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Valorisation of insect infested sweet sorghum reeds towards production of a fermented beverage

Tawanda Proceed Makopa[1,5*], Thandiwe Semumu[1,2], Mpho T. Gaaipone[1], Thato Masemola[3], Santosh Ramchuran[3], Urska Vrhovsek[4] and Nerve Zhou[1*]

Abstract

Sweet sorghum variety (*Sorghum bicolour* (L)) commonly known as sweet reeds, *Ntšhe*, in Setswana, is a valuable cash crop mostly for small scale farmers in Botswana and other southern African countries. These reeds are widely consumed as a delicacy and contribute significantly to food security, employment, and rural incomes. However, infestations by the larval stages of *Chilo partellus* (stem borer moths) lead to substantial economic losses, as consumers reject worm-infested reeds. To mitigate these losses, valorisation of condemned sweet reeds is attractive. Here, we took advantage of our understanding of yeast-insect interactions to isolate yeasts associated with larval stages of the stem borer moths and investigated their potential for use in the production of an alcoholic sweet sorghum beverage. We report the isolation of thirty-two yeast strains from the larvae and assessed their ability to ferment the simplest sugar, glucose, a constituent of the sweet sorghum juice. Out of the selected yeasts, a subset of fourteen strains belonging to *Hanseniaspora and Candida* genera were further characterised based on their capacity to ferment more sugars found in sweet sorghum juice. We further assessed the isolates for the ability to tolerate brewing/fermentation-associated stresses and production of complex aroma profiles towards the use of sweet sorghum juice as a sole feedstock to produce a commercial beverage. Our findings suggest that yeast-insect interactions offer a promising approach for converting rejected sweet sorghum stalks into a novel alcoholic beverage, adding economic value to an otherwise discarded resource.

Clinical trial number Not applicable.

Take Away

- *Chilo partellus* larvae, a major destructive stem boring pest in southern Africa, hosts fermentative yeasts with potential as starter cultures for a sweet sorghum beverage.
- Non-*Saccharomyces* larvae-associated yeasts have desirable brewing attributes such as fermentative capability; ability to tolerate brewing associated stresses and ability to produce complex and unique aroma profiles.
- This work suggests valorisation of *Chilo partellus* infested sweet sorghum stalks, through the production of a novel fermented beverage, as a way for Botswana's subsistence farmers to reduce losses.

*Correspondence:
Tawanda Proceed Makopa
makopa.tawanda@studentmail.biust.ac.bw
Nerve Zhou
zhoun@biust.ac.bw

Full list of author information is available at the end of the article

Keywords Insect-associated yeasts, Sweet sorghum fermentation, Non-*Saccharomyces* yeasts, Fermented alcoholic beverage

Introduction

"*Mo gase ntšhe tota tota*" ("This is a not a sweet reed"), a popular Setswana descriptor used by consumers who buy sweet reeds (sorghum) (*Sorghum bicolour* (L.) (Commonly known as *Ntšhe*) from the markets, infested with larvae of stem borer moths (*Chilo partellus*) (Crambidae, Lepidoptera) [1–4]. Sweet sorghum, similar to grain sorghum except for its juicy sugar-rich stalks; previously grown for fodder in southern Africa during the colonial times has become a delicacy sold by subsistence farmers who line the streets of Botswana towns during the months of January to April every year. Subsistence farming of this palatable and nourishing crop plays a dominant role in provision of income, food and employment as well as provision of economic opportunities to the rural population [5]. In addition, farmers prefer sweet sorghum farming due to its lower requirement for water in a semi-arid environment of Botswana [6]. However, heavy infestation of sweet sorghum stalks by vociferous feeding larval moths lead to significant economic losses [7, 8]. The larval stages of the moths bore into the sweet sorghum stems resulting in an extensive network of pink to brown stained frass-filled galleries rendering 25–30% of the stalks unpalatable and therefore rejected by consumers [4]. The maize (*Zea mays*) and sorghum (*S. bicolour* L.) moth is an important polyphagous pest in southern Africa Mutamiswa, Chidawanyika [9]. Maize and sorghum are the most common cereals in Botswana grown at commercial and small-scale levels. The moth lays eggs on the plant, which later hatch into vociferous, stalk boring larval stages. The stalk boring larvae tunnels into the stalks and produces tunnels with brown frass, which is associated with depreciation of the quality of the delicacy. The condemned and unpalatable sweet sorghum, "*Mo gase ntšhe tota tota*" which can result in 5–73% losses [10] is the major drawback of the business. In contrast to commercial farmers, who use chemical means to control the insect pest, the resource-poor farmers, are financially constrained [11]. As a measure to ameliorate the negative economic implications, valorisation of the condemned sweet sorghum is attractive. Crop infestation is a result of chemical control's several shortcomings as a primary pest management strategy. The pest is inaccessible inside the stalk galleries [2]. Valorisation of condemned sweet sorghum stalks in the production of value-added products is one strategy to reduce the losses incurred by farmers.

The potential for valorisation through fermentation of sweet sorghum juice in the production of fermented beverages is attractive. Taking advantage of our understanding of mutualistic insect-yeast associations [12–15], there is potential to find yeasts with ability to efficiently ferment sweet sorghum juice from the unaffected parts of the stalks. Studies demonstrating the industrial potential of yeasts transmitted to plants by insects document that yeasts possess a variety of traits applicable in food and beverage fermentations. Diverse ability to ferment different carbohydrates and tolerance to industrial stresses are among the most cited traits. Sugar-seeking insects harbouring non-conventional yeasts with potential in bread making [16], maltose negative yeasts with potential in production of low/no-alcoholic beers production [17], yeast with potential in bioflavourants production [17], and other common yeasts with potential in coffee and cocoa fermentation [18] have recently been reported. In addition, the non-conventional yeasts are known for their complex aroma profiles, which allow them to be used in co-fermentations along with *Saccharomyces* yeasts [19]. Several other insect associated yeasts have been used in other modern bioprocesses including waste degradation, biosurfactant production, biofuel production and pest management [20–24]. Yeast mutualistic interactions with larval stages of Lepidopteran larvae in sugar rich niches are widespread [25–30]. Yeasts ferment sugary juices to provide nutrition and produce a bouquet of aromatic volatiles required in olfactory recognition of food, breeding and egg laying insects [12, 14, 31, 32].

In this study, we proposed to ferment the remaining or un-tunnelled sections of the stalk, which is about 80% of the stalk, to produce a value-added product such as an alcoholic beverage (Fig. 1). All the sweet sorghum was collected from Palapye vending stalls and Malaka, a village about 30 km outside Palapye. The village subsistence and small-scale farmers produce and supply Palapye market vendors. Although other potential yeasts already described, commercialised, or domesticated exist, we took advantage of our understanding the ecology of yeasts and their interactions with insects in nature, to explore the potential of using native yeasts. Thus this work sought to investigate the presence of potential fermentative yeasts in association with larval moths and use the findings as leads into the applications of these yeasts in bioprocessing, towards a fermented sweet reed beverage. We assessed the ability of isolated yeasts to exhibit brewing traits such as the fermentative capacity and brewing-associated stress tolerance. We further assessed ethanol content and aromatic profiles of the produced beverages to highlight the potential to obtain a consumer-acceptable alcoholic beverage. This work, to the best of our knowledge, is the first work to document

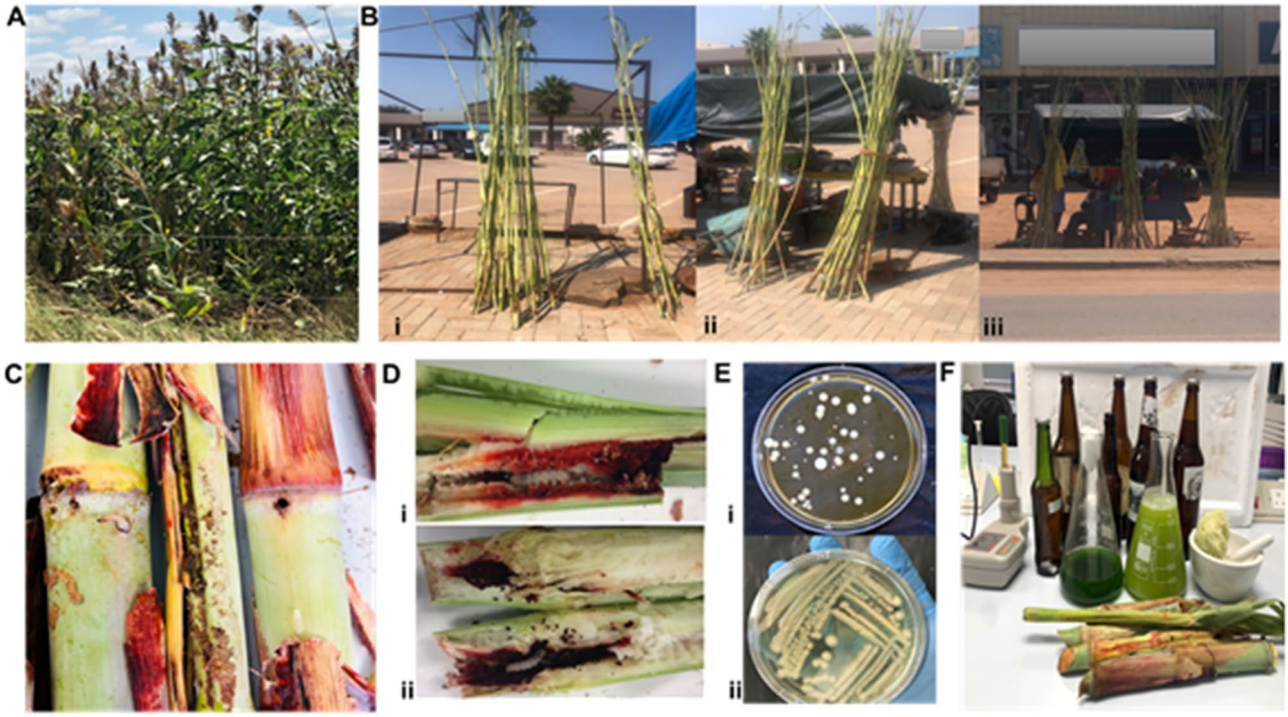

Fig. 1 Graphic summary of the steps towards production of fermented sweet sorghum alcoholic beverage. (**A**) Sweet sorghum in the field (**B**) Sweet sorghum being sold on the streets (**C**) Condemned sweet sorghum stalks with *C. partellus* larvae (**D**) Galleries and frass with *C. partellus* larvae (**E**) Yeasts comprising *Hanseniaspora* and *Candida* species isolated from larvae gut, galleries and frass (**F**) Sweet sorghum juice in conical flask and fermented sweet sorghum alcoholic beverage bottled

the fermentative potential of yeasts associated with the stem borer to valorise condemned sweet sorghum stalks.

