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Physicochemical characterization of special cassava starches and their application for bio-ethanol production through no-cook technology at very high gravity

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ABSTRACT

Various cassava genotypes were developed with distinct starch characteristics, including amylose-free and small-granule mutations. Despite these unique traits, the ethanol production potential of these starches has not been explored. Cassava starch plays a crucial role in ethanol production, particularly in tropical countries like Colombia. This study aimed to assess the physicochemical properties of different cassava starches (including the amylose-free and small granule mutations), and to evaluate their potential for ethanol production using the Simultaneous Liquefaction, Saccharification, and Fermentation process under very high gravity (SLSF-VHG) and no-cooking conditions. Comparative analysis revealed that two double mutant starches and small-granule starch (GM4694–1) exhibited lower resistant starch content than those from wild-type and amylose-free cassava, making them more susceptible to enzymatic breakdown. The amylose content for GM4694–1 and wild-type cassava was 21.9 and 16.1 %, respectively, while the remaining samples were amylose-free. In the SLSF-VHG process, GM4694–1 demonstrated a significant ethanol yield, surpassing 16 % v/v, equivalent to 80 % of the theoretical ethanol yield within 90 hours. This suggests that the GM4694–1 genotype has the potential to produce ethanol efficiently at a temperature of 30°C. Furthermore, the solid residue obtained after the SLSF-VHG process could serve as a high-quality feed additive. This study enhances our understanding of the properties of special cassava starches and their correlation with ethanol production.

1. Introduction

Bioenergy crops have the potential to play a crucial role in transitioning away from fossil fuels; however, their impact on water consumption, land use, and greenhouse gas (GHG) emissions cannot be overlooked (Hosseinzadeh-Bandbafha et al., 2021). Cassava, as a feedstock for ethanol production, represents one such bioenergy option that has been extensively explored by various research teams. Production of ethanol from cassava is less environmentally efficient than from sugarcane molasses, with higher water requirements (respectively 2.3–2.8 and 1.5–2.0 L of water per liter of ethanol; Mangmeechai and Pavasant, 2013) and higher GHG emissions (respectively 1402–2863 and 363–520 gCO_2eq/L ethanol; Papong and Malakul, 2010; Caldeira-Pires et al., 2018; Tsiropoulos et al., 2014). On a positive note, blending just 10 % of cassava-based ethanol with 90 % conventional gasoline (E10) reduces GHG emissions by an estimated 6 % compared to pure gasoline (Nguyen and Gheewala, 2008) over the whole life cycle including production and use. From an energy perspective, the net energy value (NEV) of cassava-based ethanol produced in Thailand (8.8 MJ/L), surpasses the efficiency of cassava-based ethanol in China (4.5 – 6.1 MJ/L) (Liu et al.,

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2013) and corn-based ethanol in the United States (5.9 MJ/L) (Shapouri et al., 2002), but remains lower than the NEV of sugarcane-based ethanol (Prasara-a and Gheewala, 2021). Provided the environmental costs can be reduced, cassava, as a bioenergy crop, offers several advantages, including the ability to be planted and harvested year-round, high root productivity, and a substantial carbohydrate content (Sriroth et al., 2010; Zhang et al., 2003). Estimations of ethanol yield from cassava vary, with typical values around 4100 L/ha/year, based on 25 tons of fresh roots/ha/year and a conversion factor of 6 kg of fresh roots for 1 L of ethanol (Sriroth et al., 2010; Zhu et al., 2023). This value is similar to ethanol yields from sugarcane, reported at 3600-4500 L/ha/year, based on 64-79 tons of sugarcane/ha/year (Nguyen et al., 2022; Shelar et al., 2023) and conversion factors of 10.5 kg sugarcane/kg sugar (Nguyen et al., 2022), 0.46 kg ethanol/kg sugar (i.e. 90 % of the maximum theoretical yield of 0.51 kg ethanol/kg sugar; Gombert and van Maris, 2015), and ethanol density of 0.789 kg/L. Additionally, the tolerance of cassava to poor soil quality and drought conditions makes it a viable alternative in regions where environmental conditions or soil quality are unsuitable for sugarcane cultivation (Cortés-Sierra et al., 2010).

There is a wide range of cassava varieties with diverse physicochemical and functional properties, including cooking quality (Tran et al., 2021). The International Center for Tropical Agriculture (CIAT) in Palmira, Colombia, maintains an extensive germplasm collection comprising 6000 genotypes, meticulously characterized to identify those with desirable traits for various applications, such as food, industrial processing into starch and flour, among others (Sánchez et al., 2009). For ethanol production, the preference is for varieties with high dry matter and starch content to maximize ethanol yield. A high digestibility of the starch after enzymatic treatment (using alpha-amylase and amyloglucosidase) is also crucial to maximize glucose release for fermentation. Most starches from the CIAT cassava collection share similar molecular structures and degrees of crystallinity, resulting in comparable digestibility. To improve these characteristics, CIAT has developed different breeding strategies to produce high-value cassava clones, as outlined by Ceballos et al. (2006), (2004). For example, Ceballos et al. (2007) discovered an amylose-free 'waxy' cassava through self-pollination, currently finding applications to enhance the stability of frozen and refrigerated food products (Dufour et al., 2022; Hsieh et al., 2019).

Mutation induction (exposing botanical seeds to gamma rays) was among the strategies implemented at CIAT in pursuit of suitable varieties for different industrial applications, including bioethanol production. This process led to the development of genotypes with small-granule starch (Ceballos et al., 2008). Subsequent cross-breeding with other starch mutant genotypes resulted in double mutants, particularly those combining amylose-free and small-granule traits. The small-granule cassava starch, with a mean diameter of 5.80 ± 0.33 μm, demonstrated increased sensitivity to enzymatic hydrolysis due to its higher surface-to-volume ratio (Ceballos et al., 2008; Dufour et al., 2012), in contrast to wild-type (normal granule size, mean diameter 13.97 ± 0.12–18.73 ± 0.10 μm) or amylose-free (waxy) cassava starches. This characteristic underscores its potential for ethanol production in the bioethanol industry.

Ethanol distilleries traditionally employ a conventional process for ethanol production, involving three separate steps: liquefaction (95–105°C), saccharification (60–62°C) and fermentation (30–32°C) of the starch suspension (Chu-Ky et al., 2016). Liquefaction and saccharification necessitate using high-temperature, heat-stable alpha-amylase and amyloglucosidase to gelatinize and hydrolyze starch into glucose. This energy-intensive procedure requires heating starch granule slurries above the starch gelatinization temperature (60–70°C) for prolonged periods. Lee et al. (2012) estimated that liquefaction and saccharification account for 30–40 % of the total energy consumption in ethanol production, thereby escalating production costs and environmental impacts.

In the last 15 years, more efficient ethanol production processes have emerged, employing enzymes capable of efficient liquefaction at lower temperatures (Gang et al., 2007; Wang et al., 2007). Stargen, a novel enzyme product, demonstrates the ability to hydrolyze raw starch at room temperature, containing alpha-amylase and amyloglucosidase synthesized by *Aspergillus niger* and *Aspergillus kawachi* (Wang et al., 2007). Consequently, the simultaneous liquefaction, saccharification, and fermentation (SLSF) process, or no-cook process, has been developed to hydrolyze starch at temperatures below gelatinization temperature, enhancing ethanol yield while curbing energy consumption and investment (Cinelli et al., 2015). Optimized ratios of alpha-amylase and amyloglucosidase, along with yeasts, are added to the suspension (Sakdaronnarong et al., 2018; U-thai et al., 2022; Slavić et al., 2023). SLSF is conducted in a single reactor at room temperature (30 °C) under consistent conditions (pH, stirring), so that the concomitant action of yeast and enzymes prevent glucose buildup. Since glucose is gradually produced and consumed, higher rates of ethanol production are achievable (Chu-Ky et al., 2016; Robertson et al., 2006; Xu and Duan, 2010). Very High Gravity (VHG) technology refers to a fermentation process where the initial sugar concentration in the fermentation medium is significantly higher than in traditional fermentation processes (>300 g/L versus 150–200 g/L, respectively; Thomas et al., 1993). This method is primarily used in the production of biofuels, especially ethanol, as well as in brewing and other biotechnological applications. Some key features of VHG technology have been reported (Tien et al., 2022) such as (i) high substrate concentration; (ii) increased ethanol concentration and productivity; (iii) reduced water usage; (iv) shorter fermentation times. Consequently, VHG technology offers the following benefits: (i) Cost-effectiveness: higher ethanol yields can lower production costs by reducing the need for extensive downstream processing (Devos and Colla, 2022); (ii) Sustainability: reduced water usage and higher efficiency make the process more environmentally friendly; (iii) Higher productivity: enables faster production cycles and potentially higher throughput in industrial applications. SLSF, thanks to the absence of glucose accumulation, combines well with VHG technology, allowing a dry matter content of up to 36 %, compared to 15–20 % in the conventional process. This has been demonstrated in pilot-scale processes for rice (Chu-Ky et al., 2016) and cassava flour (Nguyen et al., 2014). The elevated dry matter content enhances the production of reducing sugars and ethanol per unit volume, consequently reducing the size and operating costs of fermentation reactors (Ziska et al., 2009).

