

Integrated Optimization of Bioprocessing (Germination and Fermentation) and Ultrasound-Assisted Extraction: Maximizing Bioactive Compound Content and Antioxidant Activity for a Novel Adzuki Bean Beverage

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Received: 2 March 2026, Revised: 17 April 2026, Accepted: 24 April 2026, Published: 5 June 2026

Abstract

Adzuki beans are rich in phenolic compounds but have limited bioavailability and consumer acceptance. This study developed an integrated germination-fermentation-extraction platform to maximize bioactive content in functional beverages. Germination (27 °C, 68 h) was optimized via response surface methodology, achieving 3.5-fold phenolic enhancement. Sequential fermentation with *Saccharomyces cerevisiae* B5 (25 °C, pH 4.5, 72 h) further increased phenolics 5.1-fold versus raw beans. Ultrasonic extraction (50% ethanol, 20:1 ratio, 30 min) yielded 6.1-fold total phenolic increase (18.05 mg GAE/g DW) and 7.7-fold antioxidant enhancement. UPLC analysis revealed 16.9-fold catechin increase and 6.6-fold quercetin enhancement. The formulated beverage retained 95% bioactivity post-pasteurization with sensory scores exceeding 4.3/5.0. This platform provides a scalable strategy for transforming underutilized legumes into consumer-ready functional beverages.

Keywords: Adzuki beans, *Vigna angularis*, Response surface methodology, *Saccharomyces cerevisiae*, Antioxidant activity, Germination, Fermentation, Ultrasonic extraction, Functional beverage, Phenolic compounds

Introduction

Legumes are a cornerstone of global nutrition and food security, yet many regional varieties remain underexploited despite their rich nutritional potential. The adzuki bean (*Vigna angularis* (Willd.) Ohwi & H. Ohashi), a legume with deep roots in East Asian cuisine and traditional medicine, represents one such valuable resource [1]. Cultivated extensively in countries like Vietnam, where it is known as “Đậu huyết” the adzuki bean is prized for its excellent macronutrient profile, providing a rich source of protein, complex carbohydrates, and dietary fiber [2]. Beyond its basic nutritional value, the adzuki bean is a reservoir of diverse bioactive phytochemicals, primarily

polyphenols and flavonoids. Its seed coat, in particular, is a concentrated source of potent antioxidant compounds, including flavan-3-ols like catechin and epicatechin, proanthocyanidins, and flavanols such as quercetin and kaempferol [3,4]. These compounds are associated with a wide spectrum of health-promoting properties [5], including antioxidant, anti-inflammatory [6,7], and antidiabetic activities, positioning the adzuki bean as a prime candidate for the development of functional foods aimed at mitigating chronic diseases [8]. However, the full health potential of raw adzuki beans is often constrained, as a significant portion of these phenolic compounds exist in “bound” forms,

covalently linked to cell wall macromolecules [9], which limits their bioavailability and biological activity.

Therefore, a key challenge in food science is to develop effective processing strategies to unlock this latent bioactive potential. To enhance the nutritional and functional value of legumes, “green” bioprocessing technologies such as germination and fermentation [10] have emerged as highly effective and sustainable strategies. Conventional approaches to legume valorization typically focus on isolated unit operations - thermal processing for safety, solvent extraction for bioactive isolation, or fermentation for digestibility enhancement. However, such fragmented strategies often compromise bioactive integrity (thermal degradation) [11], sustainability (high solvent consumption) [12], or fail to yield consumer-ready products. The functional food industry increasingly demands holistic processing workflows that simultaneously enhance nutritional value, maintain safety standards, minimize environmental footprint, and deliver organoleptically acceptable products.

Germination is a natural metabolic process that awakens the dormant seed, initiating a cascade of enzymatic activities that fundamentally remodel its biochemical profile. First, it activates *de novo* phenolic biosynthesis by upregulating the phenylpropanoid pathway, particularly phenylalanine ammonia-lyase (PAL), the rate-limiting enzyme driving flavonoid production [13,14]. Second, endogenous hydrolases liberate bound phenolics through partial degradation of cell wall polymers [10,15].

Fermentation with *Saccharomyces cerevisiae*, a yeast with a long history of safe use in food production, provides complementary enzymatic biotransformation. Yeast-secreted β -glucosidases cleave glycosidic bonds on flavonoid glycosides (e.g., rutin), releasing free aglycones (e.g., quercetin) exhibiting superior antioxidant capacity and bioavailability [16].

Following bioprocessing, ultrasonic-assisted extraction (UAE) has emerged as a sustainable alternative to conventional methods. UAE reduces processing time by 67% - 83% (30 - 60 min vs. 2 - 6 h for maceration) while minimizing thermal degradation and achieved 30% - 50% solvent reduction [17]. Compared to microwave extraction, UAE offers uniform energy distribution, lower capital costs and

better scalability [18]. Acoustic cavitation (20 - 100 kHz) generates microscopic bubble collapse creating high-pressure zones that disrupt cell walls, enhancing bioactive recovery without prolonged thermal exposure [19]. While the individual efficacy of germination, fermentation, and UAE is well-documented, their optimized sequential application to generate complementary biochemical transformations in a single substrate remains an underexplored and compelling research direction, particularly for regionally significant legumes such as Vietnamese adzuki beans.

This study therefore aimed to: (1) determine optimal germination parameters for maximizing phenolic content in Vietnamese adzuki beans; (2) evaluate the effect of sequential fermentation with a newly isolated high-performance yeast strain on the bioactive profile; and (3) assess whether ultrasonic-assisted extraction can further amplify bioactive recovery from the processed material.

Using a Vietnamese cultivar (Đậu huyết), each processing stage was systematically optimized, individual phenolic transformations were characterized via UPLC-PDA chromatography, and industrial applicability was validated through beverage formulation and sensory evaluation. This work provides a reproducible, scalable framework for transforming underutilized legumes into high-value functional food ingredients.

Materials and methods

Raw materials and chemicals

Adzuki beans (*Vigna angularis*), of the local Vietnamese cultivar “Đậu huyết,” were purchased directly from farmers in Phu O Village, Hue City, Vietnam, from the Spring 2024 harvest. Before carrying out the experiments, the beans were soaked for 6 h at 35 °C.

All chemicals and reagents were of analytical grade. Gallic acid ($\geq 97.5\%$), catechin ($\geq 98\%$), chlorogenic acid ($\geq 98\%$), rutin ($\geq 94\%$), ellagic acid ($\geq 95\%$), quercitrin ($\geq 98\%$), quercetin ($\geq 95\%$), apigenin ($\geq 97\%$), 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; $\geq 98\%$), 2,2-diphenyl-1-picrylhydrazyl (DPPH; $\geq 97\%$), Folin-Ciocalteu reagent, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; $\geq 97\%$), and aluminum chloride (AlCl_3 ; $\geq 99\%$) were

purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol (99.9%) and sodium carbonate ($\geq 99.5\%$) were obtained from Merck (Darmstadt, Germany).

Yeast isolation, screening and molecular identification

Yeast strains were isolated from a sample of fermented jackfruit (*Artocarpus heterophyllus* Lam.). A 1 g sample was vortexed in 9 mL of sterile 0.9% NaCl solution. The homogenate was serially diluted and plated onto Hansen agar (1% peptone, 2% glucose, 0.1% yeast extract, 1.5% agar). Plates were incubated at 37 °C for 24 - 48 h. Colonies exhibiting typical yeast morphology were purified by sub-culturing on fresh Hansen agar.

The isolate demonstrating the highest efficacy in enhancing bioactive compounds was identified via molecular analysis using a systematic approach adapted from previous publications [20,21]. Genomic DNA was extracted from a 24-hour liquid culture using a TopPURE™ Genomic DNA Extraction Kit (ABT Biomedical Solutions), followed by a phenol-chloroform purification. The Internal Transcribed Spacer (ITS) region was amplified via PCR using universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') under conditions: initial denaturation (95 °C, 5 min); 35 cycles of 95 °C (30 s), 55 °C (30 s), 72 °C (1 min); final extension (72 °C, 10 min). Resulting amplicons were sequenced bidirectionally (1st BASE, Malaysia), assembled using SnapGene (v6.2) and compared to the NCBI GenBank database using BLAST for species identification [22]. The identified yeast strain was maintained as glycerol stocks (30%, v/v) at -80 °C in Hansen broth for further use.

Germination optimization via response surface methodology

Beans were germinated at 20, 25, 30, and 35 °C in darkness with daily moistening (Figure S1, showing characteristic changes during the germination process). Samples collected at 12-h intervals (0 - 108 h) were freeze-dried (Christ Alpha 1-2 LD Plus, Germany, -90 °C, 0.01 mbar, 24 h, ground (IKA A11 mill, < 0.5 mm), and analyzed for total phenolic content (TPC), total flavonoid content (TFC), 2,2-diphenyl-1-picrylhydrazyl

(DPPH), and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) activities to identify optimal temperature and time ranges. Based on screening results, a 5-level Central Composite Design (CCD) evaluated germination temperature (X_1 : 23.98 - 31.03 °C) and time (X_2 : 26.16 - 93.84 h) across 13 experimental runs with 5 center-point replicates (Table S1). Response variables (TPC, TFC, DPPH, ABTS) were fitted to second-order polynomial models. Numerical optimization via desirability function (Design-Expert v12, Stat-Ease Inc.) identified conditions maximizing all responses simultaneously. Optimal parameters were validated experimentally ($n = 5$).

Fermentation optimization with *S. cerevisiae* B5

Nine isolated strains (B1 - B9) were inoculated (10^6 CFU/mL) into sterile germinated bean flour broth (1:10 w/v) and fermented (30 °C, pH 5.0, 72 h, 150 rpm). The strain yielding the highest TPC/TFC/antioxidant activities was selected and identified. Using 1-factor-at-a-time (OFAT) methodology, fermentation conditions were sequentially optimized: (1) material: water ratio (1:2 to 1:20), (2) initial pH (3.0 - 5.5), (3) temperature (25, 30 and 35 °C), (4) initial cell density (10^5 - 10^9 CFU/mL), and (5) time (24 - 144 h). Each parameter was optimized while maintaining prior optimized factors.

Ultrasonic-assisted extraction optimization via Box-Behnken design

A Box-Behnken Design (BBD) was employed for UAE optimization in preference to a Central Composite Design (CCD) because: (i) BBD requires fewer experimental runs for 3-factor, 3-level studies (17 vs. 20 for face-centered CCD), reducing material and time costs [23]; (ii) BBD avoids axial (star) points at extreme factor-level combinations, which can cause degradation or undesirable reactions in food extraction systems; [24] and (iii) BBD is particularly efficient for 3-factor designs and provides robust estimation of second-order response surfaces [25]. CCD was retained for the 2-factor germination optimization, where its star points were necessary to define the precise curvature across the temperature-time response space.

Fermented bean flour (from Phase 1) was extracted using an ultrasonic bath (JEK-TUC-65, 40 kHz, 180 W; volumetric intensity: 27.7 W/L; estimated

power density at working vessel surface: 0.36 W/cm², Jeken, China). Preliminary experiments evaluated effects of ethanol concentration (10% - 90%), solvent: material ratio (5:1 to 25:1 mL/g), and extraction time (10-35 min). Extracts were rapidly cooled in an ice bath, centrifuged (5,000 × g, 10 min, 4 °C; Sigma 1 - 16 with 12150-H rotor, Germany), and supernatants analyzed for TPC, TFC, DPPH, and ABTS activities.

A 3-factor, 3-level Box-Behnken Design assessed ethanol concentration (X_1 : 30% - 70%), solvent: material ratio (X_2 : 10 - 25 mL/g), and time (X_3 : 15 - 30 min) across 17 runs with 5 center-point replicates. Response variables were modelled using second-order polynomials:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^k \sum_{j=1}^k \beta_{ij} X_i X_j \quad (1)$$

where Y = response variable (TPC, TFC, DPPH, ABTS); X_i , X_j = independent variables; $k = 3$; β_0 = intercept; β_i , β_{ii} , β_{ij} = linear, quadratic, and interaction coefficients. Model adequacy was assessed via ANOVA ($p < 0.05$), R^2 , adjusted R^2 , predicted R^2 , and lack-of-fit tests ($p > 0.05$ for adequate models). Optimal conditions were predicted and validated experimentally ($n = 3$).

Beverage formulation and thermal processing

Optimized extract (Phase 2) was blended with sucrose (6% w/v, based on preliminary taste panel preference, $n = 10$) and pasteurized at 95 °C for 2 min using a water bath (to ensure *Escherichia coli* and *Salmonella* inactivation per FDA guidelines while minimizing thermal degradation) [26]. The beverage was immediately cooled in an ice bath to < 25 °C within 5 min, bottled in sterilized glass bottles (100 mL capacity), and stored at 4 °C until analysis (maximum 7 days).

Sensory evaluation

Quantitative Descriptive Analysis (QDA) was conducted by 50 panelists (age range: 22 - 35 years, 60% female, 40% male) following ISO 4121:2003 guidelines. Panelists evaluated adzuki bean samples for color, aroma, taste, and mouthfeel using a 5-point hedonic scale, with a focus on flavor profiles and

phenolic astringency (Table S2). Samples (100 mL) were served in coded, opaque cups at 25 ± 2 °C under controlled white lighting (ISO 8589, 6,500K, 1,000 lux) in individual sensory booths. Panelists rinsed with distilled water (30 s) and consumed unsalted crackers between samples to prevent carryover effects. Presentation order was randomized. Data are presented as mean $\pm$ SD ($n = 50$).

Analytical methods

Sample extraction

Adzuki bean extracts were prepared with modifications to the methods described in [27,28]. For all analyses, extracts were prepared by sonicating 50 g of ground bean sample with 500 mL of 70% (v/v) ethanol for 30 min at 40 °C at 40 kHz and 180 W. The mixture was centrifuged (8,000 × g, 10 min, 4 °C, Sigma 1 - 16 centrifuge, Sigma, Germany), and the supernatant was filtered (0.45 µm nylon syringe filter, Millipore), concentrated via rotary evaporation (Rotavator R-100, Buchi, Switzerland; 45 °C bath temperature, 100 - 120 mbar pressure, 80 rpm rotation speed) to reach 4% (w/v), and reconstituted in 50% (v/v) ethanol before storage at -20 °C.

