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Isolation and molecular characterization of *Zymomonas mobilis* for bioethanol production from palm saps in some parts of Jos, Plateau state, Nigeria

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Abstract
The search for a suitable organism for bioethanol production is still ongoing. *Zymomonas mobilis* is a bacterium belonging to the genus *Zymomonas* and is notable for its bioethanol-producing capabilities, which surpass yeast in some aspects. In the present research work *Zymomonas mobilis* isolates were obtained from fresh palm sap and characterized using conventional biochemical tests as well as molecular techniques through the PCR detection of the alcohol dehydrogenase gene (the primer sequences used targeted 16SrRNA and 23SrRNA genes). The results of the biochemical characterization of presumptive isolates of *Zymomonas mobilis* showed that out of ten (10) isolates only four (4) isolates (40%) which include ZM2, ZM3, ZM4, and ZM5 matched with the characteristics of *Zymomonas mobilis* according to available standard techniques. For the molecular characterization of the presumptive *Zymomonas mobilis* isolates, genomic DNA was isolated from *Zymomonas mobilis* (ATCC 29191), ZM2, ZM3, ZM4 and ZM5. Two different primers were used; a fragment of 900 bp was amplified using 16SrRNA(Z16p3) and 23SrRNA (Z23p5) gene primers that were specific for *Zymomonas mobilis* and another fragment of 1200bp was amplified using ADH 1 gene (ZADH IF and ZADH IR primers) that were specific for *Zymomonas mobilis* for each of the isolates. The results of the amplification with the specific primer pairs coincided with the studies carried out by other authors. The two confirmed *Zymomonas mobilis* isolates were also screened for ethanol production using rich media and they showed relatively high ethanol yields (24-60g/l for ZM2 and 30-80g/l for ZM3). The results of this research work revealed that the two *Zymomonas mobilis* isolates obtained in this research have good potential as candidates for industrial bioethanol production.

Keywords: Bioethanol, palm sap, zymomonas, molecular

Introduction
Bioethanol is a distilled colorless liquid fuel obtained from numerous potential feedstock varieties such as sugar beet, wheat, corn, cassava, fruits, bagasse, barley, molasses, skim milk (whey), potatoes, sorghum, switchgrass and cellulose biomass such as wood, paper, straw and other cellulose wastes such as grasses, others include municipal solid wastes (Shell Global, 2001) [15].

Bioethanol is mostly obtained by fermentation of sugars which are converted into carbon dioxide and ethanol by microorganisms; *Saccharomyces cerevisiae* (yeast) is the most frequently used in this bioprocess (Dien *et al*., 2003, Lin and Tanaka 2006) [3, 8]. Another promising microorganism for the production of bioethanol is *Zymomonas mobilis*. This is a bacterium belonging to the genus *Zymomonas*. It is notable for its bioethanol-producing capabilities, which surpass yeast in some aspects. It was originally isolated from alcoholic beverages like the African palm wine, and the Mexican pulque, and also as a contaminant of cider and beer in European countries (Shiva *et al*., 2012) [16].

As an organism for bio-ethanol production, *Zymomonas mobilis* has shown some advantages over *Saccharomyces cerevisiae*, for example, higher specific rate of sugar uptake, high ethanol yield (up to 2.5 times higher), lower biomass production, higher ethanol tolerance up to 16% (v/v), non-requirement of controlled addition of oxygen during fermentation and amenability to genetic manipulations (Paneser *et al*., 2006, Sahn *et al*., 2006 and Rogers *et al*., 2007) [10, 13, 12].

Extensive fundamental studies on *Zymomonas mobilis* over the last 30 years had also made this strain a promising ethanologenic organism for large-scale bio-ethanol production. *Zymomonas mobilis* degrades sugars to pyruvate using the Entner-Doudoroff pathway. The pyruvate is then fermented to produce ethanol and carbon dioxide as the only products (analogous to yeast), (Paneser *et al*., 2006) [10].

Even though many microorganisms, including *Saccharomyces cerevisiae*, and *Clostridium* sp., have been considered as ethanologenic microbes, *Zymomonas mobilis* is considered the better candidate for industrial alcohol production (Rogers *et al*., 2007) [12].

