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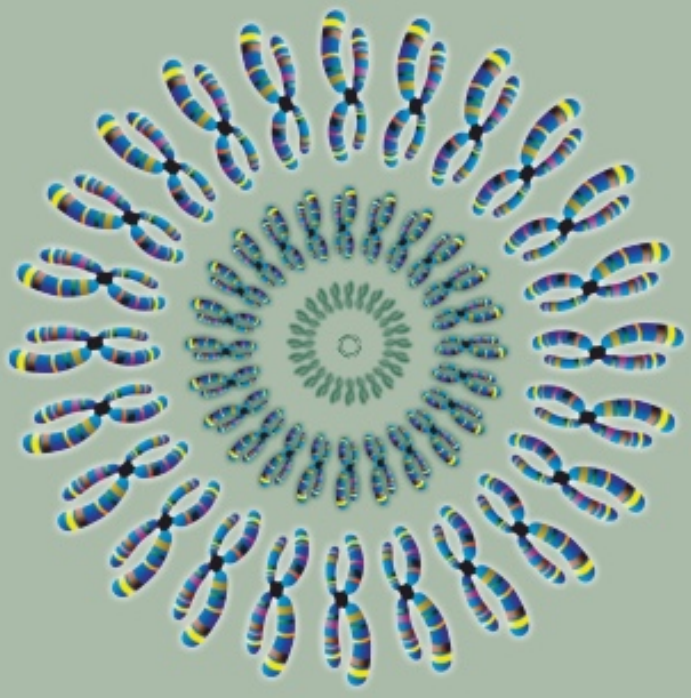

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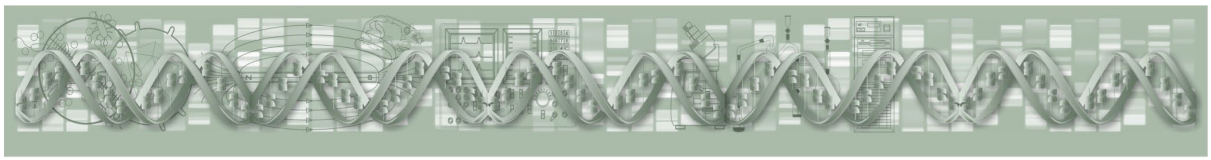

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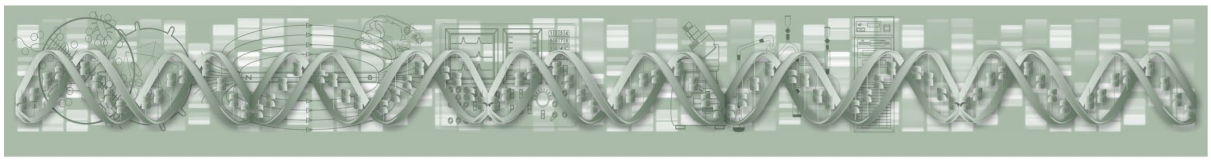

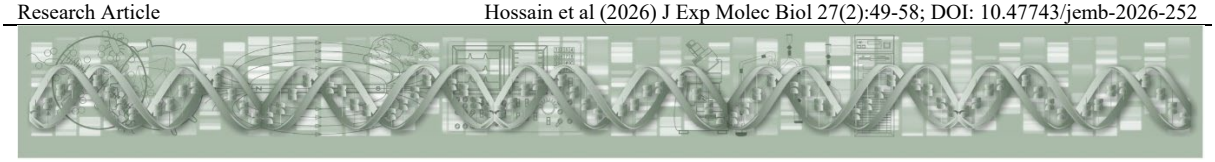

PRODUCTION OF EXTRACELLULAR RECOMBINANT PHYTASE IN YEAST AND ITS APPLICATION IN ANIMAL FEED AS ENZYME SUPPLEMENT

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Abstract

Phytase is a crucial enzyme widely employed in animal nutrition and agriculture for hydrolyzing phytic acid (phytate), the primary storage form of phosphorus in plant tissues, thereby releasing digestible inorganic phosphate. However, its commercial application is often constrained by the thermal instability of the enzyme, particularly under the elevated temperatures used in industrial feed processing. To address this limitation, the present study aimed to develop a robust and scalable yeast-based expression system capable of producing a thermostable form of phytase. A novel expression plasmid, pESC-TRP6HisHA, was constructed, featuring galactose-inducible bidirectional promoters, a 6×His-HA epitope tag for easy detection, and a secretion signal to facilitate extracellular expression. The *PhyA* gene from *Aspergillus niger*, encoding phytase, was cloned into this vector, resulting in the construct pESC-TRP6HisHAPhyA, which was subsequently transformed into *Saccharomyces cerevisiae*. Under galactose induction and phytin-supplemented medium the recombinant yeast successfully expressed and secreted active phytase, as evidenced by clear halo zones on phytin-containing agar plates. Biochemical assays demonstrated that the recombinant phytase exhibited significantly enhanced thermostability compared to the wild-type enzyme, retaining greater enzymatic activity after incubation at 30°C, 40°C, and 50°C for three hours. This improvement was supported by an aliphatic index of 83.2, suggesting moderate thermal resilience. The expression system offers both secreted and potentially surface-displayed phytase, which can facilitate purification, enhance stability, or enable direct use in feed processing. This study establishes a simple, efficient, and scalable platform for the production of thermostable phytase using genetically engineered *S. cerevisiae*. The system holds substantial promise for industrial applications, especially in animal feed manufacturing, where enzyme stability at high temperatures is critical. Future work will focus on optimizing yield, evaluating long-term stability, and conducting feed trials to validate its performance under commercial conditions.

Keywords: Enzyme stability, Genetic engineering, Phytase, *PhyA* gene, Surface display, Thermostability

Introduction

The production of recombinant proteins using microbial platforms represents a cornerstone of modern industrial biotechnology. These platforms provide scalable, cost-effective, and sustainable solutions across diverse sectors (Elazzazy et al. 2025). Among these platforms, microbial cell surface display systems have emerged as powerful tools for protein engineering,

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presenting functional proteins on the cell surface through fusion with specific cell wall-anchoring domains (Pham and Polakov 2020). This approach is widely applied in biocatalysis, vaccine delivery, environmental biosensing, and bioremediation.

Compared to bacterial systems, yeast-based display platforms offer several notable advantages, including the ability to perform post-translational modifications, improved folding of heterologous proteins, and reduced degradation due to low endogenous protease activity (Petushkova and Zamyatnin 2020). In addition to these technological advantages, yeast species such as *S. cerevisiae* are classified as generally recognized as safe (GRAS), possess robust cell wall structures for stable protein anchoring, and are already widely accepted in food and feed industries (Teparić and Mrša 2016). Over the last few decades, several yeast species including *Hansenula polymorpha* (Kim et al. 2002), *Pichia pastoris* (Wang et al. 2008), *Yarrowia lipolytica* (Yuzbasheva et al. 2012), and *S. cerevisiae* (Sun et al. 2014) have been extensively studied for recombinant protein and extracellular enzyme expression. However, many existing expression vectors are designed for specific research applications and often lack modularity, industrial scalability, or optimization for high-efficiency protein secretion during fermentation (Long et al. 2024). These limitations restrict their use in large-scale enzyme production across various industrial applications (Hossain et al. 2019). Therefore, there is a critical need for a yeast-compatible expression system that enables efficient gene insertion, tightly regulated robust expression, and high-efficiency protein secretion under industrially relevant conditions (Otto 2023).

To address this challenge, we constructed and validated a novel yeast expression vector, pESC-TRP6HisHA. This plasmid features, a bidirectional *GAL1*/*GAL10* promoter system for coordinated expression, a secretion signal sequence to direct proteins into the extracellular medium, and an HA epitope tag for immunodetection (Cevheroğlu 2015). The vector allows straightforward insertion of target genes via restriction cloning, supporting both surface display and extracellular secretion of heterologous proteins in *S. cerevisiae* (Zhao et al. 2024). As a proof-of-concept, the *PhyA* gene from *Aspergillus niger*, encoding the enzyme phytase, was cloned into this vector to generate the recombinant construct pESC-TRP6HisHAPhyA (Liao et al. 2012).

Phytase is a phosphatase enzyme that catalyzes the hydrolysis of phytic acid (myo-inositol hexakisphosphate), the principal storage form of phosphorus in plant tissues, thereby releasing inorganic phosphorus that is bioavailable to monogastric animals such as poultry and swine (Gerke 2015). The inclusion of phytase in animal feed significantly enhances phosphorus bioavailability, reduces the need for inorganic phosphate supplements, and lowers environmental phosphorus pollution (Abbasi et al. 2019). However, native microbial phytases often suffer from low thermal stability, which poses a major limitation during high-temperature feed pelleting processes (Kumar et al. 2015). Additionally, the high production and purification costs of phytase restrict its commercial application, particularly in resource-limited settings (Singh et al. 2018). In developing countries such as Bangladesh, where livestock and poultry farming are vital to the agricultural economy, thermostable and cost-effective phytase is essential (Islam et al. 2021). Using *S. cerevisiae* not only enables safe and efficient enzyme production but also provides additional nutritional benefits through its natural content of B-complex vitamins, trace minerals, and other growth-promoting factors (Hossain et al. 2019). This study aims to develop a robust yeast-based recombinant platform for extracellular phytase production, combining enhanced enzyme thermostability with the nutritional benefits of the yeast host. This dual-functional approach holds significant promise for sustainable and cost-effective feed enzyme supplementation in the animal nutrition industry.

Materials and Methods

***In silico* Design and Construction of the pESC-TRP6HisHAPhyA Expression Vector.** A novel yeast expression plasmid, pESC-TRP6HisHAPhyA, was computationally designed using SnapGene 6.3.2 (GSL Biotech) to enable inducible and efficient extracellular expression of recombinant phytase in *S. cerevisiae*. The plasmid backbone features a *GAL1*/*GAL10* promoter, allowing tight transcriptional regulation in response to galactose induction (Elison et al. 2018). An N-terminal 6×His tag and a hemagglutinin (HA) epitope tag were included to facilitate downstream protein detection and purification (Motejadded and Altenbuchner 2009). These were introduced into the pESC-TRP backbone by designing the forward PCR primer to include the sequences for the tags and signal peptide upstream of the *PhyA* coding sequence. The PCR-amplified fragment was then cloned into the multiple cloning site of the vector using NotI and EcoRI restriction sites, generating the final recombinant construct pESC-TRP6HisHAPhyA. To enable extracellular secretion, a secretion signal peptide sequence was placed upstream of the target gene. The coding sequence of the *A. niger* phytase gene (*PhyA*) was selected as the gene of interest (Li et al. 2009). Specific primers were designed to amplify *PhyA* together with its signal peptide, incorporating NotI and EcoRI restriction sites for directional cloning into the multiple cloning site of the pESC-TRP vector (Zhou et al. 2018). The resulting plasmid construct, named pESC-TRP6HisHAPhyA, was used for subsequent functional validation (Figure 1).

