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Valorization of Waste Bread by Edible Fungi Solid-State Fermentation: Physiochemical Properties, Antioxidant Activity and Flavor

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Abstract **– Bread waste poses significant environmental and nutritional challenges, necessitating sustainable valorization strategies. This study investigated solid-state fermentation (SSF) using edible GRAS molds-Aspergillus oryzae (AO) and Rhizopus oryzae (RO-to transform waste bread (particle size: 0.00636 µm, starch content: 44.03%) into value-added products. Over 168 hours of fermentation, AO rapidly acidified the substrate (pH 4.23-4.88, $p < 0.05$) and achieved peak TPC (654.89 ± 12.5 mg/g) and TFC (51.70 ± 2.1 mg/g) at 48 h, while RO showed slower acidification (pH 4.75-6.47) but higher glucose release (43.56 ± 0.05 mg/g at 72 h). Water activity (a_w) declined significantly ($p<0.05$) for both fungi (AO: 0.88→0.86; RO: 0.85→0.82), correlating with microbial metabolic activity. Principal Component Analysis (PCA) of volatile compounds revealed distinct temporal clusters (PC1: 85.89 % variance), with AO and RO producing alcohols (e.g., ethanol: 32.63 ± 0.23 mg/g) and aldehydes. SSF enhanced starch digestibility (AO: 1.47 mg/mL at 24 h; RO: 2.03 mg/mL at 72 h, $p < 0.05$). These results demonstrate SSF's efficacy in upcycling bread waste into functional ingredients, with AO optimal for rapid bioactive production and RO for sustained sugar release, contributing to food waste reduction and promoting sustainable food systems.**

Keywords **– Solid-State Fermentation, Waste Bread, Edible Fungus, Sugar Production, GRAS Mold.**

I. INTRODUCTION

Food waste is critical in global sustainability, impacting nutrition and the environment. It contributes significantly to Sustainable Development Goals (SDGs), nonetheless, the variation in food waste amongst countries, regarding embedded nutrients and ecological effects, remains poorly understood [1]. Globally, the average person wastes about 65 kg of food annually, with vegetables (25%), cereals (24%), and fruits (12%) being the largest contributors. This waste translates to significant nutrient losses, with daily wasted amounts of vitamins (C and K) and minerals (zinc, copper, manganese and selenium), reaching 25-50% of their recommended dietary intake (DRI) [2, 3]. With one-third of global food produce being lost or wasted through the supply chain, the environmental consequences are profound, driving greenhouse gas emissions, freshwater withdrawals, and land use. Reports like the EAT-Lancet Commission emphasize that halving food waste could enhance global food availability and help avoid transgressing planetary boundaries [4]. Therefore, recycling food waste is worth exploring for food security and environmental respect.

Bread, a staple food worldwide, has become a significant contributor to food waste due to modern consumer habits. With convenient packaging and easy availability, people often purchase more bread than needed, leading to large-scale wastage. Bread's nutrient composition makes it highly perishable and prone to microbial degradation. In composition, 100 g of white bread contains around 50 g of carbohydrates (47 g of starch), 37 g of water, and 8 g of protein. Bread starch gelatinizes after baking, the effects of heat and moisture, making it

more digestible and prone to microbial attack. This high nutritional value and its tendency to spoil quickly contribute to significant waste. Bread waste is particularly concerning because it is a near-complete nutritional source for microorganisms and humans. The ease with which bread spoils underscores the importance of developing strategies to minimize this waste and repurpose it for other uses [5].

Bread degradation, or staling, is predominantly a biological process, often involving filamentous fungi such as *Aspergillus species*. This natural fermentation process, while typically leading to spoilage, presents opportunities for sustainability [6]. Instead of allowing wasted bread to generate greenhouse gases like carbon dioxide or methane, this microbial activity can be harnessed for productive purposes. Solid-state fermentation (SSF) offers a promising avenue for converting stale bread into valuable products, such as enzymes or other secondary metabolites [7]. The challenge lies in preserving bread for longer periods to reduce waste. Enhancing preservation techniques, such as improved packaging and storage, could help slow down microbial degradation and extend bread's shelf life. By addressing the issue of staling with innovative solutions, it is possible to limit bread waste and reduce its environmental impact, and also a suitable substrate for microbial starters [6, 8, 9].

Solid-state fermentation (SSF) is an ancient food processing technique that involves the growth of microorganisms on a solid or semi-solid medium according to [10]. This process is commonly used in traditional fermented foods, particularly in East Asia, and is considered an important field of microbiological research. SSF has been shown to yield superior products compared to submerged fermentation, especially in terms of enzyme activity and secondary metabolites [11]. A key advantage of SSF is its ability to utilize solid food waste, such as bread, as a substrate [12]. Bread's high nutrient content and porous structure make it an ideal candidate for SSF, allowing for the creation of commercially valuable products without competing with food supplies. This innovative approach not only reduces food waste but also addresses ethical and economic challenges associated with using food-grade raw materials [13]. SSF can therefore play a pivotal role in transforming bread waste into useful products, contributing to both sustainability and economic development.

The molds *Aspergillus oryzae and Aspergillus sojae* play a pivotal role in some Asian food processing, through their use as the starter for the preparation of *koji*, a source of fungal enzymes (both amylolytic, lipolytic, and proteolytic) necessary for the assimilation of nutrients by other microorganisms in subsequent fermentations [14]. The *koji* also contributes to color, flavor, and aroma [12], which are important for the overall character of the fermented products [15, 16].