Total Phenolic Content (TPC)

Total Phenolic Content (TPC) was determined using the Folin-Ciocalteu method [29]. Briefly, 0.5 mL extract was mixed with 2.5 mL Folin-Ciocalteu reagent (10-fold diluted) and incubated (5 min, dark). After adding 2 mL Na₂CO₃ (7.5% w/v) and incubating (90 min, dark, 25 °C) with absorbance measured at 765 nm (Ultrospec 1100 pro spectrophotometer Amersham Biosciences, USA). Results were expressed as mg of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

Total Flavonoid Content (TFC)

Total Flavonoid Content (TFC) was measured using the aluminium chloride colorimetric method [7]. Extract (0.5 mL) was mixed with 1.5 mL ethanol (95%), 0.1 mL AlCl₃ (10%), 0.1 mL potassium acetate (1 M), and 2.8 mL distilled water. After incubation (40 min, 25 °C, dark), with absorbance read at 510 nm. Results were expressed as mg of catechin equivalents per gram of dry weight (mg CAE/g DW).

Antioxidant activity assays

Antioxidant capacity was assessed using 2 assays. For DPPH radical scavenging activity [30], extract (0.1 mL) was mixed with 3.9 mL DPPH solution (0.1 mM in ethanol, prepared fresh daily), incubated (60 min, dark, 25 °C), was measured at 515 nm after 60 min of incubation. For ABTS radical scavenging activity [31], ABTS radical was generated by mixing 7 mM ABTS with 2.45 mM potassium persulfate (1:1 v/v) and incubating (12 - 16 h, dark, 25 °C). The solution was diluted with ethanol to an absorbance 0.70 ± 0.02 at 734 nm. Extract (0.15 mL) was mixed with 2.85 mL ABTS solution, incubated (30 min, dark), and absorbance was measured at 734 nm. For both assays, results were expressed as μmol of Trolox equivalents per gram of dry weight ($\mu\text{mol TE/g DW}$).

UPLC-PDA analysis of individual phenolics

The identification and quantification of individual phenolic compounds were performed using a Thermo-Ultimate 3000 UPLC system (Thermo Scientific, Waltham, MA, USA), following the previous methodology [32]. In brief, chromatographic separation was achieved on a BDS Hypersil C18 column (250×4.6 mm, 5 μm) using a gradient elution with a mobile phase consisting of methanol and 0.1% aqueous phosphoric acid, delivered at a flow rate of 0.7 mL/min. Prior to analysis, extracts were filtered through a 0.45 μm nylon membrane, and a 5 μL aliquot was injected. Compounds were monitored at 265 nm, identified by comparing retention times to authentic standards, and quantified using external calibration curves. All analyses were performed in triplicate.

Statistical analysis

All experiments were conducted in a completely randomized design with 3 replicates, except sensory evaluation ($n = 50$ panelists). Data are presented as mean $\pm$ standard deviation (SD). Statistical significance was determined using 2-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference (HSD) post hoc test ($p < 0.05$; GraphPad Prism v10.0.3, La Jolla, CA, USA). Response Surface Methodology (RSM) data were analyzed using Design-Expert software v12.0.3 (Stat-Ease Inc., Minneapolis, MN, USA); model adequacy was evaluated by the coefficient of determination (R^2), adjusted R^2 , predicted R^2 , and

lack-of-fit tests ($p > 0.05$ for adequate models). All other statistical analyses were performed using GraphPad Prism (v.10.0).

Results and discussion

This study developed an integrated, 3-stage bioprocessing platform to unlock the bioactive potential of Vietnamese adzuki beans (Đậu huyết) through sequential germination, fermentation, and ultrasonic-assisted extraction. Each stage was systematically optimized using Response Surface Methodology (RSM) or one-factor-at-a-time (OFAT) approaches, with bioactive transformations characterized via UPLC-PDA chromatography. The platform culminated in a functional beverage demonstrating industrial viability through high bioactive retention and consumer acceptance.

Optimization of germination enhances phenolic content through temperature-time interaction

Raw, ungerminated adzuki beans established baseline bioactive levels: TPC of 2.94 ± 0.14 mg GAE/g DW, TFC of 0.73 ± 0.03 mg CAE/g DW (Table S3) with antioxidant activities of 5.68 ± 0.02 $\mu\text{mol TE/g DW}$ (DPPH) and 20.06 ± 0.17 $\mu\text{mol TE/g DW}$ (ABTS) (Table S4).

Preliminary screening results

Germination significantly enhanced all parameters in a temperature- and time-dependent manner ($p < 0.05$, Tables S3 - S4). Optimal accumulation occurred at 25 to 30 °C. At 25 °C, TPC peaked at 60 h, reaching 9.61 ± 0.27 mg GAE/g DW, a 3.3-fold increase over the raw beans (Table S3). This magnitude of increase aligns with germination studies on lima beans and adzuki beans showing 2-3-fold TPC elevations after 72 h [13,15]. A similar, though slightly lower, peak of 9.29 ± 0.26 mg GAE/g DW was achieved at 30 °C after just 48 h. TFC followed a similar pattern, with the highest concentrations recorded at 60 h for both 25 °C (1.70 ± 0.07 mg CAE/g DW) and 30 °C (1.57 ± 0.07 mg CAE/g DW), representing 2.3-fold and 2.2-fold increases respectively (Table S3). Germination at suboptimal temperatures (20 and 35 °C) yielded significantly lower TPC and TFC values. This differential response suggests that germination preferentially enhances both flavonoid and phenolic acid synthesis [13].

Antioxidant activities peaked correspondingly: DPPH at 60 h/25 °C (26.61 ± 0.67 $\mu\text{mol TE/g DW}$, 4.68-fold increase) and ABTS at 60 h/30 °C (35.13 ± 0.09 $\mu\text{mol TE/g DW}$, 1.8-fold increase) (**Table S4**). Sub-optimal temperatures (20 and 35 °C) yielded significantly lower enhancements. Post-72 h, all parameters declined, indicating metabolic consumption by developing seedlings.

The 25 - 30 °C optimum likely reflects balanced enzyme kinetics for phenylpropanoid biosynthesis [33], while temperatures below 20 °C provide insufficient activation energy and temperatures exceeding 30 °C accelerate respiratory consumption of newly synthesized phenolics for seedling lignification [10,13]. The differential enhancement between DPPH activity increased 4.7-fold while ABTS activity increased 1.8-fold, suggesting structural specificity in germination-generated phenolics. This interpretation is corroborated by UPLC-PDA analysis, which revealed a 16.9-fold catechin increase (a lipophilic flavanol preferentially detected by DPPH) [6] during germination, whereas the more modest ABTS enhancement reflects parallel increases in hydrophilic phenolic acids with lower DPPH reactivity.

RSM optimization

Full ANOVA decomposition for all model terms, including degrees of freedom, F-values, and *p*-values for linear, quadratic, and interaction effects, is provided in

Supplementary **Table S10** (CCD germination models). A CCD was selected for germination optimization because it accommodates 5 factor levels including axial (star) points ($\alpha = 1.41$), enabling efficient quadratic surface mapping across an extended factor space with only 13 experimental runs while maintaining near-rotatability [24], across the continuous temperature-time domain, which was essential for defining the narrow optimal range (25 - 30 °C, 60 - 72 h) [34,35].

Response Surface Methodology with Central Composite Design (13 experimental runs, **Table 1**; **Figure S2**) precisely defined optimal conditions. Second-order polynomial regression models demonstrated high predictive accuracy ($R^2 = 96.03\%$ - 99.78% , **Table 5**) with non-significant lack-of-fit tests ($p > 0.05$), confirming model adequacy. Numerical optimization via desirability function identified optimal conditions: 26.96 °C temperature and 67.89 h germination time (**Figure S2**). Experimental validation at practical conditions (27 °C, 68 h) confirmed predictions: TPC 10.33 ± 0.13 mg GAE/g DW (3.5-fold vs. raw beans, **Table S3**), TFC 1.85 ± 0.02 mg CAE/g DW (2.53-fold, **Table 2**), DPPH 27.39 ± 0.37 $\mu\text{mol TE/g DW}$ (4.9-fold, **Table S4**), and ABTS 35.65 ± 0.30 $\mu\text{mol TE/g DW}$ (1.8-fold, **Table S4**). This 3.5-fold TPC enhancement exceeds typical literature values of 1.8 - 2.5-fold for legumes under non-optimized conditions [13,36], demonstrating the value of systematic RSM optimization.

Table 1 Central Composite design matrix and experimental results for germination optimization in adzuki beans.

Factors					Result			
TT	X1	X2	Temperature (°C)	Time (h)	TPC (Y1)	TFC (Y2)	DPPH (Y3)	ABTS (Y4)
1	0	-1.41	27.50	26.06	5.04	0.93	15.85	25.36
2	-1	1	25.00	84.00	8.98	1.60	24.22	33.12
3	0	0	27.50	60.00	10.22	1.85	27.35	35.65
4	0	0	27.50	60.00	10.32	1.89	27.12	34.89
5	1.41	0	31.04	60.00	7.22	1.35	22.32	28.42
6	-1	-1	25.00	36.00	5.54	1.06	16.44	27.03
7	1	1	30.00	84.00	6.45	1.22	22.39	29.05
8	0	1.41	27.50	93.94	8.18	1.47	23.35	31.59
9	1	-1	30.00	36.00	6.08	1.22	20.05	28.34
10	-1.41	0	23.96	60.00	7.69	1.62	21.27	31.36

Factors					Result			
TT	X1	X2	Temperature (°C)	Time (h)	TPC (Y1)	TFC (Y2)	DPPH (Y3)	ABTS (Y4)
11	0	0	27.50	60.00	9.42	1.89	27.44	35.12
12	0	0	27.50	60.00	10.22	1.68	26.89	34.54
13	0	0	27.50	60.00	10.26	1.91	26.74	35.61

X₁, germination temperature (°C); X₂, germination time (h). Response variables: Y₁ (TPC), Y₂ (TFC), Y₃ (DPPH), Y₄ (ABTS).

Screening and selection of a high-performance yeast isolate

Following the optimization of germination conditions (27 °C, 68 h), 9 distinct yeast strains (B1 - B9), isolated from fermented jackfruit, were screened for their ability to further enhance the bioactive profile of the germinated adzuki bean flour. The control sample, representing optimally germinated but unfermented beans, had a TPC of 9.63 ± 0.15 mg GAE/g DW and an ABTS activity of 34.94 ± 0.50 μ mol TE/g DW. Fermentation with all 9 isolates resulted in a significant increase ($p < 0.05$) in bioactive content and antioxidant activity compared to the non-fermented control. However, isolate B5 demonstrated markedly superior performance across all metrics: TPC of 13.67 ± 0.42 mg GAE/g DW (1.4-fold increase), a TFC of 2.02 ± 0.11 mg CAE/g DW (1.2-fold), a DPPH activity of 33.45 ± 0.29 μ mol TE/g DW (1.2-fold), and an ABTS activity of 62.24 ± 0.28 μ mol TE/g DW (1.8-fold). These values were significantly higher than those achieved by any other isolate, representing a 42% increase in TPC and a 78% increase in ABTS activity over the germinated-only control (Table S5). Based on this outstanding performance, isolate B5 was selected for molecular identification and subsequent fermentation optimization experiments.

Molecular identification via ITS region sequencing confirmed isolate B5 as *S. cerevisiae*. Based on this definitive molecular evidence, the isolate B5 was identified as *S. cerevisiae* B5 (99.87% ITS sequence similarity; Table S6), a GRAS-status organism suitable for food applications, thus used for all further fermentation studies. The strain's superior bioactive-enhancing performance is consistent with β -glucosidase-mediated glycoside hydrolysis, as inferred from the pH optimum alignment (pH 4.5, within the 4.0 - 5.5 range reported for fungal β -glucosidases [37] and the UPLC-PDA-confirmed quercetin aglycone accumulation pattern, though direct enzymatic quantification was not performed in this study. Phenotypic stability (colony morphology, TPC enhancement capacity) was confirmed by re-screening 3 independently revived colonies prior to optimization experiments, with no significant variation observed ($p > 0.05$).

Optimization of fermentation parameters using *S. cerevisiae* B5

A series of single-factor experiments were conducted to optimize the fermentation conditions for maximizing the bioactive potential of the germinated adzuki bean flour using the selected *S. cerevisiae* B5 strain (Figure 1).

