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African Journal of **Biotechnology**

2 January 2019
ISSN 1684-5315
DOI: 10.5897/AJB
www.academicjournals.org

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Full Length Research Paper

Decolourization of synthetic dyes by laccase enzyme produced by *Kluyveromyces dobzhanskii* DW1 and *Pichia manshurica* DW2

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Received 11 October, 2018; Accepted 14 December, 2018

Industrialization has come with environmental challenges. Industries like paper, printing, textile, leather and so on widely use chemical dyes whose waste treatment or degradability is difficult. Among various methods employed, the use of microbial enzymes is the most effective. The study aimed at producing laccase from identified yeast strains for potential industrial use in dye decolourization. Laccase produced by *Kluyveromyces dobzhanskii* DW1 and *Pichia manshurica* DW2 were purified and immobilized up to 65.2 and 73.1%, respectively. The crude, purified and immobilized forms of the enzymes were used to decolourize malachite green and methyl red dyes each at concentrations of 0.05 and 0.1 g/L. The highest percentage decolourization by *K. dobzhanskii* DW1 was 81.50% (immobilized) and 87.50% (purified), respectively for malachite green and methyl red dyes while *P. manshurica* DW2b (crude) had 84.40 and 76.89%, respectively. The Fourier transform infrared (FTIR) spectrum of the dyes was collected within a scanning range of 4000 to 400 cm^{-1} . The spectrum of methyl red dye by the purified *K. dobzhanskii* DW1 laccase showed disappearance of some chemical groups (peak), while the crude *P. manshurica* DW2 laccase removed the main azo-group, alcohol/phenol and higher alkane (1487.17) groups. The spectrum of untreated malachite green also showed 25 peaks with one disulphide, 2 aliphatic halogens, 2 thio ethers, 3 sulphones, 4 imino groups among other chemical groups. The decolourization of the dye with the immobilized *K. dobzhanskii* DW1 laccase showed a spectrum of 17 peaks with the removal of the disulphide (420.5), one aliphatic halogen (C-I), thio ether, 2 sulphone, 4 imino and 2 higher alkanes, while the crude *P. manshurica* DW2 laccase removed the 2 aliphatic halogen, 4 imino, 1 amine, 2 alkane and 2 of the 3 alcohol/phenol chemical groups. The removal of the main components (azo chemical group) of the dyes proved their effectiveness in decolourisation and bioremediation of the textile wastes.

Key words: Decolourisation, azo-dye, laccase, immobilized, FT-IR spectra.

INTRODUCTION

Large amounts of chemically different dyes generated by textile industries are discharged into the environment and have become a major concern in wastewater treatment

(Grassi et al., 2011). The suspended dyes in water bodies influence the aquatic ecosystem (Gupta et al., 2007), pose public health risks due to bioaccumulation

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and cause soil contamination (Sriram et al., 2013). Furthermore, the reduced intermediates of azo dyes which are aromatic amines are more toxic than the dyes themselves (Gomi et al., 2011). Discharge of wastewater containing synthetic dyes have carcinogenic health effects and have biodegradable difficulty due to their complex aromatic structure therefore posing an environmentally important problem and this has persuaded environmental engineers to develop new techniques for treatment of such harmful compounds. Many methods are being used for dye removal; they include physical/chemical adsorption, oxidation, biological treatments (Akar et al., 2013), microbial biomass and enzyme treatments (Anjaneyulu et al., 2005). Among these methods, the use of microbial enzymes is most efficient for dye degradation/decolorization (Baldev et al., 2013; Pramanik and Chaudhuri, 2018). Many of the aforementioned methods are not successful in dye removals due to the following reasons: (i) the chemicals are only partially degraded; (ii) the azo-dyes are converted into the toxic metabolites, and (iii) the toxic chemicals are converted to secondary solid wastes with complex binding structure; which has to be either treated again or dumped (Behnajady et al., 2006).

Chemical dyes are widely used in many industries such as paper printing, color photography, pharmaceutical, textile and leather industries (Korbahti and Rauf, 2008; Vidya et al., 2017). Azo/Synthetic dyes contain aromatic and phenolic compounds and microbial enzymes are capable of removing phenolics and aromatic amines present in the azo dyes (Claus, 2003).

Fungal laccase are more advantageous over other sources due to their stability, their substrate non-specific nature and in oxidizing various phenolic compounds and have been widely applied in biotechnology and for various purposes (Shervedani and Amini, 2012). Due to the high sensitivity of laccase to denaturing agents, the use of immobilized laccase has proved effective in the industrial application of laccase (Gochev and Krastanov, 2007). Entrapment of enzymes on agar gel is one of the methods of immobilizing enzymes which has been very effective. Agar (agar-agar) is an agarose polysaccharide which is acid stable and has a strong gelling ability with no great significance in protein reactivity (Om and Nivedita, 2011). Moreover, the cost of this material is low when compared with other materials commonly used for immobilization.

The focus of this research is to investigate the removal of dyes by the crude, purified and immobilized laccase enzyme produced by *Kluyveromyces dobzhanskii* DW1 and *Pichia manshurica* DW2.

MATERIALS AND METHODS

Microorganisms and culture conditions

Yeast strains *K. dobzhanskii* DW1 and *P. manshurica* DW2

obtained from the previous experiments (Wakil et al., 2017) were used for this research. Stock cultures were stored in glucose yeast extract agar slants in sterile McCartney bottles and were kept in the refrigerator at 4°C.

Chemicals and reagents

2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) [ABTS] was purchased from Sigma-Aldrich, USA. Hydroquinone and tannic acid were obtained from Plus Chemical, India. Acetaminophen and catechol were obtained from May and Baker, England. Malachite green and methyl red were obtained from Himedia Chemicals, India and all other chemicals were of analytical grade.

Laccase production, purification and assay

The liquid medium containing rice bran (10 g/l) (*K. dobzhanskii* DW1), cane bagasse (10 g/l) (*P. manshurica* DW2), NaNO₃ (3 g/l), CuSO₄ (0.2 g/l), FeSO₄ (0.05 g/l), MgSO₄·7H₂O (0.5 g/l), ZnSO₄ (0.001 g/l), Na₂HPO₄ (0.2 g/l), MnSO₄ (0.05 g/l), CaCl₂ (0.01 g/l), and KCl (0.1 g/l) at pH (6.0). The liquid medium was sterilized by autoclaving at 121°C for 15 min. After cooling of the sterilized medium, then, 1 ml of yeast extract broth with actively growing *K. dobzhanskii* DW1 and *P. manshurica* DW2 isolates containing 3.1 × 10⁹ and 9.8 × 10⁹ CFU, respectively were inoculated separately and aseptically into a 250 ml Erlenmeyer flask containing 100 ml of the liquid medium. Cultures were incubated at 30 and 35°C for *K. dobzhanskii* DW1 and *P. manshurica* DW2 isolates, respectively in a shaker incubator at 120 rpm to ensure aerobic conditions. After 14 days of incubation, the cells were removed by filtration through filter paper (Whatman No. 1). The clear supernatant was stored at 4°C and used for purification. The laccase purification was performed according to the method of Ding et al. (2012).

Laccase activity was determined spectrophotometrically by measuring the oxidation of 0.02 M ABTS at 30°C according to the method of Mongkolthanaruk et al., (2012) for the crude and purified enzyme while the method of Faramarzi and Forootanfar (2011) was used for the immobilized laccase. The ABTS was the substrate, and absorbance increase in assay mixture was monitored at 420 nm ($\epsilon_{420}=36.0 \text{ mM}^{-1}\text{cm}^{-1}$), and the enzyme activity was expressed in an international units (U) defined as the amount of enzyme needed to produce 1 $\mu\text{mol product min}^{-1}$ at 30°C. Protein concentration was determined by the method of Lowry et al., (1951) with bovine serum albumin as a standard.

Immobilization of laccase

Entrapment in agar gel

The enzyme was immobilized according to the method of Om and Nivedita (2011). A 4.0% agar solution was prepared in 25 mM sodium acetate buffer (pH 6.0) by warming at 50°C. After cooling down to room temperature, a 1.0 ml enzyme (containing 0.017 mg (DW1) and 0.020 mg (DW2) protein/ml) was mixed with 9.0 ml agar solution (the total volume of matrix and enzyme mixture being 10 ml) and immediately casted on preassembled Petri dishes. After solidification at room temperature, the gel was cut into a small size of 5 × 5 mm to make beads of immobilized enzyme. The beads were stored in 25 mM sodium acetate buffer (pH 6.0) and at 4°C for further use.

Percentage immobilization of laccase enzyme

This was done according to the method of Om and Nivedita (2011).

The percentage immobilization was calculated as the:

$$\frac{\text{Total activity in immobilized gel}}{\text{Total activity in the soluble enzyme loaded}} \times 100$$

Dye decolourization experiments

The decolourization experiment was done using the forms of laccase enzyme (crude, purified and immobilized). Two dye concentrations (0.05 and 0.1 g of dye in a litre of distilled water) were prepared. Decolourization was determined by measuring the absorbance of decolourization medium at different wavelengths depending on the dye (malachite green at 615 nm and methyl red at 502 nm).

Decolourization experiment of the crude and purified enzyme was according to the method of Mirzadeh et al., (2014) while that of the immobilized enzyme was according to the modified method of Poonkuzhali and Palvannan (2013) where 5 beads were incubated with the decolourization experiment.

Ten millilitres of the different dye concentrations were dispensed into test tubes and plugged with cotton wool. The dyes in different test tubes were then sterilized at 121°C for 15 min using autoclave before aseptically adding 1 ml of the crude enzyme, 1 ml of the purified laccase enzyme and 0.5 g (5 beads/tablets) of the immobilized enzyme. The control was prepared by adding 10 ml of the dye without any of the laccase enzymes. Each test tube for both test (dye with laccase enzymes) and control (dye without any laccase enzyme) was evenly mixed using a vortex mixer at a low speed (2,000 rpm).

Aliquot (2ml) from the experimental set-up was withdrawn immediately and absorbance was taken at different wavelengths specific for each dye. This was taken as the initial absorbance before incubation at room temperature for 4 days. Subsequent absorbance readings were done every 24 h. These were taken as final absorbance at each time interval.

Percentage decolourization was then calculated according to Olukanni et al., (2010) as follows:

$$\frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy was used to examine the surface functional groups that were involved in decolourization of malachite green dye and methyl red dye treated with crude, purified and immobilized laccase enzymes. This was done according to the methodology described by Poonkuzhali and Palvannan (2013). FTIR analysis was carried out using Spectrophotometer (FTIR-8400S Shimadzu, Japan) and changes in percentage transmission at different wavelengths were observed for 4 days incubated samples. The spectra were collected within a scanning range of 4000 to 400 cm^{-1} for both malachite green and methyl red. The samples were mixed with spectroscopically pure Potassium Bromide in the ratio of 5:95 prior to analyses.

Statistical analysis

The statistical analyses of the aforementioned experiments were carried out using Statistical Package for the Social Sciences (SPSS) software (version 16.0). All the above experiments were carried out in duplicates and their significance level was analysed using the SPSS software.

RESULTS AND DISCUSSION

The laccase enzyme was immobilized on agar gel and percentage immobilization of enzyme of both *K. dobzhanskii* (65.2%) and *P. manshurica* (73.1%) showed that immobilization reduced the enzyme activity in comparison to free enzyme (100%). Many researcher has reported 65% immobilization of laccase in other substrates (Palmieri et al., 2005; Brandi et al., 2006; Chhabra et al., 2015) while the reduced activity of the immobilized enzyme may be as a result of low enzyme loading capacity or largely due to loss in laccase activity due to leaching or inactivation (Palmieri et al., 2005; Brandi et al., 2006).

Dye decolourization of malachite green and methyl red

Table 1 shows the decolourization of two dyes (triarylmethane) malachite green and (azo) methyl red by laccase enzymes of *K. dobzhanskii* DW1. The two dyes (malachite green and methyl red) were decolourized at two different concentrations (0.05 and 0.1 g/l) by the crude, purified and immobilized laccase enzymes of *K. dobzhanskii* DW1. Percentage decolourization of malachite green increases with incubation time in all the laccase enzyme forms (crude, purified and immobilized) and better percentage decolourization recorded at the lower concentration of the dye (0.05 g/l) except with crude laccase. Similar observation was reported by Pramanik and Chaudhuri (2018) where the least dye concentration of 0.5% gave maximum decolourisation. The highest percentage decolourization (81.50%) of malachite green was recorded at 96 h for immobilized laccase enzyme at concentration 0.05 g/l and the least percentage decolourization (2.20%) recorded at 24 h for crude laccase enzyme at concentration 0.1 g/l of the dye. The observed efficient decolorization by immobilised laccase in this study is similar to that reported on the purified and immobilized laccase of *Paraconiothyrium variable* which efficiently decolourised the removal of two synthetic dyes of acid blue 25 and acid orange 7 compared to the free laccase (Mirzadeh et al., 2014). Similarly for methyl red, percentage decolourization increases with incubation time in all the laccase enzyme forms. The highest (87.50%) and lowest (13.48%) percentage decolourization of methyl red was recorded at 96 h for purified laccase enzyme at 0.05 g/l concentration and at 0.1 g/l of immobilized laccase concentration after 24 h. Statistically, at all sampling times percentage decolourization of each dye significantly differs ($P \leq 0.05$) with their concentrations. Increase in concentration affected the process of dye decolorization and their efficiency depends upon their chemical structures, enzymes, and system conditions (Sun et al., 2017).

Table 2 shows the decolourization of malachite green and methyl red dyes by laccase enzymes of *P.*

Table 1. Dye decolourization with laccase enzyme of *Kluyveromyces dobzhanskii* DW1 at different incubation times.

Dye	Enzyme	Conc. (g/l)	Decolourization (%)			
			24 h	48 h	72 h	96 h
	Crude	0.05	13.40±0.20 ^c	19.48±0.20 ^e	19.86±0.04 ^d	20.28±0.14 ^d
		0.1	2.20±0.36 ^e	19.42±0.03 ^d	19.77±0.42 ^d	20.47±0.00 ^d
Malachite Green	Purified	0.05	62.38±0.00 ^a	63.36±0.14 ^b	63.86±0.14 ^b	65.84±0.04 ^b
		0.1	38.18±0.00 ^b	38.92±0.04 ^c	39.53±0.20 ^c	40.15±0.00 ^c
	Immobilized	0.05	38.72±0.00 ^b	75.73±0.00 ^a	81.40±0.14 ^a	81.50±0.02 ^a
		0.1	4.49±0.42 ^d	10.26±0.24 ^f	10.79±0.04 ^e	12.39±0.00 ^e
Methyl red	Crude	0.05	35.68±0.04 ^c	45.08±0.42 ^c	60.37±0.20 ^d	65.86±0.03 ^d
		0.1	44.04±0.20 ^b	76.20±0.35 ^a	80.63±0.14 ^b	81.70±0.04 ^b
	Purified	0.05	49.91±0.03 ^a	75.79±0.00 ^b	87.25±0.04 ^a	87.50±0.00 ^a
		0.1	13.95±0.42 ^e	45.54±0.36 ^c	70.27±0.46 ^c	73.91±0.03 ^c
	Immobilized	0.05	32.19±0.12 ^d	36.93±0.20 ^d	47.55±0.04 ^e	52.12±0.03 ^e
		0.1	13.48±0.03 ^e	16.54±0.00 ^e	22.67±0.14 ^f	27.33±0.20 ^f

Values are average of duplicate readings ± standard deviation. Means of values on the same column with the same superscript are not significantly different ($P \geq 0.05$) from each other within each dye.

manshurica DW2. The two dyes were decolourized at two different concentrations (0.05 and 0.1 g/l) by the crude, purified and immobilized laccase enzymes of the yeast isolate. With malachite green, at all incubation time and at different forms of laccase enzyme except the crude laccase enzyme, the lower concentration of the dyes (0.05 g/l) had the better percentage decolouration. Percentage decolourization of malachite green also increases with incubation time in all the laccase enzyme forms. The highest percentage decolourization of malachite green (84.40%) was recorded at 96 h and at a concentration of 0.1 g/l for crude laccase enzyme and the lowest percentage decolourization (2.56%) was recorded at 24 h for purified laccase enzyme also at 0.1 g/l concentration of the dye. Similarly for methyl red, percentage decolourization increases with incubation time in all the laccase enzyme forms. The highest (76.89%) and lowest (2.70%) percentage decolourization of methyl red was recorded at 96 h for crude (0.1 g/l) and at 24 h for purified (0.1 g/l) laccases. Generally, for both dyes and at both concentrations used, the purified form of the laccase enzyme gave the least decolourization at most times. Statistically, at all sampling times, percentage decolourization of each dye significantly differs ($P \leq 0.05$) with their concentrations.

