World Journal of Advanced Research and Reviews
eISSN: 2581-9615 CODEN (USA): WJARAI
Cross Ref DOI: 10.30574/wjarr
Journal homepage: https://wjarr.com/

(RESEARCH ARTICLE)

Check for updates

Fermenting Buckwheat Seeds (*Fagopyrum esculentum*) with *Aspergillus* and subsequent amylase treatments enhance phenolic content and antioxidant activities

Phonesavanh Phouthaxay and Insavai Sitthivohane*

Department of Plant Science, Faculty of Agriculture and Forest Resource, Souphanouvong University, Laos.

World Journal of Advanced Research and Reviews, 2024, 22(02), 1080–1094

Publication history: Received on 02 April 2024; revised on 10 May 2024; accepted on 13 May 2024

Article DOI: https://doi.org/10.30574/wjarr.2024.22.2.1452

Abstract

This research focused on investigating the effects of fermenting processed buckwheat with different *Aspergillus* species and subsequent treatment with α-amylase, β-amylase, and Glucoamylase. The study found that fermenting with *Aspergillus Oryzae* 82-3, followed by β-amylase treatment, significantly impacted saccharification within 2 hours, leading to increased total polyphenol content, rutin, and quercetin levels. High-Performance Liquid Chromatography (HPLC) results showed that Asp-82-3 with β-amylase had the highest rutin and quercetin levels. The saccharified buckwheat also exhibits elevated total protein and total carbohydrate content. DPPH analysis revealed enhanced antioxidant activity in treated samples. In vitro assays demonstrated that buckwheat saccharification with amylase after *Aspergillus* fermentation resulted in potent production, with Asp-82-3 and β-amylase showing the highest Nitric Oxide (NO) production. The study concludes that combining *Aspergillus* species and amylase for buckwheat saccharification enhances its antioxidant capacity and functional value, making it valuable in the functional food industry.

Keywords: *Fagopyrum esculentum*; Saccharification; Amylase; Rutin; Quercetin; Antioxidant Activity

1. Introduction

The bioconversion brought about by microorganisms has been shown to considerably reduce the disadvantages of traditional physical and chemical extraction methods (Ameh, et al. 2010). Fermentation processes have been studied for many decades. Solid state fermentation is a simple technique for the production of bioactive compounds (Oliveira et al., 2010; Schmidt & Furlong, 2012). Among the major microorganisms known for their ability to produce enzymes that degrade the cell wall of plants, fungi comprise the most interesting group (Hegde et al., 2006). Fungi, particularly Rhizopus and *Aspergillus*, are recognized for their enzyme production, which enhances the breakdown of plant cell walls. In solid-state fermentation (SSF), these fungi have been extensively employed to create diverse products, including easily digestible proteins, in a controlled environment without generating any toxic substances (Dartora et al., 2002). The probiotic filamentous fungus *Aspergillus Oryzae* is widely employed in fermenting soybeans, rice, grain, and potatoes. It plays a key role in the production of various fermented Asian foods such as tempeh, soy sauce, miso, sake, and rice vinegars. (Daba et al., (2021). The fermentation process yields enzymes with benefits for both humans and animals. *Aspergillus* oryzae, known to produce amylase, a crucial enzyme for promoting a healthy gut and digestion, has been used by humans for over 2,000 years, although the exact date of domestication remains unknown. (Jørgensen, 2007). Nine enzymes, including amylase, protease, and lipase, directly collaborate with probiotics. Amylase, predominantly located in the pancreas and salivary glands, partners with probiotics to absorb simple sugars it releases. Amylase plays a crucial role in breaking down sugars into simpler components, allowing bacteria to convert them into lactic acid, hydrogen peroxide, and other essential substances for maintaining microbial balance in the gastrointestinal tract. (http://nootriment.com/*Aspergillus*-oryzae/).

* Corresponding author: Phonesavanh Phouthaxay & Insavai Sitthivohane

Oates (1997) proposed that enhancing the incubation temperature to around 60 °C can improve the hydrolysis of native starch. Further increasing the conversion of native starch would be beneficial for the industrial production of fermentable sugars and bioethanol. Recent technological advances have led to the discovery of new generation enzymes, including α-amylase from *Aspergillus* kawachi and glucoamylase from *Aspergillus* niger. Enzymes collaborate to efficiently break down granular starches, directly hydrolyzing raw starch in a single step at a moderate temperature below gelatinization. The synergistic action involves glucoamylase's sharp and deep pinhole formation (exon-activity) and α-amylase's ability to widen pinholes (endo-activity). This combination facilitates the continuous release of fermentable glucose from granular starches (Franco, Preto & Ciacco, 1987). In addition, High-dextrose starch hydrolysates find extensive use in the food industry and serve as a fermentable sugar source. The industrial production of glucose syrups involves a two-step process: initial saccharification of starch to maltodextrin using α-amylase, followed by a second hydrolysis to glucose using glucoamylase. Amylases, vital for starch hydrolysis, are widely applied in the food, textile, and pharmaceutical industries. Key sources of α-amylase, such as Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus licheniformis, and *Aspergillus* oryzae, are valued for their highly thermostable enzymes (Ghimeray et al., 2014). The use of enzymes to modify the structure and improve the physicochemical and biological properties of flavonoids has been of great scientific and industrial interest due to their wide availability, high selectivity, low cost and their promotion of efficient reactions with few products (de Araújo, et al., 2013). Previous work has shown that enzymatic hydrolysis of specific glycosyl groups or the conversion of flavonoid glycosides to aglycones increases anti-inflammatory activity of naringin (Amaro et al., 2009) and antioxidant activity of kaempferol, besides improving the bioavailability of hesperidin (Nielsen et al., 2006), and of flavonoid glycosides in fruit juices and green tea.

The present study aimed to assess the functional characteristics of saccharified buckwheat (*Fagopyrum esculentum*) after *Aspergillus* species fermentation, followed by treatment with α-amylase, β-amylase, and Glucoamylase at various intervals. The investigation focused on changes in soluble solid content, rutin and quercetin content, total polyphenols, and DPPH radical scavenging activity. The results aim to offer an effective processing method for achieving more efficient saccharification of beneficial phenolics in common buckwheat products, ultimately enhancing the health benefits of these products.

2. Material and methods

2.1. Culture preparation and fermentation procedure

Figure 1 Fermentation of *Aspergillus* species of buckwheat seeds, (A) after fermented 72 hours in 500 ml Erlenmeyer flasks and (B) dried in over 45°C after fermented 72 hours.

Aspergillus Oryzae strains Acidus (KACC 46420), CF1003, and KACC-82-3 obtained from the Korean Agricultural Culture Collection (KACC) were cultivated on potato dextrose agar media. A 7-day-old active sporulating fungal culture was utilized. Common buckwheat seeds (100 g) were soaked in 300 ml distilled water for 4 hours, dehydrated for 30 minutes, and autoclaved at 121 °C for 15 minutes. After cooling, fungal spores (0.2%, 106 spores/ml) were added to each flask containing autoclaved buckwheat seeds, mixed, and incubated at 30 °C for 72 hours. The harvested samples

were dried at 45-50 °C for 48 hours in Fig. 1, ground into powder using a blender, and prepared for saccharification with amylase enzymes.

2.2. Sample preparation and enzyme experiment

Common buckwheat seed powder, following fermentation with *Aspergillus* species (*Aspergillus Acidus KACC 46420, Aspergillus Oryzae CF1003,* and *Aspergillus Oryzae 82-3*), was combined with distilled water (1:5 w/v) in 250 ml Erlenmeyer flasks. Saccharification of buckwheat involved the addition of 0.1 % α-amylase (*BAN® 480L, Novoenzymes, Denmark*), β-amylase (*Betalase1500 EL, Senson, Finland*), and gluco-amylase (*AMG 300L, Novoenzymes, Denmark*) for 2, 4, or 8 hours. The reaction was halted by boiling (100 °C) for 15 minutes, followed by freeze-drying and storage for analysis as depicted in Fig. 2. Control samples, without enzyme presence, served as the baseline for buckwheat saccharification during fermentation.

