Biochemical Evaluation of Pre-treated Unpeeled Cassava Roots (*Manihot esculenta* Crantz) and its application in Alcohol Production

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ABSTRACT

This study investigated the use of pretreated unpeeled cassava roots (*Manihot esculenta* Crantz) for industrial alcohol production and examined the associated biochemical changes. The pre-treatment process resulted in increased levels of soluble protein, glucose concentration, and reducing sugar concentration compared to the control. In addition, pre-treated solid-state fermented cassava demonstrated improved free radical scavenging and percentage inhibition capacity compared to the control. The pre-treated and autoclaved solid-state fermented unpeeled cassava hydrolysed with 0.1N sulphuric acid showed a significant increase ($p<0.05$) in amylase, phenol, and flavonoids concentration compared to the control. When unpeeled cassava roots were pre-treated and subjected to submerged fermentation with *Saccharomyces cerevisiae* for alcohol production, there was a significant increase ($p<0.05$) in soluble protein concentration and a significant reduction ($p<0.05$) in glucose and reducing sugar concentration. The results also indicated that solid-state fermented cassava roots in submerged fermentation had higher free radical scavenging activity and percentage inhibition capacity compared to the control. While there was a significant reduction in alcohol production in the pre-treated unpeeled cassava roots compared to the control, the percentage of alcohol in the pretreated solid state fermented unpeeled cassava roots in submerged fermentation with *S. cerevisiae* was significantly improved.

Key words: Cassava roots, Fermentation, *Rhizopus oligosporus*, *Saccharomyces cerevisiae*, Alcohol

INTRODUCTION

As the global population continues to grow at a rapid pace and the demand for fossil fuels increases, conventional crops such as corn, sugarcane, and switchgrass face challenges in meeting the global alcohol production requirements. As a result, there is a growing demand worldwide for sustainable and renewable biological fuels such as alcohol, which is the second-most widely used solvent in the chemical industry, after water. Alcohol has many applications, including use as a solvent in

industrial products like lacquers, dyes, paints, and oil, as well as disinfectants, raw materials in chemical synthesis, and an alternative energy substitute for fuel (Busca 2021; Amalia et al., 2021).

Alcohol, also known as bioethanol, is a renewable bioenergy source that is environmentally friendly and can be derived from sugar and starch-containing materials such as potatoes, corn, sugar cane, wheat, molasses, and more. Although the cost of producing bioenergy from cultivated crops is higher than petroleum-based energy production, we require an economical and easily available source of raw materials. One alternative source for the production of alcohol is agricultural waste and non-food crops such as lignocellulosic biomass, which is abundant and promising. The production of alcohol from lignocellulosic biomass offers significant potential to meet the present energy demand, while also reducing greenhouse gas emissions and the release of harmful pollutants during combustion (Amalia et al., 2021).

According to Okwuonu et al. (2021), Nigeria has emerged as the largest producer of cassava worldwide. Cassava, a drought-resistant tuber crop that thrives in tropical and subtropical regions, is consumed by both humans and animals and is an important food source in the areas where it is cultivated (Tonukari, 2014; Egbune et al., 2023b). It is an affordable source of energy, high in carbohydrates, and provides more calories per acre than grain crops. However, the crop contains high levels of cyanide, making it unsuitable for raw consumption. The International Fund for Agricultural Development (IFAD) and the United Nations Food and Agriculture Organization (FAO) have made efforts to increase cassava productivity globally, given its ability to grow in a range of soil types and withstand drought (Mohamed et al., 2019). In nations where cassava is a major food commodity, there is often a surplus harvest, and the crop is used as a substrate for various industrial products due to its abundance and poor shelf life (Tonukari et al., 2016, 2023; Egbune and Tonukari, 2023). It is worth noting that the non-food parts of *M. esculenta* Crantz, such as stem and root peel, contains 85% of the sugar that can be used to produce alcohol, with a yield of approximately 60% (Nuwamanya et al., 2012).

In order to increase the hydrolysis rates of complex lignocellulosic biomass, pretreatment is often necessary due to its recalcitrance and resistance to enzymatic hydrolysis (Mohapatra et al., 2017). The pretreatment step aims to break down the rigid structure of the feedstock and separate its major components, including cellulose, hemicelluloses, and lignin, in order to maximize the volumetric productivity of biofuel through subsequent enzymatic hydrolysis and fermentation steps (Li et al., 2016). Simple sugars, such as six-carbon (hexoses) sugar, can be produced from the fragmentation of cellulose by either enzymatic or acid hydrolysis, which can then be easily fermented into alcohol (Srivastava et al., 2014).

Solid state fermentation is a method in which microorganisms break down starch into simple sugars to produce alcohol and CO_2. This process has been extensively used for the production of essential products, including food, beverages, pharmaceuticals, and medical products (Crini et al., 2020). Solid state fermentation is also used to produce enzymes and animal feeds (Abdul and Webb 2017). Additionally, this method is less time-consuming and results in minimal nutrient loss.

Various pretreatment techniques are used to produce biofuels from lignocellulosic biomass, which can be either physical or

chemical, or a combination of both. Among these, the dilute acid pretreatment method, which incorporates both physical and chemical processes, is considered the most suitable for large-scale commercial application due to its high efficiency. The objective of this research is to assess the biochemical characteristics of solid-state fermented cassava that has been pretreated using both autoclaving and acid hydrolysis methods, using the microorganism *Rhizopus oligosporus*. The aim is to produce alcohol from unpeeled cassava that has undergone different pretreatment processes.

MATERIALS AND METHOD

Collection of cassava tubers and starter culture

Cassava tubers were sourced from a farm in Abraka, Delta State, Nigeria and were cleaned thoroughly to eliminate any impurities. The unpeeled cassava root was cut into pieces measuring approximately 2 cm x 1 cm and dried in the sun until a constant weight was achieved over 24 hours. The dried material was then ground into a fine powder with a particle size of 71 m and stored at 37°C. The *R. oligosporus* strains utilized in the fermentation process by PT Aneka Fermentasi Industri in Bandung, Indonesia were supplied by Tonukari Biotechnology Laboratory in Sapele, Delta State, Nigeria. The organism was preserved in a sterile glass vial filled with glycerol and stored in a refrigerator at 4°C. Prior to inoculation, cells were reconstituted in Potato Dextrose agar medium.

Preparation of substrates for Solid-state fermentation

The substrates for solid-state fermentation were prepared by homogenizing 1 g of *R. oligosporus*, which had an estimated colony forming unit (CFU) of 1.4 x 10^2 CFU per gram, obtained from Tonukari Biotechnology Laboratory, with 15 mL of 50 mM phosphate buffer at pH 6, and 10 grams of powdered, unpeeled cassava root. The resulting mixture was then sealed and allowed to ferment at room temperature for 72 h. Control samples were also prepared using mold-free, dried, and crushed unpeeled cassava with buffer alone and without cells. After fermentation, aliquots of 6 grams of the cell-cassava mixture were collected for further analysis. The aliquots were homogenized with a mortar and pestle, and 10 mL of distilled water was added to the resulting mixture. The mixture was then centrifuged for 10 minutes to collect the supernatant as crude extract. Replicate samples of crude extracts were prepared for subsequent assays (Egbune et al., 2022).