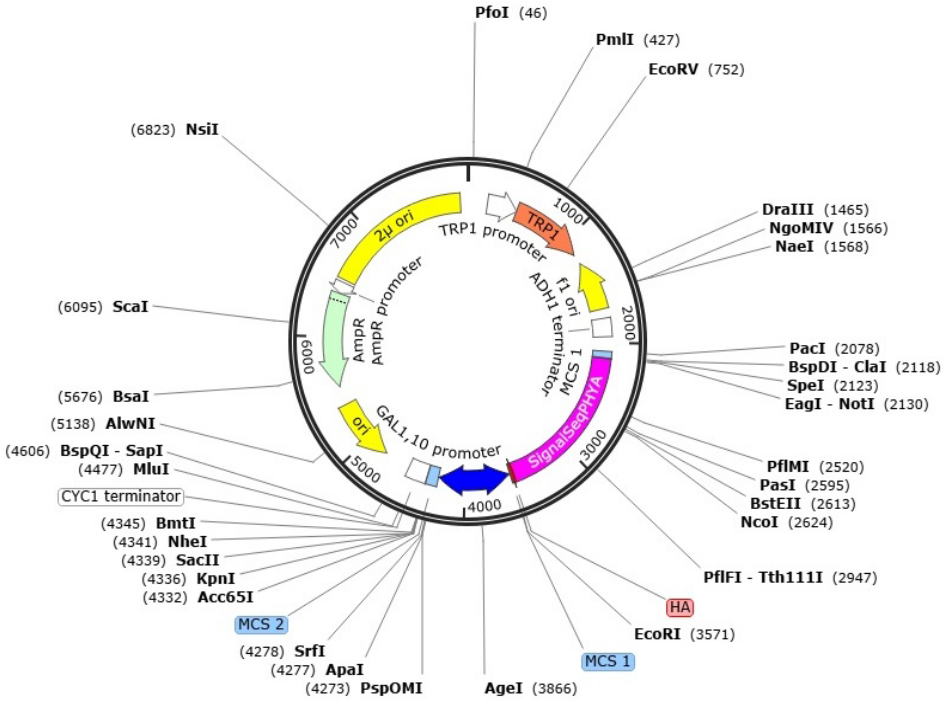


Figure 1. Genetic map of the pESC-TRP6HisHAPhyA final plasmid designed using the Snap Gene software

Fungal Strain, Culture Conditions, and Genomic DNA Isolation. Grapes were surface-sterilized with 70% ethanol for 1 min, followed by 1% sodium hypochlorite for 3 min, and rinsed three times with sterile distilled water before fungal isolation. *A. niger* was isolated from these surface-sterilized grape samples and cultured on potato dextrose agar (PDA) plates at 28°C for 5–7 days. Mycelia were harvested from actively growing cultures, and approximately

100 mg of mycelium was used for genomic DNA extraction using a modified phenol-chloroform-isoamyl alcohol protocol (Kumar et al. 2018). The phenol–chloroform–isoamyl alcohol DNA extraction protocol was slightly modified by extending the lysis step to 30 min and including an RNase treatment for cleaner genomic DNA. DNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and confirmed by agarose gel electrophoresis. Genomic DNA quality was assessed by electrophoresis on 1% agarose gel run at 100 V for 30 minutes.

Amplification and Cloning of the Phytase Expression Cassette. The *PhyA* (GenBank accession no. Z16414) gene along with its native secretion signal was PCR-amplified using high-fidelity DNA polymerase (Phusion, Thermo Fisher Scientific, USA). The *PhyA* gene was amplified using PhyA-NotI-F primer 5′-GCGGCCGCATGGGCGTCTCTGCCGTTCTC-3′ and PhyA-EcoRI-R primer 5′-GAATTCTCAAGCAAAACACTCCGCCCAATC-3′, incorporating NotI and EcoRI restriction sites, respectively. The PCR product was gel-purified, digested with NotI and EcoRI, and ligated into the similarly digested pESC-TRP plasmid using T4 DNA ligase. Recombinant plasmids were transformed into chemically competent *Escherichia coli* DH5α cells. Positive clones were screened by colony PCR and validated by plasmid restriction digestion and Sanger sequencing.

Transformation of *Saccharomyces cerevisiae*. The recombinant plasmid pESC-TRP6HisHAPhyA was introduced into *S. cerevisiae* strain BY4741 using the lithium acetate/polyethylene glycol (LiAc/PEG) transformation method (Bernardi et al. 2019). Transformed yeast cells were plated on synthetic defined (SD) medium lacking tryptophan (SD-Trp) and incubated at 30°C for 48–96 hours. Successful transformants were confirmed by colony PCR targeting the *PhyA* insert (Casado-del et al. 2019).

Isolation and Microscopic Identification of *Aspergillus niger* and *Saccharomyces cerevisiae*. *A. niger* was isolated from decomposing soil waste, while *S. cerevisiae* was obtained from grapes. Pure cultures were established by streaking samples on selective agar media. *A. niger* was cultured on potato dextrose agar (PDA) composed of potato extract (200 g/L), dextrose (20 g/L), and agar (20 g/L), and incubated at 28°C for 5–7 days. *S. cerevisiae* was maintained on yeast extract–peptone–dextrose (YPD) medium containing yeast extract (10 g/L), peptone (20 g/L), dextrose (20 g/L), and agar (20 g/L) at 30°C for 48–72 hours. Microscopic analysis of the cultures was performed using a light microscope to confirm morphological characteristics, including filamentous hyphae and conidial heads in *A. niger* and budding cells in *S. cerevisiae*.

Detection of Native Phytase Activity in *Aspergillus niger*. The phytase-producing ability of *A. niger* was evaluated using a phytin-containing screening medium, in which phytin served as the sole phosphate source. The medium was composed of glucose (15 g/L), NH_4NO_3 (5 g/L), KCl (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.01 g/L), $MnSO_4 \cdot 4H_2O$ (0.01 g/L), agar (15 g/L), and phytin (2 g/L). Fungal cultures were spot-inoculated onto the medium and incubated at 28°C for 5 days. Phytase activity was qualitatively assessed by observing clear halo zones around colonies, indicating hydrolysis of phytin and release of inorganic phosphate.

Qualitative Plate Assay for Phytase Activity. To evaluate extracellular phytase activity, both recombinant *S. cerevisiae* transformants and wild-type *A. niger* were cultured on PDA plates supplemented with 2% sodium phytate (phytin) as the sole phosphorus source (Upton 2017). Plates were incubated at 30°C for up to 5 days. Enzymatic activity was qualitatively assessed by observing clear halo zones around colonies, indicative of phytate degradation and phosphate release (Sharma and Bansal 2024).

Quantitative Thermal Stability Assay of Phytase. Thermal stability of the expressed phytase enzyme was assessed by incubating culture supernatants at 30°C, 40°C, and 50°C for 3 hours. Phytase activity was quantified using a standard colorimetric phosphate release assay, where liberated inorganic phosphate was detected using ammonium molybdate reagent, and

absorbance was measured at 700 nm using a microplate reader (Qvirist et al. 2015). Enzyme activity was expressed as the percentage of residual activity relative to untreated controls. All assays were conducted in biological triplicates.

Bioinformatic Evaluation of Aliphatic Index. To predict thermal stability from a sequence-based perspective, the amino acid sequence of the expressed phytase was analyzed using the ProtParam tool available on the ExPASy Bioinformatics Resource Portal (Naqqash 2023). The aliphatic index, a metric associated with protein thermostability, was calculated based on the relative molar content of aliphatic amino acids (alanine, valine, isoleucine, and leucine) using the formula:

$$AI=X(Ala) + aX(Val) + b[X(Ile) + X(Leu)]$$

where a and b are weighting coefficients reflecting volume contributions of respective residues.

Statistical Analysis. All experimental procedures were performed in triplicate (n = 3) unless otherwise specified. Data are presented as mean ± standard deviation (SD). Unpaired Student's t-tests were used for pairwise comparisons (e.g., wild-type vs. recombinant strains), while one-way ANOVA followed by Tukey's post hoc test was employed for comparisons across multiple temperatures. A significance threshold of $P < 0.05$ was considered statistically significant. All statistical analyses and graphical visualizations were carried out using GraphPad Prism version 9.0 (GraphPad Software, USA).

Results and discussions

Construction and Validation of pESC-TRP6HisHAPhyA Recombinant Plasmid. The recombinant expression vector pESC-TRP6HisHAPhyA was successfully constructed by inserting the PCR-amplified NotI-EcoRI flanked HA-tagged *PhyA* signal sequence into the pESC-TRP backbone via restriction-ligation. Following ligation, the plasmid was digested with NotI and EcoRI, generating two distinct bands of approximately 6503 bp and 1441 bp, consistent with the expected sizes of the vector and insert, respectively (Figure 2). This molecular confirmation was further substantiated by commercial DNA sequencing (GeneScript, China), which verified the correct orientation, frame, and integrity of the insert sequence.

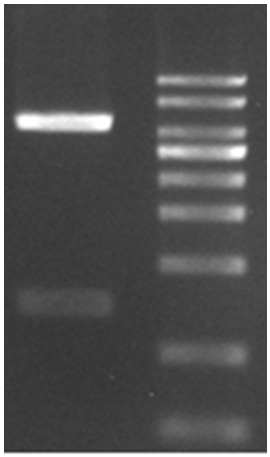


Figure 2. Restriction digestion of the pESC-TRP6HisHAPhyA plasmid with NotI and EcoRI, generating fragments of 6503 bp and 1441 bp. A 1 kb DNA ladder (Thermo Fisher Scientific, USA; 250 bp–10 kb) was used to estimate fragment sizes

Isolation and Microscopic Identification of *Aspergillus niger* and *Saccharomyces cerevisiae*

Pure cultures of *A. niger* and *S. cerevisiae* were successfully obtained. Microscopic examination revealed filamentous hyphae and globular conidial heads in *A. niger*, while budding cells were observed in *S. cerevisiae* (Figure 3).

