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Production of Bioethanol from Wild Cassava *Manihot glaziovii* through Various Combinations of Hydrolysis and Fermentation in Stirred Tank Bioreactors

Anselm P. Moshi[1,2,3], Ken. M. M. Hosea[2], Emrode Elisante[2], Anthony Manoni Mshandete[2] and Ivo Achu Nges[1*]

[1]Division of Biotechnology, Lund University, P.O.Box 124, SE-221 00 Lund, Sweden.
[2]Department of Molecular Biology and Biotechnology, College of Natural and Applied Sciences, University of Dar es Salaam, P.O.Box 35179, Dar es Salaam, Tanzania.
[3]Tanzania Industrial Research and Development Organization (TIRDO), Kimweri Avenue, TIRDO Complex, P.O.Box 23235, Dar-es Salaam, Tanzania.

Authors' contributions

This work was carried out in collaboration between all authors. Author APM designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author IAN managed the analyses of the study and participated in writing of the first draft. Authors EE, KMMH and AMM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim of the Study: The aim of this study was to evaluate three ethanol fermentation approaches namely (I) separate hydrolysis and common fermentation (II) separate hydrolysis and fermentation and (III) simultaneous saccharification and fermentation in stirred tank reactors using inedible wild cassava as feedstock.
Study Design: Tubers of wild cassava (*Manihot glaziovii)* were obtained from two districts in

**Corresponding author: Email: nges.ivo_achu@biotek.lu.se;*

Tanzania. Fermentation of hydrolysate and partially liquefied cassava flour was performed in stirred tank reactors.

Methodology: Feedstock composition analysis for structural carbohydrate was performed using acid hydrolysis and high pressure liquid chromatography technique. Analysis of total nitrogen was done by Kjeldahl acid digestion technique, total cyanide was determined using linamarase loaded picrate paper whereas macro-and micronutrients were analysed by inductively coupled plasma atomic emission spectrometry. Thermostable α-amylase and glucoamylase were used to partially hydrolyze the cassava flour to fermentable sugars prior to yeast fermentation. The hydrolysis (liquefaction) was performed at 90°C, 1h followed by saccharification using glucoamylase at 60°C, 2h for approaches I and II. For approach III, liquefaction was performed at 90°C, 1h followed by direct saccharification and fermentation. Fermentation of hydrolysate and partially liquefied starch from wild cassava was done in stirred tank reactors at 30±2°C using baker's yeast.

Place and Duration of Study: Department of Biotechnology, Lund University from January to June 2014.

Results: The wild cassava (*M. glaziovii*) tubers possessed comparable physical dimensions to the domesticated cassava, however they displayed higher average flesh proportion (76 to 79%) compared to the domesticated cassava (74%). Compositional analysis disclosed that the wild cassava possessed interesting properties for bioethanol production such as dry matter of up to 89% w/w, degradable carbohydrate up to 90% (dry weight basis), total kjeldahal nitrogen 0.8-1.6% w/w and satisfactory concentration of macro-and micronutrients. Amongst the three fermentation approaches, high ethanol titre of 10-11% (v/v) at high conversion efficiency of 97.6% was achieved for separate hydrolysis and fermentation and simultaneous saccharification and fermentation, whereas low ethanol titre (4.2% v/v) at efficiency of 39% was achieved for separate hydrolysis and common fermentation. Volumetric productivities for the three approaches; 'separate hydrolysis and common fermentation', 'separate hydrolysis and fermentation', and 'simultaneous saccharification and fermentation' were 2.0, 5.5 and 6.5 respectively.

Conclusion: The results obtained in the present study demonstrated that wild cassava has a high starch content, contain balanced nutrients required for efficient bioethanol production and that simultaneous saccharification and fermentation is the best approach for bioconversion of the wild cassava to bioethanol using stirred tank reactors.

Keywords: Bioethanol; fermentation; hydrolysis; wild cassava.

1. INTRODUCTION

The main limitation as regards the use of bioethanol in lieu of fossil fuel is its high price, which is attributed mainly to the costs of feedstock and bioconversion (efficiency) to bioethanol. During bioethanol production, feedstock cost contributes between 50 to 70% of total cost of production [1,2]. The fossil energy used in both production and processing of feedstock to bioethanol impacts negatively not only on price of the final product, but also on the prospected environmental benefits. Therefore, as far as first generation bioethanol production is concerned, research efforts are focused on novel inedible starch/sugar-rich feedstocks as well as on improved productivity and efficiency of the bioconversion technologies used for processing the traditional energy crops (such as sugarcane, sugar beet, corn, wheat, rice, sweet potato, potatoes, cassava etc.) [3-5]. Cassava has been identified as a suitable feedstock for bioethanol production because of its high starch content (70-85% on dry weight basis) and high biomass production per ha [6]. For example, cassava production is reported to be between 30-40 tons of biomass per ha which translates to 4800-7600 litres of bioethanol per ha as compared to corn, wheat and rice which are estimated at 2600, 2000 and 2850 litres of bioethanol per ha respectively [6,7].

In addition, cassava can be cultivated on poor soils wherein other crops cannot be grown profitably making it a cheaper feedstock [8]. Cassava is grown mainly in the tropics e.g. in Southern Asia, Africa and South America [9,10]. In Africa, cassava (*Manihot esculentum)* is a staple food in many countries where it is produced in large amounts, e.g. in Western and Eastern Africa cassava is synonymous to food security [11]. Accordingly, cassava cannot be prospected as an energy crop without prior improvement and expansion of the overall productivity [12]. However, some wild inedible cassava species are known to form significant

tubers [13] and these tubers can be use as cheap and readily available feedstock for bioenergy production. The wild cassava tubers are inedible because of higher cyanide content. Compared to the cultivated cassava *Manihot esculentum*, some wild cassava species are very resilient and fast growers, thrive in poor rocky soils unsuited for almost any other type of crop and resistant to insect and fungal attacks [14]. These qualities are desirable for a bioenergy crop since such crops require minimum agricultural inputs and hence reduced cost of feedstock, which is the major cost item in bioethanol production.