From Table 1, the best percentage decolourization by laccase of *K. dobzhanskii* Dw1 for methyl red was recorded by the purified laccase at 0.05 g/l concentration of the dye. Meanwhile, the best percentage

decolourization for malachite green was recorded by the immobilized enzyme at 0.05 g/l concentration of the dye. Therefore, laccase of *K. dobzhanskii* was observed to work best (highest percentage decolourization) at 0.05 g/l concentration for both dyes. The difference in the extent of decolorization of structurally different dyes by laccase at the wavelength of each dye may be due to the difference of the redox potentials and the suitability of their steric structure with the active site of the enzyme (Afreen et al., 2016).

From Table 2, the best percentage decolourization by laccase enzymes of *P. manshurica* Dw2 for methyl red was recorded by the crude laccase at 0.1 g/l concentration of the dye. Similarly, the best percentage decolourization for malachite green was also recorded by the crude laccase enzyme at 0.1 g/l concentration of the dye. However, for laccase enzymes of *P. manshurica*, it was observed that the highest percentage decolourization was recorded at 0.1 g/l concentration of both dyes and at the same crude laccase treatments.

The observed variation in the decolorization potential of laccases even on the same dye depends on the biological sources of producing microorganism. For example, 60.5% of malachite green was removed after 15 min incubation of the dye in the presence of laccase from *P. variabile* (Forootanfar et al., 2011) while Zhuo et al. (2011) reported 98% of malachite green decolorization using laccase of *Ganoderma* sp.En3 after 72 h incubation. In addition, Vaidyanathan et al. (2011)

Table 2. Dye Decolourization with laccase enzyme of *Pichia manshurica* DW2 at different incubation times.

Dye	Enzyme	Conc. (g/l)	Decolourization (%)			
			24 h	48 h	72 h	96 h
Malachite Green	Crude	0.05	59.73±0.20 ^c	62.18±0.00 ^c	62.72±0.23 ^c	63.13±0.14 ^c
		0.1	77.92±0.14 ^a	79.67±0.00 ^a	81.20±0.03 ^a	84.40±0.26 ^a
	Purified	0.05	30.35±0.35 ^d	34.19±0.14 ^d	38.34±0.23 ^d	39.94±0.03 ^d
		0.1	2.56±0.14 ^f	6.28±0.00 ^e	7.84±0.41 ^e	7.97±0.20 ^e
	Immobilized	0.05	68.81±0.00 ^b	69.28±0.03 ^b	69.40±0.24 ^b	69.49±0.41 ^b
		0.1	6.31±0.36 ^e	6.94±0.03 ^e	7.47±0.04 ^e	7.83±0.20 ^e
Methyl red	Crude	0.05	34.65±0.00 ^b	44.78±0.41 ^b	46.91±0.03 ^c	49.25±0.04 ^c
		0.1	54.43±0.04 ^a	75.74±0.04 ^a	76.69±0.20 ^a	76.89±0.14 ^a
	Purified	0.05	11.68±0.24 ^d	31.37±0.02 ^d	43.32±0.04 ^d	47.06±0.20 ^d
		0.1	2.70±0.42 ^e	14.00±0.12 ^e	15.84±0.12 ^f	22.01±0.04 ^f
	Immobilized	0.05	28.46±0.20 ^c	41.58±0.41 ^c	58.87±0.35 ^b	63.93±0.23 ^b
		0.1	11.22±0.03 ^d	12.85±0.04 ^f	16.44±0.12 ^e	25.71±0.20 ^e

Values are average of duplicate readings ± standard deviation. Means of values on the same column with the same superscript are not significantly different ($P \geq 0.05$) from each other within each dye.

reported 72.2% removal of bromophenol blue by laccase of *P. variabile*.

Based on the aforementioned observations, these concentrations of the dyes were chosen for FTIR spectroscopy analysis.

FTIR characterization

The FTIR spectrum of methyl red: untreated by laccase, treated by the purified enzyme of *K. dobzhanskii*, DW1 and treated by the crude laccase of *P. manshurica* DW2 are as shown in Figure 1a to c, respectively.

Figure 1a shows the spectrum of untreated methyl red (control dye). The spectrum shows the changes in percentage transmission at different wavelengths and the spectrum was collected within a scanning range of 4000 to 400 cm^{-1} , while peaks were observed within a scanning range of 500 to 4000 cm^{-1} precisely within 543.94 to 3410.26 cm^{-1} . From the spectrum, sharp peaks were observed between wavenumbers 500 and 200 cm^{-1} while moderate peaks were observed between wavenumbers 2000 to 4000 cm^{-1} . From the figure, 25 peaks were recorded for the undecolourized dye with the presence of chemical groups like aliphatic halogen, alkane, alkene, ether, imine, azo (azide), alkyne, amine and alcohol/phenol.

Figure 1b shows the spectrum of methyl red decolourized by the purified laccase enzyme of *K. dobzhanskii* DW1. The spectrum shows the changes in

percentage transmission at different wavelengths and the spectrum was collected within a scanning range of 4000 to 400 cm^{-1} while peaks were observed within a scanning range of 600 to 4000 cm^{-1} precisely within 603.74 to 3416.05 cm^{-1} . The FTIR spectrum of methyl red dye decolourized by the purified laccase enzyme of *K. dobzhanskii* DW1 exhibited ten intense peaks at wavenumbers 2902.96, 2360.95, 1604.83, 1427.37, 1371.43, 1151.54, 931.65, 893.07, 657.75 and 603.74 cm^{-1} . Also, two broad and intense bands were observed at wavenumber 3416.05 and 1074.39 cm^{-1} . The FTIR spectrum showed there were a total of 17 peaks for the decolourized dye with the presence of chemical groups like alkane, alkene, ether, imine, carbonyl, alkyne and amine with total disappearance of azo and halogen groups. The observed reduction in the number of peaks in the FTIR spectra of the degraded/decolourised dye indicates that the number of reactive functional groups has reduced. This is similar to the report of Chatterjee et al., (2017) on the mycoremediation of textile dyes by *Talaromyces funiculosus* JAMS1, a reaction indicating change in the chemical reaction during the decolourisation process.

Figure 1c shows the spectrum of methyl red decolourized by the crude laccase enzyme of *P. manshurica* DW2. The spectrum shows the changes in percentage transmission at different wavelengths and peaks were observed from a scanning range of 500 to 4000 cm^{-1} precisely from 543.94 to 3431.48 cm^{-1} . The FTIR spectrum of methyl red decolourized by the crude

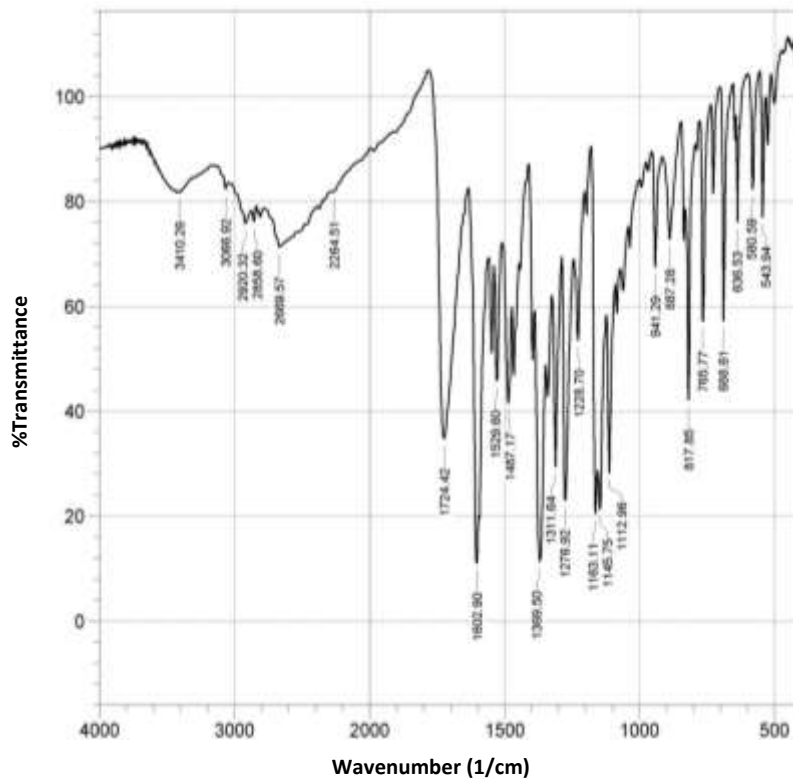


Figure 1a. FTIR spectrum of methyl red dye untreated by any laccase enzyme (Control).

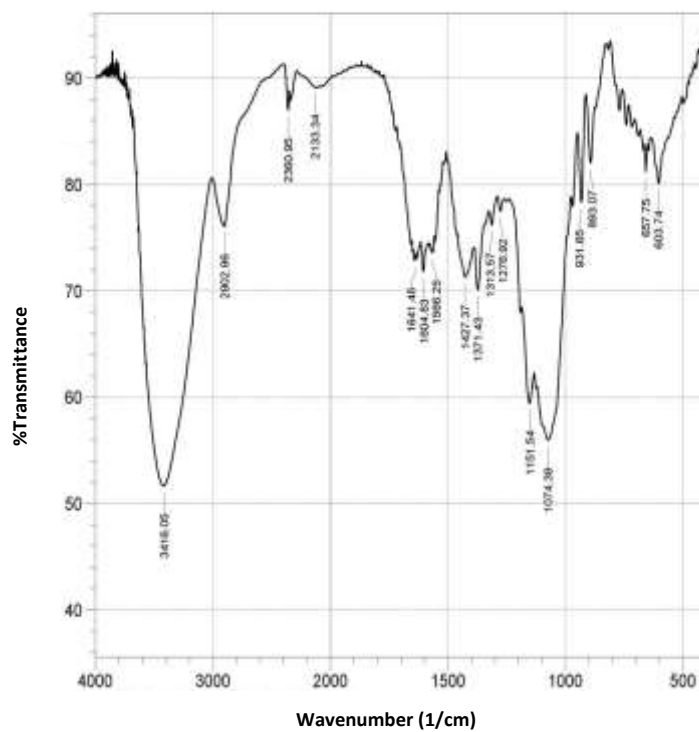


Figure 1b. FTIR spectrum of methyl red dye decolorized by the purified laccase enzyme of *Kluyveromyces dobzhanskii* strain DW1.

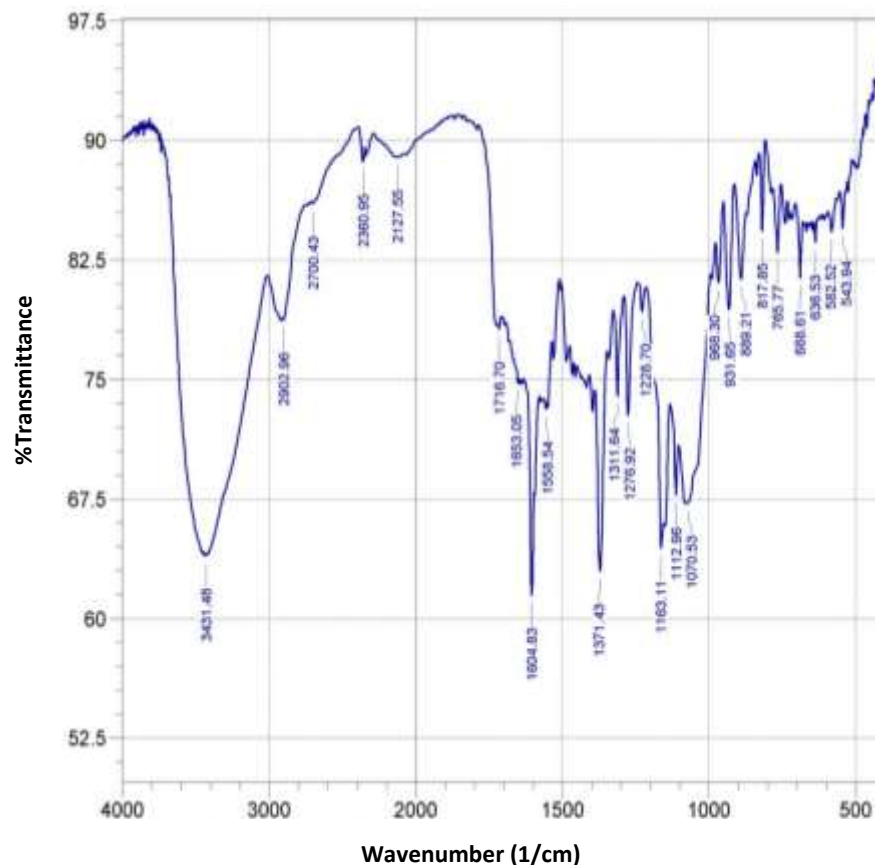


Figure 1c. FTIR spectrum of methyl red dye decolourized by the crude laccase enzyme of *Pichia manshurica* strain DW2.

laccase enzyme of *P. manshurica* exhibited sharp and intense peaks majorly between the wavelength regions of 500 and 2000 cm^{-1} with the exception of 2902.96 and 2360.95 cm^{-1} (which are unsaturated aliphatics). From the dye spectrum, a broad and intense band was observed at wavenumber 3431.48 cm^{-1} . The spectrum showed a total of 25 peaks and the presence of aliphatic halogen, alkane, alkene, ether, imine, carbonyl, alkyne and amine chemical groups.

The FTIR spectrum of malachite green: untreated by laccase enzyme, treated by the immobilized laccase enzyme of *K. dobzhanskii* DW1 and treatment by the crude laccase enzyme of *P. manshurica* DW2 are as shown in Figure 2a to c, respectively. From Figure 2a, the spectrum of untreated malachite green (control dye) shows the changes in percentage transmission at different wavelengths and the spectrum was collected within a scanning range of 4000 to 400 cm^{-1} , with peaks observed precisely from 420.5 to 3736.24 cm^{-1} . From the control dye spectrum, prominent peaks were observed mostly in the wavelength region of 500 to 2000 cm^{-1} , while moderate peaks were observed between wavenumbers 2000 and 4000 cm^{-1} . The FTIR spectrum of malachite green dye before decolourization (control) by

laccase enzyme as shown in Figure 2a revealed 25 peaks belonging to the chemical groups like disulfide, aliphatic halogen, alkane, thio ether, amide, sulfone, imino, amine, alkene, alkyne and alcohol.