Preparation of pretreated substrate for alcohol production

Following pretreatment, the solid-state fermented cassava underwent yeast fermentation using the technique outlined in Ogodo et al. (2018). Specifically, 0.8 g/l of commercial baker's yeast (*Saccharomyces cerevisiae*) was combined with 200 ml of the fermentation broth and stirred. The mixture was left to ferment for seven days. Post-fermentation, 6 grams of solid-state fermented cassava in yeast were gathered for further analysis. The aliquots were homogenized with a mortar and pestle, and 10 mL of distilled water was added to the resulting mixture. The mixture was then centrifuged for 10 min, and the supernatant was collected as crude extract. Replicate samples of crude extracts were prepared for subsequent assays.

Biochemical procedure for pretreated unpeeled cassava roots and pretreated unpeeled cassava roots in yeast fermentation for alcohol production

The total soluble protein (TSP) content of each sample was estimated at an absorbance reading of 540 nm, using bovine serum as a reference, as per the method outlined by Gornall et al. (1949). Glucose concentration, expressed in mg/dL, was monitored using a diagnostic glucose assay kit (Randox Laboratories Ltd, UK), based on the mechanistic action of glucose oxidase activity as per the manufacturer's instructions. The pH of the test samples was measured using a Mettler Toledo pH meter. The total reducing sugar (TRS) in each sample was determined using the dinitrosalicylic acid method at a wavelength of 540 nm, following Miller's protocol (1959). Alpha-amylase activity was monitored at a wavelength of 540 nm using maltose as a reference, following Nouadri et al.'s protocol (2010). The antioxidant potential of each sample was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, which uses DPPH as a hydrogen radical scavenger, resulting in the formation of a deep violet complex that was measured at 517 nm, according to Hatano et al.'s protocol (1988). Total phenolic content (TPC) was quantified using the Folin-Ciocalteu reagent, which oxidizes phenolic compounds in the sample, resulting in the formation of a blue-colored complex that was measured at 760 nm, as per the method described by Singleton and Rossi (1965). The total flavonoid content was determined at a wavelength of 510 nm using the method outlined by Jia et al. (1999).

Determination of total soluble solids

An Atago Master series handheld refractometer from Japan was utilized in this experiment. To standardize the refractometer, a single drop of distilled water was applied onto the prism. The refractometer was then positioned in such a way that sunlight could enter the prism, and the coarse and fine adjustments were properly adjusted. The eye-piece was used to observe the standardization process, as described in the AOAC (1990) guidelines.

Specific Gravity

The percentage alcohol content was calculated using the following formula based on the specific gravity (SG) obtained: % ABV = (Initial SG - Final SG) / 7.36 x 1000, where the initial and final SG values were obtained by measuring 50 mL of the sample into a measuring cylinder at 20 °C and dipping a hydrometer into it to determine the specific gravity (with appropriate temperature correction factor), in accordance with the method described by Balogu and Towobola (2017).

Statistical analysis

Data obtained were subjected to statistical analysis using one-way ANOVA (analysis of variance) and Fischer's test of least significance (LSD); values are presented as Mean ± Standard deviation. Results were considered significant at p-values less than 0.05, that is, at 95% confidence level ($p< 0.05$).

RESULTS AND DISCUSSION

Biochemical parameters of pretreated unpeeled cassava roots

Soluble proteins are proteins that are soluble in water and other polar solvents. They are typically found in the cytoplasm or extracellular fluid of cells and play a vital role in many biological processes, such as enzyme catalysis, signal transduction, and structural support. In the case of the study presented in Figure 1A, it appears that pre-treatment of unpeeled cassava has led to an increase in soluble protein concentration

compared to the control. This could be due to the breakdown of complex proteins in cassava into smaller, soluble protein fragments during the pre-treatment process (Kringel et al., 2020; Ziero et al., 2020). From the study it was observed that the pre-treatment process may have caused an increase in protein extractability from the cassava samples, leading to the observed increase in soluble protein concentration. However, it is interesting to note that there was no noticeable increase in soluble protein concentration in the autoclaved unpeeled cassava samples. Autoclaving is a high-temperature, high-pressure process that is commonly used to sterilize materials (Ma et al., 2022). It is possible that the high temperature and pressure of the autoclave may have denatured or degraded the proteins in the cassava samples, leading to a decrease in soluble protein concentration. Among the pre-treated samples, solid-state fermented unpeeled cassava had the highest soluble protein concentration at 51.23±0.4 mg/g. This may be due to the fact that solid-state fermentation is a process that involves the use of microorganisms to break down complex organic compounds, such as proteins, into simpler, more soluble forms (Banat et al., 2021; Areeshi, 2022). The microorganisms used in solid-state fermentation may have contributed to the observed increase in soluble protein concentration in the fermented cassava samples (Hawashi et al., 2019; Tan et al., 2019). The increase in soluble protein concentration observed in pre-treated unpeeled cassava may be due to the activation of endogenous proteases that break down insoluble proteins into soluble forms (Aryee & Boye, 2014; Egoamaka et al., 2021). The pre-treatment may have created conditions favorable for the activation of proteases by disrupting the cell walls and/or cellular structures of the cassava tissue (Kwiatkowska et al., 2011; Wu et al., 2022). Solid-state fermentation (SSF) of unpeeled cassava resulted in the highest soluble protein concentration compared to other pre-treatments. During SSF, microorganisms produce enzymes such as proteases, which can break down complex proteins into simpler, soluble forms (Ezedom et al., 2022; Liu et al., 2023; Patel et al., 2023). The microbial activity may have further activated endogenous proteases and increased the soluble protein concentration in the fermented unpeeled cassava (Aryee & Boye 2014; Ilango & Antony 2021).