Materials and methods

Sample collection and sorghum juice extraction

Sweet sorghum reeds were bought from street vendors from Palapye and Malaka in Central region of Botswana. Stalks with round holes evident with pink to brown colouration (Fig. 1C) revealing the damage by the larval stages of moths were cut and taken to the laboratory for isolation of yeasts. Undamaged sections of the stalks were peeled and cut into small pieces using a knife and crushed using a pestle and mortar to extract the sorghum juice. The sorghum juice was filter sterilised through a 0.22 µm nitrocellulose filters and stored at – 20 °C before use.

Isolation of fermenting yeasts

To isolate fermenting yeasts, the damaged sorghum stalks were cut open to expose the galleries and the boring insect larvae as well as the associated feeding frass. A total of fourteen insect larvae were collected and separated from the frass (together with feeding residues) and placed in sterile petri dishes. The larvae were surface sterilised by immersing them in 95% ethanol for 5 min. The guts of individual larvae were ripped open and homogenised in sterile phosphate buffered saline (PBS) using sterile forceps. The homogenates were resuspended in test tubes containing 2 mL of filter sterilised sweet sorghum juice. A cocktail of antibiotics was added into the tubes as previously described by Makopa, Modikwe [33]. These samples were divided into larvae and frass, making a total of twenty-eight tubes were then placed in a shaking incubator (MaxQ 6000, Thermo Scientific, Ohio, USA) up to 7 days at 180 rpm at 30 °C. Tubes were examined every day for gas production during incubation and fermenting yeasts were isolated and stored as previously described by Makopa, Modikwe [33].

Preliminary anaerobic growth assay

The ability to ferment the simplest sugar, glucose, was assessed by plating all isolates on YPD agar containing 10 g/L yeast extract, 20 g/L glucose, and 20 g/L peptone and 20 g/L Agar at pH 6.2. The plates were inverted and put in an AnaeroPack System (2.5-L jar) containing anaerobic sachets (AnaeroGen™ 2.5 L). The sealed jar was further incubated in an anaerobic incubator (Thermo Fisher Scientific Model 4111) set at 5% CO_2 and at 30 °C for 48 h to select for and isolate anaerobes. *Saccharomyces* industrial strains [34, 35] were used for reference (See Table S5 for their ITS sequences). *Kluyveromyces lactis*, strain CBS 2359 was used as a negative control (non-fermentative yeast). The experiments were done in triplicates and the best presentable plate was scanned and

recorded. A subset of isolates that grew under anaerobic conditions was selected for further studies.

Carbon fermentation assays

A subset of isolated strains that could ferment glucose under anaerobic conditions were further tested for their ability to ferment the other sugars found in sweet sorghum juice. YP agar plates containing 20 g/L yeast extract, 20 g/L peptone, 20 g/L agar of 2 mg/L Antimycin A and supplemented with glucose, sucrose, and fructose as sole carbon sources at 20 g/L each were used for fermentative assays. Antimycin A was added as a functional inhibitor of the mitochondrial electron transport systems. Therefore only yeasts that were able to grow using the fermentative metabolism showed comparable growth to the plates without Antimycin A (used negative controls) [36]. In brief, pure colonies of yeast isolates were picked and grown in test tubes obtaining 2 mL YPD and incubated overnight as above. The cells were harvested by centrifugation at 4000 rpm for 4 min and washed successively with 2 mL Phosphate buffered saline (PBS) twice before resuspending in 1 mL PBS. Cell densities were estimated using a spectrophotometer and adjusted to an OD_{600nm} of 0.2 (approx. 3×10^6 cells/mL) before dispensing 100 µL of each of strains into 96-well plates as described by Semumu, Gamero [37] and Motlhanka, Lebani [38]. The cell suspension was 2-fold diluted and dispensed into successive wells to obtain a series of wells with OD_{600nm} of 0.2, 0.1, and 0.05. The cells were transferred to YP agar plates containing a respective sole carbon source using an 8×6 array stainless steel replicator/stamp. Plates without sugars (containing YP only) were included to account for remnant sugars in yeast extract. The plates were then incubated at 30 °C for 3 days before qualitatively scoring growth. The above-mentioned reference strains were included as positive controls whereas *Kluyveromyces lactis* was a negative control. These experiments were done in triplicates. The most representative plates were selected from triplicate experiments, scanned, and recorded.

Fermentative capacity assay

Isolates with the ability to grow anaerobically were further investigated for their capacity at which they fermented the principal sugars in sweet sorghum juice, glucose, fructose, sucrose as well as sweet sorghum juice as a carbon source. The respective carbon sources (50 g/L) were supplemented with 10 g/L yeast of Yeast Extract and 20 g/L of peptone and pH adjustments to 6.2 except for media with sorghum juice as the sole source of carbon. Yeast inoculums were grown overnight, washed and quantified as previously described by Semumu, Gamero [37] and Motlhanka, Lebani [38]. The cells were then inoculated into 3 mL of respective media in 60 mL BD Luer-Lok™ syringes (BD® Syringes) and incubated at 30 °C at 180 rpm for 3 days as previously described by Semumu, Gamero [37] and Motlhanka, Lebani [38]. The initial concentration of cells was set at OD_{600nm} of 1. The plunger movement, as CO_2 was accumulated was recorded after every 2 h. The fermentative capacity was calculated as the rate of change of CO_2 accumulation (mL/hr). The positive and negative controls were included as above. These experiments were done in triplicates.

Brewing associated stress tolerance

We assessed for comparative tolerance of the potential isolates to brewing-associated stresses such as osmotic stress, oxidative stress, ethanol stress and thermal stress using spot assays as previously described by Semumu, Gamero [37] and Motlhanka, Lebani [38]. Overnight cultures were prepared and replica plated on YPD media (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose agar) supplemented with each of the following stress inducing agents: oxidative stress (3 mM, 4 mM, 5 mM, and 7 mM hydrogen peroxide (H_2O_2)), osmotic stress (0.5 M, 0.75 M, 1.0 M, 1.25 M and 1.50 M NaCl) and ethanol stress (5%, 7%, 8% and 9% (v/v) ethanol). Plates were incubated at 30°C for 3 days. Plates containing H_2O_2 and ethanol were sealed using parafilm before incubation. For thermal stress, the cells were plated on YPD media plates, but incubation was carried out at different temperatures (25 °C, 30 °C, 37 °C, and 40 °C). Stress tolerance was assessed qualitatively as previously described [37, 38]. These experiments were done in triplicates. The most representative plates were selected from triplicate experiments and scanned and recorded.

Identification of fermentative yeasts

To putatively identify the characterised isolates, we sequenced the ITS1-5.8S-ITS4 rDNA using ITS1 (5'–TCCGTAGGTGAACCTGCGG–3') and ITS4 (5'–TCCTCCGCTTATTGATATGC–3') [39]. Genomic DNA from each isolate was extracted using ZR Soil Microbe DNA kit™ (Zymo Research, Orange, CA, USA) according to manufacturers' recommendations. Template DNA from each isolate was used to amplify a 560–750 bp region using the Applied Biosystems Proflex Thermal cycler (Thermo Fisher, Marsiling, Singapore) on a PCR program described by Zhou, Schifferdecker [40]. PCR products were analysed by gel electrophoresis (using 1% agarose gel in 1X TBE buffer) and then purified and quantified using an ND-1000 spectrophotometer (NanoDrop; Thermo Scientific, Wilmington, DE, USA). Inqaba Biotechnical Industries in Pretoria, South Africa sequenced the purified PCR products. The removal of uncalled bases was done by using a SnapGene sequence editing tool (http://www.snapgene.com). The yeasts were identified by searching databases using BLAST sequence analysis

tool (http://www.ncbi.nlm.nih.gov/BLAST/). To confirm the identity, pairwise identification database owned by the Westerdijk Fungal Biodiversity Institute (CBS-NAW) (http://www.westerdijkinstitute.nl/) and The Yeast Trust Database (https://theyeasts.org/alignment) were used. Sequences were deposited in the GenBank NCBI database (SUB13837643). For further discrimination of the identified isolates, PCR-RFLP was done in silico using *CfoI* and *HinfI* as restriction enzymes. Using SnapGene® viewer software ver.4.2.11 (http://www.snapgene.com) all the ITS amplicons were first aligned and then trimmed to remain with the consensus regions with the comparable number of base pairs per sample and then run on a simulated 4% agarose gel with Biozym Quantitas (25 to 500 bp) as the molecular weight marker.

Phylogenetic analysis isolated yeasts

Molecular Evolutionary Genetics Analysis software (MEGA X ver.10.2.6) was used for assessing the phylogenetic relationship between the ITS1-5.8 S-ITS4 sequences of identified yeast strains [41]. In brief, multiple sequence alignment was done by log expectation (MUSCLE) [42] and the evolutionary history was inferred using the Maximum Likelihood method based on the Tamura 3-parameter model [43]. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The proportion of sites where at least 1 unambiguous base was present in at least 1 sequence for each descendent clade was shown next to each internal node in the tree. *Schizosaccharomyces pombe* CBS 356T strain was used as an out–group.

Ntšhe beverage brewing and aromatic profiling

All the yeast isolates and the control yeasts (baker's yeast, ethanol red, wine yeast, lager yeast and ale yeast) were grown in YPD. The overnight cultures were grown, harvested, washed, and quantified as stated above. The cells were inoculated into 5 mL of sweet sorghum juice to a final OD_{600nm} of 1 in 15 mL centrifuge tubes. After 2 weeks, the supernatant was centrifuged at 8000 rpm for 5 min in a microcentrifuge and sterile filtered into 2 mL microcentrifuge tubes using 0.22 µm filters before storing at – 20 °C for metabolite and aroma analyses. Initial sugar concentrations of were measured from the unfermented sweet sorghum juice and the residual sugar concentrations were measured after fermentation. Concentrations of initial and residual sugars were determined using an ultra-high-performance liquid chromatography system, (Thermo Scientific Dionex Ultimate 3000 UHPLC, Milan, Italy). Ethanol concentrations were determined using a gas chromatography instrument (Agilent 7890 A Gas Chromatography System, California, United States of America). The ethanol enzymatic kits (Megazyme) according to manufacturer´s recommendations were also used as described elsewhere [44]. Volatile organic compounds produced by yeasts were analysed as previously described by Makopa, Modikwe [33]. The results were analysed using a heatmap to best understand the variation in the aroma compounds produced. A Z-score correlating to different colours was ascribed to represent the data of the organic compounds. Unfermented sweet sorghum juice was used as the reference substrate. The results were analysed in triplicates using R package ver.1.0.12 "pheatmap" software [45] to generate a heatmap.

