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ARTICLES

Full Length Research Paper

Assessment of feeding value of vegetable-carried pineapple fruit wastes to Red Sokoto goats in Ogbomoso, Oyo State of Nigeria

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This study compared the sun-drying characteristics of five blends each (w/w; 1:1, 1:1.5, 1:2, 1:2.5, 1:3) of wheat offal-carried pineapple waste (WO:PW) and brewers' dried grains-carried pineapple waste (BDG:PW), assessed the blends for their nutrient contents and the feeding value of the optimum blends with Red Sokoto (RS) goats. Moisture contents of all the blends were reduced to between 10.95 - 14.38% and 11.73 - 14.72%, respectively for WO:PW and BDG:PW blends within 7 h. Drying was observed to be optimum at 1WO:2PW and 1BDG:2PW and their respective proximate compositions suggest their potentials as an energy source and a protein source respectively in ruminant nutrition. Free choice intake, coefficient of preference and percentage preference of the optimum blends (1WO:2PW and 1BDG:2PW) and their respective equal mixtures (w/w) with a formulated conventional concentrate (CCON) by RS goats, were subsequently evaluated alongside the CCON in a cafeteria system. Results indicated that RS goats would opt for CCON in preference to other test feeds, but would readily accept WO-carried pineapple waste as an alternative to CCON.

Key words: Acceptability, feed processing, fruit-processing by-products, seasonal nutritional stress, small ruminants.

INTRODUCTION

Small ruminants represent between 63.7 and 75% of total grazing domestic livestock in Nigeria and are widely distributed in rural, urban and peri-urban areas (Ajala et al., 2008; FMA, 2008), hence their significance in livestock agriculture and human protein nutrition. The

Red Sokoto (RS) and West African Dwarf (WAD) goats are the two most important goat breeds in the country (Yakubu et al., 2010). Even though natural pastures provide what is regarded as the "cheapest" feed for ruminants (Akinrinde and Olanite, 2014), it has long been

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recognized that they are incapable of sustaining the animals on a year-round basis as they are often deficient in nutritional quality for most of the year. In Nigeria, as observed by Bamigboye et al. (2013), rangelands only blossom in the rainy season while in dry season, they become standing hay. Thus, animals will have abundant feed in the wet season and a shortage of feed in the dry season.

Considerable research has been carried out to improve the quality and availability of feed resources, including works on sown forages, forage conservation, the use of multi-purpose trees, fibrous crop residues and strategic supplementation (Thornton, 2010) with promising results, but also with their attendant limitations. Many reasons have been adduced for the general non-adoption of sown forages in Nigeria, the most important of which is that most of the people involved are resource-poor; hence, they lack the needed financial resources to embark on improved pasture production (Akinrinde and Olanite, 2014). Other reasons (Akinrinde and Olanite, 2014), include lack of technical skills to manage such a system and relatively low prices for animal products. Although adequate levels of nutrients are retained in conserved feeds to merit their use in dry periods, the nutritive qualities differ from those of fresh materials (Asaolu et al., 2015). In Nigeria, two exotic species, *Gliricidia sepium* and *Leucaena leucocephala*, have shown appreciable forage potentials among multipurpose trees (Odeyinka et al., 2003; Fadiyimu et al., 2014). They have however been observed to have difficulty in adapting the local environment and are susceptible to pests and diseases, such as psyllid epidemic (*Heteropsylla cubana*) in *L. leucocephala* (Baumer, 1992). Furthermore, most native species shed their leaves during the dry season and majority of them possess physical structures and anti-nutritive chemical compounds, such as tannins, saponins, cyanogens, mimosine and coumarins (Leng, 1997), which are said to protect them against herbivores (Coley et al., 1985), but could reduce their palatability as well as limit their nutrient availability and digestibility (Barry, 1989). Kalio et al. (2015) identified a number of constraints to the utilization of fibrous crop residues as feed resources, including the lack of knowledge of where the crop residues could be gathered in reasonable quantities, the seasonality of their production, their alternative uses as composting and mulching materials by most crop farmers, the difficulty and expense of collecting, handling and storing large quantities of these bulky crop by-products and the lack of knowledge of the nutritive value of the materials as feed resources for ruminant livestock. Livestock have historically utilized large amounts of well-known and widely-available traditional by-products such as oil meals, bran, middlings, brewers' grains, distillers' grains, beet pulp and molasses in strategic supplementation strategies (Mirzaei-Aghsaghali and Maheri-Sis, 2008). Unfortunately, these supplements are often not fed due to their unavailability

and high costs (Nouala et al., 2006). Additionally, as the world population increases relative to arable land, an increased demand for cereal grains and oilseed meals for direct use in human diets is expected in the long run (Knutson and Stoner, 2012). However, less conventional by-products have become available, such as vegetable- and fruit-processing residues, whey and culinary wastes (Mirzaei-Aghsaghali and Maheri-Sis, 2008). One of such by-products is pineapple waste.

Pineapple wastes, occurring mainly as pineapple peels and core (Buckle, 1989), are rich in fermentable sugars, organic acids, and fibre, have high digestibility potential (Jetana et al., 2009; Migwi et al., 2001). These characteristics make fruit by-products a potential feed resource for small ruminants (Pagan et al., 2014). Pineapple wastes account for approximately 40 to 50% of the fresh fruit weight (Buckle, 1989), and are mostly dumped with the attendant acceptable safe solid-waste disposal problems (Hepton and Hogson, 2003; Makinde et al., 2011). Incidentally, large quantities of fresh pineapple fruits are produced in Nigeria. Weight composition of a typical *Cayena lisa* pineapple is pulp (33%), core (6%), peel (41%) and crown (20%) (Medina and Garcia, 2005). Pineapple (*Ananas comosus*) is the third most important tropical fruit in the world after banana (Musa spp.) and Citrus spp. (Esiobu and Onubuogu, 2014). Nigeria is number six on the list of world pineapple producers (CADP Manuel, 2012), and the leading pineapple producer in Africa with an annual production of 1,400,000 metric tons (MT) of fresh pineapple (FAOSTAT, 2011). By extrapolation, Nigeria has a fresh pineapple waste generation potential of about 560,000 to 700,000 MT per annum. Such a huge yearly generation of residue constitutes a potential pollutant, and daily disposal of the residues is sure to increase the running cost of the fruit processing industry (Makinde and Sonaiya, 2007; Karkoodi et al., 2012). Fortunately, some research results have shown the benefits of utilizing such residues in ruminant feeding (Mokhtarpour, 1996). Feeding such residues to livestock has been considered not only to lessen environmental problems, diminish dependence of livestock on grains that can support human and eliminate the costly waste management programs (Grasser et al., 1995), but also to support sustainable development among the agricultural community (Suksathit et al., 2011).

Like other fresh fruit by-products, fresh pineapple cannery wastes are rich in water (about 90%) and soluble carbohydrates and decay very quickly (Ososanya et al., 2014). Therefore, there is the need for rapid utilization of the waste, but the canneries are often not located in areas of animal production and transportation of such bulky products is expensive and may require daily visits to the cannery (Nhan et al., 2009). Efforts toward proper processing and utilization of pineapple wastes by previous investigators in Nigeria involved sun-drying (Lamidi et al., 2008; Olosunde, 2010) and ensiling

(Ososanya et al., 2014), followed by the incorporation of the processed product in animal diets with satisfactory results (Makinde et al., 2011; Ososanya et al., 2014). Either way, the high moisture content of pineapple waste was a major problem. Drying which ought to be an easy way out has been reported to last between 5 and 14 days, depending on environmental conditions. Additionally, it reportedly (Ososanya et al., 2014) allows the soluble carbohydrates dispersed in water to be evaporated, hence, the need for the development of a quicker conversion method. Building upon earlier research efforts (Makinde and Sonaiya, 2007, 2010), it was found by Makinde et al. (2011) that pineapple wastes could be rapidly dried into a potential animal feed using wheat offal as a vegetable carrier/an absorbent.

The potential value of by-products in animal feeding depends on their nutritive characteristics and energy value with palatability also being an important feature (Mirzaei-Aghsaghali and Maheri-Sis, 2008). The significance of anti-oxidant contents of feed resources in the health and productivity management of livestock on an ecologically-sustainable basis (Shiau and Hsu, 2002) cannot be over-emphasized. This study was therefore designed to compare the sun-drying characteristics of different blends of pineapple waste with wheat offals and brewers' dried grains as moisture absorbents, assess these different blends for their proximate, fibre, mineral contents, and quantify the total polyphenols and other anti-oxidants present in the experimental pineapple waste. The intake and acceptability of the optimum blends of the two vegetable-carried pineapple wastes were also assessed relative to a conventional feed concentrate with Red Sokoto goats.

MATERIALS AND METHODS

Experimental site

The study was conducted during the dry season between February and March, 2015, at the Small Ruminant Unit (SRU) of Ladoke Akintola University of Technology Teaching and Research Farm (LAUTECH T&R F), Ogbomoso, Oyo State, located within the semi-arid zone where the major nutritional limitation of small ruminants is that of bridging the gap between wet and dry seasons (Onim et al., 1985). The area is located at 8°10' North latitude and 4°10' East longitude with annual rainfall of 1270 to 2030 mm, which occurs in 7 to 10 months with a peak between July and September of the year (Olaniyi, 2006). Ogbomoso is located within a 100-km radius of Ibadan, which is home to the Lafia Canning Factory of Fumman Agricultural Products Nigeria Ltd, Moor Plantation, Ibadan; one of the major generators of pineapple fruit wastes at commercial levels in Nigeria.

Procurement of fresh pineapple fruit waste, absorbents and other feed ingredients

Fresh wet pineapple wastes (the peelings and the pulp) were collected between 8.30 and 9.00 h from the Lafia Canning Factory of Fumman Agricultural Products Nigeria Ltd, Moor Plantation, Ibadan, Nigeria; and immediately transported to the SRU of

Table 1. Ingredient composition of a conventional feed concentrate for ruminant animal supplementation (Isah and Babayemi, 2010).

Ingredient	Levels (%)
Cassava peel	34.50
Wheat offal	24.00
Maize cob	15.00
Groundnut cake	10.00
Maize	6.00
Soybean	5.00
Bone meal	3.00
Oyster shell	2.00
Premix	0.25
Salt	0.25
Calculated analysis	
Crude protein (%)	15.92
Metabolizable energy (Mcal kg^{-1}DM)	2300.65

LAUTECH T& R F, Ogbomoso. The moisture absorbents, that is, wheat offals and brewers' dried grains, as well as other as other experimental feed ingredients were obtained from a reliable feed ingredient store in Ogbomoso.

Formulation of a conventional feed concentrate

The formula of Isah and Babayemi (2010) for a conventional feed concentrate, as shown in Table 1, was adopted.

Determination of sun-drying characteristics of different blends of wheat offal- and brewers' dried grains-carried pineapple wastes

Five blends each of wheat offal-carried pineapple waste (WO:PW) and brewers' dried grains-carried pineapple waste (BDG:PW) were prepared. The blends (w/w), with each blend made in triplicates, were: wheat offal:pineapple waste blends (a) WO mixed with PW (1:1), (b) WO mixed with PW (1:1.5), (c) WO mixed with PW (1:2), (d) WO mixed with PW (1:2.5), (e) WO mixed with PW (1:3) and brewers' dried grains:pineapple waste blends (a) BDG mixed with PW (1:1), (b) BDG mixed with PW (1:1.5), (c) BDG mixed with PW (1:2), (d) BDG mixed with PW (1:2.5), (e) BDG mixed with PW (1:3).

For each of the blends, PW was thoroughly mixed with WO or BDG by hand until the fluid from pineapple waste was not superfluous (Makinde and Sonaiya, 2007). The blends were evaluated on the capacity to sun-dry to ≤10 to 12% in 7 h; as moisture content >12% is not desirable pertaining to good keeping quality (Rozis, 1997). The protocol of Makinde and Sonaiya (2007, 2010) was adopted for the drying of the blends but with a modification of the drying period from 4 to 7 h as reported by Asaolu (2013). The different blends were sun-dried by spreading thinly on polythene sheets (0.7 mm thickness) on a concrete floor, with each replicate weighing an average of 0.20 kg and covering an area of 1.42 m^2. Drying started at about 11.30 h and the mixtures were turned at about the first hour into drying. They were also turned about mid-way into the drying process. This involved rubbing handfuls together and spreading again. After the drying period, the blends were sampled for moisture contents and degrees of wetness. Wetness of each blend was estimated as the difference

between its initial and final weight. The resultant blends were subsequently dried to constant moisture content and blended with a plate (burr) mill. Dried and cooled blends were stored in high-density polythene bags and then in a freezer for later chemical analyses.

Acceptability and preference evaluation of wheat offal- and brewers' dried grains-carried pineapple wastes relative to a conventional ruminant feed concentrate using Red Sokoto goats

The blends of WO and BDG with the highest PW contents (1WO:2PW and 1BDG:2PW), respectively that dried to ≤10 to 12% moisture content within 7 h were further produced and evaluated for free choice intake alongside equal mixtures of each blend with a formulated conventional concentrate (CCON as contained in Table 1); that is {50(1WO:2PW):50CCON} and {50(1BDG:2PW):50CCON}, respectively, relative to the sole conventional concentrate (CCON). Hence, there were five experimental supplements, namely, 1WO:2PW;   1BDG:2PW;   {50(1WO:2PW):50CCON}; {50(1BDG:2PW):50CCON} and CCON, with the CCON serving as the reference supplement. Ten matured Red Sokoto goats, weighing 12.3±1.69 kg were used in a cafeteria style. The animals were housed together in a free stall with dwarf walls and concrete floors covered with wood shavings. All the animals were pre-conditioned to the experimental supplements for a period of 4 days after which the animals were offered 4 kg each (wet basis) of experimental supplements daily for a period of 10 days. Each 4 kg-serving was simultaneously presented in two separate feeding troughs, thus making a total of ten feeding troughs at a time. The positions of the feeding troughs were randomly changed on a daily basis to avoid any of the animals associating a particular experimental supplement to a particular position. Fresh water was also offered daily on a free choice basis. Intake of supplements was measured 2 h after they were offered by deducting remnants from the amount served and animals were subsequently allowed to graze for the rest of the day.

Coefficient of preference (CoP) was used as an index of acceptability while percentage preference (PP) was used as a preference index. The CoP was calculated as the ratio of individual test supplement intake to average intake of all the supplements while PP was calculated as the ratio of individual intake to total intake multiplied by 100. Test supplements were considered acceptable when the CoP was greater than one while ranking was based on PP (Ososanya and Olorunnisomo, 2015).

Chemical analyses

Proximate analyses of all the test supplements were carried out according to the methods of AOAC (2000), while the fibre components were determined according to Van Soest et al. (1991). For mineral analysis, samples were dry-ashed at 550°C for 4 h, followed by wet digestion of the resulting ash. Calcium concentration was estimated by using the Jenway Digital Flame Photometer (PFP7 Model), while phosphorus and magnesium contents were estimated using the Atomic Absorption Spectrophotometer (model Bulk 221CGP).

Beta carotene content was determined as described by Rodriguez-Amaya and Kimura (2004) and AOAC (2000). Total anthocyanins were determined by the pH deferential method (Lee et al., 2005) using a spectrophotometer (Unicam UV/VIS ATI UNICAM, Cambridge, UK). Total polyphenols were determined by the Folin-Ciocalteu method (Makkar, 2003). Ascorbic acid was determined using 2,4,6-dichloroindositol method of AOAC (2000) while total sugars were measured using Spectrophotometric method of AOAC (2000). pH was measured by a pH meter (model:

PHS-25), and this was followed by the measurement of titratable acidity with NaOH as described by Garner et al. (No date) using the equation:

Titratable acidity = (ml of NaOH used) × (Normality of NaOH used) × MF × 100 / Weight of sample (g)

where MF = Milliequivalent factor, which is taken to be 0.067 for malic acid; the predominant acid in apples.

All analyses were done in triplicates.

Data analyses

The sun-drying characteristics data were analyzed with the 2-way analysis of variance using the General Linear Model (GLM) procedure of SAS (2001) in a completely randomized block design, with vegetable carrier as the main treatment effect and mixing ratios as the block. Significant differences between means were separated using the Duncan's New Multiple Range Test (DNMRT) of the same package. Data on acceptability and preference study were subjected to 1-way analysis of variance using the GLM of SAS (2001). Significant differences between means were also separated using the DNMRT of the same package.

RESULTS AND DISCUSSION

Chemical compositions of pineapple waste, wheat offals and brewers' dried grains

The chemical compositions of PW, WO and BDG are contained in Table 2. Wheat offals and BDG contained high dry matter contents while PW contained a considerable amount of moisture. Brewers' dried grains were relatively higher in crude protein and ether extract, respectively followed by WO and PW. Total ash was least for BDG and highest for WO. Crude fibre values appeared comparable for the three feedstuffs; while nitrogen free extract was the highest for PW followed by WO and BDG, respectively. Calcium and magnesium levels were highest in WO followed by BDG and PW, respectively. Wheat offals and BDG contained comparable amounts of phosphorus which were both higher than the level in PW. The proximate components of each of the three test feeds fell within the respective reported literature values (Wondifraw and Tamir, 2013; Hemalatha and Ambuselvi, 2013). Unlike BDG and WO, PW was observed to be very high in moisture content, which compares with 80% reported by Makinde et al. (2011). Hemalatha and Ambuselvi (2013) reported an even higher moisture value of 91.35%. Such high moisture contents make pineapple waste a highly perishable material. A low energy value could also be implied in this high moisture state (Muller and Tobin, 1980). In order to optimize the energy potential of this waste therefore, treatments to minimize its moisture content becomes expedient. Additionally, its shelf life will be significantly increased. Only BDG contained a crude protein content that is higher than the range of 15 to 18% requirement for growing lambs (Aruwayo et al., 2009). Brewers' dried grains have been reported (Wondifraw

Table 2. Chemical compositions of pineapple waste, wheat offals and brewers' dried grains.

Parameter	Fresh pineapple waste	Wheat offals	Brewers' dried grains
Dry matter	21.84	89.99	90.10
% of DM			
Crude protein	5.08	13.76	31.08
Ether extract	1.18	4.65	8.20
Ash	6.21	9.43	4.88
Crude fibre	11.57	16.45	13.10
Nitrogen free extract	67.00	55.72	42.75
Calcium	0.18	0.35	0.20
Phosphorus	0.10	0.50	0.58
Magnesium	0.09	0.38	0.19
Total sugars	26.28	Nd	Nd
Total polyphenols	0.60	Nd	Nd
Total anthocyanins	0.05	Nd	Nd
Total titratable acidity	0.71	Nd	Nd
Vitamin C (mg/100g)	7.42	Nd	Nd
β-Carotene (μg/100g)	378.59	Nd	Nd
pH	4.40	Nd	Nd

nd: Not determined.

and Tamir, 2013) as valuable sources of crude protein, metabolizable energy, many of the B-vitamins, phosphorus but relatively low in calcium. They are also considered as good sources of rumen undegradable protein, fibre and water-soluble vitamins (Westendorf and Wohlt, 2002; Vasso and Winfried, 2007). The crude protein content of WO was however higher than the range of 11.00 to 13.00% known to be capable of supplying adequate protein for maintenance and moderate growth performances in goats (NRC, 1981), while PW contained even less than range of 7.00 to 8.00% recommended for efficient functioning of rumen microorganisms (Van Soest, 1994). The observed crude protein was comparable to the value of 5.11% (Adeyemi et al., 2010) for pineapple peels alone, but higher than the value of 3.69% reported by Omwago et al. (2013) for the waste. It was however lower than 6.12% reported by Aboh et al. (2013) for pineapple peels. With the crude fibre content observed in this study, Omole et al. (2011) opined that PW could be a veritable source of fibre in livestock diets. Ether extract and ash values as obtained in this study have earlier been described (Omole et al., 2011) as low and rich, respectively. The nitrogen free extract values reflect higher concentration of energy in PW than in WO and BDG in that order. Aside from compositional differences between the wastes, Aboh et al. (2013) attributed the observed chemical variations in PW to pineapple varietal differences and supply of fertilizers.

Pineapple waste was observed to be acidic with moderately low pH, titratable acidity, total polyphenols and anthocyanidins, but relatively high contents of ascorbic acid (vitamin C), total sugars and β-carotene. A pH range of 2.5 to 7.0 has been reported (Ambuselvi and Muthumani, 2014), implying that pineapple wastes are usually acidic in nature. Hemalatha and Ambuselvi (2013) reported a titratable acidity of 1.86% for PW while the value for the whole fruit ranged from 0.80 to 1.50%. Titratable acidity levels as observed in this study have been described (Hemalatha and Ambuselvi, 2013) as moderate. The acidity found in the pineapple and citrus by-products is typical of ripen fruits and results from the presence of organic acids, mainly citric, malic, ascorbic and tartaric (Falade et al., 2003). The ascorbic acid content reported for PW in this study was higher than the range of 2.50 to 3.50 mg/100 g reported by Ambuselvi and Muthumani (2014). Vitamin C has been described (Adebowale et al., 2011) as an indispensable and multifunctional micronutrient substance that is essential in minute amounts for the proper growth and metabolism of a living organism. It is the body's primary water soluble antioxidant against free radicals that attack and damage normal cells (Hossain et al., 2015). A powerful antioxidant, vitamin C supports the formation of collagen in bones, blood vessels, cartilage and muscle, as well as the absorption of iron. Vitamin C also retards the development of urinary tract infections during pregnancy (Debnath et al., 2012). Sugar is a major biochemical component of pineapple fruit and its concentration will determine the quality of the fruit (Siti Roha et al., 2013). This assertion could rightly apply to the wastes of the fruit as Correia et al. (2004) reported that PW still retains a

Table 3. Drying characteristics of different blends of wheat offal-carried pineapple waste (WO:PW) and brewers' dried grains-carried pineapple waste (BDG:PW).

Mixing ratio	Vegetable carrier/SEM	Wetness	Moisture
	WO:PW	0.06	10.95
1:1	BDG:PW	0.13	11.61
	SEM	0.01	0.07
	WO:PW	0.17	11.59
1:1.5	BDG:PW	0.18	11.73
	SEM	0.01	0.17
	WO:PW	0.22	11.88
1:2.	BDG:PW	0.23	11.90
	SEM	0.01	0.12
	WO:PW	0.27	14.36
1:2.5	BDG:PW	0.28	14.50
	SEM	0.01	0.05
	WO:PW	0.33	14.38
1:3	BDG:PW	0.38	14.72
	SEM	0.01	0.18

[abc]Triplicate mean values in each column for the same parameter at different mixing ratios with different superscripts are significantly different at $P<0.05$. Drying surface temperature range = 45 to 52°C; Ambient temperature range = 32 to 35°C; Fresh pineapple waste average % moisture = 78.16%, WO: Wheat offals; BDG: brewers' dried grains; PW: pineapple waste, SEM: standard error of the mean.

considerable amount of sugars that are contained within the fruit. The sugar content of PW was detected by Siti Roha et al. (2013) to comprise of fructose, glucose and sucrose, with sucrose being the main sugar. Of the three major components of PW (crown, peels and core), pineapple core extract has been found (Siti Roha et al., 2013) to have the highest amounts of the three sugars, irrespective of the stage of maturity. It was further reported by Siti Roha et al. (2013) that sucrose is the major sugar found in pineapple core and peel extracts. Hence, sucrose is likely to constitute the bulk of the sugars that were detected in this study. Muller (1978) reported that PW, because of its high sugar content, has long been exploited in cattle rations as a source of readily available carbohydrates. These sugars, which are principally non-reducing in nature, in association with other carbohydrates and proteins, are used as a nutrient medium for growth of microbes and fermentation using yeast to produce ethanol and single cell protein (Hemalatha and Ambuselvi, 2013). The experimental PW was also very high in provitamin A (β-Carotene) relative to the values reported by Nzeagwu and Onimawo (2010) for the popular black currant drink (1.24 mg/100 g) and juice made from *Eugenia uniflora* L. (pitanga) fruits (15.85 mg/100 g), although lower than the value of 926.55 μg/100 g reported by Asaolu (2013) for cashew apple.