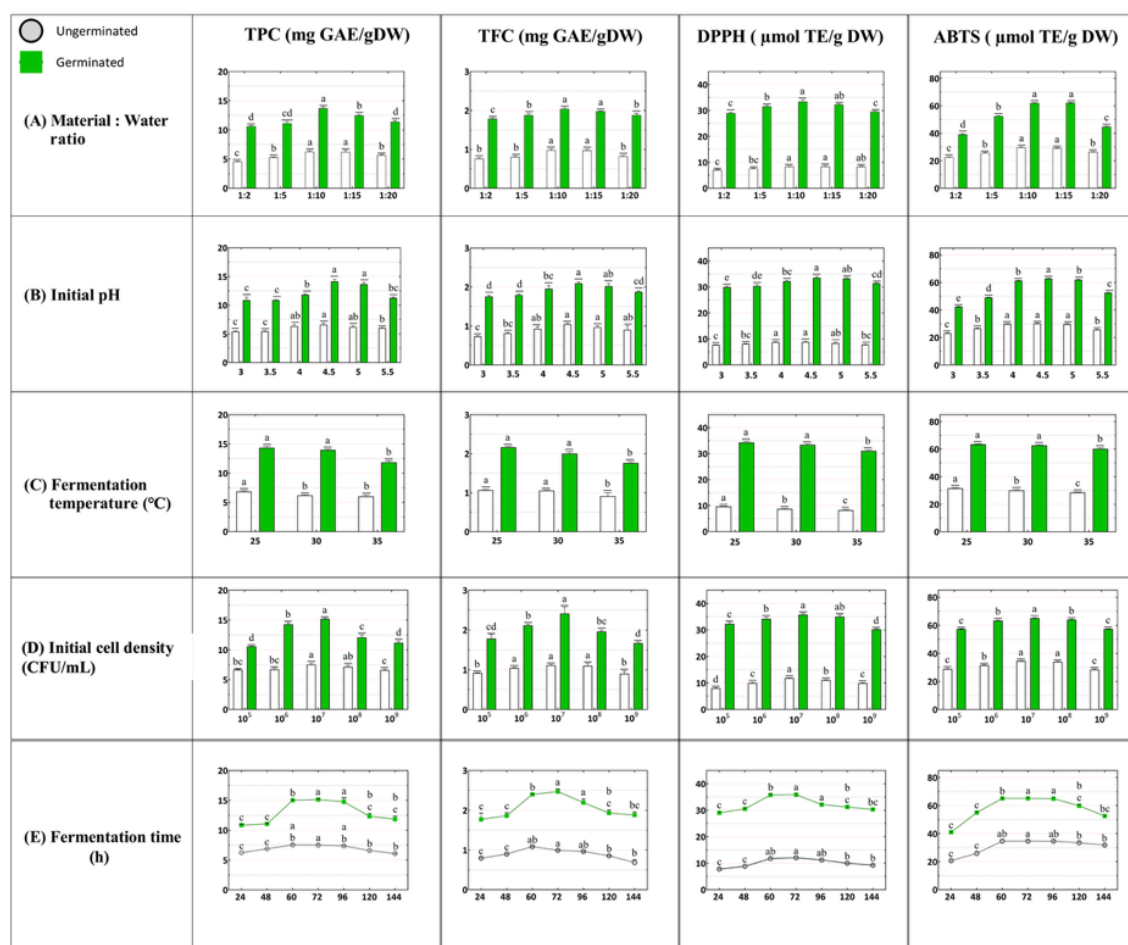


Figure 1 Sequential optimization of fermentation parameters via one-factor-at-a-time (OFAT) methodology. Effects of (A) material: water ratio (1:2 to 1:20 w/v), (B) initial pH (3.0 - 5.5), (C) temperature (25 - 35 °C), (D) initial cell density (10⁵ - 10⁹ CFU/mL), and (E) fermentation time (0 - 144 h) on total phenolic content (TPC), total flavonoid content (TFC), DPPH activity, and ABTS activity. Germinated beans (green bars/lines) and ungerminated beans (white bars/lines) fermented with *S. cerevisiae* B5. Each parameter optimized sequentially while maintaining previously optimized conditions. Data represents mean ± SD (n = 3). Different letters within each panel indicate significant differences ($p < 0.05$, Tukey's HSD). Optimal conditions: 1:10 ratio, pH 4.5, 25 °C, 10⁷ CFU/mL, 72 h. DW, dry weight; GAE, gallic acid equivalents; CAE, catechin equivalents; TE, Trolox equivalents; CFU, colony-forming units.

Effect of material-to-water ratio

The material-to-water ratio significantly influenced the extraction and biotransformation of phenolic compounds during fermentation (**Figure 1(A)**). Among the 5 ratios tested (1:2, 1:5, 1:10, 1:15 and 1:20), a ratio of 1:10 was found to be optimal for germinated beans. At this ratio, TPC reached its peak value of 13.69 ± 0.25 mg GAE/g DW. This ratio also yielded the highest DPPH (33.41 ± 0.81 μmol TE/g DW) and ABTS (62.05 ± 0.24 μmol TE/g DW) activities. While TFC was not significantly affected by the ratio, the 1:10 condition consistently produced the best overall results and was selected for subsequent

experiments. This ratio balances the substrate concentration for yeast metabolism with a sufficient aqueous medium for enzyme diffusion and metabolite solubility. Similar optimal solid-to-liquid ratios of 1:8 - 1:12 have been reported for yeast fermentation of legume substrates, where ratios below this range impair enzyme diffusion due to excessive viscosity while higher dilutions reduce substrate availability per unit microbial biomass [38,39].

Effect of initial pH

The initial pH of the fermentation medium was a critical factor, with an optimal range identified between

4.0 and 4.5. The highest TPC (14.14 ± 0.36 mg GAE/g DW), TFC (2.10 ± 0.03 mg CAE/g DW), and ABTS activity (62.84 ± 0.22 μ mol TE/g DW) were all achieved at an initial pH of 4.5. DPPH activity, however, peaked slightly lower at pH 4.0 (32.21 ± 0.36 μ mol TE/g DW), though the value at pH 4.5 (33.55 ± 0.56 μ mol TE/g DW) was not statistically different. Therefore, an initial pH of 4.5 was determined to be optimal for the overall process (**Figure 1(B)**). This pH optimum aligns with reported activity profiles for fungal β -glucosidases, which typically exhibit pH optima between 4.0 - 5.5 [37]. The slightly acidic environment simultaneously suppresses competing bacterial growth while maintaining yeast metabolic activity, providing dual benefits for fermentation control [39].

Effect of fermentation temperature

Fermentation was conducted at 25, 30 and 35 °C to determine the optimal temperature for *S. cerevisiae* B5 activity. The results showed that 25 °C was the most effective temperature for maximizing the bioactive profile. At 25 °C, the highest values for TPC (14.34 ± 0.22 mg GAE/g DW), TFC (2.17 ± 0.03 mg CAE/g DW), DPPH (34.25 ± 0.51 μ mol TE/g DW), and ABTS (63.52 ± 0.32 μ mol TE/g DW) were recorded. Fermentation at 30 °C yielded slightly lower but statistically similar results, while performance at 35 °C was significantly reduced, indicating thermal stress on the yeast viability and enzyme stability (**Figure 1(C)**). The 25 °C optimum, lower than typical *S. cerevisiae* fermentation temperatures (28 - 32 °C for ethanol production), may reflect temperature-dependent partitioning between primary metabolism (biomass/ethanol production) and secondary metabolism (phenolic biotransformation), warranting further investigation through metabolic flux analysis. The lower-than-expected optimal temperature (25 °C vs. the typical 28 - 32 °C for ethanol production) is consistent with reports that reduced temperatures favor secondary metabolic flux toward phenolic biotransformation over primary biomass accumulation in *S. cerevisiae* [20,39]. This temperature-dependent metabolic partitioning warrants further confirmation through metabolic flux analysis.

Effect of initial cell density

The initial cell density was varied from 10^5 to 10^9 CFU/mL. The optimal cell density differed among response variables. The highest TPC (15.20 ± 0.13 mg GAE/g DW) and ABTS activity (65.12 ± 0.41 μ mol TE/g DW) were achieved with an initial density of 10^7 CFU/mL. In contrast, TFC was maximized at a lower density of 10^6 CFU/mL (2.41 ± 0.16 mg CAE/g DW), while DPPH activity peaked at a higher density of 10^8 CFU/mL (35.79 ± 0.52 μ mol TE/g DW). Considering the significant impact on TPC and ABTS, the primary indicators of overall bioactivity, an initial cell density of 10^7 CFU/mL was selected as the optimal initial cell density (**Figure 1(D)**). This differential response pattern suggests that distinct enzymatic activities operate optimally at different cell densities, possibly reflecting competition for substrates or oxygen at high cell densities. The optimal initial cell density of 10^7 CFU/mL aligns with findings in fermented grain and legume systems, where intermediate cell densities balance β -glucosidase secretion rates with substrate availability; higher densities generate inter-cellular competition for soluble phenolic precursors, reducing net biotransformation efficiency [37,40].

Effect of fermentation time

Under the established optimal conditions (1:10 ratio, pH 4.5, 25 °C, 10^7 CFU/mL), the fermentation was monitored over 144 h. Bioactive content and antioxidant decline increased steadily, peaking between 60 and 72 h before gradually declining. The maximum TPC (15.13 ± 0.13 mg GAE/g DW) and ABTS activity (65.16 ± 0.25 μ mol TE/g DW) were recorded at 72 h. TFC (2.47 ± 0.07 mg CAE/g DW) and DPPH activity (35.87 ± 0.58 μ mol TE/g DW) peaked slightly earlier at 60 h, though the values at 72 h were not significantly different. Therefore, a fermentation time of 72 h was established as optimal for achieving the maximum synergistic effect (**Figure 1(E)**). The biphasic accumulation-decline pattern, peaking at 72 h, is characteristic of *S. cerevisiae* fermentation in phenolic-rich substrates and has been reported in fermented legume and fruit systems, where late-stage phenolic decline reflects microbial catabolism of accessible monomers as primary substrates become exhausted [20,39].

The cumulative 5.1-fold TPC enhancement through optimized germination followed by

fermentation substantially exceeds the 3.4-fold increase from germination alone, providing quantitative evidence of synergistic rather than merely additive effects. This synergy likely reflects complementary mechanisms: germination activates de novo phenolic biosynthesis and liberates matrix-bound compounds, while fermentation enzymatically converts glycosides to more bioactive aglycones and further releases cell wall-entrapped phenolics.

The cumulative optimization of 5 fermentation parameters through one-factor-at-a-time (OFAT) methodology identified synergistic conditions that collectively elevated bioactive content from the germinated baseline. The optimized parameters (1:10 ratio, pH 4.5, 25 °C, 10⁷ CFU/mL, 72 h fermentation) achieved a total phenolic content (TPC) of 15.13 ± 0.13 mg GAE/g DW on fermented germinated beans,

representing a 5.1-fold enhancement over raw beans (2.94 mg GAE/g DW, **Table S3**) and a 1.5-fold improvement over germinated beans alone (10.33 mg GAE/g DW, **Table 2**). Similarly, ABTS radical scavenging activity reached 65.16 ± 0.25 µmol TE/g DW, a 3.3-fold enhancement over raw beans (**Table 3**). The progression from individual treatment optimization to final fermented product demonstrates the additive nature of sequential bioprocessing: germination (3.5-fold) × fermentation multiplier (1.5-fold) = cumulative 5.1-fold enhancement. This multiplicative relationship indicates independent pathway activation rather than biochemical synergy, with each stage employing distinct enzymatic mechanisms (germination: PAL activation; fermentation: β-glucosidase secretion) that sequentially enhance bioactive content.

Table 2 Regression equations and statistical parameters from germination optimization.

Measured responses	Regression Equations and statistical parameters				
TPC (mg GAE/g DW)	$-189.22 + 12.8487 X_1 + 0.773 X_2 - 0.222 X_1^2 - 0.003 X_2^2 - 0.012792 X_1 * X_2$				
R ² (%)	Lack of fit	R ² (adj)	R ² (pred)	Optimal value	Experimental Value
97.85	0.94	96.32	91.74	10.09	10.05 ± 0.25
TFC (mg CAE/g DW)	$Y2 = -27.396 + 1.837 X_1 + 0.1396 X_2 - 0.0315 X_1^2 - 0.0006 X_2^2 - 0.00225 X_1 * X_2$				
R ² (%)	Lack of fit	R ² (adj)	R ² (pred)	Optimal value	Experimental Value
96.03	0.67	93.20	86.41	01.84	01.83 ± 0.06
DPPH (µmolTE/g DW)	$Y3 = -364.51 + 24.78 X_1 + 1.512 X_2 - 0.4229 X_1^2 - 0.0065 X_2^2 - 0.022667 X_1 * X_2$				
R ² (%)	Lack of fit	R ² (adj)	R ² (pred)	Optimal value	Experimental Value
99.78	0.27	99.62	99.45	27.11	27.2 ± 0.16
ABTS (µmolTE/g DW)	$Y3 = -331.41 + 23.80 X_1 + 1.3860 X_2 - 0.41465 X_1^2 - 0.0057 X_2^2 - 0.02242 X_1 * X_2$				
R ² (%)	Lack of fit	R ² (adj)	R ² (pred)	Optimal value	Experimental Value
98.84	1.30	98.02	95.02	35.16	35.05 ± 0.30

R², coefficient of determination; Adj. R², adjusted R²; Pred. R², predicted R²; Lack of fit, *p*-value (*p* > 0.05 indicates adequate model). Optimal values validated experimentally (n = 5).

Optimization of ultrasonic extraction parameters using response surface methodology (RSM)

Preliminary experiments evaluated effects of 3 critical extraction parameters on bioactive recovery

from fermented adzuki bean flour through one-factor-at-a-time (OFAT) screening (**Figure 2, Tables S7 - S11**): ethanol concentration 30% - 70%, solvent-material ratio 10 - 25 mL/g, extraction time 15 - 30 min.