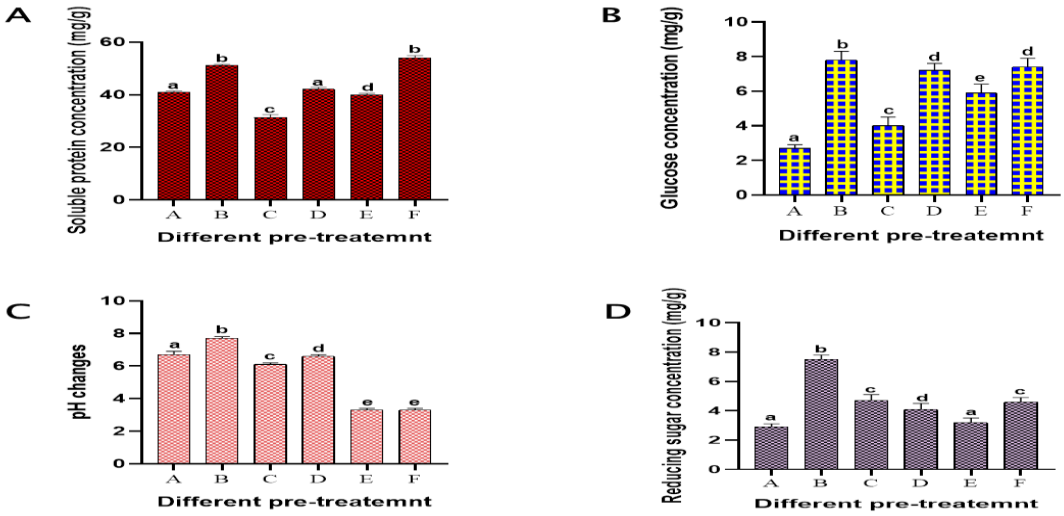

Figure 1. (A) Levels of soluble proteins, (B) Glucose concentration, (C) Effect of pH and (D) Reducing sugar concentration at different pre-treatments of unpeeled cassava. The values marked with superscripted letter a, b, c, d indicate significant differences from the control ($p < 0.05$). A) Unfermented unpeeled cassava roots, B) SSF unpeeled cassava roots, C) Autoclaved unpeeled cassava roots, D) Autoclaved SSF unpeeled cassava roots, E) Autoclaved unpeeled cassava roots with 0.1N sulphuric acid, and F) Autoclaved SSF unpeeled cassava roots with 0.1N sulphuric acid.

Figure 1B presents the results of glucose concentration analysis in autoclaved and acid hydrolyzed solid-state fermented cassava, which was treated with R. oligosporus. The pre-treatment of unpeeled cassava showed a significant increase ($p<0.05$) in glucose concentration compared to the control. The significant increase in glucose concentration observed in pre-treated unpeeled cassava may be attributed to the breakdown of starch present in the cassava tissue (Omede et al., 2017; Cereda et al., 2017; Egbune et al., 2023). Starch is a complex carbohydrate composed of glucose units linked together by glycosidic bonds. Pre-treatment may have disrupted the cell walls and structures of cassava tissue, which allowed enzymes to access and hydrolyze the starch into smaller, soluble glucose units (Phitsuwan et al., 2013; Weldemhret et al., 2020). Solid-state fermentation by microorganisms, such as R. oligosporus, produces various enzymes including amylases that can break down starch into glucose units (Egbune et al., 2022; Anigboro et al., 2023). The enzymatic breakdown of starch by microorganisms in fermented unpeeled cassava may have further contributed to the observed increase in glucose concentration (Egbune et al., 2023).

Figure 1C shows the changes in pH values of autoclaved and acid hydrolyzed solid-state fermented cassava using R. oligosporus. The pre-treated unpeeled cassava showed a noticeable decrease in pH compared to the control. This change in pH is likely due to the biochemical processes occurring during fermentation. During fermentation, microorganisms such as R. oligosporus utilize the carbohydrates in the cassava substrate as a source of energy. As they break down these carbohydrates, they produce organic acids such as lactic acid, acetic acid, and succinic acid (Behera et al., 2019; Sánchez et al., 2021). These organic acids can then cause a decrease in pH as they accumulate in the fermentation mixture (Fernández-Naveira et al., 2019). In addition, it is also possible that the pre-treatment of the unpeeled cassava may have led to changes in the composition of the substrate, such as an increase in the availability of fermentable sugars. This could have resulted in a greater production of organic acids during fermentation, leading to a more pronounced decrease in pH compared to the control (da Silva Mazareli et al., 2021). Overall, the changes in pH observed in Figure 1C are likely due to the metabolic activity of R. oligosporus during fermentation and may be influenced by the pre-treatment of the cassava substrate.

Figure 1D shows the results of the determination of reducing sugar concentration in autoclaved and acid hydrolyzed solid-state fermented cassava using R. oligosporus. The pre-treated unpeeled cassava demonstrated a significant increase ($p<0.05$) in reducing sugar concentration compared to the control. Autoclaved unpeeled cassava hydrolyzed

with 0.1N sulphuric acid did not show any significant difference from the control. These results can be explained by the biochemical processes that occur during cassava fermentation and hydrolysis. During fermentation, microorganisms such as R. oligosporus break down complex carbohydrates in the cassava substrate into simpler molecules, such as monosaccharides (Sharma et al., 2020; Anigboro et al., 2022). These monosaccharides can then be utilized by the microorganisms for energy production or can be further metabolized into other compounds, such as organic acids (Wu et al., 2021; Wang et al., 2021). As a result of these metabolic processes, the concentration of reducing sugars, such as glucose and fructose, in the fermentation mixture can increase.

In addition, pre-treatment of the unpeeled cassava, such as slicing or crushing, can disrupt the cell walls and release enzymes that can further break down complex carbohydrates into simpler molecules (Udoro, 2021). This can lead to an increased availability of reducing sugars for microbial fermentation, which may explain the higher concentration of reducing sugars observed in the pre-treated unpeeled cassava in Figure 1D. On the other hand, autoclaved unpeeled cassava hydrolyzed with 0.1N sulphuric acid did not show any significant difference from the control in terms of reducing sugar concentration. This may be due to the fact that autoclaving can break down some of the complex carbohydrates in the cassava substrate, making them more accessible to acid hydrolysis (Zakariyah et al., 2021). As a result, the acid hydrolysis may have already released most of the available reducing sugars from the autoclaved cassava substrate, leaving little difference between the acid-hydrolyzed autoclaved cassava and the control. Overall, the results in Figure 1D suggest that pre-treatment of the unpeeled cassava can increase the concentration of reducing sugars during fermentation, while autoclaving followed by acid hydrolysis may not be as effective in releasing these sugars.

The free radical scavenging activity determination using DPPH assay is a common method to evaluate the antioxidant capacity of plant extracts and natural products. DPPH is a stable free radical that can be reduced by antioxidants, resulting in a color change from purple to yellow. The extent of color alteration is in direct correlation to the antioxidant potential of the sample being evaluated. Figure 2A displays the outcomes of the assessment of free radical scavenging activity of autoclaved and acid hydrolyzed solid-state fermented cassava treated with R. oligosporus. The findings indicated that the pre-treated solid-state fermented cassava demonstrated an enhancement in its free radical scavenging activity when compared to the control. This increase may be linked to the higher concentration of antioxidant compounds present in the pre-treated sample (Tylewicz et al., 2020; Wojdyło et al., 2021). However, all other pre-treated samples exhibited significant reduction in free radical scavenging activity, which may be due to the breakdown of antioxidant compounds during the pre-treatment process. This result is consistent with previous studies that reported the degradation of antioxidant compounds in food matrices during thermal and acidic treatments (Jiang et al., 2019; Quan et al., 2020). Overall, the findings suggest that the pre-treatment process can have a significant impact on the antioxidant capacity of solid-state fermented cassava, and that optimization of the pre-treatment conditions is necessary to preserve the antioxidant compounds.

