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Natural nutrient supplements and sisal bole yeast isolate: A synergistic approach to bioethanol production from sisal bole juice

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Abstract

The increasing demand for sustainable biofuels and the need to reduce reliance on synthetic nutrients additives during yeast fermentation has prompted the search for cost effective and locally available nutrients sources. This study investigated the potential of natural nutrients supplements derived from agro-waste (orange, cassava and banana peels) as well as sisal bole yeast isolates to enhance bioethanol production from sisal bole juice. Yeast colonies were isolated from the sisal bole juice and then characterized morphologically, biochemically and through molecular identification using PCR amplification of the ITS1 and ITS4 rDNA regions. A 2^n full factorial design was employed to study the effect of natural nutrient supplements combination and dosage on the bioethanol yield. Macroscopic results revealed that the isolated colonies were either ellipsoidal or oval and gram-positive similar to *Saccharomyces cerevisiae*. Sequencing of the PCR products confirmed that all positive isolates showed high similarity (>99%) to *Saccharomyces cerevisiae*. The highest bioethanol yield achieved was 27.38% w/w with orange peels (3 g/100 ml), cassava peels (3 g/100 ml) and banana peels (1 g/100 ml) compared to 20.13% w/w for the blank and 18.71% w/w for the control. Statistical analysis confirmed a significant linear model, with notable effects from orange peels and interactions between orange and banana peels. These findings highlight the potential of natural supplements to replace expensive and less accessible artificial supplements such as Magnesium sulfate heptahydrate, Iron (II) sulfate tetrahydrate and Copper (II) sulfate pentahydrate which provide essential Mg, Fe and Cu that improved bioethanol production efficiency.

Key words: *Banana peels; bioethanol production; cassava peels; Natural nutrient supplements; orange peels; sisal bole; yeast isolate*

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Introduction

The growing demand for clean fuel for anthropogenic activities especially domestic, transportation and industrial uses has led to a surge in interest in renewable resources. Anthropogenic activities are the leading producers of greenhouse gases which contribute about 38.8% from power generation, 26.56% from transportation and 24.76 % from industries (Yoro and Daramola, 2020). Large-scale renewable energy production and use will enhance energy security and reduce climate change. The adverse effects of fossil fuels to the environment such as ozone layer depletion and global warming makes bioethanol the best alternative owing to the fact than it is considered carbon-neutral (Lamichhane *et al.,* 2021; Guimarães *et al.,* 2023).

Bioethanol feedstocks could be different types of lignocellulosic biomass that is abundantly available at low cost and rarely compete with food supply chains (Adewuyi, 2022; Mujtaba *et al.,* 2023). Sisal bole is the post-harvest waste which comprises up to 97.94% w/v organic matter and total sugar content in juice of up to 30% w/v which can be used to produce bioethanol (Msuya *et al.,* 2018b). The biomass is surrounded by many natural microorganisms that could convert it to different end products, including bioethanol (Ngonyani *et al.,* 2013). These microorganisms could be isolated, purified and used to convert the same biomass into specific end products.
The microorganisms isolated from the same biomass used for fermentation are better conditioned for the process as they play a crucial role in improving and accelerating fermentation rates and impart characteristic sensory and aromatic flavour to the fermented product (Al Daccache *et al.,* 2020; Prabhu *et al.,* 2022; Luthfi *et al.,* 2023). They are acquainted with the microbial ecosystem and metabolic activity that interacts with the substrates (Sharma *et al.,* 2020). In their study, Ngonyani *et al.* (2013) found that sisal bole contains about 15 ± 1.6% of *Saccharomyces cerevisiae* which are responsible for bioethanol production.

Fermentation feedstocks are normally deficient in essential nutrients such as minerals, vitamins and growth factors required by ethanol-producing yeast. For instance, mineral concentrations in sisal bole juice reported by Msuya *et al.* (2018b) are below the thresholds required for efficient fermentation (Kampen, 2014). These deficiencies are generally addressed through supplementation of the fermentation broth.
Artificial nutrient supplements, which are not environmentally friendly and expensive are widely used in fermentation. Natural nutrient supplements have been found to be more environmentally friendly, easily available and cheaper compared to artificial nutrients (Torres-Guardado *et al.,* 2022; Sene *et al.,* 2024). However, they are not widely used. Thus, it is important to study the effect of natural nutrient supplement types, dosages and sisal bole yeast isolates on production of bioethanol from sisal bole juice.

Materials and Methods

Study area

The primary raw material, sisal bole, was collected from Kumburu sisal farm, Tanga (7° 13′ S, 38° 53′ E), representing major sisal-producing estates. Nutrient supplements included ripe banana and orange peels and; cassava peels from matured cassava, collected from the University of Dar es Salaam cafeteria. The proximate analysis was done in the Chemical Laboratory, department of Chemical

and Process Engineering at the University of Dar es Salaam. The chemical analysis was done at the analytical laboratory of the Ministry of Water, Ubungo, Tanzania. Microbiological characterization and fermentation were carried out at the Microbiology laboratories, College of Natural and Allied Sciences at the University of Dar es Salaam.