In this study, commercially available fungi were added to stale bread flour to enhance their physicochemical properties. The objective of this study, therefore, is to investigate the potential of utilizing waste bread as a substrate for solid-state fermentation (SSF) using edible grass mold strains, *specifically Aspergillus oryzae and Rhizopus oryzae*, to improve the production of sugar and bioactive compounds such as phenolic and flavonoid content, while also optimizing the fermentation process to reduce food waste and contribute to sustainable food systems.

II. Materials and Methods

2.1. *Materials*

The following analytical-grade chemicals were procured from Hope Bio-Technology Co., Ltd. (Qingdao, China): Potassium sulfate (K_2SO_4), copper sulfate ($CuSO_4$), sulfuric acid (H_2SO_4), boric acid (H_3BO_3), sodium

hydroxide (NaOH), chromatography (HPLC)-grade methanol, ethanol, acetone, ascorbic acid, gallic acid, metaphosphoric acid (MPA), glacial acetic acid, ethylenediaminetetraacetic acid (EDTA), sodium carbonate (Na_2CO_3), Folin-Ciocalteu reagent, aluminum chloride ($AlCl_3$), ammonium molybdate, potassium ferricyanide ($K_3Fe(CN)_6$), hydrochloric acid (HCl), phosphate buffer, sodium phosphate buffer, trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), MRS agar.

The filamentous fungal strains used were *Aspergillus Niger, Aspergillus Albicans, Rhizopus Oryzae, and Aspergillus Oryzae*, all fungi strains were generally regarded as safe (GRAS), commercially available starters purchased from Shandong Hezhong Kangyuan Biotechnology Co. LTD.

2.2. *Solid-State Fermentation of Waste Bread Flour*

2.2.1. *Dried Bread Flour Preparation*

White bread safe for consumption and free from mold, was purchased from the Jiangnan University School canteen and was used in the study. The bread was left at ambient temperature and allowed to stale. The bread was further sliced into cubes and dried in a hot air oven at 70 ºC for 24 h. The dried slices were homogenized into flour using a food processor (KYS, High-speed multi-functional crusher), sterilized in an autoclave at 121 ºC for 15 min, and stored in an air-tight container for further analysis [17]. The composition and physical properties of the dried waste bread flour are presented in Table 1, highlighting nutritional profile, showcasing its significant starch content alongside modest levels of lipids and proteins. They also provide insights into the flour's physical characteristics.

Table 1. Composition and Physical properties of dried bread flour.

Particle Size & Distribution		**Nutritional Value of Waste Bread Flour %**				
Particle size (µm)	Particle size distribution	Starch	Lipids	Protein	Ash	Moisture
0.00636	0.30227	44.030	7.650	13.290	0.990	10.631

2.2.2. *Solid-State Fermentation of Bread Powder/Flour*

In the preliminary studies, four edible fungi *Aspergillus niger, Aspergillus candidus, Aspergillus oryzae and Rhizopus oryzae,* were evaluated individually and in combination for starch hydrolysis using solid-state fermentation with 4 single treatments and 3 combined treatments. The fungi were assessed based on their efficiency in sugar release, fermentation duration, and bacterial profile. Based on these parameters, *Rhizopus oryzae (RO) and Aspergillus oryzae (AO)* were suitable and consequently selected for further study.

Inoculation was performed according to [18] with slight modifications. All samples were used in a 1:1 bread and water ratio, with 100 g of bread flour per sample being done in duplicates using a conical bottle. Solid-state fermentation was carried out, 100 g of bread flour, and 10 g of commercial stater were transferred into a conical flask, and sterilized cold purified water was added to the bread flour in the bottle. The mixture was stirred with separate sterile spoons for each sample, then covered and incubated following the manufacturer's instructions. The fermentation process lasted for seven days.

2.3. *Physicochemical Characteristics of Solid-State Fermented Waste Bread Flour*

2.3.1. *Water Activity*

The water activity (aw) meter (Novasina AG, 100-240V, Switzerland), was used to analyze the aw content. The samples were retained in a closed chamber sustained at 25 °C, and the aw measurements were noted 15 min after the samples reached equilibrium.

2.3.2. *pH and Total Titratable Acidity (TTA)*

The pH and TTA were determined in the samples. For pH, 10 g sample was diluted with 90 mL of purified water and homogenized, and the resulting pH was measured using a digital pH meter (Cyberscan PC 510, UK). For TTA, 5 g of sample was diluted with 45 mL of purified water and homogenized. The TTA was measured following the standardized protocol described by [19]. It was expressed as the mL of 0.1 N NaOH needed to achieve a pH of 8.6.

2.3.3. *Microbiological Analysis*

Microbial analysis was done on all samples during the fermentation duration. 1 g of fermented bread flour was homogenized with 20 mL of sterile NaCl buffer followed by serial dilutions with sterile NaCl buffer (0.85%, w/v). 1.0 mL aliquots of the dilute were inoculated into MRS agar (Difco, Detroit, MI, USA). Single forming colonies were counted after incubating the plates at 37 ˚C, for 48 h. The total visible counts were expressed as log 10 colony forming units (CFU)/g [19].