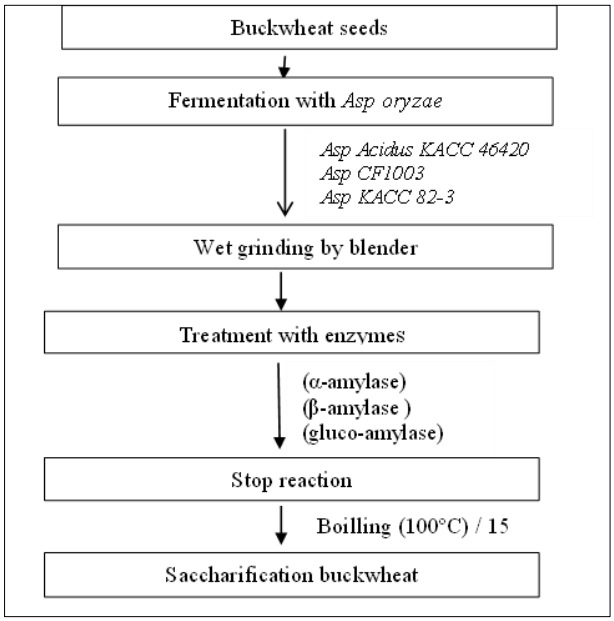

Figure 2 Process of saccharification of buckwheat after fermented by *Aspergillus* species and Subsequent Amylase Treatments

2.3. Estimation of changes in soluble solid content (°Brix) during saccharification of the buckwheat

Brix° measurement of each soluble solid sample was saccharificated from 2 hours to 8 hours. The soluble solid samples were centrifuged 14.400xg and the upper level was used to measure Brix by refractive sugar (N-50E, ATAGO, Japan).

2.4. Estimation of total carbohydrates and proteins

The total carbohydrate content of saccharified buckwheat (Fagopyrum esculentum), following fermentation with *Aspergillus* species and subsequent treatment with amylase (α-amylase, β-amylase, and glucoamylase), was assessed using the phenol–sulfuric acid assay, employing a standard glucose solution (Dubois et al., 1956). Protein content was determined through the Lowry method, utilizing bovine serum albumin (BSA) as the standard (DC Protein assay kit; Bio–Rad; Hercules, CA, USA). The uronic acid content of the polysaccharide was analyzed using a sulfamate/m-hydroxy diphenyl colorimetric assay, with glucuronic acid as the standard (Filisetti–Cozzi & Carpita, 1991).

2.5. Determination of total polyphenol content

The total polyphenol content in the saccharification samples was determined following the Folin-Ciocaltues method outlined by Singleton and Rossi (1965). In summary, 0.2 ml aliquots of the samples were mixed with 0.2 ml of 1M phenol reagent in test tubes. The volume was adjusted by adding 1.8 ml of distilled water, followed by vertexing for 3 minutes. Subsequently, 0.4 ml of Na2CO3 (10% in water, v/v) was added, and the volume was brought to 4 ml with the addition of 0.6 ml of distilled water. The mixture stood at room temperature for 1 hour, and absorbance was measured at 725 nm using a spectrophotometer (Model UV-1800, Shimadzu Corporation, Kyoto, Japan). The total phenolic content was determined from a calibration curve (R2 = 0.999) using tannic acid equivalent (TAE) per gram of dry weight (dw).

2.6. Determination of rutin and quercetin content

Rutin and quercetin quantities were assessed through a high-performance liquid chromatography (HPLC) system, following the method of Ohara et al. (1989) with minor adjustments. After enzyme treatment, saccharification buckwheat samples were filtered using a 0.45 μm ultrasonic membrane filter before being injected into the HPLC. Refer to Table 1 for details. Rutin and quercetin standards for analysis were procured from Sigma, US.

Table 1 Conditions for HPLC analysis of rutin and quercetin

Item	Condition
Instrument	YL 9100 HPLC system
Detector	UV 360 nm
Column	Agilent TC-C18
Mobile (Gradient mode)	A : 0.1% H3PO4 , B: acetonitrile 0~7 min - A 80% : B 20% 15~18 min - A 20% : B 80% 19~23 min - A 80% : B 20%
Flow rate	1 mL/min
Injection volume	50 μL
Temperature	40℃

2.7. Antioxidant activity

2.7.1. DPPH radical scavenging activity

The antioxidant activity of saccharified buckwheat (*Fagopyrum esculentum*), following fermentation with *Aspergillus species* and subsequent treatment with amylase (α-amylase, β-amylase, and glucoamylase), was assessed at various time points using the scavenging activity of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. The evaluation method, as described by Braca et al. (2003) with slight modifications, involved mixing 1 mL of different concentration saccharification samples with 3 mL of DPPH solution (0.15 mM). After vigorous shaking, the mixtures were allowed to stand in the dark at room temperature for 30 minutes. Absorbance was measured at 517 nm using a spectrophotometer against a methanol blank. The scavenging activity was determined using the equation: Radical scavenging activity (%) = (1 - A sample / A control) × 100, where A sample represents the absorbance of the experimental sample, and A control represents the absorbance of the control.

2.7.2. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of saccharified buckwheat (*Fagopyrum esculentum*), following fermentation with *Aspergillus species* and subsequent treatment with amylase (α-amylase, β-amylase, and glucoamylase) at different time points, was determined based on the method by Halliwell et al. (1987) with modifications. In brief, test tubes containing 0.1 ml of various saccharification sample concentrations were mixed with a 1:1:1 ratio solution (10 mM FeCl3, 10 mM EDTA, 10 mM H2O2) and 0.9 ml of 10 mM deoxyribose in potassium phosphate buffer (pH 7.4). The reaction mixture was incubated for 1 hour at 37 °C, followed by additional heating in a boiling water bath at 95 °C for 15 minutes after adding 0.5 ml of 10% trichloroacetic acid (TAC) and 0.5 ml of 1% 2-thiobarbituric acid (TBA). Color development was measured spectrophotometrically at 532 nm. The inhibition percentage was calculated using the formula (%) = 1 - As/Ac × 100, where As and Ac represent the absorbance of the sample group and control group, respectively.

2.7.3. Determination of reducing power

The determination of reducing power was conducted following Oyaizu's method (1986) with modifications. Samples of saccharified buckwheat (*Fagopyrum esculentum*), after fermentation with *Aspergillus species* and subsequent treatment with amylase (α-amylase, β-amylase, and glucoamylase) at different times (0.5 ml), were combined with 0.5 ml of 0.2 M sodium phosphate buffer (pH 6.6). Subsequently, 0.5 ml of 1% potassium ferricyanide was added, and the mixture was incubated at 50 °C for 20 minutes. After cooling to room temperature, 0.5 ml of 10% trichloroacetic acid was introduced. The resulting solution was centrifuged at 2000 rpm for 15 minutes, and 1 ml of the upper layer was combined with 1

ml of distilled water in a tube. To this mixture, 0.2 ml of 0.1% ferric chloride (III) was added, and color development was measured spectrophotometrically at 700 nm.

2.7.4. Determination of ferric reducing antioxidant power (FRAP)

The Ferric Reducing Antioxidant Power (FRAP) of saccharified buckwheat (*Fagopyrum esculentum*), following fermentation with *Aspergillus* species and subsequent treatment with amylase (α-amylase, β-amylase, and gluco-amylase) at various time points, was evaluated using the method outlined by Pulido, et al. (2000) with slight adjustments. A freshly prepared FRAP reagent (1.5 ml), incubated at 37 °C for 30 minutes, was combined with 0.5 µl of saccharification samples. The reaction mixture was then incubated at 37ºC for an additional 30 minutes in a water bath. The FRAP reagent consisted of 1 ml of 10 mM TPTZ solution (2,4,6-Tris (2-pyridyl)-s-triazine) in 40 mM HCl, 1 ml of 20 mM FeCl3-6H2O, and 10 ml of 0.3 M acetate buffer (pH 3.6). After incubation, absorbance readings were promptly taken at 593 nm using a spectrophotometer (Model UV-1800, Shimadzu Corporation, Kyoto, Japan).

2.8. Determination of macrophage proliferation and nitric oxide production

The RAW264.7 macrophage cell line, cultured in RPMI–1640 medium with 10% FBS, was seeded in a 96-well plate at a density of 1×105 cells/well obtained from ATCC. Triplicate wells were incubated with saccharification samples at varying concentrations (12.5, 25, and 50 µg/mL). Following a 24-hour incubation in a 5% CO2 humidified atmosphere at 37 °C, 20 µL of WST–1 solution was added, and further incubation occurred for 4 hours at 37 °C. Optical density at 450 nm was measured using a microplate reader (EL–800, BioTek Instruments, Winooski, VT, USA). Absorbance was converted to macrophage proliferation ratio (%) = At/Ac × 100, where At and Ac represent the absorbance of the test and control groups, respectively. Nitric oxide (NO) production in RAW264.7 cell culture supernatant (1×105 cells/well), exposed to saccharification of buckwheat by amylase treatments at different concentrations (12.5, 25, and 50 µg/mL) and LPS (1 µg/mL) at 37 °C for 24 hr, was assessed using the Griess reaction (Green et al., 1982), with NO production calculated based on a standard curve obtained with NaNO2 (1–200 µM in culture medium).