Juice extraction

Juice was extracted from Agave (Hybrid 11648) boles of 10-12 years according to Msuya *et al.* (2018a) method. The sisal bole was cleaned to remove leaf stubs, weighed by beam balance (D7470, 98 % accuracy, ± 0.1 kg); chopped using a chopping machine (240 V and 2880 rpm) capable of producing 5-15 mm^3 chops and its juice extracted using a hydraulic pressing machine (16 tonnes capacity). The extracted juice was hydrolyzed to release fermentable sugars according to Msuya *et al.* (2018a) method. A 1 M HCl was added dropwise into 50 ml juice in a 250 ml conical flask until the pH reached 1-2. The pH was monitored using a pH meter (HI-98130, ±0.01) whose globe was dipped in the solution. The flask was then heated in a water bath at 60°C for 30 minutes. The mixture was neutralized with 2.5M NaOH and diluted with de-ionized water to make 100 ml. A portion of extracted juice (80 ml) was used for yeast isolation.

Isolation of Saccharomyces cerevisiae

Four pyrex plates were dried in an oven (GALLENKAMP) at 165± 1.5°C for 1 hour and left to cool at room temperature for 20 min. The 0.04 g/ml of Yeast Peptone Dextrose Adenine (YPDA) media, containing yeast extract, peptone, and dextrose, was sterilized at 121°C and 1 bar for 15 minutes and used to provide essential nutrients for yeast growth (Dymond, 2013). YPDA was used over other media because of its suitability for the growth and flourishing of various yeast species from different environments, and is readily available in laboratories (Msuya *et al.,* 2024). Saline solution (250 ml, 0.009 g/ml) was prepared, aliquoted into 4 sterile 50 ml vials each containing 49.5 ml, and autoclaved at 121°C 1 bar for 15 min. The sequence of serial dilutions was made from 10^2 ml, 10^4 ml, 10^6 ml and 10^8 ml diluted solutions, and then 100 µL of diluted and undiluted juice was from the dilution series and streaked into the Petri dishes containing YPDA. The plates were covered and then sent to the incubator at 30 ±2°C for 48 hours. The colonies were isolated by a wire loop and streaked into sterile YPDA to obtain pure colonies. The sub-cultured plates were incubated at 30°C for 24 h to multiply. The pure colonies obtained were used for identification.

Microbial characterization

Morphological characteristics of the pure colonies were identified based on colour, texture, margin, elevation and other unique features using the method by Anyanwu et al. (2020). The gram staining method by Sulmiyati *et al.* (2019) was adopted for microscopic characterization of the microbes. A pure yeast colony was placed onto a clean-dry microscope slide followed by the sequence of crystal violet, iodine, alcohol and safranin. An oil emulsion drop was added onto the slide and then observed by X100 objective of the microscope (B-350 OPTIKA).

Method of biochemical characterization by Antia *et al.* (2018) was adopted to characterize the isolates by their ability to ferment hydrolysed sisal bole juice. For each subculture, 10 ml of glucose water (1% glucose) was prepared in peptone water and dispensed in test tubes with BS625 neutral glass Durham's tubes for detecting gas production

and three drops of phenol red were added as indicator. The test tubes and the contents were autoclaved (WACS-2060) at 121°C and 1 bar for 15 min. The tubes were removed and placed in a water bath for cooling to 30 ± 3°C. After autoclaving, a single colony of the yeast isolates were added to the BS625 Durham tubes. The test tubes were incubated at 30°C for 24 h. After incubation, the tubes were observed for acid and gas formation.

Method of yeast identification through molecular characterization by Antia *et al.* (2018) was adopted. The cells were incubated at 30 ± 0.1°C in a hydrolyzed sisal bole juice and placed in rotary shaker for 12 – 15 hours to facilitate the breakdown of their cell walls (Antia *et al.*, 2018; Blount *et al.*, 2016). Genomic DNA was extracted overnight using 500 µl of the prepared buffer (3% CTAB, 100 mM, 20 mM EDTA and 1.4 M NaCl, pH 7.0) with glass beads, and then centrifuged supernatant fluid discarded. The internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) was amplified using universal primer ITS1 (CGG GAT CCG TAG GTG AAC CTG CGG) and ITS4 (CGG GAT CCT CCG CTT ATT GAT ATG C). The amplification reaction was done in a 50 µL reaction mixture containing 20 pmol of primer (ITS1 and ITS4), 300 ng DNA template, 0.25 mmol/l dNTP, 1.50 mmol/L $MgCl_2$, and 0.50 U of Taq polymerase. For negative control, a gel-well labelled W was filled with water. The PCR products were visualised using a gel imaging system (Gel. LUMINAX) under UV. Samples showing single bands of approximately 500-600 bp were purified and Sanger sequenced. The sequencing results were analysed using NCBI BLAST to determine species identity based on sequence similarities.