Apart from feedstock cost, the bioconversion process contributes significantly to the final ethanol cost [15]. Therefore, extensive research has been carried out towards optimising operating conditions for the fermentation process [16]. To that effect many bioreactor types and configurations are often employed in bioethanol production. The stirred tank bioreactors (STRs) are preferred because they guarantee better heat and mass transfer and consequently high process performance [17]. In addition, during conversion of starch to bioethanol the hydrolysis and the fermentation steps may be carried out separately or combined in different ways and each of these approaches may have its own advantages and disadvantages. For instance, in separate hydrolysis and fermentation (SHF), since hydrolysis and fermentation are carried out in separate vessels, optimal conditions for both enzymes and yeast can be achieved [18]. Besides, it offers possibility of cell recycling [19]. However, glucose may accumulate during saccharification. This may inhibits ß-glycosidase which is known to catalyse the hydrolysis of cellobiose to glucose [20,21] and this can lead to reduced reaction rate. Conversely, in simultaneous saccharification and fermentation (SSF), the glucose released can be readily converted to ethanol by yeast thereby reducing the depression of enzymes activity [22]. Additionally, the use of a single bioreactor for hydrolysis and fermentation reduce costs, labour and risks of contamination [23]. Nonetheless, with complex and heterogeneous hydrolysate with high lignin content, yeast cells recirculation may become difficult [22]. Therefore, the choice of process configuration will be determined by a balance of advantages and disadvantages associated with the two concepts for any particular feedstock. This is therefore the motivation of applying both process concepts plus a third concept in the present study using cheap, inedible wild cassava species as feedstock. Hence, the present study seek to evaluate, for the first time three approaches namely (I) separate hydrolysis and common fermentation (SHCF), (II) SHF and (III) SSF in STRs using inedible wild cassava as feedstock and baker's yeast (*Saccharomyces cerevisiae*) as a fermenting microorganism. Physical dimensioning and composition of wild cassava are also reported. Additionally, the study aimed at comparing the three process technologies and singling out the best technique for bioethanol production from wild cassava in terms of ethanol concentration, yield and productivity. The process yield and productivity were also compared with that of a well known-domesticated cassava species (*Manihot esculentum*).

2. MATERIALS AND METHODS

2.1 Raw Materials

The wild cassava tubers used in this study were harvested from two districts in Tanzania namely Kisarawe in the East Coastal region (Pwani) and Muheza in North Coast region (Tanga). A photograph of these tubers denoted as MGK and MGMU for tubers obtained from Kisarawe and Muheza respectively is shown in Fig. 1.

The plants from which tubers were obtained were first identified using an identification key (dichotomous key) at the Botany Department, University of Dar-es Salaam, Tanzania [15]. Afterwards the plants were earmarked at the beginning of the rainy season when starch formation commenced concomitantly with an increase in size of the tubers. The tubers were harvested 8 months later, when the tubers had attained maximum size and starch content [24] to ensure that tubers from all plants were of similar maturity. The tubers were stored in an air conditioned room with temperature maintained at ca.16ºC and were processed within 24h. The domesticated cultivar, *Manihot esculentum* tubers were purchased from a local vendor in Dar-es-Salaam and were used as reference material. The tubers were processed as reported in a previous study [15]. Baker's yeast was purchased from a local supermarket in Lund, Sweden and cultivated according to Moshi et al. [15] and stored at 4ºC until required for use.

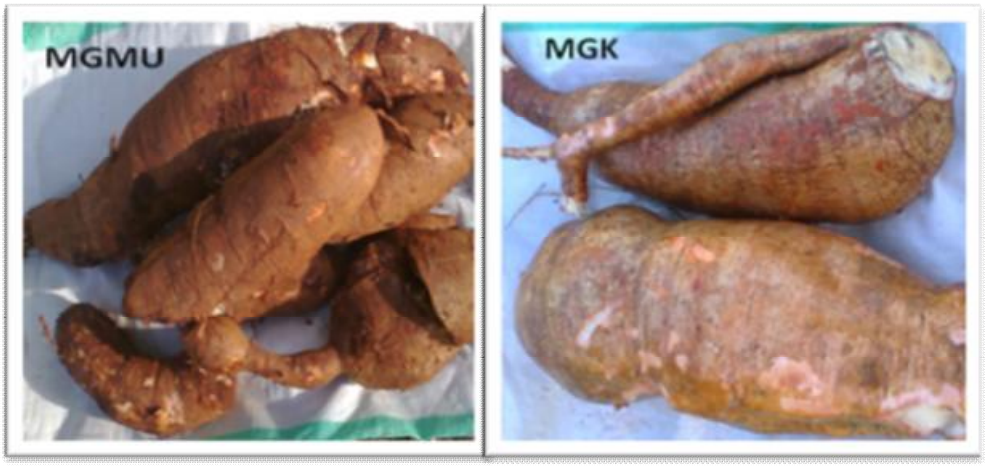

Fig. 1. Tubers of wild cassava (*M. glaziovii*) obtained from Muheza district in the North East, Tanzania (MGMU) and Kisarawe district in the East Coast, Tanzania (MGK)

2.2 Physical Characteristics of *Manihot glaziovii* Tubers

Some physical measurements were made on the tubers following the method described by Adetan et al. [25] to provide preliminary data for design of peeling equipments. A measuring tape was used to measure the lengths of tubers while the diameters of the tubers were measured using a pair of vernier callipers. The mass of each tuber, before and after peeling, was determined with an electronic balance and a micrometer screw gauge was used to measure the thickness of the peels. The tubers were peeled manually with the of a kitchen knife.

2.3 Enzymatic Hydrolysis of Wild Cassava Flour

The domesticated cultivar, *Manihot esculentum*, denoted as ME was used as reference material to establish the amount of flour that can be effectively hydrolysed prior to fermentation using commercially available thermostable α-amylase and glucoamylosidase (Novozymes, Copenhagen, Denmark). The cassava flour was hydrolysed by two stage enzyme hydrolysis as described by Moshi et al. [15]. The highest substrate concentration that yielded the highest amount of reducing sugars, as percentage of the theoretical glucose, was applied to another batch under the same conditions which included the reference material (ME) and flour from the wild cassava MGK and MGMU. Theoretical glucose was calculated from pre-determined total solids (TS) and total starch content [15] using the following equation:

$$\text{Total theoretical glucose} = \text{sample (g)(w/w)} * \%\text{TS} * \%\ \text{Starch content} * \frac{1}{0.9} \quad (1)$$

Where 0.9 is hydro-factor for starch, which is conversion factor for hydrolysis on polymerisation of glucose to starch. It refers to a factor due to difference in mass between anhydrous ring and glucose [26] and w/w is wet weight.

2.4 Bioreactor Set-up for Various Scenarios of Hydrolysis and Fermentation

Three process configurations (scenarios) separate hydrolysis and common fermentation (SHCF), separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) were set up for the hydrolysis and fermentation of starch from wild cassava (MGK and MGMU) and the reference material (ME). Fig. 2 shows the basic experimental set up used in the present study.

In all scenarios, hydrolysis was carried out in 0.5 L-glass bottle reactors of 0.2 L reaction volume and afterwards fermentation was performed in STRs (Schott Duran glass reactors, West Germany) which consisted of a water-jacketed vessel (14 cm height x 7 cm inner diameter) placed on a magnetic stirrer (stirring at 150 rpm), a pH probe which was connected to an automatic titrator, TTT60 titrator controller, pH standard meter (Copenhagen, Denmark). The titrator was calibrated to a maximum pH of 5.8 and to titrator

whenever the pH dropped below 5.5. The titrator was coupled to a peristaltic pump (BT100-2J, Stockholm, Sweden) calibrated at a flow rate of 0.03 mL/min. The pH regulating solution was NaOH (1M). The temperature was maintained at 3 0±2ºC by water circulation in the water jacket vessel. Feeding port for feeding and sampling port for sampling extended from the bioreactor. Gas tight Tygon tubes with gas sampling ports were used to transport produced gas to gas-tight balloons. The tubes were connected to a cooling system (4ºC) to condense ethanol and water vapour so as to ensure only CO_2 accumulates in the gas bag. All bioreactors, stoppers and tubing were autoclaved at 121ºC for 20 min prior to use.

