost-effective approaches of biofuel production. The results obtained from this study showed that *Saccharomyces cerevisiae* and *Zymomonas mobilis* are abundant in palm wine. Natural strains of the yeast have most often been found were fermentation occurs;

and these organisms play critical roles in the conversion of sugar to alcohol (Mortimer and Johnston, 2000). *Z. mobilis* abounds in palm wine as a dominant alcohol producing bacteria; and it has also shown a high growth rate and tolerance to ethanol as well as being amenable to engineering. *Zymomonas* species being an anaerobic Gram negative rod uses only Embden Meryerhoff pathway, and thus the organism requires no oxygen for its metabolic processes (Yanase et al., 2005).

Fermentation systems using *Zymomonas* species would require no sparging and/or aeration like yeast Katzen and Schell, 2006); and this helps to cut down on fermentation cost. *A. niger*, the best cellulolytic fungi isolated from corncob sampled from dumpsite at Abakaliki was assayed for enzyme activity; and the result showed a progressive increase in enzyme activity. Cellulase is an induced enzyme; and its production increased with increase in fungal biomass over the incubation period and as simple sugar in the substrate diminishes (Lynd, 1996). The result obtained also showed that there was increase in saccharification from 0 to 10th hr. The increase was steeper up to 6th hr than from 6 to the 10th hr. The slowdown in rate for hydrolysis may be due to the action of the enzymes been slowed down by obstacles that interfere with their path or a loss in activity and/or processivity making them less effective (Yang et al., 2006). The rate of Saccharification is directly proportional to substrate concentration up to the optimal substrate concentration. This is because random collisions between the substrate and enzyme active sites happen more frequently. As the cost of cellulase is high, the cellulase dosage should be minimized as much as possible (Cao et al., 1996). During fermentation, the ethanol in the product increased while the reducing sugar decreased as a result of the fact that during fermentation the yeast (*S. cerevisiae*) utilized the sugar as a source of carbon and energy, and ethanol is produced as a result (Gunasekaran and Chandra, 1999). There was increase in ethanol production over the period of fermentation, likewise as increase in saccharification making glucose available to the microorganisms (*S. cerevisiae* and *Z. mobilis*) for fermentation. Previous studies reported that enzymatic hydrolysis of the solid fraction has a large control over the total rate of ethanol production in simultaneous saccharification and fermentation (Olofsson et al., 2008). A high ethanol yield (49 %) was achieved in the fermentation of biomass hydrolysate by the bacterium *Z. mobilis* compared to the yield obtained with *S. cerevisiae* which (43 %) in our study. *S. cerevisiae*, however, consumes sugar for growth and production of other metabolites. Ethanol production in this study is comparable to previous studies where varying rates of ethanol where produced from local wastes (Olofsson et al., 2008; Agarwal et al., 2011; Cao et al., 1996). Moreover since growth commences during the aerobic phase, some amount of sugar gets used up before the anaerobic stage which is characterized by ethanol production.

Conclusively, ethanol was produced from corncob wastes via the combined fermentative and/or microbial activities of *A. niger, Z. Mobilis, S. Cerevisiae*; and corncobs was affirmed as viable feedstock for energy production. Also corncob hydrolyzed by cellulases of *A. niger* was productive in terms of ethanol yield and can therefore be harnessed in biofuel production. The present study also showed that palm wine is a good medium for the propagation of ethanogenic microorganisms (i.e. ethanol-producing organisms) including *Z. Mobilis*; and that ethanol could be produced from industrial/domestic or agricultural wastes like corncobs. *Z. mobilis* is the most relevant organism when compared with *S. cerevisiae* for industrial production of bioethanol as a result of its higher sugar uptake and ethanol yield, as well as its higher ethanol tolerance – which are all deficient in *S. cerevisiae*. The production of ethanol from agricultural wastes like corncobs will help in environmental sustainability especially in the area of controlling environmental pollution as well as making available sustainable and clean sources of energy such as biofuels.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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