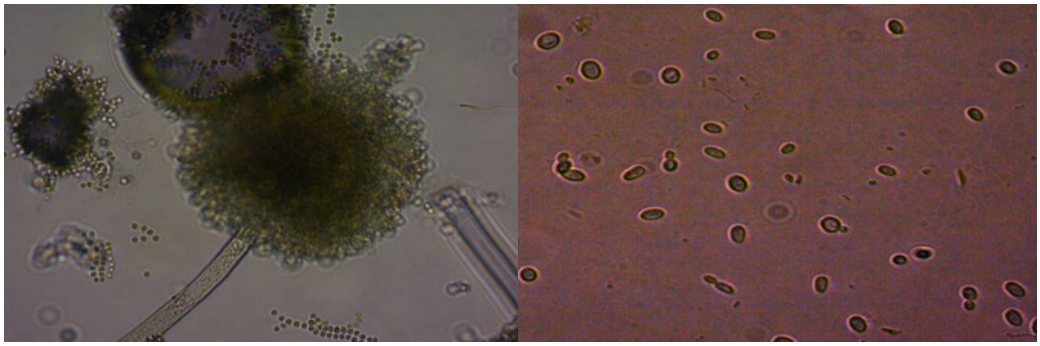


Figure 3. Microscopic view of *A. niger* (left) and *S. cerevisiae* (right) from pure culture

Detection of Native Phytase Activity in *Aspergillus niger*

A. niger exhibited clear halo zones around colonies on phytin-containing plates, indicating active phytase secretion and hydrolysis of phytin (Figure 4).

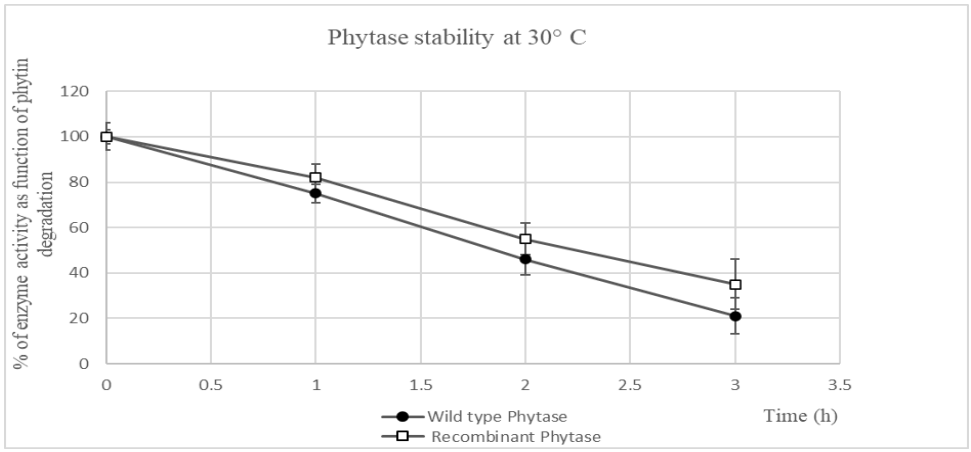
Figure 4. Clear zone around *A. niger* observed in PDA screening media containing phytin, indicating phytase activity

Functional Expression of Recombinant Phytase in *S. cerevisiae*. To evaluate heterologous expression, *S. cerevisiae* cells harboring the pESC-TRP6HisHAPhyA plasmid were cultured on YPD medium supplemented with galactose (20 g/L) and 2% phytin. The appearance of a prominent clear zone surrounding transformed colonies confirmed the secretion of active recombinant phytase and efficient degradation of phytin (Figure 5). This observation highlights the functional success of the yeast expression system and the correct targeting of the recombinant protein to the extracellular environment.

Figure 5. Clear zone around *S. cerevisiae* observed in YPD media containing phytin, confirming recombinant phytase activity

Evaluation of Thermal Stability of Recombinant Phytase. Thermostability assays were conducted to compare the residual activity of recombinant phytase with that of wild-type enzyme after 3-hour incubations at 30°C, 40°C, and 50°C. At all tested temperatures, the recombinant enzyme retained significantly greater enzymatic activity, indicating improved thermal resilience (Figures 6a–c). The enhanced stability is likely attributable to yeast-mediated post-translational modifications, particularly glycosylation, which are known to confer structural integrity under heat stress.

a)



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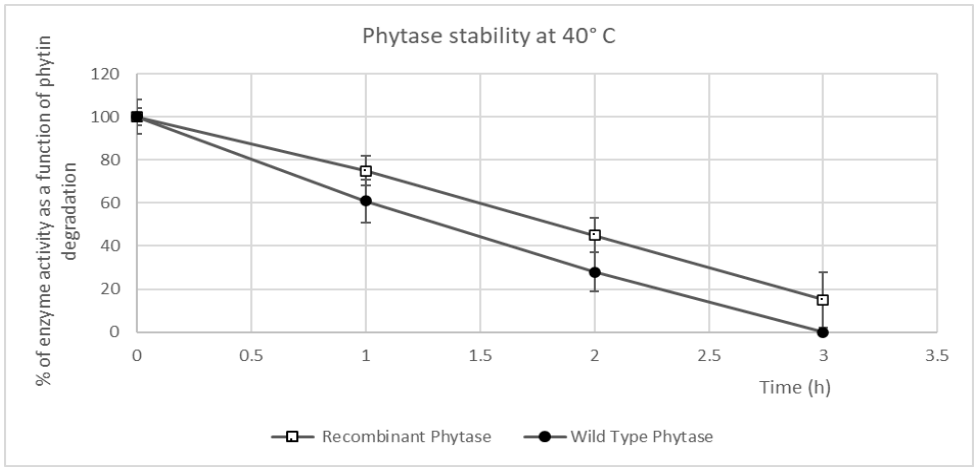

c)

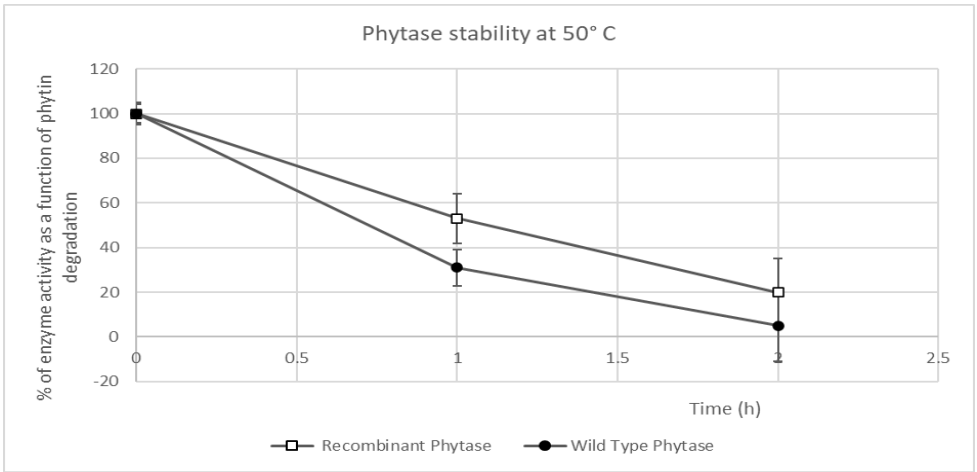

Figure 6. Temperature stability of phytase at: a) 30°C; b) 40°C; c) 50°C

These results underscore the potential of yeast-based phytase production for applications requiring moderate thermal endurance, such as feed pelleting or processing in warm environments.

***In Silico* Assessment of Phytase Thermostability via Aliphatic Index.** To further evaluate thermostability from a structural perspective, the aliphatic index of the phytase protein was calculated using the ExPASy ProtParam tool, yielding a value of 83.2. This metric, which correlates with the proportion of aliphatic side chains (e.g., Ala, Val, Ile, Leu), is often predictive of thermostability, with values above 90 typically denoting high thermal tolerance. Although the recombinant enzyme demonstrated improved thermal stability *in vitro*, its aliphatic index suggests it remains moderately thermostable and may still require protein engineering or mutagenesis for high-temperature industrial applications.

Conclusions

This study successfully demonstrates the construction of an optimized genetic cassette enabling both surface display and extracellular secretion of recombinant phytase in *S. cerevisiae*. The engineered yeast strain exhibited enhanced thermal stability of the expressed enzyme relative to its wild-type counterpart, underscoring its potential for industrial applications, particularly in the animal feed industry, where high-temperature processes such as pelleting are routinely employed. These findings align with previous reports highlighting the advantages of yeast-based expression systems in enhancing the functional properties of heterologous proteins, including improved stability and secretion efficiency. Notably, the dual localization strategy employed here, represented by simultaneous surface anchoring and secretion, offers greater operational flexibility, facilitating both direct application in fermentation processes and efficient enzyme recovery for downstream use. Despite these promising outcomes, the study acknowledges the need for further validation under real-world industrial settings, including long-term stability assays, cost-benefit analyses, and performance evaluations within complex feed matrices. In addition, animal feed trials will be essential to assess bioavailability, digestibility, and overall nutritional benefits. Such investigations will be critical for transitioning this recombinant phytase system from laboratory development to commercial-scale implementation.

Acknowledgments

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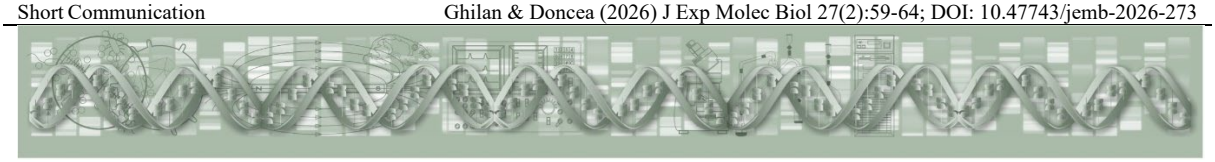

SUMMER SNOWFLAKE (*LEUCOJUM AESTIVUM* L.) – A RARE ADDITION TO THE FLORA OF "PRUTUL DE JOS" SCIENTIFIC RESERVE, REPUBLIC OF MOLDOVA

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Abstract

Currently, the summer snowflake is suffering distributional and populational declines across its range, and due to this local scarcity, it is protected by law in several countries within its distribution range. The distribution of this species in the Republic of Moldova is very little studied, and currently this species is classified as critically endangered in the 3rd edition of the national Red Data Book, with only seven known locations. This short communication aims to reveal a new location of the summer snowflake – „Prutul de Jos" Scientific Reserve, increasing the conservation importance of the reserve even more.
Keywords: Beleu lake, national distribution, new record, Red Data Book, threatened species

Introduction

The summer snowflake (*Leucojum aestivum* L.) is a native Western Palearctic species belonging to the *Amaryllidaceae* family, consisting of two valid subspecies - *L. aestivum* L. subsp. *aestivum* and *L. aestivum* L. subsp. *pulchellum* (Salisb.) Briq. currently considered threatened in many parts of its range. Although not globally threatened, it is included in many national red lists and currently benefits from legal protection status, due to its local scarcity (Parolo et al. 2011). This wetland species has recently experienced declines both in distribution and population size due to the intensive destruction of wetlands and overharvesting for alkaloid production (Parolo et al. 2011). Since the 19th century, 80% of the natural wetlands from the Danube river basin have been drained to make space for farmland (Diester 1999). Since most of the native range of the summer snowflake is situated within the borders of the Danube river basin (Parolo et al. 2011), it is expected that these populations have greatly suffered. The summer snowflake is mostly a lowland species, inhabiting floodplain forests close to waterbodies such as lakes, rivers or occasionally on the banks of canals (Parolo et al. 2011, Cassir and Ghendov 2022). It can also inhabit areas far from waterbodies, as long as sufficient soil moisture is available during the growing season, particularly in microdepressions where excess water tends to accumulate (Parolo et al. 2011). It is generally considered very tolerant of high moisture in the environment and soil tolerant (Pavlova et al. 2015), however it was shown to prefer nitrogen rich and sedimentary types of soil, such as clay or loam (Parolo et al. 2011).