Bioethanol has primarily been explored as an alternative to fossil fuels for transportation. However, the quantities achievable from starch-based crops are comparatively small when juxtaposed with the current global consumption of transportation fuel. Moreover, producing ethanol from starch crops competes with using the same crops for food, raising ethical concerns amid global food price inflation and ongoing demographic growth. Despite these challenges, bioethanol holds significant promise for applications beyond transportation. In the pharmaceutical sector, it serves as an excipient for certain medicines and functions as 70º GL antiseptic alcohol. Additionally, bioethanol can serve as a clean cooking fuel, replacing wood and charcoal, thereby potentially contributing to the reduction of preventable deaths caused by household air pollution in developing countries in Africa and other continents (Gall et al., 2013). This transition has several potential environmental benefits, including reduced deforestation and a smaller carbon footprint. From a social perspective, using bioethanol for cooking alleviates the burden of collecting firewood, which typically falls on women and girls) (Clancy et al., 2002).

In the present study, we explored the synergistic potential of integrating two recent advancements: the special cassava starches developed at CIAT and the SLSF technology to achieve high-yield bioethanol production at ambient temperature, thereby reducing production costs. The primary objectives of the study were to characterize the unique cassava starches (waxy, small granules, double-mutant), establish correlations between starch properties and ethanol production, and assess

their applicability in the no-cook process at very high gravity for ethanol production.

2. Materials and methods

2.1. Cassava genotypes and starch isolation

This study incorporated special starches from four distinct cassava genotypes. AM206–5 produces amylose-free starch, obtained through self-pollination, expressing a natural recessive spontaneous mutation (Ceballos et al., 2007). GM4694–1 resulting from gamma-induced mutation in cassava seeds, followed by self-pollination through different generations produces small-granule starch (Ceballos et al., 2008). AM1288–17 and AM1290–1, developed through conventional breeding, produce starch combining the amylose-free and small-granule traits, The study also included the Cumbre3 genotype, specifically bred for its adaptation to high-altitude environments.

With the exception of Cumbre3, all starches were extracted from plants grown and harvested at the CIAT experimental station in Palmira, Colombia, at 965 m above sea level (m.a.s.l.), using the protocol of Ceballos et al. (2007) with the following modifications: The starch suspension underwent filtration first through a 250 μm sieve, then through a 100 μm sieve to separate fibers; the sedimented starch was washed with tap water and resedimented twice. For small granule and double-mutant starches, instead of sedimentation, the filtered suspension was centrifuged (3000 rpm for 10 minutes at room temperature), followed by washing and re-centrifugation twice. The centrifugation process was necessary due to the limited sedimentation of small-granule starches. Subsequently, the starches (whether sedimented or centrifuged) were dried in a forced-ventilation oven (Binder, Model FD 23, Germany) at 40 °C for 24 h. In contrast, Cumbre3 starch was extracted in a traditional factory (*'rallandería'*) in La Agustina, Cauca, Colombia (1305 m.a.s.l.), following the protocol published by Maldonado et al. (2013). It was then transferred to CIAT (Palmira) and dried in the forced-ventilation oven like the other starches.

2.2. Physicochemical characterization of starches and hydrolysis with STARGEN

2.2.1. Dry matter content

Dry matter (DM) content was determined by drying the samples (3–5 g) in duplicates in an oven (Binder, Model FD 23, Germany) at 105°C for 24 hours (Sanchez et al., 2013).

2.2.2. Total starch content

The determination of total starch content followed the method outlined by Holm et al. (1986) with modifications. Total carbohydrates (C, % w/w, db) were assessed through incubation with thermostable α-amylase (L AYN04010 type, Novo Nordisk, Denmark) and subsequent treatment with amyloglucosidase (70 U/mg, Sigma Ref. 10115). The released glucose was measured using a spectrophotometer at 510 nm after reacting with the GOPOD solution containing glucose oxidase (Sigma, G6125–50KU) and peroxidase (Sigma; P8112–25KU). Subsequently, free glucose (FG, % w/w, db) was determined following incubation with amyloglucosidase, and the released glucose was also measured at 510 nm using a spectrophotometer after reacting with the GOPOD solution. Total starch content (TS, % w/w, db) was calculated using Eq. (1) (Moreno et al., 2021):

$$TS = C \times (162/180) - FG \quad (1)$$

2.2.3. Total protein content

Total protein content was determined by the method of Bradford (Bradford, 1976), using bovine serum albumin (Sigma, A3311) as standard.

2.2.4. Ash content

Ash content was calculated following heating at 550°C for 3 h (AOAC, 1996).

2.2.5. Amylose determination

Amylose content in the starch samples was determined following standard procedures (AOAC, 1987). The results were expressed as a percentage of the sample dry weight.

2.2.6. In vitro digestibility of starch

The *in vitro* digestibility of starches was assessed using the method described by Englyst et al. (1992) and Jiang et al. (2015) with some modifications. Starch samples (0.6 g) and guar gum (0.06 g) were combined with Milli-Q water (6 mL) in 50 mL polypropylene centrifuge tubes. Subsequently, 12 mL of pepsin solution (Sigma, P7000) in 0.01 M HCL (5 mg/mL) was added. The tubes underwent incubation at 37 °C for 30 minutes with continuous shaking (160 strokes/min). Following the initial incubation, five glass beads (diameter 3–4 mm) and 2.5 mL of sodium acetate buffer (0.5 M, pH 6) were introduced, and the tubes were vortexed for 20 seconds. The resulting dispersion was mixed with an enzyme solution (2.5 mL) comprising pancreatin extract (Sigma, P-7545), amyloglucosidase (Sigma, A7095, 300 U/mL), and invertase (Sigma, I4504, 300 U/mL). The tubes were incubated again at 37 °C. The hydrolyzed glucose content was measured at 20 and 120 minutes, using the GOPOD solution. To stop hydrolysis, 100 μL of the dispersion was quickly collected and shaken with 1.5 mL of 75 % (v/v) ethanol.

The quantification of digested starch fractions was expressed as a percentage of glucose multiplied by 0.9. Rapidly digestible starch (RDS) and slowly digestible starch (SDS) were determined based on the values measured at 20 and 120 minutes of hydrolysis. The resistant starch (RS) was calculated using Eq. (2):

$$RS = TS - RDS - SDS \quad (2)$$

where TS represents the total starch content.

2.2.7. Pasting properties

Hot starch dispersion viscosity profiles were generated using a rapid visco-analyzer (RVA) model 4 (Newport Scientific, Australia). Starch (2.5 g db) was dispersed in approximately 22.5 g distilled water, with the weight of distilled water adjusted based on the moisture content of each starch sample to maintain a constant total weight of 25 g. While RVA analysis is typically performed with 5–7 % starch suspensions, the pasting profiles of small-granule (GM4694–1) and double-mutant (AM1288–17 and AM1290–1) starches exhibited very low viscosities at this concentration, attributed to limited swelling and friction between small-starch granules. Therefore, the protocol was adapted to utilize 10 % concentrations, resulting in suitable viscosities for all starches, albeit with higher-than-usual viscosity peak values in the case of wild-type cassava starch. Viscosity was recorded with the following temperature profile: holding at 50 °C for 1 min, heating from 50 to 90 °C at 6 °C/min, holding at 90 °C for 5 min, cooling down to 50 °C at 6 °C/min, and holding at 50°C for 2 min, with continuous stirring at 160 rpm. To analyze the alterations in crystalline, molecular, and granular properties during pasting (Balet et al., 2019), the following parameters were recorded: pasting temperature (PT), peak viscosity (PV), hot paste viscosity (HV), defined as the local minimum in viscosity between peak viscosity and final viscosity, final viscosity (FV), breakdown (BD) calculated as PV-HV, and setback (SB) calculated as FV-HV.

2.2.8. Particle size distribution of starch granules

The particle size of starch granules was assessed using laser diffraction (Malvern Mastersizer 3000 with Hydro MV as the dispersion unit, UK). A starch suspension (1 g starch in 40 mL of distilled water) was

mechanically stirred and sonicated to achieve a laser light obscuration level of approximately 16 %. The starch and water were assigned a refractive index of 1.60 and 1.33, respectively. The granule size distribution was estimated based on triplicate measurements using the Fraunhofer approximation, considering opaque particles. The following parameters were recorded: median granule diameter (Dv50); mean granule size representing 10 % and 90 % of the distribution (Dv10 and Dv90); and cumulative volume fractions for smaller starch granules (diameter < 7 μm), small granules (7–20 μm), medium granules (20–40 μm), and large granules (> 40 μm).

2.2.9. Susceptibility of starch to enzymatic hydrolysis

The methodology described by Holm et al. (1985) was used to evaluate the hydrolysis pattern of different starches, with some modifications. Starch (1 % w/v, db) was suspended in Milli-Q water, and the pH was adjusted to 4.0 with 0.01 M HCl. Subsequently, 0.25 mL of Stargen 002 was added. The resulting suspension was transferred to polypropylene centrifuge tubes (50 mL/tube), which were vortexed and incubated in a water bath at 37 °C for 60 minutes with continuous shaking (160 strokes/min). Throughout the incubation period, samples were collected (100 μL) at different times (0, 5, 10, 30, and 60 minutes) and immediately vortexed with 1 mL of ethanol (50 %). The released glucose reacted with a GOPOD solution and was measured by spectrophotometry at 510 nm. A standard curve was prepared using a series of glucose solutions. The degree of hydrolysis of starch with incubation time was expressed as the weight of glucose in the solution divided by the weight of starch initially present (%).

