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Sustainable valorization of wheat bran through solid-state fermentation and enzymatic bioprocessing for enhanced antioxidant potential

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Trichoderma sp. RCK65, a potent polysaccharide-degrading fungal strain, was explored for its ability to enhance the antioxidant potential of wheat bran (WB) through solid-state fermentation (SSF). WB is a rich source of phenolic compounds (PCs) with notable antioxidant properties, but most are bound within the plant cell wall, limiting their bioavailability. This study compared unfermented WB (UWB), fermented WB before enzyme extraction (BE), and residual fermented biomass after enzyme extraction (AE), to optimize antioxidant phenolic extraction. The highest total phenolic content (TPC), DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radical scavenging activities, and ferric reducing antioxidant potential were observed in the 70% methanol extract of BE. Even AE extracts demonstrated significantly improved antioxidant activities compared to UWB. SSF with *Trichoderma* sp. RCK65 also elevated free amino acid content, notably essential amino acids lysine and threonine, reinforcing its role in nutritional fortification of cereal-based foods. Furthermore, enzymatic treatments were evaluated using commercial cellulase from *Trichoderma reesei*, Novozyme 188, and a crude enzyme extract of *Trichoderma* sp. RCK65. The crude extract led to a 3.7-fold increase in TPC (1.47 mg GAE g^{-1} WB) and the strongest enhancement in antioxidant assays: 5.3-fold (DPPH$^{\bullet}$), 2.4-fold (ABTS$^{\bullet+}$), and 2.2-fold FRAP. UPLC analysis showed notable shifts in phenolic acid composition post-treatment, with ferulic acid (648.17 μg g^{-1} WB) as the predominant compound in enzyme-treated samples. These findings underline the superior efficacy and cost-effectiveness of *Trichoderma* sp. RCK65 in releasing bound phenolics, offering a promising biotechnological strategy for WB valorization and development of functional foods and nutraceuticals.

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Sustainability spotlight

This study presents a sustainable bioprocessing strategy for wheat bran (WB), an abundant agro-industrial byproduct, using *Trichoderma* sp. RCK65 through solid-state fermentation and enzymatic treatment. By unlocking bound phenolic compounds and enhancing antioxidant and nutritional profiles, the approach transforms WB into a value-added ingredient for functional foods and nutraceuticals. The use of a crude fungal enzyme extract not only maximizes bioactive recovery but also reduces reliance on costly commercial enzymes, reinforcing the environmental and economic viability of this method. This work exemplifies circular bioeconomy principles by valorizing cereal waste into health-promoting food components.

1 Introduction

Humans obtain exogenous antioxidants from plants, animals, fungi, bacteria, and other organisms such as micro- and macroalgae, while the body also possesses its own endogenous antioxidant defense systems.[1] Vitamin C, vitamin E (tocopherols), β-carotene, lycopene, flavonoids (*e.g.*, quercetin), phenolic compounds, curcumin, and resveratrol are examples of natural antioxidants derived from plant sources. Among them, phenolic compounds are a diverse class of phytochemicals derived from phenylalanine and tyrosine through the secondary metabolism of plants. They are produced during normal development and are upregulated in response to stressors such as infection, wounding, and ultraviolet (UV) radiation.[2] In recent decades, their significance has grown rapidly across food science, clinical research, and academic fields due to their potential role in preventing chronic diseases, including cardiovascular disorders, cancer, osteoporosis, diabetes mellitus, and neurodegenerative conditions. Their antioxidant properties enable them to neutralize reactive oxygen

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species (ROS) such as O_2, H_2O_2, and $^{\bullet}OH$, thereby protecting cells from oxidative damage.[3]

In recent years, the food industry has shown a growing inclination toward the development of antioxidant-rich processed foods, driven by increasing consumer demand for health-oriented products.[4] This shift reflects a broader movement away from synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butyl hydroquinone (TBHQ), due to concerns over their potential health risks. As safer alternatives, various natural antioxidant phenolic compounds have been extracted from diverse plant sources, offering both health benefits and functionality.[5,6] The pharmaceutical and food industries alike have recognized the value of these bioactives, with applications ranging from nutraceuticals and functional foods to therapeutic agents.[4]

Wheat bran (WB), consisting of the pericarp, testa, hyaline, and aleurone layers, is the principal by-product of the wheat milling process. In recent years, its application in the food and feed industries has surged, owing to its recognized contribution to the health benefits associated with whole grains.[7] A variety of WB-enriched food products have entered the market, reflecting its growing nutritional relevance. WB typically contains 13–18% protein, 56–57% carbohydrates, and 3.5–4.3% crude fat,[7,8] along with an array of micronutrients and bioactive phytochemicals, including phenolic compounds, lignans, carotenoids, and phytosterols.

Phenolic compounds are the primary contributors to WB's antioxidant activity, yet their bioavailability is often limited due to their insoluble bound forms. These compounds are typically conjugated with polysaccharides, fatty acids, or amino acids through ester, ether, or acetal linkages, and are commonly integrated into the plant cell wall structure.[9] This binding restricts their antioxidant efficacy, as it hinders the availability of free hydroxyl groups necessary for resonance stabilization of free radicals.[10,11] Various conventional solvent extraction strategies (liquid–liquid and solid–liquid), such as Soxhlet extraction, maceration, microwave-assisted extraction (MAE), ultrasound-assisted extraction, high hydrostatic pressure extraction, pressurized hot water extraction (PHWE) and supercritical fluid extraction, have been employed for isolating phenolics from plant materials.[12,13] However, these methods often struggle to release bound phenolics without the aid of acid or base hydrolysis. In this context, alternative green and emerging extraction technologies such as enzymatic treatment and microbial fermentation have shown promise in improving phenolic release. These methods are not only more sustainable, reducing solvent usage, but also enhance extraction efficiency and product quality.[14,15]

A variety of carbohydrate-degrading enzymes can be employed in the enzymatic extraction of cell wall-bound phenolics from natural sources.[16] Solid-state fermentation (SSF) is widely favored by microorganisms for the high-yield production of such enzymes.[3] During SSF, microorganisms produce a broad spectrum of carbohydrases, including cellulases, β-glucosidases, xylanases, pectinases, β-xylosidases, β-galactosidases, α-amylases, and esterases, that facilitate the release of bound phenolic compounds.[17,18] These phenolics, known for their strong antioxidant activity, offer a promising natural alternative to synthetic antioxidants.

Numerous studies have explored the bioprocessing of cereals such as rice,[10] maize,[19] wheat,[17] buckwheat, wheat germ, barley, rye,[6] oats,[18,20] pearl barley,[21] rice bran,[22] and combinations of wheat, brown rice, maize, and oats[23] to enhance antioxidant phenolic content and bioavailability *via* SSF using various food-grade microbial strains.