This suggests that PW may probably be a very good source of provitamin A. Beta-carotene has been described as the carotenoid with the most vitamin A activity, and because of its chemical nature, it has been suggested that β-carotene may be an antioxidant within tissues protecting them from damage from free radicals (Wardlaw et al., 2004). The whole pineapple fruit is known to be high in both vitamin C and vitamin A (Joy, 2010). Even though apparently low total polyphenols and total anthocyanins were detected in this study, high amounts of phenolic compounds have been reported (Rudra et al., 2015) in PW with high antioxidant activities.

Sun-drying characteristics of different blends of wheat offal-carried pineapple waste and brewers' dried grains-carried pineapple waste

The sun-drying characteristics of the different blends of WO:PW and BDG:PW after a 7 h drying period, and the observed effects of the two vegetable carriers and different mixing ratios on these characteristics are shown in Tables 3, 4 and 5 respectively. In contrast to the reports of Lamidi et al. (2008) and Olosunde (2010) where sun drying-periods for pineapple waste ranged between 5 and 14 days, the three tables show that sun-

Table 4. Effects of vegetable carriers on the wetness and moisture contents of different blends of wheat offal-carried pineapple waste (WO:PW) and brewers' dried grains-carried pineapple waste (BDG:PW).

Vegetable carrier	Wetness (kg)	Moisture (%)
Brewers' dried grains	0.23	12.89[a]
Wheat offals	0.22	12.63[b]
SEM	0.02	0.27

[abc]Mean values in each column for the same parameter with different superscripts are significantly different at P<0.05. WO: Wheat offals; BDG: brewers' dried grains; PW: pineapple waste, SEM: standard error of the mean.

Table 5. Effects of mixing ratios on the wetness and moisture contents of different blends of wheat offal-carried pineapple waste (WO:PW) and brewers' dried grains-carried pineapple waste (BDG:PW).

Mixing ratio	Wetness (kg)	Moisture (%)
1:1	0.09[e]	11.34[c]
1:1.5	0.18[d]	11.50[bc]
1:2	0.22[c]	11.89[b]
1:2.5	0.28[b]	14.43[a]
1:3	0.35[a]	14.55[a]
SEM	0.01	0.11

[abc]Mean values in each column for the same parameter with different superscripts are significantly different at P<0.05. WO: Wheat offals; BDG: brewers' dried grains; PW: pineapple waste, SEM: standard error of the mean.

drying period in this study was significantly reduced. This observation compares with the report of Asaolu (2013) with fresh cashew apples, but the drying period was slightly longer than what was reported by Makinde et al. (2011) for pineapple waste with wheat offal. Shorter drying times have been attributed (Sonaiya, 1988; Rozis, 1997) to increased air to product surface exchange area. Table 4 shows that while there was no vegetable carrier effect (P>0.05) on the wetness of the vegetable-carried feedstuffs, it exerted a significant (P<0.05) effect on the moisture contents of these vegetable-carried feedstuffs, with the BDG-carried pineapple wastes having higher moisture contents. This is in agreement with the findings of Makinde and Sonaiya (2007) that BDG absorbed more water than WO but had lower water absorbency than WO. These observations were found to conform to the general observation that lower bulk density feedstuffs have higher water holding capacities (Sundu et al., 2005). Makinde and Sonaiya (2007) reported that BDG had a higher bulk density than WO. Mixing ratio was however observed to have significant (P<0.05) effects on both wetness and moisture contents of the two vegetable-carried feedstuffs (Table 5). Wetness and moisture levels

were observed to increase with increasing levels of fresh PW in the different blends. Blends with PW of 2.5 and 3.0 parts had comparable (P>0.05) moisture levels, but their moisture levels were significantly (P<0.05) higher than in blends containing ≤2.0 parts of PW. In line with the criterion of selecting blends that dried to ≤10 to 12% moisture in 7 h, the blend ratio with PW of 2.0 parts appeared to be the optimum for both WO and BDG to effectively dry PW, with an edge in favour of WO in view of the effect of vegetable carrier on moisture content as observed in Table 4. This confirms in part the report of Makinde et al. (2011) on the optimum drying combination of WO and PW. The high moisture level of fresh PW would most likely have been responsible for the observed trend of the effect of mixing ratio on wetness and moisture levels of the different blends. It must have exerted a high pressure by increasing the absolute amount of water (g of water) that had to be absorbed by each vegetable carrier and the corresponding absorbency (g of water/g of feed) at every higher level of PW.

Nutrient compositions of different blends of wheat offal- and brewers' dried grains-carried pineapple wastes

Tables 6, 7 and 8 show the nutrient compositions of the different blends of WO- and BDG-carried PW and the effects of the two vegetable carriers as well as the mixing ratios of these vegetable carriers with PW on the chemical compositions of the resultant blends. It can be seen from Table 6 that dry matter contents of the blends were not significantly affected (P>0.05) by the vegetable carriers at each mixing ratio, although the WO:PW blends contained slightly higher dry matter contents. The table further shows that BDG-containing blends contained significantly higher (P<0.05) levels of crude protein and ether extract at each mixing ratio while the reverse was observed with crude fibre, ash, nitrogen free extract and the analyzed fibre components. In summary, the two vegetable carriers had no significant (P>0.05) effect on the dry matter contents of the different blends of WO- and BDG-carried PW while significant (P<0.05) effects were observed in the trends for other analyzed nutrients (Table 7). These trends were as observed in Table 6. The mixing ratios of the two vegetable carriers with PW were however observed to exert significant (P<0.05) effects on all the nutrient parameters that were measured (Table 8). It can be seen from the table that with the exception of nitrogen free extract, the dry matter and all the other nutrient contents that were measured were observed to decrease significantly (P<0.05) with increasing levels of PW in the different blends. On the contrary, however, the nitrogen free extract values increased with increasing levels of pineapple waste, implying higher energy concentrations of the blends with increasing pineapple

Table 6. Nutrient compositions of different blends of wheat offal- and brewers' dried grains-carried pineapple wastes.

Mixing ratio	Veg. C./SEM	DM	CP	EE	CF	ASH	NFE	NDF	ADF	ADL
	WO:PW	89.05	11.91[b]	3.42[b]	13.54[a]	6.77[a]	53.42[a]	46.15[a]	29.76[a]	11.57[a]
1:1	BDG:PW	88.27	22.73[a]	6.01[a]	9.32[b]	4.85[b]	45.37[b]	35.08[b]	21.70[b]	6.16[b]
	SEM	0.07	0.09	0.04	0.11	0.03	0.09	0.10	0.06	0.07
	WO:PW	88.41	9.86[b]	2.60[b]	12.09[a]	6.07[a]	57.51[a]	44.22[a]	28.82[a]	10.64[a]
1:1.5	BDG:PW	88.13	22.06[a]	5.67[a]	9.51[b]	4.54[b]	46.62[b]	33.60[b]	21.09[b]	5.67[b]
	SEM	0.17	0.24	0.05	0.18	0.05	0.43	0.39	0.08	0.05
	WO:PW	88.13	9.15[b]	2.50[b]	11.75[a]	5.90[a]	57.75[a]	43.50[a]	28.10[a]	10.44[a]
1:2	BDG:PW	88.10	20.75[a]	5.33[a]	9.15[b]	4.24[b]	48.65[b]	33.38[b]	20.30[b]	5.09[b]
	SEM	0.12	0.73	0.01	0.19	0.06	0.47	0.11	0.10	0.05
	WO:PW	85.64	8.48[b]	2.36[b]	11.22[a]	5.66[a]	57.93[a]	42.72[a]	27.71[a]	10.13[a]
1:2.5	BDG:PW	85.50	19.47[a]	4.92[a]	8.60[b]	3.89[b]	48.62[b]	32.89[b]	19.38[b]	4.70[b]
	SEM	0.05	0.10	0.04	0.04	0.04	0.16	0.06	0.09	0.05
	WO:PW	85.62	7.53[b]	2.09[b]	10.58[a]	5.32[a]	60.11[a]	42.11[a]	26.76[a]	9.34[a]
1:3	BDG:PW	85.28	18.91[a]	4.50[a]	8.24[b]	3.51[b]	50.12[b]	32.28[b]	18.68[b]	4.11[b]
	SEM	0.18	0.17	0.05	0.11	0.05	0.16	0.10	0.11	0.07

[abc]Mean values in each column for the same parameter at different mixing ratios with different superscripts are significantly different at $P<0.05$, Veg. C. = Vegetable carrier, SEM = Standard Error of the Mean, DM = Dry Matter, CP = Crude Protein, EE = Ether Extract, CF = Crude Fibre, NFE = Nitrogen Free Extract, NDF = Neutral Detergent Fibre, ADF = Acid Detergent Fibre, ADL = Acid Detergent Lignin, WO = Wheat Offal, PW = Fresh Pineapple Waste.

Table 8. Effects of vegetable carriers on the nutrient compositions of different blends of wheat offal-carried pineapple waste (WO:PW) and brewers' dried grains-carried pineapple waste (BDG:PW).

Veg. C	DM	CP	EE	CF	ASH	NFE	NDF	ADF	ADL
WO	87.37	9.82[b]	2.70[b]	12.11[a]	6.06[a]	56.69[a]	44.11[a]	28.46[a]	10.46[a]
BDG	87.11	20.78[a]	5.28[a]	8.96[b]	4.21[b]	47.88[b]	33.45[b]	20.23[b]	5.14[b]
SEM	0.07	0.08	0.02	0.06	0.20	0.12	0.10	0.04	0.03

[abc]Mean values in each column for the same parameter at different mixing ratios with different superscripts are significantly different at $P<0.05$. Veg. C.: Vegetable carrier, SEM: standard error of the mean, DM: dry matter, CP: crude protein, EE: ether extract, CF = crude fibre, NFE: nitrogen free extract, NDF: neutral detergent fibre, ADF: acid detergent fibre, ADL: acid detergent lignin, WO: wheat offal, PW: fresh pineapple waste.

Table 8. Effects of mixing ratios on the nutrient compositions of different blends of wheat offal-carried pineapple waste (WO:PW) and brewers' dried grains-carried pineapple waste (BDG:PW).

Mixing ratio	DM	CP	EE	CF	ASH	NFE	NDF	ADF	ADL
1:1	88.66[a]	17.32[a]	4.71[a]	11.43[a]	5.81[a]	49.40[d]	40.62[a]	25.73[a]	8.86[a]
1:1.5	88.40[ab]	16.69[b]	4.35[b]	11.31[a]	5.51[b]	50.54[c]	39.38[b]	25.17[b]	8.40[b]
1:2	88.11[b]	15.30[c]	3.96[c]	10.62[b]	5.16[c]	53.08[b]	38.91[c]	24.56[c]	7.86[c]
1:2.5	85.57[c]	13.97[d]	3.64[d]	9.91[c]	4.78[d]	53.27[b]	37.80[d]	23.55[c]	7.41[d]
1:3	85.45[c]	13.22[c]	3.30[e]	9.41[d]	4.41[e]	55.11[a]	37.20[d]	22.72[d]	6.73[e]
SEM	0.12	0.13	0.03	0.09	0.03	0.20	0.16	0.07	0.04

[abc]Mean values in each column for the same parameter with different superscripts are significantly different at $P<0.05$. DM: Dry matter, CP: crude protein, EE: ether extract, CF: crude fibre, NFE: nitrogen free extract, NDF: neutral detergent fibre, ADF: acid detergent fibre, ADL: acid detergent lignin, WO: wheat offal, PW: fresh pineapple waste, SEM: standard error of the mean.

waste levels. All the observations on the trends of nutrient contents of the vegetable-carried PW could be attributed to the nutrient compositions of the individual feed ingredients as contained in Table 2. Although WO and BDG had comparable dry matter contents, BDG were comparatively higher in crude protein and ether extract but lower in ash and crude fibre contents. The increasing concentration of nitrogen free extract in the different blends of vegetable-carried PW with increasing proportion of PW would most likely have been due to the higher concentration of nitrogen free extract in PW relative to both WO and BDG.

The ADF and NDF contents of all the vegetable carried-PW blends at the various mixing ratios (Table 6) were low to moderate when compared with low quality roughages which ruminants effectively degrade (Okoli et al., 2003). NDF is a measure of cellulose, hemicellulose, and lignin fractions of feeds while the ADF fraction includes cellulose and lignin as the primary components (Mirzaei-Aghsaghali and Maheri-Sis, 2011). NDF is more highly correlated with feed volume and chewing activity than ADF or CF (Coppock, 1987; Varga et al., 1998). The NDF concentration could be affected by several factors such as temperature, light intensity, water availability, latitude, maturity, and harvesting and storage methods (Van Soest, 1994). On the other hand, concentrations of ADF and lignin are correlated more with digestibility (Mirzaei-Aghsaghali and Maheri-Sis, 2008). Many factors influence the relationship between ADF and digestibility, including forage variety, maturity at harvest, and storage conditions (Van Soest, 1965; Varga et al., 1998). Non-cell wall components are not influenced by lignin, but they can often be highly correlated. Therefore, lignin concentration affects mainly the availability of cell wall polysaccharides (Van Soest, 1994). According to Nagadi et al. (2000), degradability of cell wall carbohydrates is mainly limited by lignin content, accentuating its influence on feed utilization (Ahmad and Wilman, 2001). Bosch and Bruining (1995) confirmed that grass silages, with high lignin content, have a lower digestibility compared to silages that contained low levels of lignin. A possibility therefore exists of BDG-carried PW having higher digestibility values as a result of their significantly lower (P<0.05) ADL concentrations than WO:PW.

Ash and Norton (1987) demonstrated the dependence of maintenance and weight gain by goats on the protein and energy contents of feeds. The National Research Council (NRC), Animal Nutrition Sub-Committee on Feed Composition (Ash and Norton, 1987), classified feedstuffs containing averagely less than 18% CF and less than 20% CP on a dry matter basis as energy concentrates while those containing at least 20% crude protein were classified as protein sources. Based on these criteria, the selected blends of WO and BDG with PW of 2.0 parts could be regarded as an energy source and a protein source, respectively. Even though the selected wheat offal-carried pineapple waste (1WO:2PW) could be

regarded as an energy source, its crude protein level was higher than the recommended level by ARC (1980) and NRC (1985) for optimum microbial gut activities. It was also higher than the minimum 7% dietary level that is needed to meet the maintenance protein requirements of a mature cow (Hersom, 2012), and probably the requirements of cattle at various production stages at appropriate inclusion levels (Asaolu, 2013). Hence, in addition to being an energy source, 1WO:2PW could possibly meet ruminants' crude protein requirements. Gatenby (2002) however indicated that the ARC (1980) and NRC (1985) levels are too low, with a suggestion that 10 to 12% crude protein in the diet is necessary for better production in ruminants. The crude protein content of the selected BDG-carried pineapple waste (1BDG:2PW) not only met all the minimum requirements for ruminants, but was higher than the suggested level of 10 to 12% by Gatenby (2002) for better production by ruminants and also exceeded the range of 11.00 to 13.00% known to be capable of supplying adequate protein for maintenance and moderate growth in goats (NRC, 1981).

Acceptability and preference ranking of wheat offal- and brewers' dried grains-carried pineapple wastes by Red Sokoto goats

Cafeteria techniques, one of which was adopted for this study, have been used over time (Bamikole et al., 2004; Babayemi, 2007; Babayemi et al., 2009; Olorunnisomo and Fayomi, 2012; Ososanya and Olorunnisomo, 2015; Akinwande et al., 2015) to assess the acceptability of various feeds by ruminants. In this study, it can be inferred from Table 9 that Red Sokoto goats would accept CCON, 1WO:2PW and {50(1WO:2PW):50CCON} as the CoP of each of these supplements was greater than one (Ososanya and Olorunnisomo, 2015). Using the same criterion, the animals would be expected to reject the other two supplements, that is, the BDG-carried pineapple waste [1BDG:2PW] and its equal mixture with the formulated conventional concentrate [{50(1BDG:2PW):50CCON}]. However, it had been noted in some previous studies (Olorunnisomo and Fayomi, 2012; Ososanya and Olorunnisomo, 2015) that CoP may not be a realistic measure of acceptability of diets by ruminants since it does not take into consideration the previous experience of the animals or the importance of changing dietary preference of animals. On the other hand, percentage preference (PP) appears to be a more realistic index of acceptability since it does not foreclose the possibility of changing dietary preference among livestock (Ososanya and Olorunnisomo, 2015). With the range of PP values observed in this study as indices, the experimental supplements could be said to be preferred in the following order; CCON> {50(1WO:2PW):50CCON}> 1WO:2PW> {50(1BDG:2PW):50CCON}> 1BDG:2PW. In other words, the animals would opt for the conventional

Table 9. Acceptability and preference of Red Sokoto goats fed ratio one to two blends of wheat offal- and brewers' dried grains-carried pineapple waste (1WO:2PW and 1BDG:2PW), a sole conventional concentrate (CCON), and equal mixtures of each blend with the conventional concentrate{50(1WO:2PW):50CCON} and {50(1BDG:2PW):50CCON}.

Test supplement	Intake (kg, DM)	Coefficient of preference (CoP)	Percent preference (PP)	Preference ranking
1WO:2PW	0.82[bc]	1.10[bc]	20.85[bc]	3rd
1BDG:2PW	0.23[d]	0.30[d]	6.03[d]	5th
CCON	1.20[a]	1.52[a]	28.83[a]	1st
{50(1WO:2PW):50CCON}	1.06[ab]	1.35[ab]	26.61[ab]	2nd
{50(1BDG:2PW):50CCON}	0.57[c]	0.73[c]	16.73[c]	4th
SEM	0.11	0.13	2.89	-

[abc]Mean values in each column for the same parameter at different mixing ratios with different superscripts are significantly different at $P<0.05$.

concentrate in preference to any of the two vegetable-carried pineapple wastes. This is quite understandable and corroborates an earlier finding (Ikhimioya and Imasuen, 2007) that small ruminants would readily accept diets with which they have had previous experience. However, the levels of interaction between the values of PP for CCON, {50(1WO:2PW):50CCON} and 1WO:2PW (28.83[a], 26.61[ab] and 20.85[bc] %, respectively) indicate that the wheat offal-carried pineapple wastes would be more readily accepted by the animals as alternatives to CCON if and when necessary, with PP values of 16.73[c] and 6.03[d] %. The supplement 1BDG:2PW could even be regarded as a no-option for RS goats with such a very low (6.03%) percentage preference. Although palatable and readily consumed when in good condition (Heuzé et al., 2015), it is necessary to dry BDG so that they do not contain more than 10% moisture when they are intended for long storage (Boessinger et al., 2005). The moisture content of 1BDG:2PW was however greater than the 10% threshold level. Feed mixtures containing BDG spoil quite rapidly (Gohl, 1982), and the palatability decreases with storage time (Heuzé et al., 2015), particularly when stored at higher than 10% moisture levels (Boessinger et al., 2005) for more than 2 to 5 days in warm temperatures (Amaral-Phillips and Hemken, 2002; Thomas et al., 2010). The palatability and hence the acceptability, of the BDG-carried PW that were assessed in this study could have been negatively affected by the rather longer storage period under the prevailing ambient temperature at the experimental site.

CONCLUSION AND RECOMMENDATIONS

The nutritional potentials of pineapple waste as a source of energy, fibre and antioxidants in ruminant nutrition were highlighted by the results of this study. The major limitation to its utilization; high moisture content, was also highlighted. However, the blend ratio with pineapple waste of 2.0 parts was optimum for both wheat offals and brewers' dried grains to effectively dry pineapple waste for possible incorporation into a feed, with an edge in

favour of wheat offals. The proximate compositions of the selected blends of wheat offal and brewers' dried grains with pineapple waste of 2.0 parts suggest their potentials as an energy source and a protein source respectively in ruminant nutrition. This would go a long way in achieving the twin-objective of addressing the waste disposal problem associated with pineapple waste and the nutritional stress commonly experienced by ruminants in developing countries, particularly during the long periods of dry season when protein and energy deficits in feed supply are most pronounced. Fortunately, acceptability results indicated that Red Sokoto goats would readily accept wheat offal-carried pineapple wastes as alternatives to conventional concentrates that are commonly used in supplementing ruminant diets but acceptability problems were recorded with brewers' dried grains-carried pineapple wastes. These problems arose most likely from the length and conditions of storage. Further studies to address the observed problems with brewers' dried grains-carried pineapple wastes are recommended while investigations into the incorporation of wheat offal-carried pineapple wastes into practical production diets for all classes of ruminants could commence.

Conflict of Interests

The authors have not declared any conflict of interests.

Abbreviations

WO, Wheat offal; **PW,** fresh pineapple waste; **BDG,** brewers' dried grains; **BDG:PW,** brewers' dried grains-

carried pineapple waste; **WO:PO,** wheat offal-carried pineapple waste; **CCON,** conventional concentrate; **RS,** Red Sokoto; **WAD,** West African Dwarf; **NRC,** National Research Council; **PP,** percentage preference; **CoP,** coefficient of preference.

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Full Length Research Paper

Isolation and characterization of two malathion-degrading *Pseudomonas* sp. in Egypt

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Pseudomonas aeruginosa and ***Pseudomonas mendocina*** degrading malathion were studied. Morphological, biochemical and 16S rRNA genes for bacterial identification were selected. Biodegradation of some organophosphorus compounds with the 2 bacterial isolates was determined by high performance liquid chromatography (HPLC). *P. aeruginosa* strain completely removed diazinon, malathion and fenitrothion, but not chlorpyrifos within 14 days. *P. mendocina* strain was not able to degrade malathion, diazinon and chlorpyrifos completely and no significant degradation for chlorpyrifos. The bacterial growth curve showed a steady increase in the two bacterial isolates masses during malathion degradation. The highest growth rates were with yeast extract, glucose and citrate for the 2 isolates, but not with phenol. Shaked high inoculum density with incubation at 30°C of malathion bacterial cultures were found to be the optimum conditions for malathion degradation. Bacterial culture extracts subjected to liquid chromatography/mass spectrometry (LC/MS) analysis revealed that the separated products were malathion monocarboxylic acid and malathion dicarboxylic acid. Molecular characterization of carboxylesterase enzyme revealed that carboxylesterase amino acid sequences of the 2 isolates showed high identity to other carboxylesterase enzymes of *P. aeruginosa* and *P. mendocina,* respectively. Phylogenetic analysis showed that *P. aeruginosa* was localized in a separate branch from other carboxylesterase producing *Pseudomonas* sp. So, it is suggested that this enzyme is a novel esterase enzyme. Use of pesticide-degrading microbial systems for removal of organophosphorus compounds from the contaminated sites requires an understanding of ecological requirements of degrading strains. The results provided an important insight into determining the bioremediation potential of both strains. But the mentioned bacteria cannot be the aim of bioremediation due to risk of public health hazard, hence these bacteria cannot be used in bioremediation but their purified enzymes could.