2.2.10. Scanning electron microscopy

Micrographs of native starches were obtained by scanning electron microscopy as described by Yan and Zhengbiao (2010).

2.3. Simultaneous Liquefaction, Saccharification and Fermentation (SLSF) at very high gravity (VHG)

2.3.1. Enzyme and microorganism specifications

Two commercial enzymes were used as described in Chu-Ky et al. (2016): Alpha-amylase and gluco-amylase Stargen 002 (Dupont, USA) and amyloglucosidase Amigase Mega L (DSM-Food Specialties - Beverage Ingredients, Netherlands). Commercial active dry yeast *Saccharomyces cerevisiae* (Red Ethanol) was kindly provided by Leaf Technologies (France).

2.3.2. Simultaneous liquefaction, saccharification and fermentation (SLSF) at very high gravity (VHG) at laboratory scale

The SLSF-VHG process was adapted from Chu-Ky et al. (2016) with some modifications (Fig. 1). Cassava starch samples were mixed with distilled water in 1-liter fermenters to achieve a concentration of 300 g/L dry solid in a final volume of 500 mL. Alpha-amylase, amyloglucosidase (Stargen 002 at a dosage of 992 GAU/kg), and another amyloglucosidase (Amigase Mega L at a dosage of 0.05 % w/w), active dry yeast Red Ethanol (3.5×107 cells/mL), urea (32.0 mM), and KH2PO4 (8.0 mM) were simultaneously added into the mixture. The SLSF-VHG was conducted for 7 days (160 h) at 30 °C, using a water bath to maintain a constant temperature.

2.3.3. Analytical procedures

During the SLSF-VHG process, 10 mL aliquots of the fermentation slurry were sampled at regular intervals to quantify parameters such as DM, pH, sugars, and fermentation products. DM content was determined

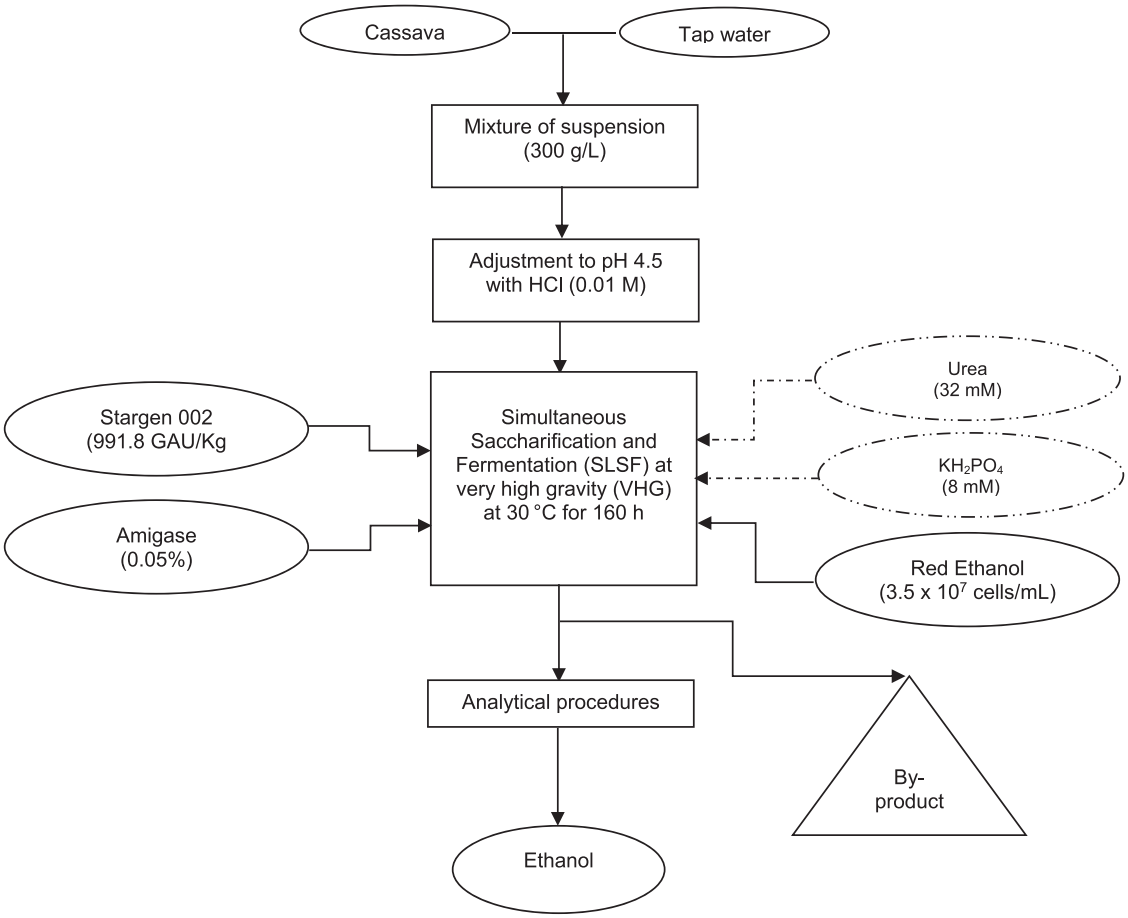

Fig. 1. SLSF-VHG process of cassava starch for ethanol production.

following the protocol in Section 2.2.1.; the interpretation of results required careful consideration. The fermentation slurry primarily consists of starch (native or partially hydrolyzed), glucose, and ethanol in varying proportions depending on the progress of the SLSF reactions, along with a mix of minority fermentation products (e.g., lactic acid, proteins, etc.). As ethanol evaporated during DM measurements, the results mainly reflected the quantities of starch and glucose remaining in the slurry and the overall progress of the SLSF process.

The pH of the fermentation slurry was determined at room temperature with a pH-meter (Fisher Scientific, AB15, USA). Ethanol, glucose, glycerol, and lactic acid were determined as follows: The fermentation slurry was centrifuged (12,101 g, 10 min, and 25 °C), and the supernatant was filtered through a membrane (Millex-GV from PVDF, 0.22 μm, Brazil). The filtrate was subjected to analysis using High-Performance Liquid Chromatography (HPLC) (Agilent 1200 series, Germany) equipped with a Biorad column, Aminex HPX 87 H, a quaternary injection valve, a UV detector (MWD T 1365D) for organic acids set at 210 nm and connected in series with a refractive index detector (RID T1362A) for glucose, ethanol, and glycerol. The samples were separated isocratically at a flow rate of 0.6 mL/min, at 30°C, with an injection volume of 15 μL. All reagents used for HPLC analysis were of analytical grade. Concentrations were calculated using standard curves correlating individual concentration to peak area (Moreno, 2011).

2.3.4. Chemical characterization of residue from the SLSF process

Once the SLSF-VHG process was completed, the remaining slurry was oven-dried (Binder, Model FD 23, Germany) at 50°C for 24 hours. The resulting residue, referred to as SLSF-Res, underwent characterization for DM, total starch content, protein content, amylose content, *in vitro* digestibility, and microscopy. The determination of these parameters followed the methodologies described in Section 2.2.

2.4. Statistical analysis

Mean values and standard deviations were calculated based on data from two independent experiments. To compare means, a one-way analysis of variance (ANOVA) was conducted, followed by an HSD test (Tukey's Honest Significant Difference test) for pairwise comparisons of samples. The level of significance was set at $p<0.05$. Additionally, correlations between variables were analyzed using Pearson's correlation test. Statistical analyses were conducted using the JMP Pro 14 program.

3. Results and discussion

3.1. Physicochemical characterization of native starches

3.1.1. Chemical composition of native starches

The moisture contents of native cassava starches fell within the range 10.5–11.5 %, with no statistically significant differences ($p<0.05$) observed between different samples (Table 1). This parameter was used to standardize the concentration of dry matter to precisely 30 % (300 g dry matter/L) in the fermentation bottles. Protein content varied from 0.02 % to 0.07 %, with AM206–5 (waxy cassava) and AM1288–17 (double-mutant) at the lower and higher end, respectively. Ceballos et al. (2007) reported an average protein content of 0.12 % for AM206–5, which is notably higher than the values obtained in the present study. Additionally, the ash content was significantly ($p<0.05$) lower for AM206–5 starch, while GM4694–1 starch exhibited the highest ash content. This elevated ash content may be correlated with a higher phosphorus content (Schirmer et al., 2013).