2.9. Statistical analysis

All results were presented as the mean ± standard deviation based on three experiments conducted using Microsoft programs. Variations among mean values across multiple groups were assessed through analysis of variance (ANOVA) using the general linear model and Tukey's method in Minitab 16.0. Significance levels were established at $p<0.05$.

3. Results and Discussion

3.1. Changes in soluble solid content (°Brix) during saccharification of the buckwheat

Buckwheat (*Fagopyrum esculentum*) underwent saccharification and fermentation with *Aspergillus species*, followed by treatment with different amylases (α-amylase, β-amylase, and glucoamylase) at varying times. Results showed that after fermenting with *Aspergillus Oryzae 82-3* and subsequent β-amylase treatment, the buckwheat exhibited higher sugar content at 13.8 Brix° in 8 hours. Another treatment, *Aspergillus Oryzae CF1003* with β-amylase, showed 13.4 Brix°, while low sugar content was observed with glucoamylase treatment at 8.6 Brix°. Overall, extended treatment time increased sugar content, with *Asp-82-3* and β-amylase yielding the highest values. The heating-moisture during saccharification, starch gelatinization, and enzyme effects contributed to sugar release. α-amylase hydrolyzed starch, leading to increased sugar content in saccharified buckwheat during fermentation and amylase treatments. Literature suggests that various amylase enzymes hydrolyze starch, converting it from poly-saccharide to mono-saccharide and enhancing sugar content.

3.2. Effect of amylase treatments on total carbohydrates and proteins

In analyzing the total carbohydrate content during the saccharification of buckwheat post-fermentation with *Aspergillus species* and subsequent amylase treatment, we observed a significant increase, especially with enzyme treatment over an extended period. Statistical analysis indicated a significant interaction ($p<0.05$) between ferment/enzyme treatment and time. Notably, Asp-82-3 with β-amylase showed a rapid and stable increase, reaching 43.95% after 8 hours. The control (fermentation-only) exhibited lower total carbohydrate levels (Fig 3). Amylase treatments, particularly *Asp-82-3* with β-amylase, significantly increased total carbohydrate compared to the control. This aligns with findings in literature, such as Bonafaccia et al. (2003) reporting 55.8% starch content in buckwheat grains. Additionally, the experiment supported the use of *Aspergillus Oryzae* amylases for enhancing total carbohydrate content in buckwheat saccharification.

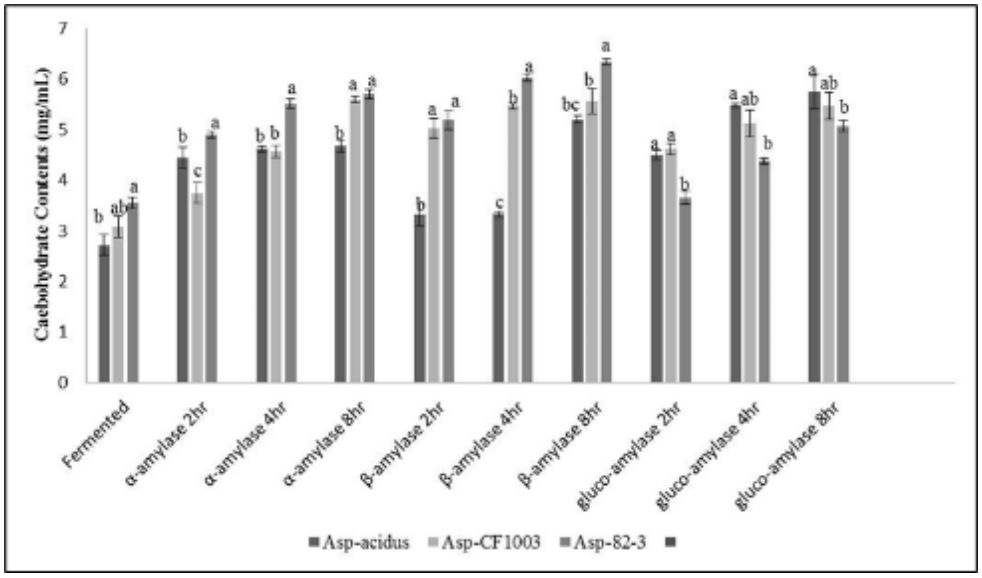

Figure 3 Effect of saccharification of buckwheat (*Fagopyrum esculentum*) after fermented with *Aspergillus species* and subsequently treated with amylases samples on Total carbohydrate contents. The results were presented in mean±standard deviation, and those with different letters in the same column are significantly different compared to controls ($p<0.05$)

Fig. 4 illustrates that saccharification of buckwheat after fermentation with *Aspergillus species* and subsequent amylase treatments significantly increased ($p<0.05$) protein content in a time-dependent manner, peaking at 8 hours. *Asp-82-3* with β-amylase treatment yielded the highest protein content (21.19mg/mL), followed by *Asp-82-3* with glucoamylase (18.13mg/mL) and *Asp-CF1003* with β-amylase (15.25mg/mL). The control exhibited decreased protein content compared to saccharification, with *Asp-82-3* (1.15mg/mL) showing the lowest value. This suggests that amylase treatments enhance protein content during buckwheat saccharification, while the control experiences a reduction, possibly due to fermentation-induced alterations in substrate composition. Similar observations have been noted in other studies, indicating the impact of microbial actions on protein content. mixture by the action of bacteria and yeasts.

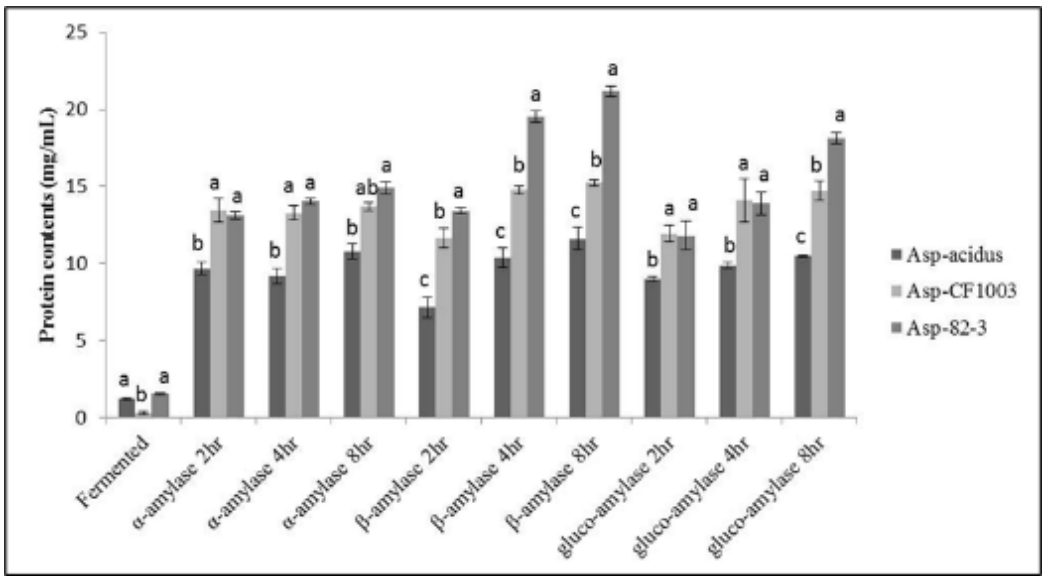

Figure 4 Effect of saccharification of buckwheat (*Fagopyrum esculentum*) after fermented with *Aspergillus species* and subsequently treated with amylases samples on protein contents. The results were presented in mean ± standard deviation, and those with different letters in the same column are significantly different compared to controls ($p<0.05$).