2.4. *Compositional Analysis of Solid-State Fermented Waste Bread Flour*

2.4.1. *Sugar Composition*

Sugars were identified and quantified according to [20] with slight modifications. Dried samples were ground and analyzed for moisture content. 5-10 g were boiled for 20 min in 100 mL of 60% ethanol, cooled, and filtered through Whatman 42 paper. Further centrifuging at 10000 rpm the eluate and sequentially filtering through a Sep-Pak Plus C18 cartridge (Waters, Milford, MA) and a 0.22 µm, membrane filter before injection onto the HPLC (modified Picha1985). Duplicate samples were extracted for each product and processing stage, and duplicate analysis was performed on the separate extractions.

2.4.2. *Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) Analysis*

The extracts obtained from the samples were used to determine the TPC and TFC. 1 g of the sample was extracted with 40 mL 80% methanol, followed by shaking at 100 × g in a gyro rotary shaker for 30 min and, centrifuged at 10000 x g for 10 min. The resultant pellet was gathered and reextracted following the same method. The supernatants were combined and stored in an amber tube, following a previously reported protocol [21]. The extraction and re-extraction process was performed in triplicate for each trial.

The TPC was determined by the Folin-Ciocalteu method, following the procedure outlined by [22], with slight modifications. 1 mL of the extracted sample was mixed with 5 mL of Folin-Ciocalteu reagent (diluted at 1:10) in a dark tube. 4 mL of a 5% Na_2CO_3 solution was added after 3 min, and the mixture was allowed to stand for 30 min in the absence of light. The absorbance was measured at 765 nm using a spectrophotometer (UV-2600, Unico Instruments Co. Ltd., Shanghai, China). TPC was calculated and expressed as gallic acid equivalents (GAE/g) on a DW basis.

The TFC was evaluated following a method described by [22]. The TFC was expressed as mg rutin equivale-

-nt/g dry weight of bread flour at 490 nm. For analysis, 1 mL of the extracted sample was mixed with 0.5 mL of 1.5% $NaNO_2$ solution, 1 mL of 1N NaOH, and 1 mL of 3% AlCl3. All treatments were agitated and stored in the dark for 5 min. The absorbance was measured at 490 nm using a spectrophotometer. TFC was quantified as g of rutin equivalent per kilogram of material (mg/g DW).

2.5. *In-Vitro Starch Digestion*

The enzymatic hydrolysis method was used for this study. 2 g of fermented bread to 40 mL of distilled water was diluted, let to stand for 6 h, and centrifuged followed at 10000 rpm for 10 min at 4 ˚C, 0.4 mL DNS reagent was added to 0.2 mL supernatant. Tubes were placed in a boiling water bath for 5 min, transferred to ice to rapidly cool down, and then brought to room temperature by placing them in a water bath at 25 °C. The absorbance was measured at 540 nm, using a Pharmacia Biotech Novaspec II spectrophotometer by [23].

2.6. *Flavor Analysis*

An electronic nose (iNose 102, Isenso, New York, NY) was used to determine the flavor of fermented samples. The electronic nose was prewashed for 3,000 s to release the previous odor. 3 g of freeze-dried fermented bread flour was transferred into a microextraction bottle with a penetrable cover (12 × 80 mm; diameter × height) and placed in an incubator at 25 °C for 60 min. The washing time was 120 s, and the detection time was 120 s for four trials. Table 2 provides a comprehensive overview of various electronic nose sensors, detailing the response materials they detect, the categories of substances they analyze and their characteristics for each chemical. The response data were analyzed using two principal component analysis (PCA) of the original variable information by [24].

Table 2. Electronic nose sensors and specific performance description.

Sensors	Response Material	Category of Substance
s1	Alkanes, Smoke, Alcohol,	Propane, Natural Gas, Smoke
s2	Aldehydes, Short-Chain Alkanes, Alcohols, Smoke, Isobutane, Formaldehyde	
s3	Ozone	
s4	Sulfide	Hydrogen Sulfide
s5	Organic	Ammonia, Methylamine, Ethanolamine
s6	Aromatic Compounds	Toluene, Acetone, Ethanol, Hydrogen, Other Organic Vapors
s7	Short-Chain Alkanes	Methane, natural gas, biogas
s8	Short-Chain Alkanes	Propane, LPG
s9	Aromatic Compounds, Alcohols, and Aldehydes	Toluene, Formaldehyde, Benzene, Alcohol, Acetone
10	Hydrogen-Containing Gas	Hydrogen
s11	Alkanes, Olefins	Liquefied Gas, Alkanes, Olefins
s12	Short-chain alkanes	Liquefied Gas, Methane
s13	Combustible gas	Methane
s14	Combustible gas	Combustible gas, smoke

Sensors	Response Material	Category of Substance
s15	Alkanes, organic gases	Smoke, isobutane, organic acid esters, aliphatic hydrocarbons
s16	Sulfide	Sulfur compounds
s17	Nitride	Nitrogen oxides
s18	Ketones, alcohols	Acetone, ethanol, organic solvents

2.7. *Statistical analysis*

Experimental results are reported as mean value ± standard deviation. Experimental results were analyzed with SPSS software, one-way ANOVA, and Duncan's test for pair comparisons of treatments at $P < 0.05$, and GraphPad Prism 9.0 software.