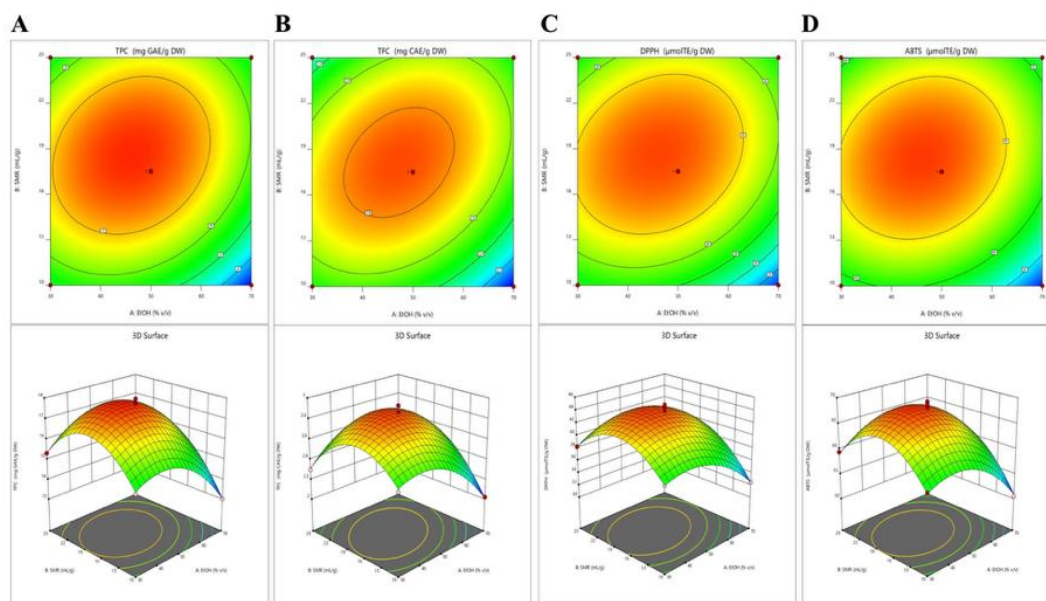


Figure 2 Response surface plots for ultrasonic extraction optimization via Box-Behnken Design. Three-dimensional surfaces showing effects of ethanol concentration (X_1 , 30% - 70%), solvent: material ratio (X_2 , 10 - 25 mL/g), and extraction time (X_3 , 15 - 30 min) on (A) total phenolic content (TPC, mg GAE/g DW), (B) total flavonoid content (TFC, mg CAE/g DW), (C) DPPH radical scavenging activity ($\mu\text{mol TE/g DW}$), and (D) ABTS radical scavenging activity ($\mu\text{mol TE/g DW}$). Optimal conditions: 50% ethanol, 20:1 ratio, 30 min (composite desirability = 0.94). Model validation: $R^2 = 97.51\% - 99.49\%$, non-significant lack-of-fit ($p > 0.05$). Colour gradient from low (blue) to high (red) response. Ultrasonic bath (Jeken TUC-65, 40 kHz, nominal power 180 W; tank volume 6.5 L; transducer area $\sim 225 \text{ cm}^2$; calculated ultrasonic power density: $\sim 27.7 \text{ W/L}$; ultrasonic intensity: $\sim 0.8 \text{ W/cm}^2$)

Effect of ethanol concentration

Ethanol concentration significantly influenced extraction efficiency. TPC and TFC peaked at 50% ethanol ($17.68 \pm 0.25 \text{ mg GAE/g DW}$ and $2.73 \pm 0.06 \text{ mg CAE/g DW}$, respectively), while declining at both lower (10%: $13.02 \pm 0.41 \text{ mg GAE/g DW}$) and higher concentrations (90%: $11.73 \pm 0.41 \text{ mg GAE/g DW}$) (Supplementary Table S7). This biphasic response reflects competing solubility and extraction mechanisms: low ethanol concentrations favor hydrophilic phenolic acids (gallic acid, chlorogenic acid) but inadequately solubilize lipophilic flavonoid aglycones (quercetin, apigenin). Conversely, high ethanol concentrations efficiently extract aglycones but precipitate polysaccharides and proteins that entrap phenolics, reducing overall recovery [7,17].

The effect of the solvent-to-material ratio (SMR)

Solvent-to-material ratio exhibited positive correlation with bioactive recovery up to 20:1 mL/g, beyond which no additional benefit occurred (Table S8). TPC increased from $15.16 \pm 0.13 \text{ mg GAE/g DW}$

(5:1 ratio) to $17.66 \pm 0.38 \text{ mg GAE/g DW}$ (20:1 ratio), with statistically equivalent performance at 25:1 ratio ($17.76 \pm 0.30 \text{ mg GAE/g DW}$). This saturation pattern indicates that 20:1 provides sufficient solvent volume to establish favorable concentration gradients for mass transfer while avoiding unnecessary dilution that would increase downstream concentration costs for industrial applications [38].

Effect of extraction time

Extraction time showed positive effects up to 30 min (Table S9), with TPC increasing from $13.08 \pm 0.61 \text{ mg GAE/g DW}$ (10 min) to $17.72 \pm 0.06 \text{ mg GAE/g DW}$ (30 min), then plateauing at 35 min ($17.71 \pm 0.59 \text{ mg GAE/g DW}$). This kinetic profile reflects initial rapid extraction driven by acoustic cavitation-induced cell disruption (0 - 15 min), followed by slower diffusion-limited mass transfer (15-30 min), and finally equilibrium establishment ($> 30 \text{ min}$) [12,17].

Response Surface Methodology (RSM) for UAE

Full ANOVA decomposition for all model terms, including degrees of freedom, F-values, and *p*-values for linear, quadratic, and interaction effects, is provided in **Tables S11** (BBD UAE models). Box-Behnken Design evaluated ethanol concentration (*X*₁: 30% - 70%), solvent-to-material ratio (*X*₂: 10 - 25 mL/g), and extraction time (*X*₃: 15 - 30 min) across 17 experimental runs (**Table 4, Figure 2**). Second-order polynomial

regression models demonstrated excellent statistical validity for all responses, with *R*² values ranging from 97.51% (DPPH) to 99.49% (TPC), non-significant lack-of-fit tests (*p* > 0.05 for all models), and close agreement between adjusted *R*² and predicted *R*² values (**Table 3**). The desirability function approach (equal weight = 1.0 for all 4 responses) is a well-established composite optimization methodology validated for multi-response food process optimization [24,34].

Table 3 Statistical validation parameters for ultrasonic extraction optimization models.

Response	R ² (%)	Adj. R ² (%)	Pred. R ² (%)	Lack of Fit (<i>p</i>)	Model (<i>p</i>)
TPC	99.49	98.84	96.29	0.88	< 0.0001***
TFC	99.07	97.87	94.47	0.58	< 0.0001***
DPPH	97.51	94.31	94.50	0.06	< 0.0001***
ABTS	98.14	95.76	93.73	0.19	< 0.0001***

Adj. R², adjusted R²; Pred. R², predicted R²; ****p* < 0.001. Non-significant lack-of-fit (*p* > 0.05) confirms model adequacy.

Experimental validation confirmed predictions with < 10% deviation: TPC 18.05 ± 0.35 mg GAE/g DW (19.3% enhancement vs. fermented flour), TFC 3.13 ±

0.13 mg CAE/g DW, DPPH 43.95 ± 0.23 µmol TE/g DW, ABTS 70.01 ± 0.40 µmol TE/g DW (**Table 4**).

Table 4 Box-Behnken Design matrix and experimental responses for ultrasonic extraction.

TT	Code			Net Value			Experimental Results			
	<i>X</i> ₁	<i>X</i> ₂	<i>X</i> ₃	EtOH (% v/v)	SMR (mL/g)	Time (min)	TPC (mg GAE/g DW)	TFC (mg CAE/g DW)	DPPH (µmolTE/g DW)	ABTS (µmolTE/g DW)
1	1	0	1	70	17.5	30	13.03	2.12	32.53	51.57
2	0	-1	1	50	10	30	13.05	2.15	31.87	51.02
3	1	1	0	70	25	22.5	15.34	2.50	38.28	58.09
4	0	0	0	50	17.5	22.5	17.73	2.86	44.24	68.76
5	0	0	0	50	17.5	22.5	17.74	2.79	41.28	64.86
6	0	0	0	50	17.5	22.5	17.52	2.86	43.74	67.98
7	0	0	0	50	17.5	22.5	17.96	2.93	44.82	68.66
8	-1	1	0	30	25	22.5	15.33	2.30	38.25	59.45
9	0	-1	-1	50	10	15	14.01	2.28	33.96	53.29
10	-1	0	1	30	17.5	30	16.42	2.68	40.98	63.69
11	1	-1	0	70	10	22.5	13.01	2.02	32.47	50.46
12	-1	0	-1	30	17.5	15	13.68	2.23	33.13	51.50
13	-1	-1	0	30	10	22.5	15.17	2.45	37.86	58.85

TT	Code			Net Value			Experimental Results			
	X ₁	X ₂	X ₃	EtOH (% v/v)	SMR (mL/g)	Time (min)	TPC (mg GAE/g DW)	TFC (mg CAE/g DW)	DPPH (μmolTE/g DW)	ABTS (μmolTE/g DW)
14	0	1	-1	50	25	15	13.90	2.21	33.69	52.36
15	0	1	1	50	25	30	15.30	2.49	38.17	59.33
16	1	0	-1	70	17.5	15	14.57	2.38	36.26	56.55
17	0	0	0	50	17.5	22.5	17.45	2.84	44.56	69.26

X₁, ethanol concentration (%); X₂, solvent: Material ratio (mL/g); X₃, extraction time (min). Data are mean of 3 replicates. TPC, total phenolic content; TFC, total flavonoid content; TE, Trolox equivalents; DW, dry weight; SMR, solvent-to-material ratio; EtOH, ethanol.

Regression equations:

$$\text{TPC (Y}_1\text{, mg GAE/g DW): } Y_1 = -18.36 + 0.3943X_1 + 0.6972X_2 + 1.7622X_3 - 0.003262X_1^2 - 0.02962X_2^2 - 0.03470X_3^2 + 0.003617X_1X_2 - 0.007130X_1X_3 + 0.01047X_2X_3 \quad (2)$$

$$\text{TFC (Y}_2\text{, mg CAE/g DW): } Y_2 = -2.446 + 0.06245X_1 + 0.1043X_2 + 0.2467X_3 - 0.000583X_1^2 - 0.005420X_2^2 - 0.004782X_3^2 + 0.001056X_1X_2 - 0.001179X_1X_3 + 0.001888X_2X_3 \quad (3)$$

$$\text{DPPH (Y}_3\text{, μmol TE/g DW): } Y_3 = -48.05 + 0.924X_1 + 1.682X_2 + 4.679X_3 - 0.00714X_1^2 - 0.07392X_2^2 - 0.09150X_3^2 + 0.00903X_1X_2 - 0.01930X_1X_3 + 0.02925X_2X_3 \quad (4)$$

$$\text{ABTS (Y}_4\text{, μmol TE/g DW): } Y_4 = -72.9 + 1.505X_1 + 2.802X_2 + 6.828X_3 - 0.01171X_1^2 - 0.1157X_2^2 - 0.1315X_3^2 + 0.01171X_1X_2 - 0.02864X_1X_3 + 0.0411X_2X_3 \quad (5)$$

where X₁ = ethanol concentration (%), X₂ = solvent: material ratio (mL/g), X₃ = extraction time (min).

ANOVA revealed that all 3 factors and their interactions significantly influenced extraction ($p < 0.05$, **Table 4**). Ethanol concentration exhibited dominant linear effects on TPC ($\beta_1 = 0.3943$, $p < 0.001$) and ABTS ($\beta_1 = 1.505$, $p < 0.001$), while extraction time showed strongest influence on antioxidant activities (DPPH: $\beta_3 = 4.679$, $p < 0.0001$; ABTS: $\beta_3 = 6.828$, $p < 0.0001$). Significant quadratic terms for all factors ($p < 0.01$) indicated non-linear relationships characteristic of cavitation-based extraction processes. Notable positive

interaction effects between solvent: material ratio and extraction time (X₂X₃: β TPC interaction = 0.01047, $p < 0.05$) suggested synergistic enhancement of mass transfer efficiency at higher solvent volumes and longer durations. This interaction likely reflects sustained cavitation bubble formation in larger liquid volumes, maintaining extraction driving force over extended periods. Three-dimensional response surface plots visualized these complex interactions, showing distinct optima within the experimental domain. Numerical optimization using the desirability function (equal weight = 1.0 for all responses) identified optimal conditions as 50% ethanol, 20:1 mL/g solvent-to-material ratio, and 30 min extraction time (composite desirability = 0.94, **Figure 2(C)**). Experimental validation ($n = 3$) yielded TPC of 18.05 ± 0.35 mg GAE/g DW (predicted: 17.81; deviation: 1.3%), TFC of 3.13 ± 0.13 mg CAE/g DW (predicted: 2.87; deviation: 9.1%), DPPH activity of 43.95 ± 0.23 μmol TE/g DW (predicted: 44.15; deviation: -0.5%), and ABTS activity of 70.01 ± 0.40 μmol TE/g DW (predicted: 68.16; deviation: 2.7) (**Table 5**). These deviations of 0.5% - 9.1% from predicted values confirm the high accuracy of the RSM model and validate the optimized extraction conditions. Response surface plots visualizing these relationships and their interactions are presented in **Figure 2(C)**, with contour projections provided in Supplementary **Figure 1**. Critically, ultrasonic extraction achieved 19.3% additional TPC enhancement beyond fermented flour alone (15.13 up to 18.05 mg GAE/g DW) and 7.4% increase in ABTS activity (65.16 up to 70.01 μmol TE/g DW), demonstrating that optimized extraction serves as more than passive recovery - it actively releases matrix-bound phenolics

generated during bioprocessing. The cumulative 6.1-fold TPC increase (2.94 up to 18.05 mg GAE/g DW) and 7.7-fold DPPH enhancement (5.68 up to 43.95 $\mu\text{mol TE/g DW}$) compared to raw beans represent

performance levels 35% - 52% higher than previously reported for germinated-fermented legumes (Starzyńska-Janiszewska *et al.*, 2021), validating the systematic optimization approach (Table 5).

Table 5 Experimental validation of optimized ultrasonic extraction conditions.

Parameter	Predicted Value	Experimental Value	Deviation (%)
TPC (mg GAE/g DW)	17.81	18.05 $\pm$ 0.35	1.3
TFC (mg CAE/g DW)	2.87	2.82 $\pm$ 0.24	9.1
DPPH ($\mu\text{mol TE/g DW}$)	44.15	43.95 $\pm$ 0.23	0.5
ABTS ($\mu\text{mol TE/g DW}$)	68.16	70.01 $\pm$ 0.40	2.7

Optimal conditions: 50% ethanol, 20:1 ratio, 30 min. Data are mean $\pm$ SD (n = 3). Deviation = [(Experimental – Predicted)/Predicted] $\times$ 100%.

Quantification of individual phenolic compounds via UPLC-PDA chromatographic analysis

To elucidate the specific chemical transformations underlying the observed increases in bioactivity, 8 key phenolic compounds were quantified via UPLC-PDA chromatographic analysis at each processing stage [32] at each processing stage. Representative chromatograms showing the progressive changes in phenolic profiles

across all processing stages are presented in Supplementary Figures S3 - S7, and final optimally extracted beans (Table 6, Figure S7). While UPLC-PDA chromatography enabled quantification of 8 major phenolic compounds, the semi-targeted approach may have missed minor bioactive metabolites generated during bioprocessing. Untargeted metabolomics (LC-MS/MS) would provide comprehensive phenolic profiling.

Table 6 Progressive phenolic transformation across bioprocessing stages: germination, fermentation and UPLC-PDA in Adzuki Beans ($\mu\text{g/g DW}$).