Figure 2b shows the spectrum of malachite green decolourized by the immobilized laccase enzyme of *K. dobzhanskii* DW1 with peaks recorded precisely from 489.94 to 3419.9 cm^{-1} . The FTIR spectrum exhibits prominent peaks around wavenumbers 1000 to 4000 cm^{-1} inclusive of the broad band peak at 3419.90 cm^{-1} . A total of 17 peaks showing the presence of aliphatic halogen, alkane, alcohol, aromatic alkane, amide, sulfone, carbonyl, alkyne and amine chemical groups were observed.

Figure 2c shows the spectrum of malachite green decolourized by the crude laccase enzyme of *P. manshurica* DW2 with peaks observed within a scanning range of 500 to 4000 cm^{-1} precisely from 536.23 to 3427.62 cm^{-1} . The FTIR spectrum of malachite green decolourized by the laccase enzyme of *P. manshurica* DW2 exhibited prominent peaks around wavenumbers 1000 to 4000 cm^{-1} inclusive of the broad band peak at 3419.90 cm^{-1} . The FTIR spectrum revealed chemical groups like disulfide, alkane, thio ether, alcohol, aromatic

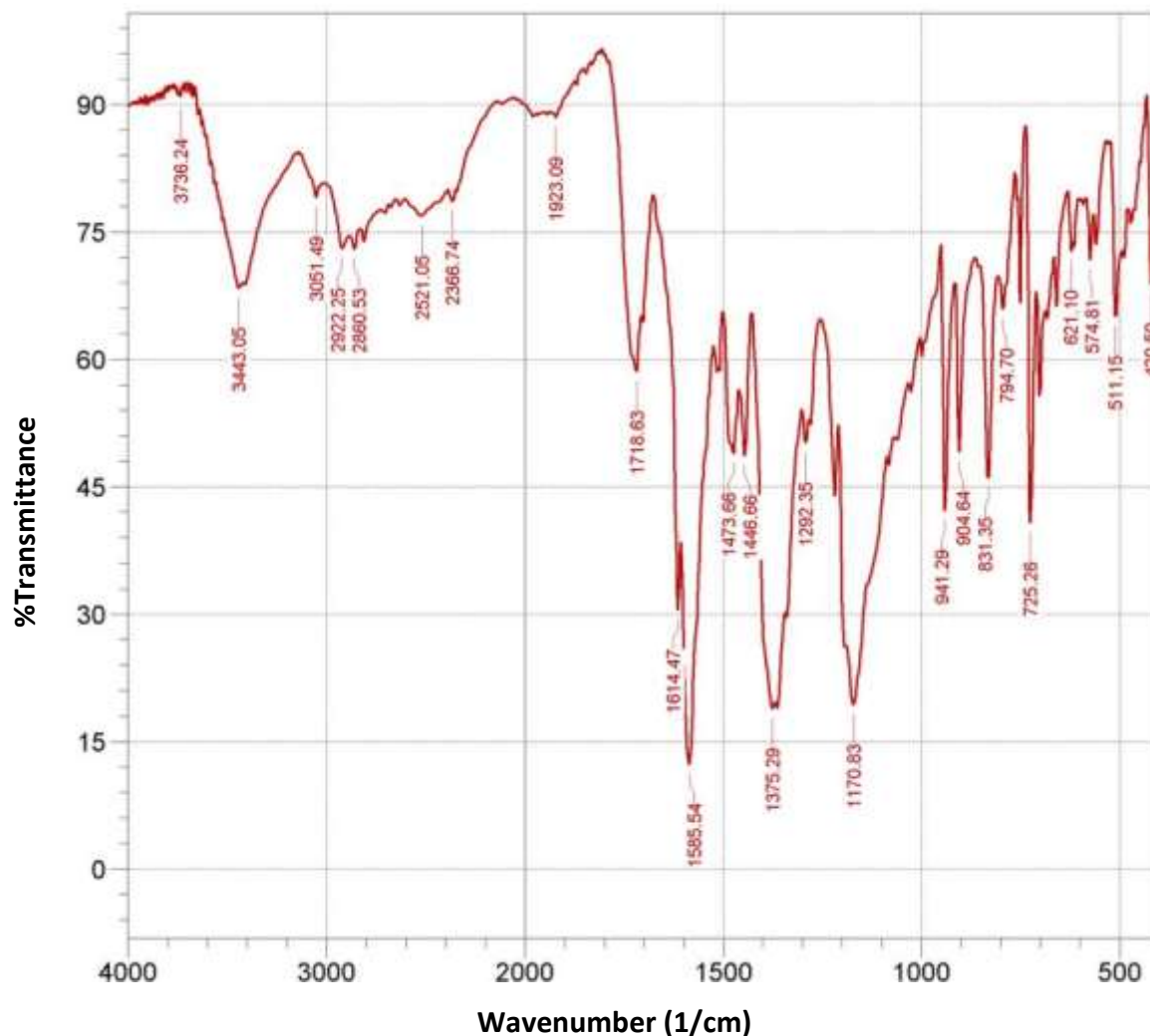


Figure 2a. FTIR spectrum of malachite green dye untreated by any laccase enzyme (Control).

alkane, amide, sulfone, carbonyl, alkyne and alcohol/phenol. The exhibited prominent peaks in the degraded dyes may be an indication on degradation of alkynes and aromatic groups to alkanes

The percentage decolourization range of both dyes (malachite green and methyl red) by the forms (crude, purified and immobilized) of laccase of *K. dozhanskii* DW1 and *P. manshurica* DW2 was between 2.2 and 87.5%. The highest percentage decolourization for malachite green by laccase enzyme of *K. dozhanskii* was 81.5% at 0.05 g/l concentration of the dye while the highest percentage decolourization for methyl red by laccase enzyme of *K. dozhanskii* was 87.5% at 0.05 g/l dye concentration. Also, the highest percentage decolourization for malachite green by laccase enzyme of *P. manshurica* was 84.4% at 0.1 g/l concentration of the dye while the highest percentage decolourization for methyl red by laccase enzyme of *P. manshurica* was 76.89% at 0.1 g/l dye concentration. Taguchi et al.,

(2018) similarly reported 82% decolorization of Orange G (an azo dye) at 0.3 mM concentration in the presence of iodide by laccase enzyme of *Iodidimonas* sp. Q-1

Similar result of 4.1 to 91.5% was observed by Zouari-Mechichi et al., (2006) when purified and crude laccase enzymes of *Trametes trogii* strain B6J was used to decolourize 0.05 g/l of azo dyes; Neolane blue, Neolane pink, Neolane yellow and Maxilon blue and the indigoid dyes Basacryl yellow and Bezaktiv S-BF turquoise. Ravikumar et al., (2013) reported that the purified laccase of *Hypsizygus ulmarius* showed maximum amount of decolourization in Remazol Brilliant Blue R (85%) followed by methyl orange (75%), Alizarin Red (73%), methyl violet (72%) and congo red (69%) without any additional redox mediator although at a reduced dye concentration of 0.025 g/l. Furthermore, with the addition of a redox mediator, 0.1 mM iodide was found to be sufficient for 71 to 99% decolorization of the azo and indigoid dyes, while 1 mM iodide was required for 78%

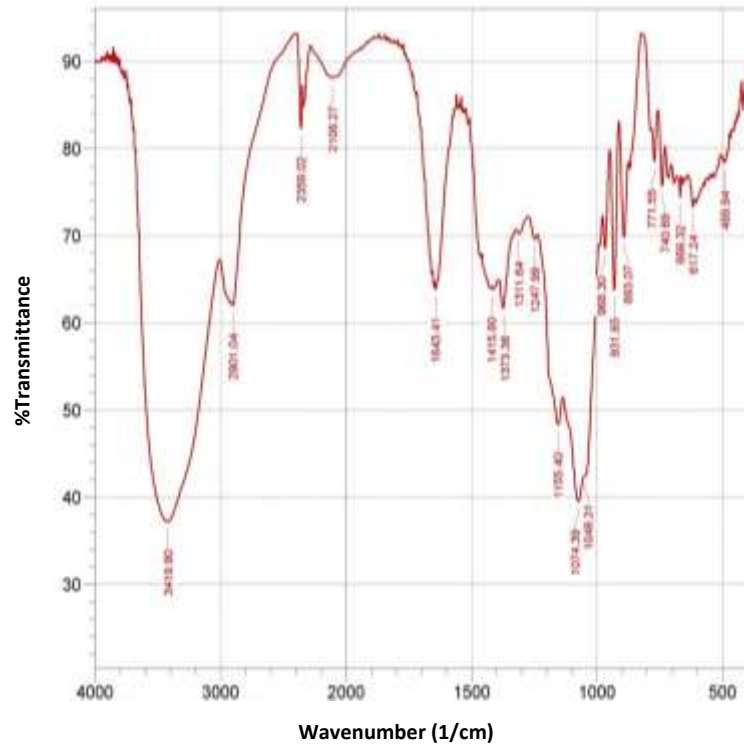


Figure 2b. FTIR spectrum of malachite green decolorized by the immobilized laccase enzyme of *Kluyveromyces dobzhanskii* DW1.

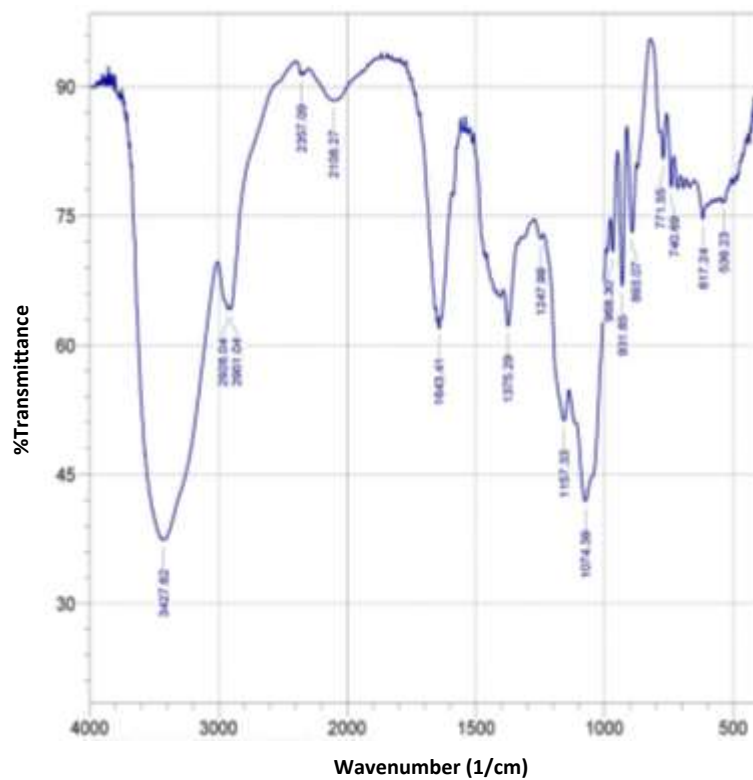


Figure 2c. FTIR spectrum of malachite green decolorized by the crude laccase enzyme of *Pichia manshurica* DW2.

decolorization of RBBR (Taguchi et al., 2018).

Conclusion

In this study, laccase enzyme from two yeast strains (*P. manshurica* DW2 and *K. dobzhanskii* DW1) were applied for the decolourisation of two synthetic dyes (Methyl red and Malachite green) and the enzyme forms (that is, crude, purified or immobilized) significantly affect the percentage decolorisation. The reduction in the peak numbers of the FTIR spectra of the decolourised dyes indicated the degradation or removal of some reactive functional groups. The removal of the main components (azo chemical group) of the dyes proved their effectiveness in decolourisation and bioremediation of the textile wastes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Enhancement of anaerobic batch digestion of spineless cacti (*Opuntia ficus indica*) feedstock by aerobic pre-treatment

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Received 14 September, 2018; Accepted 22 November, 2018

One of the best options for African countries to meet rural energy needs is to grow care-free crassulacean acid metabolism plants on a massive scale in waste lands. This can enable bioenergy production without disrupting food supplies and hence sustainable energy supply for the future. *Opuntia ficus indica* is an ideal plant for arid regimes but has barely been studied as a potential bioenergy source. This study investigated the effect of aerobic pretreatment on methane yield of *O. ficus indica* biomass. This effect was investigated in batch bioreactors which were exposed to aerobic conditions by varying time from 3 to 72 h before the start of anaerobic digestion. Reducing sugar content and dissolved oxygen levels after pretreatment period was analyzed. Reducing sugar content in bioreactors increased with increase in pretreatment time from 12.22 ± 0.69 to 59.08 ± 5.35 g/L in the untreated and 72 h pretreated batches, respectively. Methane yields after pretreatment were observed to range from 0.286 to 0.702 L/kg volatile solids at 9 and 72 h of pre-treatment, respectively. A 9 h pre-treatment of feedstock prior to anaerobic digestion yielded 123% higher methane yield when compared to that without pre-treatment. The findings that there was an increase in reducing sugar production and methane yield at 9 h of aerobic pre-treatment suggests that there was increased hydrolysis with pretreatment. Hence, short pre-treatment period could be an option to increasing solubilization of cladodes and promoting methane productivity. Therefore, pre-aeration of *O. ficus indica*, was shown to be an effective method for enhancing both its digestibility and improved methane yield during anaerobic digestion.

Key words: Anaerobic digestion, biogas, methane, *Opuntia*, pretreatment, spineless cacti.

INTRODUCTION

One requirement for sustainable development, especially in the developing countries is the availability of adequate energy to satisfy basic needs, improving social

welfare and achieving economic development (Rogner et al., 2004). Some of the issues associated with the current major sources of fuel include the fact that they are non-

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renewable and therefore can be exhausted, and that they contribute to generation of greenhouse gases leading to global warming, and consequently climate change and its negative environmental impacts (Moshi et al., 2015).

To date, corn and sugarcane are the main feedstock used in the biofuel production (Moshi et al., 2015; Cruz et al., 2018); this is attributed to their relatively simple conversion to biofuel as well as availability of infrastructure for planting, harvesting, and processing which are already in place. Nevertheless, their large scale cultivation for biofuel production is associated with issues such as decrease in food availability and dramatically increase in food prices worldwide (Calabr et al., 2017; Jigar et al., 2011).

Therefore, there is a need to find alternative sources in order to reduce competition of these natural materials which are also used as human food and animal feed. Presently, with the reality of global warming, crassulacean acid metabolism (CAM) plants that can withstand and resist drought have become more attractive as feedstock for anaerobic digestion (Cushman et al., 2015; Yang et al., 2015). Among these plants is the fast-growing *Opuntia ficus indica*, which is known to have high water use efficiency. *O. ficus indica* is the most widely distributed species of the cactus family (Nobel et al., 2002 and Bobich 2002). This plant have been reported to pose a great potential as source of lignocellulosic biomass with a yield of 10 to 50 tonne dry mass/(year·ha) (Calabr et al., 2017; Consoli et al., 2013).

These are desert plants that can survive where most of the plants cannot grow (Tarisse, 2008), hence suitable plant resource for climate change adaptation. Using spineless cacti as a potential energy-generating crop may offer serious perspectives to countries prone to drought and relying on imports for their energy consumption (Tarisse, 2008; Nobel et al., and Bobich 2002 2002). Moreover, the ability to grow on unfertile land will make use of the land that currently is not occupied with agricultural crops and hence improve land utilization. The fact that spineless cacti is not used as food in most areas would reduce the competition of food versus fuel use (Calabr et al., 2017).

