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African Journal of Biotechnology

Full Length Research Paper

Comparative studies on the antimicrobial, chemical and biochemical contents of the foliar extracts of *Capsicum fructescens* L. varieties

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Capsicum fructescens **is a variety of Capsicum with much nutritional and therapeutic usefulness. This study is aimed at investigating the antimicrobial, chemical and biochemical properties of *C. fructescens.* The biochemical, mineral and phytochemical analyses of the extract were carried out using standard methods. The organic contents of the extracts were determined by GCMS to identify its bioactive constituents which were tested for antibacterial and antifungal activities against 7 bacterial and 3 fungal isolates using disc-diffusion method. The biochemical analysis showed that the leaves were high in protein, fibre, carbohydrate and fat. Different mineral elements were detected in the leaves and they include Magnesium, Calcium, Iron, Sodium, Copper, Potassium, Zinc and Manganese. GC-MS revealed 13 different organic compounds belonging to four groups of chemicals namely alkanol, alkanoic acid, alkanoate and ester. The methanolic extracts of *C. fructescens* leaves at a dose range of 5 and 25 mg/ml showed significant antibacterial and antifungal activity on some test organisms. The presence of great quantity of dodecanoic acid among other compounds in the extract of bawa suggested the reason for its profound anti-staphylococcal and anti-candidal activities. This study concluded that *C. fructescens* foliar extract is rich in important chemical and biochemical metabolites which have shown some therapeutic properties. An advocacy in the higher consumption of these peppers among folks is hereby recommended.**

Key words: *Capsicum fructescens* extracts, chemicals and biochemical composition, antimicrobial activity.

INTRODUCTION

Plants including cryptogams have shown to be rich in bioactive constituents (Femi-Adepoju et al., 2018). *Capsicum* species with several bioactive compounds are increasingly gaining interest due to their effectiveness in improving nutrition and human health (Unuofin et al., 2017).

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The origin of the name *Capsicum* is most likely from the Latin word, “capsa” meaning to bite, in allusion to the hot pungent properties of the fruit and seeds with several universal English names, include chili, sweet pepper, hot pepper and bell pepper, belongs to the family Solanaceae (Adepoju et al., 2019). Their fruit becomes brightly colored once its seeds are mature enough to germinate thereby attracting the attention of birds that then distribute the seeds. In West Africa, the genus *Capsicum* is represented by two cultivated species, namely, *Capsicum annuum* and *Capsicum frutescens*, with numerous varieties. They are the third in Nigeria among the cultivated vegetables being utilized in the dry state as spice due to their capsaicin content. Peppers generally have been found to contain essential vitamins, minerals, and nutrients in various quantity which serves great usefulness for human health. They also contain a number of phytochemicals such as carotenoids, capsaicinoids, flavonoids, ascorbic acid, and tocopherols which have been reported to prevent inflammatory diseases associated with oxidative damages and maintain optimum health (Kim et al., 2019). Many reports have been given and published on the comparative analysis of metabolite compositions in *Capsicum* spp. and *C. frutescens* has not been left out (Kantar et al., 2016; Sarpras et al., 2016).

Capsicum belongs to a group of crops that are widely cultivated for its spicy nature and nutritional value. Five species (*C. annuum* var. *annuum, Capsicum chinense, Capsicum frutescens, Capsicum baccatum* varieties *pendulum* and *umbilicatum,* and *Capsicum pubescens*) were domesticated by American natives. After Columbus, they became widely exploited in tropical to temperate regions because of their fruits, which have high nutritional contents, especially in vitamins. Peppers are constituents of the human diet, the pungent cultivars are used as spice ("ajies," "paprika," "chilies," "hot peppers") and the sweet types as vegetables ("sweet pepper," "bell pepper," "pimiento"). Hot red peppers consist of spicy compounds called capsaicinoids which include capsaicin, dihydrocapsaicin, nordihydrocapsaicin and other compounds (Ludy et al., 2012). Capsaicin, water-insoluble derivative of homovanillic acid and the main active ingredient in capsicum fruits, is responsible for hot sensation to the tongue (Papoiu and Yosipovitch, 2010). *C. chinense* species have been reported traditionally to contain the hottest cultivars (Canton-Flick et al., 2008). According to González et al. (2004), the seed and placenta tissues of *C. chinense* are reported to contain most of the capsaicin with 37 and 62%, respectively.

The crop is employed both as condiment and food; the thick sweet fleshy or non-pungent varieties are used in salads or stuffed with meat and cooked (Adepoju et al., 2019). In addition to the use of capsicum fruits in traditional medicine and food additives, it has been reported to be useful in the treatment of sore throat, cough, healing wound, toothache, parasitic infections and rheumatism (Singletary, 2011), it has also been utilized as an antiseptic (Pawar et al., 2011), immunomodulator and antioxidant (Otunola et al., 2017; Maji and Banerji, 2016), to protect against gastrointestinal ailments (Low Dog, 2006) including dyspepsia, loss of appetite, gastroesophageal reflux disease and gastric ulcer (Maji and Banerji, 2016). Other useful effects of Capsicums include antibacterial (Neelam et al., 2016) and anticancer (Pawar et al., 2011).

The fruits of pepper contain a range of bioactive phytochemicals including flavonoids, carotenoids, phenolics, and other antioxidant compounds (Alvarez-Parrilla et al., 2011). Several classes of plant chemicals including phenolic compounds and antioxidants are sufficiently available in high quantity in vegetables and fruits; thereby forming an important part of human consumption. Since numerous studies have suggested that eating foods rich in phytochemicals reduces risk of certain forms of cancer, cardiovascular diseases and stroke, much attention has been drawn to natural foods especially spices and vegetables rich in these compounds (Kaur and Kapoor, 2001; Prior and Cao, 2000). Takahashi et al. (2018) reported high antioxidant properties of the fruit extracts of *C. frutescens* from green to red stages based on the oxygen radical absorbance capacity (ORAC) and DPPH tests (Takahashi et al., 2018). This work focused on investigating the antibacterial and antifungal efficacies vis-avis biochemical, mineral, phytochemical and organic chemical contents of three varieties of *C. fructescens*, with a view to establish any relationships between them.

MATERIALS AND METHODS

Collection and identification of plant samples

Fresh leaves of three different *C. fructescens* varieties ijosi, bawa and sombo were collected into a sterile polythene bag. The leaves were identified at the Biology Laboratory Complex, Ladoke Akintola University of Technology, Ogbomoso, Nigeria. The harvested leaves were cleaned using clean drinkable water and air-dried for four weeks in the general biology laboratory complex, Ladoke Akintola University of Technology, Ogbomoso, Nigeria. The dried leaves were crushed using porcelain mortar and pestle and the resulting powders were kept in air-tight containers placed in cool, dry environment.

Preparation of extracts

Extractions were carried out by soaking 20 g of each of the powders in 200 ml of absolute methanol in well-labelled clean conical flasks and corked. After seven days of extraction, the soaked leaf powder were removed and the decanted solutions were concentrated on a rotary evaporator. The dried extracts were kept in a refrigerator in readiness for analyses. Enough quantity of powder was kept for various analyses for which they were needed. The analyses carried out on the dried leaf samples included those of biochemical and mineral compositions, while phytochemicals and organic chemical compositions by GC-MS analysis were carried out

on the leaf extracts, which were also used for the antimicrobial testing (Sukhdev et al., 2008).

Collection, growth and maintenance of test organism

Test organisms used for this study were obtained from the microbial gene bank of Pure and Applied Biology Department, Ladoke Akintola University of Technology, Ogbomoso, Nigeria. These include bacteria cultures of different strains, such as *Bacillus cereus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Pseudomonas putida, Staphylococcus aureus* and *Klebsiella pneumoniae* maintained at 37°C; then, fungal culture of *Aspergillus niger, Aspergillus flavus* and *Candida albicans* maintained at 28°C.

Inoculum preparation

For bacteria inoculums, two loopful of overnight grown and morphologically similar colony of bacteria was inoculated into 5 ml of sterile nutrient broth and incubated for 2 h at 37°C when the turbidity was equivalent to a 0.5 $BaSO_4$ standard. For fungi inoculums, 0.2 g of yeast extract was mixed with 1 g of sucrose in 100 ml of distilled water. 5 ml was pipette in test tubes and sterilized for 15 min at 121°C. After cooling, two loopful of test fungi was inoculated into the medium and incubated for 2 h after which the suspensions were maintained at 4°C for further use.

Biochemical analysis

The biochemical compositions of the dried leaf samples were determined using standard analytical methods. All measurements were done in duplicates and their values are presented in percentages. Moisture, ash, crude fibre, crude protein, fat and carbohydrate in the leaves of all the species studied were determined in accordance with the following procedures: moisture content (AOAC, 1995); crude fibre (James, 1995); protein (Pearson, 1976); fat (Onwuka, 2005); carbohydrate (Arithmetic Difference Method, that is, %CHO = 100 - (% fat + % ash + % fiber + % protein).

Mineral content analysis

Estimation of mineral substances in dried grinded leaves was performed by using a NOVA400 atomic absorption spectrometer (ANALYTIK JENA AG, Jena, Germany) hollow cathode lamps and acetylene/air flame to measure absorbance. By using slits, wavelengths and lamp current; sodium (Na), potassium (K), manganese (Mn), magnesium (Mg), zinc (Zn), phosphorus (P), copper (Cu) and iron (Fe) were calculated. The analyzed results for Na, Mg, Ca, K, Zn, Cu and Fe contents were expressed in ppm.

Qualitative and quantitative phytochemical analysis of plant extracts

Chemotaxonomic studies on *C. fructescens* varieties ijosi, bawa and sombo focused on qualitative and quantitative analyses for phlobatannins, alkaloids, tannins, cardiac glycosides, saponins, flavonoids, terpenoids and phenols. The qualitative analysis of the bio-constituents present in the plant extracts was performed using the methods of Trease and Evans (1989).

Quantitative phytochemical screening

Determination of saponin content was done by the spectrophotometric method described by Brunner (1984), flavonoids was determined according to the method outlined by Harborne (1984). The quantity of alkaloids, tannins, total phenols and cardiac glycosides were determined by the alkaline precipitation gravimetric method described by Harborne (1984), spectrophotometric method of Makkar et al. (1993), the method described by Mahadevan and Sridhar (1982) and the use of Buljet's reagent as described by El-Olemy et al. (1994), respectively.

Analysis and identification of organic compounds in the plant extracts

The GC-MS analysis of the leaf extracts was carried out at the department of Chemical Engineering, University of Ilorin on Agilent 19091S Gas chromatograph (GC) interfaced to a mass spectrometer 433HP-5MS instrument employing the following conditions: silica capillary column fused with 100% phenyl methyl silox, (length; 30 m × 250 μm; film thickness 0.25 μm). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at constant flow rate 1.5 ml/min and an injection volume of 1 μl was employed (Split ratio of 50:1) injector temperature-300°C; average velocity of 45.67 cm/s. The oven temperature was programmed from 100°C (Isothermal for 4 min) with an increase of 4°C min^{-1} to 240°C. Total GC running time was 49 min. The relative percentage amount of each component was calculated, by comparing its average peak area to the total areas. The software adopted to handle mass spectra and chromatogram was a turbomass. The detection employed the NIST Ver. 2.0 year 2009 library[29]. After the performance of the GCMS, was the identification of the components detected using their spectra.

Identification of components

Interpretation on mass spectrum of GC-MS was done using the database of National Institute of Standard and Technology (NIST) which contains more than 62,000 patterns. The mass spectra of the unknown components were compared with the spectrum of the known components contained in the NIST library. The name, molecular weight and structure of the components of the test materials were also ascertained using the fragmentation patterns they exhibited and the information available in the library.

Antimicrobial assay of plant extracts on test organisms

The antibacterial and antifungal activities of the plant extracts were studied using the disc-diffusion method. Petri plates were prepared with 20 ml of sterile nutrient agar for bacteria and potato dextrose broth for fungi. The bottom of each plate was divided into segment 5 and 25 mgL^{-1}, respectively with the control at the centre which each name of the organisms boldly written on each plate. The test organisms were swabbed on the solidified sterile media and the perforated filter paper (4 mm) each was put into the extract at varied concentration and was put into the marked area that match the disc. Methanol was used as control. Plates were incubated at 37°C for 24 h and 25°C for 48 h for bacteria and fungi, respectively. Positive control was prepared using broad spectrum antibiotic entamicin and augmentin as the case may be. The diameters of zone of inhibition (clearance) were recorded in millimeter.

Statistical analysis

Data obtained were analyzed with IBM SPSS version 20 software and subjected to one-way ANOVA to assess significant difference

Table 1. Percentage biochemical composition of three species of *Capsicum* studied.

Parameter	Fibre	CHO	Dry matter	Fat	Moisture	Protein	total ash
IJO	$16.01^{b} \pm 0.10$	$7.88^{b} \pm 0.12$	$2.40^{b} \pm 0.02$	$12.303^{b} \pm 0.23$	$37.700^{a} \pm 0.23$	$33.100^{b} \pm 0.22$	$15.730^{a} \pm 0.11$
SOM	$16.21^{a} \pm 0.21$	$6.30^{c} \pm 0.11$	$2.36^{b} \pm 0.01$	$12.100^{c} \pm 0.22$	$36.100^{b} \pm 0.23$	$33.250^{a} \pm 0.27$	$15.300^{b} \pm 0.23$
BAW	$14.00^{c} \pm 0.22$	$12.06^{a} \pm 0.21$	$5.63^{a} \pm 0.03$	$17.106^{a} \pm 0.21$	$31.624^{c} \pm 0.23$	$30.123^{c} \pm 0.35$	$10.906^{c} \pm 0.12$

Mean values in columns with different superscripts of alphabets are significantly different at P≤0.05 while those with the same alphabets are not significantly different at P≤0.05. IJO= *C. fructescens* var. *ijosi*, SOM= *C. fructescens* var. *sombo*, BAW= *C. fructescens* var. *bawa*.

Table 2. Mineral element composition (ppm) in the three species of *Capsicum* studied.