Characterization of the peels

Proximate analysis of the peels

The peel samples were analysed for moisture content, ash content, volatile organic components and crude fat. All Analysis were done in triplicate, with the mean and standard deviations reported. A one-way analysis of variance (ANOVA) was used to assess the significant differences in proximate and mineral composition analysis (ultimate) among orange, cassava and banana ($p < 0.05$). The Data Analysis Toolpak for Microsoft Excel was used to conduct the statistical analysis.

Method by Enoch *et al.* (2022) was adopted for analyzing moisture content. The peel (2 g) was transferred into an empty crucible weighing W0 and the total weight W1 was determined. The sample was dried in an oven at 105°C for 8 h to remove moisture, giving a final weight W2. The moisture content was calculated as a percentage using Equation 1.

$$Moisture\ (\%) = \frac{W1\ (g) - W2(g)}{W1(g) - W0(g)} x100\% \quad [1]$$

Determination of ash content was done following a method by Enoch *et al.* (2022) with some modifications in heating time because the sample was dry. The crucible was dried in an oven at 100°C for 20 min. and weighed (W1). The sample was then added to the crucible and combined weight was recorded (W2). The crucible containing the sample was heated in a furnace at 550°C for 3 hours. After cooling, the final weight was recorded as W3. Ash content was calculated as a percentage using Equation 2.

$$Ash\ (\%) = \frac{W3\ (g) - W1(g)}{W2 - W1(g)} x100\% \quad [2]$$

Crude fat determination adopted the method by A.O.A.C (1999). Approximated 2 g sample was put in a beaker and weighed (W). It was mixed with 10 ml of water to form a suspension, followed by addition of 10 ml of concentrated HCl to dissolve the slurry and form a solution. The mixture was transferred to a water bath (WTB11) at 70°C and heated for 3 hours. After cooling for 5 minutes, 10 ml of ethanol was added and the mixture agitated vigorously. The ether layer was decanted into a pre-weighed flask (W1) and placed in a boiling water bath to evaporate the ether. The extraction was repeated using 50 ml diethyl ether to evaporate the ether and leave the fat behind. The flask containing the extracted fat was then weighed as (W2). The fat content was calculated using Equation 3.

$$Fat\ (\%) = \frac{W2(g) - W1(g)}{W(g)}\ x\ 100\% \quad [3]$$

Method by A.O.A.C (1999) was adopted for analysing volatile content. The wet peel (2 g) was transferred into a crucible and total weight (W) was determined. The sample was dried in an oven at 105°C for 8 h to remove moisture, giving a final weight (W1). The dry sample was further dried in a muffle furnace at 110 ± 5°C for 1 hour. The volatile content was calculated as a percentage using Equation 4.

$$Volatile\ content\ (\%) = \frac{W1(g) - W2(g)}{W(g) - W1(g)}\ x\ 100\% \quad [4]$$

Ultimate analysis of the peels

Method by Uzama *et al.* (2021) of analysing Ca, Cu, Fe, Mg and K was adopted. A sample of 0.5 g of dry peel was ground and about 0.31 g of the formed powder was treated with 10 ml mixture of concentrated HNO_3 and HCl (1:1) and left for 12 – 15 hours to facilitate mineral dissolution. Then 90 ml distilled water was added to make 100 ml of digest. The digests were filtered using Whatman filter paper, and then dissolved minerals were analysed using Inductively coupled plasma-optical emission spectroscopy (Agilent 5110 ICP-OES). The K and Ca were determined by an Atomic Absorption Spectrophotometer (AA-7000), equipped with an air-acetylene flame. All the mineral analysis were done at the analytical laboratory of the Ministry of Water, Ubungo - Tanzania.

Preparation of natural nutrients for supplementation

Method by Popalia *et al.* (2022) and Sankalpa *et al.* (2017) of peel preparation were adopted. Dirt and grit were removed from collected peels by washing them with tap water. Samples were dried in a moisture extraction oven at 70°C for 24 hours to facilitate powder formation. The dried peels were ground into fine powder using an electric blender (DW8002 – 220 V, 50 Hz, 1000 W).

Ethanol production from sisal bole juice

Fermentation was done without nutrient supplements (blank), with synthetic supplements (control) and with different proportions of the powdered peels. All fermentation broths were sterilized by autoclaving at 121°C for 15 minutes. To each 250 ml flask, 90 ml of hydrolyzed sisal bole juice containing 20% simple sugar (fructose) measured by a digital refractometer (METTLER TOLEDO, 86.8% accuracy) was added followed by supplements, where needed.