3.3. Effect of amylase treatments on total polyphenol content (TPC)

Following a fermentation-only period of 72 hours, a minor increase in phenolic content was observed, whereas in amylase treatments post-fermentation with *Aspergillus species*, a substantial rise occurred during the buckwheat saccharification process (Fig. 5). Comparing amylase treatments with the control (fermentation-only), the total polyphenol content (TPC) in buckwheat saccharification was significantly higher at $p<0.05$. Phenolic content predominantly increased with enzyme treatment after fermentation with *Aspergillus species* and over an extended time. Statistical analysis indicated a significant elevation at $p<0.05$ for enzyme treatment after fermentation with *Aspergillus* species and increased duration. The highest TPC (9.10mgTAE/g) was attained with *Asp-82-3* and β-amylase treatment after 8 hours, followed by *Asp-82-3* with α-amylase (9.00mgTAE/g) and Asp-Acidus with glucoamylase (8.44mgTAE/g) at 8 hours, compared to the control (2.58, 1.47, and 1.42mg/TAE/g for Asp-82-3, Asp-Acidus, and Asp-CF1003, respectively). While there was no significant increase in PTC with α-amylase treatment after fermentation with *Asp-Acidus* and *Asp-CF1003*, they exhibited significant ($p<0.05$) increases compared to the control. This underscores that the presence of amylase treatments post-fermentation with *Aspergillus* was the primary factor leading to increased TPC in buckwheat saccharification. The study implies that different *Aspergillus species*, when fermented and subsequently treated with distinct amylase treatments, show significantly different TPC increases in buckwheat seeds depending on time, potentially influenced by substrate type, fungus, and fermentation conditions. This aligns with findings suggesting that fermentation-induced structural breakdown of cell walls may liberate or synthesize bioactive compounds. Additionally, the increase in phenolic content in amylase-treated samples may result from both heating and amylase hydrolysis, as the latter occurs under heating conditions. The hydrolysis of α-amylase breaking α-1,4-glycosidic bonds in starch to form maltose further contributes to the release of bound phenolic compounds, leading to a time-dependent increase in phenolic content with a peak at 8 hours of enzyme hydrolysis compared to the control.

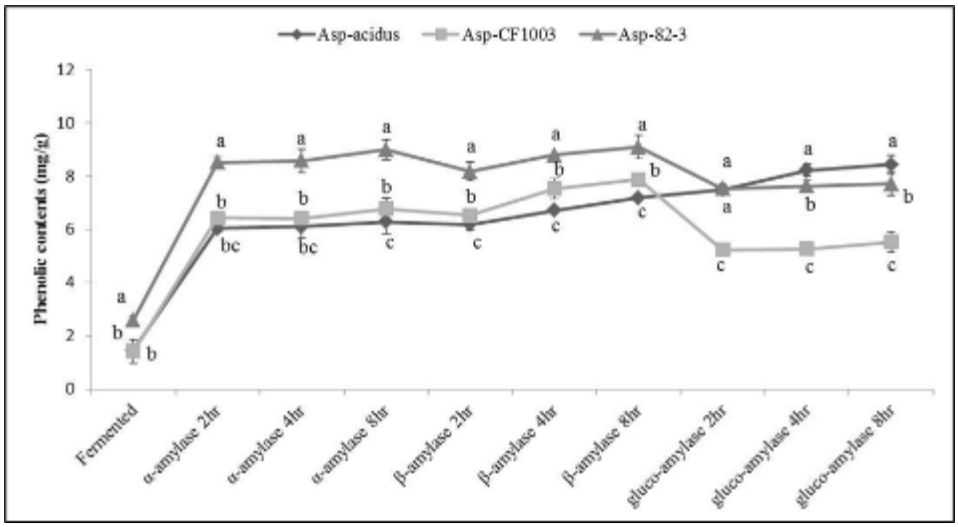

Figure 5 Effect of saccharification of buckwheat (*Fagopyrum esculentum*) after fermented with *Aspergillus species* and subsequently treated with amylases on Total polyphenol content. The results were presented in mean ± standard deviation, and those with different letters in the same column are significantly different compared to controls ($p<0.05$)

3.4. Effect of amylase treatments on rutin and quercetin content

This investigation focused on the phenolic compounds (rutin and quercetin) in the saccharification of buckwheat post-fermentation with *Aspergillus species* and subsequent amylase treatments, comparing them with the control (fermentation-only). The results indicate higher rutin and quercetin content in amylase treatments after fermentation with *Aspergillus species* compared to the control. Maximum rutin content was observed at 2 hours with *Asp-82-3* with β-amylase, *Asp-82-3* with α-amylase, and *Asp-CF1003* with α-amylase (4.79±0.13, 4.33±0.01, and 4.19±0.08mg/100g respectively). Maximum quercetin content occurred at 2 hours with *Asp-82-3*, *Asp-CF1003*, and *Asp-Acidus* with β-amylase (9.04±0.14, 8.45±0.16, and 7.76±0.13mg/100g, respectively) during buckwheat saccharification (Fig. 6 and Fig. 7). In amylase treatments after fermentation with *Aspergillus species*, rutin and quercetin content was significantly higher ($p<0.05$) compared to the control (0.27±0.1 of *Asp-82-3*, 0.16±0.03 of *Asp-CF1003*, and 0.10±0.03mg/100g of *Asp-Acidu*, respectively) for rutin, and quercetin content was 1.17±0.15 of Asp-82-3, 1.65±0.1mg/100g of *Asp-Acidus*, and 0.75±0.19 of *Asp-CF1003*, respectively. HPLC results showed that all *Aspergillus species* fermented with β-amylase had the highest quercetin content, significantly surpassing α-amylase and glucoamylase. α-amylase and glucoamylase

treatments post-fermentation with *Aspergillus species* also significantly increased rutin and quercetin content compared to the controls. The study concludes that amylase treatments, particularly after fermentation with *Aspergillus species*, significantly elevate rutin and quercetin content in buckwheat saccharification. Additionally, the lower rutin levels may be attributed to rutin-degrading enzymes activated during milling, causing losses during processing. This aligns with findings suggesting that amylase treatments release phenolic compounds, consistent with the observed increase in total polyphenol content.

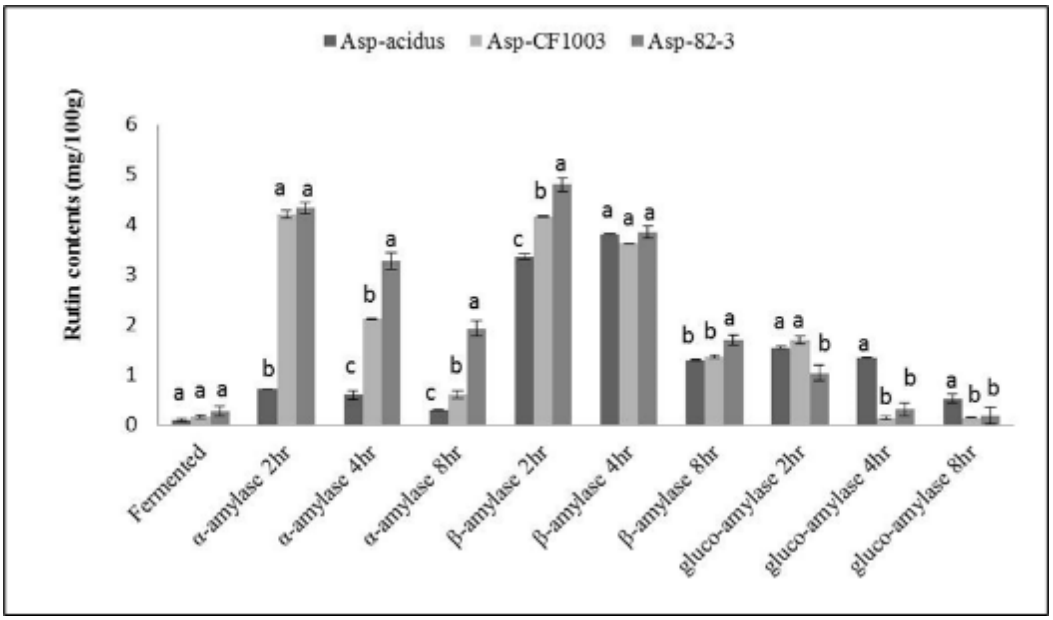

Figure 6 Rutin content of saccharification of buckwheat (*Fagopyrum esculentum*) after fermented with *Aspergillus species* and subsequently treated with amylases. The results were presented in mean ± standard deviation, and those with different letters in the same column are significantly different compared to controls ($p<0.05$)