Statistical analysis

Statistical analysis was performed using STATISTICA ver.13.2 (StatSoft Inc., Oklahoma, USA). One-way analysis of variance (ANOVA) and the Tukey's *post-hoc* test (95% confidence interval) were used for estimating the statistical differences between respective means of ethanol produced by the yeast strains.

Results and discussion

The guts of larvae and associated feeding frass harbour yeasts

The condemned sweet sorghum stalks were infested with spotted stem borer larvae (Fig. 1C and D). All the insect larvae were identified as the larval stage of the moth, *Chilo partellus* Swinhoe in the family Crambidae (Lepidoptera).

We successfully isolated a total of thirty-two yeast strains from fourteen larvae belonging to *C. partellus*. While 53% (17 out of 32) of the strains were isolated from the guts of the larvae, 47% (15 out of 32) strains were isolated the feeding frass found in the galleries of sorghum stalks (Table 1). Yeast associations with insects are widespread [13, 16, 26, 27, 46–49]. The behaviour, development and ability of Drosophilid insects' larvae to survive and reach reproductive stages is determined by presence of a specific type and respective amount of yeasts within their diet [50, 51]. The absence of yeasts result in failure of larvae to reach pupal stage [52, 53]. Other than nourishment, yeast antagonism suppresses entomopathogenic fungi [54] and reduces mortality of Lepidopteran insects [27].

Stem borer-associated yeasts have fermentative or respiratory traits

Yeasts involved in fermentative valorisation of sweet sorghum juice must be able to ferment sugars in the juice. Analysis of sugars found in sweet sorghum juice revealed that sucrose was the most abundant (20.6 g/L ± 0.089) sugars followed by glucose (14.2 ± 0.068 g/L), fructose (11.6 g/L ± 0.060) (Table S1). The results were in agreement with work from Kim and Day [55] who reported the same sugars in respective ratios. As preliminary

Table 1 Yeasts isolated from *Chilo partellus* larvae and sweet sorghum Frass

Isolate	Anaerobic growth	Source	Location
S40	+	Larva 1, gut	Palapye market
S41	–	Larva 1, gut	Palapye market
S42	–	Larva 1, gut	Malaka
S43	–	Larva 2, gut	Palapye market
S44	–	Larva 2, gut	Palapye market
S45	–	Larva 2, frass	Palapye market
S46	–	Larva 2, frass	Palapye market
S47	+	Larva 2, frass	Palapye market
S48	+	Larva 3, gut	Malaka
S49	+	Larva 4, gut	Malaka
S50	+	Larva 5, gut	Palapye market
S51	–	Larva 5, gut	Palapye market
S52	–	Larva 5, frass	Palapye market
S53	+	Larva 6, gut	Palapye market
S54	+	Larva 7, gut	Malaka
S55	–	Larva 7, frass	Malaka
S56	+	Larva 8, gut	Malaka
S57	+	Larva 9, frass	Malaka
S58	+	Larva 10, gut	Palapye market
S59	–	Larva 10, frass	Palapye market
S60	+	Larva 11, gut	Palapye market
S61	–	Larva 11, gut	Malaka
S62	–	Larva 11, gut	Palapye market
S63	–	Larva 12, frass	Malaka
S64	–	Larva 12, frass	Palapye market
S65	–	Larva 12, frass	Palapye market
S66	+	Larva 13, frass	Malaka
S67	–	Larva 13, frass	Palapye market
S68	+	Larva 13, gut	Palapye market
S69	+	Larva 14, frass	Palapye market
S70	–	Larva 14, frass	Malaka
S71	–	Larva 14, frass	Malaka
AY	+	Control	n/a
BY	+	Control	n/a
ER	+	Control	n/a
LY	+	Control	n/a
WY	+	Control	n/a

Note: The source of the yeast isolates is presented as either from the galleries and frass or from the guts of the larva. Ability to grow anaerobically on glucose is shown by (+), while (–) shows the inability

screening for fermentative ability, we assessed the ability of insect-associated yeasts to ferment the simplest sugar, glucose. Glucose fermentation is an important criterion when selecting starter cultures for a fermented beverage, as it is usually the most preferred carbon source by most industrial strains used in cereal-based beverage bioprocessing [56]. In addition, glucose is a fermentable by-product of most carbohydrate catabolism and has a key role in essential metabolic pathways like glycolytic pathway. Our results suggest that a subset of fourteen strains (44%) were capable of fermenting glucose whereas the remainder (56%) could not (Table 1). Ten of the fermenting yeasts were isolated from the guts (Table 1). The presence of yeast strains that could not ferment glucose among the isolates, despite glucose being the carbon source used for isolation, suggests the yeasts can respire it, as the alternative pathway to assimilate it. These results suggest physiological diversity of yeasts associated with Lepidopteran insects as described elsewhere [52]. The results indicate that the source from which the yeasts were isolated could also make a huge difference, as those in the insect gut may be exposed to sugars already partly broken down by the larvae [13, 26].

Moreover, the limited oxygen levels within the insect gut would require them to ferment the abundant sugars and they have to survive other stresses commonly associated with the insect gut [57, 58]. The available substrate that the insect feeds on is another major factor influencing yeast species profiles within the insect gut. Thus, the yeasts isolated from sugar seeking insects and high sugar environments are likely to ferment those sugars as carbon sources. This has been confirmed with xylose fermenting yeasts being isolated from termites and wood feeding beetles [59–61]. However, Douglas [62] argues that the yeast within the insect gut could be different from those within their environment since the insect gut environment is usually different from the external environment. Meanwhile, yeast strains with a potential to be used for production of a fermented beverage from condemned sweet sorghum stalks must be able to assimilate and ferment those sugars found in sweet sorghum.

The subset of yeasts displays a wider fermentative substrate range

We tested for the ability of the insect-associated yeasts to grow on principal sugars supplemented with a respiratory inhibiting agent, antimycin A. Yeasts that grow on such media can only do so by using the alternative fermentative pathway to accumulate biomass [36]. Our results, assessed by scoring growth on basal media supplemented with the respective sugars as the sole carbon sources, suggest that all strains that fermented glucose could also ferment sucrose (Fig. 2). In terms of the biomass accumulated, the trend shows that more biomass was produced in the absence of the inhibitor, suggestive of respiratory metabolism, compared to biomass produced when there was a respiratory inhibitor (fermentative pathway utilisation). This is in agreement with literature, which suggests that yeasts that use the respiratory pathway produce more biomass than when they use the fermentative pathway [63, 64]. Our findings suggest that strains S40–S60 could not sustain fermentative growth on sucrose, in contrast to glucose, sufficient for fermentation. The lighter extent of growth suggests that ribose found in YP [65, 66] could be sustaining minimal growth observed. All industrial strains used as controls could ferment the

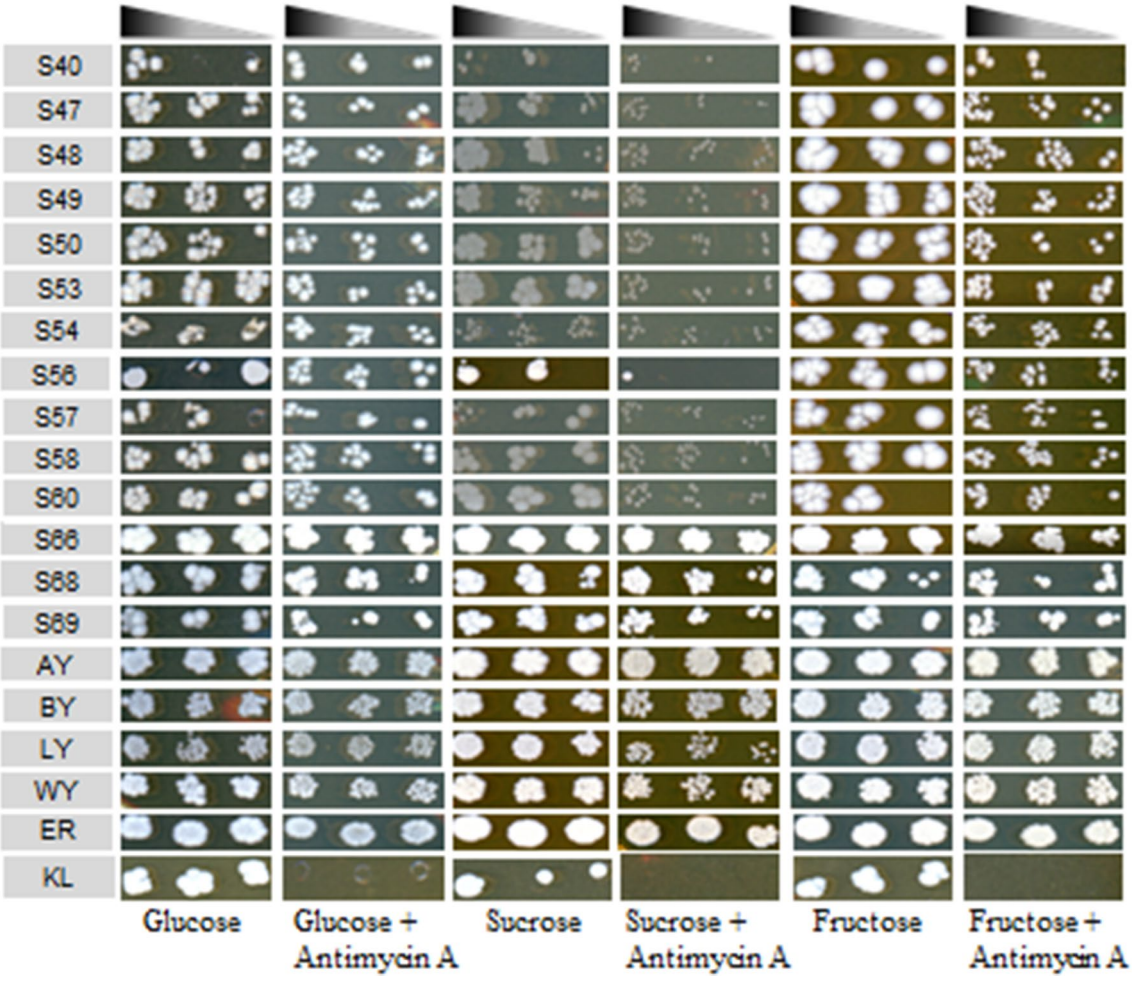

Fig. 2 Assimilation and fermentation of sweet sorghum sugars (glucose, sucrose, and fructose). Yeast colony growth in the absence of antimycin A indicates carbon source assimilation while colony growth in the presence of antimycin A indicates fermentative growth

three carbon sources assessed as expected. Strains S66, S68 and S69, revealed a pronounced fermentative growth on sucrose and fructose, but had poor growth on glucose.

The results only qualitatively confirm the ability to either grow fermentatively or not, but this test has a major drawback of not showing the extent to which they ferment. Therefore, we were further prompted to determine the rate at which these yeasts could ferment the principal sugars.