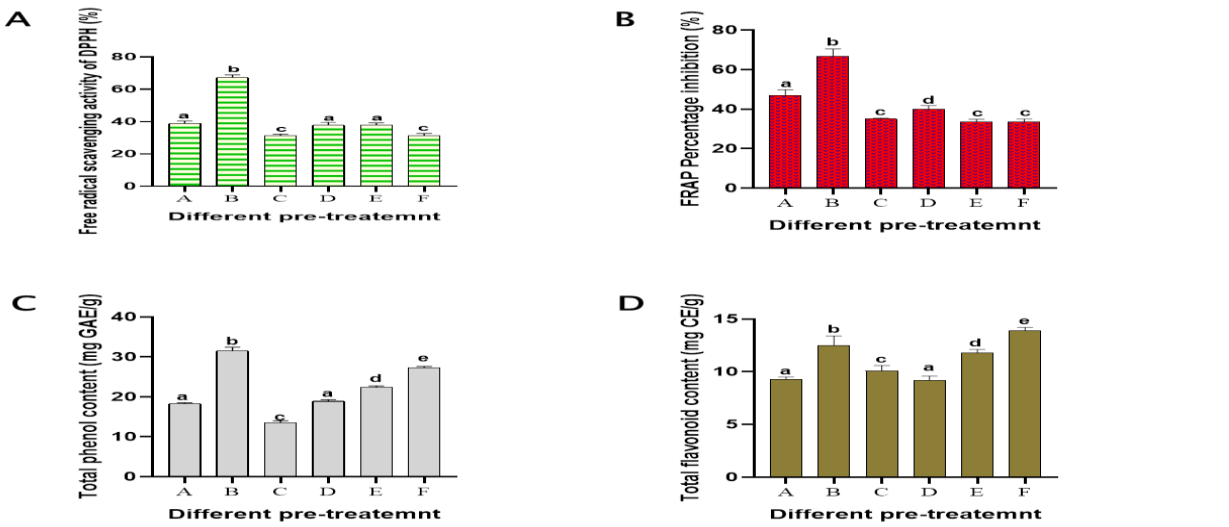

Figure 2. (A) Free radical scavenging activity of DPPH (%), (B) FRAP percentage inhibition (%), (C) Total phenolic content (TPC) and (D) Total flavonoid content (TFC) at different pre-treatments of unpeeled cassava. The values marked with superscripted letter a, b, c, d indicate significant differences from the control ($p < 0.05$). A) Unfermented unpeeled cassava roots, B) SSF unpeeled cassava roots, C) Autoclaved unpeeled cassava roots, D) Autoclaved SSF unpeeled cassava roots, E) Autoclaved unpeeled cassava roots with 0.1N sulphuric acid, and F) Autoclaved SSF unpeeled cassava roots with 0.1N sulphuric acid.

The Ferric Reducing Ability of Plasma (FRAP) assay measures the ability of a substance to reduce ferric ions (Fe3+) to ferrous ions (Fe2+) in a redox reaction (Kızıltaş et al., 2021; Nowak et al., 2021). The reduction of Fe3+ to Fe2+ is an important antioxidant mechanism in biological systems that help to prevent oxidative damage (Francoet al., 2019). In Figure 2B, the pre-treated solid-state fermented cassava showed a significant increase in percentage inhibition capacity compared to the control, indicating that the pre-treatment with R. oligosporus improved the antioxidant capacity of the fermented cassava. The increase in antioxidant activity may be due to the production of bioactive compounds during fermentation, such as phenolic compounds, which are known to have antioxidant properties (Villarreal-Soto et al., 2019; Adebo et al., 2020; Zhao et al., 2021).

Figure 2C displays the results of total phenol concentration (TPC) analysis for autoclaved and acid hydrolyzed solid-state fermented cassava treated with R. oligosporus. The pre-treated solid-state fermented cassava and autoclaved solid-state fermented unpeeled cassava hydrolysed with 0.1N Sulphuric acid showed a significant increase ($p<0.05$) in phenol concentration compared to the control. Phenolic compounds are secondary metabolites that are widely present in plants, and they play various roles, including defense against herbivores and pathogens. The increase in phenol concentration observed in pre-treated and autoclaved solid-state fermented cassava may be attributed to the activation of the phenylpropanoid pathway (Shirkavand, 2019). Phenylpropanoid pathway is responsible for the biosynthesis of phenolic

compounds, and it is activated in response to biotic and abiotic stress factors, including pathogen attack, wounding, and exposure to heat or chemicals (Jan et al., 2021; Rana & Chahal 2023). Pre-treatment and autoclaving may have triggered the activation of the phenylpropanoid pathway in cassava, leading to the synthesis of phenolic compounds (Batista et al., 2021). Moreover, phenolic compounds can also be released from their bound forms, such as cell walls and complexes, by acid hydrolysis. The increase in phenol concentration observed in acid hydrolyzed solid-state fermented cassava may be attributed to the release of phenolic compounds bound in the cell walls and complexes, which became available for detection after hydrolysis (Verduzco-Oliva & Gutierrez-Uribe 2020; Gutierrez-Uribe & Verduzco-Oliva 2019).

Figure 2D depicts the results of the determination of total flavonoid concentration (TFC) for autoclaved and acid hydrolyzed solid-state fermented cassava treated with R. oligosporus. The pre-treated cassava displayed a significant increase ($p<0.05$) in flavonoid concentration compared to the control, while autoclaved cassava was not different from the control. Flavonoids are a group of natural compounds that have antioxidant and anti-inflammatory properties (Chen et al., 2019). They are synthesized by plants as a response to environmental stress, and they play a crucial role in the plant's defense mechanism (Kumar et al., 2020). The increase in TFC in pre-treated cassava samples could be due to the conversion of flavonoid precursors into active flavonoids by the action of R. oligosporus during solid-state fermentation (Egbune et al., 2021; Egbune et al., 2022a). Solid-state fermentation has been reported to enhance the concentration of flavonoids in various food materials due to the activation of the plant's defense system and the microbial conversion of precursors into active forms (Roasa et al., 2021). R. oligosporus is known to produce enzymes such as β-glucosidase and β-xylosidase, which can hydrolyze flavonoid glycosides into aglycones, resulting in an increase in TFC (De Villa et al., 2021). The significant increase in TFC in pre-treated cassava samples could be attributed to the synergistic effect of the plant and microbial enzymes, resulting in the production of a higher concentration of flavonoids (Barani et al., 2022).