The valorization of cereal processing by-products through fermentation-based bioprocessing is increasingly recognized as a sustainable biorefinery strategy for generating functional food ingredients while supporting circular economy principles in the agri-food sector.[24–26] WB, a major cereal by-product, is rich in dietary fiber and phenolic compounds but is often underutilized in food applications. Only limited studies have specifically addressed the valorization of WB, particularly the enhancement of antioxidant properties through SSF and enzymatic treatments, and a systematic comparison of these two approaches in WB remains scarce.[16,27–29] In particular, it remains unclear which method is more effective at improving overall antioxidant capacity and which is better suited for selectively releasing valuable phenolic acids such as ferulic acid. This gap indicates a need for further exploration in this area. We hypothesized that SSF would lead to a greater enhancement of total antioxidant activity due to the synergistic effects of enzymatic release and other microbial metabolic activities, like microbial biotransformation of phenolic compounds, whereas enzymatic treatment would be more efficient for the selective liberation of bound ferulic acid *via* specific bond hydrolysis. Food technologists and biorefinery stakeholders need evidence to choose the most effective strategy depending on whether the goal is broad antioxidant enrichment or selective recovery of high-value phenolics.

In the present study, the potential of SSF using *Trichoderma* sp. RCK65 was explored for the first time to release bound phenolic compounds and enhance the antioxidant properties of WB. This organism abundantly produces cell wall-degrading enzymes, such as cellulase and xylanase, which can be effectively applied in various industrial sectors. Therefore, we have also assessed the antioxidant potential of the residual fermented biomass after enzyme extraction (AE) to utilize the extracted enzymes for commercial applications and to simultaneously use the residual fermented WB as a rich source of antioxidant compounds.

First, we compared the total phenolic content (TPC) and antioxidant properties of unfermented wheat bran (UWB), fermented wheat bran before enzyme extraction (BE), and the residual fermented biomass after enzyme extraction (AE). Second, the amino acid profiles of fermented and unfermented wheat bran were evaluated to assess compositional changes induced by fermentation. In addition, enzymatic treatment of wheat bran was performed to enhance antioxidant activity and facilitate the release of bioactive phenolic compounds, particularly ferulic acid, the predominant phenolic constituent of wheat bran. Finally, the outcomes of SSF were systematically compared with those of enzymatic treatment to evaluate their

relative effectiveness. The study also systematically evaluated solvent efficiency for phenolic extraction, ensured experimental reproducibility through biological replication, and validated the findings using appropriate statistical analysis.

2 Materials and methods

2.1 Materials

The following chemicals were procured from Sigma-Aldrich Chemicals (USA): 2,20-diphenyl-1-picryl-hydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS), Trolox, phenolic acid standards such as gallic (GA), protocatechuic acid (PCA), caffeic acid (CA), 4-hydroxy benzoic acid (HBA), 4-hydroxy 3-methoxy benzoic acid (HMBA), *trans*-cinnamic acid (TCA) and ferulic acid (FA). All other chemicals were of analytical grade.

2.2 Inoculum preparation

Four discs (8 mm diameter) excised from a 10-day-old culture of *Trichoderma* sp. RCK65 grown on potato dextrose agar (PDA) were inoculated into a 250 mL Erlenmeyer flask containing 100 mL of potato dextrose broth (HiMedia) supplemented with 0.5% carboxymethyl cellulose (Sigma). The flask was incubated under shaking conditions at 30 °C and 150 rpm for 48 hours. Subsequently, a secondary inoculum was prepared by transferring 5 mL of this primary culture into another flask containing 100 mL of the same broth composition. This was again incubated at 30 °C and 150 rpm for an additional 48 hours.

2.3 SSF of wheat bran

Fermentation was performed in a 250 mL Erlenmeyer flask containing 5 g of wheat bran (WB), moistened with 15 mL of a nutrient-rich moistening agent composed of soybean meal (2.4%), KH_2PO_4 (0.05937%), $(NH_4)_2SO_4$ (0.03125%), and yeast extract (0.5%) and adjusted to pH 4.5. The mixture was autoclaved at 121 °C for 15 minutes and cooled to ambient temperature. After sterilization, the substrate was inoculated with 1 mL of the secondary culture of *Trichoderma* sp. RCK65 and incubated under static conditions at 30 °C. On the third day of incubation, the fermented biomass was harvested for the extraction of extracellular enzymes and phenolic compounds. SSF was done in triplicate, conducted as independent fermentations in separate flasks with separate inoculations. A flask containing sterilized WB without fungal inoculation served as the control (unfermented wheat bran, UWB).

2.4 Extraction of phenolic compounds

Two types of fermented wheat bran (WB) samples were utilized for phenolic compound extraction. The first, designated as BE, refers to fermented WB without enzyme extraction. In the second set, extracellular enzymes produced by *Trichoderma* sp. RCK65 were extracted from the fermented biomass using 0.1 M citrate-phosphate buffer (pH 5.0). The biomass was incubated at 30 °C with shaking at 150 rpm for 1 hour, followed by filtration through muslin cloth; this treated biomass was designated as AE.

All samples including BE, AE, and unfermented WB (UWB), were dried in an oven at 60 °C for 24 hours, ground individually using an electric grinder, and defatted by blending with hexane (1 : 5 w/v) for 5 minutes at ambient temperature; this process was repeated three times. The defatted samples were air-dried for 24 hours and stored at −20 °C for further analysis.

Phenolic compounds were extracted from each sample using eight solvent systems: water, methanol, ethanol, acetone, ethyl acetate, 70% methanol, 70% ethanol, and 70% acetone, with a solid-to-solvent ratio of 1 : 10 w/v. Extractions were performed twice at 50 °C for 60 minutes in a water bath. The resulting extracts were filtered through Whatman no. 1 filter paper, and the filtrates were used for comparative analysis of total phenolic content (TPC), $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical scavenging activities, and ferric reducing antioxidant power (FRAP).

2.5 Enzymatic extraction of phenolic compounds from wheat bran (WB)

Three enzymatic treatments were employed to extract phenolic compounds from wheat bran:

(i) Pure cellulase derived from *Trichoderma reesei* (Sigma-Aldrich, USA) (6.5 U mg^{-1}), with an activity of 13 U mL^{-1} (*i.e.* 2 mg mL^{-1}).

(ii) Crude enzyme extract from *Trichoderma* sp. RCK65-fermented WB, exhibiting enzymatic activities as follows: FPase – 10.6 IU mL^{-1}, CMCase – 44.77 IU mL^{-1}, β-glucosidase – 39.71 IU mL^{-1}, and xylanase – 28 480 IU mL^{-1}.

(iii) Novozyme 188, applied at a concentration of 250 U g^{-1} (1 mL).

Defatting of WB samples (4 × 1 g) was carried out by blending each sample with hexane (1 : 5 w/v) for 5 minutes at ambient temperature, which was repeated three times. The defatted samples were then air-dried for 24 hours and stored at −20 °C until further use.