Currently included in the third edition of the Red Book from the Republic of Moldova (2015), the summer snowflake is classified at the national level as Critically Endangered (CR) according to IUCN standards. Until now, only seven locations from the Republic of Moldova

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are known to host this rare species (Pînzaru 2018, Cassir and Ghendov 2022, Ghereg et al. 2023, Lazarchevici 2023). Multiple factors have contributed for the scarcity of this species in the Republic of Moldova, the most relevant being the following: the presence at the northern limit of its range around the Black Sea (Parolo et al. 2011, Cassir and Ghendov 2022), the decline in availability of unfragmented and pristine floodplain forests, and the lack of dedicated field effort in appropriate and inaccessible wetlands during the flowering season. According to Ghereg et al. (2023) the summer snowflake is in bloom during April-May, detectability drastically reducing outside this time period. Nowadays, wetlands cover only 2.9% (96.900 ha) of the total area of the country, of which only 8924 ha are constituted by marshland (HG 357/2020). Floodplain forests are also very scarce and mainly along the bigger rivers such as Prut and Dniester. Woodland as a whole makes up about 13.6% of the country's land cover (National Bureau of Statistics from Moldova, 2023), of which poplar (*Populus* sp. L.) and willow (*Salix* sp. L.) represent no more than 1.3% and 1.2% respectively (Talmaci et al. 2018). This being said, the combination of little field effort in the few potential and inaccessible sites from southern Moldova where the last floodplain forests remain, makes the local scientific knowledge about the species very scarce. Until 2022, the only known population in the country was in the central west Republic of Moldova and it was comprised of only six locations from the vicinity of Prut river floodplains, close to the following localities: Călmățui, Cioara, Cotul Morii, Leușeni, Nemțeni villages, from Hîncești district, and Sărata-Răzeși from Leova district (Pînzaru 2018, Ghereg et al. 2023, Lazarchevici 2023). The habitat in which the summer snowflake was found in these locations consists mainly of white willow (*Salix alba* L.) and white poplar (*Populus alba* L.) floodplain forest. In 2022 a seventh location was found near Crihana Veche village, Cahul district. This particular location is easily accessible, and it sits inside of Lower Prut Lakes Ramsar site, but outside of Lower Prut Scientific Reserve, this way being more prone to local extinction due to human interventions. The former mentioned protected area hosts no less than 310 plant species (Postolache et al. 2017, Cassir 2022), and in this study a new and rare floristic addition has been identified.

Material and Methods

The observation was made during a field trip in the forest compartment 5 of "Prutul de Jos" Scientific Reserve. The protected area was established in 1991, comprising a total of 1775 ha, of which 168,3 are strictly protected (Postolache 2021). At least 310 rare species of vascular plants, with 15 of them included in the 3rd edition of the Red Data Book of the Republic of Moldova are found in this wetland (Postolache 2021).

Results and discussion

On 23/04/2025 a patch of flowering summer snowflakes was discovered in one of the most isolated and inaccessible forests from the vicinity of Beleu Lake (Figures 1, 2B). The species was found in a primary white willow forest, exhibiting minimal anthropogenic disturbance, with overgrown dewberry (*Rubus caesius* L.) in the shrub layer (Figure 2A), very similar to the described habitat in which the summer snowflake was found by Pînzaru (2018) and Cassir and Ghendov (2022). Pînzaru (2018) even described and proposed a new subassociation, including the summer snowflake as a characteristic species – *Salicetum albae leucojetosum aestivi*. Just a single patch numbering a few individuals was found in the floodplain forests of Beleu Lake. A total of 8 locations are now known to host the summer snowflake spanning from its most northern location, Nemțeni Natural Reserve (Hîncești district), to its most southern location, Prutul de Jos Scientific Reserve (Figures 1, 3). Even though the latter mentioned location is strictly protected, it is also heavily subject to poaching (authors' observation), making it

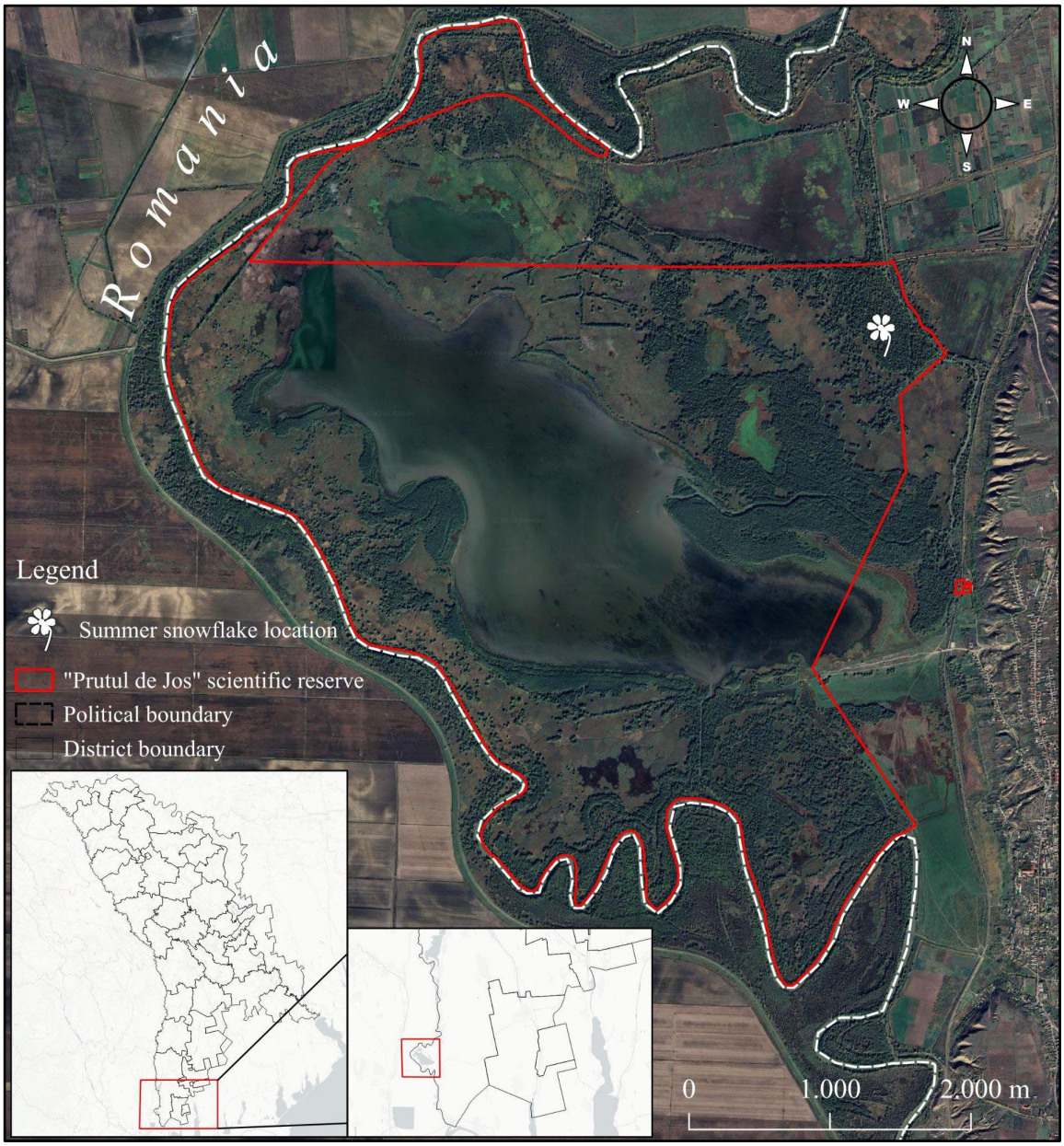

Figure 1. *Leucojum aestivum* found in the floodplain forests of "Prutul de Jos" Scientific Reserve (23 April, 2025)

possible to threaten species of fauna and flora. It is probable that the summer snowflake occurs in more locations along Prut river, resulting in a more connected distribution range. Since work for the much awaited fourth edition of the Red Book of the Republic of Moldova has started, it is important that all the mentioned locations are to be included. It is also recommended that more field work should be done, to properly assess the actual distribution and conservation status of the species in the Republic of Moldova. This is particularly important given that the summer snowflake is a perennial plant, flowering in the same location each spring [Parolo et al. 2011]. This site fidelity increases its vulnerability to habitat disturbance, making targeted protective measures crucial for ensuring its long-term conservation. Knowledge gaps could be filled if more field effort would be conducted during April-May, downstream of Nemțeni floodplain forests. Another relevant task would be to search upstream of Nemțeni, in order to find the absolute limit of the distribution range.

Figure 2. A. Floodplain forest with white willow and dewberry where *Leucojum aestivum* was found (23 April, 2024); **B.** *Leucojum aestivum* found in the floodplain forests of "Prutul de Jos" Scientific Reserve (23 April, 2025)

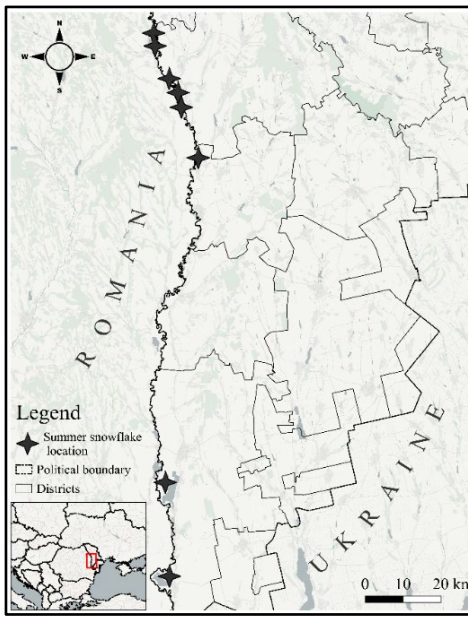

Figure 3. National distribution of *Leucojum aestivum*. Map drawn using QGIS v.3.28. (http://qgis.osgeo.org)

Conclusions

The floristic list of "Prutul de Jos" Scientific Reserve has increased by a rare addition, namely the threatened and protected summer snowflake, highlighting the importance of this protected area for biodiversity conservation. Currently a total of 8 locations is known to host the summer snowflake in the Republic of Moldova, however more field work is required to establish the whole distribution range.