There is an increasingly interest in evaluating the potential of *O. ficus indica* as feedstock for anaerobic digestion and biogas production (Jigar et al., 2011; Egigu, 2014; Calabr et al., 2017; Ramos-Suarez et al., 2014; Yang et al., 2015). Nevertheless, limited studies have dealt with pretreatment of the plant cladodes prior to anaerobic digestion and the effect they could have on both methane production and yield.

A pretreatment method of lignocellulosic material is needed for an anaerobic process to reduce the volume of material used, increase solubility and production of methane (Antonopoulou et al., 2015; Antognoni et al., 2013; Tizé et al., 2016). Pretreatment of biomass prior to anaerobic digestion has been shown to enhance hydrolysis which is known to be a rate limiting step during anaerobic digestion, without degrading carbohydrates or

forming by-products that are inhibitory to other processes downstream (Montgomery and Bochmann, 2014; Taherzadeh and Karimi, 2008; Xolisa et al., 2007).

Pretreatment of *O. ficus indica* has been investigated earlier by Calabr et al. (2017) who evaluated the effect of the thermal, alkaline and acidic pretreatments on the composition and biochemical methane potential. The authors found that only the acidic pretreatment (HCl) had significantly increased methane generation, while neither thermal nor alkaline pretreatment produced noticeably affects methane yields (an average reduction of 8% was recorded). These pretreatment methods may have some draw backs such as the use of hydrochloric acid can lead to a very low final pH, which could have a negative effect on the anaerobic digestion process (Calabr et al., 2017).

Though *O. ficus indica* have been studied for its potentials as animal feedstock and other agronomic applications, limited studies have exploited its potentials in anaerobic digestion (Jigar et al., 2011; Calabr et al., 2017). Currently, there is no scientific report on biological pretreatment of *Opuntia* prior to anaerobic digestion, most specifically aerobic pretreatment. This study therefore aims to characterize *O. ficus indica* as lignocellulosic feedstock for anaerobic digestion and evaluate effect of aerobic pretreatment on digestibility and methane potential of *O. ficus indica*.

MATERIALS AND METHODS

O. ficus indica (L.) mill raw material

O. ficus indica used as feedstock was obtained from Dar es Salaam region in Tanzania, which is located at 6°48' South, 39°17' East (World Bank Group, 2011). The cladodes (green, plate-like sections) were the plant part of interest for this study. *O. ficus indica* in this study were sampled on non-irrigated and non-cultivated land (Figure 1). The cladodes were manually chopped into pieces averaging 1 cm³ with a sharp knife, blended at maximum speed using kitchen blender (Philips HR2067/04 600 W) placed in closed plastic containers and stored at 5°C until its further use.

Substrate inoculum

Cow rumen fluid obtained from Vingunguti abattoir, Ilala Municipal Dar es Salaam, Tanzania was used as a source of inoculum for this study. The fresh rumen fluid was collected and filtered through a sieve of 2 mm pores (Endecott's Test Sieve Limited, BS 410, England) to separate solid content from the slurry. Twenty liters plastic containers with airtight lids were used to carry the inoculum at ambient temperature (31±1°C) to the laboratory. Prior to use, the inoculum was left to mature for 16 days at 31±1°C to remove the easily degradable volatile solid present in inoculum (Liew, 2011).

Determination of physico-chemical properties of the *Opuntia* biomass

Volatile solids (VS) and total solids (TS) of the substrate were determined by the oven-drying and ignition methods, respectively according to standard methods (APHA, 2006). Total carbon



Figure 1. *Opuntia ficus indica* used in this study.

determination was done by using the Walkley-Black Potassium Dichromate method as described by Nelson and Sommers (1996). Total nitrogen determination was carried out using Kjeldahl method described by APHA (2006). The total carbohydrates, cellulose and hemicellulose determination were carried out using the procedure described by Allen (1989). The determination of acid detergent fiber followed the procedure described by Van Soest et al. (1991). Reducing sugar was determined using the Hagedorn-Jenson method based on quantitative oxidation by potassium ferricyanide ($C_6N_6FeK_3$) and titration with sodium thiosulphate ($Na_2S_2O_3$) as described by Allen (1989). Determination of pH of the biomass and effluent was determined before and after anaerobic digestion using a pH meter (HANNA HI 2211).

Batch bioreactor configuration

Anaerobic digesters were constructed in bench-scale experiments where biogas was produced out of the degradation of organic matter in 500 ml bioreactors consisting of wide mouth Erlenmeyer conical flasks which was connected to gas-tight aluminium-reinforced via a gas tight-plastic tubes for biogas collection (Figure 2). Gas sampling port was fitted in the bioreactor with n-butyl

stoppers and sealed with aluminium caps as explained by Mshandete et al. (2005). Each bioreactor had a sampling septum made of rubber stopper for taking biogas samples and a gastight bag for collecting the gas.

Experimental set up of bioreactors

The experiment, which was carried out in a laboratory at a temperature of $31 \pm 1^\circ C$ was set up in twenty-six bioreactors organized into two sets. The first set had six digesters without aeration including three which had untreated cladodes; the other three had inoculum only as controls. The second set of bioreactors comprised bioreactors with aeration in five different time intervals consisting of four bioreactors each.

Determination of the effect of aeration pretreatment on methane yield

Investigation of the effect of aerobic pretreatment on anaerobic digestion of *O. ficus indica* cladodes was done in anaerobic batch bioreactors using the rumen fluid as inoculums. To examine the

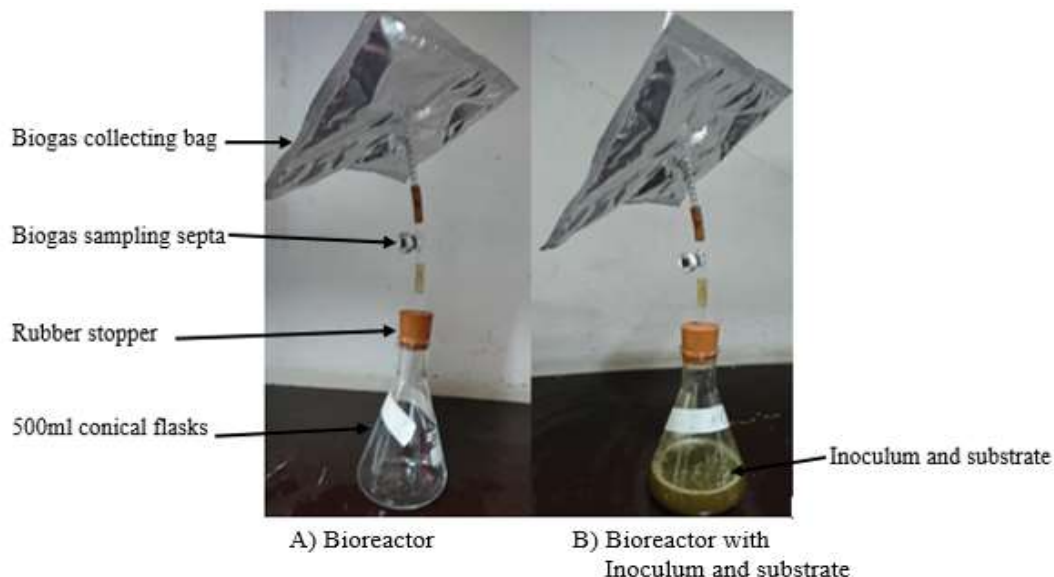


Figure 2. Bioreactor used in this study. (a) Bioreactor. (b) Bioreactor with contents (inoculum and substrate).

effect of pre-treatment on the subsequent performance of batch anaerobic digestion of *Opuntia* biomass, experiments were carried out at five different times of aerobic exposure: 3, 9, 24, 48 and 72 h.

For each of the bioreactor, 20 g of the feedstock was added followed by inoculum to make up to 250 ml of working volume. Surface aeration was achieved by using a shaking incubator (Orbital Incubator S150, Stuart Scientific, UK), whereby bioreactors were left open and shaken at 130 rpm and 31°C for the aerobic period (3, 9, 24, 48 and 72 h). This allowed regular mixing of bioreactor contents and suitable condition for growth of hydrolytic bacteria. After every pretreatment period relative amount of dissolved oxygen was determined with an oximeter (OXI 3205, Weilheim 2009, Germany) and samples for the analysis of sugars content were centrifuged at 2000 g for 5 min (Thermofisher scientific, 41930819, Germany) and the supernatant was used to analyze reducing sugars as described by Allen (1989).

Immediately after the aeration periods, the content in each digester was flushed with nitrogen (N_2) gas for 3 min to replace the oxygen and provide anaerobic conditions. Subsequently, the bioreactors' openings were closed with stoppers to ensure gas tightness. Bioreactors were kept at a temperature of $31 \pm 1^\circ C$ and shaken manually for 1 min, twice a day to provide regular substrate-inoculums distribution. These bioreactors were compared in terms of its biogas and methane content after every 72 h. All the tests were performed in triplicate 30 days.

Methane yield determination

The methane content was estimated by the concentrated alkaline absorption method described by Erguder et al. (2001) where concentrated potassium hydroxide (KOH) stock solution (20 g/L) at atmospheric pressure and 5 ml sample of the biogas was used. In this method, only methane was determined while other biogas components such as carbon dioxide (CO_2) and hydrogen sulphide (H_2S) were dissolved in the potassium hydroxide solution. The volume of biogas formed during the experiment was measured using a graduated 100 mL glass syringe (SGE International Pty Ltd., Ringwood, Australia) according to Pham et al. (2013).

Statistical analysis

Data was expressed as mean \pm standard error (SE) of the triplicate measurements. Differences between mean values were examined by one-way analysis of variance (ANOVA) and significance was set at $P = 0.05$. All statistical analyses were performed using Prism version 6.01 for Windows.

RESULTS

Physico-chemical properties of the *O. ficus indica* biomass

The concentrations of various physico-chemical parameters of *O. ficus indica* feedstock are shown in Table 1. Moisture content of the biomass was quite high, around $88.05 \pm 2.23\%$. Total solid and volatile solid contents of *O. ficus indica* biomass obtained were 11.95 and 73.95%, respectively. The *O. ficus indica* cladodes were found to have $59.65 \pm 0.36\%$ carbohydrates content. Initial pH readings for all digesters were around 7.5. Hemicellulose and cellulose were 33.10 ± 0.11 and 8.39 ± 0.01 , respectively. The amount of carbon and nitrogen content in the feedstock made a ratio of around 30.6:1.

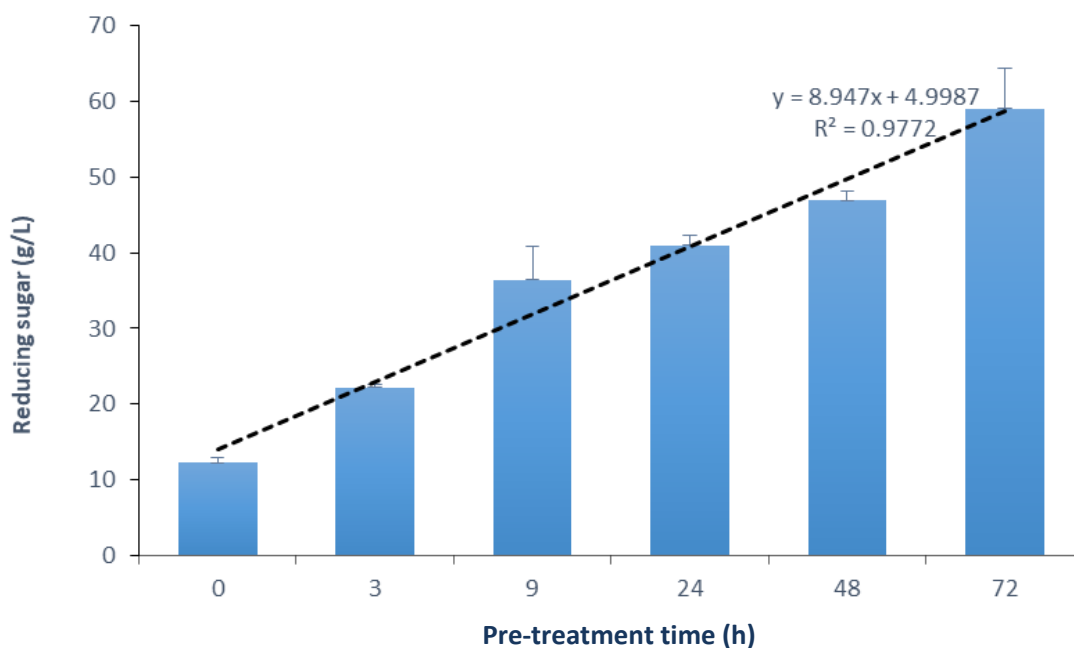
Reducing sugar contents in *O. ficus indica* biomass after pre-treatment

The amount of reducing sugar in the bioreactors during aerobic pre-treatment was between 12 and 59 g/L on the untreated and 72 h pretreated batch bioreactors,

Table 1. Physico-chemical composition of *Opuntia ficus indica* feedstock used in this study. (TS= Total solids).

Parameter	Concentrations
Moisture content (%)	88.05±2.23
Total carbohydrates (%)	59.65±0.36
Total nitrogen (% of TS)	2.08±0.20
Total solids (TS) % of fresh	11.95±2.23
Volatile solids(% of TS)	73.95±6.33
Acid detergent fiber (% of TS)	17.45±0.04
Hemicellulose (% of TS)	33.10±0.11
Cellulose (% of TS)	8.39±0.01
Reducing sugars (% of TS)	76.29±1.14
Total carbon (% of TS)	63.74±1.23
pH	7.52±0.02
Carbon: Nitrogen ratio	30.6:1

TS, Total solids.

**Figure 3.** Reducing sugar content of hydrolysate in bioreactors after each pre-treatment period.

respectively. The increase of reducing sugar amount steadily increased as the pretreatment time increased reaching the highest amount on the bioreactor pretreated for 72 h as shown in Figure 3. The rise in reducing sugar contents can be seen as early as the 3 h pretreatment time. There was slight reduction in the increase of reducing sugar content on 24 and 48 h pretreatment time. Measurements were done in triplicates and values are represented as mean plus or minus standard error (represented as error bars

Total methane production and methane yield during the incubation period

Methane yield (as meter cubic of methane per kilogram of volatile solids used) from the different pretreatment periods during the anaerobic digestion period is shown in Table 2. As seen from the results, the methane yield varied with pretreatment time of *O. ficus indica* feedstock. The highest and lowest methane yields were obtained from 9 and 72 h pretreatment, respectively. From these

Table 2. Total methane production in liter and methane yield (L/kg VS) during anaerobic digestion of aerobically pre-treated *Opuntia ficus indica* biomass in batch bioreactors for 30 days of digestion.

Time of pretreatment (h)	Total methane production (L)	Methane (L/kg VS added)
0 (Untreated feedstock)	0.56±0.038	0.315±0.016
3	0.72±0.065	0.412±0.026
9	1.24±0.130	0.702±0.053
24	0.75±0.065	0.425±0.027
48	0.60±0.046	0.341±0.019
72	0.50±0.054	0.286±0.022

VS, Volatile solids.

results, pretreatment increased the methane yield till around 9 h of pretreatment and then leveled off to around 0.286 L/kg VS for the 72 h pretreatment time. It can also be observed that there is no significant change after 24 h pretreatment in methane yield with further increase of exposure period to oxygen prior to anaerobic digestion.

Using one way ANOVA from Prism version 6.01, the difference in methane yield among the pretreated groups was found to be of statistical significance with P value < 0.05. Pairwise comparison revealed that the difference was of statistical significance between the untreated group and the group pretreated for 9 h.