There has been no report on the isolation and characterization of *Zymomonas mobilis* in Nigeria. This study seeks to isolate and characterize *Zymomonas mobilis* from palm sap, apple juice, and orange juice and to test the organisms ability to produce ethanol.

Materials and Methods

Isolation of *Zymomonas mobilis*

Media with the following compositions as described by Swings and De Ley (1977) [17] were used for isolation of *Zymomonas mobilis* from spoiled orange, fermented apple juice and fresh palm saps (1) Standard solid media that contained yeast extract 5.0 g, glucose 20 g, agar 20 g, distilled water 1 litre, pH 6.8, (2) Malt yeast peptone glucose (MYPG) medium contained malt extract 3.0g, yeast extract 5.0 g, peptone 5.0 g, glucose 20 g, distilled water 1litre, PH 4.8 was used. Each medium was treated with 10mg cycloheximide (actidion) per litre to inhibit yeast growth and autoclaved at 121 ºC for 15min.

For isolation of *Zymomonas mobilis* from rotten *Citrus aurantium* (sweet orange), fermented apple juice and fresh palm saps, the method described by Obire (2005) [9] was adopted. The orange samples were washed thoroughly with water to remove dirt and some surface contaminants. The juice were squeezed out into a beaker, using different sterile syringes 1ml of each sample (apple, orange, and palm saps) were taken and diluted with 9ml sterile distilled water in a test tube and serially diluted in sterile distilled water from 10^1-10^6 dilutions. The spread plate technique was used to inoculate the samples on the sterilized media. Plates were incubated for 2 days at 30 ºC in an anaerobic jar containing anaerobic gas packs (to exhaust the oxygen and produce CO_2 saturated atmosphere) after which presumptive *Z. mobilis* isolates were obtained. Presumptive colonies of *Zymomonas mobilis* were sub-cultured on fresh MYPG medium and incubated for 2 days at 30 ºC. Isolates were preserved at 4 ºC until required.

Identification and Characterization of Presumptive *Zymomonas mobilis*

Presumptive *Z. Mobilis* isolates were subjected to some conventional biochemical tests for identification. Gram stain, catalase test, motility, oxidase, urease, nitrate reduction, indole, carbohydrate fermentation tests as well as alcohol tolerance tests were carried out according to the method described by Cheesebrough, 2010 [1].

Confirmation of *Zymomonas mobilis* isolates using PCR

Zymomonas mobilis DNA extraction

Genomic DNA extraction was done by using DNA purification kit from Thermo Scientific Products. A single colony of *Z. mobilis* was inoculated into 10ml standard media broth and incubated overnight at 37 ºC. The bacterial cells of 2×10^9 were harvested by centrifugation for 10 minutes at 500 × g and the supernatant was discarded. The pellet was resuspended in 180 µL of digestion solution with the addition of 20µL of proteinase K solution and mixed thoroughly by vortexing to obtain a uniform solution. The sample was incubated at 56 ºC by shaking in a water bath until the cells were completely lysed for 30min. 20uL of RNase A solution was added and mixed by vortexing and incubated for 10 min at room temperature. 200ml of lysis solution was added and mixed by vortexing for about 15 s until a homogeneous mixture was obtained. To the mixture, 400 µL of 50% ethanol was added and mixed by vortexing. The DNA was purified using the GeneJET Genomic DNA Purification column with the aid of wash and elution buffers. The purified DNA was used immediately or stored at -20 ºC.

Zymomonas mobilis Polymerase chain reaction

The target primers were for alcohol dehydrogenase I gene which include *Zad HIF* and *Zad HIR* with a primer sequence; ((5'CCCTCGAGGTAATCGGCTGGCAAT CGTTTTCC 3') and 23SrRNA (5' GTTCTAGAGATAGCGGCTTATAGCAACGAGTG 3') of about 1.2-kilobase size as well as 16SrRNA gene primers which include Z16P3-F and Z23P5-R. with sequences; (5'-CAAGCCTGCAAAGGTTAG-3') and (5'-ATGCTCTTACCTCACGCT-3') of about 900bp. The PCR reaction mixture of 50µl contained 2.5 mM dNTPs, 5 µg genomic DNA template and 20 µmol of each primer. The PCR mixture was subjected to the following thermal conditions using DNA thermal cycler: The Products were isolated after 40 cycles of amplification with initial denaturation at 94 °C for 30 s, 30 seconds at 58 °C and 2 minutes at 72 °C, final extension for 3 minutes at 72 °C. Purification to obtain PCR products was done with the QIA quick PCR purification kit (Qiagen). All amplification products were separated into 1% agarose gels which were stained with ethidium bromide. The PCR products were observed under an ultraviolet (UV) light Tran's illuminator (Sambrook *et al*., 1989) [14].