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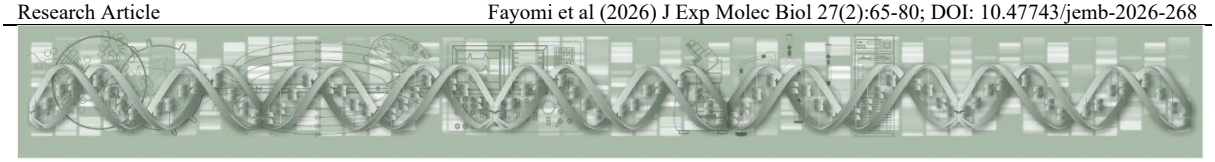

CHEMICAL ANALYSIS AND ANTINUTRITIONAL VALUE OF SELECTED INBRED LINES OF GROUNDNUT (*ARACHIS HYPOGAEA* L.)

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Abstract

This study assesses the proximate composition and anti-nutritional profiles of three groundnut (*Arachis hypogaea* L.) breeding lines: (SAMNUT-26×ICGV-91328, SAMNUT-23×ICGV-91324, and SAMNUT-24×ICGV-91328). Seed samples of these hybrid lines were obtained from the Seed Centre at the College of Agronomy, Joseph Sarwuan Tarka University, Makurdi, Nigeria. Standard analytical methods were employed to determine moisture, ash, crude fibre, lipid, protein, carbohydrate contents, and selected anti-nutritional parameters, including phytic acid, oxalate, and cyanide levels. Moisture content varied from 6.29% in SAMNUT-23×ICGV-91324 to 7.05% in SAMNUT-26×ICGV-91328. Ash content ranged between 3.26% and 4.50%, with SAMNUT-23×ICGV-91324 exhibiting the highest value. Fibre content was relatively stable, with a narrow range of 2.91%–3.14%. Lipid concentration was highest in SAMNUT-26×ICGV-91328 (47.00%) and lowest in SAMNUT-23×ICGV-91324 (46.12%). The highest protein content (22.85%) was recorded in SAMNUT-24×ICGV-91328, while carbohydrate content peaked at 18.37% in SAMNUT-23×ICGV-91324. Regarding anti-nutritional factors, SAMNUT-23×ICGV-91324 had significantly higher cyanide levels (5.10 mg/100g) compared to the other two lines ($P < 0.05$), followed by SAMNUT-24×ICGV-91328 (4.63 mg/100g) and SAMNUT-26×ICGV-91328 (2.56 mg/100g). Similarly, the highest oxalate concentration was observed in SAMNUT-23×ICGV-91324. Overall, the results reveal notable compositional differences among the groundnut hybrid lines, with SAMNUT-24×ICGV-91328 emerging as the most nutritionally advantageous candidate based on its superior protein and balanced nutrient profile.

Keywords: Peanut, fibre content, Cyanide, Phytic acid, oxalate, proximate, Proteinous Proteins food

Introduction

Groundnut (*Arachis hypogaea*), also known as peanut, is a globally important oilseed crop cultivated across a wide range of agroecological zones, from tropical to temperate regions. Africa produces an average groundnut yield of 1,007 kg/ha, with Nigeria presently contributing

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about 3.3 t/ha, a part of the 55% West African Groundnut production in the continent (Fukah et al. 2024, Njoki et al. 2024). Globally, though Nigeria's groundnut production ranks high, it is less than the 60 % combined contributions by China and India (Gelaye and Luo 2024). Its agricultural versatility and high economic value have made it an essential part of food systems in many nations of the world, particularly in Asia, Africa, and South America. Groundnut cultivation has been naturally driven by its high oil yield to supply domestic consumption and industrial processing (Abady et al. 2019). However, a recent study highlights a broader nutritional and functional profile, positioning groundnut as a promising crop for improving dietary quality and combating micronutrient deficiencies, particularly in low- and middle-income countries (Njoki et al. 2024).

In addition to its oil content, groundnut is a rich source of high-quality protein, polyphenols, flavonoids, dietary fibre, and essential micronutrients such as magnesium, phosphorus, potassium, calcium, and vitamins E, K, and the B-complex group (Musalima et al. 2019, Lakhlifi El Idrissi et al. 2024). Compounds such as resveratrol, phytosterols, and other antioxidants found in groundnuts have demonstrated potential cardiovascular benefits by inhibiting cholesterol absorption and improving lipid profiles (Ortiz and Martirosyan 2025). In food-insecure areas, where plant-based diets predominate, and animal protein is often costly, groundnut offers an affordable and nutrient-dense dietary alternative.

Beyond these mentioned nutritional values, groundnut seeds can be consumed raw, roasted, processed into oil and butter, or incorporated into a variety of conventional edibles and animal feed (Onyeike and Oguike 2016). However, processing methods can significantly influence nutrient availability. For example, roasting enhances flavour and mineral bioavailability but may degrade heat-sensitive amino acids and reduce protein digestibility, depending on temperature and exposure time (Adeyeye 2010, Eltom et al. 2023). Selecting and improving new groundnut breeding lines is essential to increase crop production, diversify products, and better match the needs of farming and the production value chain down to the consumers. Farmers' concern is getting high-yielding, disease and stress-tolerant, early-maturing, high-price, and specific-grain-colour varieties (Abady et al. 2019, Regassa et al. 2023), while others, including consumer desire for big, clean grains, oil content, storability, good price, Color, protein/nutrition, texture, flavour, healthiness (high oleic, low aflatoxin), nutritious products (Pandey et al. 2020). Studies conducted on Pakistani groundnut hybrid lines support these findings, reinforcing the crop's potential as a rich protein source with a favourable proximate composition (Atasie et al. 2009). Additionally, roasting has been linked to the generation of aromatic pyrazines, which enhance flavour but may also alter oil quality and shelf stability (Hu et al. 2021).

While groundnuts offer significant nutritional advantages, their consumption is not without concern. Like many legumes, groundnut contains several anti-nutritional factors (ANFs) that can impair nutrient absorption and utilisation. Notably, phytic acid can chelate divalent cations such as calcium, iron, and zinc, reducing their bioavailability (Gupta et al. 2015). Tannins—whose levels vary by seed colour—can inhibit digestive enzymes but may also provide antioxidant benefits when present in moderate amounts (Singh et al. 2023). Additionally, trypsin inhibitors—although present in relatively low quantities—can also reduce protein digestibility (Tibe and Amarteifio 2010).

Cyanogenic glycosides are another class of ANFs found in trace amounts in some groundnut hybrid lines. Upon hydrolysis, these compounds release hydrogen cyanide (HCN), a potent respiratory toxin. Improper post-harvest handling or storage may exacerbate cyanide volatilisation, posing a significant food safety concern (Adejoh et al. 2020). Similarly, oxalates present in groundnuts can form insoluble salts with calcium and other minerals, potentially contributing to kidney stone formation if consumed excessively (Ritter and Savage 2007). Despite these concerns, some ANFs, such as phytic acid, have demonstrated protective effects

against oxidative stress and inflammation, further complicating their classification as purely detrimental (Midorikawa et al. 2001).

Comparative data on the proximate and anti-nutritional composition of improved groundnut hybrid lines remain limited, particularly within the Nigerian context. Such insights are essential for guiding breeding efforts, dietary planning, and food processing practices that enhance nutritional benefits while reducing potential health risks. Although previous studies have explored some groundnut hybrid lines, further evaluation of newly improved hybrid lines is warranted (Olasan et al. 2024). This study aims to analyze and compare the nutritional and anti-nutritional profiles of three improved groundnut hybrid lines (SAMNUT-26×ICGV-91328, SAMNUT-23×ICGV-91324, and SAMNUT-24×ICGV-91328) to support evidence-based selection and utilization.

Materials and Methods

Study Area and Sample Collection

This study was conducted in the Biochemistry Laboratory of the College of Biological Sciences, Joseph Sarwuan Tarka University, Makurdi, Benue State, Nigeria (≈7.4–7.7° N, 8.4–8.5° E, ~100–113 m above sea level). This place has a low-elevation tropical wet-and-dry climate with mean temperatures of about 26–31 °C across cool-dry, hot-dry, and hot-wet seasons, relative humidity around 44–86%, and annual rainfall of roughly 1,000–1,250 mm concentrated between April and October (Tyubee et al. 2021). Three improved hybrid lines of groundnut seeds (SAMNUT-26×ICGV-91328, SAMNUT-23×ICGV-91324, and SAMNUT-24×ICGV-91328) were obtained from the Seed Centre at the College of Agronomy, Joseph Sarwuan Tarka University, Makurdi. These hybrid lines were selected for evaluation based on their availability and relevance to ongoing local breeding programs. Identification of the hybrid lines followed standard descriptors as outlined by Yenikalayci (2021).

Sample Preparation

Collected groundnut seeds were cleaned and air-dried at room temperature for 24 hours to reduce surface moisture and remove impurities that could cause analytical error. The dried seeds were then pulverised into fine flour using a laboratory grinder and stored at room temperature in airtight containers until analysis.

Proximate Composition Analysis

Moisture Content

Moisture content was determined using the standard oven-drying method. Clean crucibles were dried, cooled in a desiccator, and weighed. Five grams of each groundnut hybrid line were placed in the crucibles and dried in a hot-air oven at 103–105°C for two hours. Samples were cooled in a desiccator and reweighed. Drying continued until a constant weight was obtained. Moisture content was calculated as:

$$\% \text{ Moisture } \frac{w_2 - w_3}{w_2 - w_1} \; x \; \frac{100}{1}$$

$$w_1 = weight\ of\ the\ empty\ moisture\ can$$
$$w_2 = weight\ of can\ and\ sample\ before\ drying$$
$$w_3 = weight\ of\ can\ and\ sample\ after\ drying$$

Crude Protein

Protein content was determined using the micro-Kjeldahl method. Two grams of each groundnut hybrid line was digested with concentrated sulfuric acid and a selenium catalyst. The digest was diluted to 100 mL with distilled water. A 10 mL aliquot was mixed with 45% sodium hydroxide and distilled. The distillate was collected in 4% boric acid containing a mixed indicator (methyl red and bromocresol green), and then titrated with 0.02N sulfuric acid.