Effect of aeration pretreatment on methane yield potential of *O. ficus indica* feedstock

An increase in methane yield potential of 123% was recorded for the 9 h pretreatment time compared to the untreated *O. ficus indica* feedstock (Figure 4). The bioreactors which were pretreated in 3, 9, and 24 h showed high potential in increasing methane yield (greater than 30%) as compared to the bioreactor with no pretreatment on the other hand bioreactors pretreated for 48 and 72 h produced low methane yield 0.341±0.019 and 0.286±0.022 L/kg VS added, respectively compared to the control with the lowest amount on 72 h which showed potential 9% decrease in methane yield when compared with the batch without pretreatment.

DISCUSSION

Physical chemical properties

A great percentage of the *O. ficus indica* biomass obtained in this study was in moisture content of about 88.05%. The values obtained here are comparable with studies done on these plants elsewhere such as those in the study reported by Jigar et al. (2011) who worked with *Opuntia* in Ethiopia and reported the moisture content of 86%. Filho et al. (2016) evaluated moisture content of various *Opuntia* plants and reported a moisture content in

the range of 88.7±1.2 to 92.6±0.7. This result shows that the moisture content of *O. ficus indica* is relatively high, which can aid anaerobic digestion as it can increase the degree of digestion since microorganisms in the digestate can easily access liquid substrate for relevant reactions to take place (Sadaka and Engler, 2003).

As a feedstock for anaerobic digestion in this study, the ratio of carbon and nitrogen of feedstock was 30.6 (Table 1). Nitrogen and carbon are among the key components needed by microorganisms in the development of their cell structures (Zhang et al., 2011; Ajay et al., 2011). Without forgetting the role played by nitrogen in cell growth which is very crucial in methane production but there is also buffering capacity by releasing ammonium cation contributed by nitrogenous compounds (Zheng et al., 2014). This ratio does not fall far from the carbon to nitrogen ratio of 20 to 32 recommended in anaerobic digestion of organic biomasses to provide enough nutrients for microorganisms performing anaerobic digestion (Costa et al., 2012; Zeshan et al., 2012; Wang et al., 2014). Nevertheless, the ratio obtained under this study is similar to that reported by Jigar et al. (2011) who obtained a range of 24 to 28 and Calabr et al. (2017) who obtained around 27:1 when dealing with *Opuntia* in the anaerobic digestion. It has been shown that various feedstocks used for anaerobic digestion can be optimized at a C/N ratio of approximately 6:1 to 30:1 (Costa et al., 2012); the optimum point of this ratio differs from feedstock to feedstock depending on the composition of feedstock in question.

TS and VS contents of feedstock used in this study were 11.95 and 73.95%, respectively. The values obtained from experiment were similar to the values reported by Jigar et al. (2011) who obtained the total solids and volatile solids contents of cladode as 14 and 78%, respectively. Determination of total solids and volatile solids of the feedstock is one of the important steps as these measures are not only used in the substrate loading but also represents part of the feedstock that may be transformed into biogas and methane (Motte et al., 2013; Ajay et al., 2011). The volatile solids obtained here indicating that the *O. ficus indica* have relatively large fraction which is biodegradable

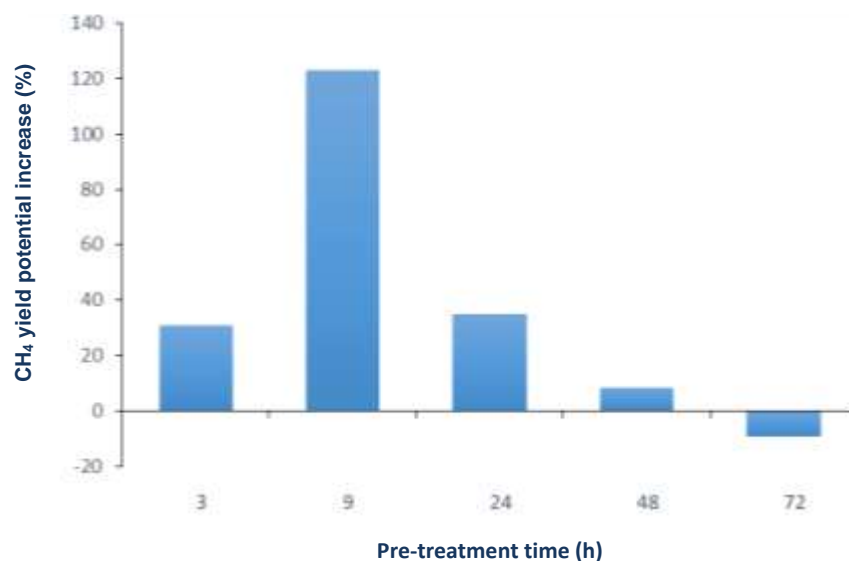


Figure 4. Potential increase in methane yield (%) of *Opuntia ficus indica* feedstock for different pre-treatment periods in comparison to the control.

suitable for anaerobic digestion. A study conducted by Costa et al. (2012) working on methane production from poultry litter reported less productivity of the digestion of substrates having 7.6% total solids.

Feedstock used in this study was found to have $59.65 \pm 0.36\%$ (Table 1) of carbohydrate which does not fall very far from that found by Malainine et al. (2003) who obtained roughly 69% total carbohydrates. Substrates hydrolyzed in the first stages of anaerobic digestion consist of carbohydrates, lipids, and proteins (Azman et al., 2015; Gerardi, 2003). In the anaerobic digestion process, structural carbohydrates are divided into their component sugars known as monosaccharides such as fructose and glucose that can be utilized by microorganisms (Gerardi, 2003; Ajay et al., 2011). Hence, the amount of carbohydrate content of feedstock is of importance in the evaluation of the viability of the biomass as a feedstock for anaerobic digestion.

Cellulose content of *O. ficus indica* in this study was found to be $8.39 \pm 0.01\%$. This value falls in close range to those reported in literature such as 7.7 ± 0.41 and 11%, reported by Calabr et al. (2017) as well as 7.95 to 13.73% reported by Ben-Thlija (2002) both of them working on *Opuntia* species. Cellulose is one among the major components of lignocellulosic plant materials contributing to about half to one third of plant tissues (Jorgensen et al., 2007). Cellulose is resistant to hydrolysis by enzymes or acids because of their structure and the lignin barrier, hence, the necessity of using pre-treatments at the initial stages of hydrolysis (Gujer and Zehnder, 1983). Pretreatment before aerobic digestion increased the accessibility of cellulose by microbial community by breaking the seal made by lignin (Antonopoulou et al., 2015).

In this study, the amount of acid detergent fiber was found to be $17.45 \pm 0.044\%$ of TS which falls in a very close range with that reported by Ben-Thlija (2002) which was in the range of 11.29 to 18.98% of TS; relatively low acid detergent fiber proportion gives them an appreciable digestibility level. Acid detergent fiber represents the least digestible fiber portion of feedstock and includes lignin, cellulose, silica and insoluble forms of nitrogen but not hemicellulose. Feedstock with high acid detergent fiber is low in energy (Von Cossel et al., 2018; Ben-Thlija, 2002).

Initial pH of the bioreactor contents for all digesters was around 7.5 (Table 1), this pH is suitable and within the range recommended for proper functioning of microorganisms in anaerobic digestion (Ward et al., 2008). This value is in agreement with a pH range of input mixture in the digester between 6.25 and 7.50, reportedly suitable for most methanogenic bacteria by Mahanta et al. (2004) and Ajay et al. (2011). While the pH of 3, 9 and 48 h pretreatment remained relative around the initial pH value (7.7, 7.62, and 7.63, respectively); there was noticeable increase in the pH of the batch without pretreatment and those of 24 and 72 h pretreatment (8.1, 8.08, and 8.15, respectively) after the completion of the incubation period.

The observed increase in pH value can be caused by various factors within the bioreactors. Amongst them being the production of alkali compounds, such as ammonium ions during the degradation of organic compounds in the digester (Gerardi, 2003). During fermentation, carbonic gas is formed and when combined with water it forms carbonates in anaerobic environment and hence can be a reason for the increase in pH. It is also possible that the formation of methane results in an

increase in pH Jensen et al. (2017). Gray et al. (1971) also showed that pH value increased by accumulation of ammonia during degradation of protein whereas accumulation of volatile fatty acids from degradation of organic matter decreased the pH value. Though microorganisms can still be functioning, values of Ph methane-forming bacteria (Gerardi, 2003; Ajay et al., 2011).

Changes in reducing sugar contents in *O. ficus indica* biomass after pre-treatment

Variations were observed in the sugar concentration during pretreatment period, where the sugar levels increased with increase in pretreatment time from 12 to 59 g/L (Figure 3); this rise can be accounted for by the hydrolysis of polysaccharides to monosaccharides (Montalvo et al., 2016). The amount of sugar formed is also affected by the hydrolytic conditions and the microbial community present (Ahn et al., 2014). The low (22.17 ± 0.47 g/L) amount of sugar content obtained during the initial hours of pretreatment can be due to the consumption of the sugars by the aerobic microorganisms, which are active due to the provided aerobic conditions (Ahn et al., 2014). As the pretreatment is stopped the amount of sugar used is reduced and hydrolytic enzymes they produced are still active and continue to make soluble sugar available for the proper functioning of aerobic microorganism for aerobic digestion (Botheju et al., 2010).

Granados-Arvizu et al. (2017) obtained the highest amount of reducing sugar after pretreatment (about 80 g/L) while using corn pericarp under acid and temperature pretreatment. Montalvo et al. (2016) also obtained increase in concentration of total sugar during the first 48 h of aeration, where an increase of 192% was obtained with respect to its initial value.

It should be well noted that even though methane producing microorganism can hydrolyze insoluble carbohydrates, lower methane yields can be observed when the lignocellulosic materials are utilized without any kind of treatment (Zheng et al., 2014).

Indeed, the pretreatment increased the solubility of polysaccharide present in the bioreactor (Antonopoulou et al., 2015), as a result, increasing sugar levels. On one hand, these consequences may facilitate the anaerobic digestion by increasing the accessibility of these sugars to microorganisms. On the other hand, if pretreatment is carried out by increasing period of time above optimum, this could become problematic in systems as significant amounts of organic material were aerobically degraded before anaerobic digestion period is started (Ahn et al., 2014). Therefore, a compromise between increasing the solubility of substrate by aerobic pretreatment and prevention of overconsumption of soluble sugars due to consumption by microorganisms prior to anaerobic

incubation needs to be found.

Effect of aeration pretreatment on methane yields

Methane yields after pre-aeration were observed to range from 0.286 to 0.704 L/kg VS, whereas for the untreated one was 0.315 L/kg VS (Table 2). Methane yields were higher with 9 h pre-aerated batch as compared to the others and lower on 72 h. When compared with the batch without pretreatment, potential increase of methane yield in the 9 h bioreactor was about 123% (Figure 4). This increase was significantly high ($P = 0.0025$) and is comparable to that which was observed by (Montalvo et al., 2016) who reported an increase of 110% in the production of methane when applied to the mixed sludge aerobic hydrolysis as compared to the digestion process of non-aerated sludge. It can be seen that pre aeration increased the methane yield till around 9th hour of pretreatment and then leveled off to around 0.286 L/kg VS a decrease which is about 9% for the pretreatment time of 72 h (Figure 4).

Since no research findings on aerobic pre-treatment of *O. ficus indica* feedstock are available, no direct comparison can be made. Nevertheless, various researchers have reported increase in methane content in biogas with the incorporation of aerobic process in anaerobic digestion (Rafieenia et al., 2017; Jang et al., 2014; Ahn et al., 2014). Results obtained in this study differ a bit from those of Ahn et al. (2014), who worked on anaerobic digestion of aerobic pretreated sewage sludge samples and obtained the highest methane yield after 24 h of pretreatment. The difference in pretreatment time which produced the highest methane yield could have been attributed to differences in the type of feedstock used, as maximum methane yield of an anaerobic digestion vary depending on several factors including feed composition (Botheju et al., 2010). Ahn et al. (2014) used sewage sludge which contains a complex mix of protein, lipids and carbohydrate. On the other hand, the results are similar to those obtained by Mshandete et al. (2005) who worked on anaerobic digestion of aerobic pretreated sisal pulp waste samples and obtained the highest methane yield after 9 h of pretreatment. Both of these studies showed no significant increase in methane potential with further increase in pretreatment time above the optimum obtained (9 and 24 h).

Increased methane yield can be attributed to improve biodegradability of aerobically pretreated *O. ficus indica* feedstock that came as a result of biological role played by microbes (Ahn et al., 2014; Conklin et al., 2007). It has been shown in some studies that short-term oxygen exposure of bioreactor does not affect methanogenic activity and methanogens can survive longer than previously reported (Conklin et al., 2007). The increase in methane yield as a result of increase of methanogenic activity can also be due to an improvement in the growth

of facultative anaerobes, which can keep a low redox potential, providing the best conditions for the growth of strict anaerobes (Montalvo et al., 2016).

The experiments revealed that the methane yield is not significantly different with further increase of exposure period to oxygen prior to anaerobic digestion. Potential decrease of 9% was observed at 72 h pretreatment (Figure 4). These results are in agreement with some author's findings who showed that long time exposure to oxygen does not significantly improve the methane yield (Xu et al., 2014; Botheju et al., 2009). In this study, dissolved oxygen measured ranged from 0.13 to 0.16 mg/L; the highest and the lowest value at 3 and 72 h pretreatment. In a study done by Ahn et al. (2014) reported that the dissolved oxygen values higher than 0.15 mg/L can start to inhibit methanogenic activities. Methane yield was the lowest on 72 h pretreatment (0.286 L/kg VS). Inhibition of the activities of methane forming bacteria and decrease in the methane yield can occur under longer exposure to oxygen as reported by Xu et al. (2014). This decrease in methane yield observed here could have been as a result of several factors such as the substrate oxidation of readily available substrates by facultative acidogenic organisms and the partial inhibition of the activity of strictly anaerobic biomass (Botheju et al., 2009). Methane consumption by aerobic methanotrophs (Fu et al., 2015) can also be a contributing factor to the decrease in methane yield observed.

The reduction of methane yield in bioreactors, which were pre aerated for longer periods have been reported by other researches (Fu et al., 2015; Ahn et al., 2014). Reduction in methane yield at 26 and 37% following pre-treatment for 48 and 72 h, respectively has been reported by Mshandete et al. (2005) while Ahn et al. (2014) obtained reduced methane yield at 48 and 96 h of pre-aeration which was linear with the period of pre-aeration. Botheju et al. (2010) also reported decreased amounts of methane generation under increased aeration conditions as a linear reduction within the oxygenation range of 0 to 10.1%.

Based on these findings, the best pretreatment time can be said to lie between 9 and 24 h as these provide enough time for the microorganisms during pretreatment to thrive and produce their effect. It can be seen that pre-aeration had an advantage in increasing the methane yield and more optimization of the pretreatment time with other factors is required to ensure maximum yield of methane from biomass and avoid the inhibition of microbial activities involved in methane production.

Conclusion

Aerobic pre-treatment of *O. ficus indica* feedstock prior to anaerobic digestion, showed significant effect on enhancing subsequent digestion of the substrate in batch anaerobic bioreactors using cow rumen fluid as an

inoculum. Nine hours of pre-treatment showed 123% more methane yield potential than with untreated *O. ficus indica* feedstock. Prolonged pre-treatment periods of 48 and 72 h showed no increase in the methane yield potential from *O. ficus indica* feedstock, rather a decrease of 9% as compared to the untreated obtained at 72 h pretreatment. Above 24 h, methane production per kg of volatile solids added decreased with increase in pretreatment periods, this is possibly due to inhibition of activities of methane forming bacteria and substrate oxidation of readily available substrates by facultative acidogenic organisms.