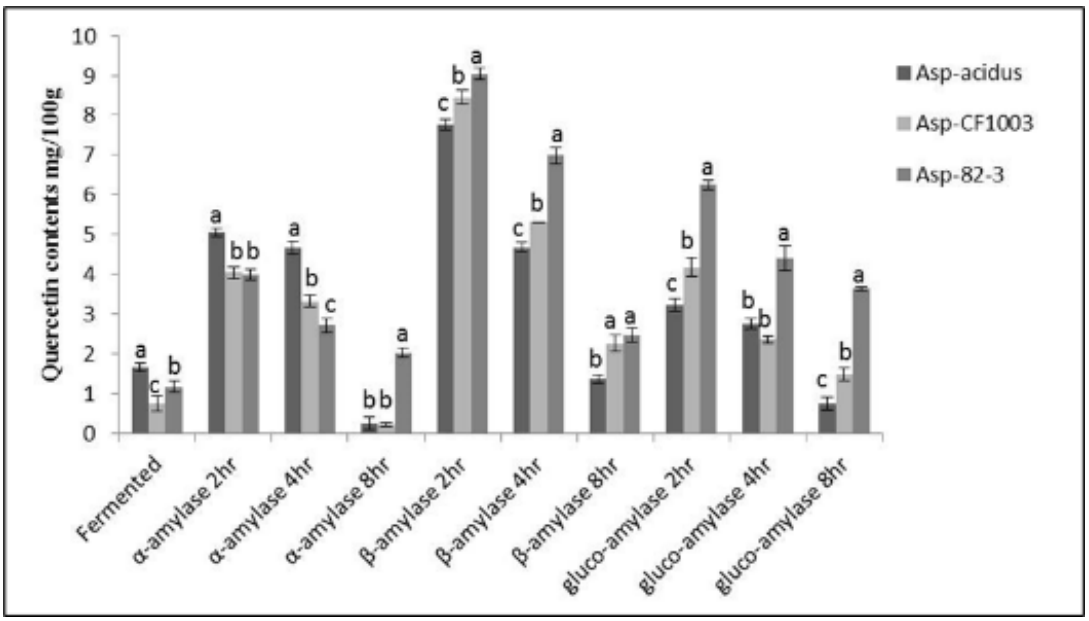

Figure 7 Quercetin content of saccharification of buckwheat (*Fagopyrum esculentum*) after fermented with *Aspergillus species* and subsequently treated with amylases. The results were presented in mean ± standard deviation, and those with different letters in the same column are significantly different compared to controls ($p<0.05$)

3.5. Effect of amylase treatments on vitro antioxidant activity

3.5.1. DPPH free radical scavenging activity

In the DPPH assay, each amylase treatment following fermentation with *Aspergillus species* in the saccharification of buckwheat seeds demonstrated potent free radical scavenging activity. Fig. 8 presents the results of the saccharification of buckwheat (*Fagopyrum esculentum*) after fermentation with *Aspergillus species* and subsequent amylase treatment (α-amylase, β-amylase, and glucoamylase). The findings indicate that all *Aspergillus species* treated with amylase (α-amylase, β-amylase, and glucoamylase) exhibited significantly higher ($p<0.05$) antioxidant activity compared to the control (fermentation-only). Furthermore, the results revealed a potential increase in antioxidant activity in enzyme treatments after fermentation with *Aspergillus species* over an extended period, with statistical significance ($p<0.05$) observed for enzyme treatment duration. The highest antioxidant activity (87.39%) was observed after 8 hours of *Asp-82-3* with β-amylase treatment, followed by *Asp-82-3* with α-amylase (85.58%) and *Asp-CF1003* with α-amylase (81.52%) at 8 hours. Notably, *Asp-82-3* with β-amylase showed the highest DPPH free radical scavenging activity, significantly different from the control (64.16%, 55.92%, and 33.03% of *Asp-82-3*, *Asp-CF1003*, and *Asp-Acidus*, respectively). The control group (*Asp-82-3, Asp-CF1003*, and *Asp-Acidus*) also displayed significant antioxidant activity at $p<0.05$. The results indicate that the increase in phenolic compounds induced antioxidant activity in amylase treatments, aligning with previous studies that associate fermentation with positive effects on total phenolic content (TPC) and antioxidative activity in cereals. The relationship between phenolic compounds, antioxidant activity, and the presence of DPPH free radicals suggests that phenolic compounds, including quercetin and rutin, contribute significantly to this antioxidative activity.

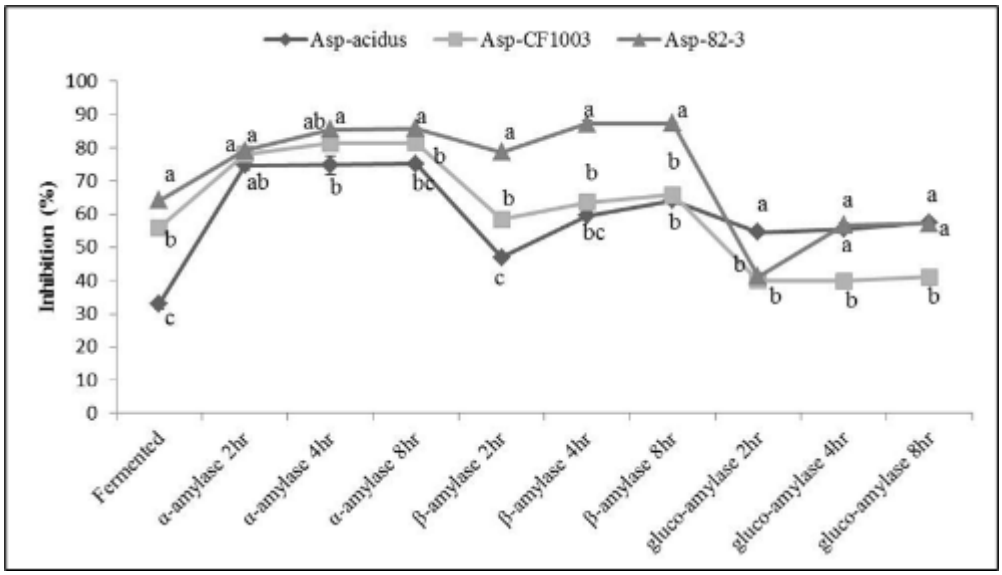

Figure 8 DPPH free radical of saccharification of buckwheat (*Fagopyrum esculentum*) after fermented *with Aspergillus species* and subsequently treated with amylases. The results were presented in mean ± standard deviation, and those with different letters in the same column are significantly different compared to controls ($p<0.05$)

3.5.2. Hydroxyl radical scavenging activity

The hydroxyl radical, known for its high reactivity and shortest half-life among reactive oxygen species, causes severe damage to adjacent biomolecules (Sakanaka et al., 2005). The impact of hydroxyl radical scavenging activity on the saccharification of buckwheat after fermentation with *Aspergillus species*, subsequent treatment with amylases, and the control (fermentation-only) was examined. The hydroxyl radical scavenging activity assay revealed that *Asp-82-3* with β-amylase exhibited significantly higher scavenging activity compared to the control, reaching 87.81% at 8 hours. *Asp-82-3* with α-amylase (82.75%) and *Asp-CF1003* with β-amylase (80.40%) also displayed notable scavenging activity at $p<0.05$. Among the treatments, *Asp-82-3* with β-amylase showed the highest hydroxyl radical scavenging activity, significantly different from the control (61.00%, 54.23%, and 28.52% of Asp-82-3, *Asp-CF1003*, and *Asp-Acidus*, respectively). The control group (*Asp-82-3*, *Asp-CF1003*, and *Asp-Acidus*) exhibited significant differences in antioxidant activity at $p<0.05$ in Fig. 9. Additionally, enzyme treatments after fermentation with *Aspergillus species* and extended duration showed potential high antioxidant activity, with statistical significance ($p<0.05$) over time. The hydroxyl radical scavenging activity mirrored DPPH free-radical scavenging ability and total phenolic content (TPC), indicating substantial differences in antioxidant activities among enzymatic treatments after fermentation with *Aspergillus species*.

The study underscores the significant role of amylase treatments post-fermentation in causing potential hydroxyl radical scavenging activity in buckwheat seed saccharification, linking the heightened presence of hydroxyl radicals to increased phenolic compounds and induced antioxidant activity with amylase treatment.