Results

The cultural and biochemical properties of the presumptive *Zymomonas mobilis* isolates (Table 1) that were gotten from palm sap, apple juice and orange juice revealed that all the isolates were rod-shaped, Gram-negative, had the ability to ferment glucose, fructose and sucrose. Only isolates ZM2, ZM3, ZM4 and ZM 5 could not ferment maltose, lactose and xylose. All the isolates were urease negative, indole negative and oxidase negative. Isolates ZM2, ZM3, ZM4 and ZM 5 were further characterized using molecular techniques (PCR Amplification of the 16SrRNA gene and Alcohol dehydrogenase (ADH I) gene of *Zymomonas mobilis*).

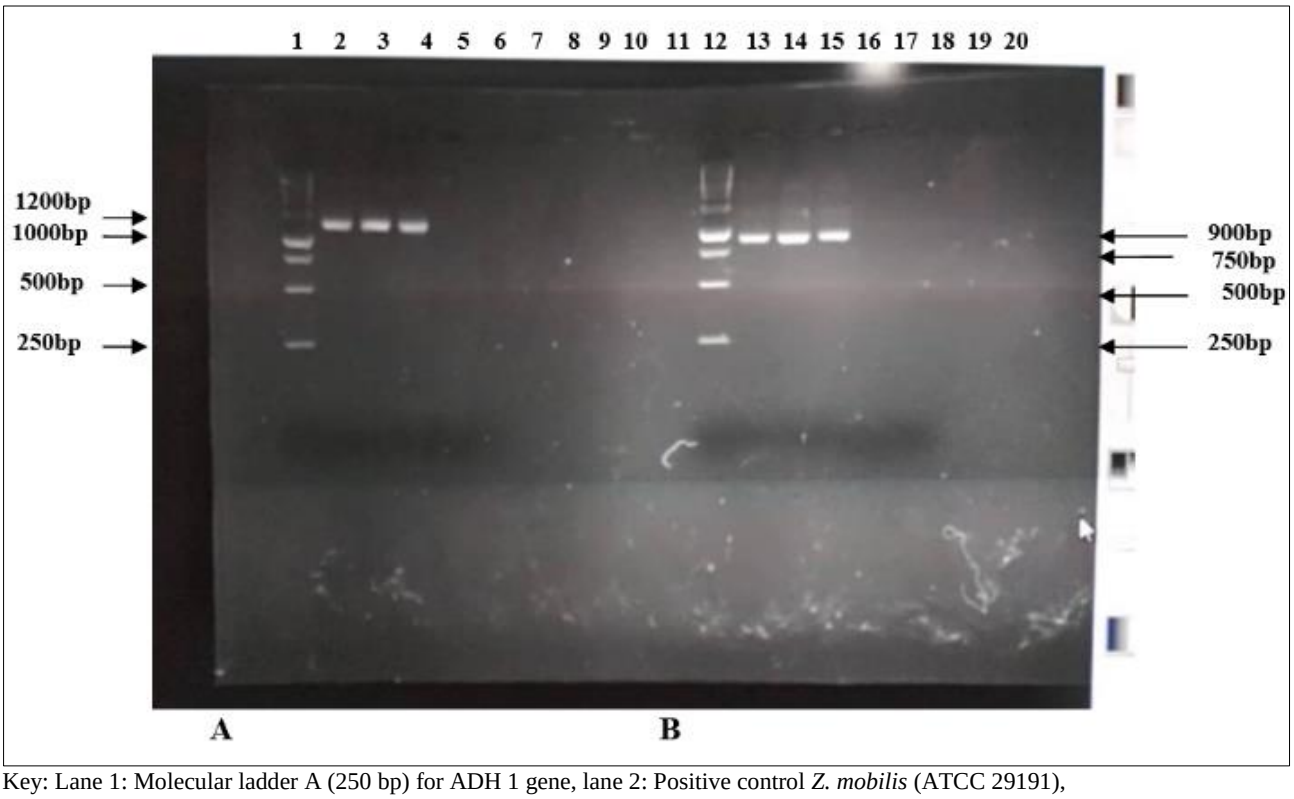
The results of the gel electrophoreses (Plate 1A and 1B) of the PCR Amplification of the Alcohol dehydrogenase gene (1200bp DNA fragment) and 16SrRNA gene (900bp DNA fragment) of *Zymomonas mobilis* ATCC 29191, bacterial isolates ZM2, ZM3, ZM4 and ZM5 showed that *Zymomonas mobilis* ATCC 29191 (positive control), isolates ZM2 and ZM3 had bands at 1200 bp position of the ladder, there were no bands for ZM4, ZM5 and the negative control. Similarly,