Using aerobic pre-treatment for the period of time around 9 to 24 h has great potential to promote hydrolysis as well as solubilization of feedstock, as a result enhance the anaerobic digestion of *O. ficus indica* feedstock. Biological pretreatment (including aerobic) of lignocellulosic material for anaerobic digestion has received increased focus during recent years driven more by negative effect of other form of pretreatments including chemicals to environment. To reducing the overall production costs and avoiding aeration expenses, low level of oxygen applied in this study can be adopted and offer an added advantage in enhancing anaerobic digestion. Further studies of this pretreatment effect can be done at pilot level and analyze its applicability at industrial scale. Integrated two stage digestion system comprising aerobic pretreatment followed by anaerobic digestion have been reported (Global Renewables, 2014; Montgomery and Bochmann, 2014). The cultivation of *O. ficus indica* in marginal and waste lands will guarantee availability of feedstock, at the same time reduce land competition with food crops. This in turn will lead to reduction in prices of staple foods used as feedstock and hence generating fuel and more food rather than choosing between the two.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the Pan African University Institute of Basic Science, Innovation and Technology for the financial support.

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Full Length Research Paper

Evaluation of the diversity in qualitative traits of Bambara groundnut germplasm (*Vigna subterranea* (L.) Verdc.) of Côte d'Ivoire

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Received 31 October, 2018; Accepted 14 December, 2018

The objectives of this study were to assess phenotypic diversity of Bambara groundnut germplasm from Côte d'Ivoire using qualitative traits and to understand the genetic diversity at different levels. Hundred and one accessions collected from four agro-ecological zones (central, eastern, northern, western) were characterized in a randomised complete block design with three replications. Thirteen qualitative traits were recorded from seedling emergence to physiological maturity of the crop species. All recorded traits were found to be polymorphic with three or four phenotypic classes. The results revealed a considerable amount of phenotypic variation in the germplasm studied. The phenotypic variation was expressed in color, shape, texture, flexibility, growth habit, pilosity and hardness in both the aerial organs and the underground pods. Cluster analysis grouped together accessions into six genetically distinct groups independently to their geographical origin, suggesting seeds exchanges between growing-zones. The chi-square analysis highlighted the presence of phenotypic variability within and between accessions from each agro-climatic zone for most of the traits evaluated indicating some adaptive forms related to the four zones. Estimates of Shanon-Weaver diversity index (H') for all agro-climatic zones ranged from 0.32 to 0.66 with a mean of 0.46. The northern zone appeared phenotypically more diversified ($H' = 0.66$) than the others. These results are useful to ensure efficient germplasm collection, conservation and management strategies.

Key words: Bambara groundnut, accession, agroclimatic zones, phenotypic diversity, qualitative traits.

INTRODUCTION

Bambara groundnut (*Vigna subterranea*) is an African crop widely grown by subsistence farmers. It is an under-utilised food legume (Azam-Ali et al., 2001) that occupies

a prominent place in the strategies to ensure food security in sub-Saharan Africa (Koné et al., 2015). Its edible seeds are an important source of calories, vitamins

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and vegetable proteins (Amarteifio and Moholo, 1998; Minka and Bruneteau, 2000). In addition to its nutritional values, the roots, leaves and seeds are used in traditional medicine (Basu et al., 2007; Jideani and Diedericks, 2014; Maphosa and Jideani, 2016). Only grown as landraces (Zeven, 1998), the crop has a potential for economic exploitation (Ahmad et al., 2016) and offers several agronomic advantages to rural communities in most sub-Saharan countries. It is adapted to various agro-ecosystems, has greater tolerance to drought and yields well on poor soils (Collinson et al., 1997; Mabhaudhi and Modi, 2013). That makes it a useful legume to include in climate change adaptation strategies (Hillocks et al., 2012). The Bambara groundnut landraces are generally grown on small areas in rotation or intercropped with cereals (maize, sorghum, pearl millet), root and tuber crops (cassava, yam). As a nitrogen-fixing legume, they contribute to the maintenance of the soil fertility. They particularly are a remedy to the intensive use of infertile soils (Kumaga et al., 1994).

In Côte d'Ivoire, Bambara groundnut is grown in contrasted agro-climatic zones including tropical rain forest and dry savannah. Rainfall and temperature are the most important climatic factors in Côte d'Ivoire, and vary significantly from one region to another (Mobio et al., 2017). Due to the mountainous zone in the west of the country, rainfall decreases along a South-west/North-east gradient while thermal gradient is oriented from the South to the North where temperatures are higher (Yao et al., 2013). These strong climatic gradients affect the genetic structure of plant populations in different agro-climatic regions (Hamasha et al., 2012).

In these zones, farmers possess considerable indigenous knowledges arising from their long habit of farming Bambara groundnut. Farmers grow the crop for immediate consumption and sale in local markets. It is therefore closely integrated in various traditional intercropping systems and plays a key role in both nutrition and the culture of peoples (Djè et al., 2005). However, the plant is now an endangered crop species (Ahoussou et al., 1995) because of the priority given to major crops such as cassava, cashew, peanut and maize in its growing areas. As a result, preliminary surveys were carried out in different agro-climatic growing regions in Côte d'Ivoire to collect Bambara groundnut germplasm. Accessions thus sampled are maintained in the seed bank of Nangui Abrogoua University. Nevertheless, the collected germplasm needs to be evaluated for various quantitative and qualitative traits. Thereby, like quantitative traits which are usually the target of the selection, qualitative traits are also essential to assess plant diversity and intraspecific variation. Furthermore, qualitative traits allow easy and rapid differentiation between phenotypes. They generally have high heritability, can be easily observed with the naked eye, and are also expressed in all environments (IPGRI et al., 2000). Both of these kinds of characters are appreciated

for different objectives, and assessed with different approaches and techniques. Genetic analysis of qualitative traits often requires the interpretation of numbers in various phenotypic classes. Statistical methods such as chi-square tests (χ^2) and Shannon-Weaver diversity index (H) or its relative index (H') are extensively used to respectively assess qualitative variation and level of diversity in several species (Ayana and Bekele, 1998; Arshad et al., 2005; Yirga and Tsegay, 2013). Morphological variability in Bambara groundnut is usually analyzed based on quantitative traits. Analysis of these quantitative traits revealed a significant genetic variability among and within indigenous accessions of Bambara groundnut from Côte d'Ivoire (Bonny and Djè, 2011; Touré et al., 2012). But little is known about their qualitative morphological traits and no work on the variability across regions and on the distribution of these traits was ever conducted. Analysis of the qualitative characteristics could also provide useful information for breeders and managers of genetic resources. So quantifying the variation and the distribution of qualitative traits of local Bambara groundnut landraces is necessary for the collection and the selection of varieties, and the effective management of its conserved germplasm. In Côte d'Ivoire, the growth habit and the color of the seed coat are the main qualitative criteria used by farmers to distinguish, to name and to easily classify Bambara groundnut landraces in the growing areas. Germplasm analysis requires however more characters. The wide range of variation in rainfall and temperature might shape the patterns of qualitative traits variation and their distribution in the different agro-climatic growing areas of Bambara groundnut in Côte d'Ivoire. It is therefore useful to know if some specially adapted forms occur, particularly in these areas. Indeed, the identification of the genetic diversity occurring between and within populations from different geographic areas and the classification of germplasm are necessary for sustainable use of a crop species for different purposes (Rao and Hodgkin, 2002).

The objectives of this research were: i) to describe the variation in 13 qualitative traits of Bambara groundnut landraces; ii) to evaluate the genetic variability and the relationship among accessions; iii) to estimate the distribution of these qualitative traits; and; iv) to assess the level of diversity linked to the main growing zones of Côte d'Ivoire in order to provide a useful qualitative database for Bambara groundnut germplasm collection and conservation.

MATERIALS AND METHODS

Plant

One hundred and one accessions of *V. subterranea* randomly selected from the seed bank of Nangui Abrogoua University were used. They were sampled in four contrasted agro-climatic zones (northern: 29; central: 24; eastern: 27; and western: 21) in Côte

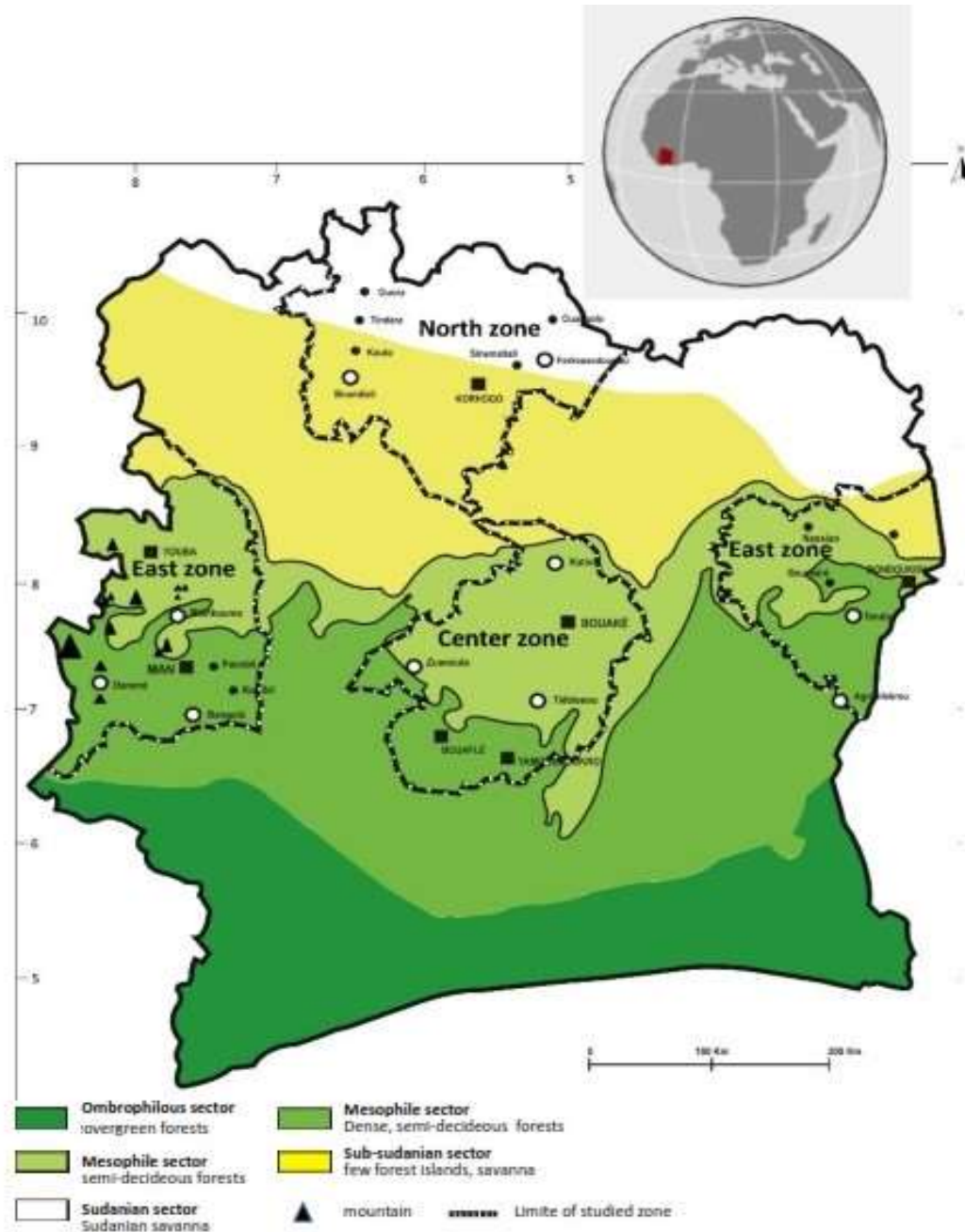


Figure 1. Map of Côte d'Ivoire depicting areas from which the bambarar groundnut germplasm was collected.

d'Ivoire (Figure 1). In this study, each accession is identified by its introduction number and geographical origin. All accessions of a given agro-climatic zone represent a population.

Study area and experimental design

Seeds from the accessions were sown at the Research Station of Nangui Abrogoua University at Abidjan, in the southern region of Côte d'Ivoire. Rainfall in that region is abundant with annual mean

greater than 2000 mm. There are two rainy seasons extending from March to July and September to November. The mean monthly temperature ranges from 25 to 28°C. Vegetation is mainly represented by the tropical rain forest (Konaté and Kampmann, 2010). The ferralitic soil is deeper, sandy, well drained and rich in organic matter.

The experiment was conducted according to a randomized complete block design with three replications. The blocks, spaced about 4 m from each other, were ploughed and leveled at the onset of the rainfall season. Each block (52 m × 10 m) consisted of rows,

10 m long spaced 1 m apart. Spacing between plants within a row was adjusted to 0.9 m. Five seeds of each accession were sown randomly in each block on June 20th, 2015. Thus, each accession was represented by 15 plants for a total of 1515 plants for all the blocks. The field was evenly kept weed-free throughout the experimentation and neither insecticide nor herbicide was applied. Harvests were done during the dry season in February 2016.

Data collection

The accessions were characterized based on 13 qualitative characters selected essentially from the descriptors of Bambara groundnut (IPGRI et al., 2000). Data were collected from seedling to harvest. The phenotypic classes found for the examined 13 qualitative traits and their codes are presented in Table 1. For each plant of an accession, the phenotypic classes were recorded according to the binary method: 1 for presence and 0 for absence. Then, each accession was scored for the most frequent phenotypic class for each qualitative trait. And phenotypic frequencies for all traits were computed for each agro-climatic zone.

Statistical analysis

Phenotypic frequency distributions of the qualitative traits were estimated using a sample of all the germplasm. Based on the qualitative data of these accessions, a cluster analysis was performed to establish groups' relationship among the accessions. The Ward method based on Euclidean distances was carried out after standardizing the data to mean zero and unity variance (Van der Berg et al., 2006). The software program Statistica version 7.1 (StatSoft Inc, 2005) was used for all statistical analyses.

Chi-square test

Data were analyzed with chi-square tests (Bolboacă et al., 2011). First, a chi-square test of independence was applied to determine whether or not characters and agro-climatic zones were dependent. Then, a chi-square test of homogeneity was performed to test the homogeneity of the populations from different agro-climatic zones. Calculations were performed using XLStat software 2016.2 (Addinsoft, 2016).

The Shannon-Weaver diversity index

The Shannon-Weaver diversity index (H') (Hennink and Zeven, 1991) is given by:

$$H' = \frac{-\sum_{i=1}^n P_i * \ln(P_i)}{\ln(n)}$$

Where, P_i is the frequency of the i^{th} phenotypic class and n is the number of phenotypic classes for a given phenotypic trait. The Shannon-Weaver diversity index was used to assess the phenotypic diversity for each trait by agro-climatic zone. Using the additive properties of H' , an analysis of variance (ANOVA) was conducted and means of H' for the agro-climatic zones were compared by the least significance difference (LSD) at 0.05 probability level using statistical software program Statistica version 7.1 (StatSoft Inc, 2005).

RESULTS AND DISCUSSION

Phenotypic diversity

The evaluation of available genetic diversity is a pre-requisite for genetic improvement in crop plants (Olukolu et al., 2012). Scoring qualitative traits is an important alternative to molecular technique to assess genetic variation on plant germplasm (Ngompe-Deffo et al., 2017). Based on 13 qualitative traits, a sample of 101 accessions was evaluated. The observations revealed a considerable amount of variation in all the traits as well as their frequency distribution (Table 1). The number of phenotypic classes observed for each trait ranged from 3 to 4 indicating that all the traits examined were polymorphic. Thus, a total of 43 phenotypic classes were identified in the current study. The number and nature of the phenotypic classes indicated a high phenotypic variability in the conserved germplasm suggesting the presence of genetically distinct accessions. Moreover, a predominance of some phenotypic classes was observed for all the traits.