Minitab (v18) was used to design the experiment and analyse the effect of natural supplements dosage on the bioethanol yield.

A 2^n full factorial design was employed to study the effect of three factors (orange peels, cassava peels and banana peels) dosage on the bioethanol production yield. Analysis of variance (ANOVA) was conducted to assess the main and interaction effects of the factors on bioethanol yield. A significance level of $p < 0.05$ was used to determine statistical significance. Main and Interaction effects were also evaluated, and results elaborated further by the Pareto chart. The experiment was done in random order using a single replicate. The basis of the supplementation was 1-3 g for all powdered peels. Whereas the low, middle and high dosage for all peels (orange, cassava and banana) were 1 g, 2 g and 3 g, respectively. The method by Wanderley *et al.* (2014) was adopted in inoculum preparation. The hydrolyzed sisal bole juice was diluted until the fructose sugar content became 5%. Supplements were then added as required (Table 1) and the mixture pH was adjusted and controlled at 4.6.

Table 1

Natural supplementation for inoculum growth

Inoculum	Peel (g)		
	Banana	Cassava	Orange
Blank	0	0	0
Inoculum 1	0.70	0.05	0.80
Inoculum 2	0.75	0.05	1.00
Inoculum 3	0.80	0.06	1.20

The four flasks with fermentation broth were covered and autoclaved at 121°C for 15 min after which they were left to cool to 30 ± 0.1°C for the next 20 minutes. Three colonies of the isolated yeasts were added to each autoclaved mixture. The flasks were placed in an incubator at 30°C for 15 hours to allow the fermentation process. Afterwards, the final brix of the mixture was determined. Cell counting method by Anyanwu *et al*. (2020) by using a Neubauer counting chamber was adopted to establish the viability of the microorganisms.

Batch fermentation was carried out at 30°C in 250 ml conical flasks tightly covered with cotton wool. The initial pH of broth was set at 4.6. About 10 ml of the inoculum was added to each flask. The brix % of samples was measured at time intervals of 3 h for the first day and then 6 hours for the subsequent days until a total of 72 hours. The bioethanol produced (% w/w) was monitored using an Abbe refractometer (93.06% accuracy, ±0.005).

Results

Juice extraction

The juice obtained was 2830 ml equivalent to 166.27 ml per kg of the cleaned sisal bole.

Macroscopic identification

The isolated colonies were either ellipsoidal or oval as shown in Table 2. The colonies were also gram-positive. The solutions changed from red to yellow indicating that acidic gases formed. However, isolates Y6 and Y8 did not

produce carbon dioxide gas during fermentation but the solutions' colour changed, suggesting that they are not *Saccharomyces cerevisiae* strains. Isolate Y0, Y2 and Y4 produced carbon dioxide gas during fermentation with Y0 producing the highest volume as shown in Table 2.

Table 2

Biochemical characterization of isolates

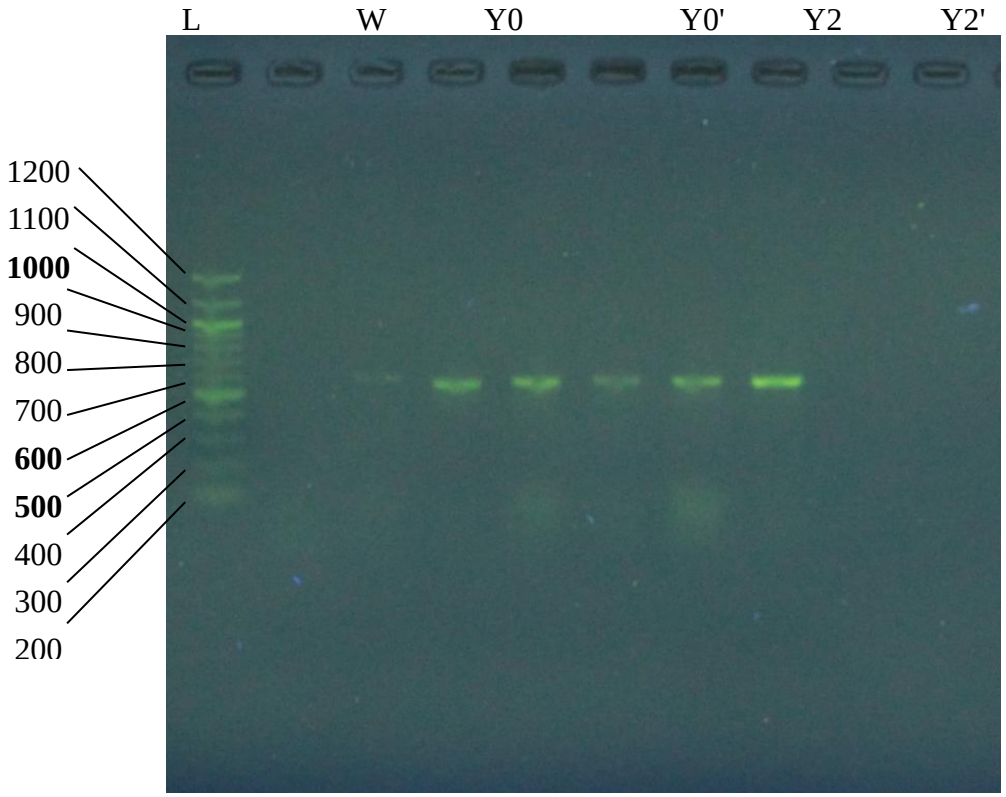
Colony code	Cell form	Gram staining (G+/-)	Juice fermentation test	Probable isolate
Y0	Ellipsoidal	G+	+++	*S. cerevisiae*
Y2	Ellipsoidal	G+	+	*S. cerevisiae*
Y4	Ellipsoidal	G+	++	*S. cerevisiae*
Y6	Oval	G+	-	*K. marxianus*
Y8	Oval	G+	-	*K. marxianus*