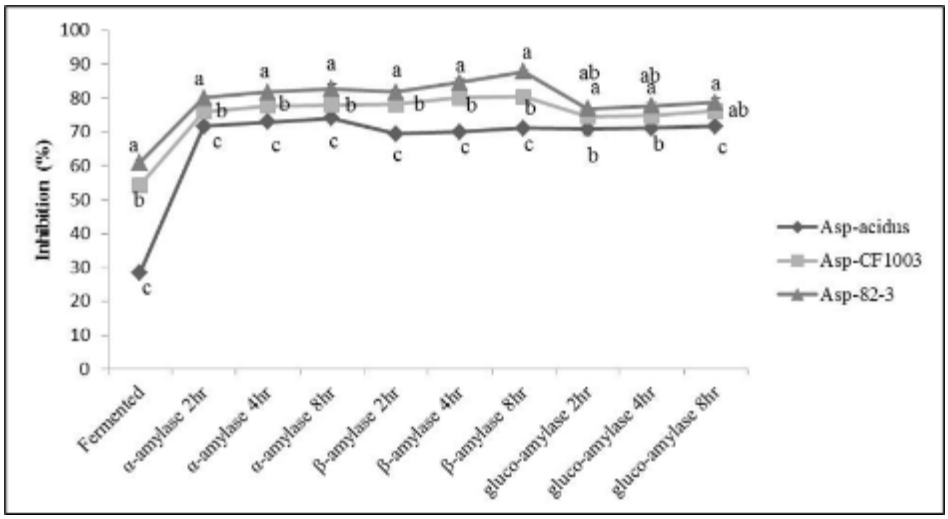

Figure 9 The Hydroxyl radical scavenging activity of saccharification of buckwheat (*Fagopyrum esculentum*) after fermented with *Aspergillus species* and subsequently treated with amylases. The results were presented in mean ± standard deviation, and those with different letters in the same column are significantly different compared to controls ($p<0.05$)

3.5.3. Production of reducing activity

In this study, the assessment of reducing activity was centered on the conversion of the Fe3+/ferricyanide complex to the ferrous form in the presence of antioxidants within the tested samples. The resulting Fe2+ was quantified by detecting Perl's Prussian blue formation at 700 nm, with an increase in absorbance indicating higher reduction capability (Oyaizu, 1986). Fig 10 illustrates the reducing activity of various amylase treatments after fermentation with *Aspergillus species* in comparison to the control (fermentation-only) treatments during the saccharification of common buckwheat. The data reveals that all amylase treatments post-fermentation with *Aspergillus species* exhibited significantly higher absorbance in saccharification, nearly or more than double that of the control. The most substantial and higher reducing activity was observed at 8 hours with *Asp-82-3* with β-amylase, displaying an absorbance of 2.45±0.03, followed by *Asp-82-3* with α-amylase (absorbance 2.47±0.03) and *Asp-CF1003* with β-amylase (absorbance 2.42±0.05). Among the treatments, *Asp-82-3* with β-amylase demonstrated the highest reducing activity, significantly different from the control (1.13±0.01, 0.95±0.01, and 0.67±0.02 of *Asp-82-3*, *Asp-CF1003*, and *Asp-Acidus*, respectively). Additionally, the control group (*Asp-82-3, Asp-CF1003*, and *Asp-Acidus*) exhibited a significant difference in antioxidant activity at $p<0.05$. The increase in reducing activity with fungal fermentation aligns with previous findings by Yang, et al. (2000), attributing the enhanced reducing power to the formation of reductants that stabilize and terminate radical chain reactions during fermentation. Although fermentation contributes to this effect to some extent, the reducing activity was lower than in amylase treatment after fermentation with *Aspergillus species* during the saccharified buckwheat period. This underscores that the presence of amylase treatment post-fermentation with *Aspergillus species* is the primary factor driving the increased reducing activity in buckwheat saccharification. Furthermore, significant differences in antioxidant activities were noted among different enzymatic treatments after fermentation with *Aspergillus species*, highlighting the noteworthy antioxidative activity of reducing power induced by enzyme processing. The study suggests that the heightened presence of reducing power is likely attributable to increased phenolic compounds, inducing antioxidant activity with amylase treatment. Correspondingly, a previous study by Li et al. (2013) explored potential correlations between total phenolic content, reducing power, and antioxidant activity in hulls, brans, and flour from buckwheat.

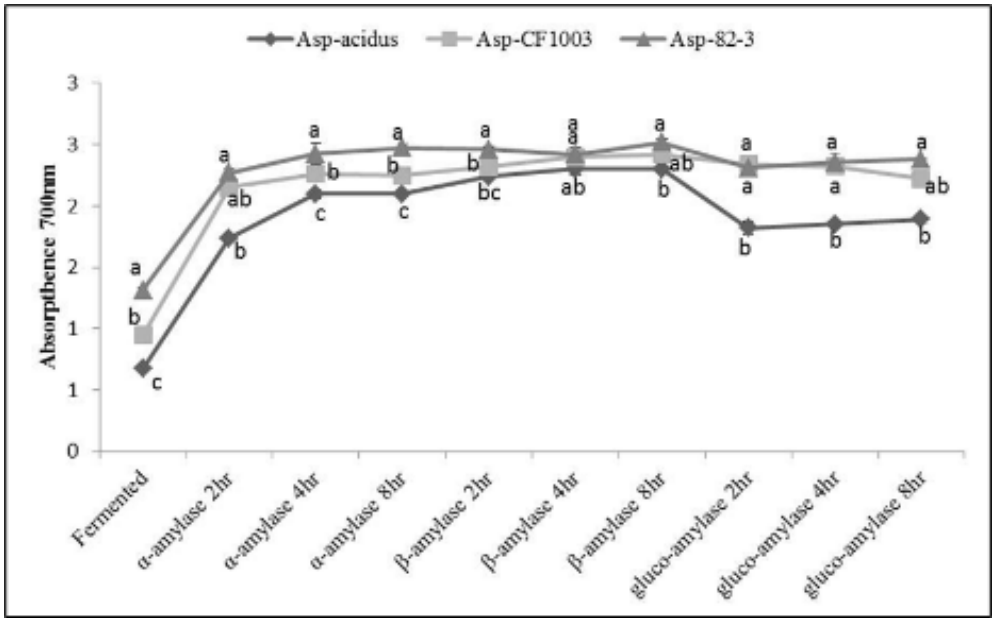

Figure 10 Reducing power activity of saccharification of buckwheat (*Fagopyrum esculentum*) after fermented with *Aspergillus species* and subsequently treated with amylases. The results were presented in mean ± standard deviation, and those with different letters in the same column are significantly different compared to controls ($p<0.05$)

3.5.4. Ferric reducing antioxidant power (FRAP)

The antioxidant potential of buckwheat saccharification, involving fermentation with *Aspergillus species* and subsequent amylase treatment, was markedly enhanced ($p<0.05$) compared to the control (fermentation-only). Results indicated superior antioxidant activity in amylase-treated samples after fermentation with *Aspergillus species*, particularly in 8 hours of *Asp-82-3* with β-amylase (2.1±0.03), followed by *Asp-82-3* with α-amylase (1.86±0.05) and *Asp-82-3* with glucoamylase (1.75±0.03). The FRAP value exhibited a rapid increase, reaching 55.67%, 44.51%, and 40.68% in 2 hours of *Asp-82-3* with β-amylase, *Asp-82-3* with gluco + amylase, and *Asp-82-3* with α-amylase, respectively, stabilizing at 59.05% in *Asp-82-3* with β-amylase after 8 hours. While the control displayed comparable antioxidant activity among *Aspergillus* species fermentations, *Asp-82-3* fermentation exhibited higher activity than *Asp-CF1003* and *Asp-Acidus* fermentations. The lowest antioxidant activity among fermented samples was noted in *Asp-Acidus* fermentation, and after fermentation with *Aspergillus species* and subsequent amylase treatment, *Asp-Acidus* treatment showed the lowest antioxidant activity compared to other *Aspergillus species* (Fig 11). Thus, the presence of amylase treatments post-fermentation with *Aspergillus species* was the key factor in increasing FRAP in buckwheat saccharification. The rise in phenolic content due to amylase treatments after fermentation with *Aspergillus species* likely contributed to the heightened FRAP values. Additionally, the potential lower sugar content in amylase-treated samples might be a partial factor enhancing FRAP values by reducing ions like Fe3+ and Cu2+, promoting TPTZ-Fe II complex formation. These findings highlight that overall antioxidant activity in buckwheat saccharification is augmented by amylase treatments after fermentation with *Aspergillus species*, a trend consistent with the relationship between antioxidant activity and phenol content reported in previous studies (Corral-Aguayo et al., 2008; Wong et al., 2006).