For enzymatic treatment, each defatted sample was mixed with 1 mL of enzyme solution and 2 mL of 0.1 M citrate-phosphate buffer (pH 5.0), followed by incubation at 50 °C for 1 hour. In the control group (untreated WB), 1 mL of buffer was used in place of the enzyme. After enzymatic hydrolysis, 7 mL of methanol was added to each sample and incubation was continued at 50 °C for another hour.

Phenolic-rich extracts were recovered *via* centrifugation at 8000×*g* for 10 minutes. These extracts were subsequently analyzed to determine antioxidant properties. Additionally, Ultra-Performance Liquid Chromatography (UPLC) was performed to characterize the phenolic acid profiles. Enzymatic treatment experiments were conducted only once due to limited material availability; consequently, no biological replicates were included, and statistical analysis could not be performed in the case of UPLC. However, TPC and antioxidant assays were conducted in triplicate.

2.6 Analytical methods

2.6.1 Determination of total phenolic content (TPC). Total phenolic content was determined following the method of Emmons and Peterson.[30] Briefly, 0.5 mL of suitably diluted

phenolic extract was mixed with 0.5 mL of the Folin–Ciocalteu reagent. Subsequently, 1.5 mL of 20% (w/v) aqueous sodium carbonate solution was added, and the mixture was thoroughly vortexed and incubated at room temperature for 15 minutes. After incubation, 5 mL of distilled water was added to each reaction mixture. The absorbance was measured at 725 nm using a spectrophotometer, with a reagent blank serving as the reference. TPC was quantified using a gallic acid standard calibration curve and expressed as milligrams of gallic acid equivalent per gram of wheat bran (mg GAE g^{-1} WB).

2.6.2 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. The free radical scavenging activity of various phenolic extract fractions was assessed using the DPPH method described by Brand-Williams *et al.*[31] A 0.1 mM solution of DPPH (Sigma-Aldrich Chemie, Steinheim, Germany) in methanol was prepared, and 0.5 mL of appropriately diluted phenolic extract was added to 2 mL of the DPPH solution. The reaction mixture was incubated in the dark at room temperature for 30 minutes. Absorbance was recorded at 515 nm using a UV-vis spectrophotometer. The percentage of DPPH radical scavenging activity was calculated using the following equation:

$$\% \text{ scavenging activity} = [(\text{Abc} - \text{Abs})/\text{Abc}] \times 100$$

where, Abc was the absorbance of the control and Abs was the absorbance in the presence of the test compound. A standard curve was prepared by using different concentrations of Trolox. The $DPPH^{\bullet}$ scavenging activities of phenolic extracts were expressed as μmol Trolox equivalent (TE) per g WB.

2.6.3 ABTS radical cation decolorization assay. The $ABTS^{\bullet+}$ [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] radical cation scavenging activity was assessed using the improved ABTS decolorization assay as described by Re *et al.*[32] $ABTS^{\bullet+}$ was generated by oxidizing ABTS with potassium persulfate and incubating the mixture in the dark until the radical cation stabilized. For the assay, 1 mL of $ABTS^{\bullet+}$ working solution was mixed with 10 μL of phenolic extract. After a reaction time of 1 minute at room temperature, the decrease in absorbance was recorded at the appropriate wavelength (typically 734 nm) using a spectrophotometer. A calibration curve was constructed using Trolox standards at varying concentrations. The $ABTS^{\bullet+}$ scavenging activity of each sample was expressed as micromoles of Trolox equivalent per gram of wheat bran (μmol TE g^{-1} WB), consistent with the reporting method used for $DPPH^{\bullet}$ assays.

2.6.4 FRAP (ferric reducing antioxidant power) assay. The ferric reducing antioxidant power (FRAP) of the phenolic extracts was assessed following the method of Wong *et al.*[33] with slight modifications. Briefly, 100 μL of phenolic extract was combined with 1.5 mL of the freshly prepared FRAP reagent, composed of 10 parts 300 mM sodium acetate buffer (pH 3.6), 1 part 10 mM TPTZ (2,4,6-tripyridyl-*s*-triazine) solution, and 1 part 20 mM $FeCl_3 \cdot 6H_2O$ solution. The mixture was incubated at 37 °C in a water bath for 30 minutes. After incubation, the absorbance was measured at 593 nm using a spectrophotometer. FRAP values were calculated from a standard curve of L-ascorbic acid and expressed as micromoles of ascorbic acid equivalent per gram of wheat bran (μmol AAE g^{-1} WB).

2.6.5 Free amino acid content. Defatted unfermented and fermented samples were extracted using a water–ethanol solution (1 : 10; w/v). A 0.2 mL aliquot of the appropriately diluted extract was mixed with 0.2 mL of 0.2% ninhydrin (in ethanol) and heated in a boiling water bath for 20 minutes. Subsequently, 1 mL of the diluent (water : *n*-propanol, 1 : 1) was added and the mixture was heated again for 15 minutes. After cooling, the absorbance was measured at 570 nm. The total free amino acid content in the supernatant was quantified using a standard curve of leucine and expressed as mg leucine equivalents per gram of the sample.[34]

Thin layer chromatography (TLC) of water extracts was carried out using the ascending technique with a developing solvent of *n*-butanol : acetic acid : water (5 : 3 : 2) containing 0.4% (w/v) ninhydrin. After development, TLC plates were dried in an oven at 90 °C for 5 minutes to facilitate color development.

2.6.6 Enzyme activities. Filter paper cellulase (FPase), carboxymethyl cellulase (CMCase), and xylanase activities were determined by quantifying the reducing sugars released from the respective substrates, Whatman no. 1 filter paper, carboxymethyl cellulose, and birch wood xylan, at 50 °C and pH 5, following the procedures described by Ghose (1987)[35] and Kapoor *et al.* (2008).[36]

β-Glucosidase activity was assessed based on the amount of *p*-nitrophenol released from *p*-nitrophenyl glucopyranoside, as per the method outlined by Wood and Bhat.[37]

One unit of enzyme activity corresponded to the formation of 1 μmol of product (*e.g.* in the case of xylanase, xylose equivalent) per minute from the substrate.

2.7 Ultra-performance liquid chromatography (UPLC)

UPLC (Waters, Milford, USA) of 70% methanolic extracts (1 μL) was performed for the separation of phenolics using a BEH 300 C-18 column (2.1 × 50 mm, 1.7 μm). The column temperature, total run time and flow rate were maintained at 30 °C, 5 min and 0.6 mL min^{-1}, respectively. Two mobile phases, consisting of water containing 0.1% TFA (solvent A) and acetonitrile containing 0.1% TFA (solvent B), were used, and gradient elution was carried out using the following program: 95% A to 90% A in 1 min, 90% A to 85% A in 1 min, 85% A to 75% A in 1 min, 75% A to 40% A in 1 min, 40% A to 0% A in 0.2 min, 0% A to 0% A in 0.6 min and 0% A to 95% A in 0.2 min. The peaks were identified by comparing their congruent retention times and UV spectra (280 nm) with those of standards (PCA: protocatechuic acid; HBA: 4-hydroxybenzoic acid; HMBA: 4-hydroxy 3-methoxy benzoic acid; CA: caffeic acid; FA: ferulic acid; TCA: *trans* cinnamic acid) and quantified based on their peak areas.