Samples Content ($\mu\text{g/g}$)	Ungerminated Control (C)	Germinated only (G)	Ungerminated Fermentation (F)	Germinated +Fermented (G+F)	Germinated +Fermented +Ultrasonic extracted (G+F+U)	Total Fold-Change
Gallic acid	38.50 ^c $\pm$ 0.31	13.36 ^c $\pm$ 0.10	36.13 ^d $\pm$ 0.30	85.56 ^b $\pm$ 0.45	89.12 ^a $\pm$ 0.44	2.3
Catechin	11.80 $\pm$ 0.10	111.65 ^b $\pm$ 0.55	49.44 ^d $\pm$ 0.36	86.20 ^c $\pm$ 0.38	199.53 ^a $\pm$ 0.62	16.9
Chlorogenic acid	7.90 ^c $\pm$ 0.08	36.08 ^c $\pm$ 0.32	11.35 ^d $\pm$ 0.16	58.092 ^b $\pm$ 0.25	72.43 ^a $\pm$ 0.28	9.2
Rutin	3.09 ^d $\pm$ 0.01	2.38 ^c $\pm$ 0.01	6.19 ^c $\pm$ 0.03	11.07 ^b $\pm$ 0.01	13.55 ^a $\pm$ 0.02	4.4
Ellagic acid	0.002 ^c $\pm$ 0.00	5.60 ^c $\pm$ 0.01	0.92 ^d $\pm$ 0.00	6.83 ^b $\pm$ 0.02	7.78 ^a $\pm$ 0.03	3889
Quercitrin	ND ^d	1.46 ^c $\pm$ 0.01	ND ^d	7.69 ^b $\pm$ 0.01	9.70 ^a $\pm$ 0.01	N
Quercetin	3.81 ^c $\pm$ 0.01	7.15 ^d $\pm$ 0.01	7.45 ^c $\pm$ 0.01	25.14 ^b $\pm$ 0.02	28.68 ^a $\pm$ 0.02	7.5
Apigenin	0.15 ^c $\pm$ 0.00	0.51 ^b $\pm$ 0.01	0.25 ^d $\pm$ 0.01	1.03 ^a $\pm$ 0.01	0.34 ^c $\pm$ 0.00	2.3
Sum	65.27	178.19	111.72	276.61	421.12	6.5

Phenolic concentrations expressed as $\mu\text{g/g DW}$ (mean $\pm$ SD, n = 3). ND, not detected ($< 0.001 \mu\text{g/g}$ limit of quantification). Superscript letters within rows indicate significant differences across processing stages within each compound ($p < 0.05$, 2-way ANOVA). Total fold-change (FC) calculated as final extract (G+F+U) $\div$ Raw beans value. UPLC-Conditions: BDS Hypersil C18 column (250 $\times$ 4.6 mm², 5 μm), methanol-0.1% H₃PO₄ gradient, 265 nm detection. See Supplementary Figures S3 - S7 for full chromatograms. C, Ungerminated raw beans as control; G, germinated; F, fermented; G+F, germinated + fermented; G+F+U, final extract.

Germination-induced transformations

Catechin content increased 9.5-fold (11.80 up to 111.65 $\mu\text{g/g DW}$), consistent with activation of flavonoid biosynthetic pathways [13]. The disproportionate 16.9-fold catechin amplification across the full bioprocessing workflow reflects a convergence of 3 mechanisms: (1) germination-induced upregulation of phenylalanine ammonia-lyase (PAL), the rate-limiting enzyme of the phenylpropanoid pathway, driving de novo catechin biosynthesis [13,14]; (2) hydrolysis of procyanidin oligomers and tannin-bound catechin monomers released during germination and UAE [15,17]; and (3) the high extraction efficiency of catechin in 50% ethanol, which closely matches its moderate log P (polarity), optimizing partitioning into the solvent phase [7]. This mechanistic convergence also explains the preferential DPPH activity enhancement observed in this study, as the DPPH assay is particularly responsive to the hydrogen-donating capacity of lipophilic flavanols such as catechin [6]. The parallel 4.6-fold chlorogenic acid increase (7.90 up to 36.08 $\mu\text{g/g DW}$) while ellagic acid surged from trace levels (0.002 up to 5.60 $\mu\text{g/g DW}$, 2,800-fold increase). Quercitrin appeared de novo (1.46 $\mu\text{g/g DW}$), suggesting glycosyltransferase activity. These transformations demonstrate that germination does not merely increase total phenolic content quantitatively-it fundamentally remodels the phytochemical profile qualitatively, generating compounds with distinct bioactivities and bioavailabilities. Conversely gallic acid decreased 65% (38.50 down to 13.36 $\mu\text{g/g DW}$) potentially due to enzymatic polymerization into hydrolysable tannins or conjugation to cell wall matrices-mechanisms requiring alkaline hydrolysis experiments for confirmation.

Fermentation-induced transformations

Quercetin aglycone increased 6.6-fold overall, a pattern consistent with β -glucosidase-mediated hydrolysis of rutin glycosides, though β -glucosidase activity was not directly measured in this study. Future work should include time-resolved enzymatic activity profiling (β -glucosidase, U/mL) across the 72-hour fermentation to confirm this proposed mechanism. The optimal pH (4.5) aligns with reported fungal β -glucosidase profiles [37,40]. Rutin simultaneously

increased 4.7-fold (2.38 up to 11.07 $\mu\text{g/g DW}$), suggesting concurrent liberation from matrix complexes or potential trans glycosylation [40]. Catechin decreased from germination peak (111.65 up to 86.20 $\mu\text{g/g DW}$), possibly reflecting polymerization into proanthocyanidins undetectable as monomers. Gallic acid rebounded dramatically (13.36 up to 85.56 $\mu\text{g/g DW}$), indicating tannase-mediated hydrolysis of gallotannins accumulated during germination. A key limitation of the current study is the absence of direct β -glucosidase and tannase activity assays during fermentation. While the observed phenolic transformations-particularly the 6.6-fold quercetin aglycone enrichment-strongly suggest yeast-mediated glycoside hydrolysis, future mechanistic studies should include time-resolved enzymatic activity measurements to establish quantitative enzyme kinetics.

UAE-induced transformations

Ultrasonic-assisted extraction (UAE) markedly enhanced phenolic recovery, achieving the highest concentrations for 7 of 8 quantified compounds, including catechin (199.53 $\mu\text{g/g DW}$; 16.9-fold vs. raw beans), chlorogenic acid (72.43 $\mu\text{g/g DW}$; 9.2-fold), and quercitrin (9.70 $\mu\text{g/g DW}$; de novo appearance). UAE also released cell-wall-bound phenolics, evidenced by a 2.3-fold increase in gallic acid and a 19% rise in total phenolics (276.61 up to 421.12 $\mu\text{g/g DW}$). Notably, apigenin decreased by 67%, likely due to oxidative degradation by ultrasound-generated hydroxyl radicals, protein-phenolic complexation, or thermal isomerization during cavitation [12,17]. Despite this loss, the overall synergistic effect was substantial, with total phenolics increasing 6.5-fold, closely matching the 6.1-fold rise in TPC measured by Folin-Ciocalteu assay, validating the efficacy of the integrated germination-fermentation-UAE workflow.

Additive enhancement via combined germination and fermentation

The sequential application of optimized germination and fermentation yielded the most profound and diverse enhancements, producing the highest concentrations for 6 of the 8 compounds analyzed. This additive process resulted in a 6.6-fold increase in quercetin (to 25.14 $\mu\text{g/g DW}$) and a 6.8-fold increase in

apigenin (to 1.03 $\mu\text{g/g}$ DW) compared to the raw beans. Furthermore, the combined treatment amplified the concentrations of gallic acid (2.2-fold), chlorogenic acid (7.4-fold), and rutin (3.6-fold). Notably, while germination alone produced the highest absolute level of catechin, the final combined process still maintained a substantial 7.3-fold increase over the initial raw bean content. These results provide clear chemical evidence of the powerful synergy between germination and fermentation for unlocking the full bioactive potential of adzuki beans [10,13].

Development of functional beverage

The optimally extracted adzuki bean flour was formulated into a consumer-ready functional beverage by blending with 6% sucrose (w/v, optimized through preliminary taste panel, $n = 10$). The beverage mixture was then filled into sterilized glass bottles (100 mL capacity), capped, and subjected to thermal pasteurization at 95 °C (water bath) for 2 min. Internal beverage temperature reached ≥ 90 °C within 60 s, as verified by a calibrated thermocouple ($n = 3$), consistent with FDA guidelines for thermal inactivation of *Escherichia coli* and *Salmonella* in plant-based beverages [26]. After pasteurization, the bottles were immediately cooled in an ice bath to < 25 °C within 5 min and stored at 4 °C until analysis (maximum 7 days). The beverage exhibited exceptional bioactive retention through thermal processing: TPC 36.20 ± 0.78 mg GAE/100 mL (equivalent to 40.2 mg GAE/g DW on dry basis, 95.0% retention), TFC 2.78 ± 0.07 mg CAE/100 mL (98.7% retention), DPPH 42.80 ± 0.45 $\mu\text{mol TE}/100$ mL (97.4% retention), and ABTS 62.95 ± 0.44 $\mu\text{mol TE}/100$ mL (99.9% retention). This thermal stability is attributed to: (1) quercetin's high decomposition

temperature (> 250 °C in dry state; aqueous solutions stable at 95 °C for short durations < 5 min), (2) sugar matrix protection where 6% sucrose forms hydrogen bonds with phenolic hydroxyl groups reducing oxidation susceptibility during heating, and (3) rapid cooling protocol minimizing post-pasteurization degradation.

Four sensory attributes were evaluated (**Table 7**): Color (1 = very poor/muddy brown, 5 = excellent/attractive reddish-brown characteristic of adzuki), aroma (1 = off-odor/fermented/ unpleasant, 5 = pleasant/characteristic nutty adzuki bean notes), taste (1 = unacceptable bitterness or sourness, 5 = well-balanced sweetness with mild acceptable astringency), and mouthfeel (1 = chalky/gritty/slimy, 5 = smooth/velvety/clean finish). Quantitative Descriptive Analysis by 50 panelists revealed strong sensory acceptance across all attributes: color 4.30 ± 0.79 (attractive reddish-brown characteristic of adzuki), aroma 4.57 ± 0.73 (pleasant nutty adzuki bean notes), taste 4.40 ± 0.81 (balanced sweetness with mild acceptable astringency), and mouthfeel 4.47 ± 0.81 (smooth, non-chalky texture). Overall acceptability scored 4.44 ± 0.75 on a 5-point hedonic scale (1 = very poor, 5 = excellent). These sensory scores compare favorably to benchmark consumer studies on legume-based beverages [41,42]: Commercial red bean milk (color 4.2, aroma 4.0), soy beverages (aroma 3.8 - 4.1, taste 3.9 - 4.2), and premium tea drinks (taste 4.3 - 4.5). The balanced sweetness (6% sucrose) effectively overcame potential phenolic astringency - a critical consumer acceptability factor often limiting legume-based functional beverages. The smooth mouthfeel (score 4.47) was superior to typical protein shakes (3.5 - 4.0), attributed to fine particle size (< 0.5 mm grinding) eliminating chalkiness.

Table 7 Sensory evaluation of adzuki bean functional beverage.

Attribute	Mean Score $\pm$ SD	Interpretation
Color	4.30 ± 0.79	Attractive reddish-brown, characteristic
Aroma	4.57 ± 0.73	Pleasant, nutty adzuki bean notes
Taste	4.40 ± 0.81	Balanced sweetness, mild astringency
Mouthfeel	4.47 ± 0.81	Smooth, non-chalky texture
Overall Acceptability	4.44 ± 0.75	High consumer appeal

5-point hedonic scale (1 = very poor, 5 = excellent). Panelists ($n = 50$) following ISO 4121:2003. Data are mean $\pm$ SD (**Table S4** for scale descriptors).

Integrated platform demonstrates cascade bioactive enhancement with qualitative phenolic remodeling

To formally assess whether the integrated platform produces genuine biochemical synergy (combined outcome > sum of independent outcomes), we compared the observed cumulative 6.1-fold TPC increase against the expected additive contribution: Germination alone yields 3.5-fold; fermentation applied to germinated beans yields an additional 1.5-fold multiplier (cumulative: 5.1-fold expected from additive sequential processing); UAE contributes a further 1.2-fold (19.3% enhancement), yielding an expected additive cumulative of approximately 6.1-fold. The observed value (6.1-fold) is therefore consistent with sequential additive enhancement rather than strict biochemical synergy in the quantitative TPC dimension. Nonetheless, the UPLC-PDA chromatographic evidence (**Figured S3 - S7**) demonstrates genuine qualitative transformations - including de novo quercitrin generation (absent in raw beans, undetectable after single-stage processing) and a progressive quercetin: rutin ratio shift from 1.2:1 (raw) to 2.1:1 (final extract) - that represent molecular remodeling unachievable through any individual processing stage and provide functional evidence of cascade-dependent biochemical transformation. The platform's modularity-with transferable optimized parameters (temperature-time relationships for germination, pH-temperature for fermentation, solvent-ratio-time for extraction)-enables diversified bioactive ingredient development from other underutilized legumes (mung beans, black beans, lentils), addressing global demands for clean-label, sustainable, plant-based functional foods.

Conclusions

This study successfully establishes an optimized, 3-phase bioprocessing strategy that systematically transforms adzuki beans from underutilized commodity to consumer-ready functional beverage through scientifically optimized sequential interventions. Phase 1 (green bioprocessing) - combining germination - induced phenylpropanoid pathway activation (27 °C, 68 h) with yeast-mediated enzymatic glycoside biotransformation (*S. cerevisiae* B5 at 25 °C, pH 4.5, 72 h)-achieved a 5.1-fold enhancement in total phenolic content. Phase 2 (optimized extraction and formulation)

employing cavitation-enhanced ultrasonic extraction (50% ethanol, 20:1 ratio, 30 min) followed by Phase 3 beverage formulation.

