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Effect of Solid-state Fermentation on Nutritional Value of Pineapple Leaves

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ABSTRACT

Pineapple leaves, a by-product of cultivation, are generally discarded and burned at the farm sites before replanting. As the demand for pineapple products increases yearly, the number of discarded leaves also increases. Valorisation of the leaves through solid-state fermentation (SSF) is a sustainable waste management approach that converts the low-nutrient substrate into valuable resources for animal feed. This study investigated the enrichment of pineapple leaf nutritional values using SSF. The process parameters such as fermentation time, inoculum type and size, additional carbon source and particle size were optimised using the one-factor-at-a-time (OFAT) method. The nutrient compositions were analysed for their total protein, total phenolic, antioxidant activity, reducing sugar and crude fibre using Bradford, Folin–Ciocalteu, DPPH (2,2-diphenyl-1-picrylhydrazyl), DNS (3,5-dinitrosalicylic acid) and AOAC978.10 methods, respectively. The optimal conditions were determined to be a 2 mm leaf particle size, 2% (w/w) *Rhizopus* sp. inoculum, and a fermentation duration of 2 days without the addition of a carbon source. Under these conditions, the nutrient enrichment resulted in a total protein content of 24.14 ± 0.31 mg/g, total phenolics of 11.81 ± 0.50 mg/g, antioxidant activity of 3.17 ± 0.04 mg/g, and a reducing sugar content of 12.03 ± 0.97 mg/g. Crude fibre content decreased from 20.37 ± 1.10% in unfermented leaves to 6.77 ± 0.44% after fermentation, potentially improving nutrient digestibility due to the reduction of indigestible fibre. These results demonstrate that SSF is a promising method to enhance the nutrient content of pineapple leaves, offering an alternative nutrient source for animal feed.

Keywords: Antioxidants, nutrient content, phenolics, pineapple leaves, solid-state fermentation

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INTRODUCTION

Malaysia is one of the pineapple-producing countries, with a total production of 289,763 metric tons from a planting area of 10,567 hectares in 2021. Production increased by 16% compared to 2020, contributing to the country's economic growth alongside other main products such as palm oil and rubber (Malaysian Pineapple Industry Board [MPIB], 2022). Along with this economic growth, the pineapple industry generates a high amount of waste every year due to the high demand for pineapple products. Moreover, the increasing tons of waste is due to the short growth and production cycle of pineapples (de Aquino Gondim et al., 2023). It contributes 45%–65% of the pineapple mass, including leaves, stems, peels, crowns and cores (Difonzo et al., 2019). Usually, the leaf waste is managed through disposal methods like open burning or natural decomposition at the farm sites. These practices raise environmental concerns, such as emissions of greenhouse gases (carbon dioxide and methane) and soil erosion, highlighting the need for a more sustainable waste management solution (Aili Hamzah et al., 2021).

Numerous studies have reported the chemical compositions of pineapple waste, including its nutritional content, volatile compounds and bioactive compounds (Baidhe et al., 2021; Polanía et al., 2023; Roda & Lambri, 2019). In particular, the nutrient content of pineapple leaves contains beneficial nutrients such as total sugar, crude fibre, crude protein, organic acids, phenolic content and antioxidants (Arampath & Dekker, 2019; Awasthi et al., 2022; Nath et al., 2023). However, despite the availability of these nutrient content, studies have reported that the leaves still contain high indigestible fibre and low protein content (Sukri et al., 2023). Current research has focused on utilising pineapple leaves for the production of bio-composite (Tripathi et al., 2022) and biofuel (Chintagunta et al., 2017; Mund et al., 2022).

Pineapple leaves should be converted using innovative approaches to increase their nutritional value, like protein before being utilised as animal feed for a sustainable agriculture system. There is an increasing demand for feed protein in animal production. Therefore, it is essential to explore alternative protein sources other than soybean meal (Kim et al., 2019). There is also a growing interest in the application of solid-state fermentation (SSF) using microbes such as fungi and yeast to improve the protein content of low-nutrient agricultural waste such as peels and pulps for animal feed production (Wang et al., 2023). Besides, SSF offers uncomplicated product recovery, allowing the resulting product to be directly utilised as animal feed (Yafetto et al., 2023). In other words, SSF can improve the quality of agricultural waste while effectively sustainably managing solid waste (Yazid et al., 2017). Therefore, SSF was used in this work to improve the nutrient content in pineapple leaves. The effects of different SSF parameters, including fermentation time, particle size, carbon source and inoculum type on the total protein, total phenolics, antioxidants, reducing sugar and crude fibre, were investigated.