Parameter	Mn	Zn	Cu	Na	K	Mg	Ca	Fe	P
IJO	0.24^{b}	0.53^{c}	0.45^{a}	1.52^{c}	27.010^{a}	13.00^{a}	27.231^{b}	0.346^{a}	0.323^{b}
SOM	0.20^{c}	0.55^{b}	0.41^{b}	1.62^{b}	27.010^{a}	13.01^{a}	7.244^{a}	0.345^{a}	0.322^{b}
BAW	0.32^{a}	0.61^{a}	0.42^{b}	1.72^{a}	27.001^{b}	$1 3.01^{a}$	26.009^{c}	0.306^{b}	0.421^{a}

Mean values in columns with different superscripts of alphabets are significantly different at P≤0.05 while those with the same alphabets are not significantly different at P≤0.05. (IJO= *C. fructescens* var. *ijosi*, SOM= *C. fructescens* var. *sombo*, BAW= *C. fructescens* var. *bawa*.

between groups followed by Tukey post-hoc test at 95% significance level and expressed in mean ± standard deviation (SDEV) using Microsoft office Excel version 2007.

RESULTS AND DISCUSSION

The result of the biochemical composition of the leaves studied is shown in Table 1 while that of the mineral elements is presented in Table 2. The leaves of all the plants were significantly rich in important nutritional factors such as crude fibre, carbohydrate, fat and protein. Of all the nutrients detected, the leaves were particularly high (30-33%) in protein, but low (2.1-2.6%) in dry matter content (Table 1). The quantities of the proximate contents (%) of the *Capsicum* spp. studied can be listed from the highest to the lowest as moisture>protein> crude-fibre>total ash> crude fat>carbohydrate>dry matter.

The mineral elements detected in the leaves analyzed were Calcium, Magnesium, Iron, Sodium, Potassium, Copper, Zinc and Manganese. The leaves were particularly high in Calcium (26.0-27.3%), Potassium (27-27.7%) and Magnesium (13%), but low as expected of trace elements, in Manganese, Zinc and Copper (Table 2). On the whole, the quantities of the mineral elements in the plants studied can be enumerated from the highest to the lowest as Potassium>Calcium>Magnesium>Sodium>Zinc> Copper>Phosphorus>Iron and Manganese.

Crude fibre reportedly increases stool mass and hastens digestion. It does not get digested in humans and animals but it aids the normal functioning of the intestinal tract. According to Bello et al. (2008), fibre assists in maintaining human health and it has been reported to function in the reduction of cholesterol levels of the body (Bello et al., 2008). Diets with low fibre contents have been associated with increased risks of heart malfunctions, cancer of the colon and rectum cells, varicose veins, phlebitis, obesity, appendicitis, diabetes and even constipation (Ibanga and Okon, 2009; Lajide et al., 2008; Saldanha, 1995) established that minerals are essential in human nutrition while O'Dell (1979) stated that minerals from plants are less readily available than those from animals. From a recent research conducted by Kim et al. (2019), the results of percentage variability for proximate analysis of some *Capsicum* spp. extract, it was reported that, the quantity of ash, crude fiber, moisture, and crude fat were significantly affected by year and location (Kim et al., 2019). The levels of each mineral across four environments showed a high variation, which indicates that these

Table 3. Quantitative phytochemical contents of the *Capsicum* plants studied (mg/g).

Parameter	Alkaloid	Saponin	Phenol	Tannin	Flavonoid	Terpenoid	Card. Glycosid	Phlobatannin
IJO	0.65^{b} ±0.001	0.60^{b} ± 0.003	0.13^{b} ± 0.009	0.33^{b} ± 0.019	0.56^{b} ± 0.002	0.64^{c} ± 0.012	0.94^{a} ± 0.002	0.32^{b} ± 0.03
SOM	0.61^{b} ± 0.012	0.73^{a} ± 0.002	0.14^{b} ± 0.02	0.41^{a} ± 0.018	0.53^{b} ± 0.001	0.77^{a} ± 0.009	0.95^{a} ± 0.030	0.33^{b} ± 0.004
BAW	0.92^{a} ± 0.001	0.61^{b} ± 0.01	0.31^{a} ± 0.001	0.40^{a} ± 0.003	0.72^{a} ± 0.02	0.70^{b} ± 0.021	0.82^{b} ± 0.001	0.40^{a} ± 0.111

Mean values in columns with different superscripts of alphabets are significantly different at P≤0.05 while those without alphabets are not significantly different at P≤0.05. IJO= *C. fructescens* var. *ijosi*, SOM= *C. fructescens* var. *sombo*, BAW= *C. fructescens* var. *bawa*.

compounds are strongly influenced by environmental factors. However, previous studies have shown that mineral contents in peppers depend on a variety of factors (Sarpras et al., 2016). Four varieties belonging to the *Capsicum* spp. were characterized by their nutritional constituents, antioxidant vitamins and capsaicin contents in another research conducted by Olatunji and Afolayan (2020). They found out that, there was variability in nutritional, vitamins and capsaicin contents among varieties and that higher levels of vitamins could be found in fresh than in dry samples. Reports have also shown that varieties of *Capsicum* spp. contain important micro- and macro-elements with antioxidative vitamins, in varying quantities which can provide significant proportions of the recommended daily intake and help improve overall health of humans (Olatunji and Afolayan, 2020)

Minerals are vital for the overall physical and mental healthiness of man as they are important constituent of bones, muscles, teeth, tissues, nerve and blood cells (Soetan et al., 2010). Calcium and phosphorus are jointly essential for growth and maintenance of muscles, bones and teeth (Okaka et al., 2006; Ladan et al., 1996). Magnesium, according to Borgert and Briggs (1975) is a component of chlorophyll and it is important for calcium metabolism in bones whose deficiency can lead to ischemic heart disease (Elegbede, 1998). Zinc is involved in normal functioning of immune system and is associated with protein metabolism. Iron is an essential trace element for the formation of haemoglobin and also for the normal functioning of central nervous system (Asaolu et al., 1997). The deficiency of these nutrients and minerals are known to adversely affect health in animals.

Phytochemicals in the leaves of Nigerian cultivars of *Capsicum*

Total of eight secondary metabolites were detected in the leaves of the three Nigerian species of *Capsicum* studied. These included alkaloids, saponin, phenols, tannins, flavonoids, terpenoids, cardiac glycosides and phlobatannins (Table 3). Of these eight, the quantities of only two, that is, cardiac glycosides and phlobatannins did not show significant differences among the taxa studied.

The presence of phytochemicals in the foliar extracts of the *Capsicum* cultivars studied, suggests possible medicinal properties in them (AOAC, 1995). Saponins, phlobatannins and steroidal glycosides were detected in all the cultivars. Saponins have also been reported in *Senna alata* and *Cajanus cajan* by Lawal et al. (2014) and also in *Lophira lanceolata* seeds by Lohlum et al. (2010). According to Harborne (1984), saponins possess anti-hypercholesterol, anti-inflammatory, cardiac health enhancement properties and also appear to mortalise or inhibit growth of cancer cells without adversely affecting the normal body cells (Okwu, 2001). Phlobatannins were reported by Asquith and Butter (1986) as inhibitors of growth in many microorganisms like bacteria, fungi and viruses. According to De-Bruyne et al. (1997), tannins are plant polyphenols which can form complexes with metals ions and with macro-molecules such as polysaccharides and proteins. Enujiugha and Agbede (2000) established that tannins usually form insoluble complexes with proteins, thereby interfering with their bioavailability.

The phytochemicals

UAMME=Undecanoic acid, 10-methyl, methyl ester; HEAME=Hexadecanoic acid methyl ester; 811- OCME=8,11- octadecanoic acid methyl ester; 710-OCME=7,10- octadecanoic acid methyl ester; 912-OCME=9,12- octadecanoic acid methyl ester (E,E); 912-OCPE=9,12,15-octadecatienoic acid, 2,3-dihydroxy propyl ester (Z,Z,Z); (Z,Z,Z); 912-OCIME=9,12-Octadecadienoic acid, methyl ester; 13-OCME=Cis-13-octadecenoic acid methyl ester; 9-OCME=9-octadecenoic acid methyl ester; HH-12-EE=Hexadecanoic acid, 1-(hydroxymethyl)-

Table 4. The percentage peak area value of some organic compounds quantified in the leaves of three Nigerian cultivars of *Capsicum*.

Compound	Class	Percentage peak area values		
		[IJO]	[SOM]	[BAW]
UAMME	Ester	19.08	0	1.75
HEAME	Ester	5.80	0	0
811- OCME	Ester	6.57	0	0
710-OCME	Ester	0	0	2.81
912-OCME	Ester	0	0	0
912-OCPE	Ester	17.40	0	0
912-OCIME	Ester	0	3.55	0
13-OCME	Ester	0	0	13.06
9-OCME	Ester	0	21.77	0
HH-12-EE	Ester	0	3.50	0
METED	Alkanoate	5.80	0	0
DODEA	Alkanoic acid	0	17.48	55.15
ET-2-OCD	Alkanol	0	11.99	0

[IJO]= *C. fructescens* var. *ijosi*, [SOM]= *C. fructescens* var. *sombo*, [BAW]= *C. fructescens* var. *bawa*.

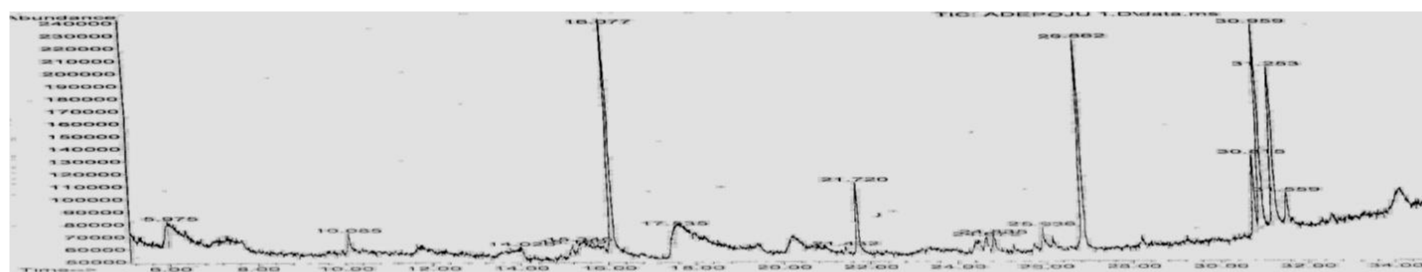

Figure 1. GC-MS Spectrum and some phytoconstituents in *C. fructescens* var. *ijosi*.

1,2-ethanediyl ester; METED=Methyl tetradecanoate; DODEA=Dodecanoic acid; and ET-2-OCD=Ethanol, 2-(9-octadecenyloxy)- Z.

The foliar organic compounds in Nigerian species of *Capsicum* via Gas Chromatography Mass Spectroscopy (GCMS)

The entries in Table 4 are the results of GC-MS analysis conducted on the leaves of the cultivars of *Capsicum* studied. A total of 13 different organic compounds were detected and these belong to four different groups of chemicals namely ester, alkanoate, alkanoic acid and alkanol. While 10 of the organic compounds were esters, only one belonged to each of the other groups (Table 4). Each of *C. fructescens* var *sombo* and *C. fructescens* var *ijosi* had five, the highest number of organic compounds detected by GCMS analysis, while *C. fructescens* var. *bawa* had four. Figures 1 to 3 present the GC-MS spectra and phytoconstituents in *C. fructescens* varieties. Neelam et al. (2016) reported that the GC-MS analysis of *n*-hexane and chloroform extracts of the seeds of *C. frutescens* revealed the presence of a total of 29 compounds from different classes. The major components found include Octadecadienal (*Z*), 3-Carene, Hexadecanoic acid, Tetracosane, Heptadec-8-ene-2,4-dione, 2(3H)-Furanone,dihydro-5-(2-octenyl)-, (*Z*) and Hexadec-8-ene-2,4-dione, in the n-hexane extract, while in the chloroform extract, Hexadecanoic acid, 9,12-Octadeca dienoic acid, 1-Hexadecene and 5-Eicosene, (*E*) were found. Octadecane, Eicosane, Docosane, 9,12-Octadecadienoic acid, methyl ester and Hexadecanoic acid, were found to be common in both the extracts (Neelam et al., 2016). Some of the compounds reported by Neelam et al. (2016) are also discovered by this study.