Figure 3 presents the outcomes of the determination of amylase activity for autoclaved and acid hydrolyzed solid-state fermented cassava treated with R. oligosporus. The pre-treated unpeeled cassava samples exhibited a significant increase ($p<0.05$) in amylase activity compared to the control. Among the samples, solid-state fermented unpeeled cassava displayed the highest amylase activity at 14.7±0.9 µg/g/min. Amylase is an enzyme that hydrolyzes starch into simpler compounds such as maltose and glucose. The increase in amylase activity observed in the pre-treated unpeeled cassava samples treated with R. oligosporus could be attributed to the production of amylase by the fungus during the fermentation process. This is supported by previous studies that have shown that R. oligosporus produces various hydrolytic enzymes during solid-state fermentation, including amylase (Egbune et al., 2022; El Sheikha & Ray 2022). Furthermore, the highest amylase activity observed in the solid-state fermented unpeeled cassava sample could be due to the presence of more available substrates for the fungus to utilize during fermentation. This is because unpeeled cassava contains more starch than peeled cassava (Sukara et al., 2020).

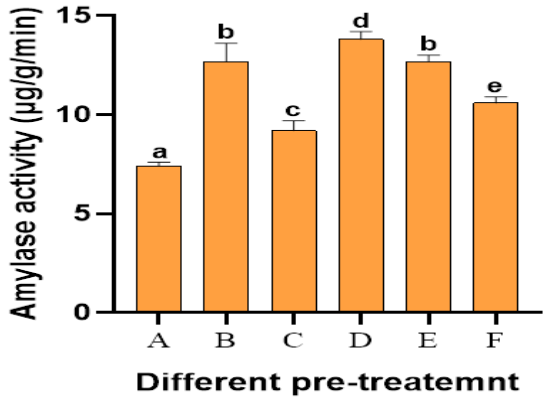

Figure 3. Amylase activity at different pre-treatments of unpeeled cassava. The values marked with superscripted letter a, b, c, d indicate significant differences from the control ($p < 0.05$). A) Unfermented unpeeled cassava roots, B) SSF unpeeled cassava roots, C) Autoclaved unpeeled cassava roots, D) Autoclaved SSF unpeeled cassava roots, E) Autoclaved unpeeled cassava roots with 0.1N sulphuric acid, and F) Autoclaved SSF unpeeled cassava roots with 0.1N sulphuric acid.

Biochemical parameters of pre-treatments of unpeeled cassava roots in submerged fermentation with *S cerevisiae* for alcohol production

The significant increase ($p<0.05$) in soluble protein concentration of pre-treated unpeeled cassava roots in submerged fermentation with *S cerevisiae* for alcohol production, as shown in figure 4A, suggests that the pre-treatment process likely caused the breakdown of complex proteins into smaller peptides and amino acids. These simpler forms of protein are more easily utilized by the yeast cells during fermentation, leading to an increase in soluble protein concentration. Additionally, solid-state fermentation may have contributed to the higher soluble protein concentration due to the production of proteolytic enzymes by the microorganisms involved in the fermentation process. Such enzymes are known to break down complex proteins into simpler forms that can be absorbed and utilized by yeast cells (Lübeck et al., 2022; Toy et al., 2022). The pre-treatment process may have also increased the availability of nitrogen, which is crucial for yeast growth and alcohol production. As nitrogen is a major component of proteins and amino acids, the breakdown of complex proteins into simpler forms may have increased the amount of available nitrogen in the cassava roots (Ziero et al., 2020). The addition of nitrogen sources, such as proteins and amino acids, is known to enhance the growth and alcohol production of *S cerevisiae* during fermentation (Gobert et al., 2019; Liu et al., 2021). Thus, the higher soluble protein concentration in the pre-treated unpeeled cassava roots may have contributed to the increased alcohol production during fermentation.

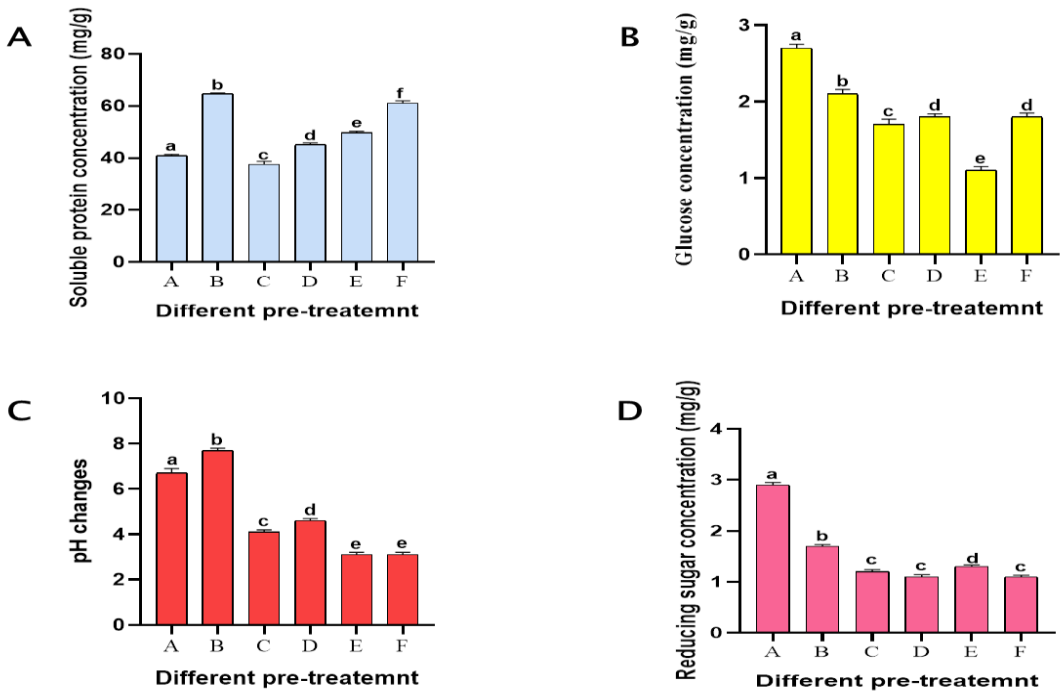

Figure 4. (A) Levels of soluble proteins, (B) Glucose concentration, (C) Effect of pH and (D) Reducing sugar concentration at different pre-treatments of unpeeled cassava roots in submerged fermentation with *S cerevisiae* for alcohol production. The values marked with superscripted letter a, b, c, d indicate significant differences from the control ($p < 0.05$). A) Unfermented unpeeled cassava roots, B) SSF unpeeled cassava roots, C) Autoclaved unpeeled cassava roots, D) Autoclaved SSF unpeeled cassava roots, E) Autoclaved unpeeled cassava roots with 0.1N sulphuric acid, and F) Autoclaved SSF unpeeled cassava roots with 0.1N sulphuric acid.