Insect – associated isolates exhibit desirable traits as starter culture for potential alcoholic beverage

A higher fermentative capacity in sweet sorghum juice

The fermentative capacity of potential starter culture yeasts is important as it influences the rate of accumulation of ethanol. The amount of gas (CO_2) evolved per time interval (CO_2 production rate), when the fourteen isolates fermented the sweet sorghum juice or the principal carbon sources found in sweet sorghum juice, was investigated. Carbon dioxide is a by-product of both respiration and fermentation and an elevated carbon dioxide production rate is an indicator of an elevated fermentative capacity [40, 67]. Although the strains S66, S68 and S69 had shown most fermentative growth on individual sugars (Fig. 2), they had the least fermentative capacity from sweet sorghum juice. These three were significantly different from the first group (i.e., S40 to S60), yet not significantly different from each other, suggesting that they might be similar. While this second group (i.e., S66, S68 and S69) were the least, they were not significantly different from the industrial strain WY (ANOVA, $p < 0.05$; Tukey´s HSD $p < 0.05$). These findings provide evidence that in terms of fermentation capacity of the sweet sorghum juice, all the fourteen strains have the potential to be used as a starter culture as they are comparable to strains currently being used in industry.

The inability of the isolates S40 to S60 to ferment sucrose would be a major drawback for these isolates to be selected as starter cultures. Sucrose, as a major sugar in sweet sorghum juice would then remain unfermented.

The presence of unfermented sugars in alcoholic drinks is a major health concern [68, 69]. The inability of these isolates in fermenting sucrose was further investigated by comparing anaerobic and aerobic growth on liquid media and suggests that this occurrence could be a delay, due to slow utilisation, rather than inability (results not shown in this study). Considering that the results from Fig. 3 were based on carbon dioxide production rate within the first 24 h of fermentation, a longer period of observation would present different results (as confirmed in Fig. 4). Furthermore, this could indicate that the isolates may be utilising extracellular enzymes and other metabolic pathways under anaerobic conditions, since they had previously shown that they were able to assimilate the disaccharide under anaerobic conditions on solid media (Fig. 2). These isolates surprisingly demonstrated a faster fermentation rate in sweet sorghum juice. This could be implying that their growth on sweet sorghum juice was because of initially utilizing other carbon sources present in sweet sorghum juice, which are fructose and glucose. Moreover, these differences in the fermentative ability by colony growth and the rate of fermentation by carbon dioxide production, would require investigation of the ethanol produced by each isolate. This is because the high rate of carbon dioxide production could be because of utilizing one sugar in the sweet sorghum juice. Thus, the ethanol yield would show how much ethanol was produced at the end of fermentation. A low ethanol yield could be important for non-alcoholic beverages, however, excess unfermented sugars in the beverage is undesirable.

Perhaps, their delay or inability could be an indirect confirmation that these yeasts benefit from the host insect larvae in breaking down sucrose into glucose monomers. Malassigné, Minard [52] described a mechanism of this interaction using the example of several phytophagous and blood-sucking insects at adult stage. Hence, this could be an indication of the symbiotic relationship between the yeasts and the associated insect considering that sucrose is the predominant carbon source in sweet sorghum juice. According to Juma, Le Ru [70] majority of lepidopteran larva prefer primary metabolites such as sucrose as phagostimulants and also contain β-fructofuranosides that hydrolyse sucrose. If this were so, it would be beneficial for both the insect and the yeast as it reduces competition over same carbon source.

The fermentative capacity of the isolates when grown in glucose, the second most abundant sugar in sweet sorghum juice was investigated. The results suggest that the rate of carbon dioxide production from glucose fermentation presented a trend similar to that of fructose and sweet sorghum juice. Since the isolates were all selected based on glucose fermentation (Table 1), we expected them to have a relatively high rate of carbon dioxide production in glucose fermentation and they all did.

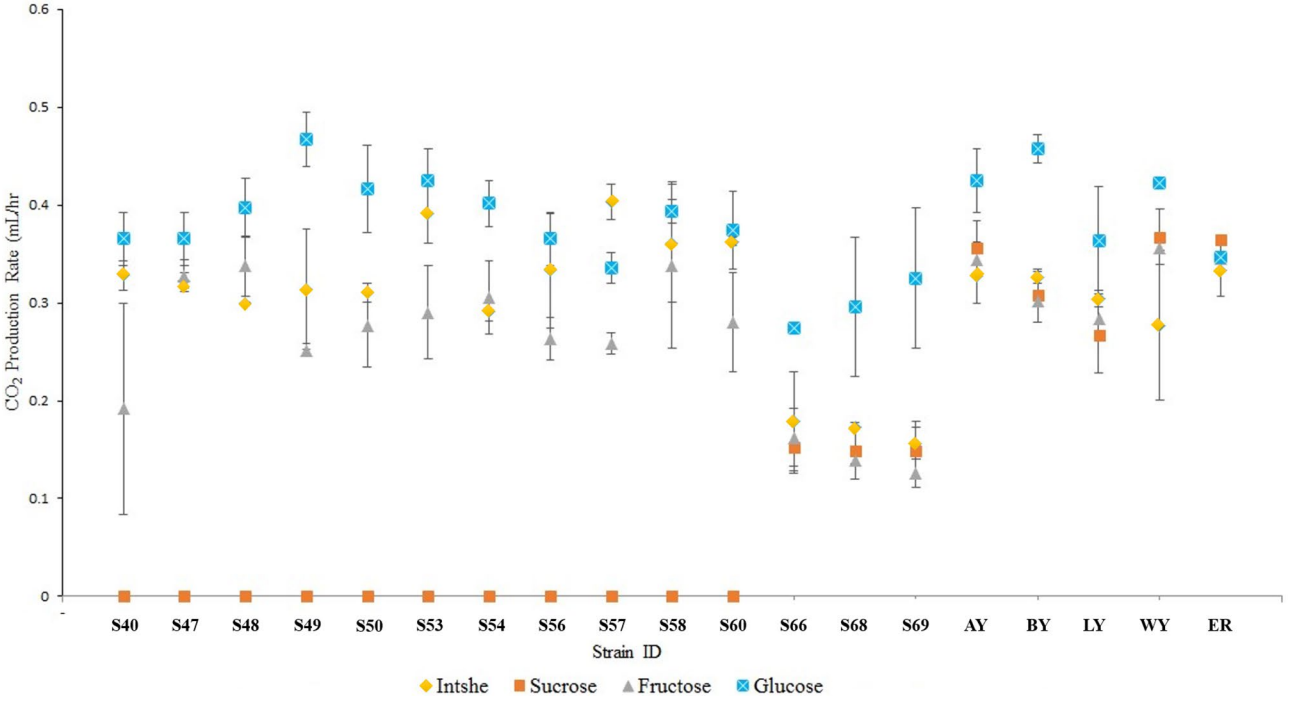

Fig. 3 Fermentative capacity for yeast isolates utilising different carbon sources. The yellow (sweet sorghum juice), orange (sucrose), grey (fructose) and blue (glucose) indicate the average CO_2 production rate when the yeasts were utilising respective sole carbon sources. A group of isolates, S66, S68 and S69 were the only isolates capable of fermenting sucrose although at a significantly lower rate than the reference strains (ANOVA, $p < 0.05$; Tukey´s HSD $p < 0.05$) (See supplementary Tables S2 & S3)

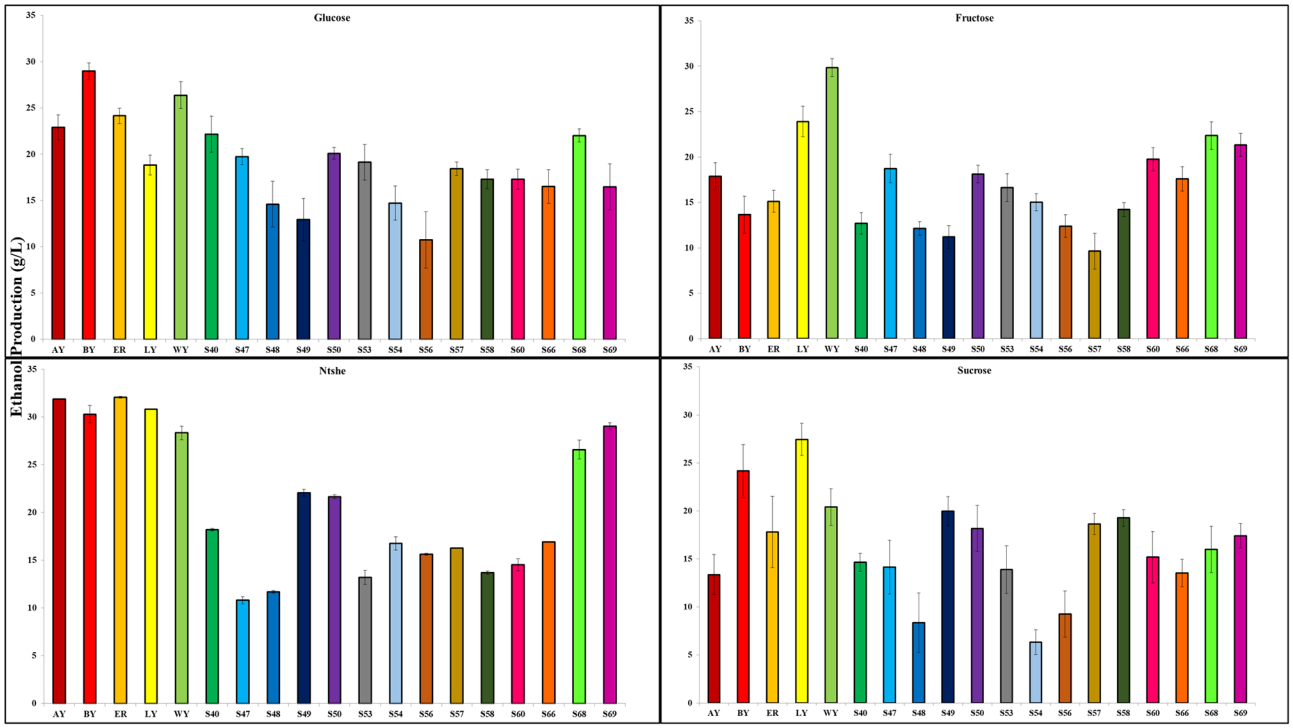

Fig. 4 Ethanol from individual carbon sources with HPLC and sweet sorghum juice with GC-MS. The HPLC data showed that there were no remaining sugars in all the strains after the fermentation period with only traces of sorbitol and mannitol

The inclusion of individual sugars in this assay helps us to know which sugar the isolates perform the most. The similarity observed in the fermentation capacity trend of fructose, the least abundant sugar in sweet sorghum juice, with glucose could be because both these sugars use the same transporters into the yeast cells during assimilation [71]. Although the strains (S40 to S60) could not ferment sucrose as much, it is clear that they can ferment well the monomers of sucrose, which are fructose and glucose.