III. Results and Discussion

3.1. *Physicochemical Properties of Fermented Waste Bread Flour pH, TTA, and Water Activity*

The pH, TTA, and water activity (aw) were measured at various fermentation times (0 h, 24 h, 96 h, and 168 h), presented in Table 3. The pH ranged from 4.23 to 4.88 for *AO*, which was significantly different ($p < 0.05$) compared to the range of 4.75 to 6.47 for *RO*, from slightly acidic to near-neutral pH levels while *AO* maintained a more acidic environment throughout the fermentation time [25]. TTA values range, from *AO* at 0 h (20.1 ± 0.26 mL), 96 h (7.6 ± 0.31 mL), and 168 h (16.6 ± 0.30 mL). RO ranged from 0 h (6.5 ± 0.15 mL), 96 h (8.3 ± 0.30mL), and (13.8 ± 0.26 mL) at 168 h. The dynamic changes in TTA indicate active microbial growth phases-lag, exponential, and stationary. *AO's* early acidity highlights its rapid fermentation, while *RO's* delayed peak suggests adaptive metabolism, consistent with [25, 26].

The Aw values, *AO* ranged from (0.88 ± 0.01 to 0.86 ± 0.01), *RO* (0.85 ± 0.01 to 0.82 ± 0.00). The decrease in Aw at 96 h is due to microbial consumption of free water during metabolic activities, including producing organic acids and enzymes [27]. This indicates significant potential to inhibit microbial growth and ability to enhance product shelf life, in correlation with [12], and are usually used in solid-state fermentation due to their efficiency in water-limited environments [14]. *RO's* gradual pH decline reflects a two-phase process: initial enzymatic starch degradation followed by acid production, as supported by [25].

The data draws a clear correlation between the physicochemical properties measured and the fermentation dynamics influenced by different fungal strains. The distinct variations in pH, TTA, and aw enhance our understanding of how these factors interrelate to impact microbial metabolism and product quality. Maintaining a lower pH, increased TTA, and decreased aw not only supports microbial activity but also optimizes conditions for fermentation, ultimately resulting in a product that is both flavorful and shelf-stable.

Table 3. Physicochemical properties of fermented waste bread flour.

	Sample[2]	Time[3]			
		0/h	24/h	96/h	168/h
Aw[4]	*AO*	0.88 ± 0.01^{c}	0.87 ± 0.00^{c}	0.81 ± 0.01^{a}	0.86 ± 0.01^{b}
	RO	0.85 ± 0.01^{c}	0.86 ± 0.00^{c}	0.81 ± 0.01^{a}	0.82 ± 0.00^{b}

	Sample[2]	Time[3]			
pH	*AO*	4.23 ± 0.11[a]	4.88 ± 0.06[c]	4.43 ± 0.11[b]	4.41 ± 0.10[b]
	RO	6.32 ± 0.15[c]	6.47 ± 0.16[c]	5.65 ± 0.17[b]	4.75 ± 0.16[a]
TTA mL	AO	20.1 ± 0.26[c]	15.7 ± 0.82[b]	7.6 ± 0.31[a]	16.6 ± 0.30[b]
	RO	6.5 ± 0.15[a]	9.2 ± 0.21[c]	8.3 ± 0.30[b]	13.8 ± 0.26[d]

[1]Means (n = 4 ± standard deviation) with different letters in the same column indicate significant differences at $p < 0.05$. [2]Sample fermented by Aspergillus oryzae – AO, and sample fermented by Rhizopus oryzae - RO. [3]Time (h) = fermentation time taken in hours (0, 24, 96, 168). [4]Aw = water activity of samples; TTA = total titratable acidity.

3.2. *Changes in Microbial Growth during Solid-State Fermentation*

The total viable count (TVC) for bacterial analysis during fermentation is expressed in Fig. 1. Fungal activity, environmental conditions, and microbial interactions influenced these results. The growth-inhibiting properties of *AO* can be attributed to its rapid acidification of the substrate environment to pH 4.5-5.5 through organic acid production, coupled with its dominant enzymatic activity in line with [28]. These conditions create an antagonistic environment for bacterial proliferation. The fungus's aggressive colonization strategy and efficient consumption of available nutrients, particularly starch and simple sugars, results in resource limitation for bacterial communities, manifesting in reduced bacterial growth rates and lower peak populations. However, bacterial growth reaches its maximum at 96 h, likely due to the accumulation of glucose from *AO* amylolytic activity, which supports acid-tolerant bacteria such as lactic acid [25, 29]. The subsequent decline in bacterial populations after 96 h can be attributed to substrate exhaustion and the accumulation of growth-inhibitory metabolites, particularly organic acids. *RO*, in contrast, supports higher bacterial growth due to its slower acidification and milder inhibitory effects. *RO* produces organic acids (e.g., lactic acid, fumaric acid) at a slower rate than *AO*, resulting in a pH range of 5.5-6.0, which is more favorable for bacterial growth Bacteria, particularly those tolerant to mildly acidic conditions, can proliferate more effectively in *RO*-dominated substrates [13]. *RO* has a slower initial colonization rate compared to AO, leaving sufficient nutrients available for bacterial growth during the early stages of fermentation the bacterial population takes advantage of the residual starch and sugars, resulting in a growth peak at 120 h.

The bacterial growth pattern observed in *RO*-dominated substrates during solid-state fermentation (SSF) demonstrates the interplay of fungal activity, pH regulation, and nutrient availability, which support higher bacterial proliferation. *AO* rapidly acidifies the substrate and suppresses bacterial growth [30].