2.5 Hydrolysis and Fermentation of Flour from Wild Cassava in STRs

Three scenarios were used in the hydrolysis and fermentation of the domesticated cassava (ME) and wild cassava (MGK and MGMU) flours. For scenario (I) SHCF, the slurry was centrifuged and the supernatant subjected to yeast fermentation; (II) SHF where at the end of saccharification the slurry (with the pulp) was subjected to yeast fermentation and (III) SSF wherein after liquefaction, the slurry was subjected to simultaneous saccharification and fermentation in one bioreactor. A layout for the conversion of cassava flour samples to bioethanol using different approaches is outlined in Fig. 3. For scenario (I) duplicate samples of 50 g for ME, MGK and MGMU were suspended in 200 mL of deionised water, and hydrolysed with α-amylase (0.1% v/w) at 90±2ºC, 110 rpm, for 1 h, followed by saccharification with amyloglucosidase at 50ºC, 110 rpm for 2 h. Afterwards the slurry was cooled to room temperature and centrifuged at 26700 g, 4ºC. Subsequently, the supernatant was pooled together to 0.25 L and inoculated with 10% (v/v) culture of *Saccharomyces cerevisiae*, and the pH adjusted to 5.7 using H_2SO_4 (1M). Fermentation was thereafter started using the bioreactor set up illustrated in Fig. 2 at 30±2ºC, with mild agitation on a magnetic stirrer.

For scenario (II) SHF, the same conditions as scenario (I) was performed except for the fact that the pulp was not centrifuged. Instead after saccharification, the pulp was cooled down to about 30ºC prior to inoculation with 10% (v/v) culture of *S. cerevisiae* and fermentation was performed as before using the bioreactor set up shown in Fig. 2 at 30±2ºC.

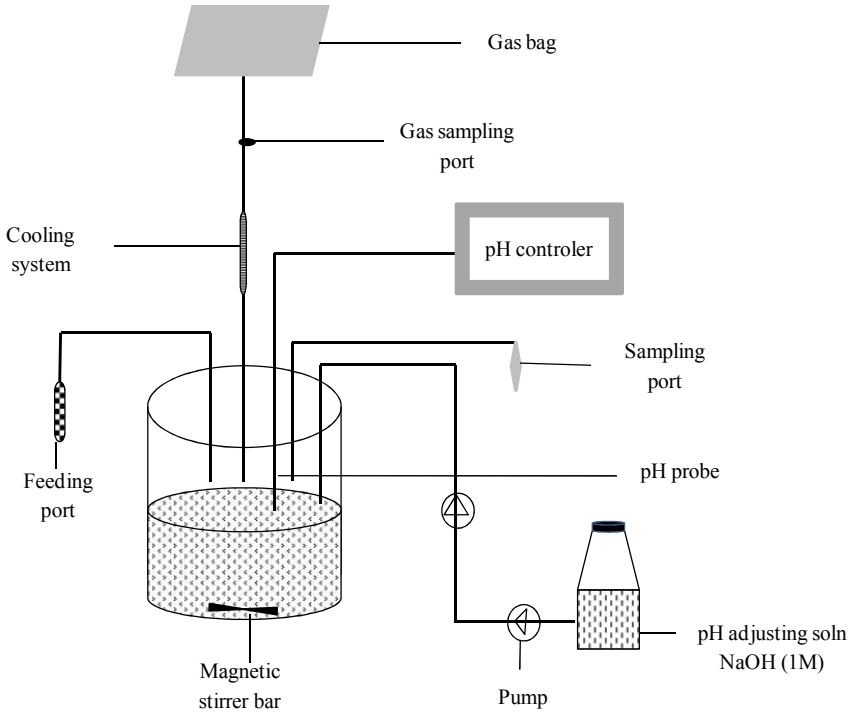

Fig. 2. Reactor set up for fermentation of cassava flour hydrolysate

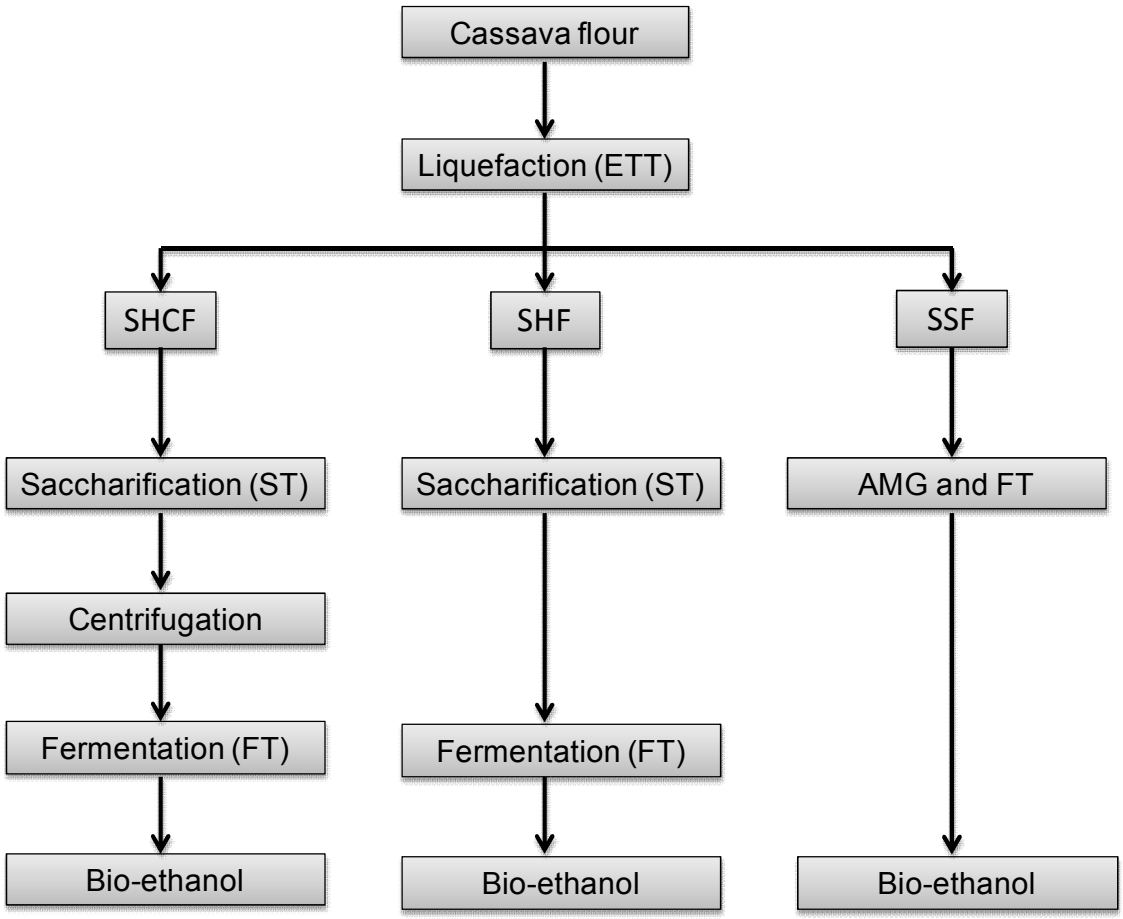

Fig. 3. Outline for preparation and bioethanol production from *M. glaziovii*. (ETT: Termamyl enzyme 0.1%, at 90ºC, 1h. AMG: Amyloglucosidase 0.1%; FT: yeast fermentation (30 ± 2ºC, 2h), ST: saccharifying enzyme AMG 0.1%, 2h

For scenario (III) SSF, after liquefaction, saccharification was combined with fermentation in which glucoamylase (0.1% v/v) and inoculum *(S. cerevisiae)* were added prior to SSF at 30±2ºC, using the same bioreactor set up as for scenario (I & II).