2.8 Statistical analysis

The mean values and the standard deviations (SDs) were calculated from the data obtained from three separate experiments (biological triplicate). The results were expressed as the mean ± SD of three sets of experiments. Analysis of variance (ANOVA) was used to evaluate the significant difference among various treatments with the criterion of $P < 0.05$.

3. Results and discussion

3.1 Solid state fermentation of WB

3.1.1 Total phenolic content (TPC). Selective extraction of biomolecules from natural sources by appropriate solvents is very important to obtain fractions with high antioxidant activity. It is noted that a solvent system for extraction is selected according to the purpose of extraction, the nature of the components of interest, the physicochemical properties of the matrix, the availability of reagents and equipment, cost and safety concerns.[38] It is generally known that alcohol/water solutions exert a better influence on the extractability of phenolic compounds in comparison to mono-component solvents.

The amount of extracted phenolic compounds obtained in this study by different solvents is presented in Fig. 1. In comparison to UWB, TPC was higher in each of the solvent extracts of fermented WB (BE and AE) except in the ethanol and acetone extracts. Maximum TPC was attained in water extract (2.23 ± 0.20 mg GAE g^{-1} WB) and in 70% acetone extract (2.14 ± 0.13 mg GAE g^{-1} WB) of UWB. Whereas, in the case of *Trichoderma* sp. RCK65 fermented wheat bran (BE), highest TPC was obtained in 70% methanol (13.09 ± 1.08 mg GAE g^{-1} WB), 70% ethanol extracts (12.19 ± 0.17 mg GAE g^{-1} WB) and water (12.10 ± 0.55 mg GAE g^{-1} WB). Among the AE samples, the highest amount of TPC was estimated in 70% methanol and 70% acetone extracts (6.37 ± 0.44 and 6.11 ± 0.29 mg GAE g^{-1} WB, respectively). Total phenolic content (TPC) was lower in all AE extracts compared to BE, likely due to the partial loss of phenolic compounds during the enzyme extraction process. If we consider the water soluble phenolics, it was clearly observed that SSF enhanced the TPC of WB by 5.5-fold in BE, and even after enzyme extraction (AE), water soluble TPC was increased by 2.5-fold compared to UWB ($p < 0.05$). A maximum of 15-fold and 7-fold enhancement was observed in BE and AE, respectively, for the 70% methanolic extracts compared to UWB ($p < 0.05$). The extent to which fermentation positively influenced the total phenolic content (TPC) and antioxidant activity of cereals was reported to vary depending on the specific microorganism employed in the process.[6,39] An increase in phenolic compounds has been observed in various cereal and cereal brans fermented by lactic acid bacteria, yeast, and mold.[23,27,29] A maximum two-fold improvement of TPC was observed by Schmidt *et al.*[22] in rice bran after SSF by *Rhizopus oryzae*. Moore *et al.*[40] found only 50 to 100% improvement of releasable TPC of WB (in 100% ethanol extract) through solid state yeast fermentation. SSF of wheat bran with *Clostridium butyricum* increased TPC from 0.45 mg GAE g^{-1} to only 0.58 mg GAE g^{-1}.[29] Maximum TPC was registered on the 3rd day of fermentation by yeast for WB (0.84 mg GAE g^{-1} DW) with a 112% increase in the TPC value compared to the control.[12] Hence, based on our current findings, SSF by *Trichoderma* sp. RCK65 appears to be a more effective approach for enhancing the extractable TPC of WB. Despite undergoing enzyme extraction, a substantial quantity of phenolic compounds remained extractable. The enhanced level of TPC observed in the fermented sample can be explained by the following facts:

(i) Following colonization of wheat by fungal strains, the structural breakdown of cell walls occurred, facilitating the release of phenolic compounds.[41]

(ii) Bound phenolics were liberated through the enzymatic activity of carbohydrate-degrading enzymes, such as cellulases and xylanases, produced by *Trichoderma* sp. RCK65 during the SSF process.

(iii) In addition, certain soluble phenolic compounds may have been biosynthesized by the microorganism because of secondary metabolic pathways.

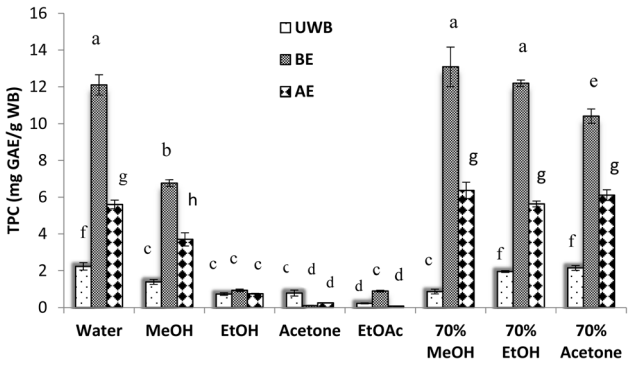

Fig. 1 TPC of different solvent extracts from unfermented (UWB) and *Trichoderma* sp. RCK65 fermented wheat bran before enzyme extraction (BE) and after enzyme extraction (AE). Data are expressed as mean ± SD ($n = 3$). Different letters in each bar are significantly different at $p < 0.05$.

3.1.2 Antioxidant status of UWB and fermented WB

3.1.2.1 DPPH• scavenging properties. The evaluation of antioxidant potential in plant-derived compounds remains a complex and unresolved issue.[42] Although several analytical mechanisms have been proposed, none can independently provide a comprehensive assessment. Currently, over 20 different indices are employed to measure antioxidant activity, yet no single assay is deemed sufficient for evaluating total antioxidant capacity.[43] Among these, the DPPH• assay is widely utilized to assess the free radical scavenging activity of various compounds. DPPH•, a purple-colored stable free radical in methanolic solution, undergoes decolorization to yellow upon interaction with antioxidant compounds *via* electron transfer or hydrogen atom donation.[16] DPPH• scavenging potential of UWB and fermented WB (BE & AE) was estimated. As shown in Fig. 2A, an enhanced level of DPPH• scavenging activity was observed in fermented wheat bran (WB), both before (BE) and after enzyme extraction (AE), when extracted using water, methanol, 70% methanol, 70% ethanol, and 70% acetone. No significant improvement was noted in extracts prepared with ethanol, acetone, and ethyl acetate. Consistent with the pattern observed for total phenolic content (TPC), the unfermented wheat bran (UWB) exhibited its highest DPPH• scavenging activity when extracted with water (4.48 ± 0.06 μmol TE g^{-1} WB) and 70% acetone (4.03 ± 0.78 μmol TE g^{-1} WB). In the case of the BE sample, the highest DPPH• scavenging activity was observed in 70% methanol (24.39 ± 1.02 μmol TE g^{-1} WB) and 70% ethanol (24.86 ± 1.10 μmol TE g^{-1} WB) extracts, whereas, in the case of the AE sample, the maximum DPPH• scavenging activity was obtained in 70% methanol extract (16.77 ± 0.59