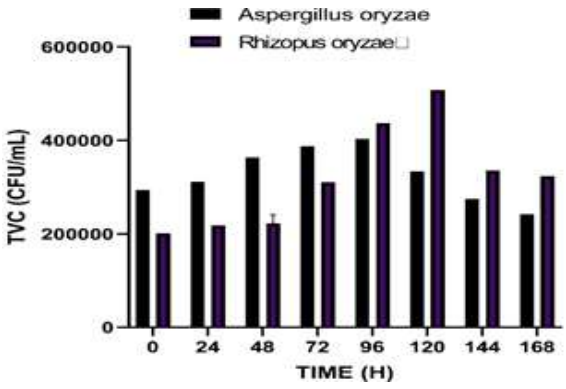

Fig. 1. Bacterial analysis during the fermentation of waste bread flour using *AO* and *RO* shows distinct growth dynamics.

3.3. *The Sugar Content and Composition in Samples during Solid-State Fermentation*

The analysis of sugar content and composition during solid-state fermentation (SSF) aimed at identifying and quantifying fructose, glucose, sucrose, and maltose at different fermentation intervals (24 h, 48 h, and 72 h). The sugars were measured based on their peak release during fermentation, and the results are shown in Table 4. The letters indicate statistically significant changes in sugar contents across samples ($p < 0.05$). The study highlights significant variations in sugar levels across samples, influenced by microbial activity, enzymatic actions, and fermentation conditions. Fructose levels were generally low across all samples. The highest fructose concentration is observed in the *RO*⁴ sample (4.31 ± 0.01). The low levels of fructose observed in the other samples suggest a limited accumulation, likely due to its rapid use as a substrate for microbial metabolism or conversion into other metabolites. The slightly higher fructose concentration in RO⁴ may imply a transient accumulation before it undergoes further metabolic processing. Glucose levels are significantly higher in *RO*⁴ (43.56 ± 0.05), this is because *RO* sustained a pH close to a neutral environment making it favorable for microbial metabolism reducing the bioactivity of phenolics showing slower phenolic release but maintaining stability allowing for enzymatic hydrolysis of starch to achieve higher glucose release, and low concentration in *AO*³ (10.69 ± 0.02) [26]. Sucrose, control (9.68 ± 0.08), *AO*² (0.32 ± 0.40), and *RO*⁴ (1.70 ± 0.01) were almost completely hydrolyzed in samples, indicating the activity of enzymes breaking down sucrose into glucose and fructose [31].

Table 4. Identification and quantification of sugars in fermented samples.

Sample	Fructose	Glucose	Sucrose	Maltose
*CTL*¹	3.28 ± 0.11^{cB}	2.64 ± 0.08^{aA}	9.68 ± 0.08^{cD}	4.75 ± 0.03^{ec}
*AO*²	3.88 ± 0.03^{dB}	7.85 ± 0.07^{cD}	0.32 ± 0.40^{aA}	6.93 ± 0.09^{fc}
*RO*²	3.11 ± 0.01^{cB}	32.63 ± 0.23^{fD}	2.63 ± 0.08^{dA}	14.67 ± 0.03^{gc}
*AO*³	2.88 ± 0.09^{bB}	10.69 ± 0.02^{dD}	1.88 ± 0.06^{cA}	3.40 ± 0.04^{dc}
*RO*³	2.78 ± 0.13^{bB}	29.29 ± 0.01^{eA}	1.79 ± 0.12^{ec}	1.75 ± 0.07^{bA}
*AO*⁴	1.36 ± 0.18^{aB}	6.40 ± 0.13^{bD}	1.08 ± 0.03^{bA}	2.50 ± 0.05^{ec}
*RO*⁴	4.31 ± 0.01^{ec}	43.56 ± 0.05^{gD}	1.70 ± 0.01^{cB}	1.42 ± 0.09^{aA}

Different lowercase letters within each column and uppercase at each represent significant differences at $p < 0.05$. Fermentation time at different hours; CTL¹- control without fungi, ²24-h, ³48-h, and ⁴72-h (AO-Aspergillus oryzae; RO- Rhizopus oryzae).

Maltose concentration ranged from *RO*² (14.67 ± 0.03), *RO*³ (1.75 ± 0.07), *RO*⁴ (1.42 ± 0.09) lower in *RO*³ and *RO*⁴, indicate maltose subsequently converted into glucose as fermentation progresses [15, 31]. This is because of the enzyme activity of Alpha-amylase, Glucoamylase, and Pullulanase, efficiency in hydrolyzing the internal α-1,4-glycosidic and α-1,6-glycosidic bonds in dextrin and maltose [13, 30]. These enzymes in starch degradation are significantly influenced by pH and temperature [16]. Additionally, the ideal temperature for fungal growth and enzymatic activity in solid-state fermentation is between 30-40°C, which is suitable for both fungi to thrive in ensuring optimal enzyme production and starch hydrolysis by maintaining stability and activity [26, 31].

Understanding these dynamics is essential for optimizing fermentation processes and enhancing the production of desired sugars, which can have significant implications for various applications in food, beverage,

and biofuel industries. The findings presented in this document underscore the complexity of solid-state fermentation and the need for careful monitoring and control of environmental conditions to achieve optimal results.