Molecular characterization

Genomic DNA was successfully isolated from five samples capable of ethanol fermentation. The colonies Y6 and Y8 were excluded from further analysis because they had a negative fermentation results. ITS region amplification using ITS1 and ITS4 primers produced clear, single bands of approximately each of size 500 – 600 bp for Y0, Y0', Y2, Y2', Y4, and Y4' as shown in Figure 1. The clear well, W, in the gel confirmed the absence of contamination. Sequencing of the PCR products followed by BLAST analysis revealed that all positive isolates showed high similarity (>99%) to *Saccharomyces cerevisiae*.

Figure 1

PCR results of the isolated DNA



Characterization of peels

The proximate analysis of the peels is shown in Table 3. Orange peels had relatively higher content of moisture (88.36 ± 0.06%), ash (3.45±0.10%) and crude fat (9.36 ±0.08%) compared to the rest of the peels. However, banana peels had relatively higher volatile matter content at 75.10±0.08% compared to the other peels based on the A.O.A.C method. The ash content is lowest for cassava peels at 1.93±0.11%, followed by banana peels at 2.24±0.12%, and orange peels at 3.45±0.10%. One-way ANOVA showed a highly significant difference in all proximate analysis among the fruit peels (p =<0.05).

Table 3

Proximate composition of the peels

Peels	Moisture content (%) (Mean ± SD)	Volatiles (%) (Mean ± SD)	Ash content (%) (Mean ± SD)	Crude fat (%) (Mean ± SD)
Cassava	73.86 ± 0.05	62.83 ± 0.12	2.24±0.12	4.39±0.10
Banana	76.87 ± 0.07	75.10 ± 0.08	1.93±0.11	8.85 ±0.12
Orange	88.36 ± 0.06	69.50 ± 0.09	3.45±0.10	9.36 ±0.08

The ultimate compositions of cassava, banana and orange peels are shown in Table 4. Orange peels have higher amount of potassium (322.00±0.87 mg/l), magnesium (88.96±0.49 mg/l) and Copper (0.25±0.01 mg/l) compared to cassava and banana peels. The

one-way ANOVA showed a highly significant difference in potassium, magnesium and copper content among orange, cassava, and banana peels ($p < 0.005$). However, iron is very high in cassava peels (34.11±0.10 mg/l) compared to both orange and banana peels. One-way ANOVA showed a highly significant difference in iron content among the peels ($p < 0.05$), confirming that at least one group mean differs significantly from the others. Banana peels have the highest Calcium content (24.40±0.10 mg/l) compared to cassava and orange peels.

Table 4

Ultimate composition of the peels

Minerals	Orange (mg/l)	Cassava (mg/l)	Banana (mg/l)
Ca	9.40±0.02	15.00±0.10	24.40±0.10
Cu	0.25±0.01	0.22±0.01	0.14±0.01
Fe	5.87±0.06	34.11±0.10	3.82±0.04
Mg	88.96±0.49	78.29±0.24	51.68±0.27
K	322.00±0.87	68.00±0.20	50.00±0.21

Fermentation

The brix drop for inoculum S1, S2 and S3 was 0.53%, 0.47% and 0.68%, respectively. Variability in the performance of the fermentation process can be caused by poorly controlled initial colony transfer and culture cultivation as observed by Webb & Kamat (1993). The inoculum S3 was selected, and the CFU per ml was 89.60 x 10^9, while for the blank sample was 44.80 x 10^9.