Key words: Biodegradation, carboxylestrase, organopgosphorus pesticides, *Pseudomonas aeruginosa*, *Pseudomonas mendocina*.

INTRODUCTION

Organophosphorus compounds (OPCs) have been used worldwide as plasticizers, petroleum additives and as pesticides since the Second World War. Although, OPCs are biodegradable, they are highly toxic to mammals and other non-target animals (Ragnarsdottir, 2000). Organophosphorus compounds inhibit the acetylcholinestrase resulting in the over-stimulation of acetylcholine receptors in the synapses (Karalliedde and

Senanayake, 1988). Overstimulation of acetylcholine receptors at the cholinergic synapses of autonomic, central nervous systems and neuromuscular junctions causes agitation, hypersalivation, confusion, convulsion, respiratory failure and finally, death (Eddleston et al., 2008).

Biodegradation is one of the natural processes that help to remove chemicals from the environment by microorganisms (Singh et al., 2004; Zhao et al., 2009). Most microorganisms, especially bacteria, have detoxifying abilities (transformation, mineralization and/or immobilization of the pollutants) and also play a crucial role in biogeochemical cycles for sustainable development of the biosphere. Organophosphorous compounds are esters; the principal reactions that occur are hydrolysis, oxidation, alkylation and dealkylation. Hydrolysis of P-O-alkyl and P-O-aryl bonds is considered the significant step in microbial detoxification. Hydrolysis of OPCs greatly reduces their mammalian toxicity (Kaneva et al., 1998).

Malathion is a wide spectrum OPC used in public health and agricultural setting. Due to extensive use of malathion, exposure risk of living organisms including human beings is very high. Malathion degradation by microorganisms has been reported by several items (Goda et al., 2010; Singh et al., 2012; Janeczko et al., 2014).

Co-metabolism is another special phenomenon; it occurs widely in microbial metabolism. Here, the microorganisms transform the desired toxicant compound even though the compound itself cannot work as the primary source of energy for those organisms. To degrade the xenobiotic, the microbes need the presence of primary substrates that can support their growth. The enzymes or coenzymes made to mineralize the primary substrate may possess some activity for other substrate that is significantly known as co-substrate (Karpouzas and Walker, 2000).

There are different types of enzymes responsible for the degradation of OPCs, the most important of them are phosphotriesterases and carboxylesterases (Goda et al., 2010). Carboxylesterases are enzymes found in the α/β-hydrolase fold family that catalyze the hydrolysis of carboxyl esters via the addition of water (Hosokawa et al., 2007). Several researchers isolated and fully characterized carboxylesterase enzymes recovered from different microorganisms (Hosokawa et al. 2007; Li et al., 2007; Goda et al., 2010). Li and colleagues (2007) were able to fuse carboxylesterase enzyme with a green fluorescent protein that made their organism easily detectable in the environment following the degradation of pesticide residues. The main objective of the present study was isolation and molecular identification of

bacterial strains capable of degrading some organophosphorus pesticides particularly, malathion and factors affecting its biodegradation. Molecular characterization of the degrading enzymes was also aimed at.

MATERIALS AND METHODS

Samples collection

Samples were collected from different environmental sites from May 2011 to May 2012, Beni-Suef City, Egypt. Agricultural soil samples were collected by removing about 5 cm of soil surface and 10 cm depth and nearly 50-100 ml of agriculture wastewater and domestic sewage samples, respectively, were collected and stored at 4°C till use.

Isolation of degrading organisms from different environmental samples

Organophosphorus compounds (OPCs) such as malathion (MAL), chlorpyrifos (CPF), diazinon (DIZ), and fenitrothion (FEN) were dissolved in the least amount of methanol and completed with water to achieve a concentration of 10 mg ml^{-1}. Ten grams of fine granular soil or 10 ml of sewage water were separately added to 20 mg MAL L^{-1} in minimal salt medium (MSM), incubated at 30°C in a rotary shaker at 150 rpm. After three successive transfers, the enrichment culture was serially diluted and spread on MSM plates containing 100 mg MAL L^{-1}. Discrete bacterial colonies based on morphological properties were selected and sub-cultured to obtain pure cultures.

Inoculum preparation for biodegradation study

As described by Anwar et al. (2009), colony forming units per milliliter (c.f.u ml^{-1}) of this suspension was quantified by the dilution plate count technique and required inoculums were prepared by adding appropriate amount of normal saline.

HPLC analysis of organophosphates degradative capacity of the recovered isolates

Degradative capacities of *Pseudomonas aeruginosa* and *Pseudomonas mendocina* were carried out by measuring the residual MAL in MSM medium. One hundred milliliter of MSM supplemented with 100 µg MAL ml^{-1} was inoculated with 1.5 ×10^8 c.f.u ml^{-1} bacterial suspension and incubated as mentioned previously for 7 days. Centrifugation to remove bacterial pellets and filter sterilization through 0.22 µm to ensure complete removal of bacterial cells was done. Control experiment was carried out in parallel by 100 ml of MSM with 100 µg MAL ml^{-1} without being inoculated with bacteria and incubated under the same conditions. Five milliliters aliquot from each of the previously mentioned media were centrifuged at 7200 g for 10 min, and extracted 2 times with 50 ml of dichloromethane by vigorous shaking. Dehydration with anhydrous Na$_2$SO$_3$ and evaporation of dichloromethane extracts with rotary evaporator at 30°C. The dry residues were dissolved

in 1 ml acetonitrile and stored at 4°C until HPLC analysis. Twenty microliter of extracted sample were injected in HPLC (Agilent Technologies 1260 Infinity, USA), using Eclipse plus C18 (4.6 ×250 mm 5 µm) and diode array detector (DAD) at λ 210 nm. HPLC working conditions were acetonitrile: water (70:30), at a flow rate 1 ml min^{-1}.

Identification of the most active degrader organisms

In addition to morphological and biochemical identification, molecular characterization of bacterial isolates was done (Goda et al., 2010). The DNA extraction was done by GenElute DNA Bacteria Genomic DNA Kit (Sigma-Aldrich, USA). The 16S rRNA gene was amplified using the universal primer pair: 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1525r (5'-AAGGAGGTGWTCCARCC-3'), ordered through, and delivered by Invitrogen, USA. Thermal profile of PCR reaction for amplification of 16S rRNA gene for the most active two isolates was done using Dream Taq Green PCR Master Mix (2X) (Thermo Fisher Scientific Inc., USA). PCR products were visualized by placing on UV transilluminator and were photographed directly. Purification of PCR products from gel were done by GeneJET Gel Extraction Kit (Thermo Fisher Scientific Inc., USA) according to manufacturer protocol and purified DNA was stored at -20°C. Sequencing reactions were performed at Clinilab in MJ research PTC-225 Peltier Thermal Cycler using ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit with AmpliTaq DNA polymerase, following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the above mentioned primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in ABI 3730 x1 sequencer (Applied Biosystem, USA). Nucleotide sequence similarities were determined using other known sequences found in the GenBank database using BLAST program of National Center for Biotechnology Information (NCBI) databases (Ye et al., 2012).

Bacterial growth curve and factors that may affect the malathion degradation by *P. aeruginosa* and *P. mendocina*

If not otherwise mentioned, the following conditions were adjusted for studying the factors affecting MAL degradation paralleled with control samples at each interval. Each treatment was performed in three replicates. Flasks containing 100 ml of MSM amended with 100 mg MAL l^{-1} were inoculated with 1.5 ×10^8 c.f.u ml^{-1} of isolate and incubated at 30°C on a rotary shaker working at 150 rpm. At different time intervals, samples were taken and then analyzed for the residual MAL concentration by HPLC. Control experiments were carried out in parallel by incubating flasks of the same composition in the same conditions but without addition of the bacteria. These factors included addition of three different carbon sources (glucose, sodium citrate and phenol) and yeast extract as a nitrogen source added separately, at a concentration of 1g l^{-1} to the culture media. Parallel to measurement of MAL biodegradation, growth curve of the 2 isolates in the presence of the different carbon sources was also measured. Aliquots of 3 ml of bacterial growth culture were withdrawn at different time intervals and monitored by measuring the OD600 with a spectrophotometer (Shimadzu, Japan). To investigate the effect of temperature on biodegradation process, three flasks were incubated at three different temperatures: 25, 30 and 37°C. The effect of inoculum density was investigated using three different inoculum densities: 1.5×10^4, 1.5×10^8 and 1.5×10^{12} c.f.u ml^{-1}. To determine the effect of agitation on MAL biodegradation, two sets were applied, a set of flasks incubated on a rotary shaker and another unshakable one.

Detection of degradation metabolites of malathion by LC/MS

One hundred milliliter of MSM supplemented with 100 mg MAL l^{-1} was inoculated with bacterial cells suspension with a concentration of 1.5 ×10^8 cf.u ml^{-1} and incubated at 30°C in orbital shaker at 150 rpm for 7 days. Fifty milliliters aliquot from medium was centrifuged at 7200 g for 10 min to remove the bacterial pellets, and extracted 2 times with 50 ml of dichloromethane by vigorous shaking. Dichloromethane extract was passed through anhydrous Na$_2$SO$_3$ for dehydration, and then evaporated to dryness using rotary evaporator at 30°C. The dry residue was dissolved in 1 ml acetonitrile and submitted for HPLC/MS analysis. The equipment was 6120 Quadrupole LCMS, Agilent Technologies with 1260 Quat Pump VL that was used for metabolites detection according to the following conditions: reversed phase C18 column (15 cm × 2.1 mm I.D., 4 µm) at flow rate of 1 ml min^{-1} with injection volume of 100 µl and isocratic mobile phase of acetonitrile/water (70/30, v/v). The mass spectrometer was equipped with electrospray ion source operated in positive and negative polarity mode. The electron spray ion mode was operated under the condition of gas temperature of 350°C and drying gas flow of 12.0 l min^{-1}. The nebulizer nitrogen gas was 35 psi with capillary voltage of 3.0 kV. Full scan signal were recorded within the m/z range of 100 to 1000.

Molecular characterization of carboxylesterase enzyme responsible for organophosphorus degradation

The sequences of the different carboxylesterase genes for *P. aeruginosa* and *P. mendocina* were taken from the NCBI using the online BLASTN program. These sequences were aligned using the multiple sequence alignment tool (ClustalW2) available online (Larkin et al., 2007). Finally, the conserved regions of the genes were determined and then used for primer design. The oligonucleotide primers used in all sets of experiments for carboxylesterase enzyme screening were designed with the online program primer 3 (Ye et al., 2012), corrected manually according to degeneracy of genetic code and ordered from Invitrogen, USA. Primer pairs used in the present study for detection and sequencing of carboxylesterase enzyme for the isolated were: *P. aeruginosa*; Forward: GTAGTCGTGCCAGCCCAC and reverse: CGAACCCCTGATCCTCGATG with product size 574 bp and annealing temperature 56°C. For *P. mendocina,* primer pairs were forward: GTG ATG ACG CAG ATG ATC GT and reverse: CCA TCG TTC CAG CAG TCT CA at annealing temperature of 51°C with product size of 766 bp. Primers preparation, PCR reaction conditions, purification of PCR products and sequencing of amplified PCR products were done as mentioned earlier.

Phylogenetic analysis of 16S rRNA gene and carboxylesterase gene for the degrading isolates

Sequences of 16S rRNA genes of the isolates were aligned with their homologous sequences using ClustalW2 with default settings (Larkin et al., 2007). Phylogeny was analyzed with MEGA version 6.06 software (Tamura et al., 2013) and distances were calculated using Kimura-2 parameter distance model (Kimura, 1980), while clustering was performed with neighbor-joining method (Saitou and Nei, 1984).

For the carboxylesterase gene sequence, ExPASY translate tool was used to convert sequenced nucleotides to their corresponding amino acids sequences (Gasteiger et al., 2003). The translated amino acids sequences were aligned with their homologous sequences using ClustalW2 with default settings (Larkin et al., 2007). Phylogeny was analyzed with MEGA version 6.06 software (Tamura et al., 2013) and distances were calculated using Poisson model (Ranola et al., 2010) clustering was performed with

neighbor-joining method (Saitou and Nei, 1984).

RESULTS

Isolation of active organophosphate-degrading bacteria from environmental samples

A total of 18 bacterial isolates were recovered from thirty-six domestic sewage, agricultural wastewater and soil samples collected from different sites in Beni-Suef City, Egypt. The selected isolates were morphologically and biochemically identified. The most common isolated bacteria were *Pseudomonas* sp. (72.2%), followed by *Enterobacter* sp. (16.6%). On the other hand, only one isolate (5.6%) was isolated from each of the following bacteria: *Serratia* sp. and *Acinetobacter* sp. Regarding the type of the sample, agricultural sewages were the most rich samples with MAL degrading bacteria (49.9%), followed by domestic sewages (38.9%).

Morphological, biochemical and molecular identification of the bacterial isolates

According to the morphological and biochemical results, the selected isolates were preliminary identified as *P. aeruginosa,* isolated from an agricultural sewage sample, and *P. mendocina*, isolated from domestic sewage, and were abbreviated as PA and PM, respectively. To confirm the identified results, their 16S rRNA genes were amplified from their genomic DNA. Sequence blast showed that strain *P. aeruginosa* PA had a high similarity (99%) with *P. aeruginosa* strain ODE5 (Accession number KF420847.1), *P. aeruginosa* strain NRRLB-59992 (Accession number KF18511.1) and *Pseudomonas* sp. strain pDL01 (Accession number AF125317.1). *P. mendocina* PM strain showed a high similarity (99%) with *P. mendocina* strain FB8 (Accession number HQ701687.1) and *P. mendocina* strain PC7 (Accession number DQ178223.1).

Phylogenetic analysis of 16S rRNA sequences of the selected bacterial isolates

Phylogenetic analysis of 16S rRNA gene sequence of *P. aeruginosa* strain showed that our strain PA was closely related to *P. aeruginosa* strain YL 84 that was isolated from compost, a chitinase-producing quorum-sensing bacterium. This study strain was also close to *P. aeruginosa* strain Pa 24 that was isolated from the Arecanut garden soil of Hannoverin coastal Karnataka, India and showed herbicide biodegradation ability. While, there was a great difference with *P. aeruginosa* strain LES like 1 (Liverpool epidemic strain) (Jeukens et al., 2014) (Figure 1a). On the other hand, phylogenetic analysis of 16S rRNA gene sequence of *P. mendocina*

strain PM showed that it was the closest to fomesafen-degrading *P. mendocina* strain FB5, which can grow on a medium with 500 mg l^{-1} fomesafen herbicide as a sole carbon source but it was not close to some extent to *P. mendocina* strain PC6, the strain that was able to degrade phenol- and *p*-cresol, as shown in Figure 1b.

Biodegradation of malathion and other organophosphorus compounds by pure cultures of *Pseudomonas* sp.

Malathion and other three OPCs (DIZ, FEN and CPF) degradation was monitored at different time intervals and their concentrations were determined using HPLC. The obtained results showed that *P. aeruginosa* strain PA grew in the presence of MAL with no lag period and the degradation began within the first 24 h from inoculation and completely disappeared on the 14th d of inoculation. The isolate approximately degraded 91% of DIZ within 7 days from inoculation with almost complete removal in the 11th day and 86 and 96% of FEN were degraded within 11 and 14 days, respectively. Only 56% of CFP was degraded within 14 days (Figure 2a). *P. mendocina* strain PM degradation was less than PA strain where after 24 h from inoculation, only 77.6, 74 and 47% of MAL, DIZ and FEN, respectively, were degraded with no significant degradation for CPF within 14 d of inoculation (Figure 2b).

Different factors affecting degradation of MAL by *P. aeruginosa* and *P. mendocina* PM

Factors affecting degradation of MAL by the selected isolates were determined to study the effect of different chemical and physical agents. The bacterial growth curve with OD measurement at 600 nm showed a steady increase in the two bacterial isolates masses. The highest growth rates were with yeast extract, followed by glucose and lastly citrate for the 2 isolates but with phenol and MAL, no significant differences was detected between them (Figure 3).

Simultaneously, the HPLC analysis was carried out to measure MAL concentration with the different carbon sources. Results of Figure 4a and b showed a substantial reduction in the level of MAL concentration in the 2 bacterial cultures. The isolates were capable of degrading MAL in the presence of other carbon sources greater than MAL consumption alone. For *P. aeruginosa* strain PA, it was found that after 4 days, PA strain degraded more than 89, 84 and 81% of MAL in the presence of yeast extract, glucose and citrate, respectively, with almost complete removal within 7 days (Figure 4a). Degradation of MAL by PM strain was also greatly enhanced by the addition of yeast extract, glucose or citrate with significant degradation of up to 90, 83 and 72%, respectively, within 7 days of incubation, with
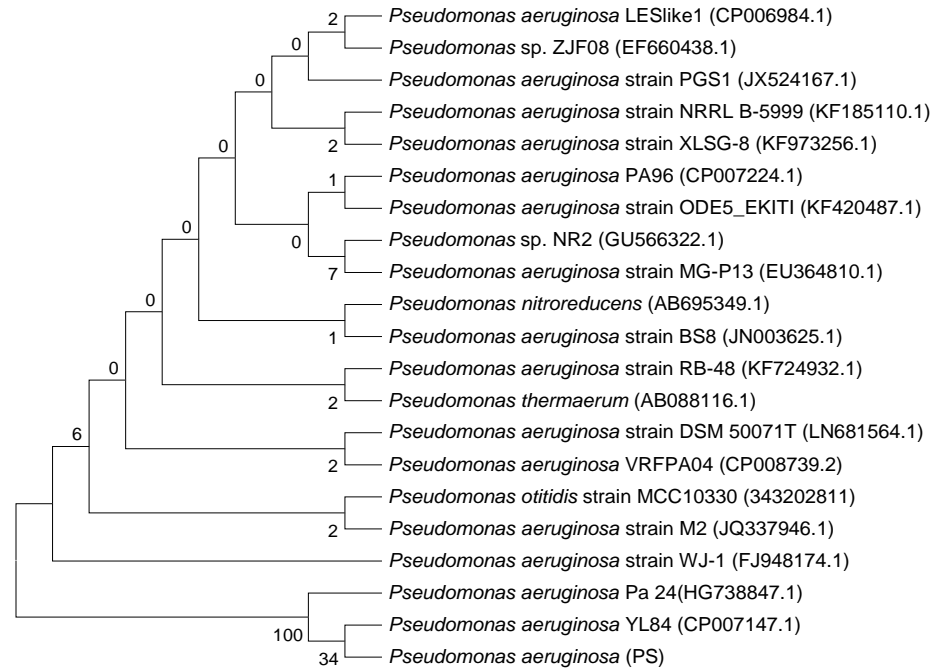
(a)
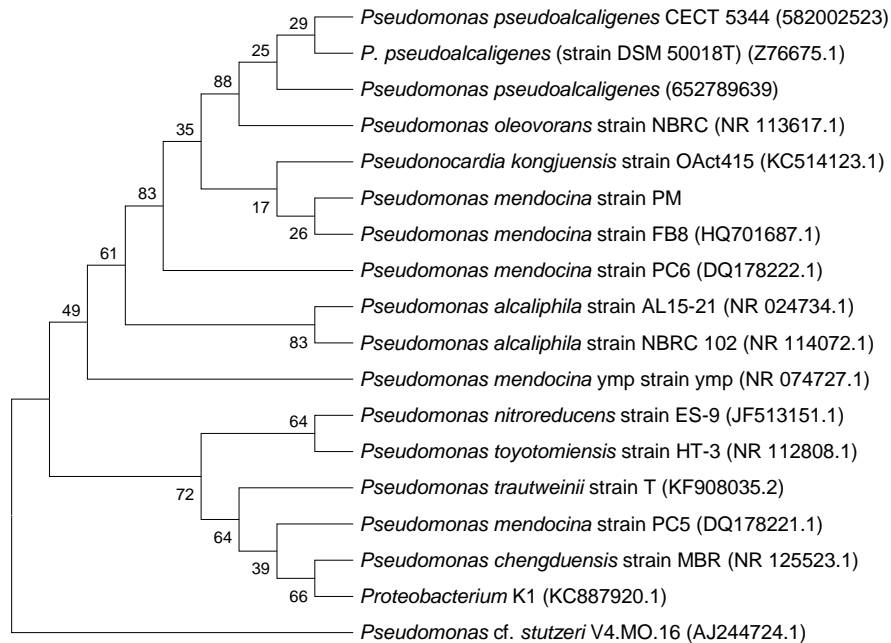
(b)

Figure 1. Phylogenetic tree of *Pseudomonas aeruginosa* strain PA (a) and *Pseudomonas mendocina* strain PM (b) based on 16S rRNA gene sequence analysis. Bootstrap values obtained with 500 replications were indicated as percentage at all branches. Gene bank accession numbers are given in parentheses.

complete removal within 14 days. For phenol, there was no significant difference in the presence or absence of

phenol with MAL at any time interval with any bacterial culture (Figure 4b).

(a)   (b)
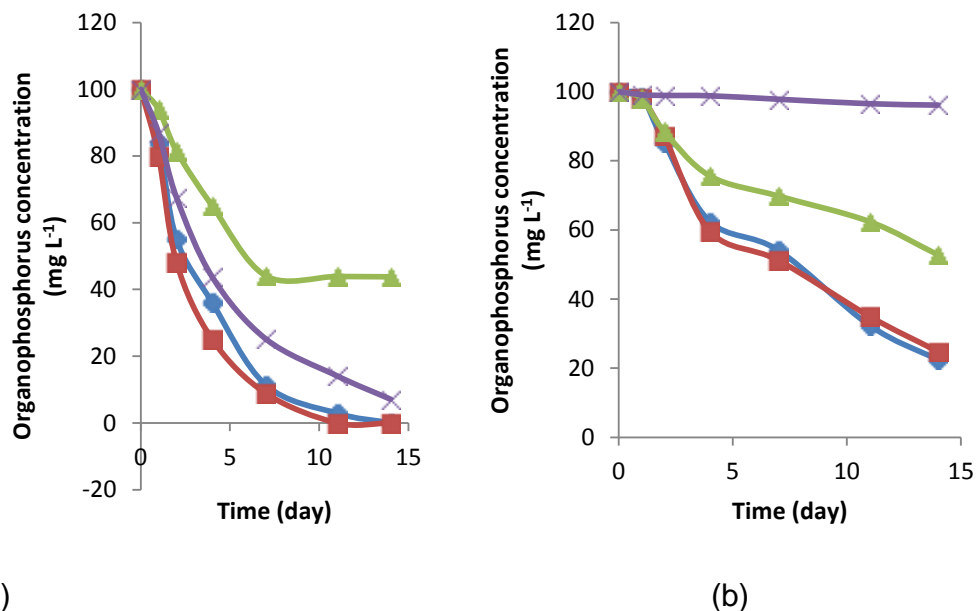
Figure 2. Biodegradation of different organophosphorus compounds by (a)Pseudomonas *aeruginosa* strain and (b)*Pseudomonas mendocina* strain in MSM containing diazinon (■), malathion (♦), fenitrothion (×) and chlorpyrifos (▲) at a concentration of 100 mg.l^{-1} in MSM using each of them as the sole carbon and energy source separately.