2.6 Analytical Methods

Total solids (TS), volatile solids (VS) and ash content were determined according to Sluiter et al. [27] and Sluiter et al. [28]. Total starch was analysed by a protocol elaborated by Holm [29] whereas total carbohydrate and total fibres were determined according to Sluiter et al. [30]. Total nitrogen was determined by micro Kjeldahl method [31]. Total cyanide was determined by linamarase loaded paper (Canberra, Australia) according to Bradbury et al. [32]. Macro-and micronutrients phosphorus (P) potassium (K), sodium (Na), magnesium (Mg), iron (Fe), zinc (Zn) copper (Cu) and manganese (Mn) were analysed by inductively coupled plasma atomic emission spectrometry (ICP-AES, Perkin Elmer Optima 8300, USA). Reducing sugars released during and after enzyme hydrolysis were analysed by DNS technique according to Miller [33]. This technique may overestimate the actual sugars released because it also measures other reducing factors [34]. However, it was chosen because it shortens time of analysis and the overall amount of sugars released was only used to ascertain the highest amount of substrate that can be liquefied by the designed amount of enzyme (i.e. 0.1% v/w), also to compare the extent of liquefaction of the wild and the domesticated cassava. Subsequently, all samples containing glucose and fermentation products were analysed by HPLC. Therefore, the samples were acidified with 2% of 3.7 M H_2SO_4

(i.e. 20 µL/mL of sample) and filtered through a polystyrene membrane pore size (0.45 µm). Thereafter, samples were analysed by HPLC, (JASCO Corporation, Tokyo, Japan) on an HPX 87H ion-exchange column (Biorad Laboratories Inc., Hercules, CA, USA) at 65ºC using MH_2SO_4 (0.5 mM) as eluent with a flow rate of 0.4 mL/min and detected with a refractive index detector (Erc Inc., Saitama, Japan). Total gas volume was measured using graduated 100 mL gas-tight glass syringe with a sample lock (Fortuna, Germany). The CO_2 produced during fermentation was collected in gas bags and analysed off line by GC (6890N, Agilent Technologies, Wilmington, USA), equipped with a thermal conductivity detector. Helium was the carrier gas at a flow rate of 29.6 mL/min. The column temperature was maintained at 70ºC whereas the injector and detector temperatures were 110 and 150ºC, respectively. Afterwards the amount of CO_2 in moles was computed based on a pre-established calibration curve.

It is worthwhile to note that at low temperatures (30-32ºC), small amount of CO_2 may dissolve in the fermentation broth and consequently the measured CO_2 may be slightly low [15]. This was corrected by regression analysis of ethanol derived from the volume of CO_2 produced compared to ethanol measured by HPLC as reported in a previous study [15]. Cell dry weight was determined according to a procedure described by Moshi et al. [15].

One way ANOVA was performed on the physical data of the tubers to determine their significant difference on the various physical properties. The statistical package was installed directly in excel using the Add-In function of Microsoft word 2007.

3. RESULTS AND DISCUSSION

3.1 Some Physical Properties of *Manihot glaziovii*

Tubers with size comparable to or bigger than the domesticated cultivar (ME) have been observed in wild cassava, *Manihot glaziovii*, plants found in Tanzania [15]. The physical dimensions of two types of wild cassava are reported for the first time (Fig. 1). These tubers as well as ME tubers differed significantly (P<0.05) in terms of weight, length, diameter, percentage of flesh, total tuber weight and percentage of peels, but no significant difference in peel thickness (Table 1).

The average length and diameter observed for ME, 29.4±4.0 and 1.9-8.9 cm respectively are in the same range as values reported in other studies [25,35] but differed significantly (P<0.05) from the wild cassava tubers (Table 1). The observed average thickness of peels (0.14±0.0 cm) from ME is lower than the 0.2 cm reported by Adetan et al. [25] but fall within the range reported by Ahwovoriole et al. [35]. These variations could be attributed to different measurement techniques used and or cultivar difference.

The proportion of peels in the ME (21±0.5%) for 50 tubers observed in this study is higher than those reported other studies [25,36]. The tuber physical parameters (tuber size, weight, flesh and peels proportions, and peels thickness) indicated that in the preparation of large amount of tubers more energy will be required to peel the wild tubers (MGMU & MGK) than the domesticated cultivar (ME). This is because thicker peels have large diameter which requires higher penetration force per unit length of knife-edge [25,37,38]. In terms of application, more biomass can be obtained where production process configuration allows the mixing of flesh and peels for MGMU because of high proportion of peels observed (Table 1), whereas MGK is more suited for scenarios that treats flesh and peels separately.

3.2 Proximate Composition of Wild Cassava (*Manihot glaziovii)*

The wild cassava species were observed to possess starch content comparable to the cultivated cultivar (ME). However, the wild cassava displayed relatively higher content of total carbohydrates, total fibre, total kjeldahl nitrogen, total cyanide (cyanogenic glycoside) and satisfactory content of macro-and micronutrients (Table 2). Considering the starch content, the wild cassava is comparable to the cultivated cultivar as feedstock for bioethanol production. In order to utilise all the carbohydrate, however, more energy will be required to hydrolyse the wild cassava which may not be economically justified since the non-starch carbohydrate is only up to 14% of total dry matter (Table 2). Thus extra energy will be required for pretreatment to break down lignin and hydrolyse the cellulosic component to release fermentable sugars. Therefore, in this study only the starch component was targeted for bioethanol production. The remaining carbohydrates in the stillage could be used for

biogas production. The relatively higher cyanide content observed in the wild cassava (Table 2) may not have negative effect on yeast fermentation [39] but may have adverse effect on bacteria fermentation [40-43]. Cassava starch is preferred as feedstock for bioethanol because it is readily hydrolysed by virtue of its low gelatinization temperature [15] and is known to offer higher solubility to α-amylase compared to other types of starch e.g. corn starch [44]. Cassava starch is also preferred for extraction of glucose syrup because of its high hydrolytic conversion rate up to 97% [45].