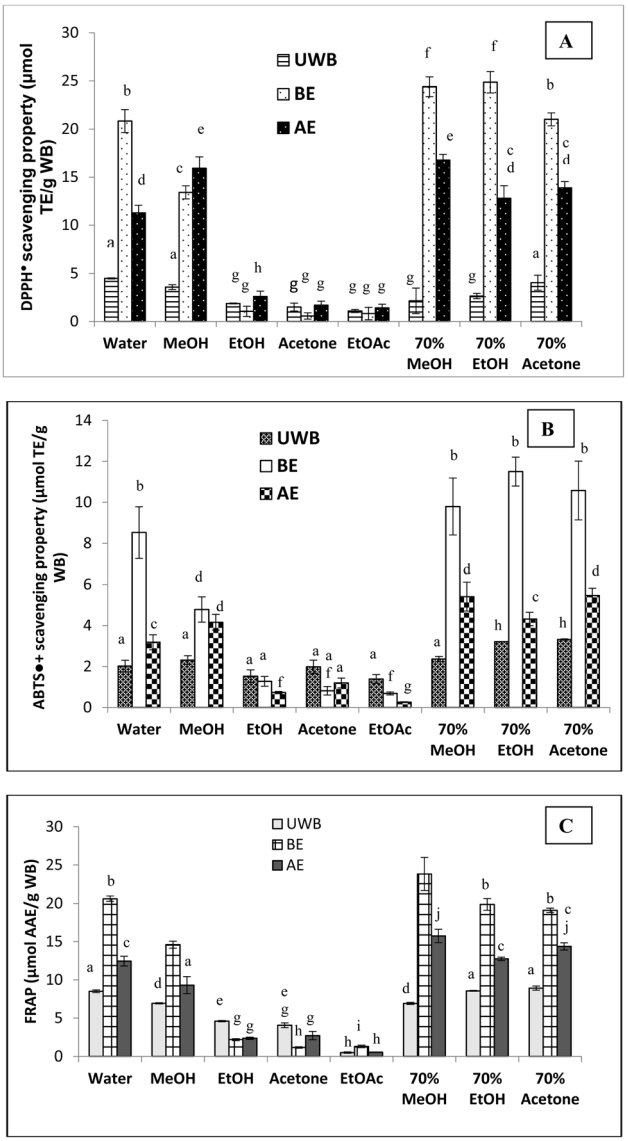

Fig. 2 DPPH• (A) and ABTS•+ scavenging properties (B) and FRAP (C) of different solvent extracts from unfermented (UWB) and *Trichoderma* sp. RCK65 fermented wheat bran before enzyme extraction (BE) and after enzyme extraction (AE). Data are expressed as mean ± SD ($n = 3$). Different letters in each bar are significantly different at $p < 0.05$.

µmol TE g^{-1} WB). Even after enzyme extraction from fermented WB, a considerable amount of DPPH• scavenging activity was retained in the fermented biomass (AE). In this study, a maximum of 11.4- and 7.8-fold improvements of DPPH• scavenging activity were observed in BE and AE samples as compared to UWB in 70% methanol extracts ($p < 0.05$), whereas, Moore *et al.*[40] obtained only 13 to 19% improvement of DPPH• scavenging activity of WB by solid state yeast treatment.

3.1.2.2 ABTS•+ scavenging activity. ABTS•+ scavenging activity of UWB, BE and AE has been shown in Fig. 2B. ABTS•+ scavenging activity of different solvent extracts of UWB followed the order of 70% acetone > 70% EtOH > 70% MeOH = MeOH > water > EtOH > EtOAc. Among different solvent extracts, there was no significant difference among 70% acetone (11.50 µmol ± 0.71 TE g^{-1} WB), 70% EtOH (10.58 ± 1.43 µmol TE g^{-1} WB) and 70% MeOH extract (9.78 ± 1.39 TE g^{-1} WB) of the BE sample and 70% acetone (5.46 ± 0.36 µmol TE g^{-1} WB) and 70% MeOH (5.41 ± 0.71 µmol TE g^{-1} WB) extracts of the AE sample. Therefore, in comparison to UWB maximum 4 and 2-fold enhancement was found in 70% methanol extract of BE and AE, respectively. Improvement of ABTS•+ scavenging activity was not observed in the ethanol, acetone and ethyl acetate extracts of BE and AE as compared to UWB. According to the result obtained by Moore *et al.*[40] solid state yeast treatments of WB increased the ABTS•+ scavenging activity only by 0 to 20%.

3.1.2.3 FRAP assay. Almost comparable amounts of FRAP were observed in 70% acetone (8.92 ± 0.27 µmol AAE g^{-1} WB), 70% EtOH (8.56 ± 0.06 µmol AAE g^{-1} WB) and water (8.51 ± 0.17 µmol AAE g^{-1} WB) extracts of UWB (Fig. 2C). There was no significant difference in FRAP values between methanol and 70% methanol extracts of UWB. Like other antioxidant properties, FRAP values of different solvent extracts increased after SSF of WB except in ethanol, acetone and ethyl acetate extracts. Among all the solvents used, 70% MeOH showed the maximum influence for the extraction of antioxidant compounds with the highest FRAP value in BE (23.81 µmol AAE g^{-1} WB) as well as in AE (15.74 µmol AAE g^{-1}) with a 3.4- and 2.3-fold increment, respectively ($p < 0.05$).

In the present study, among all the solvent systems used, 70% methanol was found to be the most efficient solvent for extraction of phenolic antioxidants from fermented WB (both BE and AE), whereas, 70% acetone was the most suitable solvent for UWB. However, EtOAc gave the lowest TPC with the lowest antioxidant properties for all the samples. According to the report of Zhou and Yu,[44] among the four solvent systems used (50% acetone, 70% methanol, 70% ethanol and ethanol), 50% acetone was proved to be the best solvent for the extraction of phenolic antioxidants from wheat bran. Various solvent systems and extracting conditions have been used for the extraction of antioxidant phenolic compounds from wheat and wheat-based products following several methods and hence with variable results.[40] Therefore, comparisons of the antioxidant properties of WB among individual research laboratories and groups are very difficult.