The results presented in figure 4B indicate a significant reduction ($p<0.05$) in glucose concentration of the pre-treated unpeeled cassava roots in submerged fermentation using *S cerevisiae* for alcohol production compared to the control. The observed decrease in glucose concentration can be attributed to two possible mechanisms. Firstly, the yeast cells utilize glucose during fermentation as it is converted into ethanol and carbon dioxide, thereby leading to a reduction in glucose concentration (Wikandari et al., 2019; Dongdong et al., 2023). Secondly, the pre-treatment process may have broken down complex carbohydrates, such as starch, into simpler forms, including glucose, which were readily utilized by the yeast during fermentation. This glucose utilization may have contributed to the decrease in glucose concentration observed in the pre-treated samples (Kim et al., 2019).

The changes in pH values of pre-treated unpeeled cassava roots in submerged fermentation with *S cerevisiae* for alcohol production, compared to the control, are shown in Figure 4C. Fermentation involves the metabolism of sugars by yeast, resulting in the production of organic acids, such as acetic and lactic acid, which can cause a decrease in pH (Mendes & Mendes-Faia

2020; Edema-Eyen et al., 2023). The observed changes in pH of the pre-treated unpeeled cassava roots may be due to the production of these organic acids during fermentation. Furthermore, the pre-treatment may have influenced the initial composition of the substrate, such as levels of buffering compounds and organic acids, which can also affect the pH (Cardoso Ribeiro et al., 2022). For instance, pre-treatment with citric acid has been shown to lower the pH in cassava-based substrates due to the presence of the acidic citric acid (Awasthi et al., 2022). Overall, the changes in pH observed in the pre-treated unpeeled cassava roots in submerged fermentation using *S cerevisiae* for alcohol production compared to the control are likely due to the production of organic acids during fermentation and the initial composition of the substrate.

Figure 4D presents the analysis of reducing sugar concentration in unpeeled cassava roots that underwent different pre-treatments and were subjected to submerged fermentation using *S cerevisiae* for alcohol production, compared to the control. The results showed a significant reduction ($p<0.05$) in reducing sugar concentration in the pre-treated samples compared to the control. The observed decrease in reducing sugar concentration in the pre-treated unpeeled cassava roots in submerged fermentation using *S cerevisiae* for alcohol production may be attributed to the utilization of glucose by the yeast during fermentation. Glucose is a primary carbon source for energy production and alcohol fermentation by yeast (Gonçalves et al., 2019; Zazulya et al., 2020). Additionally, pre-treatment may have caused the breakdown of other sugars, such as fructose and sucrose, which are present in cassava roots, into simpler forms like glucose and other monosaccharides through enzymatic hydrolysis (Zhang 2019; Ghazali & Razak 2021). Moreover, the pre-treatment may have caused the breakdown of non-sugar compounds such as amino acids and proteins, which could have contributed to the reducing sugar concentration in the control (Shi et al., 2021). Overall, the observed decrease in reducing sugar concentration in the pre-treated unpeeled cassava roots in submerged fermentation using *S cerevisiae* for alcohol production can be attributed to the utilization of glucose by the yeast during fermentation, enzymatic breakdown of other sugars, and breakdown of non-sugar compounds during pre-treatment.

The study presented in Figure 5A investigated the effect of pre-treatment methods on the free radical scavenging activity of DPPH in unpeeled cassava roots during submerged fermentation with *S cerevisiae* for alcohol production. The results showed that solid-state fermented cassava roots in submerged fermentation had higher free radical scavenging activity compared to the control, indicating the production of bioactive compounds with antioxidant activity during fermentation. However, all other pre-treatment methods resulted in a significant reduction in free radical scavenging activity, possibly due to the degradation of bioactive compounds present in the cassava roots during pre-treatment. The study highlights the crucial role of pre-treatment methods in determining the antioxidant capacity of fermented food products, and further research is needed to identify the specific bioactive compounds responsible for the observed effects.

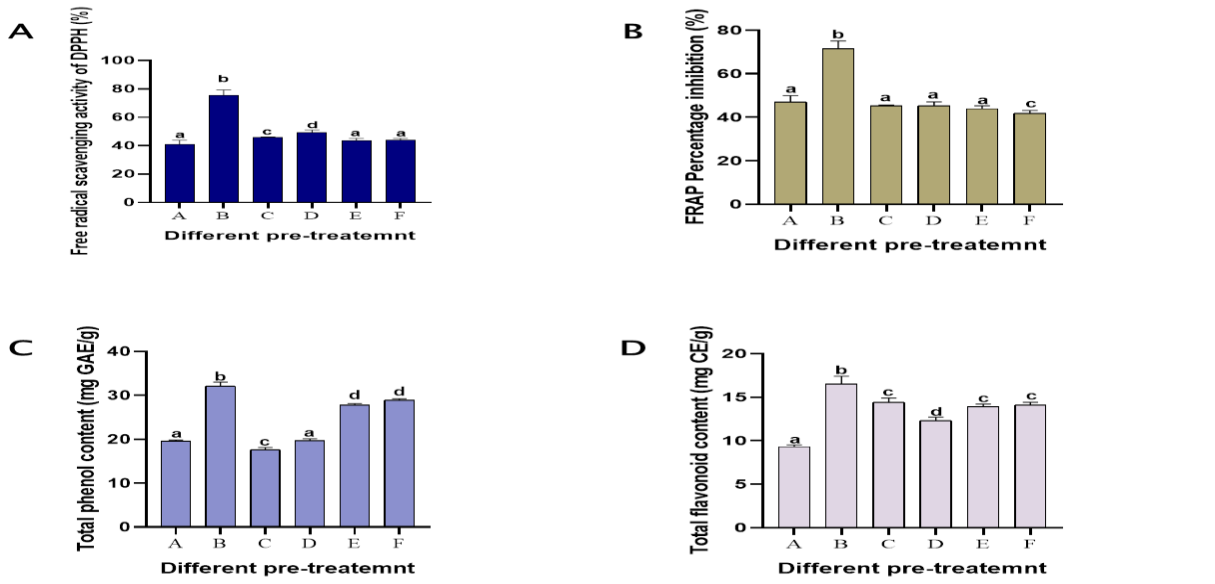

Figure 5. (A) Free radical scavenging activity of DPPH (%), (B) FRAP percentage inhibition (%), (C) Total phenolic content (TPC) and (D) Total flavonoid content (TFC) at different pre-treatments of unpeeled cassava roots in submerged fermentation with *S cerevisiae* for alcohol production. The values marked with superscripted letter a, b, c, d indicate significant differences from the control ($p < 0.05$). A) Unfermented unpeeled cassava roots, B) SSF unpeeled cassava roots, C) Autoclaved unpeeled cassava roots, D) Autoclaved SSF unpeeled cassava roots, E) Autoclaved unpeeled cassava roots with 0.1N sulphuric acid, and F) Autoclaved SSF unpeeled cassava roots with 0.1N sulphuric acid.