The wild cassava species displayed relatively higher amount of macro-and micronutrients compared to the domesticated cultivar. The wild cassava disclosed higher amount of nitrogen (N), P K, Na, Mg, Fe, Zn, Cu and Mn compared to the domesticated cultivar (Table 2). Nutrients play important role in yeast physiology and fermentation. N and P are the main nutritional requirements for the yeast growth and maximum ethanol production. P has the major role in the glycolysis cycle in yeast cell [46].

Nutrients such as K, Zn and Mg directly influence yeast cell proliferation, metabolism of yeast and subsequent ethanol production [46,47]. Zn is required by *S. cerevisiae* cells to maintain growth and metabolism and supplementation of culture with Zn has been reported to enhance ethanol fermentation [48,49]. Cu and Mn act as cofactors in various enzyme systems in yeast metabolism [50]. Therefore, the wild cassava seemed to show a balanced nutrient content; carbohydrate, protein content and these may act synergistically to enhance ethanol fermentation.

Table 1. Some physical properties of wild cassava (*Manihot* glaziovii) and the reference domesticated cassava (*Manihot esculentum*)

Parameter	ME	MGMU	MGK
Weight (whole tuber) (Kg)	2.6±0.1 (1.2-3.6)^{a}	3.1±0.1 (1.4-4.3)^{a}	0.4±0.1 (0.1-1.5)^{c}
Proportion of flesh (%)	74.0±1.4 (32.0-88.5)^{a}	75.9±0.9 (64.3-87.5)^{a}	78.9±0.9 (56.0-95.6)^{c}
Proportion of peels (%)	21.0±0.5 (16.7-29.5)^{a}	24.0±0.9 (12.5-35.7)^{b}	17.7±0.6 (3.4-28.8)^{c}
Diameter (cm)	10.1±0.2 (7.3-14.6)^{a}	12.1±0.2 (8.8-17.6)^{b}	0.6±0.1 (0.1-2.5)^{c}
Peel thickness (cm)	0.1±0.0 (0.1-0.2)^{a}	0.2±0.0 (0.1-0.2)^{b}	0.3±0.0 (0.2-0.5)^{c}
Length (cm)	29.4±0.6 (21.0-39.0)^{a}	35.5±0.7 (25.2-46.8)^{b}	25.5±1.4 (13.3-39.1)^{c}

ME Manihot esculentum; MGK Manihot glaziovii Kisarawe; MGMU Manihot glaziovii Muheza. Values are means for 50 tubers plus standard deviation, values in bracket represent the range for the 50 tubers. $^{a\text{-}b\text{-}c}$ *Means in a row without a common superscript letter differ (P < 0.05), as analyzed by one-way ANOVA*

Table 2. Characteristics of feedstocks used in the study

Characteristics	ME	MGK	MGMU
Dry matter (%)	88 (0.1)	88 (0.1)	89 (0.8)
Volatile solids (VS)	85 (0.7)	85 (0.9)	87 (1)
Moisture content (%)	12 (0.7)	12 (0.6)	11 (0.8)
Ash content (%)	3.8 (0.5)	3.9 (0.7)	3.1 (0.8)
Total starch (%)	82 (0.1)	80 (1.1)	77 (1.1)
Total carbohydrate (%)	86 (0.4)	87 (1.1)	90 (3.0)
Total fibre (%)	5 (0.4)	7.0 (0.4)	14 (3.0)
Total kjaldahl nitrogen (%)	0.41 (0.0)	0.75 (0.0)	1.62 (0.1)
Total cyanide (%)	0.20 (0.0)	0.26 (0.0)	0.41 (0.0)
Macro- and micronutrients (µg/g)			
Copper (Cu) (µg/g)	2.6 (1.9)	1.6 (0.8)	3.8 (2.0)
Iron (Fe) (µg/g)	14 (6.2)	10.0 (5.4)	50 (1.7)
Potassium (K) × 10^3	1.0 (0.9)	15.7 (1.6)	12.1 (1.5)
Magnesium (Mg) × 10^2	6.3 (1.3)	8.7 (0.8)	23.0 (1.2)
Manganese (Mn)	24 (1.6)	2.1 (1.4)	6.1 (1.2)
Sodium (Na) × 10^2	1.9 (0.7)	2.6 (1.9)	7.2 (1.6)
Phosphorus (P) × 10^2	4.2 (3.1)	18.8 (1.9)	7.5 (1.9)
Zinc (Zn)	9.6 (1.0)	6.3 (0.7)	11 (0.2)

Values in bracket are SD for triplicates except for micronutrients in which values in bracket refers to RSD (%).

3.3 Enzymatic Hydrolysis of Flour from Wild Cassava (*Manihot glaziovii)*

As mentioned earlier the target component in the wild cassava for bioethanol production is starch which constituted ca. 80% of total dry matter. Starch is a high yield feedstock for bioethanol production but its hydrolysis is required to release fermentable sugars prior to yeast fermentation. In order to achieve high ethanol yield at a process which ensure minimal production cost, first step was to establish the highest substrate concentration which can be hydrolysed with lowest enzyme dosage. The highest concentration of the domesticated cassava flour that could be hydrolysed with the smallest amount of enzymes (w/v of thermostable α-amylase) was 250 g/L (Fig. 4A). Consequently, the highest yield of reducing sugars expressed as percentage of theoretical