3.1.2.4 Total free amino acid content. Total free amino acid contents of water extract obtained from unfermented wheat, *R. oryzae* fermented wheat, unfermented wheat bran and *Trichoderma* sp. RCK65 fermented wheat bran before enzyme extraction (BE) & after enzyme extraction (AE) were 2.95, 14.16, 8.28, 22.2 and 19.25 mg equivalents of leucine per g of the sample, respectively, whereas for ethanol extract, those values were 0.28, 0.83, 0.27, 4.91, 2.65 mg equivalents of leucine per g of the sample, respectively (Table 2). A significant increase in total free amino acid content was clearly observed in wheat bran following SSF. Notably, even after enzyme extraction, the free amino acid levels remained substantially higher compared to those in unfermented wheat bran.

TLC analysis of the amino acid profile further supports the observed increase in free amino acid content, which may be attributed to microbial degradation of proteins or an increase in

protein synthesis resulting from the mycelial growth of the organisms (Fig. 5).

In general, cereal proteins are low in Lys (1.5–4.5% *vs.* 5.5% per the WHO recommendation), tryptophan (Trp, 0.8–2.0% *vs.* 1.0%), and threonine (Thr, 2.7–3.9% *vs.* 4.0%). Due to this deficiency, these essential amino acids (EAAs) are often the limiting factors in cereal-based proteins. It is thus of economic and nutritional significance to enhance the EAAs in plant proteins.[45] The fermentation process can improve those amino acid content.[46] In our study, SSF of wheat bran by *Trichoderma* sp. RCK65 clearly demonstrated an increase in the concentrations of lysine (Lys) and threonine (Thr), suggesting enhanced bioavailability and improved protein quality.

3.2 Enzymatic treatment of WB for the release of antioxidant phenolics

3.2.1 TPC, DPPH$^{\bullet}$, ABTS$^{\bullet+}$ scavenging activity and FRAP of enzyme treated WB. WB was treated with cellulase of *Trichoderma reesei* (Sigma-Aldrich), Novozyme 188 and enzyme extract of *Trichoderma* sp. RCK65. Fig. 3A shows the TPC extracted through enzymatic treatment. TPC of WB without enzyme treatment (control) was 0.4 mg GAE g^{-1} WB. The maximum amount of total phenolics (1.47 mg GAE g^{-1} WB) was released due to the action of enzyme extract obtained from *Trichoderma* sp. RCK65 with a 3.7 times improvement. Whereas pure cellulase of *Trichoderma reesei* increased the TPC only 1.7-fold. There was no effect of Novozyme 188 on the release of phenolics from WB.

As shown in Fig. 3B–D, the maximum improvement of DPPH$^{\bullet}$ (5.3-fold) and ABTS$^{\bullet+}$ (2.4-fold) scavenging properties and FRAP (2.2-fold) was observed by the treatment of enzyme extract obtained from *Trichoderma* sp. RCK65 fermented WB as compared to untreated WB (control). While the enzyme from *Trichoderma reesei* and Novozyme 188 showed no effect on the improvement of antioxidant properties of WB. Saroj *et al.*[16] observed the maximum increase in TPC and antioxidant properties (DPPH & FRAP) in cellulase (Sigma-Aldrich) treated wheat bran followed by xylanase (Sigma-Aldrich) and β-glucanase (Sigma-Aldrich).

Cellulase has been used for the extraction of phytochemicals from black currant pomace,[47] wheat bran[16,28] and oat bran.[48,49] All those previous studies have reported enhanced total phenolic content (TPC) and antioxidant properties through enzymatic action; however, high-cost commercial enzyme preparations were predominantly employed. In contrast, the present study employed a crude enzyme extract derived from a newly isolated laboratory strain, *Trichoderma* sp. RCK65, for the release of antioxidant phenolics. This approach demonstrates a potentially cost-effective method for improving the antioxidant potential of wheat bran.

Beyond antioxidant activity, phenolic compounds in WB may also exert biofunctional effects such as modulating glucose and lipid metabolism, supporting gut microbiota, and reducing inflammation.[25] The release of bound phenolics through fermentation and enzymatic bioprocessing can improve

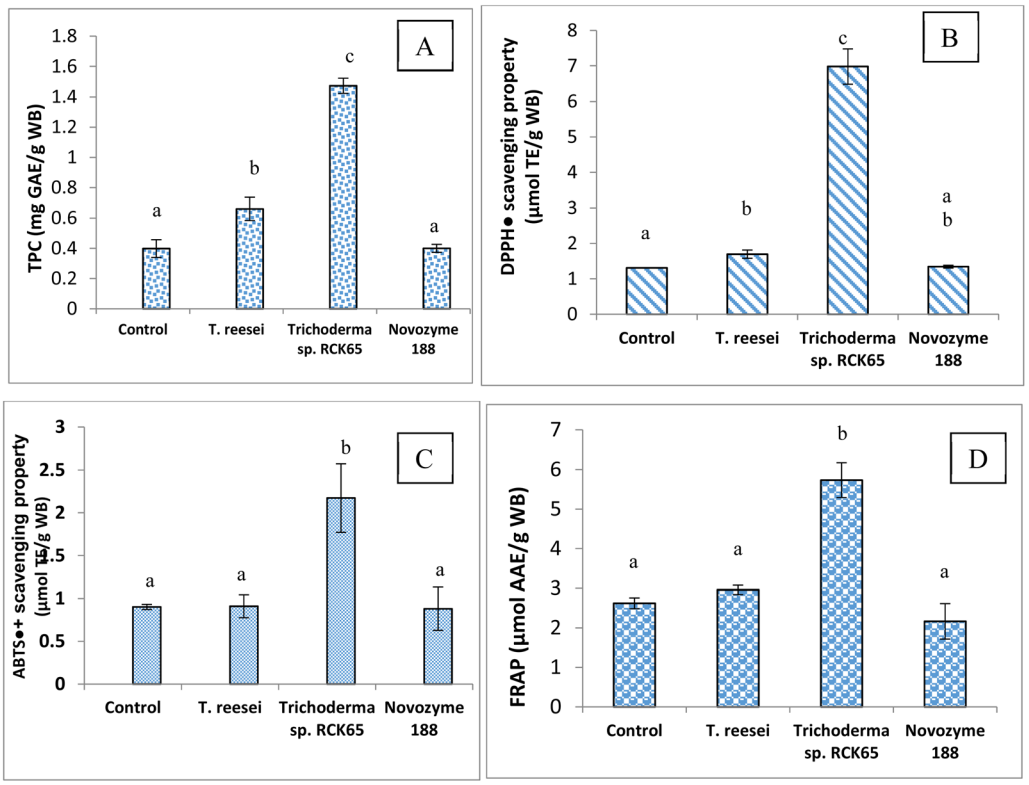

Fig. 3 TPC (A), DPPH$^{\bullet}$ (B), ABTS$^{\bullet+}$ scavenging activity (C) and FRAP (D) of enzyme treated WB. Data are expressed as mean ± SD ($n = 3$). Different letters in each bar are significantly different at $p < 0.05$.