MATERIALS AND METHODS

Materials

Bradford reagent, magnesium sulphate, potassium dihydrogen phosphate, sulfuric acid and hydrochloric acid were obtained from Sigma-Aldrich (St. Louis, USA). Folin-Ciocalteu reagent was purchased from Chemiz (Selangor, Malaysia). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), bovine serum albumin (BSA), dinitrosalisylic acid (DNS) and potassium sodium tartrate were purchased from Macklin (Shanghai, China). Glucose, sodium hydroxide, gallic acid, ascorbic acid, and ethanol were procured from HmbG Chemicals (Hamburg, Germany). Palm oil decanter cake was collected at Banting, Selangor. Effective microorganisms (EM4) and *Rhizopus* sp. were bought from BH Farm (Perak, Malaysia) and Raprima (Bandung, Indonesia), respectively. Longan syrup was obtained from KBK Greenleaf Enterprise (Kedah, Malaysia).

Preparation of Pineapple Leaves Powder

Pineapple leaves were collected at Sweetpine Pineapple farm in Kuantan, Pahang. They were dried under sunlight for three days. After that, they were cut into smaller sizes and dried in an oven at 70°C for 48 h. The dried leaves were ground before sieving through a 4 mm Retsch sieve.

Solid-state Fermentation

Experiments were carried out using the one-factor-at-a-time (OFAT) method to determine the optimum nutrient content in the fermented pineapple leaves. In the SSF, 3% (w/w) of *Rhizopus* sp. was added in a mixture containing 50 g of 4-mm leaves sample and 3% (w/w) of spent coffee ground as a nitrogen source. Subsequently, 45 ml of trace elements consisting of 1 g/L magnesium sulphate, 2 g/L potassium dihydrogen phosphate and 0.4 N hydrochloric acid were added to the mixture (Kupski et al., 2012). Then, 20% (w/v) of the bulking agent was mixed with the mixture before being incubated at 30°C for 3 days. The procedure was repeated at different fermentation parameters such as fermentation time (1–5 days), substrate particle size (2–20 mm), inoculum type (effective microorganism 4, palm decanter cake), inoculum size (1%–5% (w/w)), carbon source (sucrose, molasses, longan syrup) and carbon composition (2.5%–10% (w/w)).

Extraction of Nutrient Content

The nutrient content in unfermented and fermented pineapple leaves was analysed using 5 g of sample extracted with 50 ml of 75% ethanol. The extraction process was carried out through incubation in an ultrasonic water bath at 40°C for 1 h (Azizan et al., 2020). After that, the sample was filtered using Whatman No. 1 paper, and the filtrate was used for further analysis.

Determination of Total Protein Content

The Bradford method determined the protein content (Setti et al., 2020). Briefly, 1.5 ml of Bradford reagent was added to 0.05 ml of the sample in a cuvette. The absorbance of the mixture was measured at a wavelength of 595 nm using a UV-Vis spectrophotometer (Thermo Scientific AquaMate 7100, USA). Bovine serum albumin (BSA) concentrations (0–0.14 mg/ml) were used as the standard reference.

Determination of Total Phenolic Content

The total phenolic content (TPC) was determined using the Folin–Ciocalteu method (Azkia et al., 2023). Briefly, 0.5 ml of the sample was added to 1 ml of 10% Folin-Ciocalteu solution, followed by 1 ml of 10% sodium carbonate solution. The mixture was left in the dark at room temperature for 2 h. The absorbance of the mixture was measured at 760 nm using a UV-Vis spectrophotometer (Thermo Scientific AquaMate 7100, USA). Gallic acid concentrations (0–0.2 mg/ml) were the standard reference. The TPC in the sample was expressed as gallic acid equivalents (GAE) in mg/g.

Determination of Reducing Sugar

Reducing sugar was determined using the dinitrosalisylic acid (DNS) method (Anigboro et al., 2023). Briefly, 3 ml of the sample was added to 3 ml of 1 L solution of DNS reagent containing 10 g DNS, 2 g phenol, 0.5 g sodium sulphite and 10 g sodium hydroxide. The mixture was heated in a boiling water bath for 10 min. After that, 1 ml of a 40% potassium sodium tartrate was added to stabilise the colour developed and allowed to cool at room temperature. The absorbance of the mixture was measured at 575 nm using a UV-Vis spectrophotometer (Thermo Scientific AquaMate 7100, USA). Glucose concentrations (0–2 mg/ml) were used as the standard reference.