Table 5 shows the concentration of bioethanol produced using different nutrient supplement ratios. From Table 5, the highest average bioethanol concentration achieved was 26.62% w/w in the 12th and 23rd runs. This result was obtained using a mixture of 3 g of orange peels, 3 g of cassava peels, and 1 g of banana peels fed in 100 ml of sisal bole juice. The CFU per ml was 89.60 x 10^9. Conversely, the lowest average bioethanol content of 17.33% w/w was recorded in the 5th and 21st runs, where 3 g of orange peels, 2 g of cassava peels, and 3 g of banana peels were utilized in 100 ml of sisal bole juice. The ethanol concentration in the control and blank runs was 18.71% w/w and 20.13% w/w, respectively, which justifies that the fermentation process was more efficient using isolated *Saccharomyces cerevisiae* in sisal bole juice with no nutrients than using commercial *Saccharomyces cerevisiae* under artificial nutrients (0.08g $MgSO_4$, 0.40g KH_2PO_4 and 1g NH_4NO_3). In addition, both bioethanol concentrations are lower than most runs in Table 5 showing the importance of nutrient supplements in the fermentation process.

Table 5

Concentration of ethanol produced in different peels combinations

Run	Orange (g)	Cassava (g)	Banana (g)	Ethanol (%w/w)
Control				18.71
Blank	0	0	0	20.13
1	2	1	1	17.33
2	3	2	3	22.77
3	2	3	3	20.13
4	3	2	3	22.77
5	1	1	2	17.33
6	3	1	3	24.31
7	1	1	3	18.71
8	2	3	3	20.13
9	2	2	2	21.46
10	3	1	3	18.71
11	2	1	1	18.71
12	3	3	1	27.38
13	3	3	2	24.31
14	1	3	3	21.47
15	1	3	3	21.47
16	3	3	2	20.13
17	1	2	1	18.71
18	3	1	1	24.31
19	1	3	1	18.71
20	2	2	2	21.47
21	1	1	2	17.33
22	1	2	1	21.47
23	3	3	1	25.85
24	1	1	3	18.71
25	3	1	1	21.47
26	1	3	1	17.33

Table 6 gives the analysis of variance of the effects while Figure 2 and Figure 3 show a pareto plot of the effects of factors and the interaction plot for bioethanol (%w/w) for data means, respectively.

Table 6

Analysis of variance of the effects of factors on bioethanol production

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	7	129.75	18.54	5.38	0.00
Linear	3	77.18	25.73	7.47	0.00
A - Orange peels (g)	1	64.38	64.38	18.69	0.00
B - Cassava peels (g)	1	12.67	12.67	3.68	0.07
C - Banana peels (g)	1	1.41	1.41	0.41	0.53
2-Way Interactions	3	19.42	6.47	1.88	0.17
AB	1	1.48	1.48	0.43	0.52
AC	1	18.00	18.00	5.22	0.04
BC	1	5.94	5.94	1.72	0.21
3-Way Interactions	1	7.02	7.02	2.04	0.17
ABC	1	7.02	7.02	2.04	0.17
Error	18	62.00	3.44		
Lack-of-Fit	5	26.70	5.34	1.97	0.15
Pure Error	13	35.30	2.72		
Total	25	191.74			

Key: A = Orange peels, B = Cassava peels, C = Banana peels, DF = Degree of freedom, Adj SS = Adjacent sum of squares, Adj MS = Adjacent Mean squares.