This platform demonstrates sequential bioprocessing complementarity, with cumulative TPC improvements (6.1-fold) exceeding values typically reported for optimized germination alone (2 - 3-fold [13]) or fermentation alone (~4-fold [43]). The 6.6-fold quercetin aglycone enrichment (raw beans: 3.81 up to final: 28.68 µg/g DW), 16.9-fold catechin amplification (11.80 up to 199.53 µg/g DW), and de novo generation of quercitrin (0 up to 9.70 µg/g DW) represent qualitative transformations impossible through single-stage processing.

Critically, this bioactive enhancement was successfully translated to an industrially viable functional beverage, demonstrating strong sensory acceptance across all attributes (mean scores > 4.3/5.0 from panelists). This successful proof-of-concept translation to a consumer-ready product highlights the potential for full commercial viability, although further validation is required through pilot-scale testing, techno-economic analysis, and extended shelf-life testing. The platform's modularity, with transferable, optimized parameters applicable to other legumes, enables diversified bioactive ingredient development, addressing global demands for clean-label, sustainable, plant-based functional foods. The absence of direct enzymatic assays (β-glucosidase, tannase) represents a key limitation of the current study. Planned mechanistic follow-up experiments will directly quantify β-glucosidase activity kinetics during *S. cerevisiae* B5 fermentation to establish the enzymatic basis of the observed phenolic transformations. Additional limitations include the single-cultivar scope and laboratory scale, which preclude direct generalization to other *Vigna angularis* varieties or industrial production settings. These findings provide a robust scientific foundation for the industrial-scale production of high-value, bean-based functional food ingredients, offering a sustainable method to enhance the health-promoting properties of this important legume.

Acknowledgements

This study was financially supported by Hue University (DHH2025-02-191).

Declaration of Generative AI in Scientific Writing

No generative AI tools were used in the writing of this manuscript except grammar check.

CRediT Author Statement

Phan Thi Be: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Do Thi Bich Thuy:** Conceptualization, Methodology, Supervision, Project administration, Writing - review & editing. **Bir Bahadur Thapa:** Formal analysis, Visualization, Writing - original draft. **Nguyen Cao Cuong:** Formal analysis, Writing - original draft. **Vo Van Quoc Bao:** Writing - review & editing. **Tran Thanh Quynh Anh:** Writing - review & editing. All authors have read and agreed to the published version of the manuscript.

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Supplementary materials

Supplementary Tables

Table S1 Factors and levels used in the central composite design for optimizing adzuki bean germination.

Factors	Levels				
	$-\alpha$ (−1.41)	−1	0	1	$+\alpha$ (+1.41)
Germination temperature (°C)	23.98	25	27.5	30	31.03
Germination time (h)	26.16	36	60.0	84	93.84

Table S2 Five-point hedonic scale of sensory evaluation.

Score	Color	Odor	Texture	Taste	General acceptability
1	Clear defect	Clear defect	Clear defect	Clear defect	Very Poor, Unacceptable
2	Minor defects	Weak sensation, disharmonious	Minor defects	Weak sensation, disharmonious	Poor
3	Neutral	Neutral	Neutral	Neutral	Average, no serious defects
4	Pleasant	Pleasant	Pleasant	Pleasant, well-balanced	Good, meets standards
5	Optimal color	Optimal aroma, high complexity	Optimal texture	Optimal flavor, high complexity	Excellent

Table S3 Effect of germination temperature and time on the total phenolic content (TPC) and total flavonoid content (TFC) in Adzuki Beans.

Temp. (°C) Sample	TPC (mg GAE/g DW)				TFC (mg CAE/g DW)			
	20 °C	25 °C	30 °C	35 °C	20 °C	25 °C	30 °C	35 °C
Seeds before germination		2.94 ^{nop} ± 0.14				0.73 ^{ijklm} ± 0.03		
G00		2.70 ^p ± 0.16				0.62 ^m ± 0.06		
G12	2.64 ^p ± 0.17	3.61 ^{klmno} ± 0.20	3.72 ^{klmn} ± 0.51	4.61 ^{ghij} ± 0.04	0.69 ^{lm} ± 0.07	0.78 ^{ijklm} ± 0.06	0.70 ^{lm} ± 0.14	0.78 ^{klm} ± 0.03
G24	2.66 ^p ± 0.25	3.95 ^{ijklm} ± 0.09	4.65 ^{ghi} ± 0.24	4.81 ^{fgh} ± 0.13	0.78 ^{ijklm} ± 0.09	0.96 ^{ghi} ± 0.01	1.05 ^{ghi} ± 0.06	1.14 ^{ef} ± 0.08
G36	2.81 ^{op} ± 0.02	5.54 ^{ef} ± 0.23	6.08 ^{de} ± 0.68	5.52 ^{ef} ± 0.19	0.85 ^{ijkl} ± 0.05	1.06 ^{fgh} ± 0.05	1.22 ^{def} ± 0.07	1.09 ^{ef} ± 0.02
G48	2.83 ^{op} ± 0.05	8.75 ^{bc} ± 0.39	9.29 ^{abc} ± 0.26	5.95 ^{de} ± 0.08	0.86 ^{ijkl} ± 0.03	1.38 ^{bcd} ± 0.01	1.54 ^{abc} ± 0.08	1.28 ^{de} ± 0.05
G60	2.93 ^{nop} ± 0.11	9.61 ^a ± 0.27	8.69 ^c ± 0.06	5.43 ^{efg} ± 0.49	0.87 ^{ijkl} ± 0.04	1.70 ^a ± 0.07	1.57 ^a ± 0.07	1.27 ^{de} ± 0.06
G72	3.14 ^{mnp} ± 0.11	9.54 ^{ab} ± 0.48	6.62 ^d ± 0.17	4.59 ^{hij} ± 0.13	0.86 ^{ijkl} ± 0.03	1.69 ^a ± 0.05	1.54 ^{ab} ± 0.05	1.21 ^{def} ± 0.02
G84	3.16 ^{lmnop} ± 0.07	8.89 ^{abc} ± 0.19	6.45 ^d ± 0.29	4.38 ^{hijk} ± 0.13	0.86 ^{ijkl} ± 0.03	1.60 ^a ± 0.02	1.22 ^{def} ± 0.03	0.91 ^{hij} ± 0.06
G96	3.12 ^{mnp} ± 0.11	6.47 ^d ± 0.27	4.91 ^{fgh} ± 0.14	4.32 ^{hijk} ± 0.11	0.86 ^{ijkl} ± 0.06	1.37 ^{bcd} ± 0.03	0.90 ^{hij} ± 0.02	0.88 ^{hij} ± 0.07
G108	3.21 ^{lmnop} ± 0.13	6.44 ^d ± 0.56	3.98 ^{ijkl} ± 0.19	3.79 ^{ijklm} ± 0.18	0.85 ^{ijkl} ± 0.03	1.22 ^{def} ± 0.05	0.83 ^{ijkl} ± 0.02	0.78 ^{klm} ± 0.03

Data are presented as mean ± standard deviation (n = 3). Different superscript letters within each column indicate statistically significant differences ($p < 0.05$, 2-way ANOVA followed by Tukey's HSD test). Temperature × time interaction significant for all responses ($p < 0.0001$). TPC = total phenolic content expressed as mg gallic acid equivalents (GAE) per g dry weight (DW); TFC = total flavonoid content expressed as mg catechin equivalents (CAE) per g DW.

Table S4 The effect of germination temperature and time on the DPPH and ABTS antioxidant activities in Adzuki Beans.

Temp. (°C) Sample	DPPH antioxidant activity ($\mu\text{molTE/g DW}$)				ABTS antioxidant activity ($\mu\text{molTE/g DW}$)			
	20 °C	25 °C	30 °C	35 °C	20 °C	25 °C	30 °C	35 °C
Seeds before germination			5.68 ^r $\pm$ 0.02				20.06 ^{k 1} $\pm$ 0.17	
G00			5.48 ^r $\pm$ 0.08				18.77 ^{k 1} $\pm$ 0.28	
G12	6.40 ^{pr} $\pm$ 0.15	7.13 ^p $\pm$ 0.17	7.52 ^o $\pm$ 0.08	9.24 ⁿ $\pm$ 0.05	18.99 ^{kl} $\pm$ 0.21	18.23 ^{kl} $\pm$ 0.14	15.36 ^m $\pm$ 0.49	26.69 ^{ef} $\pm$ 0.38
G24	6.40 ^{pr} $\pm$ 0.16	12.55 $\pm$.53	13.40 ^k $\pm$ 0.22	19.23 ^h $\pm$ 0.35	18.71 ^{kl} $\pm$ 0.62	24.74 ^{gh} $\pm$ 0.31	18.63 ^{k 1} $\pm$ 0.29	26.58 ^{ef} $\pm$ 0.43
G36	6.48 ^{pr} $\pm$ 0.43	16.43 ⁱ $\pm$ 0.51	20.01 ^s $\pm$ 0.58	18.85 ^h $\pm$ 0.25	18.76 ^{kl} $\pm$ 0.63	27.02 ^e $\pm$ 0.90	20.08 ^j $\pm$ 0.12	26.29 ^{ef} $\pm$ 0.32
G48	6.35 ^{pr} $\pm$ 0.28	25.06 ^{cd} $\pm$ 0.36	25.75 ^{bc} $\pm$ 0.46	19.27 ^h $\pm$ 0.21	19.52 ^{kl} $\pm$ 0.38	34.56 ^{ab} $\pm$ 0.32	31.87 ^{cd} $\pm$ 0.72	24.11 ^{ghi} $\pm$ 0.18
G60	6.38 ^{pr} $\pm$ 0.13	26.61 ^{ab} $\pm$ 0.67	25.09 ^{cdc} $\pm$ 0.07	14.21 ^j $\pm$ 0.37	19.44 ^{kl} $\pm$ 0.67	34.68 ^{ab} $\pm$ 0.12	35.13 ^a $\pm$ 0.09	19.18 ⁿ $\pm$ 0.97
G72	6.40 ^{pr} $\pm$ 0.09	26.39 ^{ab} $\pm$ 0.12	27.12 ^a $\pm$ 0.33	9.53 ^m $\pm$ 0.26	23.90 ^{hi} $\pm$ 0.19	34.48 ^{ab} $\pm$ 0.22	31.53 ^d $\pm$ 0.53	18.55 ⁱ $\pm$ 0.11
G84	6.86 ^p $\pm$ 0.16	24.19 ^d $\pm$ 0.31	22.35 ^f $\pm$ 0.49	9.59 ^m $\pm$ 0.35	23.12 ^{gh} $\pm$ 0.50	33.24 ^{bc} $\pm$ 0.25	31.37 ^d $\pm$ 0.71	15.69 ^m $\pm$ 0.05
G96	8.30 ^{mn} $\pm$ 0.41	22.18 ^{ef} $\pm$ 0.19	13.96 ^{jk} $\pm$ 0.21	9.00 ^{mn} $\pm$ 0.43	23.29 ⁱ $\pm$ 0.29	25.30 ^{fg} $\pm$ 0.50	23.09 ⁱ $\pm$ 0.43	12.05 ⁿ $\pm$ 0.17
G108	9.17 ^{mn} $\pm$ 0.08	21.23 ^f $\pm$ 0.07	13.07 ^l $\pm$ 0.22	8.84 ^{mn} $\pm$ 0.80	18.71 ^{kl} $\pm$ 0.16	20.52 ^j $\pm$ 0.70	18.39 ^{kl} $\pm$ 0.47	11.60 ⁿ $\pm$ 0.05

Data are presented as mean $\pm$ standard deviation (n = 3). Different superscript letters within each column indicate statistically significant differences (p < 0.05, 2-way ANOVA followed by Tukey's HSD test). Temperature $\times$ time interaction significant for all responses (p < 0.0001). DPPH = 2,2-diphenyl-1-picrylhydrazyl and ABTS = 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) both expressed as μmol Trolox equivalents (TE) per g DW.

Table S5 Screening of yeast isolates (B1-B9) for bioactive enhancement in fermented adzuki beans.

Sample	TPC (mg GAE/g DW)	TFC (mg CAE/g DW)	DPPH ($\mu\text{molTE/g DW}$)	ABTS ($\mu\text{molTE/g DW}$)
Control	9.63 ^f $\pm$ 0.15	1.68 ^e $\pm$ 0.02	27.65 ^f $\pm$ 0.23	34.94 ^f $\pm$ 0.50
B1	10.68 ^e $\pm$ 0.18	1.79 ^{bcd} $\pm$ 0.03	29.13 ^e $\pm$ 0.44	43.15 ^e $\pm$ 0.23
B2	11.00 ^{de} $\pm$ 0.18	1.82 ^{bcd} $\pm$ 0.04	29.28 ^e $\pm$ 0.31	46.43 ^d $\pm$ 0.36
B3	11.75 ^c $\pm$ 0.09	1.89 ^{abc} $\pm$ 0.01	30.49 ^{cd} $\pm$ 0.17	54.20 ^c $\pm$ 0.17
B4	10.41 ^e $\pm$ 0.37	1.74 ^{de} $\pm$ 0.02	29.10 ^e $\pm$ 0.27	43.11 ^e $\pm$ 0.57
B5	13.67 ^a $\pm$ 0.42	2.02 ^a $\pm$ 0.11	33.45 ^a $\pm$ 0.29	62.24 ^a $\pm$ 0.28
B6	11.61 ^{cd} $\pm$ 0.05	1.83 ^{bcd} $\pm$ 0.05	29.71 ^{de} $\pm$ 0.12	53.15 ^c $\pm$ 0.90
B7	11.40 ^{cd} $\pm$ 0.27	1.82 ^{bcd} $\pm$ 0.04	30.11 ^d $\pm$ 0.31	54.09 ^c $\pm$ 0.76
B8	11.47 ^{cd} $\pm$ 0.04	1.90 ^{abc} $\pm$ 0.02	31.79 ^b $\pm$ 0.21	58.47 ^b $\pm$ 0.35
B9	12.58 ^b $\pm$ 0.24	1.93 ^{ab} $\pm$ 0.06	31.12 ^{bc} $\pm$ 0.27	59.08 ^b $\pm$ 0.33

Isolated strains from fermented jackfruit were inoculated (10^6 CFU/mL) into sterile germinated bean flour broth (1:10 w/v) and fermented (30 °C, pH 5.0, 72 h, 150 rpm). Control represents optimally germinated but unfermented beans. Data are mean $\pm$ SD (n = 3). Different superscript letters within each column indicate significant differences (p < 0.05, 1-way ANOVA followed by Tukey's HSD test). Isolate B5 demonstrated superior performance across all metrics and was selected for molecular identification and subsequent optimization experiments. CFU, colony-forming units.