Determination of Antioxidant Content

The antioxidant content was determined using a 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay (Buenrostro-Figueroa et al., 2017). Briefly, 100 ml of the sample was added to 3 ml of 0.55 mM DPPH solution. The mixture was left in the dark for 30 min before measuring the absorbance at 517 nm using a UV-Vis spectrophotometer (Thermo Scientific AquaMate 7100, USA). Ascorbic acid concentrations (0-0.15 mg/ml) were the standard reference. The antioxidant content was expressed in ascorbic acid equivalents in mg/g.

Determination of Crude Fibre

The crude fibre was estimated using the Association of Official Analytical Chemists (AOAC 978.10). Briefly, 1 g of sample was boiled in 1.25% (v/v) sulphuric acid with n-octanol

for 30 min. The solution was filtered, and the residue was washed four times with boiling water. After that, the residue was boiled in 1.25% potassium hydroxide for another 30 min. The solution was filtered again, and the residue was washed four times with boiling water. The residue was transferred to a crucible, dried in an oven at 130°C for 2 h, cooled in a desiccator, and weighed. The crucible containing the ash was dried in a muffle furnace at 525°C for 3 h in a desiccator before reweighing to determine the crude fibre content using Equation 1 (Rivera et al., 2023).

$$\text{Crude fibre (\%)} = \frac{\text{Weight of crucible with fibre - Weight of crucible with ash}}{\text{Weight of sample}} \times 100\% \quad [1]$$

Statistical Analysis

The results were expressed as mean ± standard deviation (SD) from duplicate experiments using Origin 2022 software. For group comparisons, one-way Analysis of variance (ANOVA) was performed using Microsoft Excel 2016 with statistical significance set at $p<0.05$.

RESULTS AND DISCUSSION

The Nutrient Content of Unfermented Pineapple Leaves

Table 1 shows the nutrient content of raw pineapple leaves before fermentation, which served as the control throughout this study. The contents of protein, phenolics, antioxidants and reducing sugar in the unfermented pineapple leaves were 5.30 ± 0.51 mg/g, 9.93 ± 0.17 mg/g, 0.29 ± 0.10 mg/g and 6.67 ± 0.67 mg/g, respectively. The leaves also contained high crude fibre of 20.37%. Previous studies have also demonstrated the low quality of unfermented pineapple waste. Pineapple pulp waste has 0.1 mg/g of total protein and 0.27 mg/g of antioxidants (Hemalatha & Anbuselvi, 2013). The waste mixture containing pulp, peel core and crown has a low phenolic content of 1.12 mg/g (Rashad et al., 2015). The peels and leaves have crude fibre of 13.96%–14.72% (Aruna, 2019; Rivera et al., 2023) and 31% (Zainuddin et al., 2014), respectively. Based on the unfermented results, the use of pineapple waste as animal feed has limitations due to its low levels of beneficial nutrients.

Table 1
Nutrient content of unfermented pineapple leaves (raw)

Nutrient	Content
Total protein (mg/g)	5.30 ± 0.51
Total phenolic (mg/g)	9.93 ± 0.17
Antioxidant activity (mg/g)	0.29 ± 0.10
Reducing sugar (mg/g)	6.67 ± 0.67
Crude fibre (%)	20.37 ± 1.10

Effect of Fermentation Time on the Nutrient Content

Fermentation time is one of the important process variables for accessing protein enrichment in the SSF method. In this study, the highest protein content (13.96 ± 0.43 mg/g) was achieved on day 2, monitored over 5 days of fermentation time, as shown in Figure 1. It represents a 2.6-fold increase compared to unfermented pineapple leaves (5.30 ± 0.51 mg/g). Previous studies have demonstrated that extending fermentation time from 0 to 3 days can maximise protein content up to 2.8-fold in pineapple waste containing peel and core using *Aspergillus niger* and *Trichoderma viride* (Omwango et al., 2013). In another study, the total protein content increased from 30.28 ± 3.52 mg/g to 44.08 ± 4.17 mg/g after 5 days of fermenting brewery spent grain with *Bacillus velezensis* (Zeng et al., 2021). Similarly, the total protein content of dehusked barley increased from 4.76 ± 0.20 mg/g (unfermented) to 9.50 ± 0.16 mg/g after 36 h of co-fermentation with *Rhizopus oryzae* and *Lactobacillus plantarum* (Wang et al., 2020). Typically, long fermentation could yield