(a)   (b)
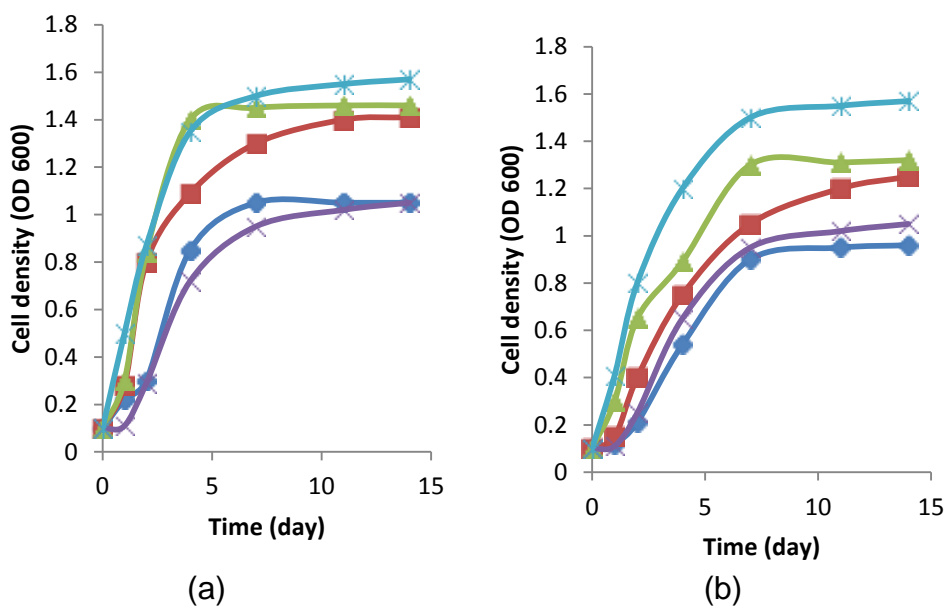
Figure 3. Microbial growth (cell density) of (a) *Pseudomonas aeruginosa* strain PA and (b) *Pseudomonas mendocina* strain PM during malathion biodegradation using it as a sole carbon source (♦) and in presence of different carbon sources as phenol (×), citrate (■), glucose (▲) and yeast extract (✕).

The extent of degradation of MAL increased and lag period decreased with increase in the inoculum density to 1.5×10^{12} c.f.u.ml^{-1} of both PA and PM (Figure 4c and d).

P. aeruginosa PA strain started MAL degradation immediately after inoculation and at the 4th day, the three bacterial inoculums degraded MAL with the same rate. *P.*

mendocina PM strain at a concentration of 1.5 ×10^4 c.f.u.ml^{-1} could not degrade MAL at all. On the other hand, bacterial concentration with 1.5×10^8 and 1.5×10^{12} c.f.u.ml^{-1} degraded MAL equally with a lag period of 1 day and after 14 days, the concentration reached 30 mg.l^{-1} of MAL (Figure 4d).

At 30°C incubation temperature, PA strain completely degraded MAL within 12 days, while in flasks incubatedat 25 and 37°C, almost 86 and 96%, respectively, of MAL were degraded within 14 d (Figure 4e). Upon incubation of PM strain at 25°C, the lag period was extended to 4 days and only 52% of MAL was degraded within 14 day while at 37°C, the lag period was about 2 days and more than 68% of MAL was degraded. At 30°C, it needed only 24 h to start MAL degradation and approximately 80% were degraded at the end of the incubation period (Figure 4f).

P. aeruginosa strain PA in an unshakable state showed a lag phase extended for 24 h, with a decreased biodegradation ability. Shaking of bacterial cultures with 100 mg MAL l^{-1} resulted in a faster degradation with no shoulder. On the 7th day of incubation, both conditions gave the same rate and extent of degradation and finally disappeared after 14 days of incubation. *P. mendocina* strain PM in resting condition was unable to show biodegradation ability until 4 days of inoculation and the degradation was hardly affected, where only 32% from the initial concentration of MAL was degraded in 14 days. While, in agitation condition, 44% of initial MAL was degraded within 4 days that increased to about 78% after 14 days of inoculation (Figure 4g-h).

Identification of metabolites produced from MAL biodegradation

Bacterial culture extract was subjected to LC/MS analysis to determine the MAL biodegradation metabolites produced from MAL degradation. The spectrum pattern of the separated products showed a molecular ion peak at m/z 330.9 which correspond to MAL. A molecular ion peak at *m/z* 301 and 274 were observed which is consistent with the molecular formula of malathion monocarboxylic acid (MMC) ($C_8H_{15}O_6PS_2$) and malathion dicarboxylic acid (MDC) ($C_6H_{11}O_6PS_2$).

Molecular characterization of carboxylesterase enzyme responsible for organophosphorus biodegradation

Malathion monocarboxylic acid, $C_8H_{15}O_6PS_2$ (MMC) and malathion dicarboxylic acid, $C_6H_{11}O_6PS_2$, (MDC) as biodegradation metabolites produced during degradation of MAL proved the breaking of ester linkage of the MAL by the bacterium. It was suggested that this isolates might contain carboxylesterase enzymes, so specific primers were designed for their detection. The forward and reverse primers successfully amplified genes of ~550 bp for PA and ~750 bp for PM strain. The translated amino acid sequences were aligned using NCBI protein blast tool on (Figure 5). Carboxylesterase amino acid sequence of *P. aeruginosa* PA showed 96% identity to carboxylesterase enzyme of *P. aeruginosa* (WP_003156353.1) and 95% identity to carboxylesterase enzyme of *P. aeruginosa* (WP_034079889.1). Carboxylesterase of *P. mendocina* PM showed 94% identity to carboxylesterase enzyme of *P. mendocina* (WP_013716910.1) and *P. mendocina* (WP_017362547.1) and 90% identity to carboxylesterase enzyme of *P. pseudoalcaligenes* (WP_004424218.1). Phylogenetic analysis with MEGA 6.0 software showed *P. aeruginosa* was localized in a separate branch from other carboxylesterase producing *Pseudomonas* sp. (Figure 6a). Therefore, it is suggested that this enzyme may be a novel esterase, which differed from those previously reported esterase enzymes. As shown in the Figure 6b, carboxylesterase enzyme of *P. mendocina* PM was closely related to carboxylesterase enzyme of *P. mendocina* (WP 013716910), heavy-metal resistant *P. mendocina* strain isolated from France vineyard soil (Chong et al., 2012).

DISCUSSION

Organophosphorus compounds poisoning is a worldwide health problem with about 3 million poisonings and 200,000 deaths annually (Karalliedde and Senanayake, 1988; Sogorb et al., 2004). Bioremediation, which involves the use of microorganisms to detoxify and degrade pollutants, has received increased attention as an effective biotechnological approach to clean up polluted environments. Several chemicals have been successfully removed from soil and aquatic environment using degrading microorganisms such as coumaphos (Mulbry et al., 1996).

In the present study, enrichment culture technique was used to isolate MAL degrading bacteria from polluted samples, where 18 isolates strains were obtained and biochemically identified. Among the isolated *Pseudomonas* sp., *Enterobacter* sp*., Serratia* sp. and *Acinetobacter* sp*., P. aeruginosa* and *P. mendocina* were were found to have the highest capacity for degradation of 100 mg MAL l^{-1}. Accordingly, the results in this study suggested that the enrichment culture technique was well suited for the isolation of physiologically and genetically different strains. *Pseudomonas* are a diverse group of bacteria that occur in large numbers in soil, where they are active in mineralization of organic matters. In this study, *P. aeruginosa* strain PA was isolated from agricultural sewage that might be exposed repeatedly to OPCs while, *P. mendocina* strain PM was isolated from domestic sewage that might not be exposed to OPCs.

(a)

(b)

(c)

(d)

(e)

(f)

(g)   (h)
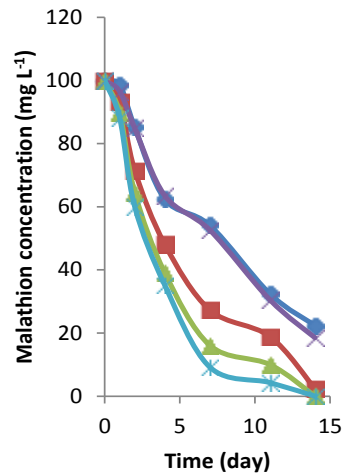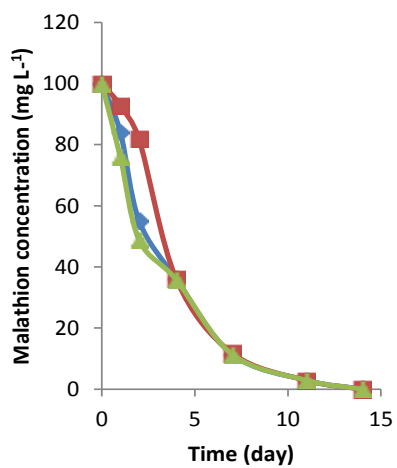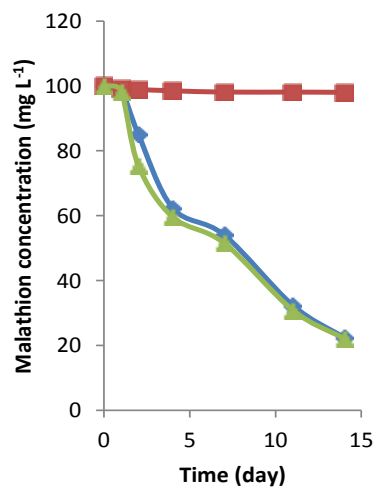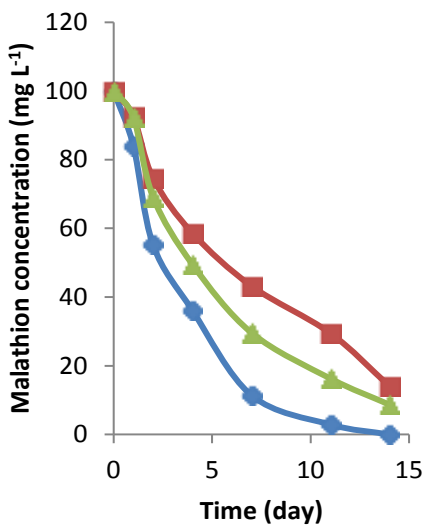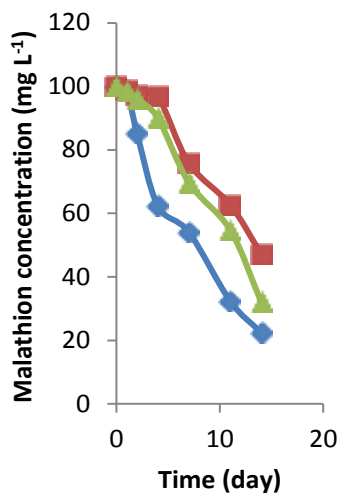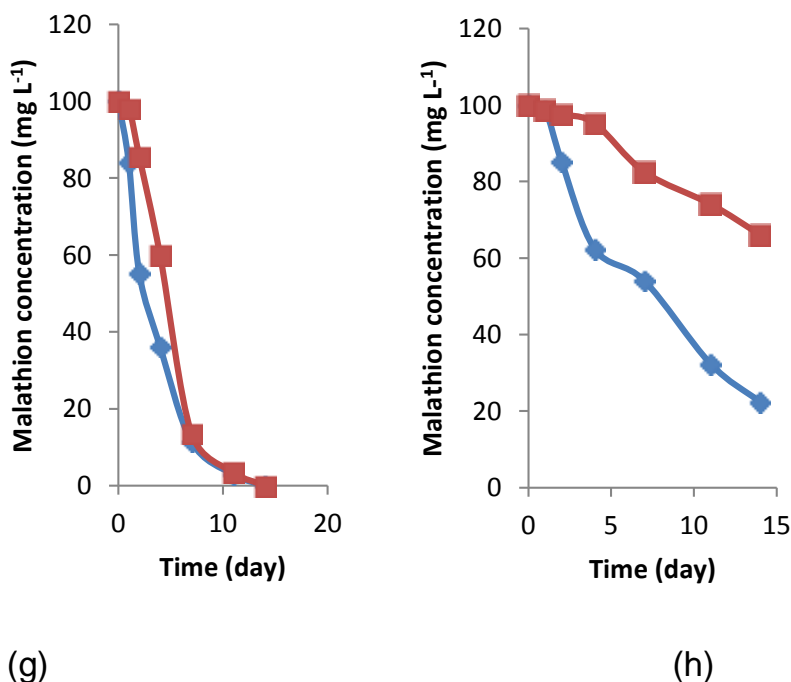
Figure 4. Effect of different factors on malathion degradation. Effect of different carbon sources on malathion biodegradat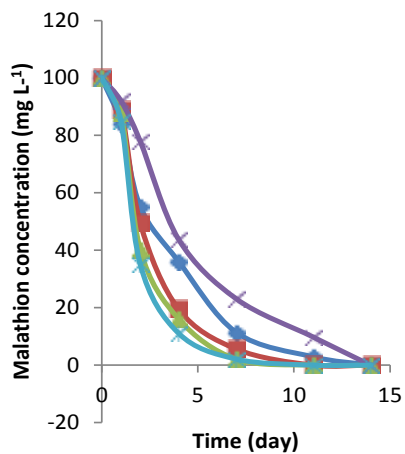ion by (a) *Pseudomonas aeruginosa* strain PA and (b) *Pseudomonas mendocina* strain PM in MSM medium containing 100 mg. l⁻¹ of malathiona sole carbon and energy source (♦), phenol + malathion (✕), malathion + citrate (■), malathion + glucose (▲) and malathion + yeast extract (✳). Effect of inoculum density on malathion biodegradation by (c) *Pseudomonas aeruginosa* strain PA and (d) by *Pseudomonas mendocina* strain PM in MSM medium containing 100 mgl⁻¹of malathion as a sole carbon and energy source 1.5×10⁴c.f.u.ml⁻¹ (■), 1.5×10⁸c.f.u.ml⁻¹ (♦), and 1.5×10¹²c.f.u.ml⁻¹ (▲). Effect of temperature on malathion biodegradation by (e) *Pseudomonas aeruginosa* strain PA and (f) *Pseudomonas mendocina* strain PM in MSM medium containing 100 mgl⁻¹ of malathion as a sole carbon and energy source at 37°C (▲), 25°C (■) and 30°C (♦). Effect of agitation on MAL biodegradation by: (g) *Pseudomonas aeruginosa* strain PA and (h)*Pseudomonas mendocina* strain PM using malathion as a sole carbon and energy source on a rotary shaker at speed 150 rpm (♦) and in stationary case (■).

VLVRLQPVAEALQVVLPSTRFILPQAPSQAVTVNGGWV**Met**PS
WYDILAFSPARAIDEDQLNASADQVIALLDEQRAKGIAAERIIL
AGFSQGGAVVLHTAFRRYGKPLGGVLALSTYAPTFDDLA

(a)

MetTAP**Met**ILQPPQTADASVIWLHGLGADRYDFLPVAE**Met**LQER
LPTTRFILPQAPTRPVTINGGWS**Met**PSWYDILA**Met**SPARAIDQA
QLDESADQVIALIEAERESAIAAERIVLAGFSQGGAVVLHTAFL
RYPETLGGVLALSTYAPTFSDD**Met**QLADTKRQLPVLCLHGRFD
DVVTPD**Met**GRAAYDRLHASGVQVQWRDYP**Met**THEVLPEEIRD
IAEWLGQLLA

(b)

Figure 5. Deduced amino acid sequence of carboxylesterase enzymes of (a) *Pseudomonas aeruginosa* PA; (b) *Pseudomonas mendocina* PM, using ExPASY translate tool of sequenced nucleotides.

Carboxylesterase [*Pseudomonas alcaliphila*] (WP 017675246.)
94
Carboxylesterase [*Pseudomonas mendocina*] (WP 021488710.)
100
56
Carboxylesterase *[Pseudomonas pseudoalcaligenes* AD6] (EZH84060.1
37
Carboxylesterase [*Pseudomonas stutzeri*] (WP 003293649).
53
Carboxylesterase [*Pseudomonas taeanensis*] (WP 025165016).
Carboxylesterase [*Pseudomonas cremoricolorata*] (WP 028696585).
39
Carboxylesterase [*Pseudomonas fuscovaginae*] (WP 017904199.)
63
Carboxylesterase [*Pseudomonas fluorescens*] (WP 011332570).
65
98
Carboxylesterase [*Pseudomonas putida*] (WP 033044035).
89
Carboxylesterase [*Pseudomonas mandelii* JR-1] (AHZ69511.1)
Carboxylesterase [*Pseudomonas aeruginosa*] (WP 003156353.)
Carboxylesterase [*Pseudomonas psychrotolerans*] (WP 007161882).
Carboxylesterase [*Pseudomonas aeruginosa* strain PS]

(a)

Carboxylesterase [*Pseudomonas mendocina* strain PM]
99
Carboxylesterase [*Pseudomonas mendocina*] (WP 013716910.)
54
Carboxylesterase [*Pseudomonas* sp. 1-7] (KFJ93309.1)
92
Carboxylesterase [*Pseudomonas pseudoalcaligenes*] (WP 004424218)
100
50
Carboxylesterase [*Pseudomonas alcaliphila*] (WP 017675246)
Carboxylesterase [*Pseudomonas oleovorans*] (WP 037055996)
Carboxylesterase [*Pseudomonas resinovorans*](WP 028628594)
56
Carboxylesterase [*Pseudomonas thermotolerans*] (WP 027897314)
100
Carboxylesterase [*Pseudomonas stutzeri*] (WP 014821255)
78
Carboxylesterase [*Pseudomonas chloritidismutans*] (WP 023446800)
Carboxylesterase [*Pseudomonas alcaligenes*] (WP 021700291)
69
Carboxylesterase [*Pseudomonas taeanensis*] (WP 025165016)
52
Carboxylesterase [*Pseudomonas chlororaphis*] (WP 038357647)
100
Carboxylesterase [*Pseudomonas fluorescens*] (WP 034148976)

(b)

Figure 6. Phylogenetic analysis of carboxylesterase enzyme of (a) *P. aeruginosa* PA (b); *P. mendocina* PM and their homology proteins from various bacterial genomes using neighbor-joining method. The bootstrap values obtained with 500 replications were indicated as percentage at all branches. Gene bank accession numbers are given in parenthesis.

Prakash and his colleaagues (1996) observed that a pesticide can be degraded rapidly in the soil from a field to which it had never been applied before but which had been exposed to a pesticide from the same chemical group; this phenomenon is known as cross-adaptation.

Phylogenetic analysis of 16S rRNA gene sequence of *P. aeruginosa* strain PA showed that this strain PA was closely related to *P. aeruginosa* strain YL 84 (a chitinase-producing quorum-sensing bacterium) (Chan et al., 2014) and *P. aeruginosa* strain Pa 24 isolated from Areca nut

garden soil and showed herbicide biodegradation ability. The present study results were confirmed by Singh (2009) who reported that the bacteria with the capacity to degrade OPCs were more closely related than those without degrading capability. Phylogenetic analysis of 16S rRNA gene sequence of *P. mendocina* strain PM showed that it was the closest to fomesafen-degrading *P. mendocina* strain FB5. The phylogenetic tree showed sister linage between sequences of xenobiotic degrading microorganisms. The same results were obtained by Cabrera et al. (2010) who reported that fenamiphos degrading bacteria were more closely related than others, and suggested that nematicide degrading bacteria were grouped within a common microbial population which can be of great significance for the soil ecology.

The two bacterial strains isolated in this study were able to utilize MAL as a sole carbon and energy source. In this study of MAL biodegradation, in the presence of yeast extract, glucose or citrate as additional carbon sources, there was a marked increase in the bacterial biomasses for the two bacterial isolates. The findings revealed that *P. aeruginosa* strain PA and *P. mendocina* strain PM preferred to utilize MAL even in the presence of rich nutrient environment and its degrading ability was positively influenced by the presence of supplementary nutrient sources. The preferrential use of MAL in the presence of additional carbon sources was attributed to: 1) MAL degrading enzymes in bacteria were expressed even in the presence of readily available carbon sources; 2) The presence of favorite carbon sources accelerate cell growth, reduce the toxicity of MAL and drastically promoted the degradation of pesticides and 3) when a culture medium contained two compounds, the microorganisms preferrentially utilize the most assimilable and the most concentrated until the concentration approached equality, at which time both sources were utilized simultaneously. In the present case, the first sample was taken after 24 h which was considered as a sufficient time for the complete removal of favourable nutrients, at this time, the equillibrium state was reached with high inoculum density. On the other hand, Goda et al. (2010) found that MAL degradation in the presence of other carbon sources was relatively lower than when MAL was present as a sole carbon source for the first 2 days and after 4 days of the degradation was the same in both cases. They explained the preferrential use of favorable carbon sources instead of MAL. Similar findings with this results had been reported for *P. putida* epII (Karpouzas and Walker, 2000), where the degradation of ethroprophos was inhibited by succinate or glucose. In other studies, the addition of sodium acetate or glucose did not affect the degradation of pentachlorophenol by *P.mendocina* NSYSU (Kao et al., 2005), neither did supplementation with glucose improved degradation of phenanthrene (Zhao et al., 2009). Thus, inclusion of other carbon sources may or may not affect the biodegradation of the insecticides by bacteria. The gradual consumption

of MAL was accompanied by a consistent increase in culture density, which reached maximum once MAL had been completely consumed, suggesting that bacterial growth was driven by MAL catabolism. This result was similar to those reported by Zhao et al. (2009).

The obtained results showed that MAL was degraded by *P. aeruginosa* strain PA during incubation with the three intial inoculum densities tested (1.5×10^4, 1.5×10^8 and 1.5×10^{12} c.f.u.ml^{-1}), while MAL degradation by *P.mendocina* strain PM in cultures at a concentration of 1.5×10^4 c.f.u.ml^{-1} could not. Similar results were obtained by Karpouzas and Walker (2000) who found that *Sphingomonas* strain could not degrade cadusafos when the organism was introduced in soil at a density of 4.3×10^4 cells g^{-1}.