In the early stages of seedling emergence, three categories of seedling color were detected in the accessions studied. The phenotypic variance for this trait showed significantly higher frequencies of purplish red (48.52%) and green (47.52%) seedlings than dark green (3.96%) seedlings (Table 1). Regarding the leaflet color, four phenotypic classes were observed. Among them, a high frequency of accessions with green leaflets (69.31%) was recorded, followed by accessions with light green (12.87%) and dark green (11.88%) leaflets. Only 5.94% of accessions had yellowish green leaflets. In addition, three phenotypic classes were identified for leaflet flexibility.

The majority of the accessions studied had soft leaflets (89.11%), while accessions with moderately soft and hard leaflets were poorly represented at 5.94 and 4.95%, respectively. The leaflet shape of the accessions also differed during plant growth. It was found that most of the accessions produced elliptic leaflets (74.26%) followed by the accessions with oval leaflets (17.82%). Only 6.93% of the accessions had lanceolate leaflets and accessions with round leaflets were rare (0.99%). The variation in the colors of the petioles also was noticeable. Out of the accessions analysed, 76.24% had light green petiole, 22.77% showed green petiole and only 0.99% had pale green petiole. Observed variations found among the local landraces regarding the leaflet color, shape and flexibility may be a useful tool in future collection missions.

The majority of the accessions in the sample showed a dark green stem color (78.22%). The two other stem colors observed were green and pale green in the respective proportions of 19.80 and 1.98%. Furthermore, three distinct types of stem hairiness were recorded among the accessions. With the exception of some accessions which showed absent hairiness (6.93%) or

Table 1. Qualitative traits, codes and phenotypic classes and frequency (%) distribution of Bambara groundnut accessions.

Qualitative trait	Code and phenotypic class		Frequency
Seedling color at emergence	1	Purplish red	48.52
	2	Green	47.52
	3	Dark green	3.96
Fully expanded leaflet color	1	Dark green	11.88
	2	Green	69.31
	3	Ligth green	12.87
	4	Yellowish green	5.94
Leaflet flexibility	1	Soft	89.11
	2	Moderately soft	4.95
	3	hard	5.94
Terminal leaflet shape	1	Round	0.99
	2	Oval	17.82
	3	Lanceolate	6.93
	4	Elliptic	74.26
Petiole color	1	Green	22.77
	2	Pale green	0.99
	3	Ligth green	76.24
Stem color	1	Green	19.80
	2	Pale Green	1.98
	3	Dark green	78.22
Stem hairiness	1	Absent	6.93
	2	Sparse	2.97
	3	Dense	90.10
Growth habit	1	Bunch type	31.68
	2	Semibunch type	8.91
	3	Spreading type	59.41
Pod shape	1	Round without point	22.77
	2	Rounded base with a point at the top	76.24
	3	Elongated with rounded base and a point at the top	0.99
Pod color	1	Yellowish-brown	2.97
	2	Brown	89.11
	3	Ligth brown	2.97
	4	Beige	4.95
Pod texture	1	Smooth	6.93
	2	Wrinkled with little grooves	12.87
	3	Wrinkled with much grooves	53.47
	4	Rough	26.73
Pod hardness	1	Low	11.88
	2	Moderately hard	41.58
	3	Hard	46.54

Table 1. Contd.

Seed shape	1	Round	10.89
	2	Oval	86.14
	3	Oblong	2.97

sparse hairiness (2.97%), most of the accessions produced dense hairiness stem (90.10%).

Bambara groundnut varied in vegetative growth. Our observation was in agreement with those of Doku (1969) who categorized the vegetative growth of Bambara groundnut in three distinct growth habits, namely, bunch, semi-bunch and spreading types. These three growth habits were found in our germplasm in different proportions. Among the accessions studied, the spreading type was the most observed (59.41%) followed by the bunch type (31.68%). The semi-bunch type was less frequent (8.91%). The growth habit of crops is of high significance to the cropping system (Egbadzor et al., 2014). Each type of growth habit could be subject to different cultivation procedures in the traditional cropping system. They could be chosen judiciously as cover crop in phytotechnical procedures involving various cultivated species of locale tubers and cereals crops.

The pod of the Bambara groundnut is an important underground organ of the crop species. The pod was a qualitative trait that differed according to genotype. The accessions showed phenotypic variations with respect to color, shape, texture and hardness of pods. In this study, the majority of accessions expressed brown pod color (89.11%). The color of the pods of the remaining accessions was almost equally distributed as yellowish-brown (2.97%), light brown (2.97%) and beige (4.95%). The pod texture varied considerably among the accessions. Four patterns of pod texture were identified in the present study: smooth, rough, and wrinkled with little grooves or more grooves. Most of the accessions had wrinkled pods with more grooves (53.47%). These were followed by accessions producing rough pods (26.73%), wrinkled pods with little grooves (12.87%) and smooth pods (6.93%). Pod shape varied from accession to accession and phenotypic variations included three patterns. Accessions producing pods characterized by rounded base with a point at the top were most frequent (76.24%) followed by those producing rounded pods without point (22.77%). Accessions producing elongated pods with rounded base and a point at the top were rare (0.99%). Concerning the proportion of pod hardness in all the material studied, three categories of hardness were observed as follow: 46.54% of the accessions showed hard pods, while 41.58% had moderately hard pods. Only 11.88% of the accessions had soft pods.

With regard to seed shape, three categories were observed. A large proportion of the accessions produced oval seed (86.14%), while 10.89% of accessions had

round seed. Only 2.97% of the accessions studied produced oblong seed.

The phenotypic variations expressed in color, shape, texture, flexibility, growth habit, pilosity and hardness in both the aerial organs and underground fruits of Bambara groundnut landraces suggest that a high degree of diversity is still maintained within this species. According to Joshi and Baniya (2006), the existence of such diversity in cultivated crops may be due to genetic drift, spontaneous variations and natural or human selections. These preliminary results point to the need for more studies in order to identify useful germplasm with known characteristics.

Cluster analysis

The dendrogram (Figure 2) resulting from the cluster analysis shows the phylogenetic relationships between the accessions from all the zones. This analysis helped to identify related and genetically distinct groups of accessions. Six groups were formed at 30% similarity level. Except, the other traits, the phenotypic traits related to seedling color, petiole color and stem color were observed in all groups.

For example, group I contained 15 accessions from all collection zones and was further subdivided into two subgroups. The common traits of the accessions of both subgroups were green and soft leaflets. Furthermore, their plants had a bunch type growth habit with dense hairiness stems and produced oval seed. The pods of these accessions have a rounded base with a tip at the top, and wrinkled with more grooves on their surface. These pods have a low hardness and a brown color. The difference between the two subgroups involved the leaflet shape, which was oval for the first subgroup and lanceolate for the second subgroup.

Group II consisted of 21 accessions collected in three zones (central, eastern, and western zones). Those accessions clustered into two subgroups. Accessions from group II are similar for their oval seed and differ in the pod shape. The pods of the first subgroup have a rounded base with a tip at the top while those of the second subgroup are rounded without a tip at the top. Moreover, all the accessions of group II have a green color, soft and elliptic leaflets, produced plants with a spreading growth habit and dense hairiness stems. The pods of this group had more grooves, were hard and brown in color.

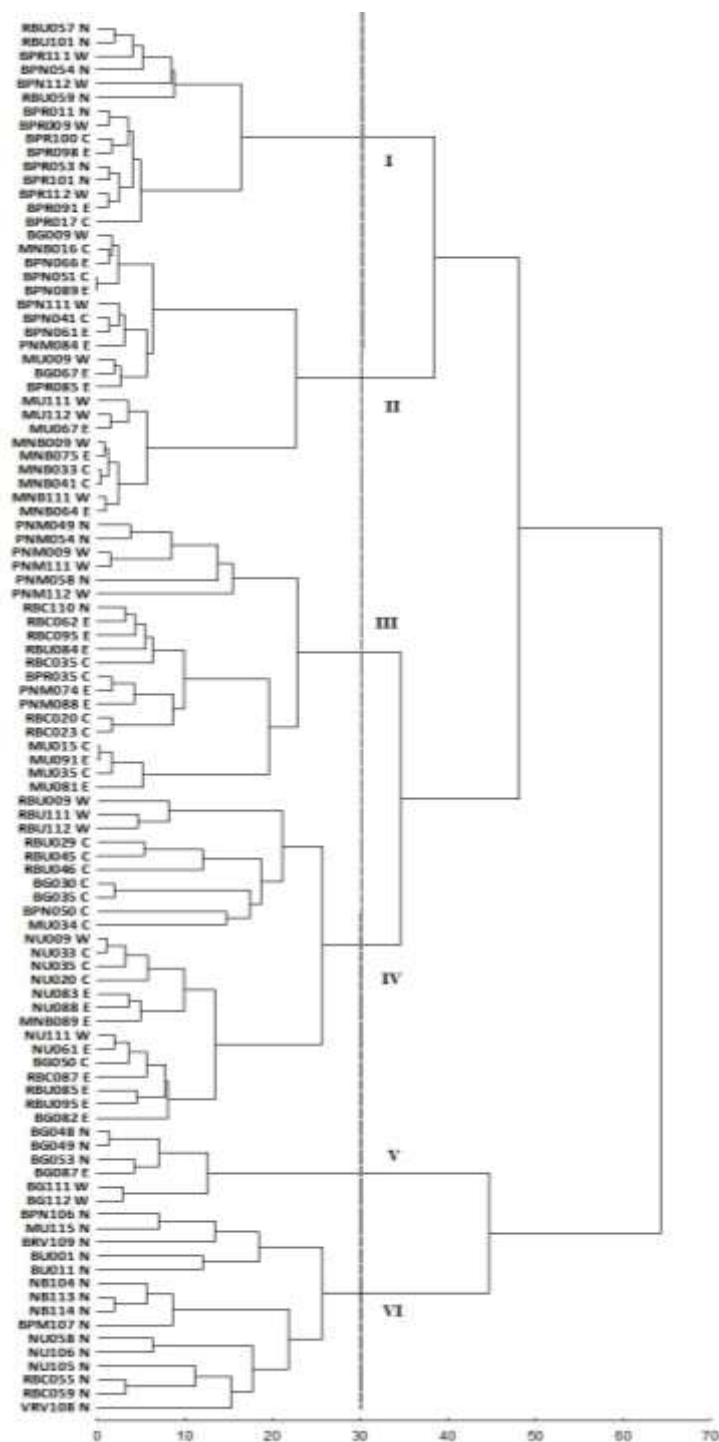


Figure 2. Dendrogram for the 101 accessions of *Vigna subterranea* from four agroclimatic zones [central (C), eastern (E), northern (N), western (W)] of Côte d'Ivoire, constructed by Ward's method.

Group III included 20 accessions from the four collection zones. It is subdivided into three subgroups. Accessions from these subgroups were identical for the following phenotypic traits: green, soft and elliptic leaflet, spreading

growth habit, dense hairiness stem, brown and hard pod, and oval seed. The first subgroup additionally contains accessions with oval leaflets, a bunched growth habit, rounded base pod with a tip at the top and rough.

Accessions from the second subgroup produced rounded base pods with a tip at the top but wrinkled with more grooves. Accessions from the third subgroup produced only rounded pods without a tip at the top and wrinkled with more grooves.

Group IV consisted of 24 accessions originating from the western, central and eastern zones. It is structured into three subgroups. All the accessions that belong to this group have mainly light green color, soft and elliptic leaflets, a spreading growth habit, dense hairiness stem and produce oval seeds. Pods produced by these accessions are brown, rough and hard but differ in their shape. Thus, accessions in the first and second subgroups have rounded base pods with a tip at the top while those in the third subgroup have rounded pods without a tip at the top.

The group V gathered 6 accessions originating from three zones (northern, western and eastern). The leaflets of these accessions are yellowish-green, relatively hard and oval. Their plants have semi-bunch growth habit and dense hairiness stems. Their pods have a rounded base with a tip at the top, wrinkled with little grooves on the surface, hard and brown in color. The pods contained oval seeds.

The group VI was more heterogeneous. It was composed of 15 accessions collected exclusively in the northern zone. It is further subdivided into two subgroups. Accessions belonging to this group are phenotypically more varied with respect to the leaflet color and shape, and also the color and texture of the pod. For example, the leaflet color varied from light green to dark green, the leaflet shape was oval, elliptic or lanceolate. All the plants of the accessions had a bunch growth habit, hairiness stems and produced rounded base pods with a tip at the top. The differences between accessions of the two subgroups concerned mainly the texture, the hardness and the color of the pods and the shape of the seed. In the first subgroup, the pods of the accessions have a smooth surface, a beige color and were soft; their seeds were round. In the second subgroup, the pods had a roughed surface, a light brown color and were moderately hard; the seeds shape was round or oblong.

The cluster analysis revealed that qualitative traits are also useful markers to distinguish and classify local Bambara groundnut accessions. Genetic patterns obtained from this study of Bambara groundnut germplasm could be of great importance. It can help Bambara groundnut breeders to make better choices during selection and gene bank management. The qualitative traits evaluated in this study could be used to easily distinguish varieties with simple and inexpensive selection methods if their high heritability was proven.

The accessions from different geographical origins were clustered into the same group, and those from the same geographical zone were clustered into different groups. These results suggest that for a given traits, farmer's selection criteria could be uniform. That could also be due to seed exchanges among the different

growing-regions.

Distribution of traits in the agro-climatic zones

Characterizing the geographical patterns of variation is useful for efficient conservation strategies and use of agricultural genetic resources (Dulloo et al., 2010). In this study, the frequency distribution for the thirteen qualitative traits was evaluated among the main growing zones of Bambara groundnut landraces. Tables 2 and 3 show the observed and relative frequency distributions of phenotypic classes by agro-climatic zones of collection. Out of the thirteen qualitative traits examined, four traits, namely seedling color, leaflet flexibility, petiole color and stem color showed an independent distribution of the agro-climatic zones ($\chi^2_{gk} < 12.59$ for a $ddl = 6$ and $\chi^2_{gk} < 16.92$ for $ddl = 9$; $P > 0.05$). These results indicate that collection zones shared strong similarity in reference to all phenotypic classes for these traits. The nine other traits, which expressed together 31 phenotypic classes, presented a dependant distribution of the agro-climatic zones ($\chi^2_{gk} > 12.59$ for a $ddl = 6$ and $\chi^2_{gk} > 16.92$ for $ddl = 9$; $P < 0.05$). Overall, these data demonstrated the existence of different populations of Bambara groundnut in Côte d'Ivoire related to agro-climatic zones. This spatial distribution of populations could be an essential factor for the long-term maintenance of genetic variation (Cruzan, 2001).

The homogeneity tests corroborated the differences among accessions of the different zones with regard to the expression of 31 phenotypic traits. Geographical origin likely contributed to the genetic variability among the accessions (Geleta et al., 2005). Out of the 31 phenotypic classes, the most remarkable results were achieved with only 15 phenotypic traits ($\chi^2_g > 7.81$ for $ddl = 3$). The identified phenotypic classes were dark green (for Leaflet color), oval, lanceolate and elliptic (for leaflet shape), absent (for stem hairiness), bunch, semi-bunch and spreadind (for growth habit), round without point at the top (for pod shape), light brown and beige (for pod color), smooth (for pod texture), low and hard (for pod hardness) and round (for seed shape). These phenotypic traits that showed different occurrences depending on the agro-ecological zone could serve as morphological markers during future collection missions.