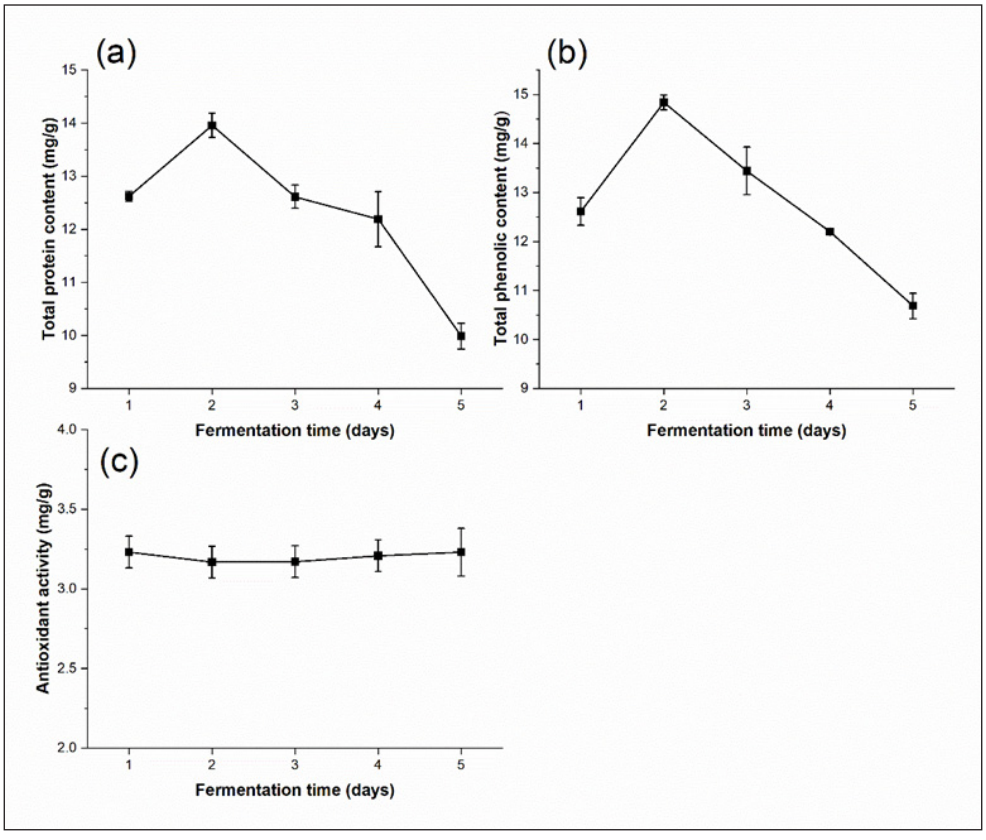

Figure 1. Effect of fermentation time on the nutrient content of fermented pineapple leaves. (a) Total protein content, (b) total phenolic content and (c) antioxidant activity. The value was expressed as the mean ± SD of duplicate experiments

optimal protein content due to fungal growth that secreted more extracellular enzymes during metabolism. However, a prolonged fermentation period may reach a plateau or even decline due to depletion of nutrients, changes in the microbial community or the accumulation of inhibitory by-products (Olorunnisola et al., 2018).

Furthermore, fungal treatment through SSF releases bioactive compounds such as phenolics and antioxidants. This enhancement was due to the degradation of lignocellulosic components, which breaks open phenol rings trapped within the lignin fraction, releasing phenolic compounds with antioxidant activity (Buenrostro-Figueroa et al., 2017). In their study investigating the effect of time, the maximal release of phenolics (4.77 mg/g) and antioxidants (0.53 mg/g) was reported at 36 h of fermentation with *A. niger*. A similar trend was observed in the present study, with total phenolic content increasing from 9.93 ± 0.17 mg/g (unfermented) to 14.84 ± 0.15 mg/g on day 2 of fermentation. Moreover, antioxidant activity on day 2 was 11-fold higher (3.17 ± 0.11 mg/g) compared to unfermented leaves (0.29 ± 0.10 mg/g). Extending the fermentation beyond day 2 did not result in a significant difference in antioxidant activity ($P>0.05$). Previous research has consistently shown that extending fermentation time enhances soluble protein, phenolic content, and antioxidant activity. For example, during SSF using ragi tape and black glutinous rice as substrates, total phenolic compounds increased from day 0 (4.65 mg/g) to day 3 (6.94 mg/g), leading to a 35.24% increase in antioxidant activity (Azkia et al., 2023). Similarly, co-fermentation with *Rhizopus oryzae* and *Lactobacillus plantarum* in dehusked barley from 0 to 36 h demonstrated an increase in soluble protein, phenolics, and antioxidants (Wang et al., 2020).