Incubation temperature is an important factor affecting the survival of the organism and degradation of the organophosphorus. Incubation temperature of 30°C could be suggested to be the optimum for the growth of the organism and production of degrading enzymes of *P.aeruginosa* and *P. mendocina* giving higher extent of MAL degradation by these isolates. Studies by Hong and colleagues (2007) reported that *Pseudomonas* sp. and *Burkholderia* sp. were able to degrade fenitrothion over a temperature range of 20-40°C with optimum degradation at 30°C. Yang et al. (2008) showed that 30°C is the optimal temperature for heterologous expression of methyl parathion hydrolase enzyme (MPH). They found that 30°C is optimal for the formation of properly folded enzyme and inhibition of ATP-dependent and ATP-independent proteolysis. Also, Kaneva and co-workers (1998) found that cultures expressing organophosphate hydrolase enzyme (OPH) constitutively on the surface did not survive at 37°C and could only be grown at 30°C or lower temperatures.

In the present study, analysis of MAL bacterial culture by HPLC/MS was carried out to identify the metabolite(s) produced due to MAL degradation. It was found that the spectrum pattern of the separated products showed molecular ion peaks, suggesting that the primary degradative pathway of MAL might be due to the activity of esterase enzyme (Goda et al., 2010). From HPLC/MS analysis for identification of the metabolites produced from MAL degradation, it was concluded that carboxy-lesterase enzyme might be the enzyme responsible for the degradation of MAL.

Sequence analysis in the present study of carboxylesterases enzymes of *P. aeruginosa* strain showed a high similarity with carboxylesterase enzyme of *P. aeruginosa*. However, there was no homology with the previously described carboxylesterase enzyme or with other recognized hydrolases that were isolated from mammals or microorganisms. The finding suggested that carboxylesterase in *P. aeruginosa* strain is a novel esterase enzymes that differed from those previously mentioned.

This study carboxylesterase enzyme maybe coded by

chromosomal genes, suggesting that it should be a stable trait, once more emphasizing the potential for use of these isolates in bioremediation process. The suggestion was almost confirmed by the stability of its degradation power even in the presence of other favorable carbon sources. A recent study by Farrugia et al. (2013) confirmed the result that carboxylesterase enzyme was encoded by chromosomal genes. On the other hand, many of the genes for xenobiotic transformation were carried on mobile elements such as plasmids (Pieper and Reineke, 2000) or megaplasmid of 60 Kb in *Bacillus* sp. (Niazi et al., 2001).

For future work, there is a need for further research on some other parameters that may affect the degradation of organophosphorus compounds such as pH and use of different concentrations of the same organic substrate. As shown, these bacterial enivronmental biohazards to carboxylesterase enzyme purification and stability is one of the aims of future research and attempt of enzyme cloning in a competent *Escherichia coli* will also be the authors' concern.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Analysis of the impact of domestication of *Warburgia ugandensis* (Sprague) on its genetic diversity based on amplified fragment length polymorphism

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***Warburgia ugandensis* Sprague (Canellaceae) occurs in East and Central Africa and is an important multipurpose tree species. Over-exploitation of natural forests for medicinal purposes and clearance for farming threaten the species survival. Cultivation of the tree species would ensure sustainable medicinal source and its conservation. However, on-farm genetic diversity of the species is currently unknown. The genetic diversity of the on-farm *W. ugandensis* populations and their proximate natural populations were analyzed using the amplified fragment length polymorphism (AFLP). Four primer combinations produced a total of 223 polymorphic bands. Both the natural and on-farm populations had high genetic diversity ranging from *H* = 0.2892 to *H* = 0.1278. Principal co-ordinates analysis and dendrogram separated the ten populations into two major groups corresponding to Kenyan and Tanzanian populations, respectively. Ugandan populations were shared between the two major groups; this is probably because Uganda is believed to be the centre of diversity for *W. ugandensis*. Close genetic relationships between the on-farm and their proximate natural population were revealed. Analysis of molecular variance (AMOVA) revealed that a total of 54% AFLP variation resided within populations with 46% reside among populations. The high genetic diversity of *W. ugandensis* on-farm populations could be useful in germplasm collection and conservation strategies.**

Key words: *Warburgia ugandensis,* amplified fragment length polymorphism (AFLP), domestication, genetic diversity, on-farm, natural.

INTRODUCTION

Warburgia ugandensis Sprague (Canellaceae) is a multi-purpose tree found in the lowland rainforest and upland dry evergreen forest of Eastern and Southern Africa (Orwa et al., 2009) and is highly valued for its medicinal

properties. It has a mixed mating system, predominantly outcrossing and pollinated by small bees (e.g. *Trigona*) and other insects (Muchugi et al., 2008). Natural forests inhabited by this species are under threat of destruction by clearing for farming and unsustainable exploitation for medicinal purposes (Maundu and Tengnas, 2005). Cultivation of the species in plantations and on-farm has been advocated to ensure sustainable supply of the herbal products and its conservation (Okigbo et al., 2008). The species is propagated through seeds, stem cuttings (Akwatuilina, 2011) and tissue culture (Kuria, 2012). A prerequisite for the efficient use of genetic resources in any planting program is a detailed understanding of the genetic diversity and the extent of distribution of genetic variation available within a species (Muchugi et al., 2012). A wide genetic base provides the ability to withstand potential inbreeding depression through future generations of farmer propagation (Frankham, 2010). However, little is known about the influence of cultivation on genetic diversity within the species and whether on-farm stands are suitable sources of material for further planting.

Molecular markers have proven to be invaluable tools for assessing plants' genetic resources by improving our understanding with regards to the distribution and the extent of genetic variation within and among species (Ilga and Yousry, 2014). Previously, genetic diversity studies on *W. ugandensis* have been carried out using random amplified polymorphism (RAPDS) (Wamalwa et al., preliminary report) and amplified fragment length polymorphism (AFLP) (Muchugi et al., 2012, 2008).

AFLP technique is a powerful method for marker analysis genetic diversity investigations. It targets large loci due to several primer pair combination that can be used. In addition, AFLP markers are highly polymorphic reproducible and do not require prior sequence information of the species under investigation.

In the present study, the genetic diversity in on-farm and proximate natural populations of *W. ugandensis* was investigated. Genetic partitioning among 10 populations in East Africa was evaluated using AFLP markers in an attempt to determine possible effects of domestication on the genetic structure of this species and to provide backing for management and conservation of these populations. The data will be important for future exhaustive germplasm core collections for breeding and conservation purposes.

MATERIALS AND METHODS

Sample collection

Samples were collected from natural forest populations and their proximate on-farm stands in East Africa (Table 1). Sampling for the natural trees was done at random, separated by a minimum distance of 100 m while farm trees were collected from single individuals from a series of separate small farms and did not have a minimum distance requirement. Three young leaves were collected from each tree, cleaned using 70% ethanol, dried with silica gel and stored at -20°C, prior to DNA isolation.

DNA extraction and AFLP analysis

Total genomic DNA was extracted from approximately 30 mg of silica-dried leaves following a modified cetyltrimethyl ammonium bromide (CTAB) (Doyle and Doyle, 1987). The integrity and quality of the DNA was checked using 0.8% (w/v) agarose gel and NanoDrop^R nd-1000 spectrophotometer (Thermo Fischer Scientific, Wilmington, DE,USA) and diluted in TE buffer (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA) to a final concentration of 200 ng/µl. The AFLP method was carried out following the standard procedure described by Vos et al. (1995) and adapted in the AFLP^{TM} Plant mapping protocol of the Applied Biosystems (ABI), USA. First, 200 ng of genomic DNA was digested with *EcoRI* and *MseI* restriction enzymes, followed by ligation of adapters to the restriction fragments. A pre-amplification of the primary templates was done with *EcoRI and MseI* primers having three selective nucleotides at the 3'end. A selective amplification was performed on the pre-amplified fragments with fluorescent-labeled *EcoRI* primers having three selective nucleotides at the 3' end and *MseI* primers having three selective nucleotides at the 3' end. The fluorescent-labeled primers were synthesized by Applied Biosystems (ABI), USA. The selective amplification was performed in a MJ Research PTC-200 DNA Engine thermocycler using the following amplification profile. The selective PCR Amplification was programmed for an initial 2 minutes at 94°C followed by one cycle of 94°C for 20 s, 66°C for 30 s and 72°C for 2 min. This cycle was repeated 12 times with a lowering of the temperature of 1°C per cycle. This was followed by 20 cycles of 94°C for 20 s, 56 °C for 30 s and 72°C for 2 min and a further hold time of 30 min at 60°C. The AFLP product was prepared for analysis in ABI prism 3730 DNA analyzer by making a 1:3 dilution. A loading buffer mix was prepared by adding 10 µl of gene scan 500 LIZ internal size standard ABI to 990 µl deionised formamide. Nine microliters (9 µl) of the size standard mix was added to 1 µl of the diluted selective amplification products in a micro-Amp PCR plate. The amplification products were first denatured by heating at 95°C then cooling in ice for 5 min before loading onto the genetic analyzer. From the ABI PRISM 3730*,* the sample data was directed to the GeneMapper ^{TM} software to analyze and display the sizing results as electrograms and tabular data. The four *EcoRI:MseI* primer combinations were selected based on the previous AFLP analyses in *W. ugandensis* (Muchugi et al., 2008).

Statistical analysis

The resulting fragments from the ABI Prism 3730 automated sequencer (Applied Biosystems) were described and screened for quality using Gene Mapper 3.7 software. GeneMapper generated allele frequency data for all individuals as product presence (1) or product absence (0) which were then converted into Microsoft Excel spread sheet. Population allele frequency diversity values and genetic distance (D) between population were computed in TFPGA 1.3 (Miller, 1997). Principal coordinates analysis (PCoA) was performed using GenAlEx 6.3 (Peakall and Smouse, 2009) software to a provide visual representation of genetic structure. Cluster analysis using an unweighted pair-group method with arithmetic averaging (UPGMA) (Sneath and Sokal, 1973) was used to generate a dendrogram showing relationship among populations based on genetic distance (Nei,1978). The analysis of molecular variance (AMOVA) (Excoffier et al., 1992) based on phipT-statistic was generated by GenAlEx 6.3 using 999 permutations.

Table 1. Details of ten populations sampled for *W. ugandensis* for AFLP analysis

Country	Population	GPS reading	Altitude (M)	Population type	Number of samples	Population analysis
Kenya	Cherangani	N 01° 00′ E 035° 01′	2189	On-farm	15	1
	Rumuruti	N 00° 07′ E 036° 25′	2100	On-farm	15	2
	Kitale	N 01° 00′ E 035° 01′	2189	Natural	15	3
	Rumuruti	N 00° 07′ E 036° 25′	2100	Natural	15	4
Uganda	Mabira	N00° 22.798′ E033° 04.804′	1215	Natural	22	5
	Mabira	N00° 22.798′ E033° 04.804′	1215	On-farm	12	6
	Kibale	N00°13' E30° 19'	1300	Natural	20	7
Tanzania	Lushoto	S 04° 35' 05"E 38° 11' 33"	1780	Natural	16	8
	Kagera	S 01° 05' 07"E 31° 31' 06"	1167	Natural	16	9
	Lushoto	S 04° 38' 54" E 38° 14' 42"	1950	On-farm	17	10

Table 2. Mean diversity estimates *(H)* for 10 populations of *W. ugandensis* generated from 223 AFLP markers (the percentage polymorphic loci and sample size (N) are shown).

Country	Provenance	N	*H*	Polymorphic loci (%)
Kenya	Rumuruti on-farm	15	0.1278	37.22
	Rumuruti natural	15	0.1787	59.64
	Cherangani on-farm	15	0.2119	69.96
	Kitale natural	15	0.1464	50.22
Uganda	Mabira natural	22	0.1946	63.23
	Mabira on-farm	12	0.1963	57.85
	Kibale natural	20	0.2892	88.79
Tanzania	Lushoto natural	17	0.2210	63.68
	Lushoto on-farm	16	0.2431	74.89
	Kagera natural	15	0.2195	61.88

RESULTS

Estimation of genetic diversity

The mean Nei's genetic diversity for all the 223 loci in the 10 populations ranged from $H = 0.1278$ (Rumuruti on-farm) to $H = 0.2892$ (Kibale natural) as shown in Table 2. The percentage of polymorphic loci was proportional to the genetic diversity estimate; populations with higher diversity estimates showed higher percentages of polymorphic loci.

Generally, higher genetic diversity was observed for on-farm stands than in their proximate natural counterparts. For instance, Cherangani on-farm had higher genetic diversity estimate ($H = 0.2119$) than its proximate Kitale natural population ($H = 0.1464$), same results were shown for Lushoto on-farm ($H = 0.2467$) and Lushoto natural ($H = 0.2210$). Mabira on-farm ($H = 0.1963$) was slightly higher than proximate Mabira natural ($H = 0.1946$) while Rumuruti natural ($H = 0.1787$) was higher than proximate Rumuruti on-farm ($H = 0.1278$). Among all the natural populations, Kitale had unusually low genetic diversity estimate ($H = 0.1464$) with Kibale natural having the highest ($H = 0.2892$). The level of genetic diversity as shown by Shannon's diversity index was average for most of the populations ranging from 0.1920 (Rumuruti on-farm) to 0.4000 (Kibale natural) (Table 2). Based on the mean polymorphism of the loci within population, the diversity ranged from 37.22 (Rumuruti on-farm) to 88.79% (Kibale natural) (Table 2).

Genetic relationships and population structure

Principal coordinates analysis (PCoA) was performed to visualize regional genetic structure of *W. ugandensis* based on the 10 populations. PCoA resolved the genetic groups on two axes, accounting for 73.93% of the overall variation (Figure 1). The first and second axes explained 60.70 and 13.23%, respectively, of the total variation
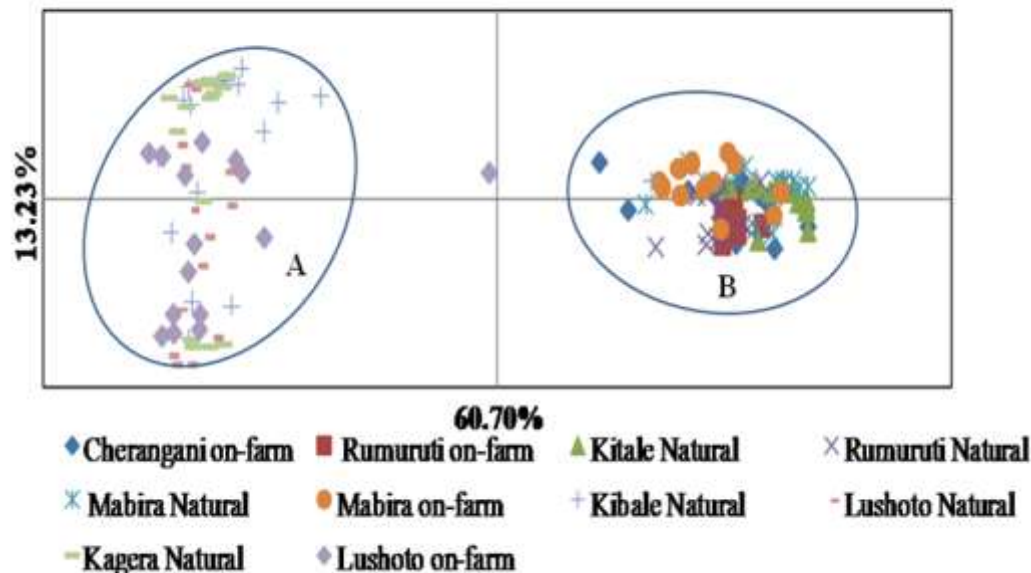
Figure 1. Principal co-ordinate analysis of 163 *W. ugandensis* from Kenya, Uganda and Tanzania.

Table 3. Matrix of unbiased genetic distance according to Nei (1978) among 10 populations of *W. ugandensis* based on 223 AFLP markers.

Population	Cherangani-on-farm	Rumuruti-on-farm	Kitale-Natural	Rumuruti-Natural	Mabira-Natural	Mabira-on-farm	Kibale-Natural	Lushoto-Natural	Kagera-Natural
Cherangani-on-farm									
Rumuruti-on-farm	0.1622								
Kitale-Natural	0.0485	0.1472							
Rumuruti-Natural	0.1498	0.0531	0.1399						
Mabira-Natural	0.0612	0.1428	0.0425	0.1014					
Mabira-on-farm	0.0929	0.1813	0.1182	0.1215	0.0463				
Kibale-Natural	0.4367	0.5231	0.5342	0.4979	0.4959	0.4823			
Lushoto-Natural	0.6269	0.6721	0.7587	0.6424	0.7059	0.6651	0.0642		
Kagera-Natural	0.6042	0.6621	0.7412	0.6282	0.6866	0.6442	0.0445	0.0496	
Lushoto-on-farm	0.5796	0.6565	0.7185	0.6221	0.6718	0.6333	0.0566	0.0246	0.0365

between cluster A and B. One cluster consisted of Lushoto (natural), Lushoto (on-farm) and Kagera (natural) while the other consisted of Mabira (on-farm), Mabira (natural), Rumuruti (on-farm), Rumuruti (natural), Cherangani (on-farm), Kitale (natural), with Kibale population splitting in the two groups (Figure 1).

Genetic variation within and among the 10 populations of *W ugandensis* populations was quantified by analysis of molecular variance (AMOVA). Partitioning of genetic variability by AMOVA revealed that most of the AFLP variation was found within populations (54% *P*<0.01) while the remaining resided among populations (46%

P<0.01) (Table 3).

**Genetic differentiation**

A dendrogram generated using Nei's genetic distance (Table 4) showed two major clusters (Figure 2). The first cluster consisted of Kibale natural, Kagera natural, Lushoto natural and Lushoto on-farm while the other comprised Cherangani on-farm, Kitale natural, Mabira natural, Mabira on-farm, Rumuruti on-farm and Rumuruti natural. Each of the two major clusters had two distinct

Table 4. Analysis of molecular variance (AMOVA) for AFLP among *W. ugandensis* sampled from Lake Victoria region in Kenya, Uganda and Tanzania.

Source of variation	Df	SS	MSD	Total variance (%)	P-value
Among populations	9	2882.06	320.22	46	0.01
Within populations	131	3277.38	25.01	54	0.01
Total	140	6159.39	345.24		0.01

Analysis based on all stands. Df=degrees of freedom, SS=sums of squares, MSD=mean square deviations and P=significance of variance. Significant values were based on the random permutations 99 times.
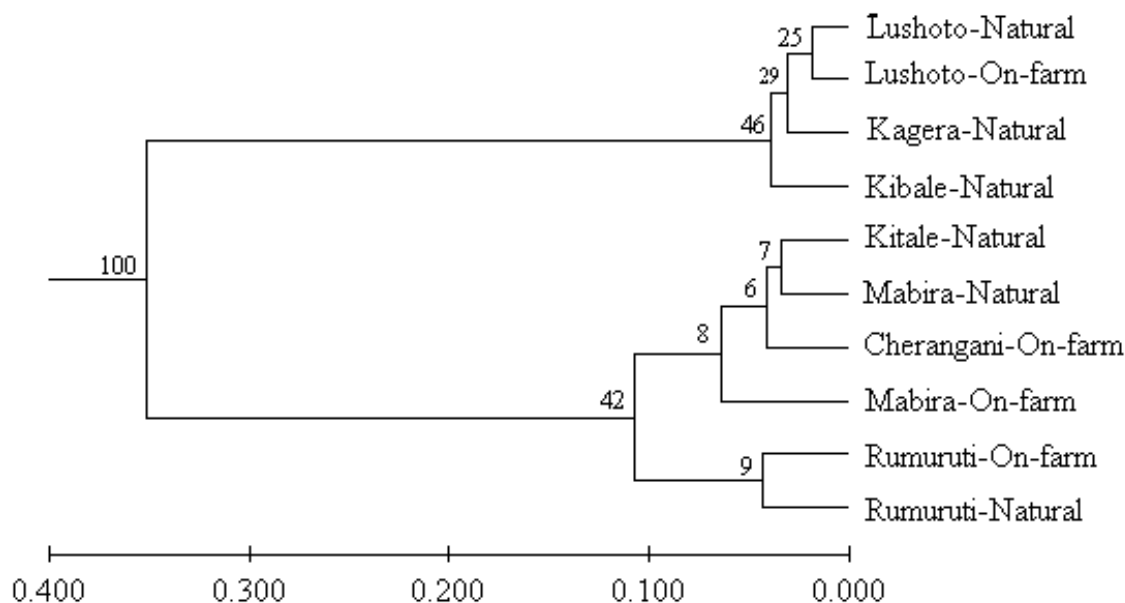
Figure 2. Phenogram based on Nei (1978) genetic distance for 10 populations of *W. ugandensis* from Kenya, Uganda and Tanzania generated using 223 AFLP markers.

subgroups with the first cluster having Lushoto natural and Lushoto on-farm separated from Kagera and Kibale natural and the second having Rumuruti on-farm separated from Kitale natural, Cherangani on-farm, Mabira natural and Mabira on-farm. Most on-farm populations clustered with the proximate natural populations. The clustering pattern based on unweighted pair-group method with arithmetic averaging (UPGMA) was consistent with the PCoA results (Figure 1). The shortest genetic distance was observed between Lushoto natural and Lushoto on-farm (0.0246), while the most distant populations were Lushoto natural and Kitale Natural (0.7587) (Table 4).

DISCUSSION

Evaluation of genetic diversity

Genetic diversity estimation is useful for optimization of

sampling strategies, conserving and management of genetic diversity of trees (Hamrick and Godt, 1996). AFLP markers are able to detect high levels of polymorphism, thus making it a powerful tool for assessing genetic diversity in many species (Bensch and Åkesson, 2005).

In the present study, the genetic diversity estimate of the sampled populations was relatively high, consistent with Muchugi et al. (2008) who found high genetic diversity among the sampled *W. ugandensis* populations. Levels of genetic diversity observed within a population may be influenced by population size, isolation and gene flow (Godt and Hamrick, 1989). Cherangani on-farm population showed higher genetic diversity than its proximate population Kitale natural. A significant difference was observed in genetic diversity between Rumuruti on-farm and Rumuruti natural populations. This may be an indication that the planting materials were sourced from a few mother plants from the natural population. However, during the collection, most farmers

clearly stated that they did not plant the trees and that the on-farm trees had regenerated from remnants of natural forest trees. In this case, the loss in genetic diversity in on-farm trees can be linked to selection pressure (e.g. and firewood) in the now cultivated land, resulting to significant loss of alleles (Ratnam and Boyle, 2000). In addition, restricted gene flow could have contributed to the difference as the barrier between the two populations is expected to increase with increased farming and settlement, resulting in differences in genetic diversity. On-farm populations analyzed in this study remain relatively diverse; therefore genetic erosion concerns that could result to loss of adaptability and potential inbreeding did not appear to have occurred.