Antimicrobial activities of methanolic extracts of *C. fructescens* varieties on test organisms

The antimicrobial activity of methanolic extracts of

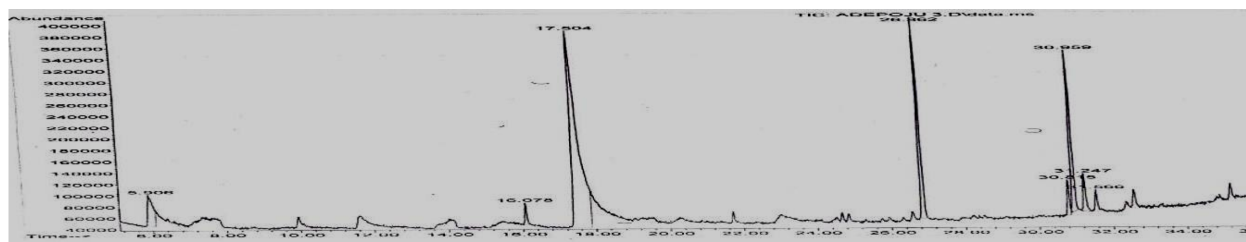

Figure 2. GC-MS Spectrum and some phytoconstituents in *C. fructescens* var *sombo.*

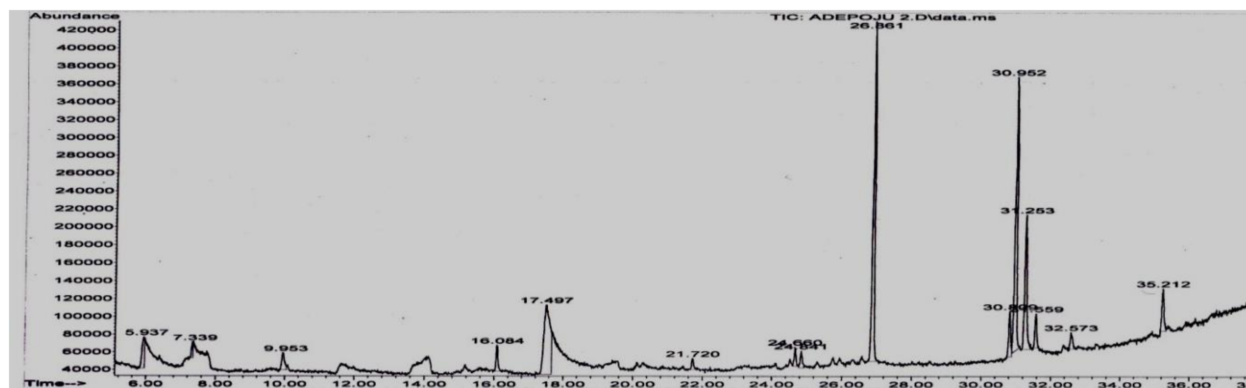

Figure 3. GC-MS Spectrun and some phytoconstituents in *C. fructescens* var *bawa.*

Capsicum leaf were investigated against the selected pathogens *P. aeruginosa, P. putida, B. cereus, K. pneumonia, B. subtilis, E. coli, S. aureus, A. niger, A. flavus* and *C. albicans* by disc diffusion method. The antimicrobial effects of leaves extracts of *C. fructescens* as shown by zone of inhibition in millimeters (mm) on selected bacteria and fungi are presented in Figure 4. The antibacterial activity of the extracts was found to be the highest at 5 mg/ml against *C. albicans* which produced an inhibition zone of 13 mm for ijosi; 5 and 25 mg/ml produced the highest zone of inhibition of 12 mm for sombo and 17/15 mm for bawa against *C. albicans*. *S. aureus* was found to be susceptible most to the foliar extract of bawa.

Significance of organic, proximate, mineral and phytochemical contents on antimicrobial efficacy of the extracts

In the present study, most of the identified volatile compounds belong to the class ester, alkanol, alkanoic acid and alkanoate. These compounds have been reported to be pharmacologically active. For example, hexadecanoic acid is known to have potential antibacterial and antifungal activities (McGraw et al., 2002); unsaturated fatty acids are also suggested to be responsible for the anti-inflammatory activity (Li et al., 2004); long-chain unsaturated fatty acids, such as linoleic acid, also show antibacterial activity and are the key ingredients of antimicrobial food additives and some antibacterial herbs (Chang et al., 2005), and hexadecanoic acid, methyl ester and 9,12-octadecadienoic acid (*Z*,*Z*)-, methyl ester have shown antioxidant and anticancer properties, respectively (Wei et al., 2011).

According to Ishida et al. (2000), crude fibers in the diet are necessary for digestion and effective to eliminate the risk of coronary heart disease, constipation, hypertension, colon, and diabetes and breast cancer (Ishida et al., 2000). Thus, these medicinal plants are regarded as a valuable source of dietary fiber in human nutrition. There is a strong correlation between fibre and moisture contents, as the fibre are easily digested and disintegrated which could be of interest to human health (Hussain et al., 2010). Minerals are required for normal growth, activities of muscles and skeletal development, copper and iron are responsible for cellular activity and oxygen transport, respectively, magnesium for chemical reaction in the body and intestinal absorption, sodium and potassium used in fluid balance and nerve transmission. Manganese plays a major role in production of energy and in supporting the immune system (Muhammad et al., 2011). Deficiency of these nutrients and minerals are known to affect the performance and health in both humans (MERCK, 2005).

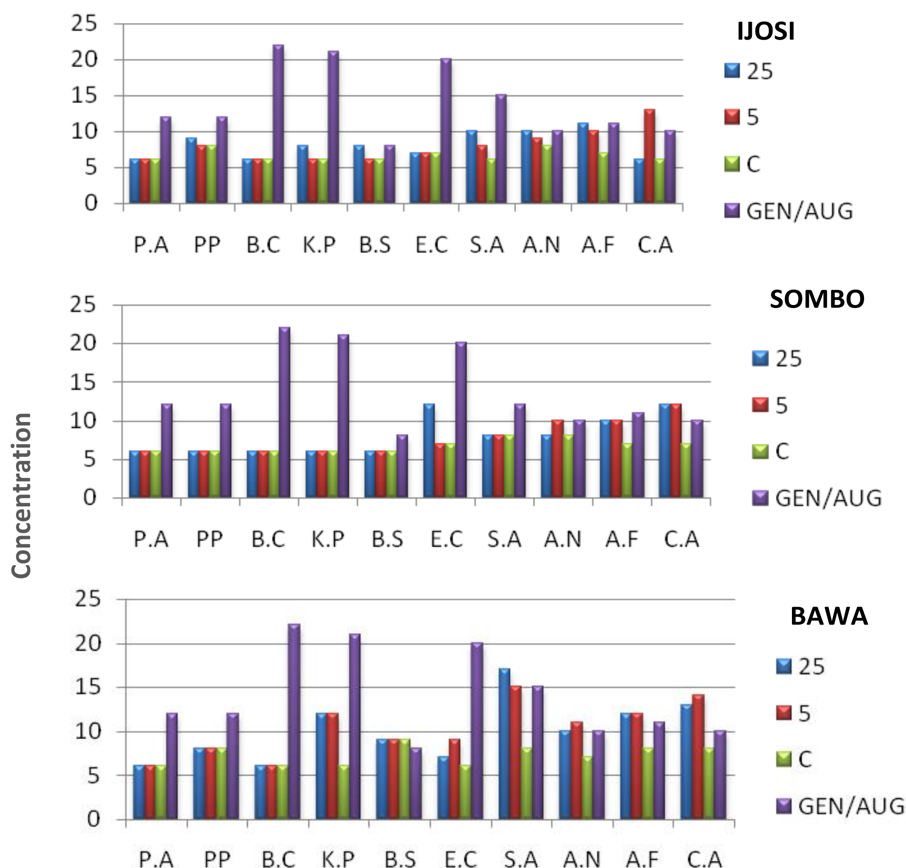

Figure 4. The antibacterial and antifungal potentials of leave extracts of *C. fructescens* on test organisms at varying concentrations in mg/ml. P.A= *P. aeruginosa*; P.P= *P. putida*; B.C= *B. cereus*; K.P= *K. pneumonia*; B.S= *B. subtilis*; E.C= *E. coli*; S.A= *S. aureus*; A.N= *A. niger*; A.F= *A. flavus* C.A= *C. albicans*; C = Control.

In earlier studies, the antimicrobial activity of ethanolic, methanolic and aqueous extracts of *C. frutescens* has already been reported against a number of microorganisms (KoffiNevry et al., 2012; Shariati et al., 2010). In a study by Vinayaka et al. (2010), even the foliar methanolic extract of *C. frutescens* showed dose-dependent antibacterial activity against *S. aureus*, *K. pneumoniae* and *P. aeruginosa*. Aqueous extracts of the leaf and fruit of *C. frutescens* have also exhibited potentials to prevent growth of seed-borne fungi (Soumya and Nair, 2012). In the study carried out by Neelam et al. (2016), broad spectrum activity was shown to be exhibited by low polar *n*-hexane and chloroform extracts of *C. frutescens*. This significant antimicrobial activity could be attributed to the compounds identified in the GC-MS spectrum. Isolation and proper identification of these antimicrobial agents from capsicum can lead to an important improvement in the area of food safety and can be used in the prevention of certain human diseases (Neelam et al., 2016). Also, the antimicrobial property of silver nanoparticles fabricated from the extract of *C. frutescens* was higher than that of the other two spices (Otunola et al., 2017).

Flavonoids have been studied to be very useful as an antimicrobial agent, inhibitor of mitochondrial adhesion, as an anticancer agent and antiulcer agent (Biju et al., 2014). It has been confirmed that consumption of food and beverages rich in phenol prevent diseases, such as cancer (Chalise et al., 2010). In this study, the presence of flavonoid and phenolic compounds in *C fructescens* var. leaf extracts confer health benefits associated with it. The significant antimicrobial activity of methanolic extracts of *C. fructescens* var. might be due to the synergistic effects of compounds identified in the proximate, organic, mineral and phytochemical analyses of the plant part. Something striking to note is that bawa extract was found to be most active against *S. aureus* and *C. albicans* with a zone of inhibition that suggests a better performance than the test drugs.

Conclusion

The leaves of the Nigerian species of *Capsicum* studied

are rich in important mineral elements such as calcium, magnesium, iron, sodium, potassium, copper, zinc and manganese; and are also sufficiently rich in some notable nutritional factors such as protein, crude fibre, total ash, crude fat and carbohydrates. The data obtained from the antimicrobial tests, as well as those of phytochemical constituents and organic compounds in the leaf extracts have revealed that the extracts contain important bioactive compounds. The significance of this is that an advocacy for more consumption of these peppers among folks can deal with infections associated with these pathogens. They can also be useful in the formulation of disinfectants and antiseptics as well as in chemotherapy. Relative abundance of the foliar nutrients in the species of the genus studied has far reaching implications in an effort to diagnose the species as potential leafy vegetables and as components of herbal formulations.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

First report of mortality in *Phoenix canariensis* associated with subgroup 16SrIV-D phytoplasmas in Coahuila, Mexico

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In 2015, a disease of unknown origin appeared in Torreon, Coahuila, in the Northeast of Mexico, causing great mortality in *Phoenix* palms, especially in *Phoenix canariensis*. Until early 2019, around 1300 palms died from this disease. The aim of this study was to determine its etiology. The symptoms registered in affected palms were similar to those described for Texas Phoenix Palm Decline (TPPD). Phytoplasmas were detected in samples from nine *P. canariensis* individuals using a TaqMan/real-time PCR assay specific for group 16SrIV detection. DNA of positive samples was amplified by nested PCR using primer pair P1/P7 followed by LY16Sf/LY16-23Sr and R16F2n/R16R2. *In silico* analysis of the sequences obtained revealed the presence of phytoplasmas associated with TPPD, belonging to subgroup 16SrIV-D. This is the first report of a disease associated with subgroup 16SrIV-D phytoplasmas in the Northeast of Mexico, further extending the known geographical range of this pathogen.

Key words: Texas Phoenix palm decline, 16SrIV-D phytoplasmas, nested polymerase chain reaction (PCR), sequence analysis.

INTRODUCTION

Lethal yellowing-type diseases (LYDs) associated with group 16SrIV phytoplasmas are among the most important diseases affecting palms worldwide due to the significant economic losses that they have caused throughout the years, particularly in coconut (*Cocos nucifera* L.) plantations (Gurr et al., 2016). Since no successful method for culturing group 16SrIV phytoplasmas on artificial media exist, these pathogens are currently studied using a variety of detection techniques that target phytoplasma DNA in infected host tissues, such as nested PCR (Gundersen and Lee, 1996), which is useful for phytoplasma characterization and *in silico* sequence analysis, and real-time PCR (Córdova et al., 2014), which increases sensitivity and reduces sample-processing time.

In addition to *C. nucifera*, LYDs are known to affect

several other palm species including edible date (*Phoenix dactylifera* L.) and Canary Island date (*Phoenix canariensis* hort. ex Chabaud) (Harrison and Elliott, 2016). These two species of palms have been used as ornamentals for more than sixty years in the city of Torreon, Coahuila, Mexico. In 2015, some of these plants began showing symptoms suggestive of a phytoplasma disease, similar to both lethal yellowing (LY) and one of its variants, the Texas Phoenix palm decline (TPPD) (McCoy et al., 1980; Harrison et al., 2008). By early 2019, the disease in Torreon was associated with the death of around 1300 *Phoenix* species palms throughout the city, mostly affecting mature *P. canariensis* individuals.

The phytoplasmas associated with LY and TPPD, subgroups 16SrIV-A and D, respectively, are the most commonly reported group 16SrIV strains affecting palm and non-palm species in the United States (Harrison et al., 2008; Bahder et al., 2019), Mexico (Oropeza et al., 2020), Central America and the Caribbean region (Ntushelo et al., 2013; Myrie et al., 2014). Although *P. canariensis* and *P. dactylifera* can be affected by both subgroups (Harrison et al., 2008), observations made in Florida suggest that they are more susceptible to subgroup 16SrIV-D (Harrison and Elliott, 2016). Also, both phytoplasma subgroups have been detected in natural populations of one of their candidate vectors, the planthopper *Haplaxius crudus* Van Duzee (Narváez et al., 2018), whose occurrence in the urban area of Torreon has been reported recently (Hernández-Rodríguez et al., 2019). However, despite the fact that mortality in *P. canariensis* in association with subgroup 16SrIV-A and D phytoplasmas has been reported on numerous occasions in Southern United States (Harrison et al., 2002c, 2008; Ong and McBride, 2009; Singh, 2014), so far, there are no reports in scientific journals of either of these phytoplasmas affecting *P. canariensis* palms in Mexico, nor has an outbreak of LY or TPPD occurred in the country's entire Northeast region (comprising the states of Coahuila, Nuevo Leon and Tamaulipas). Therefore, the objective of this study was to determine, by means of nested PCR and real-time PCR detection, followed by *in silico* sequence analysis, if the disease affecting *Phoenix* palms in Torreon, Coahuila, is associated with a phytoplasma enclosed in group 16SrIV.

MATERIALS AND METHODS

Sampling and evaluation of palms

Between December 2015 and October 2018, trunk samples were collected from eleven Canary Island date palms with symptoms suggesting a LYD at different locations in the city of Torreon, state of Coahuila (25°32′22″N, 103°26′55″W). *P. canariensis* was selected for our study as it became evident that most symptomatic palms in Torreon belonged to this species. All sampled individuals as well as other *P. canariensis* showing identical symptoms were monitored closely in order to record and describe symptom progression. Samples were obtained using a portable electric drill following Oropeza et al. (2010). After collection, samples were stored on an ice box for transportation to the laboratory, where they were refrigerated (usually 1 to 3 days) until nucleic acid extraction. Samples from five asymptomatic *P. canariensis* were also included in this study for comparative purposes.