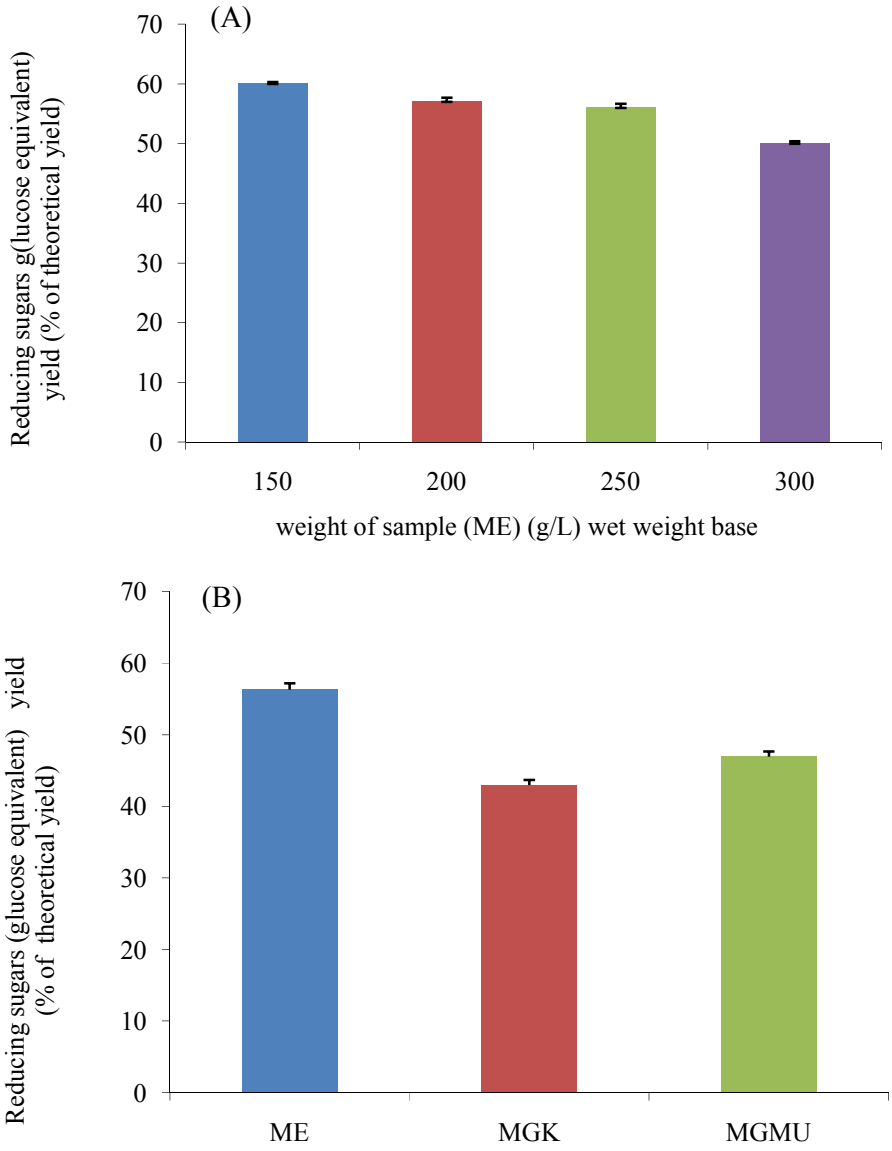

Fig. 4. Hydrolysis of *M. glaziovii* compared to *M. esculentum* using 0.1% of thermostable α-amylase at 90ºC, 1 h and 0.1% glucoamylase at 50ºC, 2 h (A) Effect of substrate concentration using ME; (B) Substrate type with optimal substrate concentration chosen Error bars indicate standard error of the mean of the replicates

glucose was 56% (Fig. 4A). Under the same conditions, the flour from the wild cassava yielded 43% and 47% reducing sugars as percentage of theoretical glucose for MGK and MGMU respectively (Fig. 4B). This disparity could be attributed to high ratios of cellulose and lignin in the wild species which tend to increase the resistance to hydrolysis [51]. These results indicated that under these conditions ca. 44% and 53-57% of reducing sugars for domesticated and the wild cassava respectively were not released at the end of hydrolysis. However, it was assumed that hydrolysis would continue in the fermentation step and that the presence of low initial sugars concentration could be advantageous to the yeast as it will mitigate substrate inhibition due to high osmotic pressure [15].

Therefore, it was decided not to optimise the process for complete hydrolysis of the cassava starch prior to fermentation but rather to ferment the partially hydrolysed wild cassava starch in a combined saccharification and fermentation process as discussed later in section 3.4.

3.4 Impact of Various Process Configuration on Fermentation of Raw (*Manihot glaziovii*) Flour on Bioethanol Concentration, Yield, Fermentation Efficiency and Productivity

In reference to the results described in section 3.2, three scenarios for hydrolysis and fermentation were evaluated and compared. The results of these experiments are presented in Table 3.

For SHCF it was observed that most of the sugars were not released from the pulp, as demonstrated by very low yield (Table 3).

This could be attributed to the end product inhibition of glucoamylase. Liquefaction of cassava starch and afterwards saccharification by glucoamylase at 60^{o}C resulted in fast accumulation of glucose, which could have inhibited the enzyme. Consequently, most of the glucose remain unreleased from the pulp and were removed during centrifugation. This fact was confirmed by analysis of glucose residue at the end of fermentation which revealed that there was no glucose residue in the broth.

A better process performance was achieved with SHF and SSF as compared to SHCF. When the SHF and SSF were compared in terms of ethanol concentration (g/l), yield (g/g), conversion efficiency (%) and productivity (g/L/h), no considerable difference was noted except for the fact that a better productivity was achieved for the SSF process. Since hydrolysis and fermentation were performed in one vessel, SSF took a shorter time and resulted in an overall better process performance (Table 3). Therefore, it was further evaluated on yield of initial reducing sugars (after liquefaction), fermentation efficiency, ethanol concentration (Fig. 5 A-C), carbon recovery and stoichiometrical mass balance (Table 4) and fermentation kinetics (Fig. 6).

During liquefaction by amylases, yield of reducing sugars was only 46, 29 and 28% for ME, MGK and MGMU respectively (Fig. 5A). However, at the end of fermentation, ethanol concentrations of 84, 81 and 77 g/L for ME, MGK and MGMU respectively were achieved (Fig. 5B). Conversion efficiency ranged from 91 to 97% (Fig. 5C). Conversion efficiency was evaluated as percentage of achieved ethanol (g/L) compared to the expected theoretical ethanol (g/L) pre-determined from the dry matter and starch content of the cassava flour. The differences in ethanol concentration observed could be ascribed to the differences observed in dry matter and total starch content in the samples. Stoichiomentric carbon balance revealed that carbon recovery was in the range of 92-101% (Table 4). Carbon recovery is the sum of carbon mole allocated to different end products (ethanol, acetic acid, CO_2 and biomass).

The high carbon recovery is also reflected in the high conversion efficiency (Fig. 5C) and can be attributed to the inherent properties of the substrate (i.e. readily hydrolysable, balanced macro and-micronutrients (Table 2) and optimal conditions in the SSF, especially optimal sugar concentration. The ethanol yield obtained was based only on the starch component in the flour. As mentioned earlier, the structural carbohydrates which accounted for about 14% dry matter were not hydrolysed for the sake of process simplicity. Instead it is proposed that the fermentation residue which contain cellulose, hemicelluloses, lignin, protein, recycled enzymes and biomass to be reserved for a more robust bioconversion process e.g. anaerobic digestion (AD) for biogas production. AD of the residues will be evaluated in our next studies.