3.4. *Changes in Total Phenolic (TPC) and Flavonoid (TFC) Content during Solid-State Fermentation*

The TPC values of the treated samples over 0, 24, 48, and 72 h of phenolic compound release and transformation (Fig. 2a). Day 0 reflects the baseline phenolic content of the substrate at 430.91 mg/g before treatment. The levels increased rapidly, indicating early enzymatic activity [12]. TPC value for *AO* ranged from 519.20 mg/g, 654.89 mg/g, and 604.09 mg/g, for days 24, 48, and 72. The production of cellulases, hemicellulases, and feruloyl esterase, which start degrading the lignocellulosic matrix and releasing bound phenolic compounds could be the reason for TPC released [32, 33]. The moderate increase suggests that *AO* is beginning to colonize the substrate and activate its enzymatic machinery, but the full enzymatic potential has not yet been reached. Its peak at 48 h, coincides with the maximum enzymatic activity of *AO*-producing ligninolytic enzymes that degrade lignin or cellulose (polysaccharides of the cell wall) polymers that encapsulates many bound phenolics [34, 35].

The breakdown of the cell wall releases free phenolics into the substrate, significantly increasing the TPC, secondary metabolism may also contribute to TPC by synthesizing fungal-derived phenolic metabolites [36]. This peak reflects the optimal conditions for phenolic compound release, with high enzymatic activity and sufficient substrate availability. The slight decline in TPC after 48 h is likely due to oxidative degradation of phenolics [37]. Substrate exhaustion and reduced enzymatic production at this stage also limit further phenolic release. The decline suggests that prolonged fermentation may not be beneficial for maximizing TPC with *AO* [38].

The values for *RO* for days 24, 48, and 72 ranged from 601.36 mg/g, 631.36 mg/g, and 631.36 mg/g. *RO* starts with a higher baseline TPC (545.45 mg/g) compared to *AO* (430.91 mg/g), possibly due to differences in substrate composition or the initial extractable phenolic content. *AO* shows a steeper increase in TPC (+51.9% from day 0 to day 48), indicating its superior enzymatic potential for releasing bound phenolics and synthesizing fungal-derived phenolics. *RO* exhibits a slower and more gradual increase in TPC (+15.8% from day 0 to day 48), reflecting its weaker enzymatic efficiency, particularly in cell wall degradation. *AO* experiences a slight decline in TPC after 48 h (-7.8%), likely due to oxidative degradation by polyphenol oxidases.

RO maintained stable TPC after 48 h, suggesting minimal phenolic degradation, possibly due to limited oxidative enzyme production. *AO* production of ligninolytic and hydrolytic enzymes enables it to release more phenolics from the substrate, making it more effective for enhancing TPC during fermentation. *RO* enzymatic activity is primarily cellulolytic, with limited ligninolytic or cellulosic potential, resulting in slower phenolic release and lower TPC values over time [11]. *AO* is highly effective at releasing and synthesizing phenolic compounds, making it an ideal candidate for fermentation processes aimed at maximizing TPC.

The optimal fermentation time for *AO* is 48 h, as TPC peaks at this stage. Prolonging fermentation beyond this point may lead to phenolic degradation. *AO* is suitable for applications requiring high phenolic content, such as the production of functional foods, nutraceuticals, and natural antioxidants. *RO* exhibits a slower and more stable release of phenolics, which may be advantageous for longer fermentation processes where phenolic

stability is desired. However, its minimal phenolic degradation at later stages may be beneficial for a selected application [21, 26].

Similar trends were observed for TFC levels over 0, 24, 48, and 72 h after enzymatic degradation, in Fig. 2b. These flavonoids may be present as free flavonoids or loosely bound forms that are more readily extractable. A notable decrease in TFC is observed after 24 h (34.06 mg/g) of fermentation. This decline may be due to the oxidative degradation of free flavonoids by *RO* metabolic activity. Unlike *AO, RO* produces lower cellulolytic and ligninolytic enzymes during the early stages of fermentation, resulting in the limited release of bound flavonoids [36, 39].

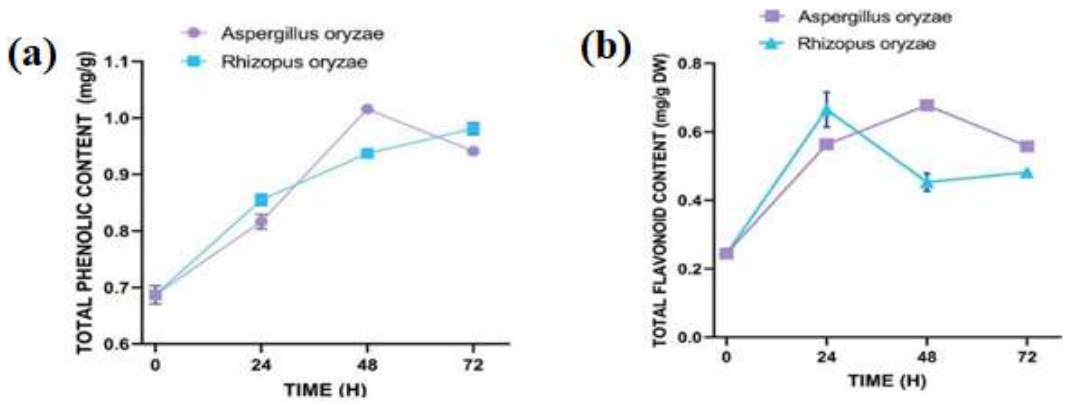

Fig. 2. (a) Total phenolic content during fermentation (b) Total flavonoid content.