Titration was done from the initial green colour to a deep red or pink endpoint. The total nitrogen was calculated and multiplied by the factor 6.25 to obtain the crude protein content (Sáez-Plaza et al. 2013).

$$\% \, Crude \, protein = \%N6.25$$

$$\% \, N_2 = \frac{(100x)Nx14xV_f xT}{w \, x \, 100 \, x \, V_A}$$

$$W = weight \, of \, the \, sample$$

N = Normality of filtrate ((H_2S0_4) = 0.02N
V_f = Total volume of the digest = 100mL
V_A = Volume of the digest distilled

Crude Fat

Fat content was extracted using a Soxhlet apparatus with petroleum ether as the solvent. Five grams of each groundnut sample were wrapped in filter paper, placed in the extractor, and subjected to reflux for approximately 4 hours. After extraction, the solvent was recovered, and the flask containing the oil was dried, cooled, and weighed. Fat content was calculated as:
Formula for the Calculation:

$$\% \, of \, fat = \frac{w_2 - w_1}{Ww_1} x \frac{100}{1}$$

Where: *W = weight of the sample*
W_1 weight of empty extraction flask
W_2 = weight of flask and oil extract

Ash Content

Ash content was determined by weighing 5 grams of dried groundnut sample of each breeding line into pre-dried crucibles and igniting in a muffle furnace at 550–600 °C for 2 hours until all organic matter burns off, leaving inorganic residue (ash). The crucibles were cooled in a desiccator and re-weighed; ash % = (ash residue ÷ original sample) × 100 (Quirino et al. 2023). Ash content was determined by incinerating 5 grams of each groundnut sample in a muffle furnace at 700°C for 2 hours. Pre-weighed crucibles were used, and after cooling in a desiccator, the residual ash was weighed. The percentage ash content was calculated as:

$$\% \, Ash = \frac{w_2 - w_3}{w_2 - w_1} x \, \frac{100}{1}$$

W_1 = weight of the crucible
W_2 = weight of sample crucible
W_3 = weight of crucible + ash

Crude Fiber

Crude fiber was determined using the Weende method. Defatted Groundnut samples (from fat analysis) were boiled in 1.2% sulfuric acid for 30 minutes, filtered, washed, and then boiled in 1.25% sodium hydroxide for another 30 minutes. The residue was washed, dried in an oven at 150°C, cooled, and weighed. It was then incinerated at 550°C in a muffle furnace, cooled, and reweighed (Medina-Saavedra Tarsicio et al. 2018). Crude fiber was calculated using:

$$\% \; Crude \, fibre = \frac{loss \, in \, weight \, incinertion}{weight \, of \, sample} x \, \frac{100}{1} = \frac{w_2 - w_3}{weight \, of \, sample}$$

W_2 = weight of crucible sample after washing and drying in oven

W_3 = weight of crucible + sample ash

Carbohydrate Content

Carbohydrates were estimated by difference, using the nitrogen-free extract method:

$$\% \text{ NFE} = 100 - \% (a+b+c+d+e)$$

where: *a= protein*, *b= fat*, *c= fibre*, *d= ash*, *e= moisture*

Anti-Nutritional Factor Analysis

Oxalate Content

Approximately 0.5g of each sample was extracted with 15 mL of 3M sulfuric acid for one hour. The mixture was filtered, and 5 mL of the filtrate was titrated hot with 0.05M potassium permanganate until a faint pink color persisted for at least 30 seconds. Oxalate content was calculated using the factor that 1 mL of 0.05M $KMnO_4$ equals 2.2 mg oxalate (Ijarotimi and Keshinro 2013).

Phytic Acid

Two grams of the finely ground sample were extracted with 25 mL of 0.1 mol L^{-1} HCl (≈2% v/v) for 3 h and filtered to obtain a clear chloride-containing filtrate. A 25 mL aliquot of this extract was then treated with 0.1 mol L^{-1} ammonium thiocyanate (NH_4SCN) indicator and titrated with 0.014 mol L^{-1} $FeCl_3$ solution until a stable brownish-yellow ferric thiocyanate colour persisted for 5 min, following a Volhard-type argentometric/thiocyanate titration principle for chloride determination (Cai et al. 2020). Two grams of the sample were soaked in 2% HCl for three hours and filtered. A 25 mL aliquot of the filtrate was reacted with ammonium thiocyanate (NH_4SCN) as an indicator and titrated with iron (III) chloride solution until a stable brownish-yellow colour persisted for 5 minutes (Yahaya et al. 2022a). The phytic acid content was calculated using:

$$\text{Phytic Acid} = \text{Titre Value} \times 1.15 \times 1.19 \times 3.55$$

The **titre value** from the titration of phytic acid-P and $FeCl_3$.

The factor **3.55** is the classical chemical conversion from phytic acid-P to phytic acid ($C_6H_{18}O_{24}P_6$).

The **1.15** and **1.19** are constants to account for extraction dilution, sample aliquot, or moisture basis (Romero-Aguilera et al., 2017).

Cyanide Content

A 2.5 g portion of the ground sample was soaked in 50 mL of distilled water overnight. After filtration, 1 mL of the extract was mixed with 4 mL of alkaline picrate and incubated at 100°C for 5 minutes. The absorbance was read at 450 nm against a blank, and cyanide content was determined from a standard curve.

Statistical Analysis

Data The data collected were subjected to one-way analysis of variance (ANOVA) using SPSS version 2016. Mean differences were separated using Duncan's Multiple Range Test at a 95% confidence level ($p < 0.05$).

Results

Proximate Composition in three Groundnut Breeding lines

Table 1 presents the proximate composition of three groundnut breeding lines: SAMNUT-26×ICGV-91328, SAMNUT-23×ICGV-91324, and SAMNUT-24×ICGV-91328. Moisture

content ranged from 6.29% to 7.05%, with SAMNUT-26×ICGV-91328 showing the highest value and SAMNUT-23×ICGV-91324 the lowest (6.29 ± 0.13%), giving a mean of 6.62%. Ash content varied between 3.26% and 4.50%, highest in SAMNUT-23×ICGV-91324 and lowest in SAMNUT-26×ICGV-91328, with an overall mean of 3.84%.
Fiber content ranged from 2.91% in SAMNUT-24×ICGV-91328 to 3.14% in SAMNUT-23×ICGV-91324, averaging 3.05%. Lipid content was highest in SAMNUT-26×ICGV-91328 (47.00%) and lowest in SAMNUT-23×ICGV-91324 (46.12%), with a mean of 46.70%. Protein content was highest in SAMNUT-24×ICGV-91328 (22.85 ± 0.07%) and lowest in SAMNUT-23×ICGV-91324 (21.59 ± 0.07%), yielding a mean of 22.32%. Carbohydrate content ranged from 16.99% to 18.37%, with the highest in SAMNUT-23×ICGV-91324 and the lowest in SAMNUT-24×ICGV-91328, averaging 17.47%. The grand means are presented in Figure 1.

Table 1. Proximate composition in three groundnut breeding lines

Varieties	Moisture (%)	Ash (%)	Fiber (%)	Lipid (%)	Protein (%)	Carbohydrate (%)
SAMNUT-26x1CGV-91328	7.05±0.04[a]	3.26±0.03[a]	3.11±0.03[a]	47.00±0.28[a]	22.52±0.52[a]	17.06±0.83[a]
SAMNUT-23x1CGV-91324	6.29±0.13[a]	4.50±0.01[a]	3.14±0.06[a]	46.12±1.31[a]	21.59±0.07[a]	18.37±1.46[a]
SAMNUT-24xICGV-91328	6.52±0.02[a]	3.76±0.28[a]	2.91±0.07[a]	46.97±0.64[a]	22.85±0.07[a]	16.99±0.58[a]
Grand mean	6.62±0.23	3.84±0.36	3.05±0.07	46.70±0.29	22.32±0.38	17.47±0.45

Moisture: χ^2 (Variety Vs Moisture content) = 0.046, P=0.977 (P>0.05)
Ash: χ^2(Variety Vs Ash content) = 0.203, P=0.904 (P>0.05)
Fiber: χ^2 (Variety Vs Fiber content) = 0.01, P=0.995 (P>0.05)
Lipid: χ^2 (Variety Vs Lipid content) = 0.011, P=0.995 (P>0.05)
Protein: χ^2 (Variety Vs Protein content) = 0.038, P=0.981 (P>0.05)
Carbohydrate: χ^2 (Variety Vs Carbohydrate content) = 0.069, P=0.966 (P>0.05)
Carbohydrate: χ^2 (Variety Vs Carbohydrate content) = 0.069, P=0.966 (P>0.05)

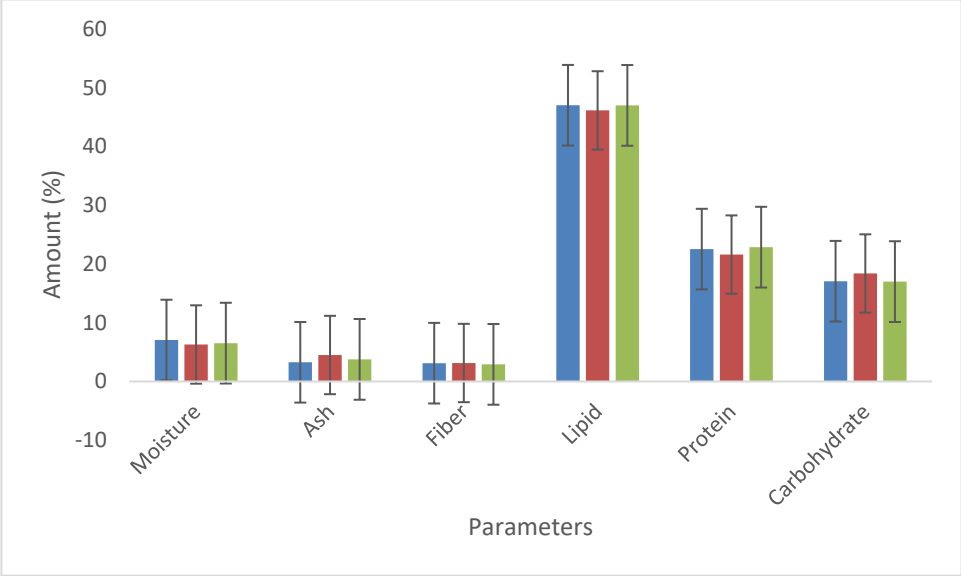

Figure 1. Grand mean of proximate composition in groundnut breeding lines

Anti-nutritional factors in three groundnut breeding lines

Table 2 with figures 2-4 presents the levels of three anti-nutritional factors—cyanide, oxalate, and phytic acid—in the groundnut breeding lines SAMNUT-26×ICGV-91328, SAMNUT-23×ICGV-91324, and SAMNUT-24×ICGV-91328. Figure 5 shows the Comparative analysis of anti-nutritional factors in the groundnut hybrid lines. Cyanide content was highest in SAMNUT-23×ICGV-91324 (5.10 ± 0.10 mg/100g), followed by SAMNUT-24×ICGV-91328 (4.63 ± 0.08 mg/100g), and lowest in SAMNUT-26×ICGV-91328 (2.56 ± 0.02 mg/100g). These differences were statistically significant ($F = 322.37$, $p < 0.05$).