Table S6 Molecular identification of yeast isolate B5 via ITS sequencing.

Yeast strain No.	Accession numbers of the ITS sequences	Species	Similarity (%)
B5	LC007671.1	<i>Saccharomyces cerevisiae</i>	99.87

ITS, internal transcribed spacer. Top BLAST matches from NCBI GenBank database.

Table S7 Effect of ethanol concentration (10-90%) on ultrasonic extraction efficiency (preliminary OFAT screening).

EtOH (%)	TPC (mg GAE/g DW)	TFC (mg CAE/g DW)	DPPH ($\mu\text{molTE/g DW}$)	ABTS ($\mu\text{molTE/g DW}$)
10	13.019 ^c $\pm$ 0.414	2.0355 ^c $\pm$ 0.0131	36.74 ^d $\pm$ 0.241	65.953 ^c $\pm$ 0.317
30	17.274 ^a $\pm$ 0.127	2.6409 ^{ab} $\pm$ 0.0534	40.576 ^b $\pm$ 0.445	69.231 ^b $\pm$ 0.262
50	17.677 ^a $\pm$ 0.245	2.7265 ^a $\pm$ 0.0641	43.798 ^a $\pm$ 0.147	70.197 ^a $\pm$ 0.334
70	16.356 ^b $\pm$ 0.25	2.5228 ^b $\pm$ 0.0311	38.626 ^c $\pm$ 0.643	59.103 ^d $\pm$ 0.126
90	11.733 ^d $\pm$ 0.414	1.4927 ^d $\pm$ 0.0559	32.013 ^c $\pm$ 0.423	55.783 ^c $\pm$ 0.443

Table S8 Effect of SMR (solvent: material ratio: 5 - 25 mL/g) on ultrasonic extraction efficiency of TPC, TFC, DPPH and ABTS (preliminary OFAT screening).

SMR (mL/g)	TPC (mg GAE/g DW)	TFC (mg CAE/g DW)	DPPH ($\mu\text{molTE/g DW}$)	ABTS ($\mu\text{molTE/g DW}$)
5	15.157 ^c $\pm$ 0.131	2.150 ^b $\pm$ 0.083	35.087 ^c $\pm$ 0.562	48.177 ^d $\pm$ 0.510
10	16.499 ^b $\pm$ 0.274	2.305 ^b $\pm$ 0.086	41.919 ^b $\pm$ 0.519	57.170 ^c $\pm$ 0.146
15	17.397 ^a $\pm$ 0.188	2.574 ^a $\pm$ 0.081	43.067 ^a $\pm$ 0.482	66.121 ^b $\pm$ 0.405
20	17.658 ^a $\pm$ 0.375	2.716 ^a $\pm$ 0.083	43.792 ^a $\pm$ 0.057	70.239 ^a $\pm$ 0.073
25	17.758 ^a $\pm$ 0.302	2.724 ^a $\pm$ 0.055	43.852 ^a $\pm$ 0.255	70.113 ^a $\pm$ 0.193

Table S9 Effect of extraction time (10-35 min) on ultrasonic extraction efficiency (preliminary OFAT screening)

Extract time (min)	TPC (mg GAE/g DW)	TFC (mg CAE/g DW)	DPPH ($\mu\text{molTE/g DW}$)	ABTS ($\mu\text{molTE/g DW}$)
10	13.075 ^c $\pm$ 0.609	1.4908 ^c $\pm$ 0.097	28.58 ^c $\pm$ 1.86	54.564 ^d $\pm$ 0.126
15	14.178 ^c $\pm$ 0.532	1.849 ^b $\pm$ 0.034	35.103 ^b $\pm$ 0.267	57.884 ^c $\pm$ 1.087
20	16.016 ^b $\pm$ 0.837	1.9071 ^b $\pm$ 0.024	35.387 ^b $\pm$ 0.486	59.229 ^c $\pm$ 0.655
25	16.661 ^{ab} $\pm$ 0.149	2.711 ^a $\pm$ 0.011	43.206 ^a $\pm$ 1.270	62.801 ^b $\pm$ 0.955
30	17.719 ^a $\pm$ 0.062	2.7168 ^a $\pm$ 0.008	43.81 ^a $\pm$ 0.1250	70.197 ^a $\pm$ 0.883
35	17.705 ^a $\pm$ 0.585	2.7192 ^a $\pm$ 0.134	42.725 ^a $\pm$ 0.976	70.029 ^a $\pm$ 0.291

Table S10 ANOVA tables - Central Composite Design (CCD) - germination optimization.

Source	df	Sum of squares	Mean square	F-value	p-value	Sig.
Response 1: TPC (mg GAE/g DW)						
Model	5	43.8004	8.7601	63.85	0.0000	***
A (Temperature)	1	0.8822	0.8822	6.43	0.0389	*
B (Time)	1	8.5072	8.5072	62.01	0.0001	***
AB	1	2.3562	2.3562	17.17	0.0043	**
A ²	1	13.2877	13.2877	96.85	0.0000	***
B ²	1	22.6715	22.6715	165.25	0.0000	***
Residual	7	0.9604	0.1372			
Lack of Fit	3	0.3959	0.1320	0.94	0.5020	ns
Pure Error	4	0.5645	0.1411			
Total (corrected)	12	44.7607				
R ²		97.85%				

Source	df	Sum of squares	Mean square	F-value	p-value	Sig.
Adjusted R ²	96.32%					
Std Dev	0.3704					
Model p-value	0.000011					
Lack-of-fit p	0.5020 (> 0.05 = adequate)					
Response 2: TFC (mg CAE/g DW)						
Model	5	1.2941	0.2588	33.89	0.0001	***
A (Temperature)	1	0.0452	0.0452	5.92	0.0452	*
B (Time)	1	0.2123	0.2123	27.80	0.0012	**
AB	1	0.0729	0.0729	9.54	0.0176	*
A ²	1	0.2673	0.2673	35.00	0.0006	***
B ²	1	0.7984	0.7984	104.53	0.0000	***
Residual	7	0.0535	0.0076			
Lack of Fit	3	0.0179	0.0060	0.67	0.6118	ns
Pure Error	4	0.0355	0.0089			
Total (corrected)	12	1.3475				
R ²	96.03%					
Adjusted R ²	93.20%					
Std Dev	0.0874					
Model p-value	0.000092					
Lack-of-fit p	0.6118 (> 0.05 = adequate)					
Response 3: DPPH (μmol TE/g DW)						
Source	df	Sum of Squares	Mean Square	F-value	p-value	Sig.
Model	5	192.1503	38.4301	636.59	0.0000	***
A (Temperature)	1	1.3328	1.3328	22.08	0.0022	**
B (Time)	1	53.6951	53.6951	889.46	0.0000	***
AB	1	7.3984	7.3984	122.55	0.0000	***
A ²	1	48.1852	48.1852	798.19	0.0000	***
B ²	1	96.8481	96.8481	1604.29	0.0000	***
Residual	7	0.4226	0.0604			
Lack of Fit	3	0.0707	0.0236	0.27	0.8461	ns
Pure Error	4	0.3519	0.0880			
Total (corrected)	12	192.5729				
R ²	99.78%					
Adjusted R ²	99.62%					
Std Dev	0.2457					
Model p-value	0.000000					
Lack-of-fit p	0.8461 (> 0.05 = adequate)					
Response 4: ABTS (μmol TE/g DW)						
Source	df	Sum of Squares	Mean Square	F-value	p-value	Sig.
Model	5	151.9546	30.3909	119.56	0.0000	***
A (Temperature)	1	5.9784	5.9784	23.52	0.0019	**

Source	df	Sum of Squares	Mean Square	F-value	p-value	Sig.
Model	9	1.4105	0.1567	82.56	0.0000	***
A (EtOH concentration)	1	0.0493	0.0493	25.97	0.0014	**
B (Solvent:material ratio)	1	0.0441	0.0441	23.23	0.0019	**
C (Extraction time)	1	0.0143	0.0143	7.52	0.0288	*
AB	1	0.1002	0.1002	52.77	0.0002	***
AC	1	0.1250	0.1250	65.83	0.0001	***
BC	1	0.0452	0.0452	23.79	0.0018	**
A ²	1	0.2294	0.2294	120.86	0.0000	***
B ²	1	0.3915	0.3915	206.24	0.0000	***
C ²	1	0.3045	0.3045	160.42	0.0000	***
Residual	7	0.0133	0.0019			
Lack of Fit	3	0.0040	0.0013	0.57	0.6618	ns
Pure Error	4	0.0093	0.0023			
Total (corrected)	16	1.4237				
R ²	99.07%					
Adjusted R ²	97.87%					
Std Dev	0.0436					
Model p-value	0.000003					
Lack-of-fit p	0.6618 (> 0.05 = adequate)					
Response 3: DPPH (μmol TE/g DW)						
Model	9	332.5686	36.9521	30.45	0.0001	***
A (EtOH concentration)	1	14.3166	14.3166	11.80	0.0109	*
B (Solvent:material ratio)	1	18.7180	18.7180	15.42	0.0057	**
C (Extraction time)	1	5.2959	5.2959	4.36	0.0751	ns
AB	1	7.3333	7.3333	6.04	0.0436	*
AC	1	33.5125	33.5125	27.61	0.0012	**
BC	1	10.8274	10.8274	8.92	0.0203	*
A ²	1	34.3664	34.3664	28.32	0.0011	**
B ²	1	72.8018	72.8018	59.99	0.0001	***
C ²	1	111.5295	111.5295	91.90	0.0000	***
Residual	7	8.4954	1.2136			
Lack of Fit	3	0.3786	0.1262	0.06	0.9771	ns
Pure Error	4	8.1168	2.0292			
Total (corrected)	16	341.0639				
R ²	97.51%					
Adjusted R ²	94.31%					
Std Dev	1.1016					

Source	df	Sum of Squares	Mean Square	F-value	p-value	Sig.
Model p-value	0.000085					
Lack-of-fit p	0.9771 (> 0.05 = adequate)					
Response 4: ABTS ($\mu\text{mol TE/g DW}$)						
Model	9	748.3858	83.1540	41.12	0.0000	***
A (EtOH concentration)	1	35.3767	35.3767	17.49	0.0041	**
B (Solvent:material ratio)	1	30.5137	30.5137	15.09	0.0060	**
C (Extraction time)	1	17.7459	17.7459	8.77	0.0210	*
AB	1	12.3341	12.3341	6.10	0.0429	*
AC	1	73.8139	73.8139	36.50	0.0005	***
BC	1	21.3444	21.3444	10.55	0.0141	*
A ²	1	92.3005	92.3005	45.64	0.0003	***
B ²	1	178.4576	178.4576	88.24	0.0000	***
C ²	1	230.2585	230.2585	113.85	0.0000	***
Residual	7	14.1568	2.0224			
Lack of Fit	3	1.7799	0.5933	0.19	0.8970	ns
Pure Error	4	12.3769	3.0942			
Total (corrected)	16	762.5426				
R ²	98.14%					
Adjusted R ²	95.76%					
Std Dev	1.4221					
Model p-value	0.000031					
Lack-of-fit p	0.8970 (> 0.05 = adequate)					

Significance codes: *** $p < 0.001$ ** $p < 0.01$ * $p < 0.05$ ns $p \geq 0.05$.

SS = Sum of Squares; MS = Mean Square; df = degrees of freedom.

Supplementary Figures

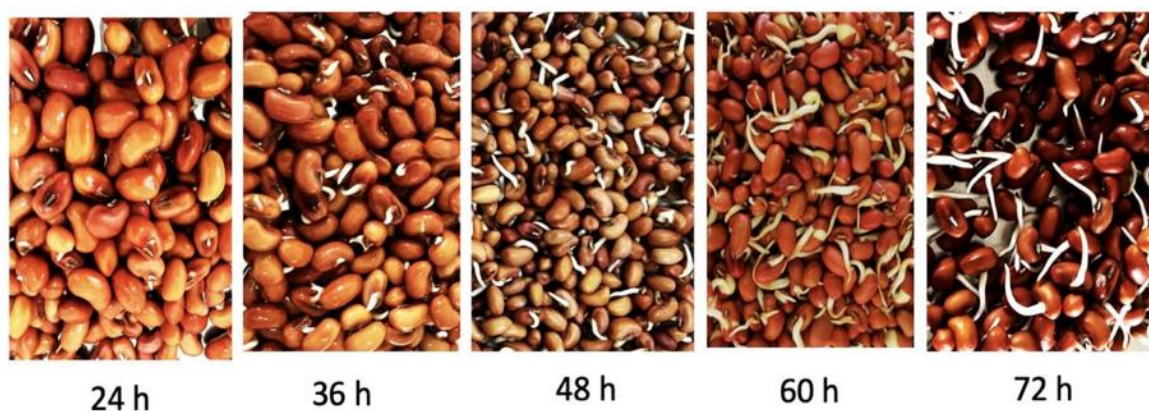


Figure S1 Morphological changes in adzuki bean (*Vigna angularis* cv. “Đậu huyết”) during the germination process.