The amount of ethanol accumulated at the end of fermentation is an equally important attribute in addition to the fermentative capacity of potential starter culture yeasts. The accumulated ethanol determines the final amount of ethanol in the alcoholic beverage whereas the fermentative capacity is important for determining the efficiency and subsequently the productivity process. Assessment of the amount of ethanol accumulated and the sugars before and after fermentation was done using an HPLC and GC-MS. Analyses of the accumulated ethanol show that most of the isolates were highly competitive as potential starter culture yeasts when compared to industrial yeast strains (Fig. 4).

In fructose fermentation, the strains S47, S50, S60, S68 and S69 had high ethanol yield which was comparable to the industrial strains. Although majority of the isolates had high ethanol yield from glucose, none of them exceeded the industrial strains on this sugar. These findings from the HPLC analysis indicate that although the fermentative growth and the rate of fermentation may be high, it does not mean that the ethanol produced at the end of fermentation will be high. Furthermore, the findings for ethanol yield from sucrose as a sole carbon source also confirm this as the strains that had shown poor rate of carbon dioxide production begin to show ethanol accumulation after being allowed to ferment sucrose over an elongated period. This could confirm our claim from the observation of rate of carbon dioxide production. There was no significant difference in the ethanol yield of S40, S47, S49, S53 with S66 and S68 which had demonstrated a higher fermentation capacity in Fig. 3. Wang, Nayak [72] explains that the conversion of sucrose by extracellular invertase prior to assimilation results in elongated lag phase for yeasts fermenting sucrose as a sole carbon source.

Finally, the ethanol accumulated from the sweet sorghum juice fermentation revealed three separate groups among the isolates: high ethanol producers (S49, S50, S68 and S69), intermediate ethanol producers (S40, S54, S56, S57 and S66) and low ethanol producers (S47, S48, S53, S58 and S60). Of all the carbon sources, it is in fermentation of the sweet sorghum juice that the isolates were most comparable to the industrial strains. This overall result backs up the ecological understanding on which the exploitation of this niche was based and creates avenues for production alcoholic beverages with

varying alcohol concentrations which could satisfy the wide customer preferences. The ethanol produced is affected by the tolerance of the yeast isolates of industry related stresses as some yeast begin to die once the ethanol exceeds a threshold which they can bear. This can be detrimental to the brewing process as it results in incomplete fermentation and increases oxidative stress from the dying yeast cells [73]. Thus, we went on to investigate the stress tolerance of the isolated yeast strains.

Insect-associated yeasts exhibit desirable brewing stress tolerance

Industrial strains often encounter various stressors during fermentation [73]. Despite their capability to ferment, their tolerance to metabolic and environmental stressors is a key factor for the selection of starter cultures [74]. The main types of stress assessed in our study include alcoholic stress, thermal stress, osmotic stress, oxidative stress as well as nutritional stress. A total of fourteen selected isolates were exposed to different amounts of stressors and the results are presented in Figs. 5, 6, 7 and 8.

Alcohol stress tolerance Industrial strains encounter increasing amounts of ethanol as fermentation progresses. Ethanol is toxic to cells as it damages the cell membrane and deactivates enzymes required for metabolic processes [73]. All isolates were able to grow in the presence of 5% (v/v) ethanol except S66, S68 and S69, (Fig. 5). This was in stark contrast to the industrial strains which tolerated up to 9% (v/v) ethanol except the lager yeast. The strains S40, S47, S48, S49 and S57 are the only ones that indicated a low tolerance to 7% (v/v) ethanol. However, at 10% (v/v) ethanol there was no growth at all including the positive controls. The porosity of the yeast plasma membrane is affected by high alcohol concentration, resulting in the

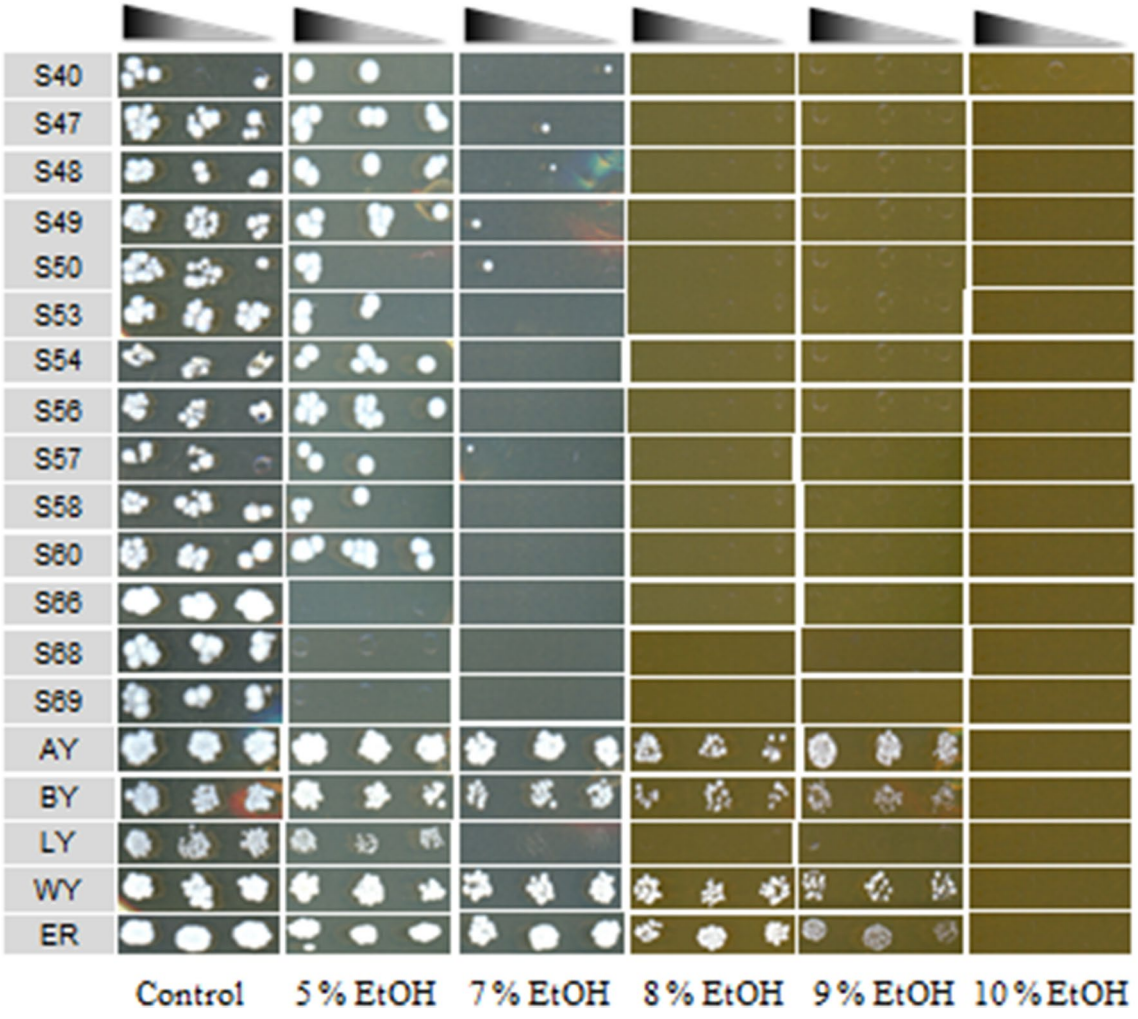

Fig. 5 Ethanol stress tolerance of yeast isolates. Yeast isolates were stamped in three different dilutions onto YPD media supplemented with varying concentrations of ethanol from 5% (v/v) up to 10% (v/v). Isolates S40 to S60 exhibited higher tolerance by growth in 7% (v/v) ethanol, while S66 to S69 could not grow at 5% (v/v). Some industrial stains went up to 9% (v/v) ethanol concentration

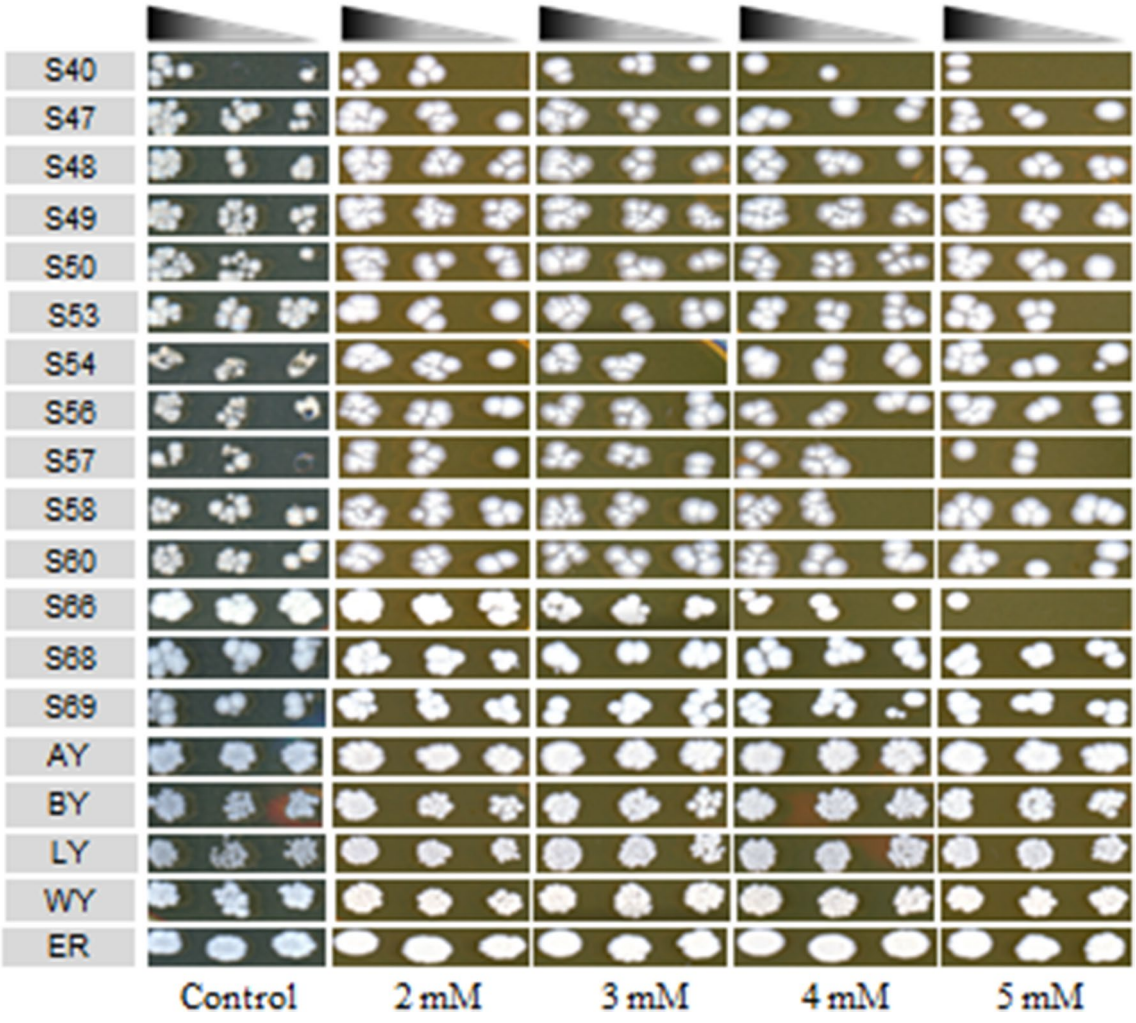

Fig. 6 Oxidative tolerance of yeast isolates. Yeast isolates were stamped in three different dilutions onto YPD media supplemented with varying concentrations of H_2O_2 from 2 mM up to 5 mM. Based on colony growth S40 and S66 had the least oxidative stress tolerance. However, isolates S48, S49, S56, S58, S68 and S69 were able to grow well up to 5 mM H_2O_2 and were comparable to industrial strains

yeast cell failing to transport nitrogen and sugar into the cell despite their presence in the fermentation medium which causes fermentation to top [75]. The ethanol tolerant strains may have a plasma membrane make up that gives them the ability to survive high ethanol concentrations as such this ability is highly dependent on the yeast strain.