Oxalate levels ranged from 0.51 ± 0.07 mg/100g in SAMNUT-24×ICGV-91328 to 1.32 ± 0.55 mg/100g in SAMNUT-23×ICGV-91324. However, the variation was not statistically significant ($F = 1.83$, $p > 0.05$). For phytic acid, SAMNUT-24×ICGV-91328 showed the highest concentration (85.10 ± 0.30 mg/100g), followed by SAMNUT-23×ICGV-91324 (74.66 ± 0.27 mg/100g) and SAMNUT-26×ICGV-91328 (71.98 ± 0.55 mg/100g), with statistically significant differences across the hybrid lines ($F = 313.85$, $p < 0.05$).

Table 2. Anti-nutritional factors in three groundnut varieties

Varieties	Cyanide (µg/100g d.w)	Oxalate (mg/100g)	Phytic acid (mg/100g)
SAMNUT-26x1CGV-91328	2.56±0.02^{c}	1.21±0.05^{a}	71.98±0.55^{c}
SAMNUT-23x1CGV-91324	5.10±0.10^{a}	1.32±0.55^{a}	74.66±0.27^{b}
SAMNUT-24xICGV-91328	4.63±0.08^{b}	0.51±0.07^{a}	85.10±0.30^{a}
FAO/WHO limit			

F (Cyanide content) = 322.37, P= 0.000 (P<0.05)

F (Oxalate content) = 1.83, P=0.24 (P>0.05)

F (Phytic acid content) = 313.85, P=0.000 (P<0.05)

Means that do not share a letter are significantly different.

F (Cyanide content) = 322.37, P= 0.000 (P<0.05)
F (Oxalate content) = 1.83, P=0.24 (P>0.05)
F (Phytic acid content) = 313.85, P=0.000 (P<0.05)

Means that do not share a letter are significantly different.

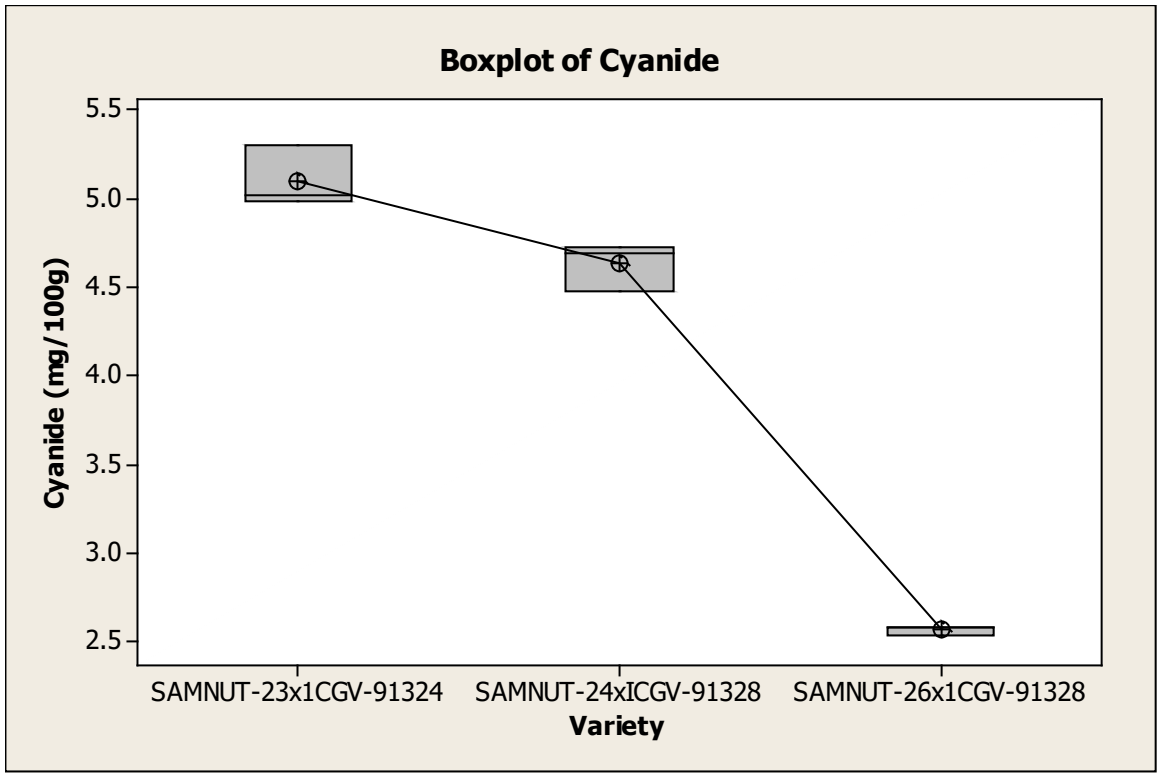

Figure 2. Boxplot of cyanide content in three groundnut breeding lines

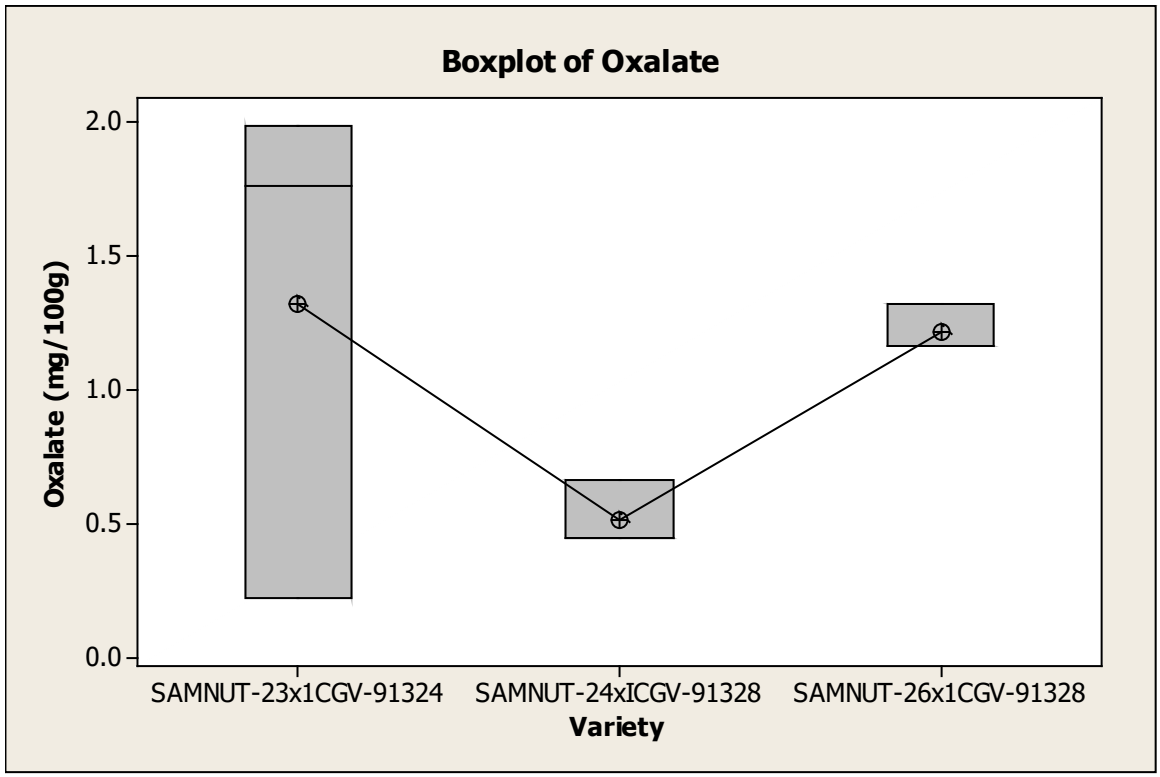

Figure 3. Boxplot of oxalate content in three groundnut breeding lines

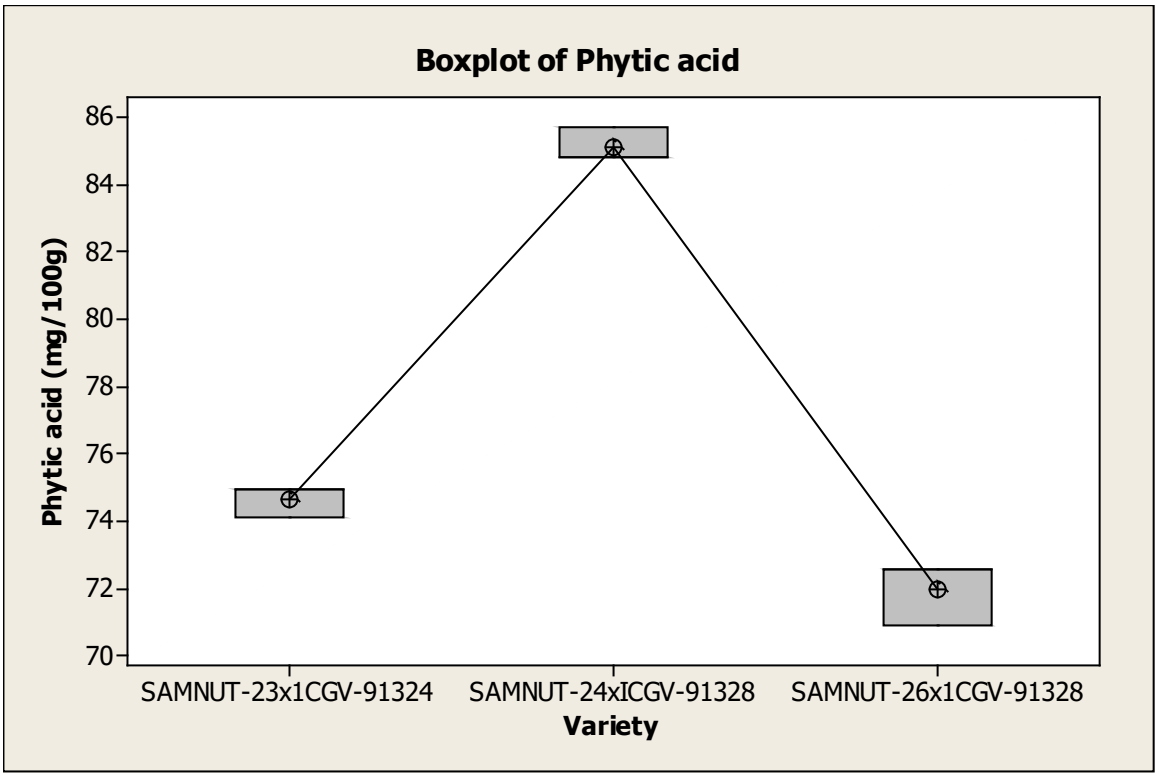

Figure 4. Boxplot of phytic acid content in three groundnut breeding lines

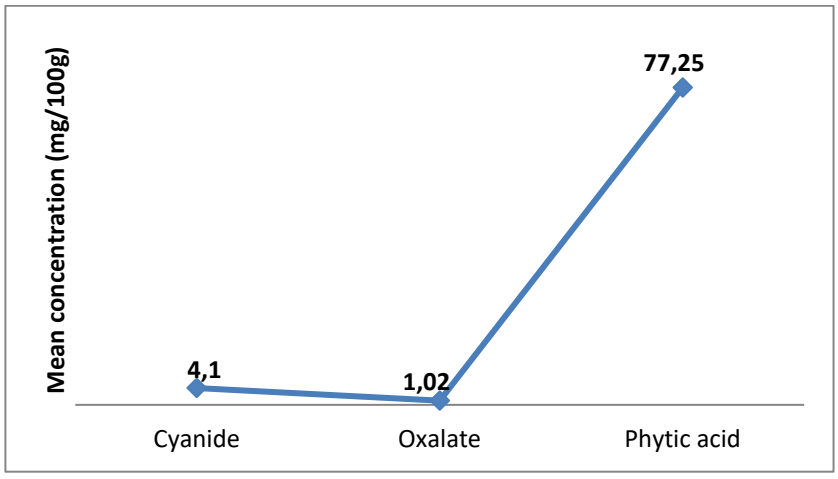

Figure 5. Comparative analysis of anti-nutritional factors in groundnut breeding lines

Correlation among Nutritional and Anti-Nutritional Components

Table 3 summarises the Pearson correlation coefficients among the nutritional and anti-nutritional parameters of the groundnut hybrid lines. Moisture content showed no significant correlation with any other component. Ash content was negatively correlated with moisture (r = -0.810) and moderately correlated with carbohydrate (r = 0.345), but showed weak or no meaningful relationships with other components.