Double-mutant starches isolated from genotypes AM1288–17 and AM1290–1 exhibited extremely low amylose content, as anticipated, with absorbances falling below the limit of quantification (0 % amylose). Similarly, the amylose content in the waxy starch (AM206–5) was notably low, although not reaching zero, with average values of 2.9 %. The detection of a small amount of amylose using the colorimetric method may be attributed to the presence of some long-chain amylopectin branches that can bind similarly to amylose in the colorimetric tests (Bertoft, 2004). In contrast, other cassava starches examined in this study displayed average amylose contents of 16.05 % and 21.95 % for Cumbre3 and GM4694–1 (small-granule), respectively. These findings are consistent with the range reported by Sánchez et al. (2009), who documented amylose contents in 4044 wild-type cassava accessions ranging from 15.2 % to 26.5 %.

All starches exhibited high purity, with total starch content surpassing 94 % on a dry basis (db) (Table 1). This reaffirms the effectiveness of the starch isolation method. Notably, cassava starches derived from AM1288–17 and GM4694–1 showed the highest and lowest average purity, respectively 97.70 % and 94.06 %. Nevertheless, there were no statistically significant differences in total starch content among all the evaluated samples ($P=0.4562$, Table 1).

3.1.2. Digestibility of native starches

The digestibility characteristics of native starches were assessed based on the proportions of three fractions (Table 1): rapidly digestible starch (RDS) digested within 20 minutes, slowly digestible starch (SDS) digested in the 20–120 minute range, and resistant starch (RS) not digested within 120 minutes. Notably, small-granule and double-mutant genotypes exhibited higher susceptibility to hydrolysis compared to other genotypes, evident in their elevated RDS (46.0–54.4 %) and reduced RS (2.9–4.9 %) fractions (Table 1). In contrast, the RS fractions of waxy and wild-type cassava ranged from 39.8 % to 43.8 %, respectively. The likely explanation for this behavior can be attributed to the larger surface-to-volume ratio of starch granules in small-granule and double-mutant genotypes, a conclusion supported by observations from scanning electron microscopy of starch granule sizes. Additional contributing factors may include the degree of crystallinity and the length of amylopectin chains (You et al., 2014). SDS fractions exhibited less contrast, ranging from 35.90 % to 47.96 % across all genotypes.

3.1.3. Pasting properties of native starches

Small granule and double-mutant starches exhibited distinctive

Table 1
Chemical characterization of native starches.

Sample	Moisture (%, wb)	Protein (%, db)	Ash (%)	Amylose (%, db)	Starch content (%, db)			
					TS	RDS	SDS	RS
AM1288–17	10.61 ± 0.22^{a}	0.07 ± 0.004^{a}	0.21 ± 0.02^{b}	$< LQ^{d}$	97.70 ± 1.48^{a}	46.04 ± 0.14^{b}	47.96 ± 1.01^{a}	3.70 ± 0.87^{b}
AM1290–1	11.50 ± 0.29^{a}	0.05 ± 0.003^{b}	0.23 ± 0.01^{b}	$< LQ^{d}$	95.39 ± 1.29^{a}	54.36 ± 2.61^{a}	36.14 ± 2.96^{b}	4.89 ± 0.35^{b}
AM206–5	10.59 ± 0.25^{a}	0.02 ± 0.001^{d}	0.01 ± 0.00^{d}	1.59 ± 0.10^{c}	96.93 ± 0.57^{a}	16.91 ± 0.85^{c}	40.26 ± 2.25^{ab}	39.77 ± 3.10^{a}
GM4694–1	10.97 ± 0.46^{a}	0.05 ± 0.001^{b}	0.29 ± 0.03^{a}	21.95 ± 0.05^{a}	94.06 ± 1.88^{a}	48.01 ± 0.64^{b}	43.14 ± 2.05^{ab}	2.91 ± 1.41^{b}
Cumbre3	10.54 ± 0.13^{a}	0.03 ± 0.002^{c}	0.10 ± 0.00^{c}	16.05 ± 0.09^{b}	96.70 ± 3.37^{a}	16.95 ± 0.93^{c}	35.92 ± 1.08^{b}	43.83 ± 2.01^{a}
ANOVA	0.0850	0.0001	<0.0001	<0.0001	0.4562	<0.0001	0.0079	<0.0001

<LQ: less than the limit of quantification, which is the first concentration level of the calibration curve (0 %); TS: total starch; RDS: rapidly digestible starch; SDS: slowly digestible starch; RS: resistant starch. Data shown are the mean ± standard deviation from two determinations. Within each column, means with different superscript letters are significantly different ($p < 0.05$).

pasting properties (Fig. 2, Table 2), characterized by low pasting temperatures (PT) coupled with generally low viscosity parameters (PV, BD, FV, etc.). Conversely, the waxy starch (AM206–5) displayed a high PT and overall elevated viscosity parameters. Notably, Cumbre3, a wildtype starch, demonstrated a unique combination of a low PT, similar to small granule and double-mutant starches, along with exceptionally high viscosity parameters (Table 2). These variations highlight the specific pasting behavior imparted by the small granule mutation to cassava starches (Ceballos et al. in, 2008). This underscores the potential of these starches for innovative applications in food technology and bio-refinery.

3.1.4. Particle size distribution of starch granules

Small-granule and double-mutant genotypes (GM4694–1, AM1288–17, and AM1290–1) exhibited significantly smaller starch granules compared to wildtype (Cumbre3) and waxy (AM206–5) genotypes (Table 3). According to the categorization by Lindeboom et al. (2004), these two groups correspond to small (5–10 μm) and medium (10–25 μm) granule sizes, respectively, based on Dv50. Correspondingly, small-granule and double-mutant genotypes had a higher proportion of small granules (Dv50 < 7 μm) and a lower proportion of intermediate and large granules (Dv50 between 20 and 40 μm and above 40 μm) compared to wildtype and waxy genotypes (Table 3). In line with these findings, Ceballos et al. (2008) reported a small-granule cassava clone with an even smaller median size of 5.8 μm.

All starch samples exhibited a bimodal distribution (Fig. 3), featuring a population of small particles with a mean size of 1–2 μm and a population of larger particles with a mean size of 9.9–11.2 μm (small-granule and double-mutant) or 16.4 μm (wildtype and waxy). The small-granule and double-mutant starches displayed a larger population of small particles, aligning with the notion that these starches contain more small granules than the wildtype and waxy starches. Comparably, wheat also contains two populations of starch granules (A-type and B-type) with a bimodal distribution of particle size (Kim and Huber in, 2010). However, in cassava, a monomodal distribution of starch granule particle size is more commonly reported (Sriroth et al., 1999; He et al., 2020). Therefore, the possibility cannot be ruled out that the small particles observed in this study may be an artifact caused by broken granules generated during the milling of the samples, as suggested by Rao and Tattiyakul (1999), who observed a similar bimodal distribution in cassava starch.

3.2. Hydrolysis kinetics of native starch

Double-mutant and small-granule starches exhibited higher initial rates and the highest percentages of hydrolysis (Fig. 4). This observation aligns with the easier enzymatic attack attributed to a higher surface-to-volume ratio, as previously noted in the digestibility experiments (Section 3.1.2). Notably, a lower initial rate of hydrolysis was associated with a lower final percentage of hydrolysis for most genotypes, except for waxy starch (AM206–5), which started with a high initial rate but concluded with an intermediate percentage of hydrolysis. The maximum hydrolysis observed was less than 50 %, as sub-optimal experimental conditions were deliberately chosen to better discern the range of hydrolysis behaviors among different starches, rather than maximizing glucose production. Other studies have also reported that small-granule cassava starch, such as in genotype 5G160–13, exhibited a high hydrolysis rate compared to starches from other cassava genotypes and botanical sources (Dufour et al., 2012). However, differences in hydrolysis rates cannot be solely attributed to particle size, since taro starch with a mean granule size of 4 μm showed a low hydrolysis rate (Dufour et al., 2012). Factors such as the degree of crystallinity and type (A, B or C), along with amylose content, may also contribute hydrolysis behavior (Tester et al., 2006, 2004).

When observed under scanning electron microscopy (SEM), granules from native cassava starches displayed diverse shapes, including oval, truncated, or spherical forms (Fig. 5A-E). The surfaces of these native starch granules were smooth, showing no evidence of cracks, indicating minimal starch damage during the isolation process. However, starch granules that were incubated with Stargen 002 exhibited significant variations in hydrolysis patterns, both in terms of shape and size (Fig. 5F-J). The enzymatic hydrolysis seemed to predominantly occur on the granule surface, a phenomenon also noted by Franco et al. (2002), with few discernible differences among starches.