DNA extraction

Total DNA was extracted by modifying the previously described method of Doyle and Doyle (1990). 1 g of trunk tissue ground to a powder with liquid nitrogen was incubated in 5 mL of CTAB extraction buffer (2% CTAB/100 mM Tris-HCl pH 8.0/20 mM EDTA pH 8.0/1.4 M NaCl/1% PVP) added with 0.1% 1-Thioglycerol, for 30 min at 65°C. A volume of chloroform-isoamyl alcohol (24:1) was added and the mix was stirred. To precipitate the supernatant obtained after centrifugation, isopropanol and sodium acetate (3 M) were used, followed by incubation at -20°C for 1 h. Lastly, the DNA pellet was re-suspended in 30 to 50 µL of TE buffer (10 mM Tris/1 mM EDTA).

Phytoplasma detection by real-time PCR

Detection analyses for group 16SrIV phytoplasmas were performed by a TaqMan/real-time PCR assay following the protocol described by Córdova et al. (2014). Reactions were carried out using a Rotor-Gene® Q thermocycler (QIAGEN). The following amplification parameters were used: an initial phase of 2 min at 50°C followed by 10 min at 95°C, then 40 cycles of amplification, each with a first denaturation step at 95°C for 15 s and a second alignment step at 61°C for 1 min. The Ct (cycle threshold) value of each sample was assigned by manually adjusting the threshold line to intersect with the exponential phase of the amplification curves and automatically setting the baseline using the Rotor-Gene Q® - Pure Detection software version 2.0.2 (QIAGEN). A Ct cut-off value of 32 was used for discriminating positive and negative samples in accordance with Narváez et al. (2017). All samples were assessed in duplicate. Experimental controls consisted of DNA from a LY-infected *Pritchardia pacifica* Seem. and H. Wendl. palm (positive) and ultrapure water (negative).

Phytoplasma detection by nested PCR

Samples that were positive for the presence of phytoplasma DNA by the TaqMan/real-time PCR assay were subjected to nested PCR in order to obtain amplicons for sequencing purposes. For the first amplification, phytoplasma universal primers P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) were used, followed by a second round of amplification with universal primers

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R16F2n/R16R2 (Lee et al., 1993; Gundersen and Lee, 1996) or the group specific set LY16Sf/LY16-23Sr (Harrison et al., 2002c, b). All reactions were performed in a T100™ thermocycler (Bio-Rad). The amplification programs for the reactions with the P1/P7 and R16F2n/R16R2 primer pairs were carried out following the specifications of Cordova-Lara et al. (2017), while the reactions with the LY16Sf/LY16-23Sr pair were performed according to Harrison et al. (2002a). The P1/P7 products were diluted 1:20 or 1:40 with ultrapure water prior to the second amplification, for both sets of primers. Positive and negative controls were included in all reactions as described previously.

Cloning and sequencing

Amplification products were purified with the QIAquick® Gel Extraction Kit (QIAGEN) and cloned into the pGEM®-T Easy Vector System I (Promega) following the manufacturers' instructions. To purify the recombinant plasmids, the QIAprep® Spin Miniprep Kit (QIAGEN) was used. Cloned inserts were sent to the University of California, Davis (The College of Biological Sciences ^{UC}DNA Sequencing Facility) for subsequent sequencing.

In silico analysis

The F2nR2 fragment of all sequences was analyzed using the *i*PhyClassifier interactive online tool (Zhao et al., 2009). Virtual RFLP profiles were generated and similarity coefficients were calculated to classify the phytoplasma strains detected based on previously established criteria (Wei et al., 2008). Subsequent molecular analyses were performed with MEGA software version X (Kumar et al., 2018). A phylogenetic tree was constructed with representative phytoplasma 16S rRNA gene sequences applying the maximum likelihood method based on the General Time-Reversible model for nucleotide substitution. The reliability of the analysis was subjected to a bootstrap test with 1000 replicates.

RESULTS

Symptom description

P. canariensis palms with symptoms suggestive of a LYD (Figure 1) were monitored in Torreon, Coahuila. The observed progression of symptoms was as follows: in healthy or asymptomatic fruit-bearing palms usually all leaves were green (except those going through natural senescence) and inflorescences were bearing fruit (Figure 1A). At the onset of symptoms, infected palms started showing discoloration of the oldest leaves; yellowing was observed first, quickly followed by bronzing and desiccation (Figure 1B). This decay process continued to the middle canopy leaves (Figure 1C). During this period of foliar decay there was also premature fruit drop (Figure 1D), with inflorescences becoming fruitless and dry (Figure 1E), and in some cases, death of the spear leaf (Figure 1F). Finally, leaf deterioration reached the upper leaves (Figure 1G) and ended with the decay of the entire foliage (Figure 1H) and collapse of the crown (Figure 1I). In some plants, roots were revised, and decay was also observed (not shown).

Phytoplasma detection in date palms

Nine out of eleven symptomatic *P. canariensis* palms that were sampled tested positive to the group 16SrIV phytoplasma detection analysis with the TaqMan/real-time PCR assay; sampling dates and Ct values obtained for each palm are shown in Table 1. Five asymptomatic palms were also analyzed and no amplification was obtained. The DNA from the nine positive *P. canariensis* was then subjected to nested PCR to obtain amplicons for subsequent sequencing and phytoplasma identification. Positive amplifications were obtained for two samples with primer pair LY16Sf/LY16-23Sr and for three others with primer pair R16F2n/R16R2; no amplification was obtained for the rest of the samples.

Analysis of phytoplasma rDNA

Five phytoplasma sequences from *P. canariensis* were obtained and deposited in the GenBank® database of the National Institutes of Health (NIH). Primers R16F2n/R16R2 produced three 1249 bp sequences (Accession numbers MN384667, MN384668 and MN384670) and LY16Sf/LY16-23Sr produced two 1745 bp sequences (Accession numbers MN389510 and MN607700). BLAST® comparison of these sequences showed that the highest sequence identity matches were with sequences of subgroup 16SrIV-D phytoplasmas (Table 2).

Phytoplasma identification at the subgroup level was confirmed by analyzing in detail the F2nR2 fragment of the sequences obtained with *i*PhyClassifier. All sequences from Coahuila generated an identical virtual RFLP profile. This pattern was most similar to the one generated by the reference sequence of subgroup 16SrIV-D (Accession number AF237615), with a similarity coefficient of 0.98. Closer examination of the virtual gels revealed that the only difference between both patterns is produced by the *Bfa*I enzyme (Figure 2). Based on the criteria established by Wei et al. (2008), the phytoplasmas detected in the *P. canariensis* palms that were included in this study all belong to subgroup 16SrIV-D.

A phylogenetic analysis was carried out with phytoplasma 16S rRNA gene sequences (Figure 3). All Coahuila sequences were grouped in the same clade, which in turn was grouped into a larger one shared with other members of the 16SrIV-D and B subgroups as well. When examining the alignment used for the construction of the phylogenetic tree, a single base substitution was found at position 1286 of the 16S rRNA gene (in the reference sequence of subgroup 16SrIV-D, Acc. AF237615) that distinguishes the Coahuila strains from the rest of the subgroup 16SrIV-D phytoplasma sequences that were included in the matrix (Figure 4).

DISCUSSION

In the Americas, LYDs affecting *C. nucifera* and several

Figure 1. Symptom progression of the LYD observed in *P. canariensis* palms in Torreon, Coahuila. (A) Healthy *P. canariensis*. (B) Palm showing unusual discoloration of oldest leaves, leading to bronzing and desiccation. (C) Foliar decay continues to the middle leaves. (D-F) Premature fruit drop and inflorescence atrophy observed in mature palms during foliar decay, with death of the spear leaf detected in some instances depending on crown visibility. (G-I) Upper leaves become affected and die and the crown finally collapses.

other palms, including important ornamental species like *P. canariensis*, have been associated with group 16SrIV phytoplasmas (Ntushelo et al., 2013). In 2015, a disease with symptoms suggestive of a LYD appeared in Torreon, Coahuila, causing great mortality in *Phoenix* palms, especially in *P. canariensis* individuals around fifty to sixty years old. In this study, detection protocols based on nested PCR and real-time PCR were used to investigate the presence of group 16SrIV phytoplasmas in symptomatic *P. canariensis* individuals sampled in Torreon during 2015 to 2018. Our results showed that this disease is associated with phytoplasmas belonging to subgroup 16SrIV-D, thus representing a new outbreak of TPPD rather than LY. This diagnosis is consistent with

Table 1. Real-time PCR detection of group 16SrIV phytoplasmas in *P. canariensis* palms sampled in Torreon, Coahuila, showing LYD-type symptoms.

Sample	Sampling date	Real-time PCR	
		Ct	Diagnosis
1	December 2, 2015	19.5	+
2	December 2, 2015	21.1	+
3	December 2, 2015	21.6	+
4	December 2, 2015	21.4	+
5	December 2, 2015	22.3	+
6	December 2, 2015	18.3	+
7	October 14, 2018	ND	-
8	October 14, 2018	18.8	+
9	October 14, 2018	19.2	+
10	October 14, 2018	16.8	+
11	October 14, 2018	32.5	-
PC	NA	23.1	+
NC	NA	33.0	-

ND: No amplification detected; PC: positive control; NC: negative control; NA: not applicable.

Table 2. Standard nucleotide BLAST® comparison of Coahuila *P. canariensis* phytoplasma sequences with GenBank® database accessions, showing those accessions with the highest sequence identity.

Coahuila sequences		Phytoplasma sequences in the nucleotide collection			
Palm	GenBank accession	Sequence description	Classification	% Identity	GenBank accession
3	MN607700	Texas Phoenix palm phytoplasma	16SrIV-D	99.4	AF434989
6	MN389510	Texas Phoenix palm phytoplasma	16SrIV-D	99.4	AF434989
8	MN384670	Uncultered bacterium clone 3.2	16SrIV-D	99.5	KU714840
9	MN384668	Uncultered bacterium clone 3.2	16SrIV-D	99.8	KU714840
10	MN384667	Uncultered bacterium clone 3.2	16SrIV-D	99.9	KU714840

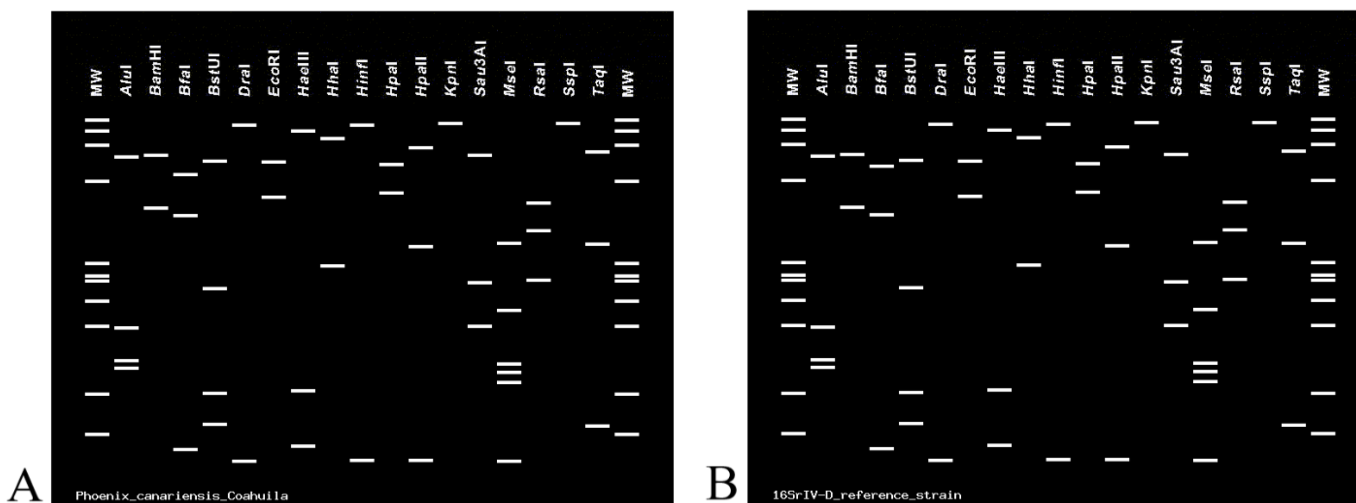

Figure 2. Virtual RFLP profiles of the F2nR2 fragment generated with *i*PhyClassifier. (A) Digestion pattern of the *P. canariensis* phytoplasma sequences obtained from Coahuila. (B) Digestion pattern of the reference sequence of subgroup 16SrIV-D (Acc. No. AF237615).