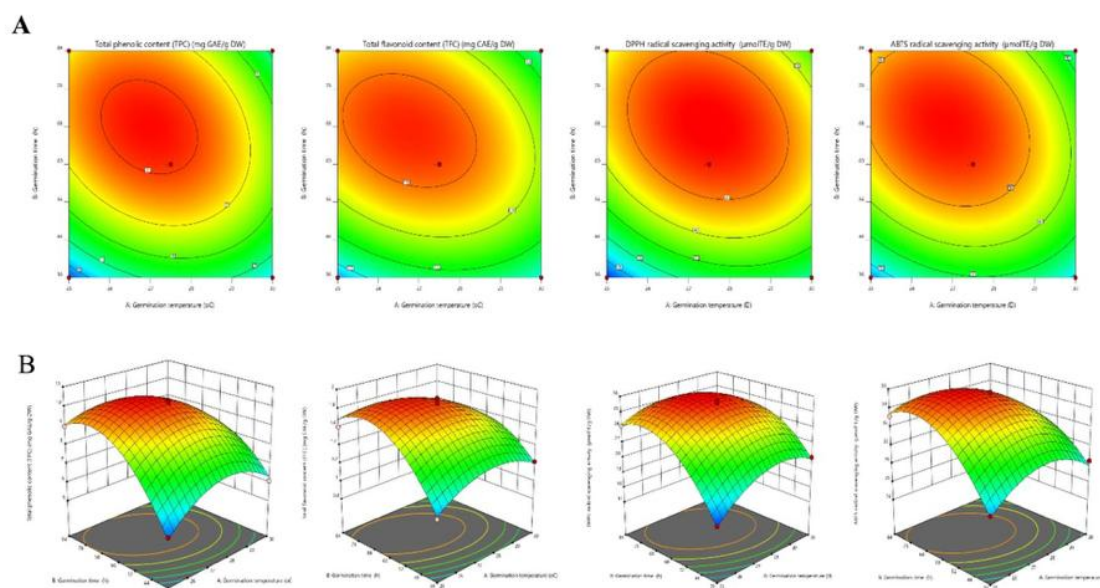


Figure S2 Response Surface Methodology (RSM) Optimization of Adzuki Bean Germination Parameters Effects of germination temperature (X_1 : 23.98 - 31.03 °C) and time (X_2 : 26.16-93.84 h) on (A) total phenolic content (TPC, mg GAE/g DW), (B) total flavonoid content (TFC, mg CAE/g DW), (C) DPPH radical scavenging activity ($\mu\text{mol TE/g DW}$), and (D) ABTS radical scavenging activity ($\mu\text{mol TE/g DW}$). Top panels show 2-dimensional contour plots with interaction zones; bottom panels show 3-dimensional response surface plots. Central Composite Design with 13 experimental runs and 5 centre-point replicates ($n = 5$). Optimal conditions identified: 26.96 °C, 67.89 h (validated experimentally at 27 °C, 68 h). Model adequacy: $R^2 = 96.03\% - 99.78\%$, non-significant lack-of-fit ($p > 0.05$), adjusted R^2 and predicted R^2 difference < 0.20 for all responses. Colour gradient indicates response intensity from low (blue) to high (red). DW, dry weight; GAE, gallic acid equivalents; CAE, catechin equivalents; TE, Trolox equivalents.

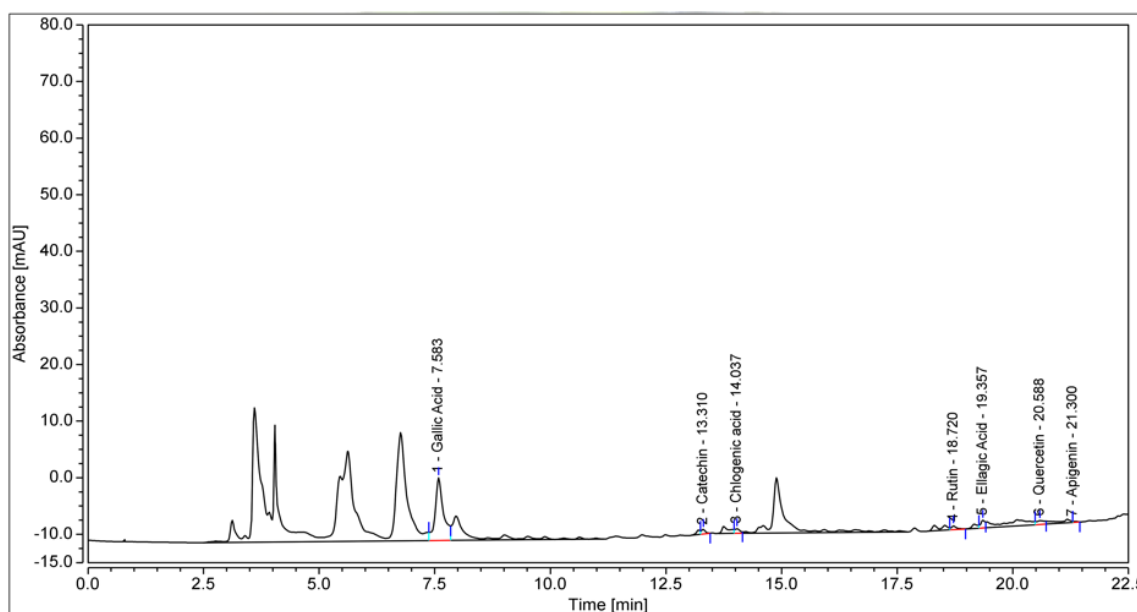


Figure S3 Complete UPLC-PDA chromatograms (265 nm) for raw beans.

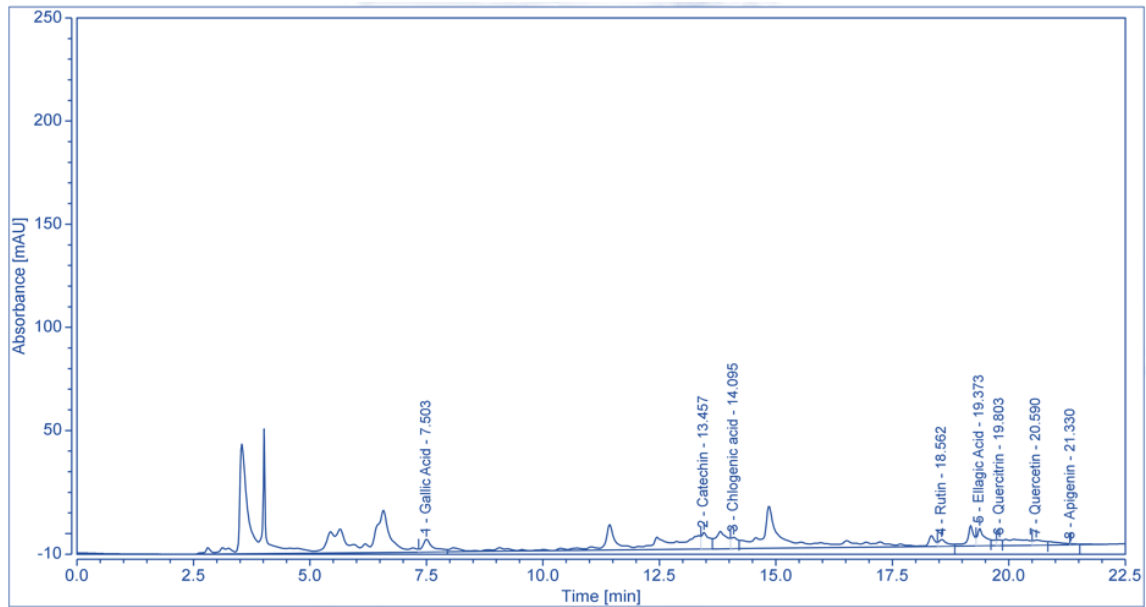


Figure S4 Complete UPLC-PDA chromatograms (265 nm) for germinated beans.

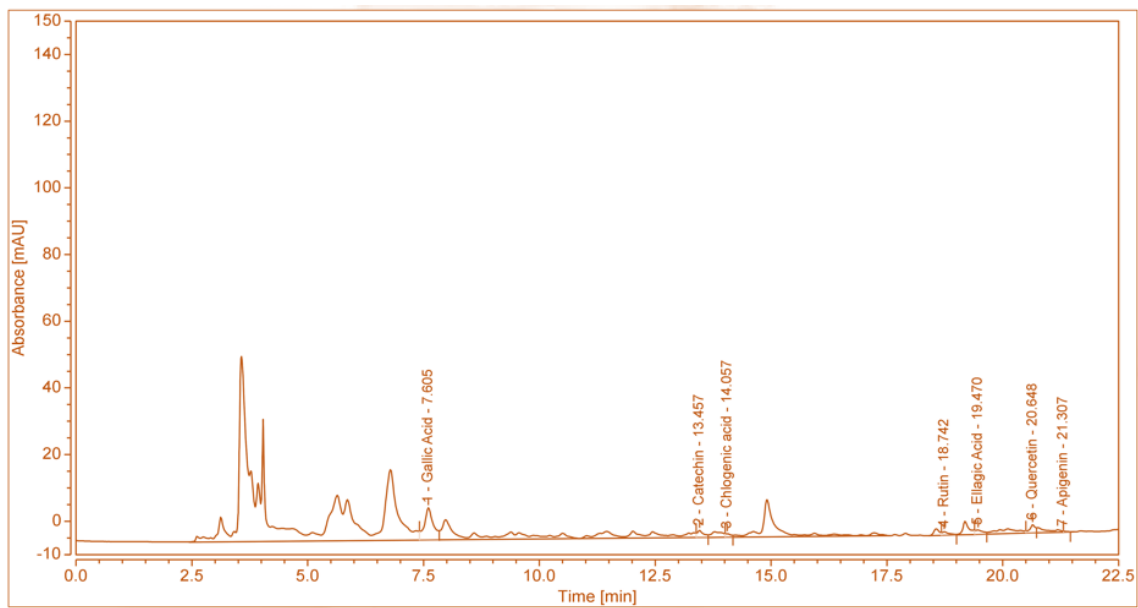


Figure S5 Complete UPLC-PDA chromatograms (265 nm) for Ungerminated but fermented beans.

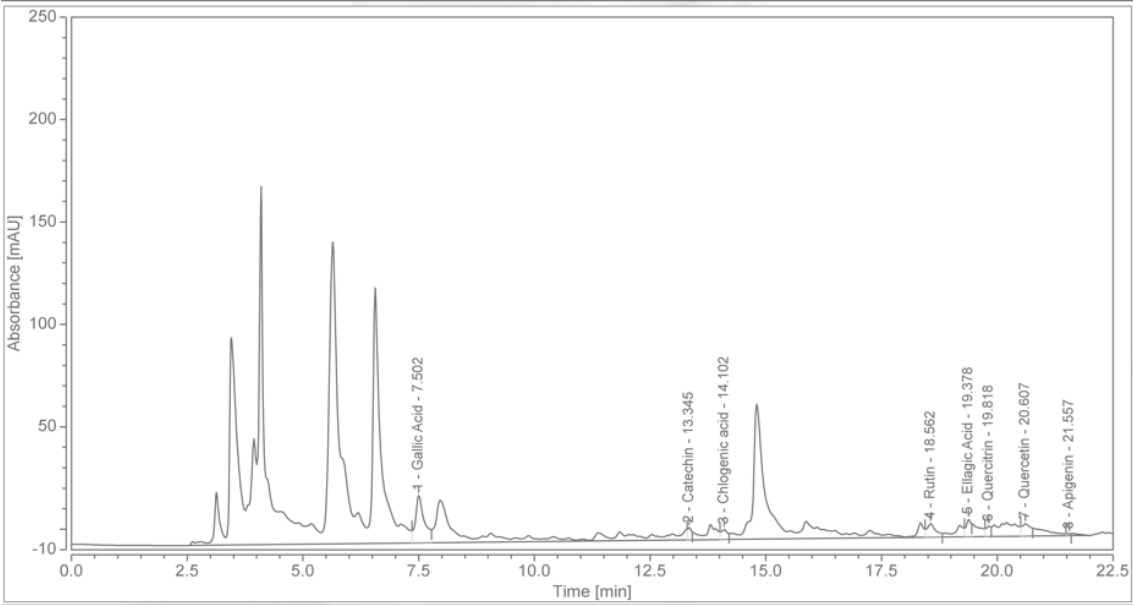


Figure S6 Complete UPLC-PDA chromatograms (265 nm) for Germinated + Fermented beans.

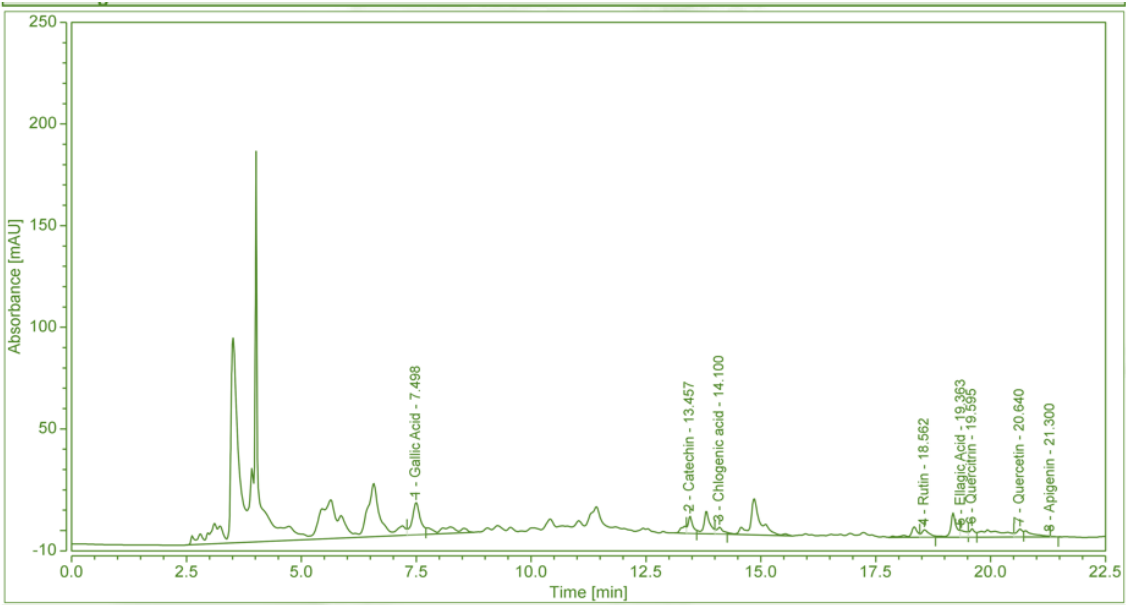


Figure S7 Complete UPLC-PDA chromatograms (265 nm) for optimally extracted beans.