Oxidative stress tolerance Oxidative stress tolerance is one of the desirable traits in industrial yeast strains. During propagation and early stages of fermentation, oxygen is supplied to generate yeast biomass and ensure efficient fermentation respectively [73]. Despite the importance of oxygen in brewing, its presence increases the accumulation of reactive oxygen species such as hydrogen peroxide, superoxide radical and hydroxyl radicals which can cause damage to the yeast cell components and ultimately to cell death [76]. Furthermore, it is not only the presence of oxygen that can result in oxidative stress but it can also be as a result of thermal shock, osmotic and ethanol stress [76]. Isolates S48, S49, S56, S58, S68 and S69 exhibited tolerance of up to 5 mM H_2O_2 concentrations, indicating great potential of tolerance to oxidative stress (Fig. 6).

Osmotic stress tolerance Osmotic stress tolerance is another trait of importance in selecting starter culture yeast strains. A high content of sugars in the beginning of fermentation can stall fermentations [77, 78]. The stressor causes an imbalance in the osmotic potential, creating a concentration gradient for the solutes inside the yeast cell resulting in dehydration as well as diffusion of essential solutes out of yeast cell [79]. Isolates S66, S68 and S69 exhibited the most tolerance to osmotic stress as they were able to grow well at relatively high concentrations of sodium chloride (1.25 M and 1.5 M) (Fig. 7). The results show that isolates from S40 to S60 only tolerated up to

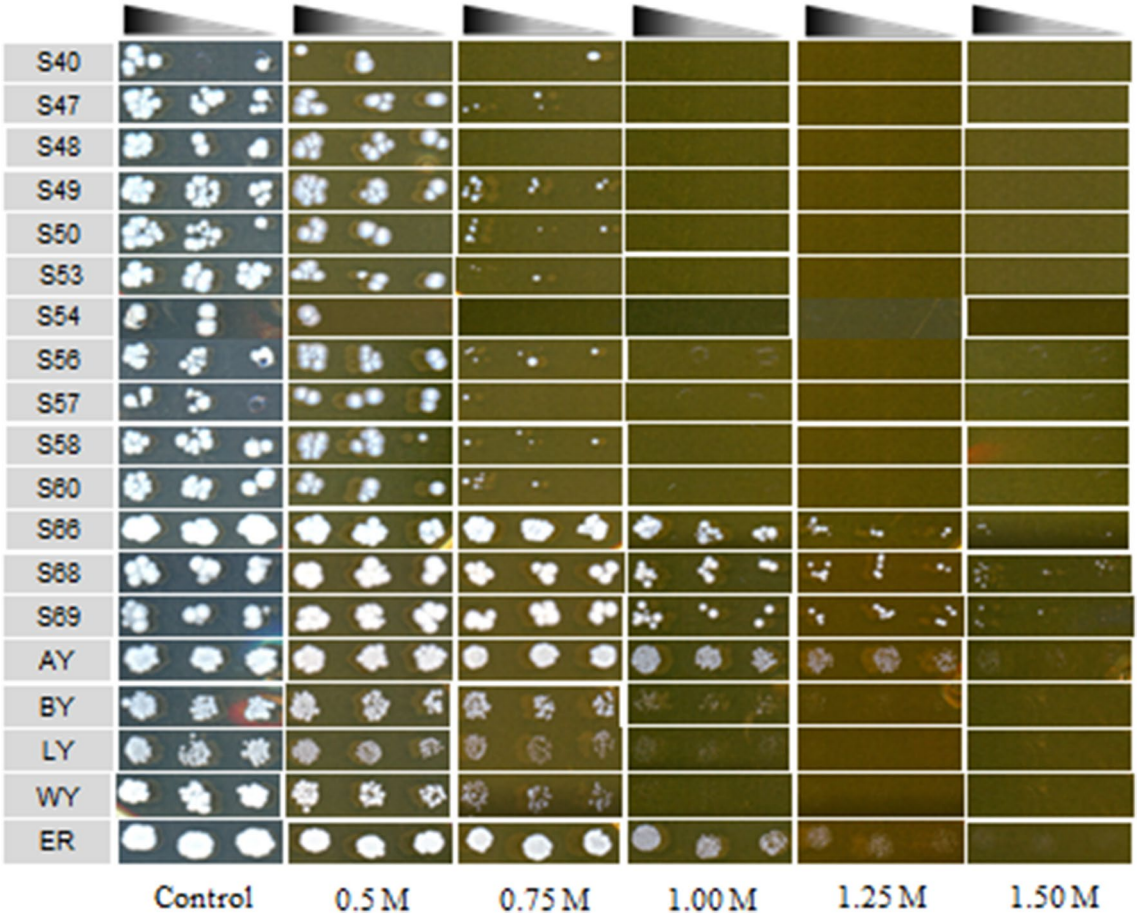

Fig. 7 Osmotic tolerance of yeast isolates. Isolates S66, S68 and S69 exhibited most growth, at the highest NaCl (1.5 M) concentration followed by industrial strains AY and ER. The rest of the isolates were not able to grow beyond 0.75 M NaCl, while the industrial strains, AY, and ER, exceeded that concentration

0.75 M of NaCl, which suggests that they may not perform well in media with a higher concentration of initial sugars. The control strains showed that ale yeast (AY) and ethanol red (ER) were more tolerant to osmotic stress, while wine yeast (WY) was the least tolerant of the industrial strains used (Fig. 7). Sodium chloride was used because of the known links between ion toxicity and osmotic stress, both of which are brought on by salt exposure [80].

Thermal stress tolerance The final selection criteria we assessed was thermal stress tolerance. As brewing yeasts are transported in various packaging to different locations and stored in different conditions by customers, they could be exposed to a wide range of temperature changes and these could exert thermal stress to the yeasts [73]. For this reason, brewing yeasts need to be tolerant to thermal stress. While growth temperature for brewing yeasts can be as low as 12–18°C [81, 82], highlight the optimal temperature range for *Saccharomyces cerevisiae* as 30–35°C and further suggest that any temperatures above this range could help increase the fermentation rate. Our findings reveal that the temperature range for the growth of these yeast isolates ranges between 25°C and 37°C (Fig. 7).

Isolates S56 and S60 grew well at 37°C and were the most thermal tolerant among all the isolates. There was no growth at 40°C except for the positive controls, AY, and ER, which displayed high thermal tolerance. Although high temperature fermentation in other application of yeasts such as bioethanol production, is associated with reduction in cooling costs, improved fermentation rate and reduction in contamination [61], in brewing it can be a disadvantage due to altered beer flavour at the end of primary fermentation [83]. This implies that as much as higher temperatures are beneficial, it is important to regulate this selection criterion and overall impact it would have on our fermented beverage.

Many strains produce sweet sorghum beverages with diverse aromatic profiles

The production of a quality fermented beverage relies on the activity of fermenting yeasts that are qualified not only for good fermentation yield efficiency, but also