Zymomonas mobilis ATCC 29191, isolates ZM2 and ZM3 had bands at 900bp position of the ladder but there were no bands for ZM4, ZM5 and the negative control.

Some parameters were checked to choose the best *Zymomonas mobilis* isolate to use for bioethanol production. They include the growth of the isolates in the presence of varying ethanol concentrations and the isolate with the highest ethanol yield. The result (Table 2) showed the growth of *Z. mobilis* isolates at varying ethanol concentrations while Table 3 showed the ethanol yield in Rich medium. ZM2 had optical densities that ranged from 1.36-0.34, as the ethanol concentrations increased from 2.5-15.0% while ZM3 had optical densities that ranged from 1.40-0.66 as the ethanol concentrations increased from 2.5-15.0%. ZM3 had the highest ethanol yield of 80 g/l, while ZM2 had the lowest ethanol yield of 60 g/l. The result of the statistical analysis (two samples T-Test) carried out showed that statistically significant difference exists for the ethanol yields of ZM3 and ZM2 after 48 h and 72 h fermentation with $p \leq 0.05$.

Table 1: Cultural and Biochemical properties of presumptive *Zymomonas* spp from palm sap, apple juice and orange juice samples

Isolate	Cell shape	Colony Morphology	Gram reaction	Lactose	Maltose	Xylose	Glucose	Fructose	Sucrose	Motility	Oxidase	Indole	Urease
1	Rod shape	Moist and whitish	_	_	+	_	+	+	+	+	_	_	_
2	Rod shape	Moist and whitish	_	_	_	_	+	+	+	+	_	_	_
3	Rod shape	Moist and whitish	_	_	_	_	+	+	+	+	_	_	_
4	Rod shape	Moist and whitsh	_	_	_	_	+	+	+	+	_	_	_
5	Rod shape	Moist and whitish	_	_	_	_	+	+	+	+	_	_	_
6	Rod shape	Moist and creamy	_	_	+	_	+	+	+	+	_	_	_
7	Rod shape	Moist and creamy	_	_	+	_	+	+	+	+	_	_	_
8	Rod shape	Moist and creamy	_	_	+	_	+	+	+	+	_	_	_
9	Rod shape	Moist and creamy	_	_	+	_	+	+	+	+	_	_	_
10	Rod shape	Moist and creamy	_	_	+	_	+	+	+	+	_	_	_
11	Rod shape	Moist and creamy	_	_	+	_	+	+	+	+	_	_	_



Key: Lane 1: Molecular ladder A (250 bp) for ADH 1 gene, lane 2: Positive control *Z. mobilis* (ATCC 29191),
Lane 3: ZM2, Lane 4: ZM3, Lane 5:ZM4, Lane 6:ZM5, Lane 7: Negative control.
Plate 1B: Amplification of 16SrRNA gene (900bp) of *Zymomonas mobilis*
Key: Lane 12: Molecular ladder 2(250bp) for 16SrRNA gene, Lane 13: *Z. mobilis* (ATCC 29191), Lane 14: ZM2, Lane 15: ZM3, Lane 16: ZM, Lane 17:ZM5 and Lane 18: Negative control.