The observed reduction indicates that the oxidative degradation of free flavonoids outpaces their release during this phase [40]. A slight recovery in TFC is observed at 48 h (36.31 mg/g), likely due to the gradual release of bound flavonoids as *RO* enzymatic activity increases. *RO* produces cellulases and hemicellulases, though at lower levels than *AO*, which may contribute to the modest increase in TFC during this period [27]. The slower enzymatic activity of *RO* limits its ability to release flavonoids as effectively as *AO*. The TFC stabilizes at 36.31 mg/g, indicating that the release of bound flavonoids and their degradation has reached equilibrium. The lack of further increase suggests that *RO* has a limited capacity to release additional flavonoids from the substrate after 48 h. *RO* starts with a higher baseline TFC (50.76 mg/g) compared to *AO* (17.65 mg/g), reflecting differences in substrate composition or initial flavonoid availability. *AO* shows a significant increase in TFC (+193% from day 0 to day 48), highlighting its superior enzymatic potential for flavonoid release and synthesis. *RO* exhibits a net decrease in TFC (-28.5% from day 0 to day 48), signifying that the oxidative degradation of flavonoids outweighs the ability to release bound flavonoids. *AO* experiences a decline in TFC after 48 h (-18.2%), likely due to oxidative degradation, while *RO* TFC stabilizes, reflecting its limited enzymatic and oxidative activity [26, 41].

3.5. *In-Vitro Starch Digestion during Solid-State Fermentation of Bread Flour Substrate*

The *in-vitro* starch digestibility of the inoculated strains during fermentation is presented in Fig. 3. The results indicated that both strains exhibit enzymatic activity early in fermentation, able to hydrolyze starch at 24 h, however, a rapid decline was observed in *AO* with a mean of 1.471 mg/mL. This is because the enzymatic activity is enhanced by acidic pH and high TTA a significant amount of phenolic and flavonoid content released. The peak of TPC content coincides with the rapid decline by inhibiting amylolytic enzymes for starch hydrolysis. *RO* pH ranges from 4.75 to 6.47 mg/mL making it favorable for microbial metabolism reducing the

bioactivity of phenolics release but maintaining stability. TPC (631.36 mg/g**)** at 72 h, allowing for enzymatic hydrolysis of starch to achieve higher glucose release. With a mean of 1.783 mg/mL at 24 h, increasing to 2.029 mg/mL at 72 h, before declining, the subsequent decline [26]. These patterns reflect differences in enzymatic profiles and metabolic pathways, which can inform their use in industrial processes requiring rapid or sustained glucose production [15, 16, 42].

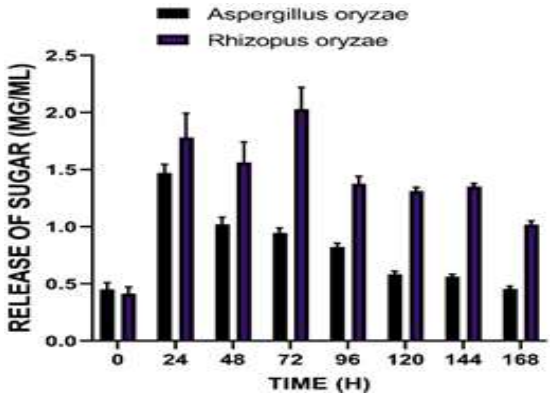

Fig. 3. The release of sugar content observed in waste bread flour during fermentation exhibits rapid enzymatic activity early in stages with, *AO* (mean, 24 h = 1.471) at, and *RO* (mean, 24 h = 1.783 and 72 h = 2.029).

3.6. *Flavor Analysis*

The E-nose analysis and PCA evaluation results in Fig. 4 (a, b, c) provide critical insights into the evolution of volatile compounds in the two samples (*AO* and *RO*) during fermentation at 24, 48, and 72 h, compared to the control (zero time). The PCA scores plot in Fig. 4b reveals that PC1 accounts for 93.6% of the variance, while PC2 explains 4.8%, indicating that PC1 captures most of the differences in volatile profiles. The control group is distinctly separated from the fermented samples, demonstrating substantial changes in volatile profiles due to fermentation. *AO* and *RO* samples exhibit temporal shifts along PC1 and PC2, reflecting dynamic changes in their volatile compositions over time. While *AO* and *RO* share overlapping regions, suggesting similarities in their volatile profiles, some differentiation is evident [43].

The PCA biplot (Fig. 4c) further elucidates the contributions of specific volatiles to sample differentiation. The vectors representing individual volatiles indicate their relative influence on sample clustering. Longer vectors signify stronger contributions to the separation of samples. Temporal progression in *AO* and *RO* samples suggests that fermentation drives significant changes in specific volatiles [44].

During fermentation, the volatile profiles evolve as follows: at 24 h, early-stage fermentation likely generates alcohols, aldehydes, and esters due to microbial activity. By 48 h, secondary metabolites such as acids and ketones emerge, contributing to flavor complexity as observed in Fig. 4a radar chart. At 72 h, the profiles stabilize or shift further due to extended microbial metabolism or environmental factors like pH changes. The distinct separation of the control group from the fermented samples underscores the transformative impact of fermentation on volatile compounds. Differences between *AO* and *RO* may result from variations in substrate composition or microbial communities influencing fermentation pathways. However, their overlapping regions suggest shared metabolic processes or volatiles. These findings align with methodologies described in related literature for analyzing E-nose data using PCA. Principal components explain most of the variance, while clustering patterns help identify temporal or treatment-based differences. Biplots link specific volatiles with

observed trends. In conclusion, E-nose analysis combined with PCA effectively tracks the evolution of volatile compounds during fermentation. The results highlight clear temporal trends for both *AO* and *RO* samples and significant differentiation from the control group. These insights provide a foundation for understanding flavor development during fermentation and can guide further exploration of key volatiles responsible for these changes [45].