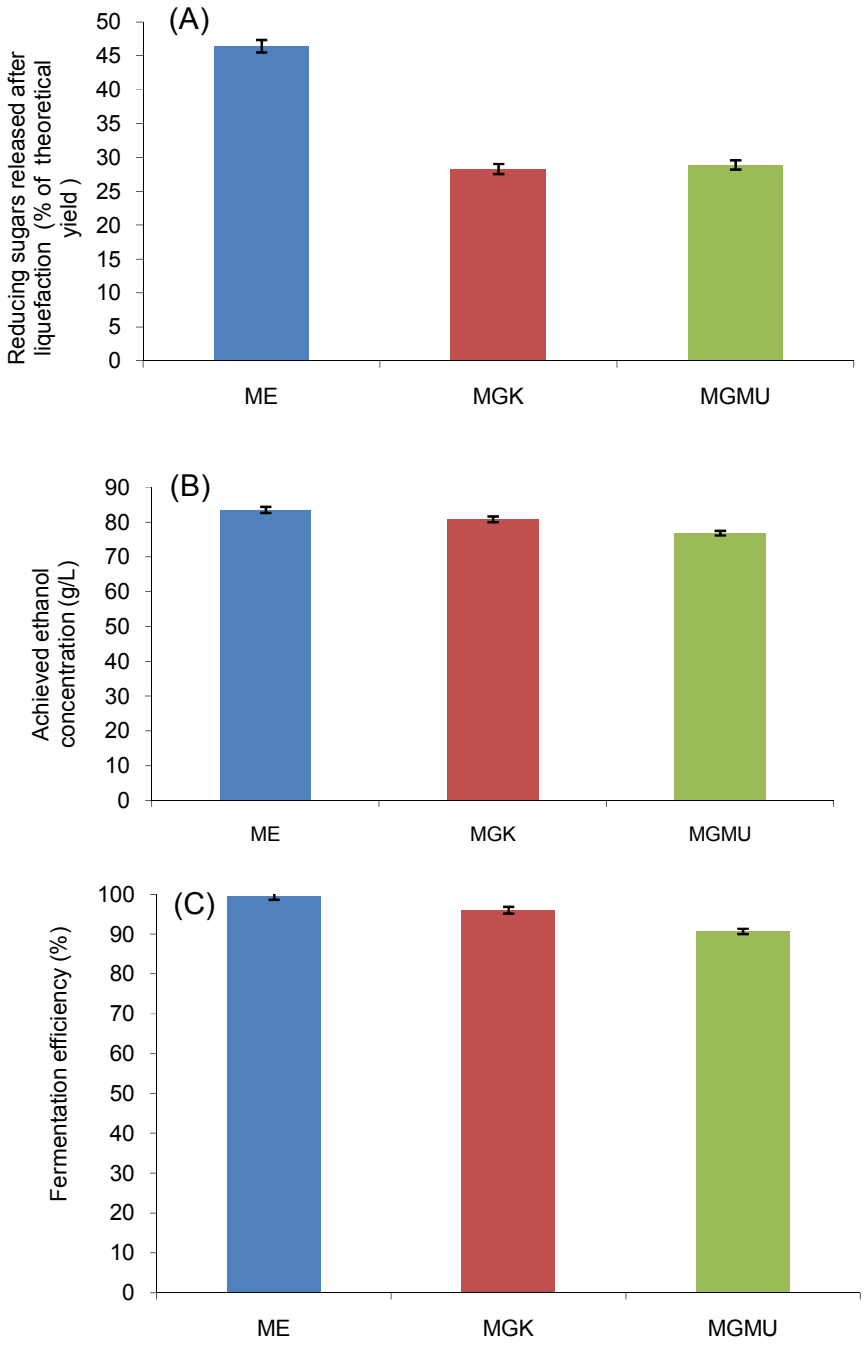

Fig. 5. Hydrolysis and simultaneous saccharification and fermentation of wild cassava (*M. glaziovii*) flour (A) Reducing sugars released after liquefaction, (B) Ethanol concentration achieved in SSF, (C) Fermentation efficiency during SSF

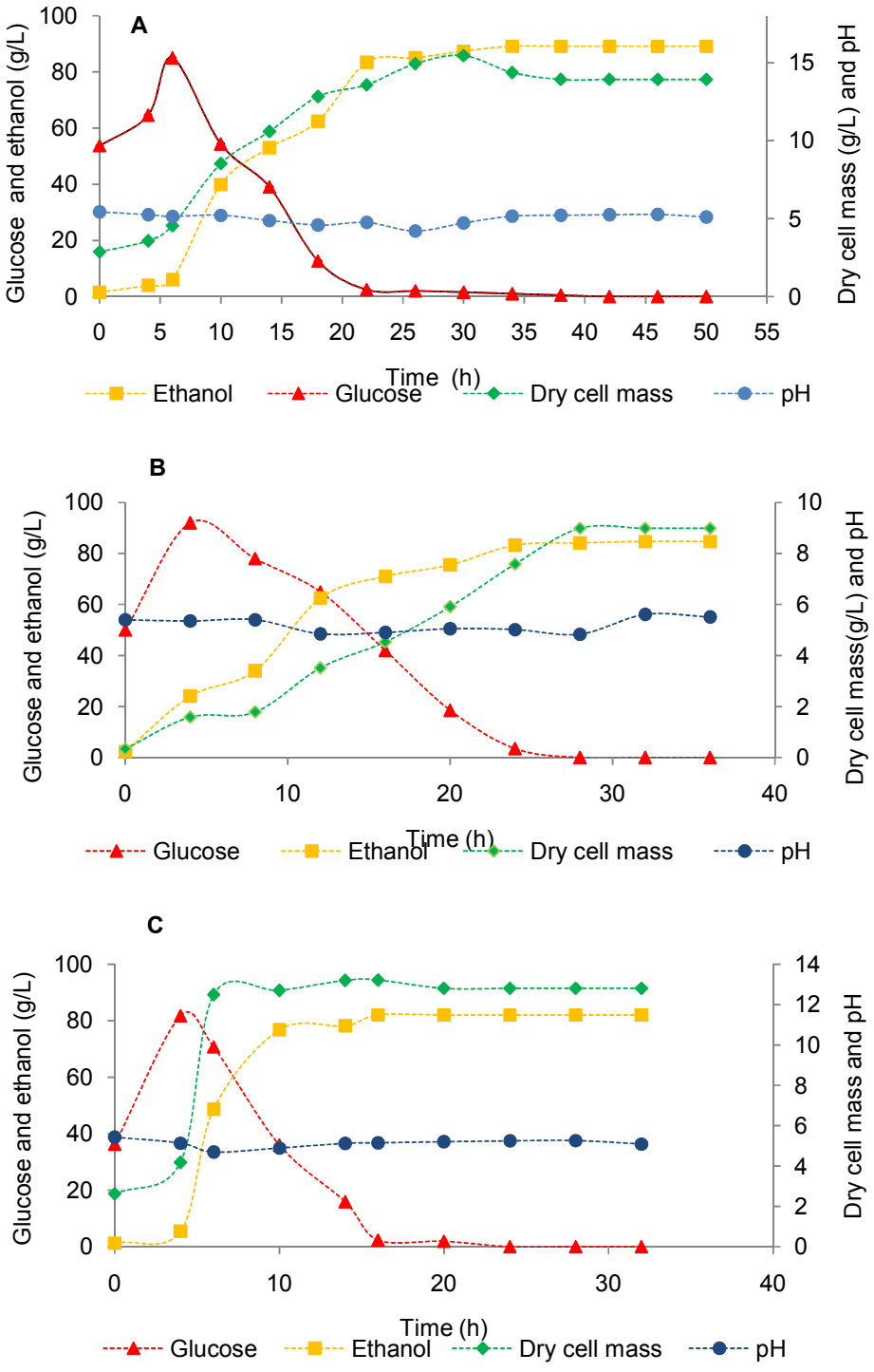

Fig. 6. Simultaneous saccharification and fermentation pattern of wild cassava *M. glaziovii* (A) ME, (B) MGK and (C) MGM