Plate 1A: Amplification of Alcohol dehydrogenase gene I (ADH I, 1200bp) of *Zymomonas mobilis*

Table 2: Alcohol tolerance of ZM2 and ZM3

Isolate	Growth (Optical Density at 540nm) at Varying Ethanol Concentrations					
	2.5%	5.0%	7.5%	10.0%	12.5%	15.0%
ZM2	1.36 ± 0.02^{b}	1.23 ± 0.01^{b}	1.22 ± 0.01^{b}	1.09 ± 0.01^{b}	1.00 ± 0.02^{b}	0.34 ± 0.01^{b}
ZM3	1.40 ± 0.02^{ab}	1.24 ± 0.01^{b}	1.21 ± 0.01^{b}	1.11 ± 0.01^{b}	1.06 ± 0.01^{b}	0.66 ± 0.01^{b}

Mean±SD that do not share at least a superscript are statistically significant ($p \leq 0.05$).

Table 3: Ethanol yield of ZM2 and ZM3 after fermentation of glucose in Rich medium

Ethanol yield in rich medium (g/l) Mean±*SD*			
Isolate	24 hr.	48 hr.	72 hr.
ZM2	24.0±2.83	48.0±0.00[b]	60.0±8.49[b]
ZM3	30.0±2.83	69.0±0.14[a]	80.0±7.07[a]

Mean±SD that do not share at least a superscript are statistically significant ($p \leq 0.05$)

Discussion

A total of thirty bacterial isolates were obtained from palm sap, apple juice and orange juice. Of the thirty presumptive *Zymomonas* species isolated, only isoloates ZM2, ZM3, ZM4 and ZM5 had morphological and biochemical characteristics that were similar to that of *Zymomonas mobilis* that were in agreement with the characteristics of *Zymomonas mobilis* published by other authors (Laura *et al*., 2014, Zhang *et al*., 1995 and Dworkin *et al*.,1996) [7, 18, 20]. The four isolates were therefore further characterized using molecular approach.

Genomic DNA was isolated from *Zymomonas mobilis* ATCC 29191, ZM2, ZM3 and ZM4. A fragment of 900bp that encodes the 16SrRNA gene that was specific for *Zymomonas mobilis* was amplified. Another fragment of 1200bp that encodes the alcohol dehydrogenase I gene that was specific for Z*ymomonas mobilis* was also amplified. Isolates ZM2, ZM3 and *Zymomonas mobilis* ATCC 29191 all had clear bands at 900 bp and 1200 bp regions for the two genes amplified whereas isolate ZM4, ZM5 and the negative control did not have any bands at all. The results of the amplification of the two genes specific for *Zymomonas mobilis* further confirmed the identity of isolates ZM2 and ZM3 as *Zymomonas mobilis.* The result coincided with the studies carried out by other authors (Laura *et al*., 2014; Coton *et al*., 2005 and Zhang *et al*., 2014) [7, 2, 19].

The two confirmed *Zymomonas mobilis* isolates were tested for ethanol tolerance by measuring their growth at different ethanol concentrations and their ethanol yields from fermentation of glucose in rich media to select the best isolate for bioethanol production. From the results, ZM3 grew better in the presence of different alcohol concentrations which were evident by the higher optical densities the isolate had as compared to ZM2. The isolate had a better alcohol tolerance than ZM2. High alcohol tolerance in microbes that would be used for fermentation is an important characteristics that is been considered when selecting strains for fermentation. ZM3 also had the highest ethanol yields from the fermentation of glucose when compared to the ethanol yields of ZM2. The result of this research work revealed that ZM2 and ZM3 both have good potential as candidates for bioethanol production.

Conclusion

Zymomonas mobilis were isolated and characterised morphologically, biochemically and by using molecular techniques through amplification of the 16SrRNA gene and alcohol dehydrogenase gene specific for *Zymomonas mobilis*.

Zymomonas mobilis isolate ZM2 and ZM3 demonstrated relatively high abilities to withstand high concentrations of ethanol and abilities to produce ethanol using glucose. The two isolates therefore have great potential for industrial bioethanol production.

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