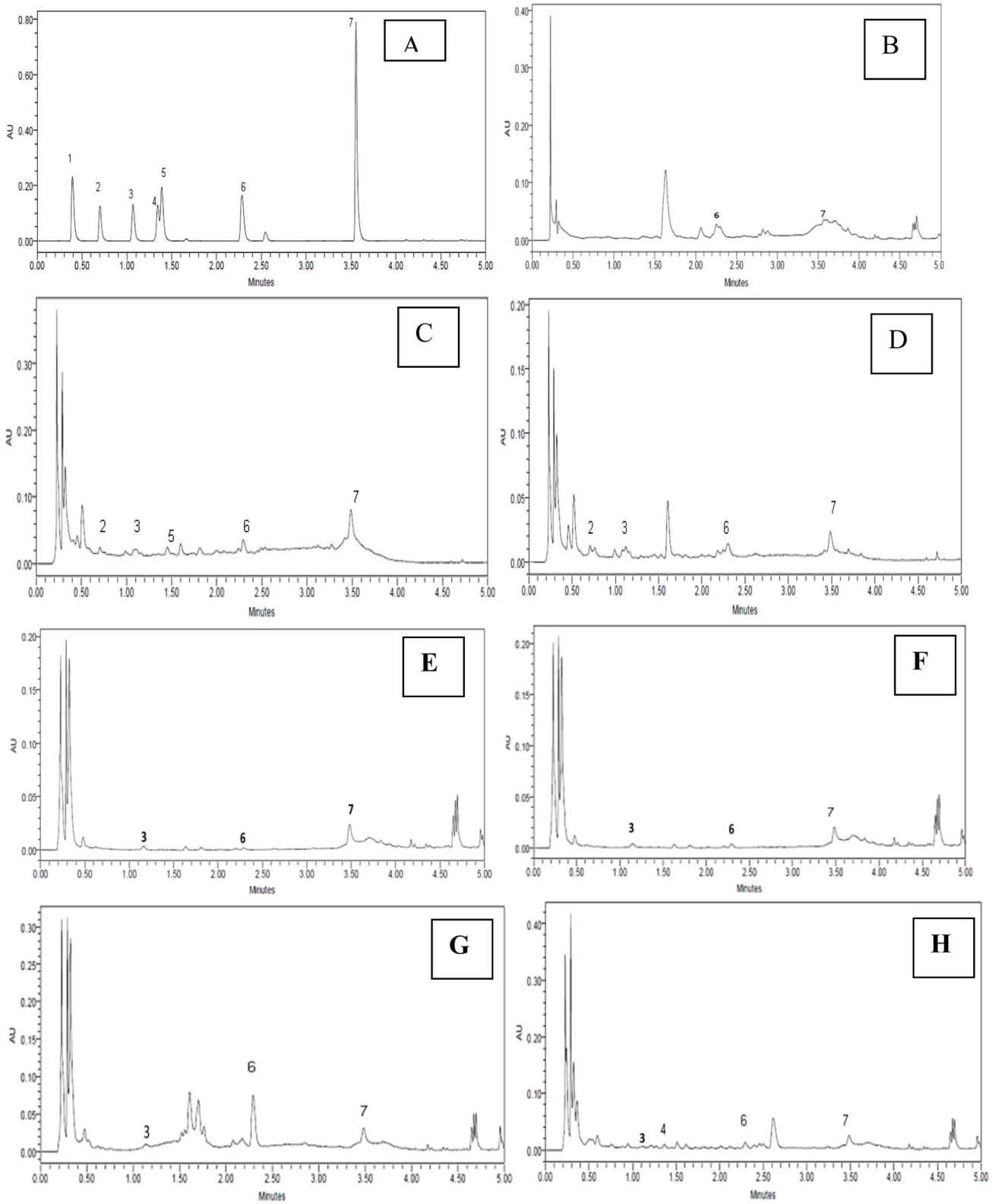

Fig. 4 UPLC profile of standard phenolic compounds (A), UWB (B), BE (C), AE (D), control WB (E), *T. reesei* cellulase treated WB (F), *Trichoderma* sp. RCK65 enzyme treated WB (G), and Novozyme 188 treated WB (H). [Phenolic acid standards were gallic acid (1), protocatechuic acid (2), 4-hydroxybenzoic acid (3), 4-hydroxy 3-methoxy benzoic acid (4), caffeic acid (5), ferulic acid (6) and *trans* cinnamic acid (7)].

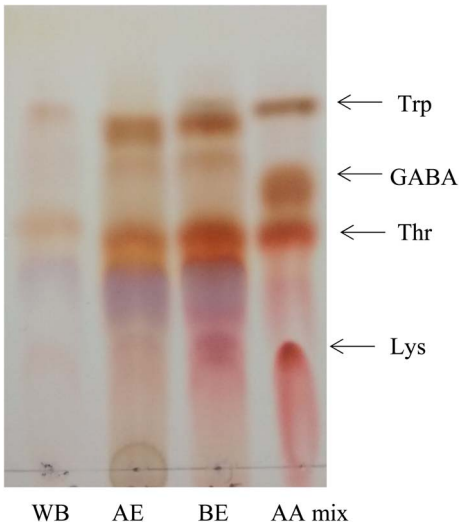

Fig. 5 Free amino acid profiling: TLC of fermented and unfermented samples.

bioavailability, thereby enhancing their potential health relevance.

3.3 Profiles of the phenolic compounds

The present study demonstrated that 70% MeOH is the best solvent for the extraction of antioxidant phenolics from both BE and AE samples. Therefore, this solvent extract was used for the compositional analysis of phenolic acids through UPLC. Seven phenolic acid standards were separated in a UPLC system within 5 min (Fig. 4A). The chromatographic profile of the phenolic acids extracted from UWB, BE and AE is shown in Fig. 4(B–D), which clearly showed the improvement of phenolic acid content in BE and AE as compared to UWB. Significant changes were observed in the UPLC profiles of BE and AE. As shown in Table 1, the main phenolic acid detected in the UPLC profile of BE was TCA (407.98 μg g^{-1} of WB) and in AE it was PCA (277.02 μg g^{-1} of WB). FA content of BE and AE was 233.62 and 204.09 μg g^{-1} of WB, respectively. The result shows that even after enzyme extraction from fermented WB, the loss of FA was very less in the AE sample.

Fig. 4E–H shows the UPLC profile of phenolic acids present in the different enzyme treated and untreated WB. Three major phenolic acids (4-hydroxybenzoic acid, ferulic acid and *trans* cinnamic acid) were detected in the extract of WB without enzyme treatment (Fig. 4E). Any significant change was not observed in the phenolic acid profile after treatment with pure cellulase from *Trichoderma reesei* (Fig. 4E and F), whereas treatment with enzyme extracts from *Trichoderma* sp. RCK65 modified the UPLC profile (Fig. 4G). Free phenolic acid content of enzyme treated and without enzyme treated WB is given in Table 1. The amount of TCA was maximum for each of the samples except *Trichoderma* sp. RCK65 enzyme treated WB, where FA (648.17 μg g^{-1} of WB) was the major phenolic acid.