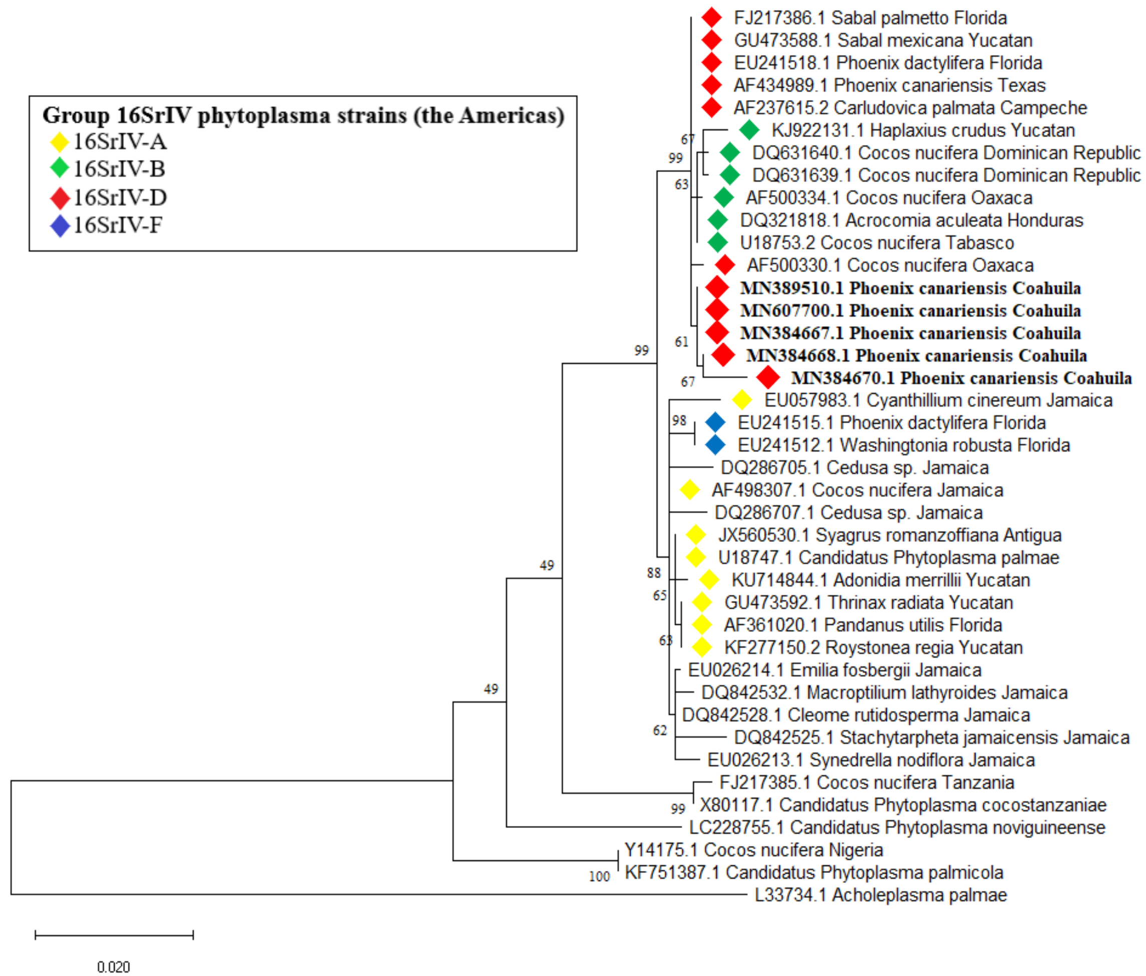

Figure 3. Molecular phylogenetic analysis of phytoplasma 16S rRNA gene sequences by maximum likelihood. Phytoplasma strains sequenced in this study are highlighted in bold. For the construction of the tree, representative sequences of group 16SrIV (shown in colored rhombi) as well as other related phytoplasma were used, indicating Acc. Nos., plant or insect host and the strain's country of detection (for USA and Mexico, states are specified instead); the sequence of *Acholeplasma palmae* was used to root the tree. The percentage of trees (from a 1000 bootstrap replicates) in which the associated taxa clustered together is shown next to the branches.

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Coahuila MN607700.1 1207 AACGCGAGTTTTTGGCAAATCTCAAAAAAATAGTCCTAGTTCGGATTGAA
Coahuila MN389510.1 1207 AACGCGAGTTTTTGGCAAATCTCAAAAAAATAGTCCTAGTTCGGATTGAA
Coahuila MN384667.1 1103 AACGCGAGTTTTTGGCAAATCTCAAAAAAATAGTCCTAGTTCGGATTGAA
Coahuila MN384670.1 1103 AACGCGAGTTTTTGGCAAATCTCAAAAAAATAGTCCTAGTTCGGATTGAA
Coahuila MN384668.1 1103 AACGCGAGTTTTTGGCAAATCTCAAAAAAATAGTCCTAGTTCGGATTGAA
Oaxaca   AF500330.1 1251 AACGCGAGTTTTTGGCAAATCTCAAAAAAATAGTCTTAGTTCGGATTGAA
Texas    AF434989.1 1251 AACGCGAGTTTTTGGCAAATCTCAAAAAAATAGTCTTAGTTCGGATTGAA
Campeche AF237615.2 1251 AACGCGAGTTTTTGGCAAATCTCAAAAAAATAGTCTTAGTTCGGATTGAA
Yucatan  GU473588.1 1167 AACGCGAGTTTTTGGCAAATCTCAAAAAAATAGTCTTAGTTCGGATTGAA
Florida  FJ217386.1 1155 AACGCGAGTTTTTGGCAAATCTCAAAAAAATAGTCTTAGTTCGGATTGAA
Florida  EU241518.1 1155 AACGCGAGTTTTTGGCAAATCTCAAAAAAATAGTCTTAGTTCGGATTGAA
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Figure 4. Sequence alignment of the 16S rRNA gene, from nucleotide positions 1251 to 1300 (in the reference sequence of subgroup 16SrIV-D, Acc. No. AF237615), of the subgroup 16SrIV-D phytoplasma strains used for the construction of the phylogenetic tree presented in this study. A single base substitution located at position T/C[1286] is shown for the Coahuila strains (non-shaded).

the symptoms observed in affected palms (Figure 1) according to the descriptions given by Harrison et al. (2002c) and Harrison and Elliott (2016). This is the first report of a disease associated with subgroup 16SrIV-D phytoplasmas in the Northeast of Mexico. Also, *P. canariensis* is reported for the first time as a host of this subgroup outside of the United States.

The majority of the *P. canariensis* sampled in our study (82%) were positive to the group 16SrIV detection analyses according to real-time PCR results. To classify these phytoplasma strains, five nested PCR products obtained with primer pairs LY16Sf/LY16-23Sr and R16F2n/R16R2, were cloned and sequenced. BLAST® analysis of these sequences suggested an identity with subgroup 16SrIV-D phytoplasmas. All sequences produced an identical virtual RFLP profile when analyzed with *i*PhyClassifier; this pattern was more similar to that of the reference sequence of subgroup 16SrIV-D (similarity coefficient of 0.98), with the only difference between them exposed by enzyme *Bfa*I in the virtual digestion. This change is likely the result of a single base substitution located at position T/C^{1286} of the 16S rRNA gene (Figure 4), since it provides an extra C^TAG recognition site for the enzyme and thus shortens the largest fragment produced in the virtual digestion (Figure 2). Information gathered so far suggest this substitution could be used to further differentiate between subgroup 16SrIV-D strains present in different regions, but more exploration is needed to support this hypothesis. In addition, a phylogenetic tree was constructed with phytoplasma 16S rRNA gene sequences. Coahuila sequences were grouped in the same clade, which, in turn, grouped into a larger one with other members of the 16SrIV-D and B subgroups present in other regions. This shows the genetic proximity of the phytoplasmas detected in Coahuila, which although they do not constitute a new subgroup within group 16SrIV, they can be clearly distinguished from other members of the 16SrIV-D subgroup in a phylogenetic tree based on differences in the virtual RFLP profile and the presence of point mutations in the sequence corresponding to the F2nR2 fragment of the 16S rRNA gene, a similar case to the geographical variants of subgroup 16SrIV-B strains reported affecting *C. nucifera* palms in the Dominican Republic (Martinez et al., 2008).

P. canariensis was the first species to show TPPD-like symptoms in Torreon, also the most affected during the outbreak, therefore our focus on this palm, however, later inspection of *P. dactylifera* individuals also present in the city and displaying similar symptoms (data not shown) suggest that TPPD is affecting this species as well, a prospect that needs confirmation. As of early 2019, this disease has been associated with the death of around 1300 *Phoenix* spp. palms in several parts of the city, where approximately 2000 *Phoenix* palms were estimated to be present before the onset of the disease (Samaniego-Gaxiola et al., unpublished data). To our knowledge, this is also the first reported outbreak of a LYD associated with subgroup 16SrIV-D phytoplasmas in Mexico with an unusually high apparent mortality rate.

Subgroup 16SrIV-D was first detected in the mid-1990s affecting *Carludovica palmata* Ruiz and Pav. plants at Calkini, Campeche (Cordova et al., 2000). In the late 1990s, it was detected again infecting coconuts palms on the Pacific coast of Mexico (Harrison et al., 2002b). This was followed by subsequent reports in a number of regions including South Texas (Harrison et al., 2002c), West Central Florida (Harrison et al., 2008) and the Ticul-Merida area in Yucatan (Vázquez-Euán et al., 2011). This pathogen is now widely distributed throughout Mexico and Southern United States according to available literature (Figure 5), however, it remains difficult to explain its presence in places as distant as the Miami-Dade County in Florida (Bahder et al., 2019) and the Bay of La Paz in Baja California Sur (Poghosyan et al., 2019), considering its absence in intermediate locations like the majority of Mexico's northern states.

The origin of the Torreon outbreak is difficult to infer, however, due to the ample presence of *P. canariensis* in the city for more than sixty years, it seems unlikely that subgroup 16SrIV-D phytoplasmas have been established in the area for a long time, given the high susceptibility of this host to TPPD (Harrison and Elliott, 2016). The pathogen could have arrived with a recent introduction of contaminated plant material (possibly ornamental palms), or through natural spread by a vector. In 2009, a syndrome with similar characteristics was observed in *P. dactylifera* and *Sabal mexicana* Mart. palms located in the state of Guanajuato, Mexico (Aviña-Padilla et al., 2011). It should be noted that the phytoplasma sequences obtained by the authors of that study (Accession Numbers JF431249 and JF431250) also have a single base substitution located at position T/C^{1286} of the 16S rRNA gene, unfortunately, the length of the Guanajuato sequences is significantly shorter than those of Coahuila, so they could not be included in the phylogenetic analysis conducted in this study to establish a possible relationship between them. It remains to be seen whether these two cases of TPPD are connected in some way, or if the disease reached Torreon from somewhere else, given also its relative proximity to the Cameron, Hidalgo and Willacy counties in Texas, where TPPD is considered active (Ong and McBride, 2009).

Since 2015, declining *Phoenix* palms in Torreon have been removed or treated with antibiotics in an effort to mitigate the impact of the disease. These measures continue to be relevant in light of the recent discovery of a putative vector of TPPD, *H. crudus,* in the urban area of Torreon (Hernández-Rodríguez et al., 2019). This implies that the disease could affect other susceptible palms in the city, like the already mentioned *P. dactylifera* as well as *Syagrus romanzoffiana* (Cham.) Glassman, which has been reported on multiple occasions as a host of subgroup 16SrIV-D in Florida (Harrison et al., 2008;

Figure 5. Known distribution of subgroup 16SrIV-D phytoplasmas in Mexico and Southern United States based on available literature (Supplementary Table 1). Timelines of reported outbreaks are indicated for each state.

Bahder et al., 2018, 2019). In the meantime, ornamental species with similar characteristics to date palms but no prior history of susceptibility to TPPD can now be suggested for reforestation purposes, such as *Washingtonia filifera* (Linden ex André) H. Wendl. and *Washingtonia robusta* H. Wendl.

The finding of subgroup 16SrIV-D in Coahuila, as well as recently in Louisiana (Singh and Ferguson, 2017), Baja California Sur (Poghosyan et al., 2019) and Tabasco (Ramos et al., 2020), greatly extends the known geographical range of this pathogen (Figure 5), indicating an increased spread of TPPD in recent years. This situation requires the immediate attention of competent authorities and the scientific community in the United States and Mexico in view of the potential spread of TPPD to major date production states like California (USA) or Sonora (Mexico) (Wright, 2016; Ortiz-Uribe et al., 2019) or to other cities within those countries that use *Phoenix* palms as ornamentals.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary Table 1. Documented cases of subgroup 16SrIV-D phytoplasma associated diseases occurring in the United States and Mexico.

Location	Sampling date	Host plants	Record status	Notes	Reference
USA					
Corpus Christi, Texas	2001	*Phoenix canariensis*	Confirmed	Complete F2nR2 sequence available	Harrison et al. (2002c)
Hillsborough, Manatee and Sarasota counties, Florida	2006	*Phoenix canariensis, Phoenix dactylifera, Phoenix sylvestris, Syagrus romanzoffiana*	Confirmed	Complete F2nR2 sequences available	Harrison et al. (2008)
Hillsborough and Manatee counties, Florida	2008	*Sabal palmetto*	Confirmed	Complete F2nR2 sequence available	Harrison et al. (2009)
Cameron, Hidalgo, Kleberg and Willacy counties, Texas	2009	*Phoenix canariensis, Phoenix dactylifera, Sabal palmetto*	Confirmed	RFLP diagnosis of nested PCR products	Ong and McBride (2009)
Hillsborough County, Florida	Not specified (2011 report)	*Phoenix roebelenii*	Confirmed	Complete F2nR2 sequence available	Jeyaprakash et al. (2011)
New Orleans, Louisiana	2013	*Phoenix canariensis*	Confirmed	RFLP diagnosis of nested PCR products	Singh (2014)
Baton Rouge, Louisiana	2017	*Trachycarpus fortunei*	Confirmed	Complete F2nR2 sequences available	Singh and Ferguson (2017)
Manatee County, Florida	2017	*Bismarckia nobilis*	Partial diagnosis	BLAST sequence identity match of *gcp* gene	Dey et al. (2018)
Florida (widespread)	2016-2019	*Butia capitata, Carpentaria acuminata, Cocos nucifera, Livistona chinensis* and others	Confirmed	Complete F2nR2 sequences available	Bahder et al. (2019)
Mexico					
Calkini, Campeche	1994	*Carludovica palmata*	Confirmed	Complete F2nR2 sequence available	Cordova et al. (2000)
Coasts of Oaxaca and Guerrero	1997-1998	*Cocos nucifera*	Confirmed	Complete F2nR2 sequences available	Harrison et al. (2002b)
Merida and Ticul, Yucatan	2004-2008	*Pseudophoenix sargentii, Sabal mexicana, Thrinax radiata*	Confirmed	Complete F2nR2 sequences available	Vázquez-Euán et al. (2011)
Abasolo, Irapuato and Salamanca, Guanajuato	2009	*Phoenix dactylifera, Sabal mexicana*	Partial diagnosis	BLAST sequence identity match (incomplete F2nR2 fragment)	Aviña-Padilla et al. (2011)
Tala, Jalisco	2013	*Agave tequilana*	Partial diagnosis	BLAST sequence identity match (incomplete F2nR2 fragment)	González-Pacheco et al. (2014)

Supplementary Table 1. ContD.