3.6. Effect of saccharification of buckwheat by amylase treatments on macrophage proliferation and nitric oxide production

The saccharification of buckwheat (*Fagopyrum esculentum*) after fermentation with *Aspergillus species*, followed by amylase treatment, exhibited elevated rutin and quercetin content at the 2-hour mark, prompting an investigation into their impact on proliferation and nitric oxide (NO) production in lipopolysaccharide (LPS)-activated murine macrophage RAW 264.7 cells. Cell proliferation in response to amylase treatments after *Aspergillus species* fermentation, compared to the control (fermentation-only), was assessed at concentrations of 12.5–50 μg/mL. The results demonstrated significantly higher ($p<0.05$) cell proliferation in amylase-treated samples, with *Asp-82-3* and β-amylase leading with 124.2% at a concentration of 12.5 μg/mL, followed by *Asp-82-3* with α-amylase (122.34%) and *Asp-CF1003* with β-amylase (118.58%). Notably, *Asp-82-3* with β-amylase displayed the highest cell proliferation, significantly surpassing the control (96.76%, 90.88%, and 88.36% for *Asp-82-3*, *Asp-CF1003*, and *Asp-Acidus*, respectively). The control group also exhibited significant differences in antioxidant activity. This suggests that amylase

treatment post-fermentation with *Aspergillus species* did not impart toxicity to RAW264.7 cells within the tested concentration range (Fig 12).

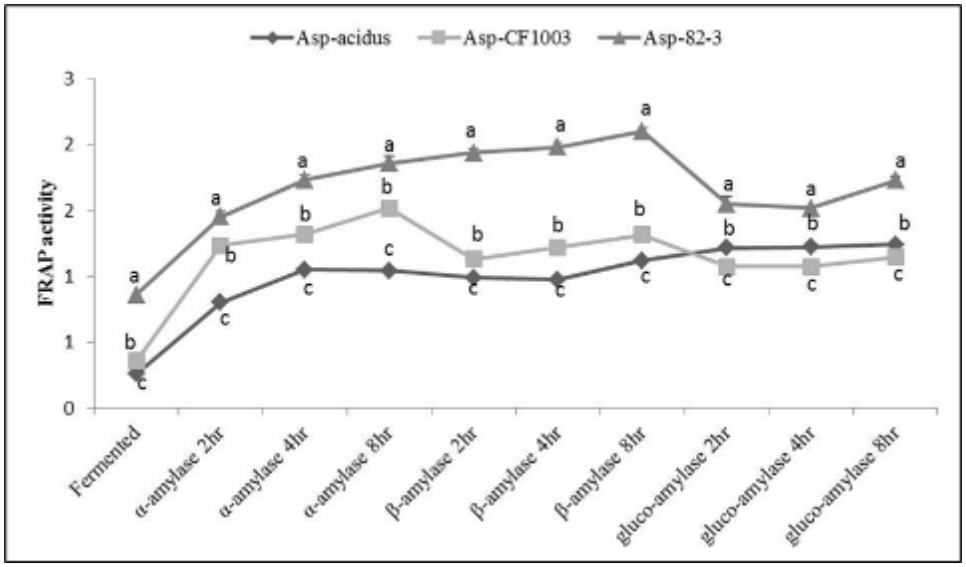

Figure 11 Ferric reducing antioxidant power (FRAP) of saccharification of buckwheat (*Fagopyrum esculentum*) after fermented with *Aspergillus species* and subsequently treated with amylases. The results were presented in mean ± standard deviation, and those with different letters in the same column are significantly different compared to controls ($p<0.05$)

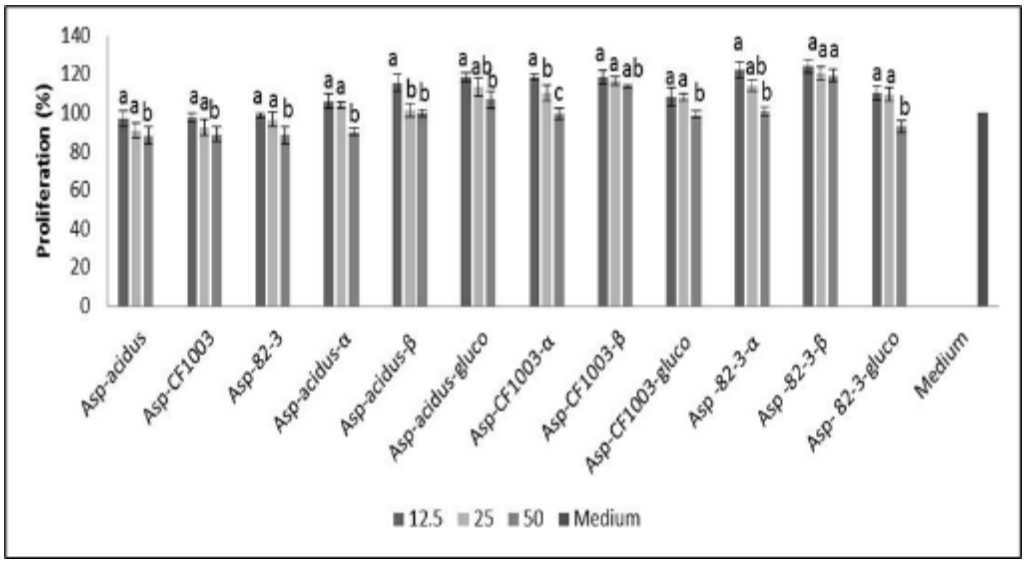

Figure 12 Cell proliferation of saccharification of buckwheat (*Fagopyrum esculentum*) after fermented with *Aspergillus species* and subsequently treated with amylases. The results were presented in mean ± standard deviation, and those with different letters in the same column are significantly different compared to controls ($p<0.05$)

Moreover, nitric oxide (NO) production, evaluated using the lipopolysaccharide (LPS) method, significantly increased ($p<0.05$) in saccharification samples subjected to amylase treatments after *Aspergillus species* fermentation compared to the control. *Asp-82-3* with β-amylase treatment demonstrated potent NO production against LPS-induced nitric oxide release, registering a value of 52.21 µM, followed by *Asp-82-3* with α-amylase (48.85 µM) and *Asp-CF1003* with β-amylase (46.1 µM) at a concentration of 12.5 µg/mL (Fig 13). All treatments markedly elevated NO levels from RAW264.7 cells compared to the control (31.44 µM, 30.46 µM, and 30.44 µM for *Asp-82-3, Asp-CF1003*, and *Asp-Acidus*, respectively) at 12.5 µg/mL, comparable to the positive control (LPS) at 1 µg/mL. The experiment suggested that the

use of amylase after fermentation with *Aspergillus species* could enhance nitric oxide (NO) in buckwheat saccharification. Previous literature suggests that various enzymes in amylase hydrolyze starch to monosaccharides, increasing nitric oxide (NO) production. Additionally, the study implied that saccharification of buckwheat by amylase treatment after fermentation with *Aspergillus species* had the potential to act as a NO producer from RAW264.7 cells.

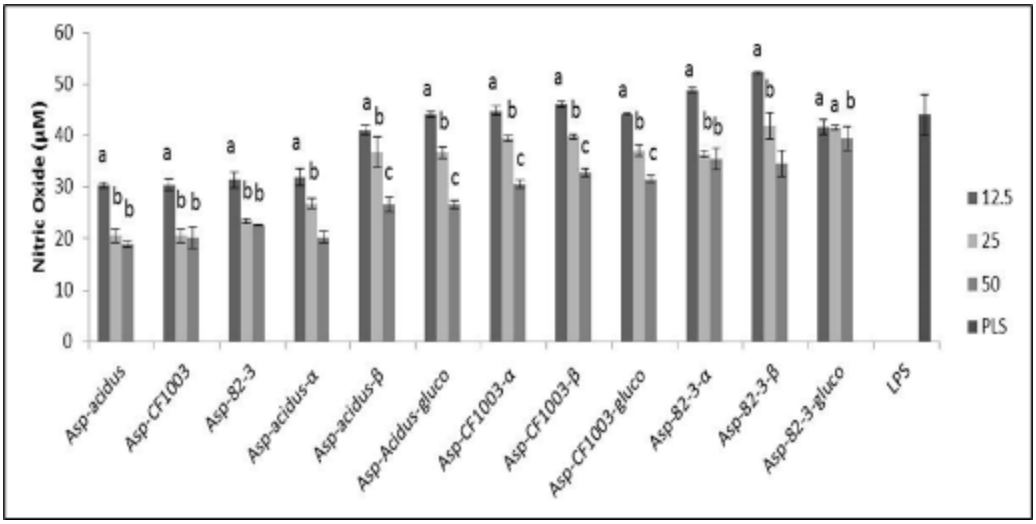

Figure 13 Nitric Oxide (NO) of saccharification of buckwheat (*Fagopyrum esculentum*) after fermented with *Aspergillus species* and subsequently treated with amylases. The results were presented in mean ± standard deviation, and those with different letters in the same column are significantly different compared to controls ($p<0.05$)