According to a previous report by Napolitano *et al.*,[50] FA content of durum wheat fiber was increased from 1.1 mg kg^{-1} to 4.6 mg kg^{-1} by the enzymatic treatment (from *Trichoderma* spp.). Faulds *et al.*[51] achieved an FA recovery of 5.7 g kg^{-1} or 5700 mg kg^{-1} from de-starched WB using *Trichoderma* xylanase and *Aspergillus niger* FAE-III after 16 h of incubation. An FA yield of 1.05 g kg^{-1} bran or 1050 mg kg^{-1} bran was achieved through a multistep biorefinery process. That method involved rehydrating bran *via* autoclaving or steam explosion followed by enzymatic pre-treatment with Alcalase and Termamyl to remove proteins and sugars, and final hydrolysis with Pentopan and feruloyl esterase to release phenolics.[52] Another notable approach utilized a recombinant bifunctional enzyme

Table 2 Total free amino acid content

	Free amino acid content (mg equivalents of leucine per g of the sample)	
Name of the sample	Water extract	Ethanol extract
WB control	8.28 ± 1.34	0.27 ± 0.03
BE	22.20 ± 1.10	4.91 ± 0.49
AE	19.25 ± 0.34	2.65 ± 0.20

Table 1 Phenolic acid composition of fermented and enzyme treated WB

	Free phenolic acid content[a] (μg g^{-1} of WB)					
Name of the sample	PCA	HBA	HMBA	CA	FA	TCA
1. SSF of WB						
UFW	ND	ND	ND	ND	201.71	248.70
BE	144.39	166.08	ND	64.32	233.62	407.98
AE	277.02	138.80	ND	ND	204.09	77.45
2. Enzymatic treatment of WB						
Untreated WB (control)	ND	56.71	ND	ND	14.32	112.95
T. reesei cellulase treated WB	ND	86.78	ND	ND	26.86	104.96
Trichoderma sp. RCK65 enzyme treated WB	ND	267.28	ND	ND	648.17	234.89
Novozyme 188 treated WB	ND	103.95	88.66	ND	141.44	155.41

[a] PCA: protocatechuic acid; HBA: 4-hydroxybenzoic acid; HMBA: 4-hydroxy 3-methoxy benzoic acid; CA: caffeic acid; FA: ferulic acid; TCA: *trans* cinnamic acid; ND: not detected. Based on the UPLC data free phenolic acid contents were estimated. No biological or technical replicates were performed and therefore, statistical analysis could not be applied.

(rXyn10A/Fae1A) with xylanase and feruloyl esterase activities, which yielded 1.82 mg g^{-1} FA or 1820 mg kg^{-1} from de-starched WB.[11] In our experiment, FA content was increased by 45 times with only 1 h of enzymatic treatment, and the amount was high: 648.17 mg kg^{-1}. This amount can be increased by optimizing enzyme doses, incubation time, WB particle size, *etc.* In addition to that, in combination with esterase from *Aspergillus* sp., the FA content can be increased many-fold. FA has various potential applications in various industrial sectors including health (antioxidant, antimicrobial, and anti-inflammatory), food (preservative agent, gel-forming properties, and flavor precursor) and cosmetic (photoprotecting agent) industries.[53,54] Therefore, *Trichoderma* sp. RCK65 can be a suitable source of enzymes for the extraction of commercially valuable FA from WB.

SSF proved more effective than enzymatic treatment in enhancing the overall antioxidant capacity of wheat bran due to the diverse biochemical actions of fermenting microorganisms. During SSF, microbes produce cell wall-degrading enzymes that release bound phenolic compounds, while also biotransforming them into more bioactive forms and generating additional antioxidant metabolites such as peptides and organic acids. This leads to a broad and synergistic increase in total antioxidant activity. The lower ferulic acid content observed after SSF compared to enzymatic treatment may be attributed to microbial metabolism and biotransformation of ferulic acid into other phenolic derivatives, as well as its possible incorporation into complex or polymerized forms that are not detected as free ferulic acid despite contributing to overall antioxidant activity. In contrast, enzymatic treatment primarily uses specific enzymes like cellulase, xylanase, and feruloyl esterases to selectively hydrolyze bonds and release ferulic acid (FA) from the bran matrix. This targeted mechanism enables efficient liberation of intact FA with minimal secondary modification, thereby improving yield and purity but does not significantly enhance the overall antioxidant profile. Therefore, SSF is more suitable for producing antioxidant-rich functional ingredients, whereas enzymatic treatment is preferable for efficient extraction of valuable ferulic acid. Additionally, SSF presents a cost-effective approach compared to traditional enzymatic treatments primarily because it eliminates the need for downstream enzyme purification.

4. Conclusion

This study proved SSF as an economical and convenient method to improve antioxidant potential of wheat bran. *Trichoderma* sp. RCK65 was established as a powerful organism for the enhancement of antioxidant properties of wheat bran within a short time period. Even after enzyme extraction, the fermented WB could be a good source of antioxidants as compared to unfermented WB. So SSF can be a useful method for simultaneous production of commercially useful enzymes as well as production of antioxidant rich WB. Moreover, SSF using *Trichoderma* sp. RCK65 significantly enhanced the free amino acid content, particularly essential amino acids like lysine and threonine, in wheat bran, demonstrating its potential to nutritionally enrich cereal-based foods. At the same time, through enzymatic treatment antioxidant properties of WB can be increased and free FA content can be improved very efficiently. If we compare the efficiency of the SSF process and enzymatic treatment, SSF was proved to be the most suitable for the development of antioxidant rich WB, whereas, enzymatic treatment was appropriate for the extraction of valuable FA.

The proposed SSF and enzymatic approach not only enhances the release of bound phenolics from WB but also aligns with broader valorization frameworks by offering a sustainable route to value-added compounds. Given that the same methodology can be adapted to existing wheat-bran biorefinery operations, the process shows potential for scalability and integration, with implications for improved processing efficiency and circular bioeconomy practices. However, releasing phenolics from wheat bran through SSF and enzyme treatment may be limited by variability in substrate composition, incomplete liberation of bound compounds, and challenges in reproducibility and scale-up, as well as the absence of bioavailability testing. Future studies should focus on downstream stabilization, cost-benefit analysis, and regulatory compliance to enable large-scale implementation of wheat bran valorization strategies within biorefinery frameworks.

Author contributions

Tapati Bhanja DE: investigation, formal analysis, data curation, writing – review & editing, writing – original draft, methodology, conceptualization. Subhojit Chakroborty: methodology. Ramesh Chander Kuhad: supervision.

Conflicts of interest

There are no conflicts to declare.

Data availability

Data are provided within the manuscript.

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