Table 3. **Hydrolysis and fermentation of *M. glaziovii* flour in stirred tank reactor: Effect of different approaches on process performance**

Approach	Sample	TG (g/L)	TE (g/L)	AE (g/L)	Yield (g/g)	PT (h)	CE (%)	VP (g/L/h)
SHCF	ME	168.7	86.2	21.9	0.1	17.0	25	1.4
	MGK	164.3	83.9	33.5	0.2	17.0	40	2.0
SHF	ME	168.7	86.2	83.5	0.5	17.0	97	4.8
	MGK	164.3	83.9	82.5	0.5	17.0	98	5.5
SSF	ME	168.7	86.2	84.3	0.5	16.0	98	5.4
	MGK	164.3	83.9	81.0	0.5	16.0	97	6.5
	MGMU	165.6	84.6	77.0	0.5	10.0	91	6.5

NB: TG = theoretical glucose, TE = theoretical ethanol, AE = achieved ethanol, PT = processing time, VP= volumetric productivity

Table 4. Simultaneous saccharification and fermentation of *M. glaziovii* flour: stoichiometrical balance and carbon recovery

Sample code	Stoichiomentric mass carbon	% CR
ME	$C_6H_{12}O_6$ --> $1.9CH_3CH_2OH + 1.9CO_2 + 0.05C5H7NO2P_{0.074}$	100
MGK	$C_6H_{12}O_6$ --> $1.9CH_3CH_2OH + 1.8CO_2 + 0.1\ C_5H_7NO_2P_{0.074}$	101
MGMU	$C6H_{12}O_6$ -->$1.8CH_3CH_2OH + 1.8CO_2 + 0.05C5H_7NO_2P_{0.074}$	92

CR = Carbon recovery

The fermentation kinetics was monitored offline by HPLC analysis. The results presented in Fig. 6A-C disclosed that all samples started with low initial fermentable sugars; however more sugars were released and assimilated by the yeast in the course of fermentation. The ME and MGMU exhibited a lower rate of fermentation for about 4-5 h, whereas higher rate of fermentation was observed for MGK from the beginning. The pH was maintained in a range of 4.7 to 5.5, which is optimal for both glucoamylase and the yeast [52]. Furthermore, it was evident that glucose concentration increased in the bioreactors during 4-6 h of fermentation. However, afterwards when biomass had built up, though hydrolysis continued the concentration of glucose in the bioreactors decreased rapidly (Fig. 6A-C). This rendered high process efficiency and productivity, since osmotic pressure could not build up to impose substrate inhibition on the yeast cells.

During SSF, the exponential phase was completed within 22 h, 25 h and 12 h for ME, MGK and MGMU respectively (Fig. 6). This observation may be attributed to both inherent differences in composition of these substrates, artefact of the experiment and hence the difference observed in ethanol formation and cell growth which was more pronounced in MGMU (Fig. 6C).

To that effect and to further discuss the impact of feedstock characteristics and process configuration/design on ethanol fermentation, different studies employing SHF and SSF with different feedstocks and operation parameters are compared with the results obtained in this study in terms of processing time, ethanol yield and productivity (Table 5).

It is evident from data presented in Table 5 that the higher efficiency and productivity obtained in this study are attributable partly to inherent properties of the feedstock and partly to the employed operation conditions. This is evident from the shorter duration 16-17 h for complete conversion of feedstock to product in this study compared to other starch rich feedstock e.g. waxy and non-waxy corn, waxy and non-waxy wheat of which complete feedstock conversion to product took 72-168 h. Moreover, higher volumetric productivities were observed for SSF compared to SHF in all the studies (Table 5).

Table 5. Comparison of SSF and SHF during bioconversion of different feedstocks to bioethanol

		SHF			SSF		
Feedstocks	**Operation conditions**	**PT (h)**	**EtOH yield (%TY)**	**PVT (g/L/h)**	**PT (h)**	**EtOH yield (%TY)**	**PVT (g/L/h)**
[1]Cassava flour (*M. esculentum)*	250 g/L liquefied and fermented with	17	96.8	4.8	16	97.6	5.4
[1]Wild cassava flour *(M. glaziovii, MGK*)	*S. cerevisiae* at 30ºC	17	98.0	5.5	16	96.9	6.5
[2]Wheat straw	Steam explosion, enzymatic hydrolysis, fermentation with *S. cerevisiae* at 37ºC	96	68	0.31	30	81	0.84
[3]Herbaceous crop Arundo donax	10% (w/w), steam pre-treated, hydrolysis fermented with *S. cerevisiae* at 45ºC	168	53	0.12[a]	96	47	0.20 [a]
[4] Wheat Straw	Steam explosion fermentation with *S. cerevisiae* at 32ºC	168	90	0.26	56	98	0.30
[5]Corn stove	8% WIS, steam pre-treated, fermented with *S. cerevisiae* at 35ºC	144	59	0.10 [a]	120	72	0.20 [a]
[6]Waxy corn	Gelatinised at 120ºC,	72	96	0.86 [a]	72	97	1.24 [a]
[6]Non-waxy	liquefied and fermented	72	94	0.80 [a]	72	98	1.20 [a]
[7]Waxy wheat	with *S. cerevisiae* at 30ºC	72	86	0.80 [a]	72	96	1.32 [a]
[7]Non-waxy wheat		72	84	0.74 [a]	72	92	1.25 [a]

TY =Theoretical yield, PT= processing time, PVT= productivity, [a]Derived from the author's article data, [b]Given in the author's article References : [1]Present study, [2][53], [3] [18] [4][19], [5][22], [6,7][39]

4. CONCLUSION

Higher proportion of degradable carbohydrates up to 90% (w/w) and higher nutrient content makes the wild cassava tubers an attractive feedstock for bioethanol production. The starch from the wild cassava tubers could be readily transformed to fermentable sugars using low dosage of enzymes (0.1%). *Manihot glaziovii* like most other Manihot species, grow on poor soils where most other crops do not thrive yet with low requirement of agricultural inputs. In addition, the use of the inedible wild cassava for production of bioethanol has added advantage in that it will not compete with other applications such as food and feed and is prospected to have a great advantage over other feedstock.

The use of partially hydrolysed slurry with more than 50% of the glucose released slowly during SSF proved to be appropriate technique for this type of feedstock as demonstrated by high efficiency, (up to 97.6%), volumetric productivities (6.5 g/L/h) carbon recovery (92-101%). This approach saves cost via small amount of enzyme used and energy saving (by skipping gelatinisation at ca.110-130ºC and saccharification at ca. 60-70ºC) and shortened reaction time. Moreover, since high ethanol titre (10-11% v/v) was obtained, further cost reduction can be gained in downstream process such as distillation and ethanol recovery and hence improved profitability. Therefore, both the feedstock and the process are of industrial interest since they lead to reduced costs of production.

Further process configuration for higher ethanol concentration and sequential bioethanol and biogas production for maximum fuel energy value from this feedstock is prospected as our future goals.

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COMPETING INTERESTS

There were no competing interests linked to this study.

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