Submerged fermentation with *S cerevisiae* for alcohol production is a process that involves the degradation of carbohydrates and other organic compounds present in the substrate, which may lead to the production of reactive oxygen species (ROS) and free radicals. These molecules can cause oxidative stress and damage cellular components, leading to cell death. Pre-treatment of cassava roots before fermentation can significantly affect the antioxidant capacity of the resulting product. The study found that solid-state fermented cassava roots in submerged fermentation had higher free radical scavenging activity, suggesting the formation of bioactive compounds during fermentation. This result is consistent with previous studies reporting an increase in antioxidant capacity in fermented food products due to the formation of bioactive compounds during fermentation (Ziemlewska et al., 2021; Suarti & Budijanto 2021; Ndego et al., 2023). In contrast, all other pre-treatment methods led to a significant reduction in free radical scavenging activity, likely due to the degradation of bioactive compounds during pre-treatment. Studies have reported similar results in the loss of phenolic compounds and antioxidant capacity in fruits and vegetables after peeling and/or blanching (Rickman et al., 2007; Rojas-Bravo et al., 2019).

Figure 5B shows the results of a study on the percentage inhibition activity of ferric reducing ability of plasma (FRAP) in pre-treated unpeeled cassava roots during

submerged fermentation with *S cerevisiae* for alcohol production. The study found a significant increase ($p<0.05$) in percentage inhibition capacity in solid-state fermented cassava roots compared to the control, suggesting the production of antioxidant compounds during fermentation. However, all other pre-treatment methods resulted in a significant reduction in percentage inhibition capacity, indicating the degradation of bioactive compounds in the cassava roots during pre-treatment. One possible explanation for these effects is the production of phenolic compounds during fermentation, which are known to have antioxidant properties (Degrain et al., 2020). Phenolic compounds are produced during fermentation through the action of enzymes such as polyphenol oxidase and peroxidase on phenolic precursors present in the cassava roots (Selo et al., 2021; Aganbi et al., 2023). Solid-state fermentation has been shown to enhance the production of phenolic compounds in cassava roots (Ojo et al., 2022), which may explain the increase in antioxidant activity observed in solid-state fermented cassava roots in submerged fermentation.

In contrast, pre-treatment methods such as peeling and slicing can lead to the loss of phenolic compounds due to oxidation and enzymatic degradation (Alegria et al., 2022), resulting in decreased antioxidant activity. Other pre-treatment methods such as blanching and boiling can also result in the loss of bioactive compounds due to leaching (Martínez et al., 2020). In summary, the results suggest that the choice of pre-treatment method can significantly impact the antioxidant activity of the resulting fermented cassava product.

The phenylpropanoid pathway is responsible for the synthesis of flavonoids, a group of natural compounds with antioxidant and anti-inflammatory properties that play a role in plant defense mechanisms. During the submerged fermentation of pre-treated unpeeled cassava roots with *S cerevisiae* for alcohol production, there was a significant increase ($p<0.05$) in the total flavonoid concentration (TFC) compared to the control, as shown in Figure 5D. This increase in TFC could be attributed to the activation of enzymes involved in the flavonoid biosynthetic pathway, which occurs during the fermentation process. This could be a response to the oxidative stress generated during fermentation.

Studies have shown that flavonoid concentration in plants can increase in response to various stresses such as drought, high salinity, and oxidative stress (Hossain et al., 2019). Similarly, the increase in TFC observed in pre-treated unpeeled cassava roots during submerged fermentation could be due to stress responses. Furthermore, previous research has reported an increase in flavonoid concentration during fermentation of other plant materials, such as tea leaves (Guo et al., 2020) and cocoa beans (Ooi et al., 2020). This increase has been attributed to the breakdown of larger flavonoid molecules into smaller, more bioavailable ones during fermentation, as well as the formation of new flavonoid derivatives. Thus, the observed increase in TFC during the fermentation of pre-treated unpeeled cassava roots could be due to the activation of flavonoid biosynthetic enzymes and the breakdown of larger flavonoid molecules into smaller, more bioavailable ones.

The study in Figure 5C analyzed the total phenol concentration (TPC) of pre-treated unpeeled cassava roots during submerged fermentation with *S cerevisiae* for alcohol production, and revealed a significant increase ($p<0.05$) compared to the control. Phenolic compounds are secondary metabolites present in plants that possess several biological activities, such as

antioxidant, anti-inflammatory, and anticancer effects (Tanase et al., 2019). The increase in TPC observed in the study suggests that the fermentation process led to the release and accumulation of phenolic compounds. The activity of enzymes, such as β-glucosidase, during fermentation can lead to the release of bound phenolic compounds in the plant matrix (Sousa et al., 2022).. Additionally, the degradation of polysaccharides in the cassava root by the yeast may result in the release of phenolic compounds that were previously trapped in the cell walls (Verduzco-Oliva & Gutierrez-Uribe 2020). Furthermore, the yeast may synthesize new phenolic compounds during fermentation, contributing to the increase in TPC (Leonard et al., 2021). The increase in TPC is consistent with previous studies that have reported an increase in phenolic content in fermented foods, which can enhance their antioxidant and health-promoting properties (Adebo & Gabriela 2020). Overall, the study suggests that the fermentation process can enhance the release and accumulation of phenolic compounds in cassava roots, contributing to their potential health benefits.

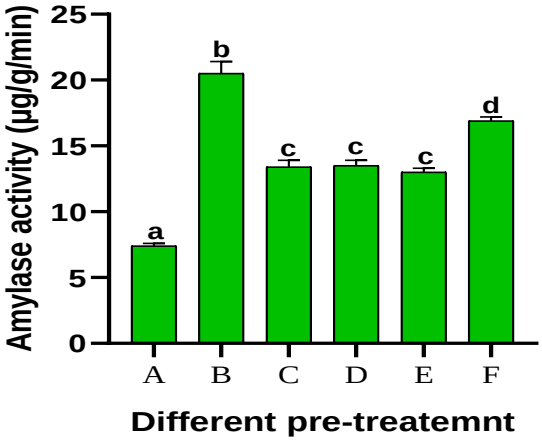

Figure 6. Amylase activity at different pre-treatments of unpeeled cassava roots in submerged fermentation with *S cerevisiae* for alcohol production. The values marked with superscripted letter a, b, c, d indicate significant differences from the control ($p < 0.05$). A) Unfermented unpeeled cassava roots, B) SSF unpeeled cassava roots, C) Autoclaved unpeeled cassava roots, D) Autoclaved SSF unpeeled cassava roots, E) Autoclaved unpeeled cassava roots with 0.1N sulphuric acid, and F) Autoclaved SSF unpeeled cassava roots with 0.1N sulphuric acid.