Figure 2

The Pareto plot of the effects of factors on bioethanol production

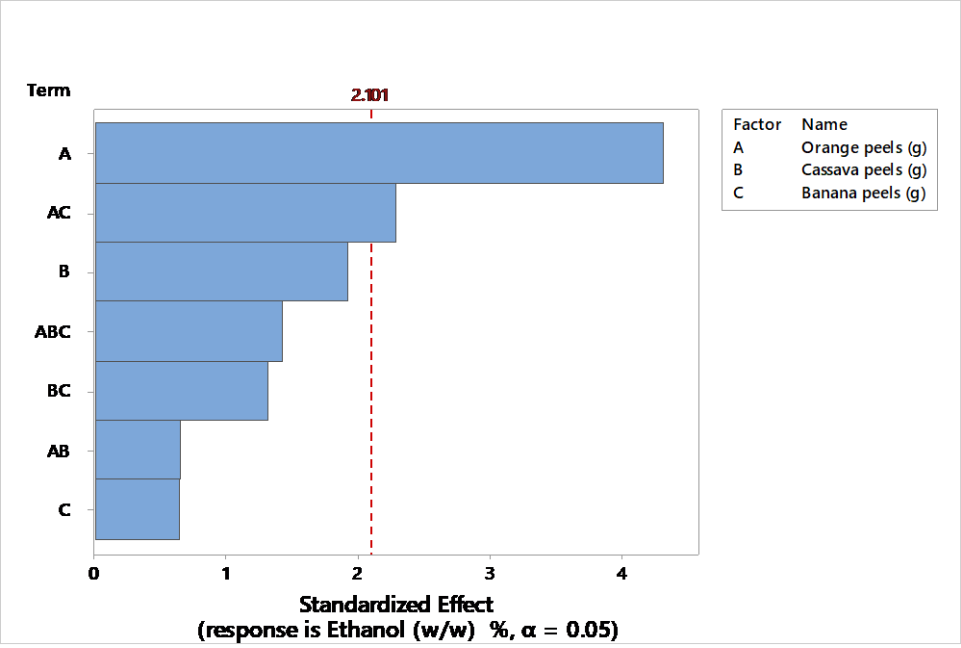
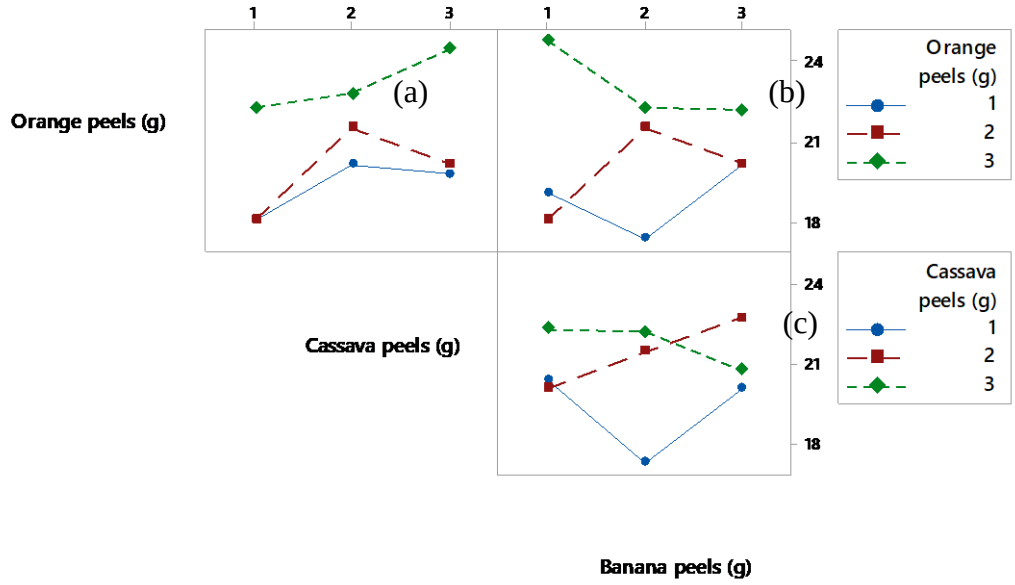


Figure 3

The interaction plot for bioethanol (%w/w) for data means



Discussion

The juice extracted in this study was very low compared to 567 ± 3 ml per kg by Msuya *et al.* (2018a), for the similar feedstock, Agave (Hybrid 11648), 10 – 12 years old. The variation might be due to ineffective chop pressing process which caused a loss of pressure control leading to self-retraction of hydraulic jack, the source of the sisal bole and

its age. The mineral content analysis of sisal boles juice by Msuya *et al.* (2018b) indicates that except for potassium (K), levels of other essential minerals such as calcium (Ca), copper (Cu), iron (Fe) and magnesium (Mg) are below the required level for effective fermentation (Kampen, 2014). This highlights the importance of using natural nutrients supplements in this study.

The biochemical characterizations in Table 2 are similar to those of Yadav and Tiwari (2016), Antia *et al.* (2018), Sulmiyati *et al.* (2019), and Okechukwu *et al.* (2020) who observed *Saccharomyces cerevisiae* to be gram-positive and the cells are ellipsoidal, oval or spherical in shape. They also observed that the *Saccharomyces cerevisiae* produce carbon dioxide gas under anaerobic fermentation. The extent of fermentation depends on the number of isolate cells (Khattab *et al.,* 2016). Nurcholis *et al.* (2019) concluded that yeast of strain *Kluyveromyces marxianus* can ferment glucose to produce lactic acid and acetic acid but with an insignificant volume of the gas. The results in Figure 1 are similar to those of Manter and Vivanco (2007) who reported that, the size of *Saccharomyces cerevisiae* was 420 – 825 bp under ITS1 and ITS4 primers. They further noted that these primers amplified all fungi when tested using PCR. Therefore, the isolates Y0, Y0', Y2, Y2', Y4, and Y4' are concluded to be *Saccharomyces cerevisiae* strains. The study by Msuya *et al.* (2024) demonstrated a positive bioethanol fermentation test for the *Saccharomyces cerevisiae* strains from sisal bole juice although expensive synthetic supplements were used. This study explored the use of natural locally available supplements to enhance the bioethanol production yield from sisal bole juice.