3.3. Simultaneous liquefaction, saccharification and fermentation (SLSF) at very high gravity (VHG) at laboratory scale

3.3.1. Evolution of dry matter and glucose content during the SLSF-VHG process

DM decreased during the SLSF-VHG process for all evaluated genotypes, revealing significant differences among them ($P<0.01$). Starch was converted to ethanol more rapidly and completely in the double-mutant (AM1288–17) and small-granule (GM4694–1) genotypes, with

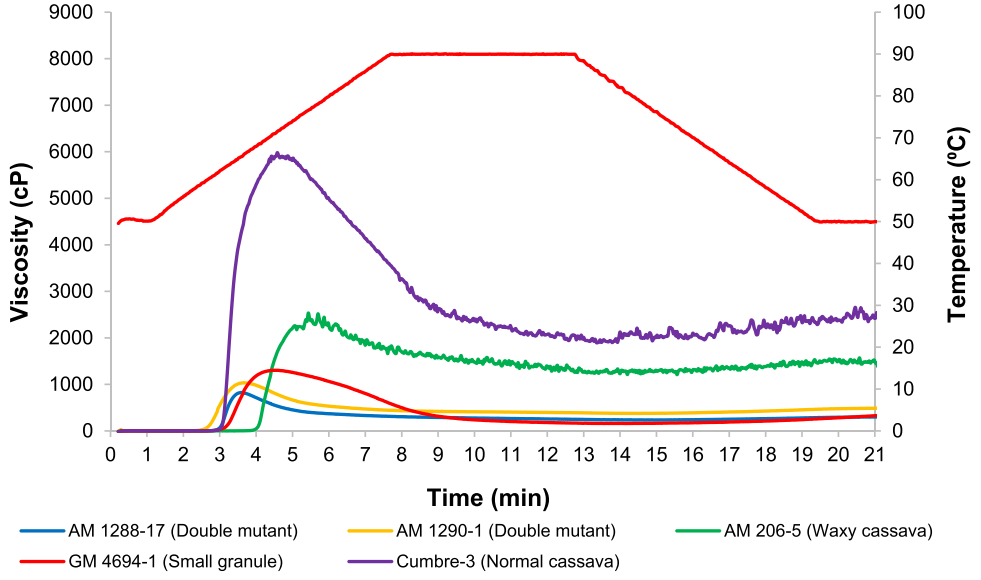

Fig. 2. Pasting curve of native starches using visco analyser in water solution.

Table 2
Pasting properties of native starches.

Sample	Pasting temperature PT (°C)	Peak viscosity PV (cP)	Hot paste viscosity HV (cP)	Breakdown BD (cP)	Final viscosity FV (cP)	Setback SB (cP)
AM1288–17	61.0 ± 0.0^{b}	827.7 ± 3.6^{e}	235.2 ± 0.1^{d}	592.5 ± 3.5^{d}	297.7 ± 0.7^{d}	62.5 ± 0.7^{b}
AM1290–1	58.0 ± 0.0^{c}	1029.6 ± 4.5^{d}	375.6 ± 0.3^{c}	654.0 ± 4.2^{d}	486.6 ± 1.7^{c}	111.0 ± 1.4^{b}
AM206–5	67.0 ± 0.0^{a}	2512.8 ± 26.8^{b}	1282.8 ± 2.9^{b}	1230.0 ± 29.7^{b}	1394.8 ± 83.3^{b}	112.1 ± 2.9^{b}
GM4694–1	61.0 ± 0.0^{b}	1291.0 ± 17.1^{c}	157.0 ± 3.0^{e}	1134.0 ± 14.1^{c}	341.0 ± 1.3^{d}	184.0 ± 4.2^{b}
Cumbre3	61.5 ± 0.7^{b}	6005.4 ± 36^{a}	1896.4 ± 2.2^{a}	4109.0 ± 38.2^{a}	2568.9 ± 70.8^{a}	672.5 ± 68.6^{a}
ANOVA	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Data shown are the mean ± standard deviation from two determinations. Within each column, means with different superscript letters are significantly different ($p < 0.05$).

Table 3
Granule size parameters of special starches.

Genotype	Dv(10) µm	Dv(50) µm	Dv(90) µm	<7 µm %	7–20 µm %	20–40 µm %	> 40 µm %
AM1288–17	1.4 ± 0.0 ^{cd}	9.1 ± 0.1^{d}	15.9 ± 0.1^{d}	38.4 ± 0.4^{b}	61.1 ± 0.2^{b}	0.5 ± 0.1^{e}	0.0 ± 0.0^{c}
AM1290–1	1.5 ± 0.0^{c}	10.2 ± 0.0^{c}	17.9 ± 0.0^{c}	31.7 ± 0.1^{c}	64.8 ± 0.0^{a}	3.5 ± 0.1^{c}	0.0 ± 0.0^{c}
AM206–5	8.2 ± 0.1^{a}	17.6 ± 0.1^{a}	31.0 ± 0.7^{a}	9.4 ± 0.1^{e}	56.6 ± 0.8^{d}	31.2 ± 0.2^{a}	2.7 ± 0.7^{a}
GM4694–1	1.2 ± 0.0^{d}	8.3 ± 0.0^{e}	16.4 ± 0.1^{d}	45.1 ± 0.2^{a}	52.5 ± 0.1^{e}	2.4 ± 0.1^{d}	0.0 ± 0.0^{c}
Cumbre3	7.3 ± 0.1^{b}	17.3 ± 0.1^{b}	29.7 ± 0.3^{b}	10.1 ± 0.0^{d}	57.8 ± 0.4^{c}	30.6 ± 0.1^{b}	1.5 ± 0.4^{b}
ANOVA	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Data shown are the mean ± standard deviation from three determinations. Within each column, means with different superscript letters are significantly different ($p < 0.05$).

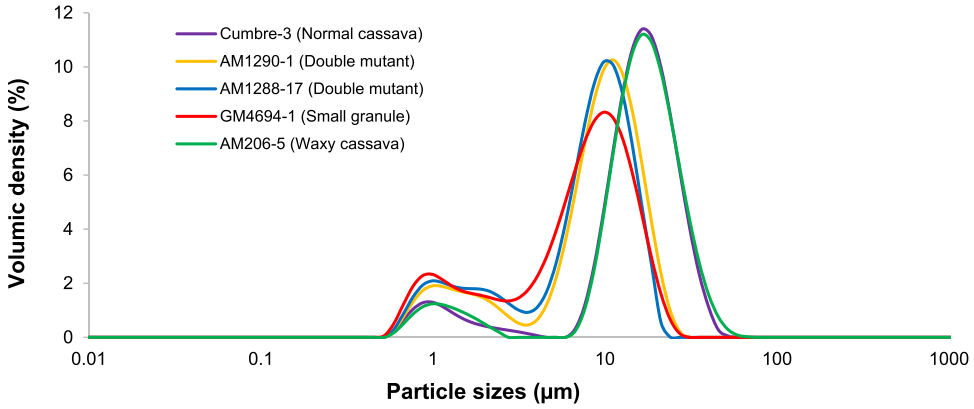

Fig. 3. Granule size distribution of GM4694–1 (small granule) compared to other special genotypes such as AM1290–1 and AM1288–17 (double-mutant), AM206–5 (waxy cassava) and Cumbre3 (wild-type cassava).

a 91 % reduction in dry matter with respect to the initial concentration after 160 hours (Fig. 6). Conversely, in the case of waxy cassava (AM206–5), the decrease was only 42 %, indicating either low starch hydrolysis and/or limited conversion of glucose to ethanol under the SLSF experimental conditions.

The glucose concentration significantly increased ($p<0.01$) during the initial 18 hours of the process, attributed to enzymatic hydrolysis of starch, and subsequently decreased due to fermentation (Fig. 7). In the three samples exhibiting the highest reduction in dry matter (small-granule GM4694–1, double-mutant AM1288–17, wild-type Cumbre3, Fig. 6), glucose was nearly fully utilized after 160 hours, indicating efficient progression of both hydrolysis and fermentation processes. The higher concentrations of glucose reached by GM4694–1 and AM1288–17 at 18 hours suggested that small granules were more sensitive to enzymatic attack, as also indicated by the starch digestibility and hydrolysis kinetics results, and released glucose faster than wild-type starch (Cumbre3), at least initially before fermentation set in and brought glucose levels back down. In contrast, in waxy starch (AM206–5), glucose concentration initially increased and reached a plateau instead of the expected decrease due to fermentation reactions (Fig. 7), confirming the earlier observation of low conversion of dry matter to ethanol.

A hypothesis to account for the suboptimal conversion rate of AM206–5 is that the low susceptibility of waxy starch to enzymatic hydrolysis (Sections 3.1.2 and 3.2) resulted in a slower production of glucose during the initial hours of the experiment. This, in turn, may have limited the initial growth of yeasts, leading to sub-optimum fermentation reactions. The subsequent accumulation of glucose could have further impeded enzymatic hydrolysis and inhibited yeasts due to excessive osmotic pressure (Puligundla et al., 2011; Szambelan et al., 2018).