Fiber content displayed weak, non-significant correlations across the board. Lipid content had a moderate negative correlation with ash (r = -0.345) and a weak positive correlation with fibre (r = 0.099). Protein content correlated moderately with lipid (r = 0.410) and negatively with ash (r = -0.583).

Carbohydrate content showed strong negative correlations with lipid (r = -0.944) and protein (r = -0.624), and a moderate positive correlation with ash (r = 0.345). Cyanide content was strongly negatively correlated with moisture (r = -0.938) and positively with ash (r = 0.797). Oxalate showed a weak positive correlation with fiber (r = 0.805) and a weak negative correlation with lipid (r = -0.567). Phytic acid was moderately positively correlated with protein (r = 0.472), and negatively correlated with moisture (r = -0.370), ash (r = -0.552), and carbohydrate (r = -0.191).

Table 3. Pearson's Correlation Matrix

	Moisture	**Ash**	**Fiber**	**Lipid**	**Protein**	**Carbohydrate**	**Cyanide**	**Oxalate**	**Phytic acid**
Moisture	1								
Ash	-0.810	1							
Fiber	0.083	0.073	1						
Lipid	0.330	-0.345	0.099	1					
Protein	0.480	-0.583	-0.462	0.410	1				
Carbohydrate	-0.408	0.345	-0.007	-0.944	-0.624	1			
Cyanide	-0.938	0.797	-0.237	-0.253	-0.334	0.287	1		
Oxalate	0.143	0.099	0.805	0.567	-0.298	-0.461	-0.192	1	
Phytic acid	-0.370	0.083	-0.720	0.118	0.472	-0.191	0.506	-0.552	1

Strength of correlation:
0.00-0.39 = Weak correlation
0.40-0.69 = moderate correlation
0.7-0.9 = High correlation
>0.90 = Very high correlation

Discussion

Proximate Composition in three Groundnut Breeding lines

Groundnut seeds are valued for their dense energy and nutrient content, but composition varies with cultivar, environment, and processing methods. In this study, the moisture content of 6.29%–7.05% aligns closely with ranges reported in Nigeria (6.0%–7.9%) and elsewhere (6.5%–8.6%) (Shokunbi et al. 2012). Recent analyses of improved SAMNUT lines—23, 24, 26—also showed moisture around 8.6%, supporting the consistency of these findings (Olasan et al. 2024).

The lipid content observed (46.1%–47.0%) is higher than the 40.9%–43.2% seen in Chinese hybrid lines (Huang et al. 2022) but closely matches the 44%–50% range reported in Nigerian hybrid lines, including SAMNUT-23 with about 49.7% oil (Huang et al. 2022). Similarly, protein levels (21.6%–22.8%) are comparable to the 20%–26% commonly observed in Nigerian groundnuts, though slightly lower than the 24%–28% range noted in other studies (Mshelmbula et al. 2017).

Fibre content (2.91%–3.14%) remains within expected norms (2.6%–3.8%), reflecting typical groundnut roughage levels (Pardeshi 2019). Ash content (3.26%–4.50%) also falls within standard soil mineral ranges (2.6%–5.0%) (Abdulbaki et al. 2022), indicating consistent mineral composition suited for both nutrition and soil quality implications.

Anti-nutritional factors in three groundnut breeding lines

Anti-nutritional factors (ANFs) are naturally occurring compounds that can impair nutrient absorption or exert toxic effects when consumed in excess (Gemede and Ratta 2014). In groundnuts, their presence and concentration are influenced by genetic variation, environmental factors, and processing techniques (Okereke et al. 2025). This study assessed the levels of three common ANFs—cyanide, oxalate, and phytic acid—in three improved groundnut breeding

lines: SAMNUT-26×ICGV-91328, SAMNUT-23×ICGV-91324, and SAMNUT-24×ICGV-91328.

Cyanide, a known metabolic toxin, interferes with cellular respiration and can pose serious health risks when ingested at high concentrations (Park et al. 2024). Among the hybrid lines studied, cyanide levels ranged from 2.56 mg/100 g in SAMNUT-26×ICGV-91328 to 5.10 mg/100 g in SAMNUT-23×ICGV-91324—the highest among the three. These values are comparable to those previously reported in groundnut and similar legume studies (Adejoh et al. 2020). While the observed levels are not immediately alarming, monitoring and minimising cyanide content in breeding selections is important for food safety.

Oxalate levels also varied across the genotypes, ranging from 0.51 mg/100 g to 1.32 mg/100 g, with the highest again recorded in SAMNUT-23×ICGV-91324. Oxalates reduce calcium bioavailability by forming insoluble calcium oxalate complexes, which may contribute to kidney stone formation in sensitive individuals (Mitchell et al. 2019). These results align with previous findings in groundnut hybrid lines (Dele et al. 2020), suggesting that while levels are within expected ranges, reducing oxalate content through breeding or processing could improve nutritional safety.

Phytic acid, another prominent ANF in legumes, was detected in amounts ranging from 71.98 mg/100 g to 85.10 mg/100 g, with SAMNUT-24×ICGV-91328 exhibiting the highest level. Phytates are known to chelate essential minerals such as iron, zinc, and calcium, limiting their intestinal absorption. Although phytic acid can also exert antioxidant and anticancer properties at moderate levels, its high concentration in certain hybrid lines may warrant nutritional balancing in breeding targets (Pires et al. 2023). These findings are in line with those from earlier studies on Nigerian groundnut lines (Solanki et al. 2021).

The variation in ANF content across the three groundnut breeding lines suggests opportunities for genetic selection. SAMNUT-23×ICGV-91324 showed the highest levels of cyanide and oxalate, while SAMNUT-24×ICGV-91328 contained the most phytic acid. These results can inform breeding strategies aimed at reducing ANF concentrations while maintaining or enhancing nutritional quality. Furthermore, applying traditional processing methods—such as soaking, roasting, and boiling—has been shown to significantly reduce ANF levels and improve mineral bioavailability in groundnut products (Yahaya et al. 2022b).

Pearson's correlation matrix

The Pearson correlation matrix presented in Table 3 offers insights into how various nutritional and anti-nutritional components in the groundnut samples relate to one another. Understanding these relationships is important for improving both the nutritional quality of groundnut and its suitability for food processing and breeding programs.

Moisture content did not show any significant relationship with other components, except for a perfect correlation with itself. However, a strong negative correlation was observed between ash and moisture contents. This suggests that as moisture decreases, the concentration of minerals (represented by ash) increases—likely due to the concentration effect during drying. Ash also showed a weak positive relationship with fibre, indicating that samples with slightly higher fibre content may also carry more mineral matter.

Fibre content, on the other hand, showed no notable correlation with most other nutrients, implying it remains relatively unaffected by the changes in other components. A weak positive relationship between fibre and lipid content was noted, suggesting a slight tendency for higher-fat samples to also contain more fibre. Lipid content also had a moderate negative correlation with ash, possibly due to the inverse relationship between dense mineral matter and the lighter lipid fraction during sample processing.

Protein content was moderately correlated with lipid levels, indicating that hybrid lines richer in fat might also be higher in protein. However, protein showed a strong negative relationship

with ash, implying that as mineral content increases, protein levels tend to decrease. This might reflect the lower inorganic content of protein-rich tissues.
Carbohydrates showed a strong inverse relationship with lipids, and a moderate negative correlation with protein. These patterns may reflect a natural nutrient trade-off in seed composition—where higher fat and protein content come at the expense of carbohydrate reserves. A weak positive correlation between carbohydrates and ash was also observed.
Among the anti-nutritional factors, cyanide content had a strong negative correlation with moisture, suggesting that drier samples tend to concentrate more cyanide. It also had a weak positive correlation with ash, possibly reflecting a co-accumulation of certain compounds in mineral-rich tissues.
Oxalate levels were weakly correlated with fiber (positive) and lipids (negative), indicating that oxalate presence may be slightly higher in fibrous samples but lower in those with high fat content. Phytic acid levels showed several associations: a weak negative correlation with moisture, a moderate positive correlation with protein, and negative correlations with ash and carbohydrate content. This suggests that phytate may accumulate more in protein-rich samples but decrease in carbohydrate- or mineral-rich ones.
Together, these correlations reveal how nutrient and anti-nutrient profiles in groundnut interact. This information is valuable for selecting hybrid lines with balanced compositions and for optimizing processing techniques to enhance nutritional value while minimising potential health risks. It also provides a useful foundation for breeding programs focused on developing groundnut lines with improved health benefits and functionality in food products.

Conclusions

This study has demonstrated that the nutritional and anti-nutritional profiles of the three evaluated groundnut breeding lines—SAMNUT-26×ICGV-91328, SAMNUT-23×ICGV-91324, and SAMNUT-24×ICGV-91328—vary notably across key components. Among the hybrid lines, SAMNUT-24×ICGV-91328 stood out with the most favourable nutritional composition, including higher levels of protein and lipid. In contrast, SAMNUT-23×ICGV-91324 recorded the highest levels of cyanide and oxalate, while SAMNUT-24×ICGV-91328 also showed the highest phytic acid content.
These findings highlight the importance of selecting groundnut hybrid lines not only for their nutrient density but also for their lower levels of anti-nutritional factors. The results can guide breeders, nutritionists, and food processors in identifying and developing hybrid lines that offer improved health benefits and food safety. Further research on processing methods to reduce anti-nutrients in high-performing hybrid lines like SAMNUT-24×ICGV-91328 is recommended to fully harness their nutritional potential.

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