Natural populations are expected to have high genetic diversity as shown by Kagera and Kibale populations; however, Kitale showed a relatively low genetic diversity. The same population showed the lowest genetic diversity among the populations during an earlier study (Muchugi et al., 2008). The low genetic diversity in Kitale population could be as a result of stochastic loss of alleles probably as a result of strong selective pressures due to deforestation for settlement causing changes in landscape, cutting down of big trees for timber, habitat destruction altering population density, diversity and abundance of pollinator communities thereby impinging on the mating systems (Lowe et al., 2005). These factors could further lead to increased genetic differentiation and potentially increase inbreeding (Lowe et al., 2004). However, some forest tree species are able to meliorate these population genetic pressures through a variety of mechanisms such as extensive gene flow in many cases over tens of kilometers (Bacles et al., 2005), which serves to maintain connectivity even in highly fragmented and degraded landscapes where trees are at very low densities (Lander et al., 2011). The long lived nature of trees and existence of overlapping generations in a single site serves to retard the loss of genetic diversity (Davies et al., 2010). Flexible mating systems in some species circumvent self incompatibility to allow selfed progeny to form (Ward et al., 2005) particularly when faced with lack of compatible mates within a landscape. The impact of fragmentation varies by species and context, but not merely simple loss of genetic diversity and increased genetic differentiation among populations (Lowe et al., 2015). The key consequence of fragmentation has been identified as increased inbreeding depression in progeny sired in fragmented landscape (Breed et al., 2012b) and fitness loss due to low numbers of partners and low pollen diversity (Breed et al., 2014). Therefore, in the case of *W. ugandensis*, a study focusing on the progeny would be more suitable to explain whether the on-farm stands have experienced any inbreeding depression. Studies that compared genetic diversity of both natural populations of tropical trees and cultivated trees Meru oak (*Vitex fischeri,* synonym *Vitex keniensis*) using RAPDS (Lengkeek et al., 2005b) and sheanut (*Vitelleria*

paradoxa) using microsatellite markers (Kelly et al., 2004) found little difference in diversity levels between agroforestry trees and the natural populations stand categories, with no evidence of genetic bottleneck events in agroforestry. In both cases, majority of trees tested from agroforests stands were expected to represent remnants or dispersed natural regenerants, thus providing an appropriate comparison with the present study. However, studies on *Inga edulis* comparing natural and planted stands at five locations in Peruvian Amazon, found limited but significant bottlenecks in the planted trees (Hollingsworth et al., 2005).

Population genetic structure

Analysis of molecular variance analysis (AMOVA) indicated low genetic differentiation between natural and cultivated populations. Partitioning genetic variation of populations of *W. ugandensis* showed a slightly higher within-population component as compared to the among-population component, which is characteristic of many widely distributed, abundant out-breeding tree species (Hamrick et al., 1992). *W. ugandensis* has a mixed mating system which is predominantly out-crossing. Thus, the transfer of pollen by insects between the different individuals and dispersal of seeds by wild animals (Muchugi et al., 2007) may increase the possibility of sexual recombination and subsequently increase within-population genetic variance. The high levels of variation within populations suggest that sampling from a few localities for either breeding or conservation could capture a large proportion of the variation within the species.

Genetic differentiation

The dendrogram and principal coordinates' analysis clustered the on-farm populations together with their proximate natural populations, implying that they are genetically related. When considered separately, the natural populations did not necessarily follow the geographical distance trend. This also does not agree with the predicted vegetation classification. Mabira and Kagera fall in the Lake Victoria Mosaic while the Kibale and Kitale fall in the Guineo-Congolian phytochoria (White, 1983). The Kibale and Kitale samples displayed this affinity previously (Muchugi et al., 2008). The clustering of geographically distant populations of Lushoto, Kibale and Kagera suggests a different migration theory from the previous study (Muchugi et al., 2008). A probable explanation is the effect of Lake Victoria as a gene flow barrier, especially in the case of Mabira and Kagera populations. Interestingly, the genetic disjunction revealed among Kenyan populations in Muchugi et al. (2008) is also revealed here with the

Rumuruti populations clustering separately from the Kitale population despite their geographical closeness, pointing to the Rift valley as the most probable barrier between the two populations hence separate evolution. In addition, the Kitale population belongs to the Guineo-Congolian vegetation block and Rumuruti falls outside this vegetation. It has been found that isolated populations evolve separately as they adapt to new ecological habitat leading to changes in allele frequencies hence the genetic differentiation (Epperson, 1992). This study further confirmed the implied theories of African floral evolution (White, 1983), which considers western Kenya as the most eastern remnant of the Guineo-Congolian phytochoria while eastern Kenya populations fall within the Somalia-Maasai centre of endemism. Such genetic differentiation reflects the different evolutionary histories of the species in the different ecological niches combined with different gene dispersal mechanisms (White, 1983).

This revelation calls for more genetic analysis in understanding the species migration and colonization from its centre of diversity which is suggested to be Uganda (Muriira, 2012). Human activities such as logging, slash-and burn agriculture, roads construction and cities together with human mediated dispersal of seeds could have also played an important role in the distribution of genetic diversity in this species as much as natural barriers which could have resulted in the current differentiation observed.

Conclusion

Despite the concerns that *W. ugandensis* trees currently on-farm might be of low genetic base, these results showed that they have high genetic diversity comparable to their proximate natural populations. Therefore, materials currently on-farm can be used as seed sources and conservation of the species. It is however, crucial to conserve the genetic variation of *W. ugandensis* by protecting the existing natural forests. High genetic structuring was evident among the populations with most genetic variation residing within populations, thus sampling intensively from a few populations would capture most genetic diversity.

Conflict of interest

The authors declare that there is no conflict of interest.

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Full Length Research Paper

Optimization, purification and characterization of recombinant L-asparaginase II in *Escherichia coli*

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We studied optimal L-asparaginase sequence from GenBank accession number X12746 encoding for L-asparaginase from *Erwinia chrysanthemi* NCPPB1125. The expression level of recombinant L-asparaginase was determined as 78% of the total proteins. The purified L-asparaginase had a molecular mass of 37 kDa with specific activity of 312.8 U/mg. Kinetic parameters, K_m, V_{max}, K_{cat} and K_{cat}/K_m of purified enzyme were found to be 0.5 mM, 500 U/mg, 14.9×10^3 s^{-1}, and 29.9×10^3 $mM^{-1}s^{-1}$, respectively. Temperature and pH optimum were observed at 45ºC and pH 7.5, respectively. The enzyme exhibited about 20 and 60% retention of activity following 100 min incubation at 55 or 40°C, respectively. The activity of enzyme was inhibited by EDTA, Hg^{2+}, Cu^{2+}, Ni^{2+}, and enhanced by Mg^{2+}. Detergents (Tween 20, Tween 80, Triton X-100, and Triton X-114) decreased enzyme activity. DTT and DMSO at appropriate concentrations enhanced enzyme activity. *In vitro* anti-cancer activity was performed using different tumor cell lines. Concentration of recombinant L-asparaginase at 50 μg/ml inhibited 45.32, 48.22, 53.68, 51.22% with HL-60, P388, P3X63Ag8, SP2/0-Ag14 cell lines. Recombinant L-asparaginase was expressed successfully in *Escherichia coli* with high expression level, had a high specific activity and antiproliferative effect on several tumor cell lines.

Key words: Characterization, *Erwinia chrysanthemi*, L-asparaginase, purification, tumor cell line.

INTRODUCTION

Acute lymphocytic leukemia (ALL) is a type of blood cancer that results when abnormal white blood cells (leukemia cells) grow quickly and crowd out the bone marrow preventing the normal red blood cells, white blood cells, and platelets that body needs. ALL incidences occur most frequently in people under the age of 15 or over 45. L-asparaginases are a cornerstone of treatment protocols for ALL (Silverman et al., 2001;

Pieters and Carroll, 2008). L-asparaginase is also used in treatment of acute myeloid leukemia (AML) (Emadi et al., 2016; Tagde et al., 2016b). Beside, L-asparaginase induced significant growth inhibition and apoptosis in K562 and KU812 cells so it might be a promising new therapeutic strategy for chronic myeloid leukemia (CML) (Song et al., 2015). Normal cells can synthesize L-asparagine by asparagine synthetase. In contrast, tumor

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cells slowly synthesize L-asparagine and are dependent on an exogenous supply. So L-asparaginase destroys extracellular source of L-asparagine, inhibits protein synthesis in lymphoblasts inducing their apoptosis (Duval et al., 2002). L-asparaginase (EC 3.5.1.1, L-asparagine amidohydrolase) catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonium. The enzyme is considered to play a significant role in L-asparagine metabolism in normal cells. There are two types of L-asparaginases: L-asparaginase I, was used to reduce the level of acrylamide in food industry (Friedman, 2003; Yano et al., 2008). Whereas, L-asparaginase II was used to treat leukemia. There are many sources of L-asparaginase such as bacteria, fungi, yeast, actinomycetes, algae and plants (Verma et al., 2007). To date, L-asparaginase gene had been cloned from variety of host such as *Escherichia coli* (Wang et al., 2001), *Erwinia chrysanthemi* (Kotzia and Labrou, 2007), *Erw. carotovora* (Pourhossein and Korbekandi, 2014), *Yesinia pseudotuberculosis* (Sidoruk et al., 2011), *Thermococcus kodakarensis* (Hong et al., 2014), *Saccharomyces cerevisiae* (Ferrara et al., 2010), and expressed in different expression systems including *E. coli* (Kotzia and Labrou, 2007; Magdy and Mohammed, 2008), *Bacillus subtilis* (Jia et al., 2013), and *Pichia pastoris* (Ferrara et al., 2006). At present, clinically useful L-asparaginases are obtained from either *E. coli* or *Erw. chrysanthemi.* In 2002, Duval compared *E. coli*-asparaginase with Erwinia-asparaginase in the treatment of childhood lymphoid malignancies, the study showed that *E. coli*–asparaginase can be recommended for first-line therapy reserving Erwinia-asparaginase for high sensitive *E. coli*-asparaginase patients (Duval et al., 2002). Here, we examined optimization of rASPG expression, along with the purification and characterization of the recombinant L-asparaginase from *Erw. chrysanthemi* in *E. coli.* We used pET21a+ vector with highly-inducible T7 promoter and induced by isopropyl-β-D-thiogalactopyranoside (IPTG) to express L-asparaginase. The results suggest that rASPG was purified with high activity and had high potential for antiproliferative application.

MATERIALS AND METHODS

Plasmid, bacterial strains and cell lines

The L-asparaginase gene based on L-asparaginase sequence from GenBank accession number X12746 was optimized codon for expression in *E. coli*, produced and inserted into vector pUC57 (pUaspg) by GenScript (USA). The DNA fragment (981 bp) encoding the mature L-asparaginase (without the signal peptide of 21 N-terminal amino acids) from pUaspg was inserted into vector pET21a(+) resulting in plasmid pEaspg to express in *E. coli.*

E. coli BL21(DE3) cells (F⁻ ompT gal dcm lon hsdS$_B$(r$_B$⁻ m$_B$⁻) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) (Fermentas). Luria-Bertani medium (LB) containing 1% (w/v) bacto tryptone; 0.5% (w/v) yeast extract; 1% (w/v) NaCl; pH 7-7.5 was used for cultivation of *E. coli*. The LB agar plates contained additionally 2% (w/v) agar and100 µg ampicillin/ml. Four tumor cell lines: human promyolecytic leukemia HL-60, mouse lymphocytic leukemia P388, mouse

myeloma P3X63Ag8 and Sp2/0-Ag14 and a normal cell line mouse mus musculus NIH/3T3 were obtained from the Bioassay group (Institute of Biotechnology, Vietnam).

Chemicals

L-asparagine, Nessler reagent and RPMI-1640 media were from Sigma (Louis, USA). IPTG, trichloroacetic acid, bactotryptone and yeast extract were from Bio Basic Inc (New York, USA). DEAE-sepharose and Sephacryl S200 were supplied by Pharmacia Co. (GE Healthcare. SDS was supplied Sigma Aldrich Co. (St, Louis, USA). Tween 20 and Tween 80 were from Bio Basis Inc. (New York, NY, USA), and Triton X-100, Triton X-114 and EDTA by Merck (Darmstadt, Germany). All chemicals were used in the experiments in their purified forms.

Plasmid construction

The L-asparaginase gene based on L-asparaginase sequence (1044 bp) from GenBank accession number X12746 was optimized codon for expression in *E. coli*, synthesized and inserted into vector pUC57 (pUaspg) by GenScript. The DNA fragment (981 bps) encoding the mature L-asparaginase (without the signal peptide of 21 N-terminal amino acids) from pUaspg was amplified using pUaspg as template and two oligonucleotides, 21 ASP -F (5'- GCC ATA TGG ATA AAC TGC CGA -3') and LASP-his R (5'- AAG CTC GAG TCA GTA GGT ATG GAA G -3') were designed as primers for introduction of the underlined *Nde*l and *Xho*l restriction sites, respectively. The PCR mixture contained 2.5 µl 10 × PCR buffer; 2 µl of 2 mM dNTP; 2.5 µl of 25 mM MgCl$_2$; 1 µl plasmid pUaspg (50-100 ng); 0.25 µl 5 unit *Taq* polymerase and 1 µl each primer (10 pmol), supplemented with 14.75 µl distillated water to fulfill 25 µl. The thermocycler conditions were as follows: 95°C/4'; 30 cycles of (95°C/45″, 55°C/45″, 72°C/45″); 72°C/10'. The polymerase chain reaction (PCR) products amplified from the pUaspg with both primer 21 ASP-F and L-ASP-his R were digested with *Nde*l and *Xho*l and purified using Gel Extraction Kit (Qiagen) in accordance with the manufacturer's instructions. It was followed by ligation of the *Nde*l-*Xho*l digested *aspg* products with pET21(a+) linearized by the same enzymes, resulting in pEaspg under the control of the T7-promoter induced by IPTG (isopropyl-β-D-thiogalactopyranoside) and possessing the ampicillin marker. The L-asparaginase encoded by the plasmid pEaspg contains the mature L-asparaginase without the 6 × histidine-tag and no leader sequence. The pEaspg plasmid was transformed in *E. coli* DH5α and BL21 (DE3) cells by heat shock method as described previously (Quyen et al., 2007).

Soluble rASPG expression

The transformant *E. coli* BL21/pEaspg was cultivated in 5 ml of LB medium with 100 µg/ml ampicillin at 37°C with agitation at 220 rpm overnight. This culture was used to inoculate 250 ml of the same media, and grown to an optical density at 600 nm (OD$_{600\,nm}$) 0.4 - 0.6 at 37°C with shaking at 220 rpm. IPTG was then added to 1 mM final concentration, the culture was continuously incubated at 28°C with agitation of 220 rpm for 6 h of induction. Cells were harvested by centrifugation at 8000 rpm and 4ºC for 5 min.

Enzyme assay

Activity analysis of L-asparaginase II was performed according to Chung's report (Chung et al. 2010) comprising the following steps: The 100 µl samples were mixed with 900 µl 0.01 M L-asparagine in 50 mM Tris buffer, pH=8.6, and 1 mL of assay mixture were incubated for 10 min at 37°C for enzymatic reaction. The reaction

was interrupted with 100 µL of 1.5 M trichloroacetic acid and the samples were centrifuged before the addition of 100 µL Nessler's reagent to measure the released ammonia after L-asparagine hydrolysis. All the measurements were done spectrophotometrically at 480 nm. The enzyme activity of recombinant protein was determined using an ammonium sulphate calibration curve. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µM of ammonia per minute.

Effect of IPTG concentration

IPTG control T7 *lac* promoter so that IPTG concentration may be affected expression level of recombinant protein. To assess the effects of IPTG concentration on the enzyme specific activity, eight flasks 100 ml contain 25 ml per flask of the recombinant clone culture was grown to OD_{600nm} of about 0.4 - 0.8 (for approximately 4 h), and induced by adding IPTG in final concentrations of 0; 0.2; 0.4; 0.6; 0.8; 1; 1.2; 1.4 mM, respectively. After 6 h of induction, bacterial cells were harvested and analyzed for the enzyme specific activity. The best IPTG concentration was selected and applied for the next stage.

Effect of amp concentration

Six flasks 100 ml were contained 25 mL LB broth were prepared. Amp with final concentrations of 25; 50; 100; 150; 200 and 250 µg/ ml were added to each flask, respectively. Half ml of the overnight culture was inoculated into each of flasks. The culture was cultivated at 37°C with agitation at 220 rpm until an OD_{600nm} of 0.4 - 0.8 was reached (for approximately 3 h) then IPTG was added. After 6 h of induction, bacterial cells were harvested to analyze for the enzyme specific activity. The best Amp concentration before induction was selected and applied for the next stage.

Effect of inoculum size

To evaluate the effect of inoculum size on the enzyme expression, the overnight culture were inoculated, inoculum of different sizes 0.5%; 1; 2, and 5% (v/v) into four flasks 100 ml which contained 25 ml LB, the recombinant clone culture was grown at 37°C in LB medium for 3 h, and then induced by adding IPTG. After 6 h of induction, enzyme specific activity was evaluated. Inoculum size with higher protein production was determined.

rASPG purification

The rASPG was expressed in *E. coli* BL21(DE3). To purify rASPG, 0.7 g cells from a 100 ml culture in LB medium were harvested by centrifugation at 8000 rpm and 4°C for 5 min, and resuspended in 8 ml of 50 mM Tris HCl buffer pH 8.6, sonicated and centrifuged at 12000 rpm and 4°C for 15 min.

Gel filtration

The supernatant cell free extract containing the crude L-asparaginase was loaded into Sephacryl S-200 column (2.6 × 6 cm) equilibrated with 50 mM potassium phosphate (pH 8) and eluted with the same buffer at the flow rate of 0.5 ml per minute. Fractions showing L-asparaginase activity were pooled and concentrated with bench top protein concentrator at 4°C. The homogeneity of the protein was checked by SDS -PAGE.

DEAE chromatography

The concentrated enzyme solution was added on the top of

Diethylaminoethyl Sepharose ion exchange column (DEAE - Sepharose) (2.6 × 6 cm) equilibrated with 50 mM Tris HCL (pH 8.6). The column was washed with 2 volumes of starting buffer and the protein was eluted with linear gradient of NaCl (0 - 1 M) prepared in 50 mM Tris HCL (pH 8.6) at the rate of 30 ml per hour. The eluate was collected with 1.5 ml per fractions. The fractions showing L-asparaginase activity were stored at 4°C.

The molecular mass of the rASPG was determined by 12.5% SDS polyacrylamide gel electrophoresis with Biometra equipment (Laemmli, 1970). Proteins were visualized by staining with 0.1% (w/v) Coomassie Brilliant Blue R-250. Protein concentrations were estimated by the method of Bradford with the bovine serum albumin as standard (Bradford, 1976).

Kinetic parameters determination

Aliquots of 100 µl of reconstituted enzyme were prepared and added with different concentrations of L-asparagine ranging from 1 mM to 4.5 mM prepared in 50 mM Tris HCl. The apparent kinetic parameters (K_m, V_{max}, K_{cat} and K_{cat}/K_m) of enzyme for L-asparagine were determined by Lineweaver-Burk plots method.

Temperature and pH optimum

The pH and temperature optimum of rASPG were determined by measuring the activity as described above using 100 mM potassium acetate buffer (pH 4-6), potassium phosphate buffer (pH 6.5-8), and Tris HCl buffer (pH 8-10) at 37°C for 30 min, and in the temperature range of 20 - 65°C at pH 8.6 for 30 min.

Temperature and pH stability

For the determination of temperature and pH stability, the purified enzyme (0.7 µg for each reaction) was incubated at 40 and 50°C, and pH 6; 7; 8 the activities were measured at various time intervals of 20; 40; 60; 80 and 100 min. Percentage of residual activities was calculated based on the untreated control activity, which is taken as 100%.

Effect of metal ions and EDTA, detergents, DTT, DMSO

The purified enzyme (0.7 µg protein for each reaction) was preincubated in presence of 10 mM of various metal ions (Ca^{2+}, Cu^{2+}, Fe^{3+}, Fe^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+}, K^+, Hg^+, Pb^{2+}), ethylenediamine tetraacetic acid (EDTA), in presence of 1-5% (w/v) of various detergents (Tween 20, Tween 80, Triton X-100, and Triton X-114), in presence of 0.1-5 mM dithiothreitol (DTT) and in presence of 0.1-2% (w/v) dimethyl sulfoxide (DMSO) at 37°C for 1 h. The residual activity was then determined at pH 8.6 and 37°C.

Cell culture and proliferation assay

Cells were routinely cultured in RPMI 1640 media. It were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate and 50 units/ml penicillin, 50 µg/ml streptomycin. Cells were cultivated in a humid atmosphere (5% CO_2, 37°C) (Takahashi et al., 2015; Hasegawa et al., 2016; Rajabi et al., 2016; Tagde et al., 2016a). Cells were seeded in 96-well plates at 1×10^4 cells per well. rASPG was added at concentrations of 0.4; 2; 10 and 50 µg/ml. After 72 h of continuous enzyme exposure, 10 µl of MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was added in each well. The plates were incubated for 1 - 4 h at
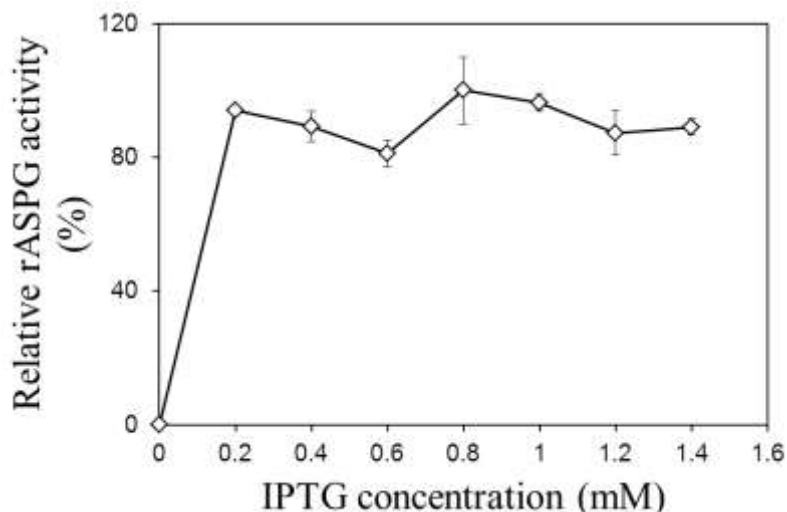
Figure 1. Effect of IPTG concentrations on rASPG activity. Relative activity was expressed as a percentage of control (100% rASPG activity was 31.7 U/mg).

37°C and the formazan product was measured at 490 nm. The experiments were performed in triplicate in three independent sets. Cell survival was calculated by subtracting the background absorbance of media alone and then dividing the absorbance of experimental wells by the absorbance of the control (untreated) wells (Neisius and Moll, 1989; Sonawane et al., 2014; Tagde et al., 2014).