The results for cassava peels in Table 3 differ slightly from Khail (2022), who obtained 72.98 – 76.20 % (wet basis) moisture content, 16.70 - 31.76 % (dry basis) of ash content and 1.45 – 1.48 % (dry basis) of crude fat. Youseff *et al.* (2024) obtained 87.11-90.2 % (wet basis) moisture content, 15.5-17.39 % (dry basis) of ash content and 7.5-8.35 % (dry basis) of crude fat for banana peels. Uzama *et al.* (2021) obtained 3.66 % (dry weight) ash content and 4.83% (dry weight) of crude fat from sweet oranges. Czech *et al.* (2020) obtained 68.1% (wet basis) moisture content in oranges. The differences are attributed to variations in cultivars, geographical origin, environmental conditions and agricultural practices. From Table 4, the higher ash content in orange peels indicates a higher mineral composition than in cassava and banana peels, making it a more suitable source of mineral supplement such as Mg, K and Cu in fermentation. Additionally, the orange peels have the highest crude fat content which is important in cell membrane functioning by allowing selective permeability, hence a better source of fat in fermentation (Kampen, 2014). From Tables 5 and 6, mixing orange, banana, and cassava peels in correct ratios can sufficiently supply Mg, Fe, and Cu. Any of the peels is a good supplier of Fe. The sisal bole juice alone can sufficiently supply K. None of the peels is a good source of Ca; hence, it is necessary to supplement nutrients using all three peels at the correct ratio to avoid overdosing or underdosing.

The ANOVA on the effects of supplementation on bioethanol (%w/w) production suggests that the model is linear and significant (p-value < 0.05) as shown in Table 6.

The linear relationship indicates that either the amount of nutrients supplied was too low such that the microbial cells were not yet

saturated or there were no inhibitory or limiting effects in the fermentation process. The same might be possible if one of the nutrients is limiting the fermentation and others are in excess (Bailey and Ollis, 2018; Portero Barahona *et al.,* 2019; Wang *et al.,* 2024). From the table it is evident that the main effect of orange peels (A) ($p = 0.000$) and interaction between orange and banana (AC) ($p = 0.035$) are significant, whereas the main effect of cassava (B) and banana (C) peels are not significant and so are two-way interactions of orange and cassava (AB), cassava and banana (BC) and the three-way interaction of orange, cassava and banana (ABC). These observations are cemented by the Pareto chart shown in Figure 2.

The Pareto plot of effects factors on bioethanol w/w% suggests that the effect of orange peels (A) and interaction between orange and banana peels (AC) extends past the reference line at significance level $\alpha = 0.05$. Less effect is shown by the interaction between orange and banana peels (AC) than orange peels alone (A). There was no significant effect on bioethanol w/w% by either cassava peels (B) and banana peels (C) or interactions of orange-cassava peels (AB), cassava-banana peels (BC) and a combination of orange-cassava-banana peels (ABC).

The interaction plot (Figure 3) suggests a strong interaction between orange-cassava peels, orange-banana peels and cassava-banana peels to bioethanol w/w% which is justified by the interaction lines being not parallel. From Figure 3(a), the high bioethanol w/w% is produced when orange peels are at 3 g and Cassava peels are at 3 g. The lowest bioethanol w/w% is at both Cassava and orange peels at 1 g. When orange peels are increased from 1 – 2 g its effect is lesser than that at 3 g. From Figure 3(b), the high bioethanol w/w% is produced when orange peels are at 1 g and banana peels are at 1 g, on increasing both banana and orange peels beyond 1 g the bioethanol w/w % decreases. From Figure 3(c), the high bioethanol w/w % is produced when Cassava peels are at 2 g and Banana peels are at 3 g. The bioethanol w/w % decreases when both Cassava and banana peels are at 3 g or 1 g.

Conclusion

The study showed that orange peels and a combination of orange and banana peels significantly enhance bioethanol yield. These peels serve as effective nutrient supplements for sisal bole yeast isolates by supplying essential minerals required for yeast growth. They can potentially replace synthetic nutrients such as Magnesium sulfate heptahydrate, Iron (II) sulfate tetrahydrate and Copper (II) sulfate pentahydrate that supplies Mg, Fe and Cu in bioethanol production.

Higher bioethanol production can be obtained when nutrients are supplied in the correct minerals proportions. The results suggest that natural nutrient supplements can substitute for costly and hard-to-find synthetic supplements. This study revealed that effective utilization of natural nutrients and sisal bole yeast isolate can produce a high amount of bioethanol in fermentation process. On this basis, further researches are recommended to explore alternative natural supplements that offer natural nutrients sufficient to replace all synthetic nutrients. Also, further study for selecting the best inoculum size for the fermentation process involving sisal bole yeast isolates and natural nutrients is highly recommended.

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