Effect of Substrate Particle Size on the Nutrient Content

Figure 2 shows the effect of pineapple leaves particle sizes on the nutrient content during SSF. The smallest particle sizes of 2 mm resulted in the highest enrichment of total protein (23.19 ± 0.52 mg/g) and total phenolics (12.61 ± 0.17 mg/g). In contrast, fermentation with the largest particle size led to only a 1.7-fold increase in total protein (9.03 ± 0.48 mg/g) compared to unfermented pineapple leaves (5.30 ± 0.51 mg/g). Similarly, there is no significant difference in antioxidant activity when using different particle sizes for fermentation ($p>0.05$). Smaller particle sizes enhance the surface area-to-volume ratio and improve the accessibility of the substrate to microorganisms, thereby promoting microbial activity and growth (Tosuner et al., 2019). Moreover, the smaller particle of the substrate sizes allows for better mass transfer between solid substrate and air surfaces, leading to a more efficient fermentation rate (Wang et al., 2023). Similarly, studies have shown that smaller particles favour higher nutrient enrichment, such as protein and phenolics in rice bran, whereas larger particles tend to generate more fungal biomass in SSF (Schmidt & Furlong, 2012).

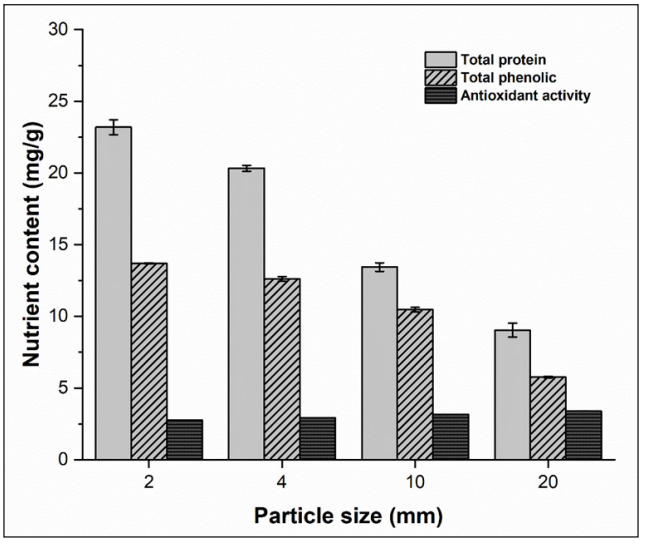

Figure 2. Effect of different particle sizes of substrate on the nutrient content of fermented pineapple leaves. The value was expressed as the mean ± SD of duplicate experiments

Effect of Inoculum Type and Size on the Nutrient Content

The impact of different types of inoculums for nutrient enrichment was evaluated using *Rhizopus* sp., effective microorganism (EM) and palm decanter cake. The results showed that *Rhizopus* sp. produced the highest protein content, followed by EM and decanter cake (Figure 3). The results suggest that all studied microbial inoculants have the potential to enhance protein content in the pineapple leaves. Additionally, raw decanter cake is rich in Indigenous microbes, making it an effective inoculum for promoting nutrient enrichment in the natural substrate (Kanchanasuta & Pisutpaisal, 2016). Besides, EM contains numerous species of microorganisms that can improve the quality of nutrients by utilising agricultural waste as a natural substrate (Hidalgo et al., 2022). The effect of inoculum size, ranging from 1% to 5% (w/w), was also studied to determine their effects on the nutrient content. However, no significant difference in the total protein content was observed despite the varying inoculum sizes within the studied range (p>0.05). The maximum protein content was achieved at 2% (w/w) of *Rhizopus* sp. with 24.14 ± 0.31 mg/g, 3% (w/w) of EM with 16.47 ± 1.19 mg/g and 1% (w/w) of decanter cake with 13.22 ± 0.65 mg/g, representing an increase of 4.5, 3.1 and 2.5 folds, respectively, compared to unfermented leaves. Several studies have shown the enrichment of low protein content in pineapple waste by SSF. The total protein content increased 4.2-fold in pineapple peels after 4 days of SSF using *Trichoderma viride* (Aruna, 2019). At the same time, a 3.5-fold increase in protein content was produced after 2 days of SSF of pineapple pulp by *Saccharomyces cerevisiae* (Correia et al., 2007).