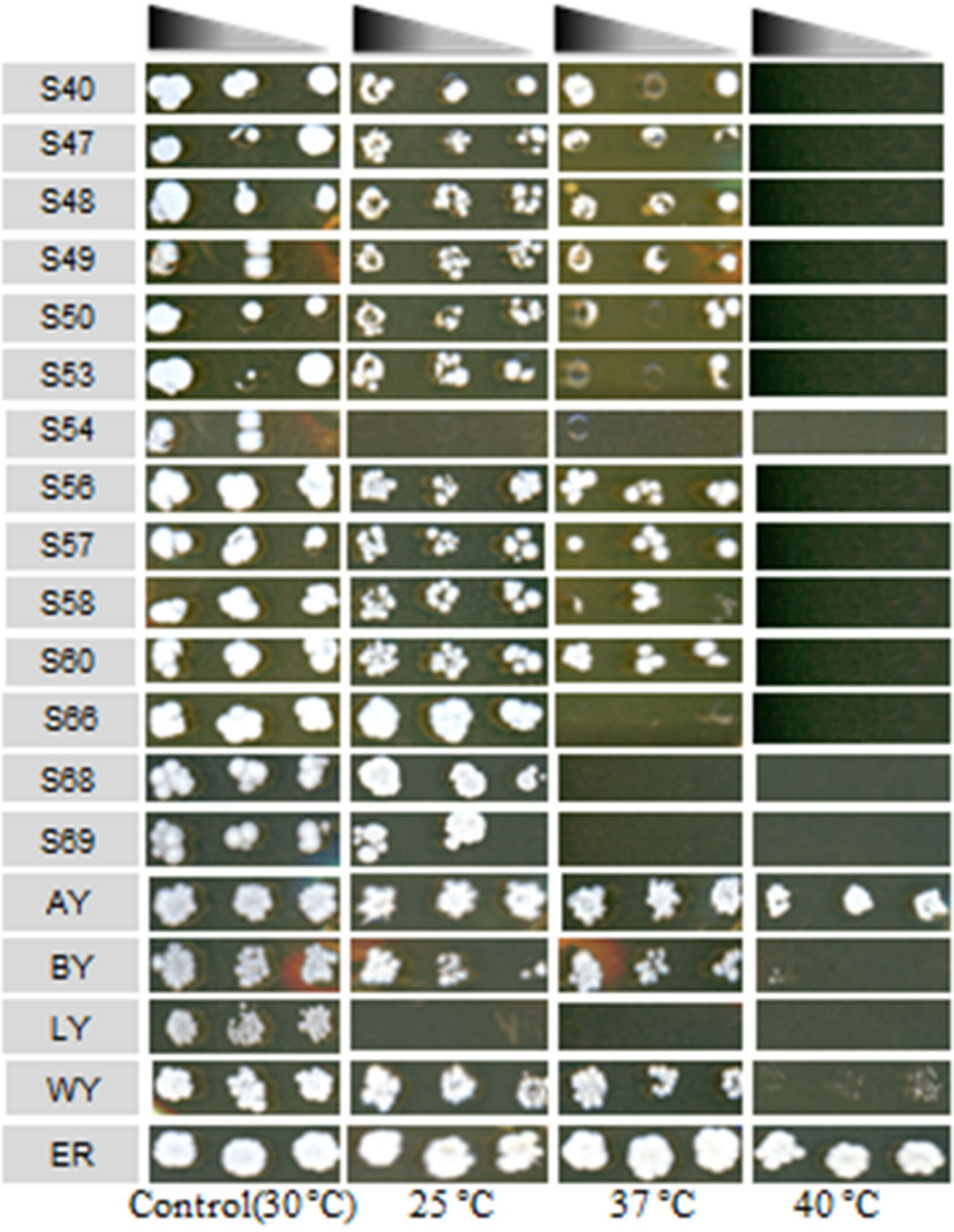

Fig. 8 Thermal stress tolerance of yeast isolates. All Isolates were able to grow well at 30°C which was the control temperature. Isolates S56 and S60 indicated significant growth at 37°C while most isolates showed none to poor growth. None of the isolates grew at 40°C, while AY and ER were capable of growing at this high temperature

for affecting aroma and flavour of the beverage [84]. The production and quality of these compounds not only depends on the available nutrients and external conditions, but also on the yeast strains involved in fermentation process, as such, their choice is of fundamental importance. Our findings suggest that our isolates produced a wide bouquet of aromatic compounds (Fig. 9). The volatiles produced included esters, higher alcohols, fatty acids, terpenes, volatile phenols, glyols, ketones and aldehydes.

Group A, which was composed of the industrial strains AY and WY produced relatively high amounts of diverse higher alcohols, acids, and esters. From the AY, we find 2-methyl propanol (alcoholic flavour), phenethyl alcohol

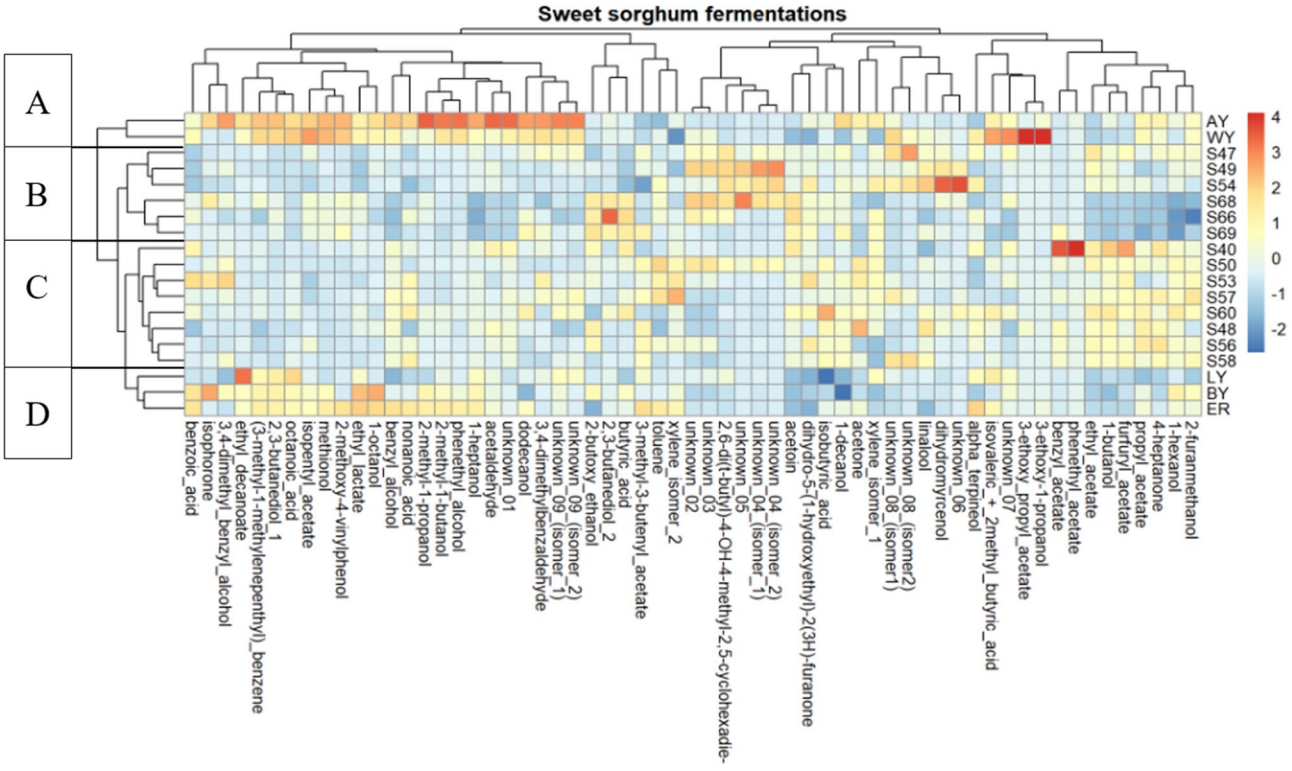

Fig. 9 Aroma profile of yeast isolates from larval moth and sweet sorghum galleries and frass. Isolates were divided into four groups (**A**, **B**, **C** and **D**) based on similarity in aromas produced by the strains (see supplementary Table S4)

(rosey flavour), dodecanol (floral aroma) and 2-methyl butanol (solventy off-flavours). The WY had, among many acids and esters, benzoic acid (causes oral prickling), isopentyl acetate (fruity aroma) and high concentrations of 3-ethoxy propyl acetate (fruity) [14, 85, 86]. While esters are produced by the yeasts, they are also a result of chemical esterification and some are already present in the substrate, being continuously subject to change depending on several external factors like pH, temperature, and alcohol levels. Thus, for a more alcoholic beverage the AY would be of choice in Group A, whereas the WY would produce a complex aroma and taste.

In Group B, which subdivided into two clades (Clade 1: S47, S49, S54) and (Clade 2: S66, S68, S69), suggestive of the fact that they would be different species. In comparison with Clade 2, Clade 1 produced higher amounts of esters, including ethyl acetate (fruity aroma), furfural acetate (floral aroma), and alcohols, including butanol (alcoholic) and hexanol (herbaceous). Furthermore, Clade 1 isolates S49 and S54 produced more terpenoids, linalool and dihydromyrcenol known for floral-citrusy aroma, than any of the Clade 2 isolates. Isolates S49 and S68, which were both from different clades produced several unidentified compounds which were not produced by the industrial strains, and these could contribute to unique flavours and taste of the fermented beverage. Clade 2 isolates had a few higher notes of desirable flavours like isopentyl acetate, but they raised a concern as they produced high amounts of butyric acid, acetoin, and butoxy-ethanol, which are associated with off-flavours, especially in high concentrations beyond the threshold. Therefore, Clade 1 isolates would be the best choice for a high flavour fermented sweet sorghum beverage.

From Group C, we have isolate S40 which produced high concentrations of desirable fruity and floral esters including benzyl acetate, phenethyl acetate and furfuryl acetate among others which were not produced by the industrial strains. The rest of the isolates in this group had similar profiles, with all producing moderate (Score 1–2, Fig. 9) concentrations of desired esters, alcohols, and acids. On the contrary, the industrial strains LY, BY and ER in Group D produced esters, isopentyl alcohol (alcoholic and banana flavour) and ethyl decanoate (grape flavour) which the isolates could not produce. The aroma profiles for Groups A and D were more similar to each other and although few, there were similarities with the Groups B and C. For example, Methyl-3-butryl acetate (fruity flavour) was produced by S69 (Group B) and S60 (Group C) as well as industrial strain ER (Group D). Similar trends were observed for other compounds like terpenes and volatile phenols which were produced in all the four groups and these are common overlapping compounds in plant-microbe associations [14]. Our findings

seem to suggest that the non-*Saccharomyces* yeasts produced more complex blends of the volatile compounds. This could be attributed to the highly conserved role played by volatile compounds in the insect-yeast niche where they act as communication signals, attracting insects and repelling some [87]. Juma, Le Ru [70] explains how some of these compounds observed from the yeasts are similar to lepidopteran insect-preferred secondary metabolites produced by the sorghum plant.

Hanseniaspora spp. Dominate the insect associated yeasts with potential for production of sweet sorghum beverages

Molecular identification of the fourteen isolated strains revealed that the potential starter cultures were composed of species nearest to *Hanseniaspora* and *Candida* genera, with the former being the most abundant (Table 2). Of these, only one isolate (S40) belonged to *H. guilliermondii* whereas ten isolates (S47-S60) *belonged* to *H. opuntiae* (Table 2; Fig. 10A). It is noteworthy that isolates S66-S69 belonged to the *Candia intermedia* species, which formed their own clade as, depicted on the phylogenetic tree. To assess the genetic diversity of the isolates under the same species an in silico RFLP was conducted. The results indicate that the *Hanseniaspora opuntiae* isolates had a similar restriction fragment banding pattern (Fig. 10B). Considering the similar physiological studies, these isolates are probably genetically similar.

Table 2 Species identification of yeast isolates from larval moth and sweet sorghum galleries and Frass based on Genbank database

Strain ID	Nearest Species Match	[a]Accession number	Percentage Match (%)	[b]Submission number
S40	*Hanseniaspora guilliermondii*	KY103518.1	100	OR544619
S47	*Hanseniaspora opuntiae*	MN268780.1	100	OR544620
S48	*Hanseniaspora opuntiae*	MN268780.1	100	OR544621
S49	*Hanseniaspora opuntiae*	MN268780.1	100	OR544622
S50	*Hanseniaspora opuntiae*	MN268780.1	100	OR544623
S53	*Hanseniaspora opuntiae*	MN268780.1	100	OR544624
S54	*Hanseniaspora opuntiae*	MN268780.1	100	OR544625
S56	*Hanseniaspora opuntiae*	MN268780.1	100	OR544626
S57	*Hanseniaspora opuntiae*	MN268780.1	100	OR544627
S58	*Hanseniaspora opuntiae*	MN268780.1	100	OR544628
S60	*Hanseniaspora opuntiae*	MN268780.1	100	OR544629
S66	*Candida intermedia*	KX371579.1	99	OR544630
S68	*Candida intermedia*	KX371579.1	98	OR544631
S69	*Candida intermedia*	KX371579.1	99	OR544632

[a] Accession number of the closest type strain [b] Submission number of the deposited sequence in Genbank (SUB13837643) accessible through the following: (https://www.ncbi.nlm.nih.gov/nuccore/OR544632.1#sequence_OR544632.1)

Ecologically, yeasts belonging to the genera *Hanseniaspora* and *Candida* are the most common of the apiculate yeasts found on various fruits, flowers, and bark as their primary habitat [88]. This work suggests that insects serve as their dispersal vectors and thus not very surprising that most of our isolates were of this genus. In fermented beverages produced from fruit musts, the natural fermentation is initiated by non-*Saccharomyces* yeasts, such as *Hanseniaspora*, *Candida* among many others, and they are known to contribute to the aroma of the beverage [89]. For *Hanseniaspora* species, these habitats are also consistent with their assimilation profiles because they can utilize carbon sources that are available in tree bark, such as cellobiose, arbutin, and salicin. This parallels our results as our isolates were able to ferment well two of the principal sugars in the sweet sorghum juice. Additionally, *Hanseniaspora* yeasts have unusually high vitamin requirements, which further suggests their close association with plant material [88].