3.3.2. *Evolution of ethanol content and yield during SLSF-VHG process*

The ethanol concentration increased gradually throughout the fermentation period, revealing significant differences ($p<0.001$) among the evaluated genotypes (Fig. 8). Ethanol production was most pronounced and rapid for the small-granule genotype (GM4694–1), reaching 16.0 % v/v at 90 hours of fermentation, two to three days earlier than other genotypes. The wild-type cassava (Cumbre3) and one double-mutant (AM1288–17) genotype achieved similar concentrations but only at 160 hours, requiring an additional 70 hours. Other genotypes (AM1290–1, AM206–5) reached lower ethanol concentrations

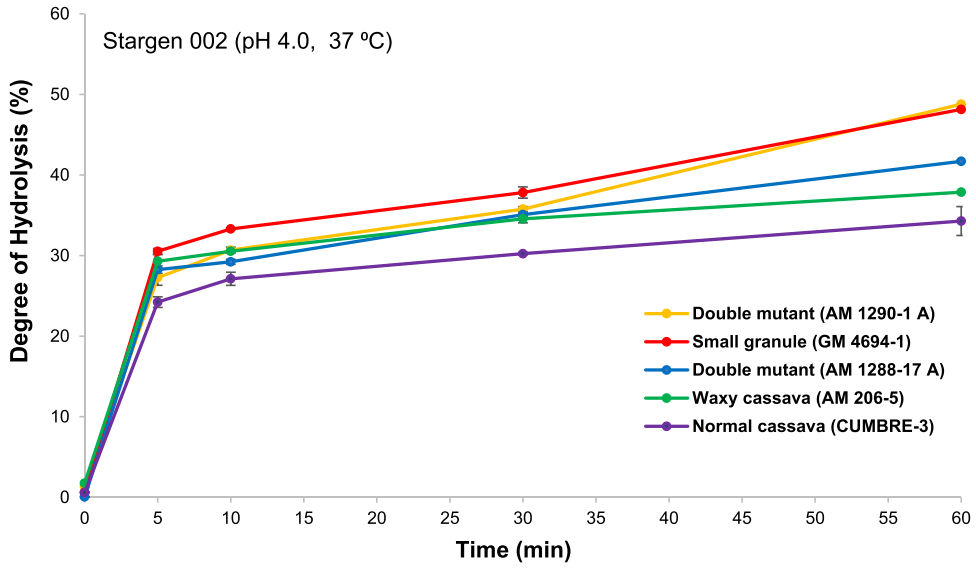

Fig. 4. Hydrolysis patterns of different cassava genotypes, using Stargen 002.

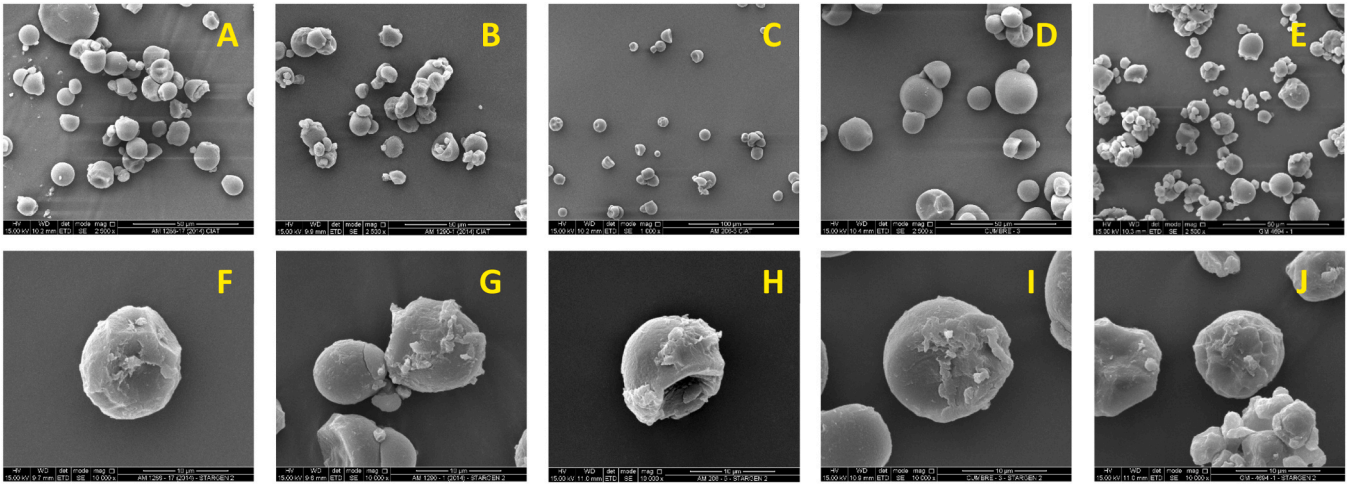

Fig. 5. A-E SEM images of native starch granules (50 μm). A: AM1288–17 (A); B: AM1290–1 (A); C: AM206–5; D: Cumbre3; E: GM4694–1. F-J SEM images of starch granules incubated with Stargen 002 (10 μm). F: AM1288–17 (A); G: AM1290–1 (A); H: AM206–5; I: Cumbre3; J: GM4694–1.

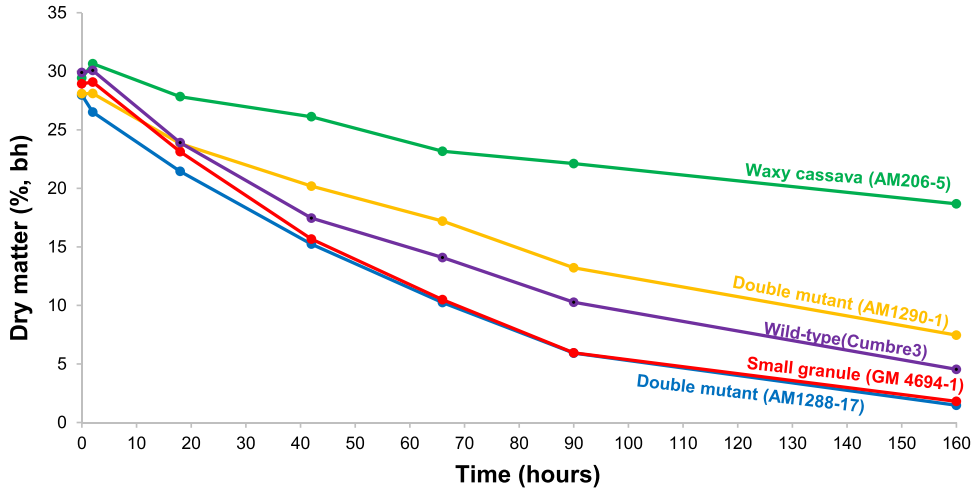

Fig. 6. Evolution of dry matters during SLSF-VHG process.

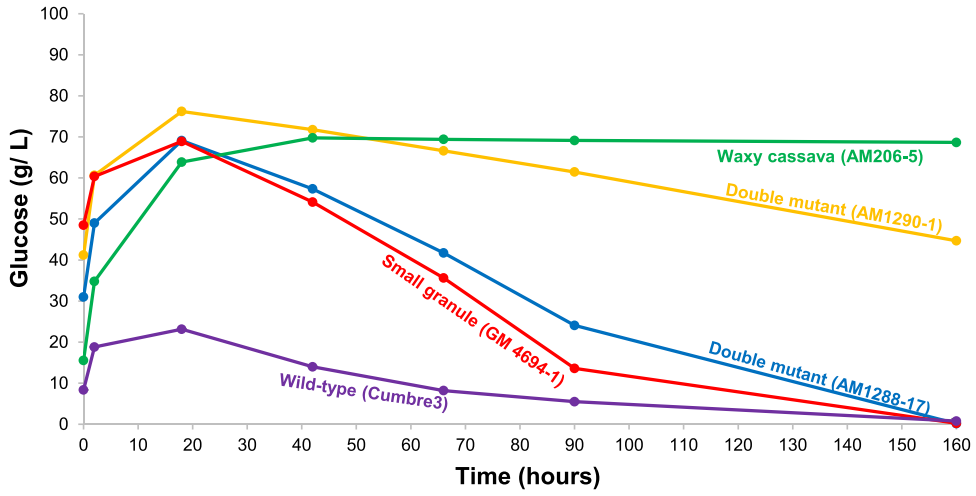

Fig. 7. Evolution of glucose contents during SLSF-VHG process.

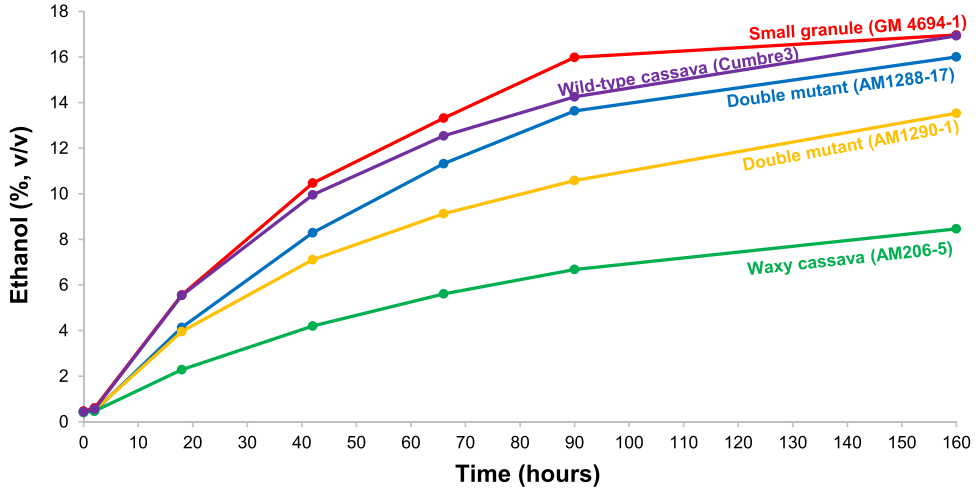

Fig. 8. Evolution of ethanol contents during SLSF-VHG process.