Figure 3 also demonstrates the influence of inoculum type and size on phenolic and antioxidant content in pineapple leaves. All tested inoculum types were able to increase the bioactive compounds concerning the unfermented leaves. However, the inoculum size did not significantly impact the levels of these compounds (p>0.05). Moreover, EM inoculation created favourable conditions for greater phenolic release compared to *Rhizopus* sp. and decanter cake. A recent study has also reported increased phenolic and antioxidant contents in the pineapple peel fermented with *Rhizopus oryzae*, with phenolic content rising from 30 to 52.70 mg GAE/g and antioxidant activity increasing from 37.02% to 61.46% (Rivera et al., 2023). Additionally, SSF of pineapple peel using *A. niger* HT3 resulted in 1.5 times higher DPPH activity (60.28 ± 1.74%) compared to the unfermented peel (Casas-Rodríguez et al., 2024).

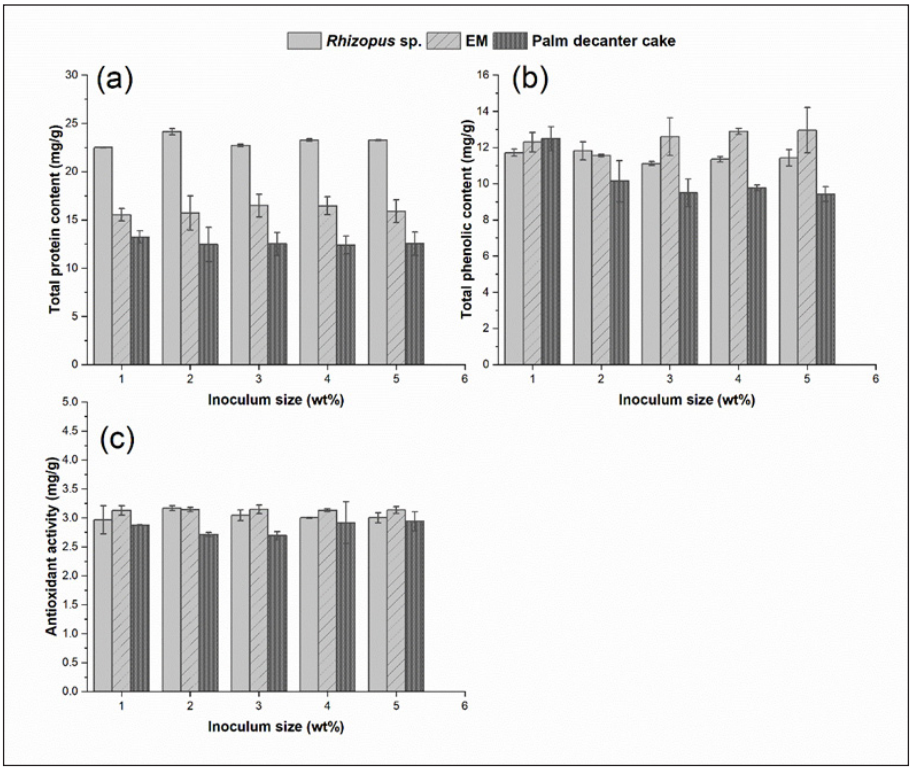

Figure 3. Effect of inoculum types and sizes on the nutrient content of fermented pineapple leaves. (a) Total protein content, (b) total phenolic content, and (c) antioxidant activity. The value was expressed as the mean ± SD of duplicate experiments

Effect of Carbon Source on the Nutrient Content

The impact of different carbon sources, including sucrose, molasses, and longan syrup, on nutrient enrichment in the SSF system using pineapple leaves was examined at varying

compositions (Figure 4). The highest yield of total protein content was achieved at 2.5% (w/w) sucrose with 24.41 ± 0.62 mg/g, followed by molasses (22.09 ± 1.91 mg/g) and longan syrup (17.17 ± 0.12 mg/g), both at 5% (w/w). The availability of sucrose serves to be a more efficient energy source for microorganism growth compared to longan syrup and molasses.