Merida, Yucatan	2013-2014	*Pritchardia pacifica*	Confirmed	Complete F2nR2 sequences available	Narváez et al. (2017)
Merida, Yucatan	2014-2015	*Adonidia merrillii*	Confirmed	Complete F2nR2 sequences available	Cordova-Lara et al. (2017)
El Centenario, Baja California Sur	2014	*Brahea brandegeei*	Confirmed	Complete F2nR2 sequence available	Poghosyan et al. (2019)
Miahuatlán, Tabasco	2017	*Adonidia merrillii, Attalea butyracea, Cocos nucifera*	Confirmed	Complete F2nR2 sequences available	Ramos et al. (2020)

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Comparative performance of different yeast strains in the production of bioethanol using simultaneous liquefaction saccharification and fermentation (SLSF)

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The performance of a hybrid of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* (Yeast B) compared with an industrial strain of *S. cerevisiae* (Yeast A) was studied. The two strains of yeasts: Yeast A obtained from the Bioresources Development Centre, National Biotechnology Development Agency Ogbomosho and Yeast B obtained from Scotch Whisky Research Laboratory in Edinburgh, Scotland (Strain 63M) were studied using the SLSF method for bioethanol production at various initial starch concentrations (20, 30 and 50%). The cassava starch used was extracted from Tropical *Manihot esculenta* (TME 419) cassava strain while the SLSF method for bioethanol production was initiated by adding granular starch hydrolysing enzyme with the yeast strains into the starch solutions at room temperature. The results obtained showed that Yeast B has higher bioethanol yield and tolerance generating 70.34, 87.34 and 120.53 ml/L compared to the Yeast A which produced 63.4, 72.73 and 112.6 ml/L at 20, 30 and 50% starch concentrations respectively. The study suggests that the hybrid strain of *S. cerevisiae* and *S. paradoxus* out performs the industrial strain and can favorably substitute or displace the industrial strains for bioethanol production.

Key words: Bioethanol, cassava, fermentation, hybrid, simultaneous liquefaction saccharification and fermentation (SLSF), yeast, *Saccharomyces cerevisiae, Saccharomyces paradoxus*.

INTRODUCTION

Biofuel has been known to be an excellent replacement for fossil fuels. This is because the latter are by nature non-renewable and have numerous environmental hazards associated with its extraction and utilization (Solomon et al., 2018). Nigeria with a population approximated to be 200 million (National Bureau of Statistics, 2018) has been faced with challenges of sufficiently meeting its ever-increasing energy demands.

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With an average daily fuel consumption of about 51 million liters in 2016 to about 54 million litres in the first quarter of 2018 (Petroleum Products Consumption Statistics, National Bureau of Statistics, Qtr 2, 2018), there is absolute need for an alternative fuel to supplement and curtail our over reliance on fossil based energy sources as a Nation. Bioethanol is the most popular alternative fuel that has found applications both as transportation fuel and in industries.

Bioethanol can be produced from various biomass feedstocks such as sugarcane, corn, cassava, etc. Among the feedstock, cassava gives the best starch and has the highest volume per unit of raw materials (Sarocha et al., 2018). The production of bioethanol from cassava has a very high potential in Nigeria because of its ability to give moderate yields even in poor soils. Nigeria has been producing the largest quantities of cassava in the world for the past decades, amounting to about 54 million tons annually (FAO, 2012). The Nigerian Biofuels Policy and Incentives was drafted in 2007 by NNPC with the view of integrating agricultural activities with oil and gas exploration and production (Ben-Iwo et al., 2016).

The conventional method of bioethanol production involves two steps: the hydrolysis of starch which is the conversion of starch to sugar by cooking the starch granules (gelatinization), liquefaction via alpha amylase enzyme and saccharification using amyloglucosidase enzyme. The second step is the fermentation of the sugar produced from hydrolysis using yeast with all processes taking place at known temperatures, pH, concentrations and time. These processes take more time, energy, equipment and labour. In recent times however, the Simultaneous Liquefaction Saccharification and Fermentation (SLSF) process has been employed to produce bioethanol with significant reduction in processing time and energy consumption which have hitherto been major constraints to investors (Chu-Ky et al., 2016) in the ethanol industry. Here, both the starch hydrolysis and fermentation are carried out in one step at room temperature using granular starch hydrolyzing enzymes (Sriroth et al., 2012). This process has been reported to increase the hydrolysis rate and consequently decrease product inhibition in addition to reduction in the number of processing units.

Fermentation process is carried out by yeasts, which are majorly known to rapidly and proficiently convert sugars into ethanol and carbon dioxide (Hossain et al., 2017). *Saccharomyces cerevisiae* was the first microorganism known to possess the ability to ferment sugars for the production of ethanol and carbon dioxide both aerobically and anaerobically (De Haas and Kreuger, 2010). Currently, most ethanol production systems use strains of *S. cerevisiae* that are highly adapted to industrial process of converting feedstock to ethanol. These yeast strains combine to efficiently convert sugars into ethanol, and exhibit important industrial characteristics that influence productivity such as low nutrient requirement, ethanol resistance, tolerance to pH, and general robustness (Bharti and Madhulika, 2016). In order to increase the genetic diversities and enhance yeast performance, hybridization mechanisms, which may involve dissimilar strains of same species, several species of same genus or strains of different genera, is often used. The overall advantage is that the new species exhibit better performance than its precursor strains (Steensels et al., 2014).

This work therefore, compares the performances of a hybrid yeast strain of *S. cerevisiae* and *S. paradoxus* with that of an industrial yeast strain of *. cerevisiae*, in terms of bioethanol production and alcohol tolerance. This is with a view to lowering the cost and time of bioethanol production from cassava starch, using SLSF process.

MATERIALS AND METHODS

Fresh TME 419 cassava roots and granular starch hydrolysing enzyme (STARGEN 002^{TM}) procured from GENECOR International, Sweden were obtained from the Bioresources Development Centre (BIODEC), National Biotechnology Development Agency (NABDA) in Ogbomoso, Oyo State.

The growth media used for the organisms composed of soluble glucose, ammonium sulphate $NH_4(SO_4)_2$, Yeast Extract, potassium di-hydrogen phosphate (KH_2PO_4), magnesium sulphate $MgSO_4.7H_2O$. Compositions of the fermentation media used for the bioethanol production were soluble glucose, yeast extract, ammonium sulphate ($NH_4(SO_4)_2$), potassium di-hydrogen phosphate (KH_2PO_4), magnesium sulphate ($MgSO_4.7H_2O$), copper sulphate ($CuSO_4$. $5H_2O$), zinc sulphate ($ZnSO_4$), iron sulphate $FeSO_4$, manganese sulphate ($MnSO_4$), with starch concentrations ranging from 1 - 10% used when testing for the effect of different substrate concentrations. Sodium acetate buffer was used to adjust the pH of the fermentation media to 4.5. All chemicals and reagents used in this study were of analytical grade.

The yeast strains used for fermentation were a new hybrid strain of *S. cerevisae* and *S. paradoxus* (Strain 63M) collected from Scotch Whisky Research Laboratory at Edinburgh, Scotland (labeled as Yeast B) and an industrial strain of *S. cerevisae* collected from the NABDA's BIODEC at Ogbomoso, Oyo State which is being used at the bioethanol plant at the Centre (labeled as Yeast A).

Cassava starch extraction

The method of starch extraction as described by Oyewole and Obieze (1995) was used to extract starch from fresh cassava roots, in this study. Fifty-eight kilograms of fresh TME 419 cassava roots were peeled, washed in water, shredded, sieved and the resultant pulp allowed to sediment for 12 h. The resultant starch was then sun-dried to constant weight.

Yeast activation and sub-culturing

Five millilitre of malt extract broth was dispensed in two McCartney bottles and placed in an autoclave for sterilization at 15 psi, 121°C for 15 min after which the broth was allowed to cool. Thereafter, 0.1 g each of an industrial strain of *S. cerevisiae* (Yeast A) and the hybrid of *S. cerevisiae* and *S. paradoxus* (Yeast B) were inoculated into each of the 5 ml broth that were labeled accordingly and

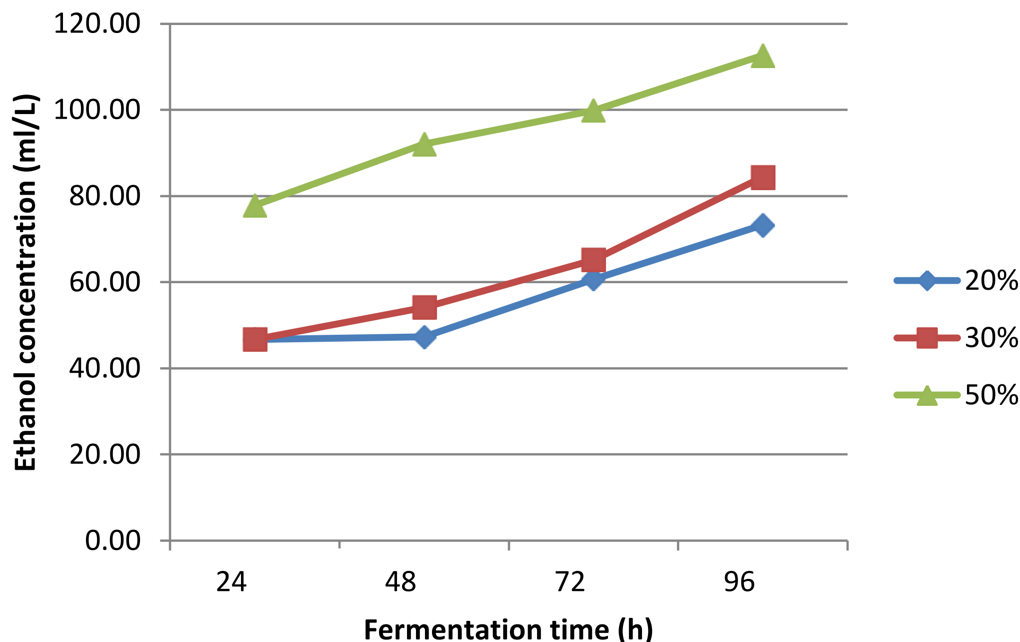

Figure 1. Ethanol concentration using the industrial yeast (Yeast A) at various initial starch concentrations.

incubated at room temperature (30°C) for 48 h. Each isolate was then sub-cultured into sterile malt extract agar medium and incubated at 30°C for 24 h accordingly to confirm viability. Subsequently, each organism was sub-cultured in agar slant (Malt Extract Agar), appropriately labeled and incubated accordingly. The slant cultures were preserved at 4°C.

Fermentation (SLSF)

Starch concentrations of 20, 30 and 50% were prepared by weighing 20, 30 and 50 g in 100 ml of distilled water inside 250-ml conical flasks. The pH of the starch solution was adjusted to 4.5 using sodium acetate buffer solution. The mixture was stirred thoroughly to ensure that all the dust particles were dissolved. Granular Starch Hydrolyzing Enzyme concentration of 2% w/v and 10 ml of yeast inoculum was then added simultaneously into the prepared starch solution for fermentation reaction to commence. At regular intervals, samples were taken in order to monitor the reaction progress.

Ethanol determination

In analyzing the ethanol produced, 10 ml of the fermenting broth was taken and centrifuged to separate the yeast as sediment; the supernatant was then decanted and the biomass kept for further analyses. Acid dichromate method was used to analyze for the ethanol content of the supernatant. The solution was gently shaken for 1 min and allowed to stand for 2 h at room temperature (Caputie et al., 1986). The formation of a light green coloured reaction product was observed. The absorbance of the solution was read at a wavelength of 576 nm on the Libra S21 UV-visible spectrophotometer (Caputie et al., 1986). The value of absorbance obtained was read off a standard curve prepared with known ethanol concentrations, to obtain the corresponding values of ethanol concentration in the sample.

Glucose determination

Glucose concentration in the supernatant was determined using the DNS method according to Miller (1959). One mililiter of supernatant was mixed with 3 ml of DNS solution in a test tube, the mixture was then placed in boiling water for 15 min after which, the test tube was removed and allowed to cool before measuring the optical density using the Libra S21 Ultra Violet visible spectrophotometer at 540 nm wavelength and the value read off a standard curve previously prepared with known glucose concentrations, to obtain the corresponding value of glucose concentration in the sample.

Ethanol tolerance determination by biomass concentration

Each of the yeasts (the industrial yeast, Yeast A and the hybrid yeast, Yeast B) used in this work was grown in sterile nutrient broth base media with 50 g/l glucose and varying quantities of absolute ethanol added aseptically at concentrations of 5, 10, 15, 20 and 25% in 250-ml conical flasks respectively. Ten ml samples of the nutrient broth from each flask were taken every 4 h from the various flasks. These samples were centrifuged, supernatant decanted and the biomass sediments oven dried to constant weight so as to monitor biomass concentrations. The dry weight was indicative of the growth of yeasts at the various ethanol concentrations.