Table 2 Changes in soluble solid content (Brix°) during saccharification of the buckwheat

Treatments	***Aspergillus Aciduc 46420***			***Aspergillus Oryzae CF1003***			***Aspergillus Oryzae KACC 82-3***		
	Hours								
	2	**4**	**8**	**2**	**4**	**8**	**2**	**4**	**8**
α-amylase	12	12.6	12.6	12	12.4	12.6	12.4	12.6	12.8
β-amylase	9.7	11.5	13.3	11	12.6	13.6	11.4	13	13.8
glucoamylase	12.1	13.1	14.4	8.6	9.8	11	9.8	10.6	11.8

4. Conclusion

This study examined the saccharification of buckwheat (*Fagopyrum esculentum*) following *Aspergillus species* fermentation and subsequent treatment with amylases to enhance phenolic compounds and antioxidant activity. The results suggest that using amylase after *Aspergillus* fermentation boosts bioactive compounds in buckwheat saccharification, altering the ratio of phenolic compounds, antioxidant activity, rutin, and quercetin content. Amylase treatment influences the potential bioactive constituents, providing a method to produce high-antioxidant phenolic compound buckwheat products. These findings contribute to understanding underutilized buckwheat and improving its biological activity, offering valuable insights for enhancing buckwheat saccharification or formulating functional food ingredients.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

References

[1] Amaro, M. I., Rocha, J., Vila-Real, H., Eduardo-Figueira, M., Mota-Filipe, H., Sepodes, B., & Ribeiro, M. H. (2009). Anti-inflammatory activity of naringin and the biosynthesised naringenin by naringinase immobilized in microstructured materials in a model of DSS-induced colitis in mice. Food Research International, 42(8), 1010-1017.

[2] Ameh, S. J., Obodozie, O. O., Inyang, U. S., Abubakar, M. S., & Garba, M. (2010). Current phytotherapy-A perspective on the science and regulation of herbal medicine. Journal of Medicinal Plants Research, 4(2), 072-081.

[3] Braca, A., Fico, G., Morelli, I., De Simone, F., Tomè, F., & De Tommasi, N. (2003). Antioxidant and free radical scavenging activity of flavonol glycosides from different Aconitum species. Journal of Ethnopharmacology, 86(1), 63-67.

[4] Bonafaccia, G., Marocchini, M., & Kreft, I. (2003). Composition and technological properties of the flour and bran from common and tartary buckwheat. Food chemistry, 80(1), 9-15.

[5] Corral-Aguayo, R. D., Yahia, E. M., Carrillo-Lopez, A., & González-Aguilar, G. (2008). Correlation between some nutritional components and the total antioxidant capacity measured with six different assays in eight horticultural crops. Journal of Agricultural and Food Chemistry, 56(22), 10498-10504.

[6] Daba, G. M., Mostafa, F. A., & Elkhateeb, W. A. (2021). The ancient koji mold (*Aspergillus* oryzae) as a modern biotechnological tool. Bioresources and Bioprocessing, 8(1), 52.

[7] Dartora, A. B., Bertolin, T. E., Bilibio, D., Silveira, M. M., & Costa, J. A. (2002). Evaluation of filamentous fungi and inducers for the production of endo-polygalacturonase by solid state fermentation. Zeitschrift für Naturforschung C, 57(7-8), 666-670.

[8] de Araújo, M. E. M. B., Franco, Y. E. M., Alberto, T. G., Sobreiro, M. A., Conrado, M. A., Priolli, D. G., & de Oliveira Carvalho, P. (2013). Enzymatic de-glycosylation of rutin improves its antioxidant and antiproliferative activities. Food chemistry, 141(1), 266-273.

[9] Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. T., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. Analytical chemistry, 28(3), 350-356.

[10] Filisetti-Cozzi, T. M., & Carpita, N. C. (1991). Measurement of uronic acids without interference from neutral sugars. Analytical biochemistry, 197(1), 157-162.

[11] Franco, C. M., Preto, S. J. D., & Ciacco, C. F. (1987). Studies on the susceptibility of granular cassava and com starches to enzymatic attack part I: study of the conditions of hydrolysis. Starch-Stärke, 432-435.

[12] Ghimeray, A. K., Sharma, P., Phoutaxay, P., Salitxay, T., Woo, S. H., Park, S. U., & Park, C. H. (2014). Far infrared irradiation alters total polyphenol, total flavonoid, antioxidant property and quercetin production in tartary buckwheat sprout powder. Journal of Cereal Science, 59(2), 167-172.

[13] Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., & Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [15 N] nitrate in biological fluids. Analytical biochemistry, 126(1), 131-138.

[14] Halliwell, B., Gutteridge, J. M., & Aruoma, O. I. (1987). The deoxyribose method: a simple “test-tube” assay for determination of rate constants for reactions of hydroxyl radicals. Analytical biochemistry, 165(1), 215-219.

[15] Hegde, S., Kavitha, S., Varadaraj, M. C., & Muralikrishna, G. (2006). Degradation of cereal bran polysaccharide-phenolic acid complexes by *Aspergillus* niger CFR 1105. Food Chemistry, 96(1), 14-19.

[16] http://nootriment.com/*Aspergillus*-oryzae/ (28.04.2023). *Aspergillus* Oryzae: Health Benefits of this Probiotic Enzyme, NOOTRIMENT.

[17] Jørgensen, T. R. (2007). Identification and toxigenic potential of the industrially important fungi, *Aspergillus Oryzae* and *Aspergillus* sojae. Journal of food protection, 70(12), 2916-2972.

[18] Li, F. H., Ya, Y. U. A. N., YANG, X. L., TAO, S. Y., & Jian, M. I. N. G. (2013). Phenolic profiles and antioxidant activity of buckwheat (Fagopyrum esculentum Möench and Fagopyrum tartaricum L. Gaerth) hulls, brans and flours. Journal of integrative Aricuture, 12(9), 1684-1693.

[19] Nielsen, I. L. F., Chee, W. S. S., Poulsen, L., Offord-Cavin, E., Rasmussen, S. E., & Frederksen, H. (2006). Bioavailability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: A randomized, double-blind, crossover trial. Journal of Nutrition, 136, 404–408.

[20] Oates, C. G. (1997). Towards an understanding of starch granule structure and hydrolysis. Trends in Food Science & Technology, 8(11), 375-382.

[21] Ohara, T., Matsumoto, Y., & Ohashi, H. (1989). The film formation dynamics in spin coating. Physics of Fluids A: Fluid Dynamics, 1(12), 1949-1959.

[22] Oliveira, M. D. S., Feddern, V., Kupski, L., Cipolatti, E. P., Badiale-Furlong, E., & de Souza-Soares, L. A. (2010). Physico-chemical characterization of fermented rice bran biomass Caracterización fisico-química de la biomasa del salvado de arroz fermentado. CyTA–Journal of Food, 8(3), 229-236.

[23] Oyaizu, M. (1986). Studies on product browning reaction prepared from glucoseamine. Japanese Journal of Nutrition, 44, 307–315.

[24] Pulido, R., Bravo, L., & Saura-Calixto, F. (2000). Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. Journal of Agricultural and Food Chemistry, 48, 3396-3402.

[25] Sakanaka, S., Tachibana, Y., & Okada, Y. (2005). Preparation and antioxidant properties of extracts of Japanese persimmon leaf tea (kakinoha-cha). Food chemistry, 89(4), 569-575.

[26] Schmidt, C. G., & Furlong, E. B. (2012). Effect of particle size and ammonium sulfate concentration on rice bran fermentation with the fungus Rhizopus oryzae. Bioresource Technology, 123, 36-41.

[27] Singleton, V. L., & Rossi, J.A. (1965). Calorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture, 16, 144–158.

[28] Wong, S. P., Leong, L. P., & Koh, J. H. W. (2006). Antioxidant activities of aqueous extracts of selected plants. Food chemistry, 99(4), 775-783.

[29] Yang, J. H., Mau, J. L., Ko, P. T., & Huang, L. C. (2000). Antioxidant properties of fermented soybean broth. Food chemistry, 71(2), 249-254.