(8.46–13.53 % v/v) at 160 hours of fermentation, consistent with the earlier observation of high glucose levels (Fig. 7) and residual dry matter in the reaction medium after fermentation (Fig. 6).

Ethanol conversion yields (Table 4) were calculated as the ratio of ethanol obtained after 90 and 160 hours of SLSF to the theoretical maximum ethanol that would be obtained if the initially present starch was fully hydrolyzed and converted to ethanol. The small-granule genotype (GM4694–1) exhibited a faster release of sugars and more rapid fermentation, achieving 80.1 % of the theoretical maximum ethanol yield in 90 hours (under the conditions of the trials). In comparison, the double-mutant (AM1288–17) and wild-type cassava starch (Cumbre3) required 160 hours to reach similar ethanol yields. Ethanol concentrations and yields obtained with the small-granule (GM4694–1) and wild-type (Cumbre3) genotypes were comparable to those reported in the literature for wild-type cassava starch using the conventional high-temperature liquefaction and saccharification process (84 % ethanol yield in 48 hours) (Pervez et al., 2014), as well as the simultaneous liquefaction, saccharification, and fermentation process (82.3 % and 99.6 % ethanol yield in 120 hours) (Ueda et al., 1981). Consequently, the SLSF-VHG process achieved similar ethanol production, in these genotypes, as the conventional process, with the added advantage of operating at lower temperatures and reducing heat requirements. On the downside, the SLSF process required longer fermentation times. However, this drawback was partially mitigated by combining SLSF with small-granule cassava starch, which reduced fermentation time by 40 % (from 160 to 90 hours), thereby increasing production capacity compared to SLSF with wild-type starch.

Table 4
Ethanol yield during SLSF process.

Starch	Genotype	Characteristic	Ethanol yield (%)*	
			90 hours	160 hours
Cassava	GM4694–1	Small granule	80.1[a]	84.9[a]
Cassava	Cumbre3	Wild-type	71.4[b]	84.7[a]
Cassava	AM1288–17	Double-mutant	68.3[c]	80.1[b]
Cassava	AM1290–1	Double mutant	53.0[d]	67.7[c]
Cassava	AM206–5	Waxy	33.5[e]	42.3[d]

* The conversion efficiency was calculated from the theoretical yields. Within each column, means with different superscript letters are significantly different ($p < 0.05$).

3.3.3. By-products

The main by-products, aside from CO_2, were glycerol and lactic acid, and their concentrations increased with fermentation time (Table 5). Glycerol accumulated steadily throughout the entire process, and the final quantities after 160 h were proportional to ethanol production ($R^2 = 0.98$). In contrast, lactic acid accumulated concurrently with the increase in ethanol production but tended to stabilize or decrease in the

Table 5
Evolution of Glycerol and Lactic acid during SLSF-VHG.

Parameter	Time (h)	AM1288-17	AM1290-1	AM206-5	GM4694-1	Cumbre3	*p-ANOVA*
Glycerol (g/L)	0	0.00 ± 0.0^{a}	0.00 ± 0.0^{a}	0.00 ± 0.0^{a}	0.0 ± 0.0^{a}	0.00 ± 0.0^{a}	0.0950
	2	0.00 ± 0.0^{b}	0.01 ± 0.0^{b}	0.00 ± 0.0^{b}	0.09 ± 0.0^{a}	0.00 ± 0.0^{b}	0.0008
	18	3.75 ± 0.9^{a}	3.42 ± 0.1^{a}	1.66 ± 0.0^{b}	4.50 ± 0.0^{a}	4.64 ± 0.1^{a}	0.0045
	42	6.83 ± 0.3^{a}	5.32 ± 0.2^{b}	2.99 ± 0.1^{c}	7.27 ± 0.0^{a}	7.21 ± 0.1^{a}	<0.0001
	66	8.37 ± 0.1^{a}	6.60 ± 0.2^{b}	3.57 ± 0.0^{c}	8.74 ± 0.0^{a}	8.53 ± 0.2^{a}	<0.0001
	90	9.08 ± 0.2^{a}	7.35 ± 0.2^{b}	4.10 ± 0.1^{c}	9.52 ± 0.0^{a}	9.09 ± 0.3^{a}	<0.0001
	160	9.47 ± 0.3^{ab}	8.49 ± 0.0^{b}	4.64 ± 0.6^{c}	10.09 ± 0.0^{a}	9.90 ± 0.3^{a}	<0.0001
Lactic acid (g/L)	0	0.09 ± 0.0^{bc}	0.16 ± 0.0^{bc}	0.02 ± 0.0^{c}	0.49 ± 0.0^{a}	0.18 ± 0.0^{b}	0.0003
	2	0.11 ± 0.0^{bc}	0.23 ± 0.1^{b}	0.01 ± 0.0^{c}	0.48 ± 0.0^{a}	0.17 ± 0.0^{b}	0.0001
	18	0.52 ± 0.1^{bc}	0.78 ± 0.2^{ab}	0.28 ± 0.0^{c}	1.14 ± 0.0^{a}	0.67 ± 0.0^{bc}	0.0033
	42	0.84 ± 0.2^{b}	0.94 ± 0.2^{b}	0.44 ± 0.0^{b}	1.48 ± 0.0^{a}	0.94 ± 0.0^{b}	0.0041
	66	1.04 ± 0.1^{bc}	1.16 ± 0.3^{b}	0.64 ± 0.0^{c}	1.68 ± 0.0^{a}	1.06 ± 0.1^{bc}	0.0042
	90	1.14 ± 0.0^{bc}	1.36 ± 0.2^{ab}	0.75 ± 0.1^{c}	1.70 ± 0.0^{a}	1.16 ± 0.2^{bc}	0.0027
	160	1.06 ± 0.1^{b}	1.64 ± 0.1^{a}	1.20 ± 0.1^{b}	0.65 ± 0.0^{c}	1.15 ± 0.0^{b}	0.0003

Data shown are the mean ± standard deviation from two determinations. Within each row, means with different superscript letters are significantly different ($p < 0.05$).

final stages of fermentation (between 90 and 160 h) for genotypes that reached near their maximum ethanol production earlier than 160 h (GM4694–1, AM1288–17, Cumbre3). This observation suggests that as the glucose-to-ethanol reaction slowed down due to glucose depletion, the yeast may have started to utilize part of the lactic acid as an alternative source of energy.

3.3.4. Chemical characterization of residue obtained from the SLSF process

SLSF-Res primarily contained residual starch left after the hydrolysis and fermentation process (Table 6). Small-granule and double-mutant genotypes exhibited the lowest total starch content, aligning with their higher starch conversion rates, such as 73 % observed in the case of GM4694–1. In contrast, the waxy genotype AM206–5 had the highest starch content (89.1 %) and, correspondingly, a very low starch conversion rate (8 %). Amylose content in SLSF-Res was notably lower than in native cassava starch, typically comprising 18–20 % of the starch fraction, except in the case of Cumbre3. This decrease may be attributed to the preferential attack by Stargen 002 and Amigase enzymes on the amorphous regions of the starch granules, which tend to be richer in amylose (Zhang and Oates, 1999). SLSF-Res also contained a modest amount of proteins resulting from yeast metabolism (Table 5), as well as fractions of rapidly and slowly digestible starch. These characteristics suggest that SLSF-Res may serve as an ingredient for animal feed, complementing other ingredients richer in proteins.

Scanning electron microscopy (SEM) micrographs of SLSF-Res (Fig. 9) revealed partially hydrolyzed starch granules for all five cassava genotypes. The amylase enzymes seemed to attack the granules from the outside, leaving partially hydrolyzed external layers. Notably, no pores opening towards the inside of the granules were visible, in contrast to other types of starches, particularly cereal starches, that had undergone similar enzymatic treatment (Demirkan et al., 2005; Jung et al., 2017). The small-granule genotype GM4694–1 appeared more damaged (Fig. 8E), with granules having lost more of their shape compared to others. This observation is consistent with previous results indicating that small-granule cassava starch is more sensitive to attack by amylolytic enzymes.

3.3.5. Correlation analysis among physicochemical properties of native starch and the properties of the slurry at the end of the SLSF process

Parameters related to native starch digestibility, such as RDS and RS, exhibited correlations (Fig. 10) that were positive and negative, respectively, with hydrolysis kinetics (H-Stargen). Starch granule size (Dv50) also showed positive correlation with RS and negative correlation with RDS and hydrolysis kinetics, supporting the hypothesis that small-granule starch is more susceptible to enzymatic attack due to a higher surface-to-volume ratio. Additionally, ash content was associated with higher hydrolysis kinetics and lower PT from RVA. A possible explanation is the presence of phosphate groups grafted onto starch molecules, which may reduce the crystallinity of starch granules and, consequently, increase susceptibility to hydrolysis and gelatinization.

Starches with higher digestibility (RDS) and small granules were expected to foster better fermentation, resulting in increased ethanol production and reduced DM and glucose in the slurry at the end of fermentation. This was only partially verified, with Pearson correlation coefficients between Dv50 (starch granule size) and ethanol as well as DM being −0.52 and −0.68, respectively. On the contrary, ash content, indicating potentially lower crystallinity of native starch, correlated significantly with ethanol production ($R = 0.71$) and reduction in DM ($R = -0.80$). Pasting temperature also seemed to predict the extent of the SLSF process, where higher PT (indicating lower susceptibility to gelatinization or enzymatic fixation) correlated with higher DM and lower ethanol content in the final slurry.