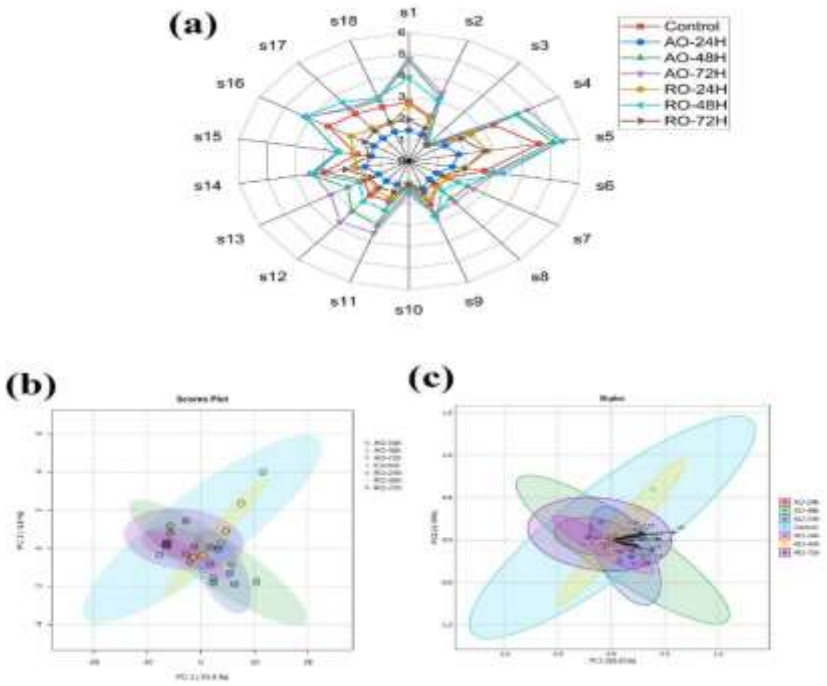

Fig. 4. Flavor analysis of fermented bread flour on e-nose at 24h, 48h, and 72h. (a) Flavor response intensity at different fermentation times on radar chat, (b) principal component analysis (PCA); PC 1 score plot (c) PC1 at different fermentation times.

IV. CONCLUSION

This study demonstrated the potential of utilizing waste bread as a substrate for solid-state fermentation (SSF) using edible GRAS molds, specifically *Aspergillus oryzae (AO)* and *Rhizopus oryzae (RO)*, a cost-effective approach. The findings highlight the effectiveness of edible fungi in enhancing the sugar content, total phenolic content (TPC), and total flavonoid content (TFC) of the fermented product, with *AO* showing superior enzymatic activity and phenolic release, while *RO* displayed a slower but sustained starch hydrolysis and phenolic stability. *AO* exhibited a rapid and significant increase in TPC and TFC, peaking at 48 h, making it optimal for short-term fermentation processes aimed at maximizing antioxidant content. *RO*, while slower in enzymatic activity, demonstrated a stable release and minimal degradation of phenolics during prolonged fermentation, making it suitable for applications requiring sustained phenolic stability. Both fungi efficiently hydrolyzed starch into sugars, with *RO* achieving a higher glucose concentration at 72 h, indicating its potential for industrial processes requiring sustained sugar production, volatile profiles generated, alcohols, aldehydes, esters and secondary metabolites such as acids and ketones emerge, contributing to flavor complexity effectively identified on e-nose.

The study provides an innovative approach to valorizing bread waste, with edible fungi and addressing environmental concerns. By optimizing fermentation conditions, waste bread can be transformed into high-value

products such as sugar additives and functional food ingredients. These findings contribute to the development of sustainable food systems and support the circular economy by repurposing food waste into commercially valuable products. Future research should focus on scaling up this process and exploring additional applications of fermented bread in the food and nutraceutical industries.

Credit Authorship Contribution Statement

Miriam Dede Doku: Conceptualization, Investigation, Data Curation, Writing - original draft, Funding acquisition. Khin Su Su Hlaing: Review, Visualization, Data Curation. Mouhamed Fall: Methodology, Review and Editing, Visualization. Steven Suryoprabowo: Software and Visualization. Yuliang Cheng: Data Curation, Review. Hang Yu: Project Administration, Validation. Shaofeng Yuan: Review and Editing, Validation. Yahui Guo: Conceptualization, Supervision, Review and Editing. Weirong Yao: Supervision, Review, and Editing.

Abbreviations:

AC	*Aspergillus candidus*
AN	*Aspergillus niger*
AO	*Aspergillus oryzae*
Aw	Water activity
DW	Dry weight
DNS	3,5dinitrosalicylic acid
E-nose	Electronic nose
HPLC	High-performance liquid chromatography
rpm	Revolution per minute
μm	Micrometer
PCA	Principal component analysis
RO	*Rhizopus oryzae*
SSF	Solid-state fermentation
TCA	Trichloroacetic acid
TFC	Total flavonoid content
TPC	Total phenolic content
TTA	Total titratable acid

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Declarations of Interest Statements

The authors declare no conflict of interest.

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