RESULTS

The performance of the hybrid yeast of *S. cerevisiae* and *S. paradoxus (*Yeast B*)* was studied in the production of bioethanol from cassava starch slurry using SLSF method and compared with that of an industrial yeast strain (Yeast A) which was used as a control strain. From Figures 1 and 2, it can be observed that there was a steady

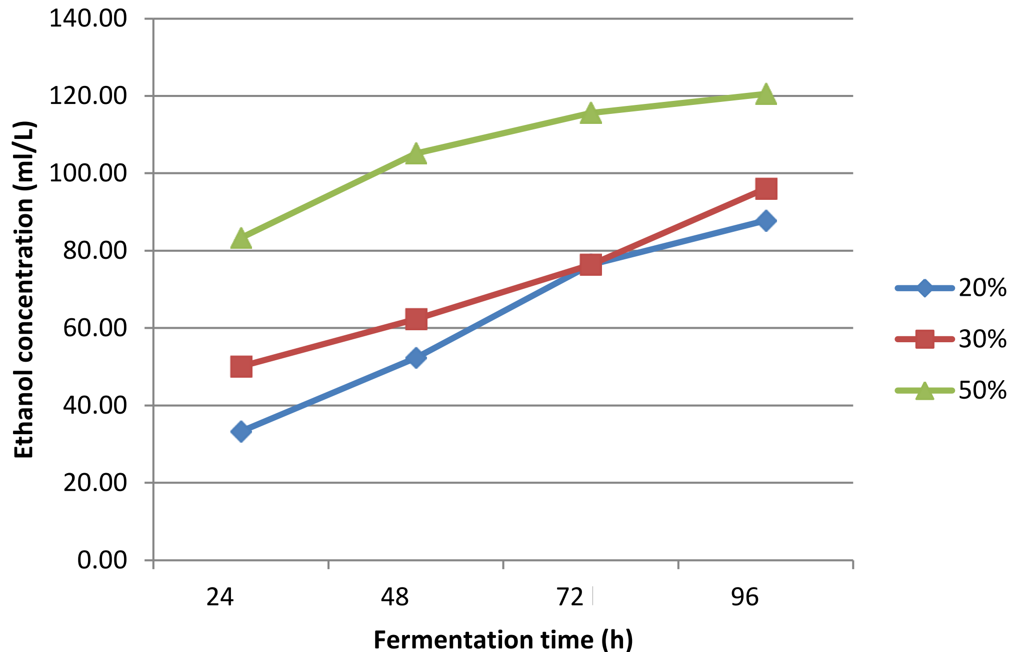

Figure 2. Ethanol concentration using the hybrid yeast (Yeast B) at various initial starch concentrations.

increase in bioethanol concentration as fermentation time increased from 24 to 96 h and as the initial starch concentrations increased from 20 to 50% during the anaerobic fermentation by both yeasts. The results in these Figures 1 and 2 show that after 96 h, the corresponding maximum bioethanol production (in ml per litre starch slurry) at 20, 30 and 50% initial starch concentrations for Yeasts A and B were 73.25, 84.37, 112.61 and 87.81, 96.08 and 120.53 v/v basis respectively, demonstrating the superior ability of Yeast B over Yeast A.

Figure 3 shows the percentage increase in bioethanol concentration after 96 h for both yeasts and it can be observed that bioethanol concentration increased by 56.9 at 20% initial starch concentration, 80.7 at 30% initial starch concentration and 44.7 at 50% initial starch concentration respectively for Yeast A while for Yeast B, ethanol concentration increased by 44.65, 91.86 and 163.64% correspondingly at 20, 30 and 50% initial starch concentrations. The data obtained show that highest rate for bioethanol conversion was attained using 30% starch concentration for Yeast A while only Yeast B performed well at 20% initial starch concentration.

Figures 4 to 6 illustrate the performance of both yeasts at various starch concentrations. It can be observed that at 20% initial starch concentration, Yeast A and the Yeast B had maximum bioethanol concentrations of 73.25 ml/L, increasing from 46.7 and 87.81 ml/L, increasing from 33.31, respectively after 96 h. At 30% initial starch concentration, the performance of the industrial Yeast A and the hybrid Yeast B were compared every 24 h for 96 h. It was observed that hybrid Yeast and the Industrial Yeast attained maximum bioethanol concentrations of 84.37 and 96.08 ml/L respectively. Within the period of fermentation, the concentration increased from 46.7 to 84.37 ml/L for Yeast A while for Yeast B, the bioethanol concentration generated was higher, increasing steadily from 50.08 to 96.08 ml/L during the same period. Using 50% initial starch concentration, a similar comparison of the two yeasts demonstrates that the industrial Yeast A and the hybrid Yeast B attained maximum bioethanol concentrations of 112.61 ml/L increasing from 77.85 and 120.53 ml/L increasing from 83.32 respectively after 96 h.

Figure 7 summarizes the comparative performance of the studied yeast strains in the conversion of cassava starch slurry into bioethanol, based on the volume of ethanol produced per liter of starch slurry. In all cases of starch concentrations, it can be observed that the hybrid Yeast Strain B outperformed the industrial Yeast Strain A. For starch concentrations of 20, 30 and 50%, ethanol yield were 73.25, 84.37 and 112.61 ml/L for yeast A as compared with 87.81, 96.08 and 120.53 ml/L for Yeast B. Maximum difference in ethanol yield occurred in the 20% concentration starch fermentation medium.

As illustrated in Figures 8 and 9, glucose concentrations were also observed to decrease for the various starch concentrations as the bioethanol concentration rises and fermentation time increases from 24 to 96 h. This trend was observed for both yeasts, indicating that they both metabolize glucose. Hybrid Yeast Strain B consumed more glucose than the industrial Yeast Strain A respectively at 20 and 30%

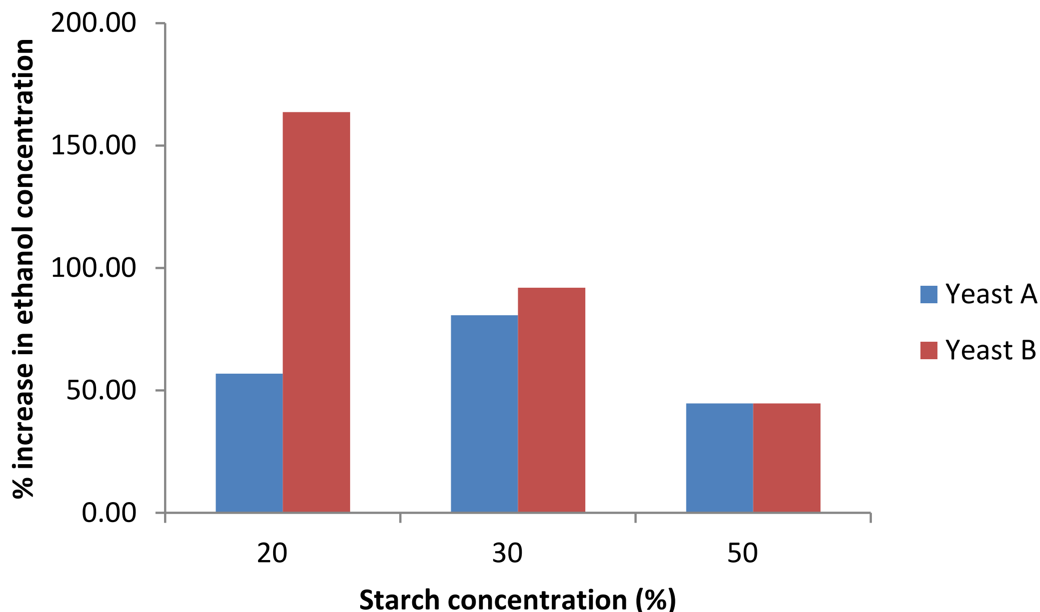

Figure 3. Percent Increase in bioethanol production after 96 h of fermentation for Yeasts A and B.

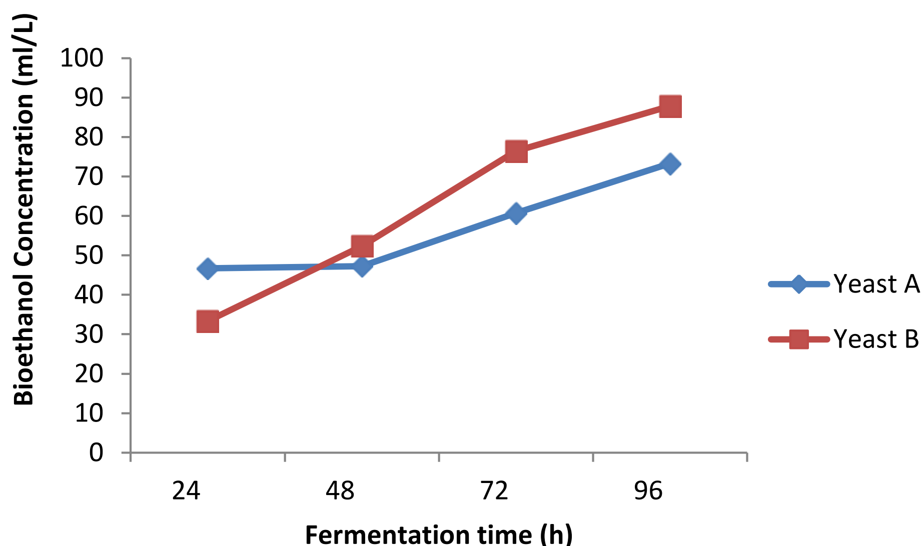

Figure 4. Ethanol concentration of Yeast A and B at 20% starch concentration.

starch concentration but the reverse was the case for 50% starch concentration where the latter consumed 3.7 moles of glucose as opposed to the 1.85 moles consumed by the hybrid yeast. Even at this, Figure 10 which compares the performance of both yeast strains based on ethanol yield show that the hybrid Yeast Strain B produced 138.06 ml of ethanol per mole glucose consumed as opposed to 117.01 for the Yeast Strain A and 65.06 as opposed to 30.45 ml/mole respectively for 20 and 50% starch concentrations.

Although, for 30% starch concentration, Yeast Strain B consumed 0.7 mole more glucose than Yeast Strain A and produce 112.4 moles less ethanol, its best performance was observed to be at the 50% starch concentration medium where it consumed half glucose consumption of Yeast Strain A and produced more than 200% of its ethanol per mole glucose consumed.

The results obtained from the measurement of biomass concentrations for the determination of ethanol tolerance of both yeasts generally show that biomass concentration

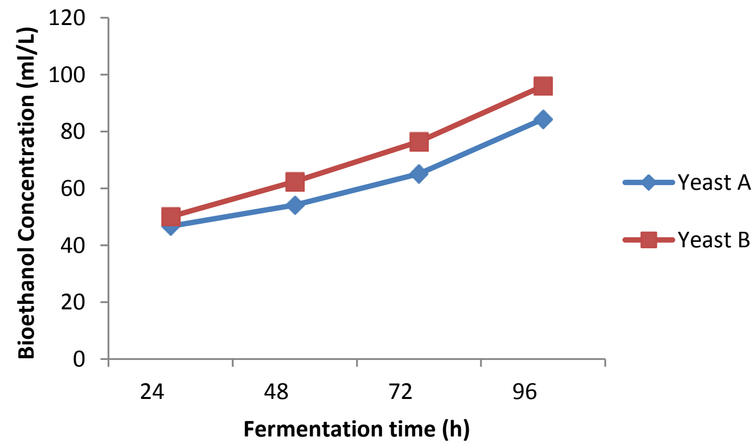

Figure 5. Ethanol concentration of Yeast A and B at 30% starch concentration.

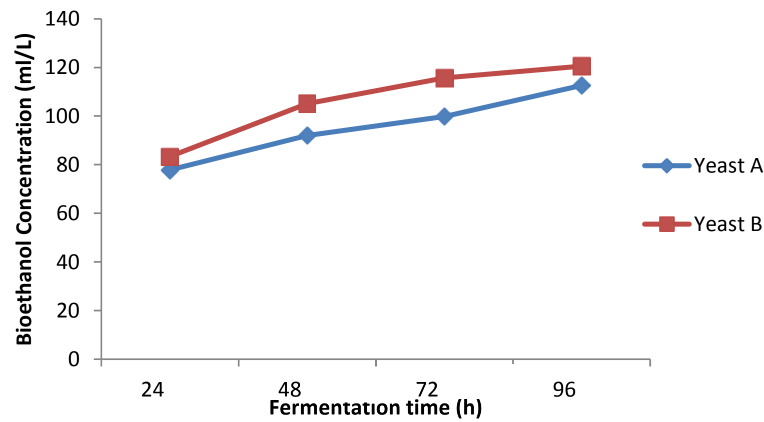

Figure 6. Ethanol concentration of Yeast A and B at 50% starch concentration.

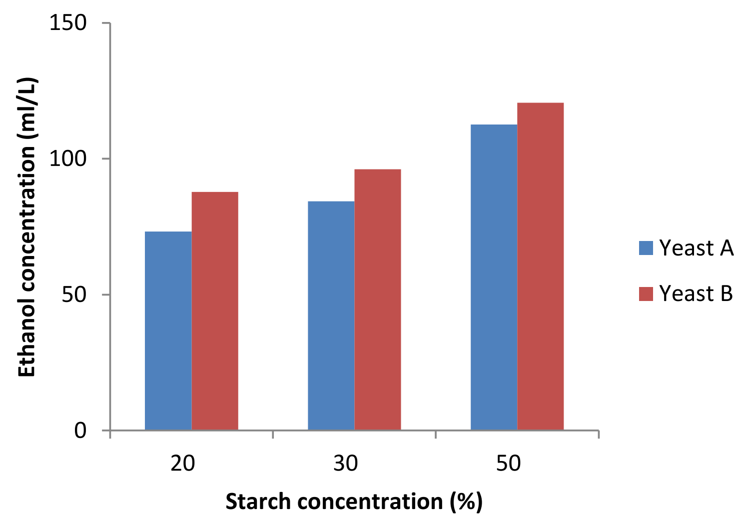

Figure 7. Ethanol concentration of yeast A and B after 96 h of fermentation at various starch concentrations.

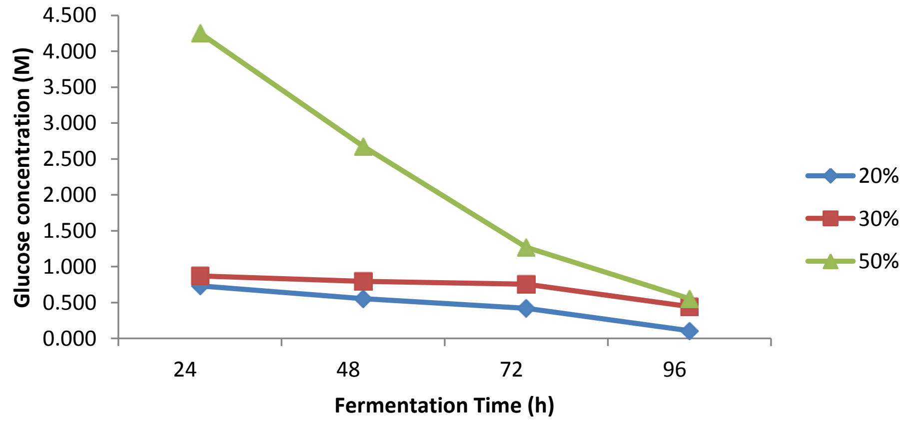

Figure 8. Glucose concentration of Yeast A at various starch concentrations.