4. Conclusions

The potential for high-gravity (300 g/L), low-temperature (30°C)

Table 6
Chemical characterization of the residues obtained from the SLSF process (SLSF-Res).

Sample	Moisture (%, wb)	Protein (%, db)	Amylose (%, db)	Starch content (%, db)			
				TS	RDS	SDS	RS
AM1288–17	11.4 ± 0.8^{b}	0.4 ± 0.00^{d}	$< LQ^{c}$	36.1 ± 1.4^{d}	14.0 ± 1.8^{ab}	19.9 ± 0.0^{cd}	2.2 ± 1.8^{c}
AM1290–1	8.3 ± 0.6^{a}	1.0 ± 0.04^{a}	$< LQ^{c}$	49.2 ± 2.1^{c}	15.6 ± 1.7^{ab}	26.3 ± 3.4^{bc}	7.2 ± 1.7^{c}
AM206–5	7.7 ± 0.1^{a}	0.2 ± 0.00^{e}	0.7 ± 0.06^{b}	89.1 ± 1.6^{a}	20.0 ± 2.1^{a}	34.7 ± 0.4^{a}	34.4 ± 1.7^{a}
GM4694–1	10.7 ± 0.3^{b}	0.5 ± 0.01^{c}	$< LQ^{c}$	25.5 ± 2.1^{e}	9.0 ± 1.7^{b}	16.0 ± 1.7^{d}	0.2 ± 0.0^{c}
Cumbre3	8.0 ± 0.1^{a}	0.7 ± 0.01^{b}	9.6 ± 0.00^{a}	60.2 ± 1.5^{b}	15.5 ± 1.4^{ab}	28.2 ± 1.4^{ab}	16.5 ± 2.8^{b}
ANOVA	0.0018	<0.0001	<0.0001	<0.0001	0.0131	0.0009	<0.0001

<LQ: less than the limit of quantification, which is he first concentration level of the calibration curve (0 %); TS: total starch; RDS: rapidly digestible starch; SDS: slowly digestible starch; RS: resistant starch. Data shown are the mean ± standard deviation from two determinations. Within each column, means with different superscript letters are significantly different ($p < 0.05$).

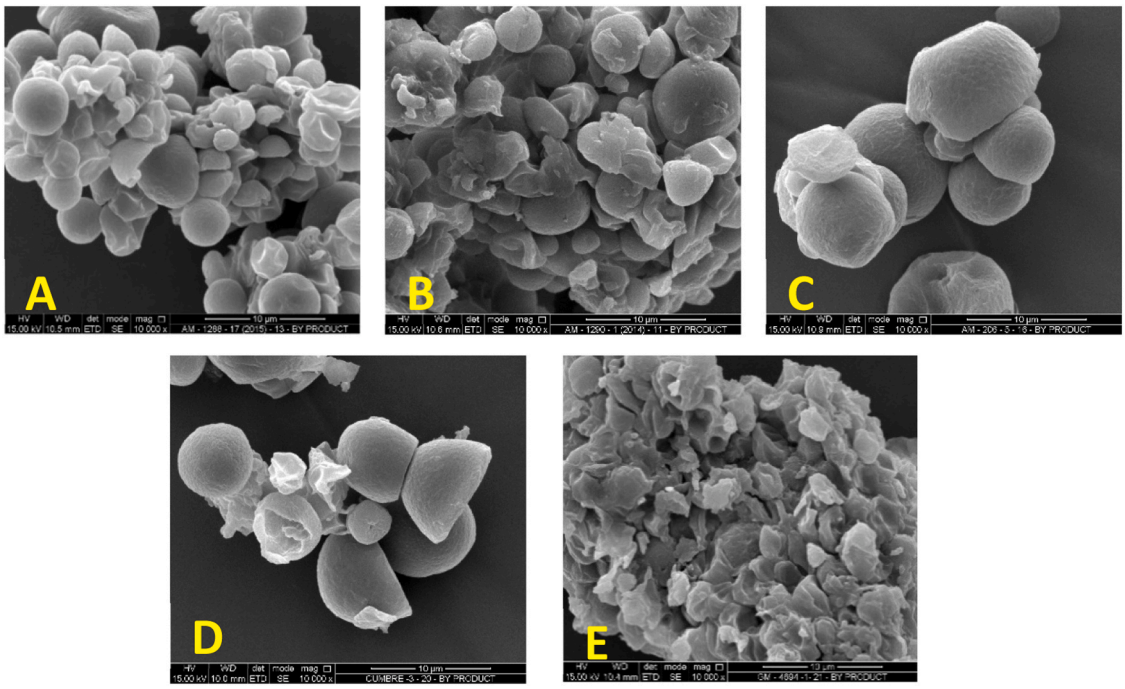

Fig. 9. A-E SEM images of SLSF-Res residue. A: AM1288–17; B: AM1290–1; C: AM206–5; D: Cumbre3; E: GM4694–1.

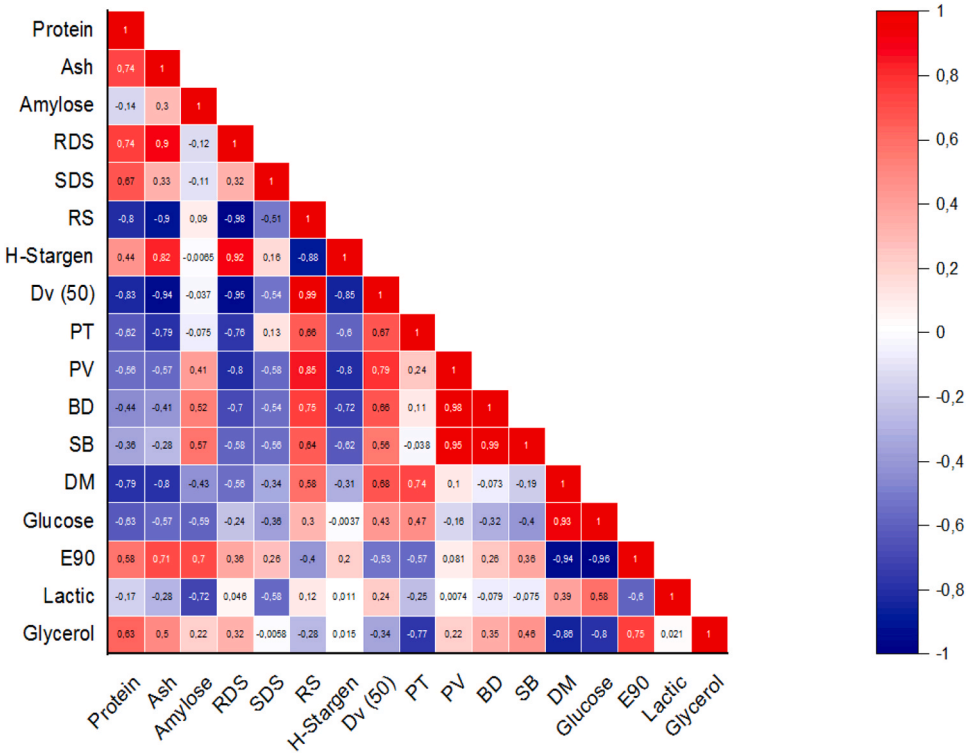

Fig. 10. Pearson correlation between the physicochemical properties of native starch (Protein, Ash, Amylose, RDS, SDS, RS, H-Stargen (starch hydrolysis), PT, PV, BD, SB) and properties of the slurry at the end of the SLSF process (DM, Glucose, Ethanol (at 90 h), Lactic acid, Glycerol).

SLSF of various cassava starches from the Cassava Breeding Program (Alliance Bioversity CIAT) with special traits (waxy, small-granule, double-mutant) was evaluated. Genotype GM4694–1 (small-granule) hydrolyzed faster and produced ethanol (17 % v/v) in a shorter time compared to other genotypes (90 hours instead of 160 hours). This makes GM4694–1 a promising feedstock for ethanol production and potentially other fermentations, such as organic acids (e.g. lactic acid). Although SLSF requires longer processing times (up to 160 hours per batch) than conventional fermentation, it offers the advantages of a single step (instead of three) conducted at higher concentrations, lower temperatures (30–35°C instead of 60–90°C), and consequently significantly lower heating requirements. Combining SLSF with the shorter processing time observed with small-granule cassava starch seems therefore a promising option to minimize production costs and

environmental impacts of bioethanol production, to be further tested at pilot-scale and industrial-scale.

CRediT authorship contribution statement

Jhon Larry Moreno Alzate: Writing – original draft, Validation, Investigation, Data curation. **Hernan Ceballos:** Writing – review & editing. **Chinh Nghia Nguyen:** Writing – review & editing, Writing – original draft. **Thierry Tran:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Son Chu-Ky:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Tien Cuong Nguyen:** Writing – review & editing, Writing – original draft. **Xiaofei Zhang:** Validation, Conceptualization. **Jonathan Newby:** Validation, Conceptualization. **Dominique Dufour:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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