RESULTS AND DISCUSSION

Optimization of rASPG expression

In previous studies, we expressed a plasmid pET22b(+) containing the full-length *aspg* gene in *E. coli* BL21(DE3) but no expression was detected by SDS-PAGE analysis, and recombinant plasmid pEaspg containing *aspg* gene and expression vector pET21a(+) was previously constructed without signal peptide with his tag, pEaspg was transformed in *E. coli* BL21(DE3) and expressed at 28°C, recombinant protein was higher levels expressed but had low activity (data not show). In this study, we constructed plasmid without the $6 \times$ histidine-tag and no leader sequence. The DNA fragment encoding the mature L-asparaginase with stop codon, truncated 21 N-terminal amino acids was inserted into pET21(a+) vector resulting in the recombinant plasmid pEaspg. The transformant *E. coli* BL21/pEaspg was grown in LB medium for the rASPG production. After IPTG induction, the cells were collected used for enzyme activity assay. The *E. coli* BL21/pEaspg transformant was showed high production of L-asparaginase (data not show). To increase the specific as well as volumetric yield of recombinant L-asparaginase, a variety of independent cultivation parameters such as inducer concentration,

ampicillin concentration and inoculum size were optimized.

Effect of IPTG concentration

IPTG concentration did not affect enzyme activity. Although, IPTG concentration increase from 0.2 to 1.4 mM but there are significant changes observed in enzyme activity (Figure 1). The maximum enzyme activity was at 0.8 mM IPTG (100%), but no significant decrease at 0.2 mM IPTG (95%). Thus, 0.2 mM IPTG was selected for the next stages. The similar results were reported in the study of Sidoruk et al. (2011) and Bahreini et al. (2014). In 2011, Vidya demonstrated that enzyme activity was decrease with the increase of IPTG from 10 µM to 50; 100 and 400 µM (Vidya et al., 2011)

Effect of Amp concentration

Amp was supplemented in culture medium to prevent the overgrowth of plasmid-free cells. Amp also affects the number of plasmid per cell. Chong reported that increase in the concentration of Amp in cultures causing the increase of the plasmid copy number in cells (Chong et al., 2003). Bahreini assumed that the increase in plasmid copy number corresponding to the rise of protein expression, but Bahreini's research has demonstrated that the higher levels of Amp had no effect on the L-asparaginase activity (Bahreini et al., 2014). We have found a similar result. It seems that the increase of Amp level higher than 25 µg/ml slightly decrease specific enzyme activity (Figure 2).
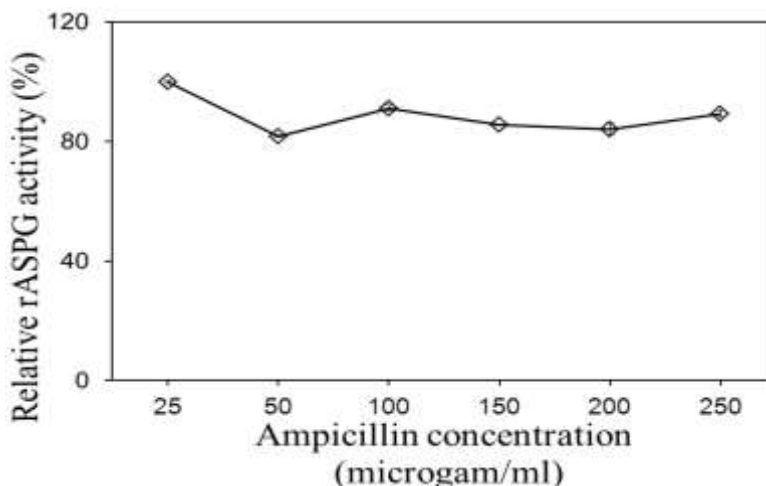
Figure 2. Effect of Amp concentration on rASPG activity. Relative activity was expressed as a percentage of control (100% rASPG activity was 35.9 U/mg).
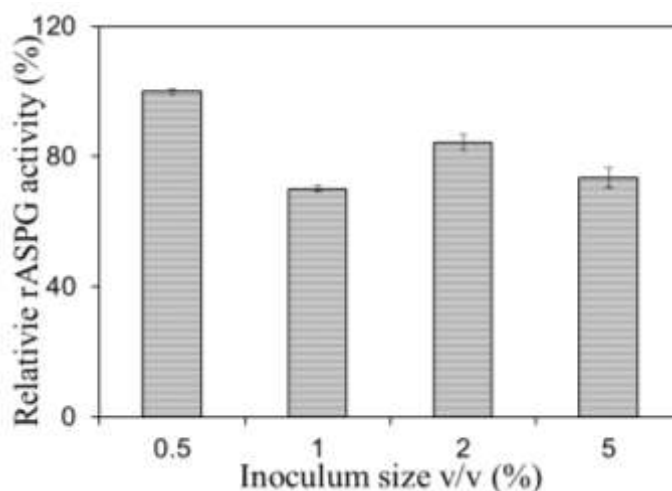

Figure 3. Effect of inoculum size on rASPG activity. Relative activity was expressed as a percentage of control (100% rASPG activity was 37.2 U/mg.

Effect of inoculum size

After 4 h culture, four flasks with different inoculum size 0.5; 1; 2 and 5% (v/v) and the value of $OD_{600\ nm}$ reached 0.5; 0.6; 0.7; 0.8, respectively. Our study showed that the inoculum size of 0.5% were found to be the most suitable condition for maximum enzyme activity, the increase in inoculum size is the reason for the decrease in enzyme activity (Figure 3). In general, the increase in cell density of bacterial expression enhances recombinant protein production. Khushoo et al. (2004) reported that induction IPTG during late log phase ($OD_{600\ nm}$= 4.5) resulted in maximum secretion of the recombinant asparaginase and specific activity (Khushoo et al., 2004). Later, Kenari et al.

(2011) optimized inoculum size of 10% (Kenari et al., 2011). Bahreini et al. (2014) reported that in L-asparagi nase production level with the maximum production at the highest cell density of $OD_{600\ nm}$= 10 (Bahreini et al., 2014). A simple explanation of these findings that inoculum sizes can be attributed to decrease in the concentration of the medium components, such as O_2 level, pH, and nutrients.

Purification of rASPG

The expression level of rASPG in optimized conditional expression was 78% of the total cellular protein by
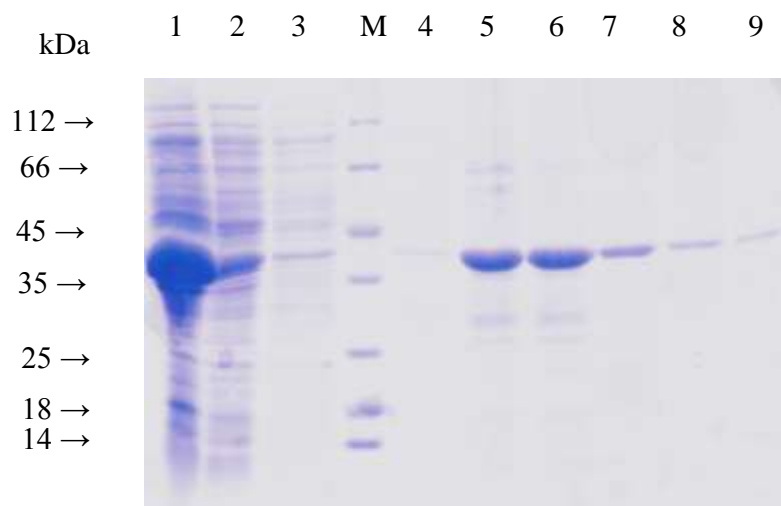
Figure 4. (A) SDS-PAGE of the overexpressed and purified of rASPG in *E. coli* BL21(DE3) (Lane M: molecular mass of standard proteins (Fermentas, Thermo Fisher Scientific Inc.,Waltham, USA); lane 1-2: *E. coli* pEaspg cell lysate after (1) and before (2) optimal conditional expression; lane 3: *E. coli* pEaspg cell lysate before IPTG induction lane 4-5-6: fractions of purified rASPG eluted from Sephacryl S-200, lane 7-8-9: fraction of purified rASPG eluted from DEAE-sepharose.

Table 1. Purification procedure of rASPG from the cell lysate of *E. coli* BL21/pEaspg.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Cell lysis	385.1	9.6	39.9	100	1
Sephacryl S-200	360.1	2.5	141.3	93.5	3.5
DEAE-Sepharose	68.5	0.2	312.8	17.8	7.8

densitometry scanning, resulting in 10% increase in the production compared to the original condition. The expression level of rASPG in *E. coli* system was reported to be approximate 50% in JM105, TG1, DH5α, AS1.357 and 75% in JM109 (Wang et al., 2001). The rASPG was purified from the cell lysis of *E. coli* BL21(DE3) by filter chromatography Sephacryl S-200 and DEAE Sepharose showed only one protein band about 37 kDa on SDS-PAGE (Figure 4, lane 7-9). The specific activity of recombinant L-asparaginase after two step purification obtained by 312.8 U/mg with a yield of 17.8% and purification factor of 7.8 (Table 1). The specific activity was very different: The activity of purified recombinant L-asparaginase II from *E. coli* K-12 express in *E. coli* BLR(DE3) was 190 U/mg (Khushoo et al., 2004), recombinant L-asparaginase II from *Erw. chrysanthemi* 3937 express in *E. coli* BL21(DE3) pLysS was 118.7 U/mg (Kotzia and Labrou, 2007), L-asparaginase II from *B. subtilis* express in *E. coli* JM109 (DE3) was 45.5 U/mg (Onishi et al., 2011) L-asparaginase from *Rhizomucor miehei* express in *E. coli* was 1,985 U/mg (Huang et al.,

2014) and activity of purified L-asparaginase from *B. licheniformis* was 697.09 U/mg (Mahajan et al., 2014).

Characteristic of rASPG

Temperature and pH optimum

The recombinant L-asparaginase from *Erw. chrysanthemi* had optimum temperature of 45°C (Figure 5A) and optimum pH of 7.5 in 100 mM Tris-HCl buffer (Figure 5B). It was similar to recombinant L-asparaginase II from the *B. subtilis* B11−06 in *B. subtilis*168 which had an optimum temperature of 45ºC and pH 7.5 (Jia et al., 2013). It was different from that of E. coli MTCC739 in *E. coli* BL21(DE3), which were 37°C and pH 6 (Vidya et al., 2011). The optimal temperature and pH of rASPG from a thermotolerant strain *E. coli* KH027 in *E. coli* DH5α was 43°C and pH 6 (Muharram et al., 2014). It was 37°C and pH 7.5 for rASPG from *E. coli* W3110 in *E. coli* BL21 (DE3) (Magdy and Mohammed, 2008) and that of rASP
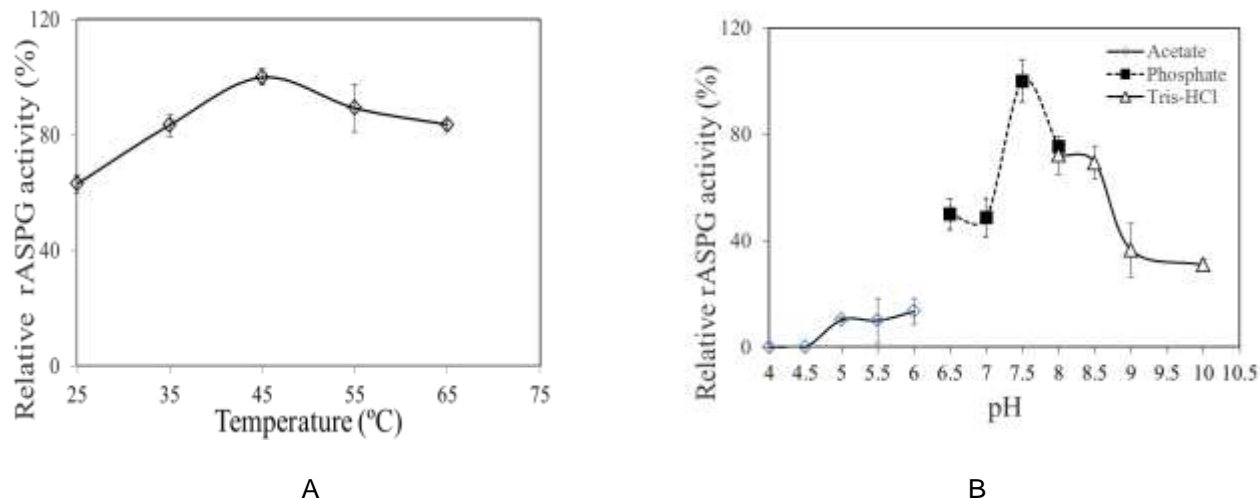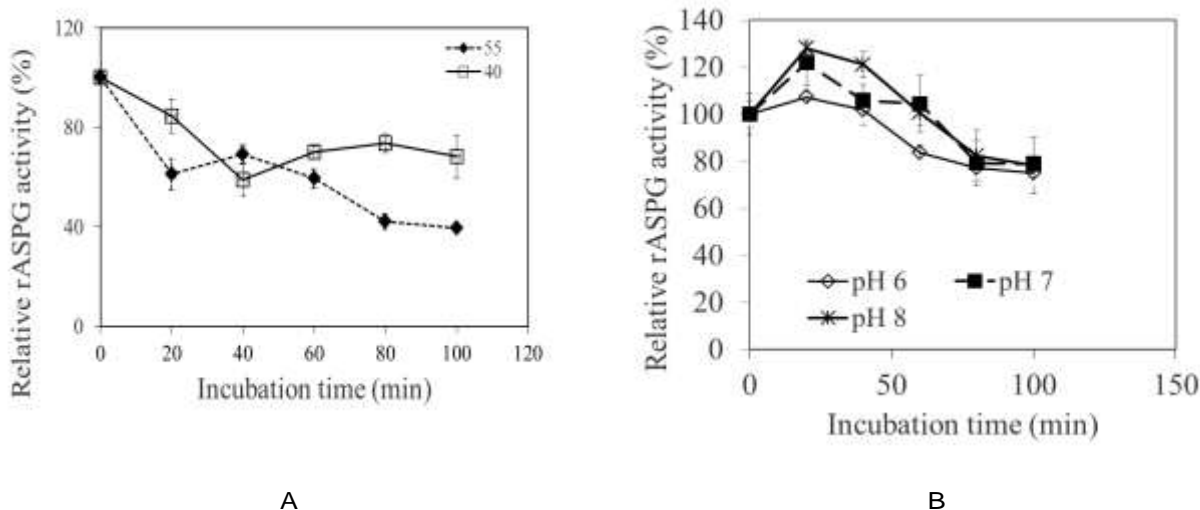
A

B

Figure 5. Temperature optimum of rASPG (A) and pH optimum of rASPG (B).

A
Relative activity was expressed as a percentage of control (100% rASPG activity was 293.7 U/mg)

B
Relative activity was expressed as a percentage of control (100% rASPG activity was 321.4 U/mg

Figure 6. Temperature stability of rASPG (A) and pH stability of rASPG (B).

G from *Withania somnifera* in *E. coli* BL21(DE3) was 37°C and pH 8 (Oza et al., 2011). The optimum temperature and pH of the wild L-asparaginase from *Cladosporium sp.* were 30°C and 6.3, respectively (Kumar and Manonmani, 2013). Consequently, the optimal temperature and pH of the recombinant enzyme is not same with different bacterial sources and different expression host.

pH and thermo stability

The thermal stability of the purified enzyme at 40 and 55°C was studied to find out the extent of temperature

resistance of the enzyme. Around 60 and 30% of the initial activity was retained by the purified enzyme after 100 min of incubation at 40 and 55°C, respectively (Figure 6A). The earlier reports on the thermostability of different L-asparaginase preparations indicate that the native enzymes were unstable at high temperatures. Wild L-asparaginase from *Erw. chrysanthemi,* which expressed in *E. coli* BL21(DE3) retains ~40% of its initial activity after 7.5 min of incubation at 50°C and on mutagenesis, around 20% increase in activity retention was achieved (Kotzia and Labrou, 2009). The wild enzyme from *E. coli* W3110 that expressed in *E. coli* BL21 (DE3) retained about 22% of its activity at 60°C after an incubation of 30 min, the remaining activity of the

Table 2. Effect of metal ions on the rASPG activity.

Additives[a]	Residual activity (%)[b]
Control (no additive)	100 ± 6
$Al_2(SO_4)_3$	82 ± 1.7
$Ba(NO_3)_2$	109 ± 7
$CaCl_2$	88 ± 6
$CuSO_4$	77 ± 6
$FeSO_4$	91 ± 6
$HgCl_2$	115 ± 2
$MgSO_4$	72 ± 2
$MnSO_4$	88 ± 6
$NaCl_2$	108 ± 3
$NiCl_2$	123 ± 3
$Pb(NO_3)_2$	92+7
$ZnSO_4$	86 ± 6
$Al_2(SO_4)_3$	107 ± 2
$Ba(NO_3)_2$	100 ± 5
$CaCl_2$	72 ± 7

[a] The final concentration of additive (EDTA or inorganic salt) in the reaction mixture was 10 mM. [b] Relative activity was expressed as a percentage of control (100% rASPG activity was 307.4 U/mg).

Table 3. Effect of detergent on the rASPG activity.

Detergent	Residual activity (%)	
	1%	5%
Tween 20	87.65 ± 8	0
Tween 80	54.35 ± 2	0
Triton X-100	47.91 ± 5	31 ± 12
Triton X-114	45.76 ± 2	0

Relative activity was expressed as a percentage of control (100% rASPG activity was 207 U/mg).

Table 4. Effect of DTT on the rASPG activity.

Concentration of DTT (mM)	Residual activity (%)
0.1	98.10 ± 1.39
0.5	109.89 ± 4.17
1	146.57 ± 11.58
5	119.06 ± 9.73

Relative activity was expressed as a percentage of control (100% rASPG activity was 203.67 U/mg).

immobilized enzyme after similar treatment was 66.8% (Magdy and Mohammed, 2008). rASPG showed pH stability at a pH range 6-8. The residual rASPG activity was above 80% in comparison to the original activity after 100 min of treatment (Figure 6B).

Effect of metal ions and EDTA, detergents, DTT, DMSO

The rASPG activity was inhibited by EDTA (Table 2). This results is in agreement with results reported for L-asparaginase from *Actinomycetes* (Basha et al., 2009) and L-asparaginase from *Thermococcus kodakarensis* KOD1 in *E. coli* (Hong et al. 2014). But several researches reported that EDTA enhanced the enzyme activity (Raha et al., 1990; Warangkar and Khobragade, 2010).

Metal ions showed that rASPG inhibitory activity of 92-72% in order of Na^+ > K^+, Fe^{3+} > Ca^{2+} > Ni^+ >Al^{3+} >Cu^{2+} >Hg^{2+} while Mn^{2+} enhancer activity of the enzyme by 123%. And Pb^{2+}, Mg^{2+}, Ba^{2+} showed a slightly enhance effect on rASPG activity, the residual activity was accounted for 108% -109% of the original activity and Zn^{2+} was not effect enzyme activity (Tab.2). The same results was also found in the report that the activity of native L-asparaginase from *Cylindrocarpon obtusisporum* MB-10 was inhibited by Zn^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+} and Ni^{2+} (Raha et al. 1990) and the activity of rASPG from *Thermococcus kodakarensis* KOD1 was inhibited by Ca^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, enhanced by Mg^{2+} (Hong et al.,

2014). Warangkar and Khobragade, (2010) reported that L-asparaginase form *Erw. carotovora* was loss of activity with Hg^{2+}, Ni^{2+}, Cd^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+}, too, but Na^+ and K^+ acting somewhat as an enhancer and Mg^{2+} inhibited enzyme activity. All detergents (Tween 20, Tween 80, Triton X-100, and Triton X-114) showed an inhibitory effect on rASPG activity. The higher concentration, the detergents are more inhibited enzyme activity (Table 3).

Interestingly, the addition of DTT at low concentration of 0.1 - 0.5 mM was not significantly effected enzyme activity, but at higher concentration of 1 - 5 mM enhanced the enzyme activity by 46% and 19%, respectively (Table 4). L - asparaginase of activity from *Erw. carotovora* was aslo reported to enhance in presence of thiol protecting reagents like DTT and it has been explained by asparaginase possesses the thiol group binding domain with high affinity towards free-SH group containing effectors (Warangkar and Khobragade, 2010). The addition of lower concentration DMSO (0.1 and 0.5%), enzyme activity was not effect, but the rASPG activity remained much higher 152% by the addition of DMSO at higher concentration (1 mM). When concentrations of DMSO was higher (1.5; 2%), the level of increased enzyme activity were decreased (Table 5).

Kinetic parameters

The K_m, V_{max}, K_{cat} and K_{cat}/K_m obtained for rASPG *Erw. chrysanthemi* expressed in *E. coli* with L-asparagine

Table 5. Effect of DMSO on the rASPG activity.

Concentration of DMSO (%)	Residual activity (%)
0.1	101.70 ± 0.93
0.5	108.25 ± 0.93
1	152.46 ± 10.65
1.5	122.01 ± 12.97
2	112.84 ± 7.41

Relative activity was expressed as a percentage of control (100% rASPG activity was 203.67 U/mg).

Table 6. Cell death of four tumor cell lines after treatment with rASPG.

Concentration (µg/ml)	Cell death (%)				
	NIH/3T3	HL-60	P388	P3X63Ag8	SP2/0-Ag14
0.4	-0.22	-2.05	0.98	0.56	0.98
2	1.76	6.91	8.23	12.79	8.23
10	4.92	16.09	19.45	27.18	19.45
50	18.02	45.32	48.22	53.68	51.22
IC_{50}	> 50	> 50	> 50	41.67	48.09

substrate were 0.5 mM, 500 U/mg, 14.9×10^3 s^{-1}, 29.9×10^3 $mM^{-1}s^{-1}$, respectively. The K_m, V_{max}, K_{cat} and K_{cat}/K_m value of the recombinant L - asparaginase from *Thermococcus kodakarensis* KOD1 expressed in *E. coli* BLR(DE3) were found to be 2.6 mM, 1121 U/mg, 694 s^{-1}, and 266.9 $mM^{-1}s^{-1}$, respectively (Hong et al. 2014), of recombinant L-asparaginase from *B. subtilis* in *E. coli* were 2.06 mM, 45.5 U/mg, 98.6 s^{-1}, and 48 $mM^{-1}s^{-1}$ respectively (Onishi et al. 2011). The K_m, K_{cat} and K_{cat}/K_m value of rASPG from *Erw. chrysanthemi* 3937 in *E. coli* BL21(DE3) pLysS were 0.058 mM, 23.8×10^3 s^{-1}, and 411.8×10^3 $mM^{-1}s^{-1}$ respectively (Kotzia and Labrou, 2007). It was found that rASPG had low K_m value, high V_{max}, K_{cat} and K_{cat}/K_m value. So rASPG is highly specific for the substrate L-asparagine.