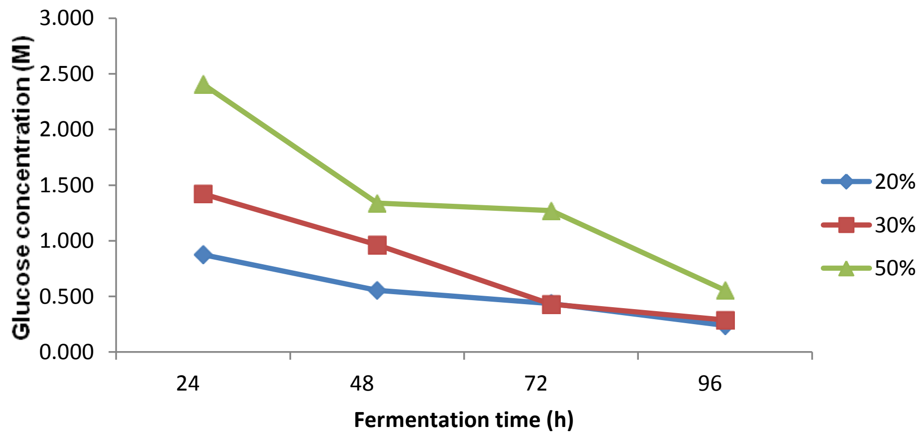

Figure 9. Glucose concentration of Yeast B at various starch concentrations.

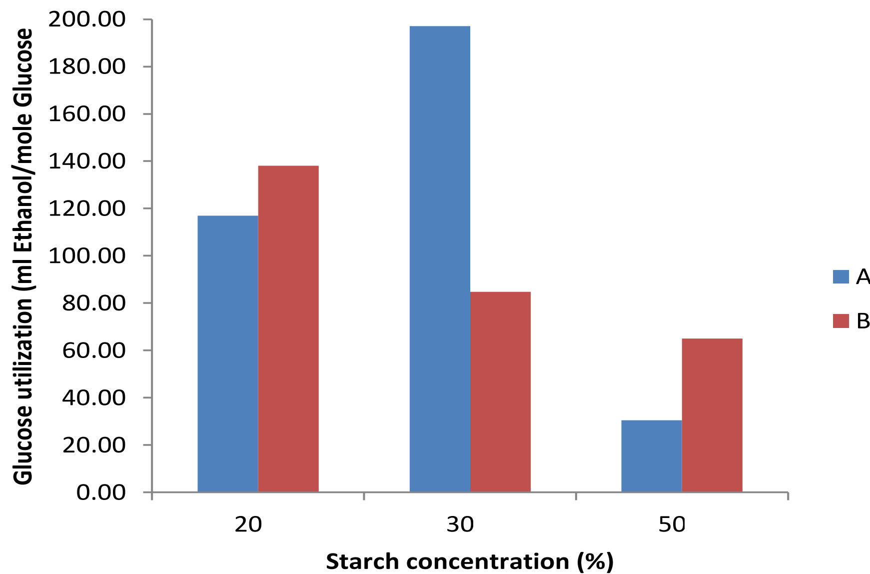

Figure 10. Ethanol yield by Yeast A and Yeast B at various starch concentrations.

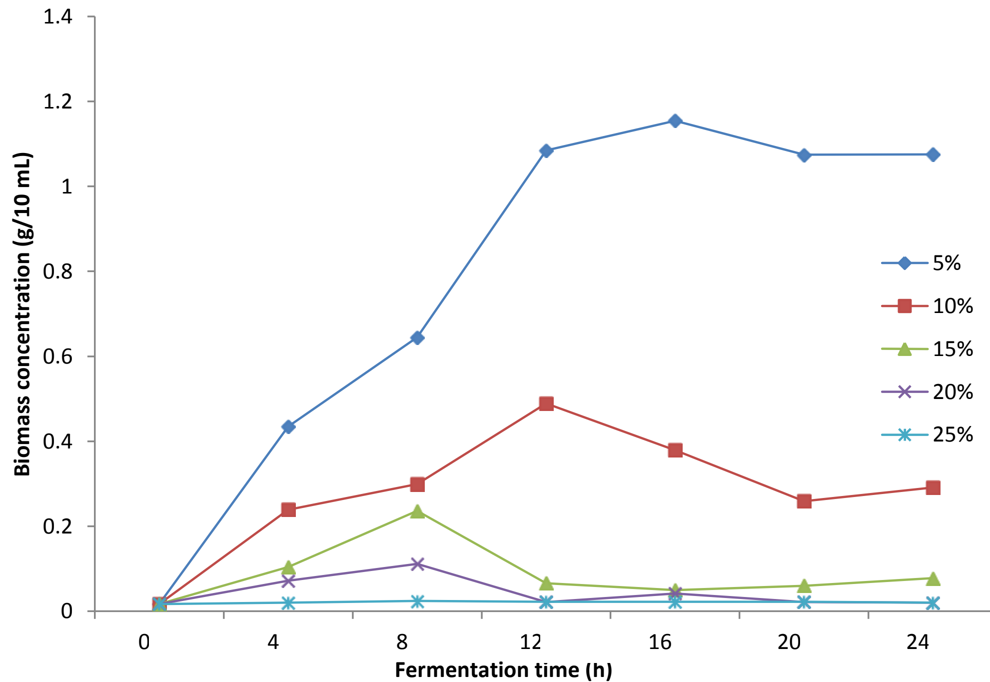

Figure 11. Biomass concentrations of Yeast A at various ethanol concentrations.

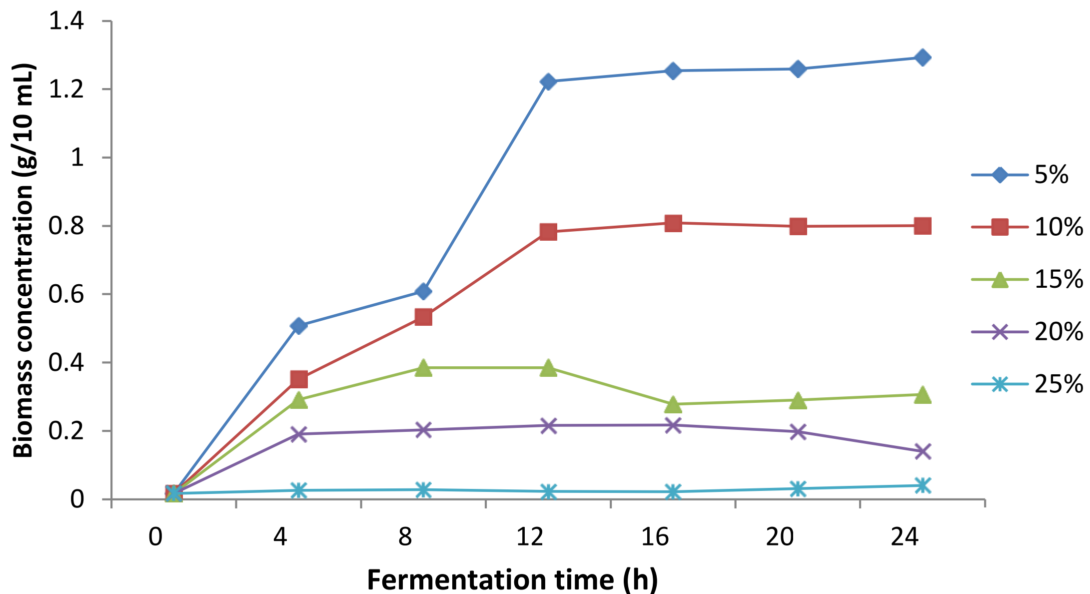

Figure 12. Biomass concentrations of Yeast B at various ethanol concentrations.

of Yeast A and B increased with inoculation time but decreased as ethanol concentration in the inoculum media increased, as described in Figures 11 and 12. Figure 13 compared the performance of both yeasts. For the Yeast Strain A, peak biomass concentration was attained after 16, 12 and 8 h of inoculation respectively in the growth medium that contained 5, 10 and 15, 20, and 25% ethanol concentration while the biomass concentration of Yeast Strain B peaked after 24 h in 5, 10 and 25% ethanol concentration and 16 h in the 20% ethanol concentration. In all cases, Yeast B had higher biomass concentration than Yeast A after 24 h of

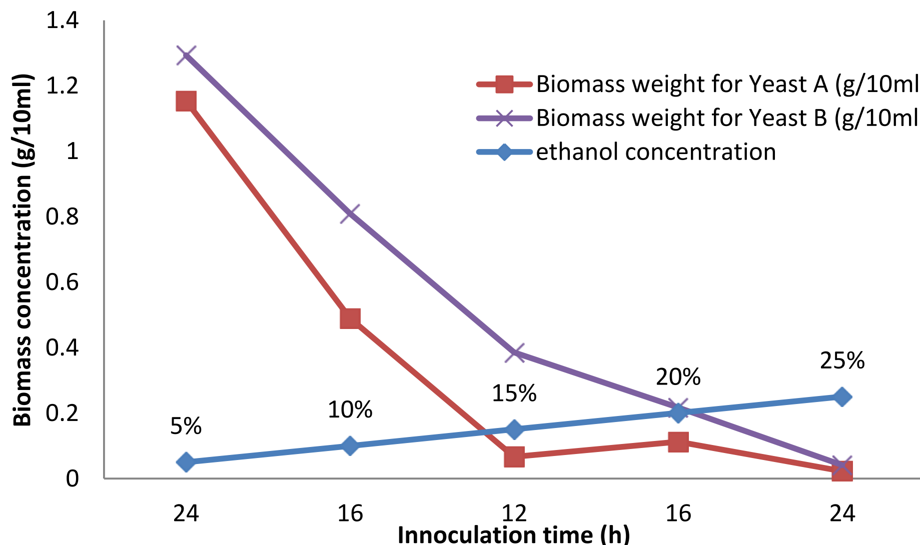

Figure 13. Biomass growth peaks for Yeast A and B at various ethanol concentrations.

inoculation, producing four, seven and two-fold more biomass respectively in the 15, 20 and 25% ethanol concentration, than the latter.

DISCUSSION

For cassava starch concentrations in the range 10 to 100% w/v, Phakping et al. (2014) reported on the effects of uncooked slurry on bioethanol yield using SLSF method. It was reported that higher concentration of cassava starch slurry led to an increase in ethanol concentration as obtained in this study (Figures 1 to 3). From the results displayed in Figure 3, it can be deduced that both yeasts performed optimally at 30% starch concentration giving 80.67 and 91.86% increase in bioethanol production after 96 h. At 50% starch concentration, both yeasts have similar percent increase in ethanol production of around 44.65% even though, Yeast B had higher substrate utilization and produced more bioethanol within the same time frame. Previous reports have been made with regards to very high gravity (VHG) fermentation with substrate concentration of > 30%. Zang et al. (2010) reported higher fermentation efficiency with ethanol concentration of around 18% v/v but also encountered problems of highly viscous starch substrate during fermentation which include handling difficulties, poor solid-liquid separation, incomplete conversion of starch into fermentable sugars, leading to a drastic drop in the efficiency of fermentation (Puligudla et al., 2011). As a result, Sriroth et al. (2010) suggested that the combination of SLSF with VHG (SLSF-VHG) is more efficient and reduces energy consumption by 18.5%. Glucose concentration was observed to decrease as fermentation time increases. This is as a result of increase in the rate of conversion of sugars formed to ethanol, which is higher with the use of the hybrid Yeast B. Biomass concentration also increases rapidly utilizing the nutrients in the fermentation media including the glucose formed for its cell growth (Ajibola et al., 2012).

The hybrid of *S. cerevisiae* and *S. paradoxus* performed better than the industrial yeast as higher bioethanol concentration was obtained. The ethanol tolerance of the hybrid yeasts, as indicated by biomass concentration, was higher for Yeast B than that of the industrial yeast A (Figures 11 to 13). Yeast growth is acknowledged to be highly inhibited by ethanol even at relatively low concentrations as it interferes with cell division, decreases cell volume and specific growth rate. At high ethanol concentrations, cell vitality reduces while cell death increases (Siti et al., 2017). Although, fermentation in this study was carried out at room temperature made possible by the use of the granular starch hydrolyzing enzyme in the SLSF process, the hybrid Yeast B performed better than the commonly used industrial Yeast Strain A, producing seven-fold more biomass than Yeast A even at 20% ethanol concentration. Previous work has also suggested that *S. paradoxus* exhibits its highest growth rate at 37°C or higher and that *S. cerevisiae* may exhibit its highest growth rate at lower temperatures. Thus, a hybrid of *S. paradoxus* and *S. cerevisiae* will likely result into a hybrid strain with a high temperature and ethanol tolerance (Hanyao et al., 2010).

Conclusion

The study revealed that the hybrid strain of *S. cerevisiae* and *S. paradoxus* performed better in terms of bioethanol

production and ethanol tolerance than the industrial strain of *S. cerevisiae.* Furthermore, the performance of this hybrid strain should be exploited further in the bioethanol production via the SLSF-VHG process which is believed to be even more energy efficient and produces around 18% v/v ethanol concentration.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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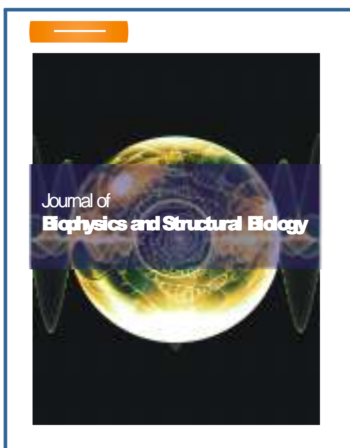

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