All phenotypic classes of a given qualitative trait occurred with varied frequencies within and between accessions from collection zones. Overall, the central, eastern and western zones showed, in general, the same trend of frequency distribution for almost all phenotypic classes, unlike the northern zone.

Within each zone, an unequal frequency distribution of phenotypic classes was recorded among accessions ($\chi^2_g > 7.81$ for a $ddl = 3$ or $\chi^2_g > 9.48$ for $ddl = 4$, data not

Table 2. Frequencies of Bambara groundnut accessions from four agroclimatic zones (central, eastern, northern, western) of Côte d'Ivoire by phenotypic classes for thirteen qualitative traits.

Qualitative trait		Northern	Western	Central	Eastern	χ^2_g	χ^2_{gk}	<i>p</i>
Seedling color at emergence	1	10	12	13	14	1.72	3.69	0.718
	2	18	8	10	12	1.91		
	3	1	1	1	1	0.06		
Fully expanded leaflet color	1	10	1	0	1	17.74	27.20	0.001
	2	14	17	19	20	2.69		
	3	2	1	5	5	3.73		
	4	3	2	0	1	3.05		
Leaflet flexibility	1	23	18	24	25	0.70	6.73	0.347
	2	3	1	0	1	2.98		
	3	3	2	0	1	3.05		
Leaflet shape	1	1	0	0	0	2.48	43.74	< 0.0001
	2	10	7	0	1	14.65		
	3	7	0	0	0	17.38		
	4	11	14	24	26	9.22		
Petiole color	1	8	4	5	6	0.47	3.25	0.777
	2	1	0	0	0	2.48		
	3	20	17	19	21	0.30		
Stem color	1	6	4	5	5	0.05	5.20	0.518
	2	2	0	0	0	4.97		
	3	21	17	19	22	0.18		
Stem hairyness	1	7	0	0	0	17.38	19.55	0.003
	2	0	1	1	1	1.25		
	3	22	20	23	26	0.92		
Growth habit	1	21	4	2	5	21.85	54.78	< 0.0001
	2	7	1	0	1	10.91		
	3	1	16	22	21	22.02		
Pod shape	1	0	5	10	8	10.93	16.12	0.013
	2	28	16	14	19	2.70		
	3	1	0	0	0	2.48		
Pod color	1	1	0	2	0	3.77	28.33	< 0.001
	2	23	18	22	27	0.72		
	3	0	3	0	0	11.43		
	4	5	0	0	0	12.41		
Pod texture	1	7	0	0	0	17.38	31.12	< 0.001
	2	2	5	0	6	7.68		
	3	10	10	17	17	3.90		
	4	10	6	7	4	2.17		

Table 2. Contd.

	1	12	0	0	0	29.79		
Pod hardness	2	15	11	5	11	3.80	49.82	< 0.001
	3	2	10	19	16	16.23		
	1	11	0	0	0	27.31		
Seed shape	2	15	21	24	27	5.59	40.35	< 0.001
	3	3	0	0	0	7.45		

$\chi^2_{G} > \chi^2_{\alpha} = 7.81$ ($ddl= 3$) is significant at the level $\alpha = 0.05$ and indicates that column (i.e. zone) frequencies are significantly different for a particular phenotypic trait; $\chi^2_{Gk} > \chi^2_{\alpha} = 12.59$ ($ddl= 6$) or $\chi^2_{Gk} > \chi^2_{\alpha} = 16.92$ ($ddl= 9$) is significant at the level $\alpha = 0.05$ and indicates that zone frequencies are significantly independent for a particular trait.

presented here) with a preponderance of certain phenotypic traits. These results would undoubtedly be the consequence of anthropic pressure resulting from a localized propagation of certain types of Bambara groundnut seeds. But for some phenotypic classes, the difference of frequency distributions among the four collection zones could be attributed to the unequal sizes of samples of accessions used in this study (Bolboacă et al., 2011). For all material, a wide range of variation was highlighted by varied degrees of polymorphism and irregular frequency distributions of qualitative traits among the collection zones. Concerning seedling color, three colors were distinguishable in this work. Most of the accessions from the four agro-climatic zones were purplish red or green in seedling color, except one accession for each zone, which was dark green in seedling color. However, the green color had the highest frequency among accessions from northern zone while the purplish red color was the dominant one in the other zones.

It was found that accessions from each agro-climatic zone presented different expression for the examined qualitative traits associated with the leaves. Four different leaflet colors were found for all the zones, except the central zone where only green and light green leaflets were observed. However, the green leaflet was the most frequent in all zones, with a predominance in the western (80.95%), central (79.17%) and eastern (74.07%) zones. The dark green leaflet was more observed in the northern accessions than the central, western and eastern ones. The proportions of light green and yellowish green leaflets were relatively low among accessions from each agro-climatic zones studied.

The three phenotypic classes (soft, moderately soft and hard) for leaflet flexibility detected in this study were found among accessions from northern, western and eastern zones. Accessions of the central zone only had the soft leaflets. Soft leaflet was the dominant leaflet flexibility over all zones, unlike the moderately soft and hard leaflets which were less frequent.

The four leaflet shapes (round, oval, lanceolate and elliptic) were present in accessions taken from the northern zone while only two (oval and elliptic) and one (elliptic) were recorded among samples from western and eastern zones, respectively. The elliptic leaflet was the most frequent in all zones, with preponderance in accessions from central (100%) and eastern (96.3%) zones. The lanceolate and round leaflets were observed only in the northern zone. These two shapes may have evolved due to adaptation to the northern zone.

Green, light-green and pale-green colors were the petiole colors found among all samples. The light-green type was predominant in all agro-climatic zones (Table 2) followed by the green type. The pale-green type was less present in the northern zone and absent among accessions from the other zones.

Dark-green stem was predominant in the samples of each agro-climatic zone, followed by green stem. The pale-green stem was only observed among accessions from northern zone and was less frequent. Stem hairiness were classified as absent, sparse and dense. In all samples from the four agro-climatic zones, the dense type of stem hairiness was the most abundant. In this study, absent stem hairiness was limited to the northern zone whereas sparse stem hairiness was present in the other zones.

As noted earlier, the three types of growth habits (bunch, semi-bunch and spreading types) were present in accessions from northern, western and eastern zones but with a mix of different proportions. The sample of central zone contained only bunch and spreading types. The frequency distributions of the three phenotypes differed within and among the four agro-climatic zones. The majority of the accessions (21 out of 29) from the northern zone were the bunch type, while the semi-bunch and spreading types were less represented. The three other agro-climatic zones showed more or less the same trend of distribution for the three growth habits with a relatively high frequency for the spreading type followed by the bunch and semi-bunch types (Table 2).

Table 3. Percentage of Bambara groundnut accessions from four agroclimatic zones of Côte d'Ivoire by phenotypic classes for thirteen qualitative traits.

Qualitative trait		Agro-climatic zones			
		Northern	Western	Central	Eastern
Seedling color at emergence	1	34.48	57.14	54.17	51.85
	2	62.07	38.1	41.67	44.44
	3	3.45	4.76	4.17	3.7
Fully expanded leaflet color	1	34.48	4.76	0	3.7
	2	48.28	80.95	79.17	74.07
	3	6.9	4.76	20.83	18.52
	4	10.34	9.52	0	3.7
Leaflet flexibility	1	79.31	85.71	100	92.59
	2	10.34	4.76	0	3.7
	3	10.34	9.52	0	3.7
Leaflet shape	1	3.45	0	0	0
	2	34.48	33.33	0	3.7
	3	24.14	0	0	0
	4	37.93	66.67	100	96.3
Petiole color	1	27.59	19.05	20.83	22.22
	2	3.45	0	0	0
	3	68.97	80.95	79.17	77.78
Stem color	1	20.69	19.05	20.83	18.52
	2	6.9	0	0	0
	3	72.41	80.95	79.17	81.48
Stem hairyness	1	24.14	0	0	0
	2	0	4.76	4.17	3.7
	3	75.86	95.24	95.83	96.3
Growth habit	1	72.41	19.05	8.33	18.52
	2	24.14	4.76	0	3.7
	3	3.45	76.19	91.67	77.78
Pod shape	1	0	23.81	41.67	29.63
	2	96.55	76.19	58.33	70.37
	3	3.45	0	0	0
Pod color	1	3.45	0	8.33	0
	2	79.31	85.71	91.67	100
	3	0	14.29	0	0
	4	17.24	0	0	0
Pod texture	1	24.14	0	0	0
	2	6.9	23.81	0	22.22
	3	34.48	47.62	70.83	62.96
	4	34.48	28.57	29.17	14.81
Pod hardness	1	41.38	0	0	0
	2	51.72	52.38	20.83	40.74
	3	6.9	47.62	79.17	59.26
Seed shape	1	37.93	0	0	0
	2	51.72	100	100	100
	3	10.34	0	0	0

Lule et al. (2012) reported that besides genetic factors, edaphic factors or other environmental conditions can influence the adaptive role of some qualitative traits.

Therefore, it is probable that differences in the phenotypic expression of some qualitative traits across contrasted environments could be more or less induced by climatic factors. For example, the predominance of bunch type in the dry northern zone compared to the predominance of spreading type in the western, central and eastern zones indicates adaptive characters. It can be concluded that variation for growth is under the control of genetic and environmental factors. This could also be the case of other aerial traits such as leaflet shape and color.

The pods' qualitative traits showed divergences from one agro-climatic zone to the other. In this work, out of the three pod shapes identified, two were observed in each sample from the agro-climatic zones. Pods characterized by a rounded base with a point at the top were the most frequent in all four zones with predominance in accessions from the northern zone. The rounded pods without point were expressed only in few accessions from central, eastern and western zones (Table 2). Only one accession from the northern zone had elongated pods with rounded base and a point at the top.

Among the four pod colors, brown pods were the most frequent in the accessions of all collection zones (Table 2). Yellowish brown pods were present only among the accessions from the northern and central zones. Beige and light brown pods were present only in accessions from the northern and western zones, respectively.

All the four types of pod texture were present in the sample from the northern zone. Eastern and western zones comprised three types and the central zone, two types (Table 2). Wrinkled pods with more grooves were the most frequent among the accessions from central, eastern and western zones, whereas wrinkled and rough pods with more grooves were the most abundant among accessions from the northern zone. However, smooth pod was limited to the northern zone where pods with little grooves were less frequent.

In the central and eastern zones, most of the accessions had hard pods followed only by accessions showing moderately hard pods. Accessions collected from the northern zone produced all three textures of pods. But, the accessions with moderately hard pods were the most frequent, followed by the accessions with the soft and the hard pods. Only moderately hard and hard pods were found among accessions from central zone with equal proportions.

The three phenotypic classes of seed shape (round, oval and oblong) were observed among the accessions from the northern zone with the predominance of oval seed followed by round and oblong ones (Table 2). In the present study, round and oblong seeds were absent from all the accessions from central, eastern and western zones.

Estimates of Shannon-Weaver diversity

The estimates of the Shannon-Weaver diversity index (H') for the thirteen traits by agro-climatic zone are shown in Table 4. For all the accessions sampled, the mean value of H' varied from 0.21 for seed shape to 0.74 for seedling color with an overall mean of 0.46. The qualitative traits also showed different levels of diversity within and between the four agro-climatic zones. The northern zone presented the highest H' values for all traits, except for the seedling color and the pod shape, which were higher in the western ($H' = 0.71$) and the central ($H' = 0.62$) zones, respectively. Moreover, all traits appeared polymorphic in the northern zone. Conversely, a monomorphism ($H' = 0$) was recorded in three traits (leaflet flexibility, leaflet shape, and seed shape) for the central zone, two traits (pod color and seed shape) for the eastern zone and one trait (seed shape) for the western zone. According to Hammer et al. (1996), monomorphism of some traits occurring within a given zone could be either a drift or a loss of genetic integrity caused by selection forces.

A highly significant difference ($F = 5.06$; $P = 0.003$) of mean values of H' was depicted between the agro-climatic zones. The level of genetic diversity in northern zone ($H' = 0.66 \pm 0.21$) was high compared to the other zones. Relatively, intermediate values of H' were recorded for western ($H' = 0.46 \pm 0.21$) and eastern ($H' = 0.40 \pm 0.26$) zones, while the lowest was recorded for the central zone ($H' = 0.32 \pm 0.24$).

The Northern zone with the highest genetic diversity appeared genetically more diversified than the other zones. That is demonstrated by the specific phenotypic traits that it contained. Nevertheless, mean values of H' of the central, eastern and western zones appeared statistically identical (Table 4), suggesting a similar genetic diversity.

Bambara groundnut is a self-pollinated species. Therefore, the level of diversity found in each zone could result from farmers' agricultural practices and seed management methods such as recycling, sorting, exchange and new introductions. According to Alvarez et al., (2005), Robert et al. (2005) and Thomas et al. (2012), these factors may favor a diversifying selection leading to the maintenance, or even the creation of a significant morphological diversity. The overall species vitality and the potential for evolutionary responses to environmental change rely on the level of genetic variation within populations (Ellstrand and Elam, 1993). Based on this assumption, a particular attention should be given to all these Côte d'Ivoire's main growing-regions. The northern zone specially seems to be the appropriate region for maximum diversity and *in situ* genetic conservation of Bambara groundnut landraces.

Conclusion

The present study revealed high phenotypic variability of

Table 4. Estimates of the Shannon-Weaver diversity index (H') for thirteen qualitative traits in Bambara groundnut by agroclimatic zones.

Character	Agroclimatic zones				H' values
	Northern	Western	Central	Eastern	Mean ± SD
Seedling color	0.71	0.76	0.75	0.75	0.74 ± 0.02
Leaflet color	0.82	0.49	0.37	0.56	0.56 ± 0.19
Leaflet flexibility	0.59	0.46	0.00	0.29	0.33 ± 0.26
Leaflet shape	0.86	0.46	0.00	0.11	0.36 ± 0.39
Petiole color	0.66	0.44	0.47	0.48	0.51 ± 0.10
Stem color	0.68	0.44	0.47	0.44	0.51 ± 0.12
Stem hairyness	0.50	0.17	0.16	0.14	0.24 ± 0.17
Growth habit	0.63	0.61	0.26	0.57	0.52 ± 0.17
Pod shape	0.14	0.50	0.62	0.55	0.45 ± 0.22
Pod color	0.44	0.30	0.21	0.00	0.23 ± 0.18
Pod texture	0.91	0.76	0.44	0.66	0.69 ± 0.20
Pod hardness	0.81	0.63	0.47	0.62	0.63 ± 0.14
Seed shape	0.86	0.00	0.00	0.00	0.21 ± 0.43
Mean ± SD (H')	0.66 ± 0.21 ^a	0.46 ± 0.21 ^b	0.32 ± 0.24 ^b	0.40 ± 0.26 ^b	0.46 ± 0.17

Côte d'Ivoire local Bambara groundnut germplasm. This work indicated that accessions can be clustered into six groups based on the qualitative traits. The distribution of most of the phenotypic traits was related to agro-climatic zones and indicated the adaptive forms in the expression of these traits. In addition, highly significant difference of levels of diversity was found among the agro-climatic zones of origin. The results represent an important database for many purposes. Phenotypic traits could be useful tools for identifying varieties in breeding programs. Data could serve for future prospection and collection missions. They will largely determine the location of areas and conservation strategies, as well as the management of Bambara groundnut genetic resources in Côte d'Ivoire.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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