Anti-cancer activity of the rASPG

Abakumova et al. (2013) provided convincing evidence that L-asparaginase induced apoptosis as its principal process of causing death of leukemic and solid tumor cells (Abakumova et al., 2013). In our study, four tumor cell lines with increasing amounts of rASPG resulted in a significant increase in the number of dead cells (Table 6). At dose 0.4 µg/ml, rASPG did not significantly affect the growth of cells but at dose 50 µg/ml, rASPG inhibited HL-60 (45.32%), P388 (48.22%), P3X63Ag8 (53.68%), and SP2/0-Ag14 (51.22%). Normal cells were not significantly affected by rASPG at dose 50 µg/ml rASPG inhibited 18.02 % for NIH/3T3.

Muharran et al. (2014) indicated that recombinant L-asparaginase in *E. coli* caused a reduction of 50% in cell viability of RS4 at a dose of 100 µg/ml after 96 h of incubation, and a reduction of 50% in cell viability of HL-60 at a dose of 200 µg/ml after 72 h of incubation (Muharram et al. 2014). L- asparaginase from *Streptomyces acrimycini* NGP (130 U/mg) inhibits the gastric stomach cancer cells with an IC_{50} of 49.11 µg/ml (Selvam and Vishnupriya, 2013). L-Asparaginase from bean (*Vicia faba)* and white kidney bean (*Phaseoulus vulgaris)* seeds (2.75 and 1.47 U/mg) had low IC_{50} values 217.71 µg/ml and 187.86 µg/ml with Hep-G2 cells (Sanaa et al., 2012). IC_{50} value of L-asparaginase from *Aspergillus flavus* on MCF-7 cells was 120.875 µg/ml (Rani et al., 2011)

Conclusion

In conclusion, L-asparaginase was expressed in *E. coli* BL21 (DE3) at high level expression. After purification, a single band indicative of purified protein was recorded. The purified enzyme was 7.8 folds with a final specific activity of 312.8 IU/mg protein and about 17.8% yield recovery. Temperature and pH optimum for rASPG were 45°C and 7.5, respectively. The activity of enzyme was enhanced by Mn^{2+}, Pb^{2+}, Mg^{2+}, Ba^{2+} and inhibited by EDTA, Na^+, K^+, Fe^{3+}, Ca^{2+}, Ni^+, Al^{3+}, Cu^{2+}, Hg^{2+} and detergents (Tween 20, Tween 80, Triton X-100, and Triton X-114). Also, DTT at a concentration of 1 mM and DMSO at a concentration of 1% enhanced the enzyme's activity. Recombinant enzyme had high anti-cancer activity. The number of apoptotic cells significantly

increased after rASPG treatment experimental wells by the absorbance of the control (untreated) wells.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Ploidy level of the banana (*Musa* spp.) accessions at the germplasm collection centre for the East and Central Africa

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Banana Germplasm Collection serves as a source of useful genes for banana breeding. However, insufficient and/or inaccurate information on the ploidy level of the germplasm renders its utilization in breeding difficult. The objective of this study was to determine and validate the ploidy level of 120 banana accessions in the *ex situ* germplasm collection centre for the East and Central Africa, located in Mbarara, Uganda. Flow cytometric analysis of the nuclear DNA content was used to determine the ploidy level of the accessions. Results indicate that accessions: Bura, Diana, Kambani-Rungwe, Paji and Pagatau, and Rungwe that were previously classified as diploids are actually triploids, whereas Selangor previously known to be a diploid is a tetraploid. Accessions such as Galeo, Mwitupemba and Ntindi 1 that were previously classified as triploids were found diploids. GT, FHIA 25 and Muzungu Mwekundu that were considered as tetraploids, were found triploids. The information generated will guide correct placement of these accessions in the regional germplasm collection centre for the East and Central Africa and their utilization in banana breeding.

Key words: Banana germplasm, breeding *ex situ* germplasm collection, flow cytometry, ploidy.

INTRODUCTION

The regional *ex situ* Banana Germplasm Collection (BGC) Centre for the East and Central Africa (ECA), located in Mbarara district, Uganda, was established in 1998 to serve as the banana reference collection for the ECA and to house duplicate banana accessions of the formerly Uganda National Agricultural Research Organization (NARO)-Kawanda banana collection. The collection was re-established in 2008 to serve as a

back-up and regional repository for genetic improvement of banana and plantain in the region. This was after an agreement reached by the Banana Research Network for East and Southern Africa (BARNESA) steering committee meeting held in Dar-es-Salaam, Tanzania in 2007 (Bioversity International, 2007).

The BGC is managed and maintained by Bioversity International in collaboration with NARO on behalf of BARNESA. Its main goal was to conserve maximum banana diversity from the East African region and to provide genes of desired traits for the banana genetic improvement activities initiated by Bioversity International in early 2009 (Bioversity International, 2010, 2011). Since 2009, the BGC in Mbarara has been enriched through collecting and planting new and unique accessions from both the East African region and International banana research Institutes. In addition, since its re-establishment, the collection has acquired 120 accessions, both local and exotic, with most of the accessions being landraces. Priority for acquisition of new accessions was given to the areas in the region that were not well represented in the BGC in Mbarara (Karamura et al., 2013). After acquisition, the accessions are evaluated for traits of interest such as resistance to pests, diseases, environmental stresses and agronomic attributes. The results of evaluation are thereafter forwarded to breeders for subsequent utilization in the banana breeding programs. Accessions from the collection are also disseminated to different stakeholders, especially students and researchers in the region, particularly to provide a platform for support to people interested in gaining knowledge of identification and description of banana.

A number of ploidy levels exist in *Musa* spp. (Tenkouano et al., 2011). Knowledge of ploidy level in *Musa* accessions is vital for breeding, conservation and tissue culture as they are affected by ploidy (Suman et al., 2012). Accurate determination of ploidy of germplasm has practical implications for breeding a perennial crop like banana that has got a long generation time and extensive land requirements (Pillay et al., 2006). Ploidy level influences fertility of banana. For instance, most triploids are sterile while diploids and tetraploids are fertile (Tenkouano et al., 2011). Depending on ploidy information, breeders are able to decide on the materials to evaluate for banana variety development. Banana breeding usually involves the transfer of useful genes from diploids to triploids by carrying out 3x by 2x crosses. Such a cross can generate a variety of progeny with ploidy levels ranging from diploid, triploid, tetraploid, aneuploidy and hyperploid progeny (Pillay et al., 2002). It is important that the ploidy of banana accessions be verified prior to using them for breeding.

Whereas, the ploidy level of most accessions in the regional *ex situ* BGC in Mbarara was reported (Karamura et al., 2016), the results were not exhaustive. Accessions in this collection are planted in three separate blocks based on three ploidy levels (2x, 3x and 4x). The first block of the collection is planted with diploids, the second; with triploids (excluding the East African Highland bananas and tetraploids, and the third with triploid East African Highland bananas, which are the major local cultivars in the East African region.

Ploidy level of the accessions that were added to the BGC since 2010 collection missions was determined from accessions' morphological appearance. Studies have however revealed that ploidy level of banana determined primarily by morphological characteristics may not be reliable (Pillay et al., 2003, 2006). Banana ploidy level is determined by other several methods, of which flow cytometry has been found user-friendly, faster and reproducible for screening a large number of accessions (Takayama et al., 2011). In addition to chromosome counting, which is slow and labour intensive, there are other indirect methods such as estimation of stomata size and density, which are not accurate (Vandenhout et al., 1995; Dolezel et al., 1998). The present study assessed the ploidy level of additional 120 accessions in the collection using the flow cytometry method. This was done to verify the ploidy level of these accessions in order to guide their placement in the correct blocks at the collection centre, as well as to provide accurate ploidy information of these 120 banana accessions to breeders.

MATERIALS AND METHODS

Plant material

Fresh midrib tissue samples of approximately 100 mg from cigar leaves of 120 accessions were collected from the *ex situ* BGC in Mbarara. These samples were individually chopped using a sharp razor blade in a disposable Petri dish containing 0.5 ml of cold OTTO I buffer (0.1 M citric acid monohydrate and 0.5% Tween 20) to form a homogenate. An additional 0.5 ml of cold OTTO I was added to the homogenate and mixed thoroughly. The homogenate was filtered through a nylon filter of 50 μm pore size into a polystyrene tube. The samples were incubated for 1-5 min with occasional shaking. Prior to analysis, 2 ml of OTTO II (0.4 M anhydrous disodium phosphate, 4 μg/ml (2 mg/500ml) of 4,6–diamidino-2-phenylindole (DAPI) and 1 μl/ml of β-mercaptoethanol) were added to each sample. This is the staining solution that allows measurement of fluorescence due to the presence of DAPI.

Flow cytometric analysis

Relative fluorescence intensity of stained nuclei was analysed using a Partec Ploidy Analyser (Partec GmbH, MÜnster, Germany) with a mercury arc lamp. The distribution of fluorescence intensities (relative DNA content) obtained after flow cytometric analyses are usually given as channel numbers (arbitrary units). For ploidy screening, the instrument was calibrated using "Calcutta 4" as a reference (standard) diploid (2x) with its peak set at channel 50. TMB4x660K-1 on the other hand was used as the reference tetraploid (4x) with its peak set at channel 100, while Enyeru, an EAHB was used as a reference triploid (3x) with its peak set at channel 75. The peaks of the unknown samples were determined by examining the position of their peaks relative to the reference accessions. All the samples with peaks at channel 50±5 were

Table 1. Expected ploidy levels of the 120 banana accessions before analysis and the observed ploidy levels after analysis using the flow cytometry method.

S/N	Accession name	Expected ploidy	Observed Ploidy	CV (%)	S/N	Accession name	Expected ploidy	Observed ploidy	CV (%)
1	Rwoyalwansega	3x	3x	5.7	61	Butuhan	2x	2x	5.6
2	Ilalyi	3x	3x	4.5	62	Mjenga Michael	2x	2x	5.4
3	Cula	3x	3x	4.6	63	Gashulie	3x	3x	5.0
4	Namutobisho	3x	3x	3.9	64	Nalwezinga	3x	3x	2.5
5	Luholele	3x	3x	4.3	65	Kasenene	3x	3x	5.4
6	Haahaa	3x	3x	3.5	66	Kambani-Rungwe	2x	3x	6.7
7	Kalasa	3x	3x	5.3	67	Bitambi	3x	3x	7.7
8	Mlema	3x	3x	4.5	68	Kirun	2x	2x	6.3
9	GT	4x	3x	4.7	69	Cultivar Foce	2x	3x	6.1
10	Ntindi 1	3x	2x	6.7	70	Mlambichi	2x	2x	6.5
11	Logiri 1	3x	3x	6.7	71	Pisang Mas	2x	2x	5.6
12	Paji	2x	3x	4.6	72	Oruhuna	3x	3x	6.4
13	Ntebwe	3x	3x	6.5	73	Obutsipa	3x	3x	6.6
14	Kabila	3x	3x	6.1	74	Katejurantamere	3x	3x	6.0
15	Kikonjekonje	3x	3x	5.3	75	Inyumbu	3x	3x	7.5
16	Ekitabwila	3x	3x	4.9	76	Mlelembo	2x	2x	6.5
17	Kanjabu	3x	3x	5.2	77	SH-3362	2x	2x	8.2
18	Engotte	3x	3x	5.2	78	Ingoromora	3x	3x	5.1
19	PV 0344	4x	4x	5.5	79	Paka	2x	2x	6.9
20	Babyesala	3x	3x	6.6	80	9722-1	2x	2x	8.8
21	Opu (Nyakisangani)	3x	3x	5.2	81	Kabana 6H	3x	3x	4.1
22	Nyamabere	3x	3x	5.3	82	Kahuma	3x	3x	5.9
23	Short Gros Michel	3x	3x	6.9	83	Ndiibwabalangira	3x	3x	5.1
24	Ibwi	3x	3x	7.3	84	Nyalambya	3x	3x	4.5
25	FHIA 25	4x	3x	6.7	85	Galeo	3x	2x	9.3
26	Kitarasa	3x	3x	6.4	86	Green Red	3x	3x	6.5
27	TMB x 25511/2	3x	4x	4.8	87	Logiri2	3x	3x	7.1
28	Menvu	3x	3x	6.6	88	Enkongo	3x	3x	5.7
29	Suu	3x	3x	9.2	89	Intariho	3x	3x	6.6
30	Marimbi	3x	3x	4.8	90	548/4 PITA 1	3x	3x	6.4
31	Ensika	3x	3x	6.0	91	Nyerere	3x	3x	5.3
32	Selangor	2x	4x	5.5	92	Nyamahwa	3x	3x	6.2
33	Mbiya	3x	3x	4.5	93	Maganya	3x	3x	5.7
34	Kikundi	3x	3x	6.8	94	Eti Kehel	2x	2x	6.9
35	Enyanshenyi	3x	3x	6.3	95	Halahala	2x	2x	7.3

Table 1. Contd.

36	Nansaba	3x	3x	6.3	96	Pitu	2x	2x	8.1
37	548/9/Pita 2	3x	4x	3.9	97	Ndyali	2x	2x	7.7
38	Saba	3x	3x	4.3	98	Red bogoya	3x	3x	6.2
39	Galeo	3x	2x	8.9	99	Namaliga	3x	3x	7.2
40	Itoke	3x	3x	3.9	100	Bura	2x	3x	6.9
41	Nyarwewunzika	3x	3x	9.0	101	Enyamanshari	3x	3x	5.7
42	M17	3x	3x	5.9	102	Enyamara	3x	3x	6.4
43	Majabaga	3x	3x	3.7	103	Entudde	3x	3x	4.3
44	Kijakazi	3x	3x	6.0	104	Inyarwanda	3x	3x	4.9
45	Enyabongere	3x	3x	3.6	105	Pagatau	2x	3x	5.2
46	KM5	3x	3x	5.3	106	Siira (-EA)	3x	3x	8.1
47	Morong Princesa	2x	2x	8.9	107	Namwezi	3x	3x	5.8
48	Poyo	3x	3x	7.0	108	TMB x 1378/Bita 2	4x	4x	4.5
49	Mwitupemba	3x	2x	6.3	109	Kitombo	3x	3x	4.5
50	Muvubo	3x	3x	6.5	110	Nakinyika	3x	3x	5.4
51	1652-4	3x	3x	4.7	111	Kattabunyonyi	3x	3x	6.4
52	8386S-19	3x	3x	5.8	112	Umburasika	3x	3x	6.3
53	Mtahato	3x	3x	4.9	113	Mukazi-alanda	3x	3x	6.8
54	Rutare	3x	3x	5.4	114	Empuramura	3x	3x	5.4
55	Ntindi 2	3x	3x	7.4	115	Nakhaki	3x	3x	5.4
56	TMB x 5295-1/Bita 3	4x	4x	4.3	116	Nante	3x	3x	4.1
57	Mzungu Mwekundu	4x	3x	6.0	117	Inkurura	3x	3x	4.4
58	Isha	3x	3x	5.6	118	Namunwe	3x	3x	5.6
59	Diana	2x	3x	5.0	119	Enyanja	3x	3x	5.7
60	TT2	2x	2x	6.9	120	Nabuyobyo	3x	3x	5.6

considered diploids, while those at channel 75±5 were triploids and those at channel 100±5 were tetraploids.

RESULTS AND DISCUSSION

The coefficients of variation for the samples analysed were less than 10% (Table 1), indicating the quality of preparation of samples and reliability of the results. Ploidy levels of the 120 accessions analyzed are shown in Table 1. Most of the accessions were triploids (83%), followed by diploids (16%) and tetraploids (1%). Following these results, accessions were reallocated to their respective correct blocks at the regional germplasm collection centre as shown in Table 2.

It is interesting to note that Selangor (*Musa acuminata*) previously reported as a diploid (Pillay et al., 2006; de Jesus et al., 2013), displayed tetraploid nuclei in the present study (Figure 1).

The inter-study result differences could be ascribed to the presence of different cytotypes in banana. Possibly chromosome counting from a large number of accessions from a wide geographical range is necessary to determine the existence of cytotypes in Selangor. Changes in ploidy levels in bananas may occur when plants are maintained under *in vitro* conditions (Dolezelova et al., 2005).

Banana taxonomists have always assigned

Table 2. Properly rearranged *Musa* accessions based on the confirmed ploidy levels after ploidy analysis.

S/N	Accession	Ploidy level	Blocks where accessions were before determination of ploidy level	Blocks where accessions are after determination of ploidy level
1	Diana	3x	Block 1	Block 2
2	Bura	3x	Block 1	Block 2
3	Kambani Rungwe	3x	Block 1	Block 2
4	Pagatau	3x	Block 1	Block 2
5	Galeo	2x	Block 2	Block 1
6	Ntindi 1	2x	Block 2	Block 1
7	Mwitu Pemba	2x	Block 2	Block 1
8	Mzungu Mwekundu	3x	Block 2	Block 2
9	GT (tetraploid)	3x	Block 2	Block 2
10	Cultivar Foce	3x	Block 2	Block 2
11	FHIA 25	3x	Block 2	Block 2
12	Selangor	4x	Block 2	Block 2
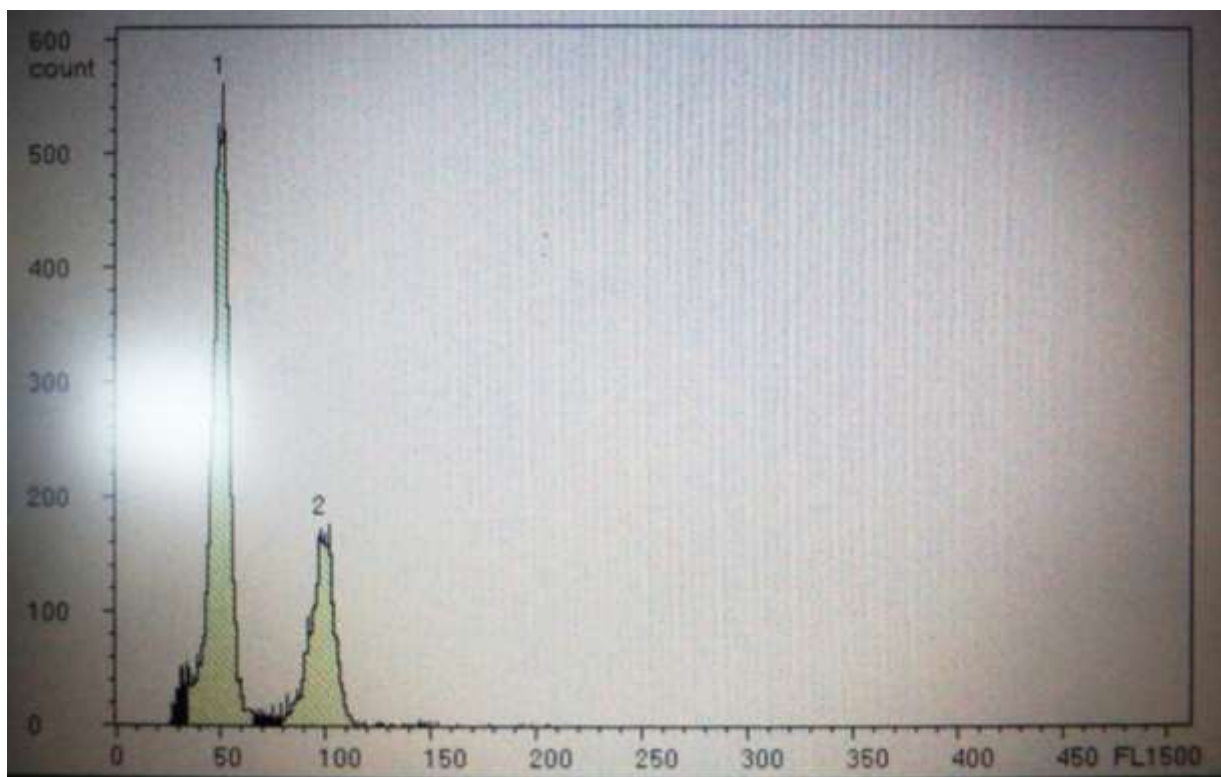
Figure 1. Histogram showing Selangor as a tetraploid with its peak at channel 100 and Calcutta 4, a diploid (control genotype) with its peak set channel at 50.

ploidy levels to different accessions on the basis of morphological traits such as leaf orientation, and on the basis of physiological, cellular and biochemical aspects (Mustafa, 2013). Polyploids such as bananas are often apparent by their distinct and robust morphology (Briggs and Walters, 1984). However, diploids are delicate in nature, lean in size and even perish in harsh weather conditions. Following this system, plants with erect leaves are considered diploids while those with drooping leaves as tetraploids, and those with an intermediate leaf orientation as triploids. This method of ploidy determination is subjective and not always reliable. It

becomes even more unreliable when evaluating hybrids from a breeding program that consist of a mixture of ploidy levels including aneuploids.

The various indirect methods of determining banana ploidy level, for example by estimating stomata size and density (Vandenhout et al., 1995) or measurement of pollen grain sizes were reported (Tenkouano et al., 1998). While these methods depend on statistical analysis to determine ploidy, they are not accurate because the measured parameters are greatly influenced by changes in the growth environment (Xu and Zhuo, 2008). Therefore, chromosome counting remains the only accurate method of ploidy level determination in banana. However, the method requires cell synchronization to metaphase stage for easy visualization of chromosomes. The technique is not routinely used because it is labour intensive and obscure by the low quality of squash slide preparations (Dolezel et al., 1998). The ploidy of plants with large chromosomes can easily be determined by chromosome counting but bananas present a challenge due to its small chromosomes which are always hard to spread out during squash preparations (Dolezel et al., 1998; Pillay and Tenkouano, 2011). Flow cytometry is a user-friendly technique, considering the fact that it is faster and reproducible for screening large number of accessions.

With the flow cytometry ploidy analysis methods, the banana ploidy level is determined by measuring the cell nuclear DNA content and subsequently comparing the relative position of the sample   peak to that of the reference accession. With this approach, synchronization of cells is not required. Cells in G_1 and G_2 phases can be differentiated easily including aneuploids. The amount of fluorescence given off by the cell nucleus is directly proportional to the DNA content, which in turn positively correlates with the number of chromosomes. Therefore, increase in ploidy level is perceived as a shift in peak position to the right. This method together with chromosome counting was used to confirm chromosome number of Sukali ndizi, which for a long time was reported as diploid AB instead of triploid AAB (Pillay et al., 2003). Unfortunately, plant cytogenetic does not appear to be the *forte* of many researchers since the advent of molecular biology, yet it is key in answering basic questions for breeders. Misallocation of ploidy levels to different accessions remains a challenge in banana germplasm collections, which calls for deliberate efforts to embrace cytogenetic tools.

Using flow cytometry, previous studies have shown inconsistencies in ploidy levels of banana accessions whose ploidy was determined based entirely on morphological traits (de Jesus et al., 2013; Dolezel et al., 1994; Irish et al., 2009; Pillay et al., 2006; Nsabimana and van Staden, 2006). Knowledge of the ploidy of bananas is valuable for banana breeding schemes as it involves interploidy crosses leading to several possible ploidy levels in the progeny. Flow cytometry provides a rapid way of determining ploidy levels in this crop.

Conflict of Interests

The authors have not declared any conflict of interests.

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