The enzyme amylase plays a critical role in the breakdown of starch into smaller molecules, including glucose and maltodextrin, during the fermentation process. This breakdown generates fermentable sugars that yeast can convert into ethanol. The results presented in Figure 6 demonstrate that pre-treated unpeeled

cassava roots in submerged fermentation with *S cerevisiae* had a significantly higher amylase activity compared to the control. This finding implies that pre-treatment methods can potentially enhance amylase production, leading to an increase in the availability of fermentable sugars.

One plausible explanation for the increased amylase activity is the activation of endogenous enzymes in cassava roots during pre-treatment. Studies have shown that soaking cassava roots in water before processing can activate endogenous enzymes such as amylase, which results in an increased availability of fermentable sugars (Wang et al., 2022). Another possible explanation is the production of exogenous amylase by microorganisms during fermentation. Certain strains of *S cerevisiae* have been reported to produce amylase and other hydrolytic enzymes during fermentation (Cripwell et al., 2019).

Table 1. Physiochemical changes pre-treated unpeeled cassava roots in submerged fermentation with *S. cerevisiae* for alcohol production for 96 hours

Various pre-treatments	Total soluble solids (Initial)	Total soluble solids (24 hours)	Total soluble solids (48 hours)	Total soluble solids (72hours)	Total soluble solids (96 hours)	Specific gravity (Initial)	Specific gravity (24 hours)	Specific gravity (48 hours)	Specific gravity (72 hours)	Specific gravity (96 hours)
A	2.10	1.20	1.10	1.20	1.20	1.15	1.03	1.01	0.99	1.02
B	2.40	1.70	1.60	1.40	1.20	1.25	1.04	0.98	0.87	0.99
C	1.90	1.10	1.10	1.10	1.10	1.06	1.03	1.02	1.02	1.02
D	1.90	1.20	1.20	1.20	1.20	1.03	1.02	1.02	1.02	1.02
E	1.10	1.30	1.20	1.20	1.20	1.04	1.03	1.03	1.03	1.03
F	1.60	1.10	1.10	1.10	1.10	1.05	1.03	1.03	1.03	1.03

A) Unfermented unpeeled cassava roots, B) SSF unpeeled cassava roots, C) Autoclaved unpeeled cassava roots, D) Autoclaved SSF unpeeled cassava roots, E) Autoclaved unpeeled cassava roots with 0.1N sulphuric acid, and F) Autoclaved SSF unpeeled cassava roots with 0.1N sulphuric acid.

In Table 1, the results demonstrate a significant decrease in the total soluble solids after 96 hours of submerged fermentation of pre-treated unpeeled cassava roots with S. cerevisiae for alcohol production. Furthermore, the specific gravity of the samples declined after 72 hours of fermentation, however, it increased again at the 96-hour mark. Notably, the highest percentage of alcohol was detected in SSF Cassava after 72 hours, while the alcohol production reduced at the 96-hour stage Figure 7.

The decrease in total soluble solids observed in the fermented cassava roots can be attributed to the consumption of sugars by yeast cells during the process of alcoholic fermentation (Madaleno et al., 2020; Gantumur et al., 2022; Jackson et al., 2023). The yeast cells utilize the sugars such as glucose present in the cassava roots as a carbon source for energy and convert them into ethanol and carbon dioxide through the process of alcoholic fermentation (Tse et al., 2021). The decrease in total soluble solids is therefore an indication of the consumption of the sugars present in the cassava roots.

The change in specific gravity observed in Table 1 can be explained by the changes in the composition of the fermentation broth during the fermentation process. The decrease in specific gravity after 72 hours of fermentation can be attributed to the production of carbon dioxide gas, which is less dense than the solution (Sani et al., 2021). The increase in specific gravity at the 96-hour mark can be explained by the accumulation of ethanol, which is denser than water (Hedgpeth et al., 2021).

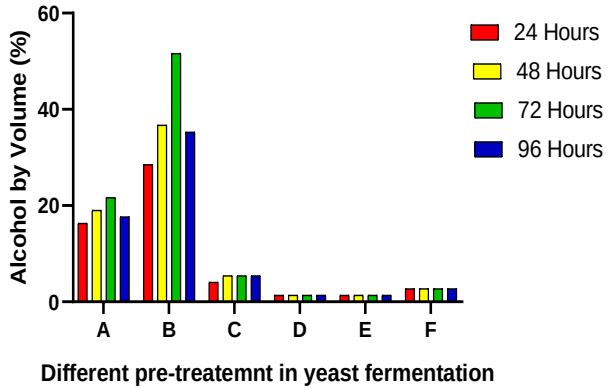

Figure 7. Alcohol percentage by volume at different pre-treatments of unpeeled cassava roots in submerged fermentation with *S cerevisiae* for alcohol production. A) Unfermented unpeeled cassava roots, B) SSF unpeeled cassava roots, C) Autoclaved unpeeled cassava roots, D) Autoclaved SSF unpeeled cassava roots, E) Autoclaved unpeeled cassava roots with 0.1N sulphuric acid, and F) Autoclaved SSF unpeeled cassava roots with 0.1N sulphuric acid.

The highest percentage of alcohol detected in SSF Cassava after 72 hours is consistent with the typical pattern of alcoholic fermentation, where peak alcohol production occurs in the early stages of fermentation, after which it gradually decreases (Coelho et al., 2020). This decrease in alcohol production may be attributed to the

inhibitory effects of ethanol on the yeast cells, which reduces their ability to continue fermenting sugars into ethanol (Wikandari et al., 2019). The improvement in alcohol percentage in the SSF fermented unpeeled cassava in yeast fermentation suggests that the pre-treatment process may have enhanced the availability of fermentable sugars or improved the overall fermentation conditions for *S. cerevisiae*. The observed reduction in alcohol production in the pre-treated unpeeled cassava roots may be due to the loss of fermentable sugars during pre-treatment or inhibition of yeast growth and fermentation by the pre-treatment process (Oliva et al., 2022).

Conclusion

The findings of this study demonstrate that the pre-treatment methods used had a positive impact on the nutritional and antioxidant properties of unpeeled cassava roots. The results showed that the amylase activity of pre-treated unpeeled cassava roots increased during submerged fermentation with *Saccharomyces cerevisiae* for alcohol production. This suggests that the pre-treatment of cassava roots can enhance the production of amylase, leading to an increase in the availability of fermentable sugars for the yeast cells to convert into ethanol.

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Ethics declarations

Conflict of interest

The authors have no conflicts of interest to declare.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Contributions

JOO and UDE contributed to data collection, methodology, and writing; OCO contributed to draft preparation and software management; TE curated the data, EOE contributed to reviewing and editing, NJT and AAA contributed to conceptualization, visualization, and investigation.

Data availability

Data will be made available on request

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