Molasses, a by-product of the sugar industry, is a suitable co-substrate due to its rich carbon content, which includes sucrose (30%–35%), fructose (10%–20%) and glucose (10%–25%) (Jamir et al., 2021). On the other hand, longan syrup contains lower levels of sucrose (14.21%), fructose (3.91%) and glucose (2.77%) (Surin et al., 2014). However, when comparing the SSF without the addition of any carbon source, the total protein content (24.14 ± 0.31 mg/g) showed no significant increase, suggesting that the native carbon sources present in pineapple leaves may be sufficient for microbial activity under certain conditions. The phenolics and antioxidants did not show positive effects in the SSF when additional carbon sources were introduced. Instead, the reduced sugar increased significantly as the carbon content increased.

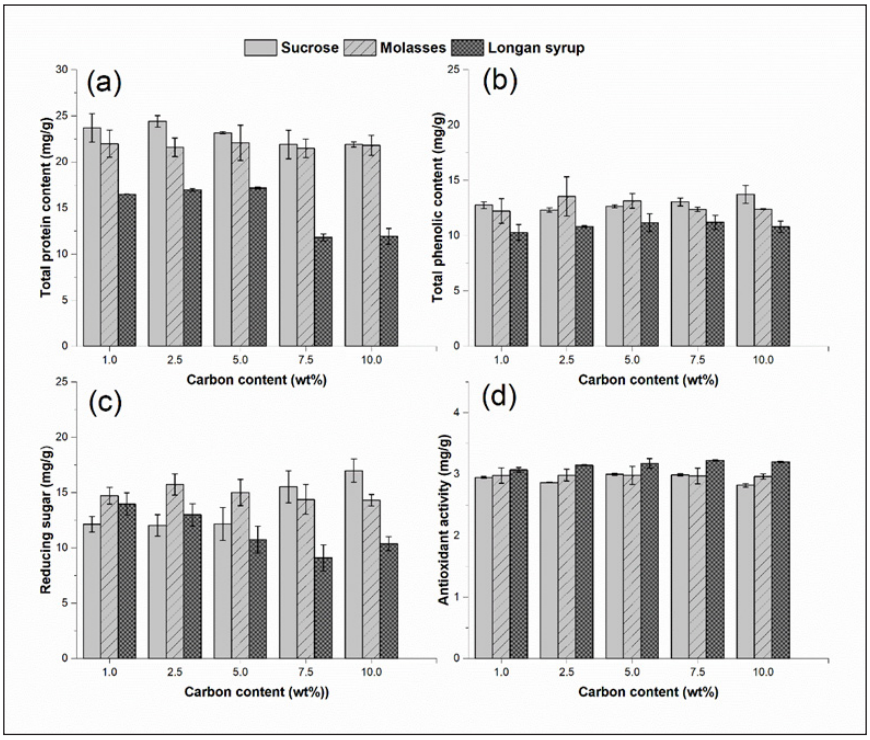

Figure 4. Effect of carbon types and size on the nutrient content of fermented pineapple leaves. (a) Total protein content, (b) total phenolic content, (c) reducing sugar, and (d) antioxidant activity. The value was expressed as the mean ± SD of duplicate experiments

CONCLUSION

The present study investigated the nutrient enrichment in pineapple leaves through solid-state fermentation. The optimal SSF conditions, determined using the OFAT method, were achieved to be 2 mm particle size of pineapple leaves, 2% (w/w) *Rhizopus* sp. inoculum, and a fermentation time of 2 days without the need for an additional carbon source. Under these conditions, the nutrient enrichment resulted in a total protein content of 24.14 ± 0.31 mg/g, total phenolic of 11.81 ± 0.50 mg/g, antioxidant activity of 3.17± 0.04 mg/g and reducing sugar of 12.03 ± 0.97 mg/g. Additionally, crude fibre was reduced from 20.37 ± 1.10% in unfermented leaves to 6.77 ± 0.44% after 2 days of fermentation. The results provide a potential approach to enhance the nutritional value of pineapple leaves through solid-state fermentation, which can be subsequently used for animal feed. Moreover, SSF offers a sustainable solution for improving the quality of agro-industrial waste, addressing solid waste management issues.

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