It is important to study yeasts in their natural environments because it helps us understand their niches, why different species live in certain places and how they can be efficiently exploited for industrial applications [90]. Yeasts that initially colonize sugary plant material, such as *Hanseniaspora* species, have limited physiological abilities, hence the definition, physiological specialists [53]. *Hanseniaspora* spp. have been found to play an essential role in the nutrition physiology and host attraction of associated insects, and the physiological behaviour of the yeast strains could be a result of co-evolution to maintain a mutualism with the associated insect [48]. The predominance of the same species could also be attributed to larval discrimination in order to maintain mutualism [14].

The two genera, *Hanseniaspora* and *Candida*, exhibited most of the desirable physiological credentials in different their different capacities as presented in the previous sections. These physiological differences could point us to a diverse evolutionary history, even among strains of the same species. We observed in Fig. 10A how *H. opuntiae* and *C. intermedia* are in different clades from their type strains. Among *H. opuntiae*, though closely related, we see three different groups: S49 and S53; S57, S48, S47 and S50; S58, S56 and S54.

The minor differences among the *C. intermedia* strains (S66, S68 and S69) are highlighted in Fig. 10B, where different fragments were obtained from consensus region. These three strains were similarly able to ferment sucrose, although they were the least in capacity to

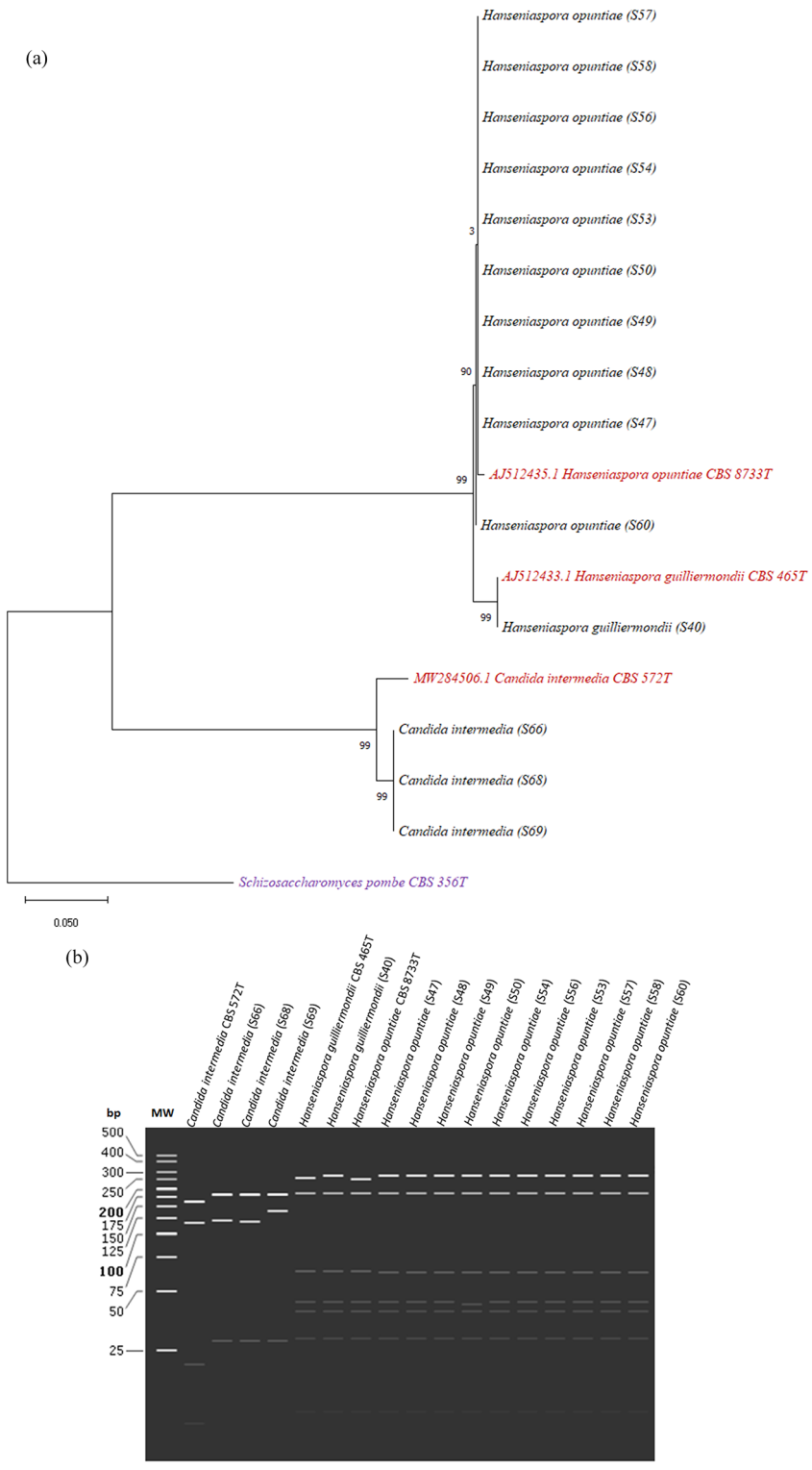

Fig. 10 (**A**) phylogenetic tree showing relationship of isolates from larval moth and sweet sorghum galleries and frass (Genbank sequence read archives SUB13837643). (**B**) In silico RFLP results for isolated yeasts using *CfoI* and *HinfI* as restriction enzymes (see supplementary Table S6 for fragment list)

ferment sweet sorghum juice. The three strains could not tolerate 5% (v/v) ethanol but exhibited relative tolerance oxidative and osmotic stress. Their aroma profiles were similar as well, as they had the highest acetoin levels, as well as butyric acid, 2,3 butanediol and 2 butoxy_ethanol. On the contrary, these strains had some physiological differences for example, *C. intermedia* (S66) had poor growth at 5 mM H_2O_2, indicating lesser tolerance to that level of oxidative stress compared to *C. intermedia* (S68 and S69). Moreover, the aroma profiles showed differences in isophorone, 2, 6 di(t-butyl)-4-OH-methyl-2,5-cyclohexadie and other unknown compounds. The RFLP results in Fig. 10B were able to pick differences in the sequences as they revealed that all three *C. intermedia* strains had slight differences in the restriction enzyme fragments, which could explain the physiological differences exhibited.

A similar trend was observed for the dominating *Hanseniaspora* species and could be attributed to the different environments (i.e., insect gut, frass, and galleries) from which they were found. For example, in aroma profiles *H. opuntiae* (S53) had high ethyl acetate and the other *H. opuntiae* strains had very lower levels. *H. opuntiae* (S60) had high butyric acid level while the other strains had lower levels. These are important aromas in brewing and are normally considered as off-flavours, more especially if they exceed a certain threshold [91]. These differences were also observed across other tested parameters, including fermentative capacity (Figs. 3 and 4) and stress tolerance (Figs. 5, 6, 7 and 8). It is highly likely that these differences are attributed to the varying environmental conditions in nature, and these intrinsic characteristics (chemical, physical and physiological) help the yeast strain's ability to exist and persist in a specific habitat, fundamental niche. Overall, the best strains for the sweet sorghum juice fermentations were S57 *(H. opuntiae)*, S53 *(H. opuntiae)* and S68 (*C. intermedia*). However, the results show that all the fourteen strains can be used as pure starter cultures and/or as co-cultures which may be for production of high flavour and low alcohol or high flavour and high alcohol, respectively.

Conclusion

Sweet sorghum infestations by larval stages of stem borers are a major concern for food security in Southern Africa. Together with other strategies of pest management, valorisation of condemned sweet sorghum stalks in the production of value-added products is an attractive strategy to reduce the losses incurred by farmers. Our research shows that stem boring larvae is associated with yeasts, particularly those from the two genera *Hanseniaspora* and *Candida*. The two yeasts revealed their ability to ferment sugars found in the sweet sorghum juice, tolerate industrial stressors, and produce volatile organic compounds comparable to the domesticated industrial yeasts. These attributes present a potential for a novel sweet sorghum beverage, a first of its own kind inspired by pest infestation.

Abbreviations

PBS	Phosphate Saline Buffer
YPD	Yeast extract Peptone Dextrose
AY	Ale Yeast
BY	Baker's Yeast
LY	Lager Yeast
ER	Ethanol Red
WY	Wine Yeast
HPLC	High Performance Liquid Chromatography
GCMS	Gas Chromatography Mass Spectrometer
ANOVA	Analysis of Variance
Turkey's HSD	Tukey's Honestly Significant Difference

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03857-0.

Supplementary Material 1

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Author contributions

N.Z. conceived, designed the analysis. M.T.G. performed preliminary experiments. T.P.M., T.S., T.M., U.V., and N.Z. collected the data, performed the experiments, visualization and interpretation of results. All authors reviewed the manuscript.

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Data availability

Sequence data that support the findings of this study are openly available in GenBank Direct Submission accessible through the following URL: https://submit.ncbi.nlm.nih.gov/subs/?search=SUB13837643 and the sequences correspond to the following NCBI accession numbers OR544619:OR544632[accn].

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]Department of Biological Sciences and Biotechnology, Faculty of Sciences, Botswana International University of Science and Technology, Private Bag 16, Palapye, Botswana
[2]Department of Biological Sciences, Faculty of Science, University of Botswana, Private Bag, Gaborone 0022, Botswana

[3]Chemicals Cluster, Council for Science and Industrial Research, Meiring Naude Road, Pretoria 0184, South Africa
[4]Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach, San Michelle All'Adige, Italy
[5]Department of Biotechnology, School of Health Science & Technology, Chinhoyi University of Technology, Chinhoyi, Zimbabwe

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