RESEARCH ARTICLE
TRENDS IN BIOTECHNOLOGY AND PLANT SCIENCE
ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
Open Access Journal

BIOAUGMENTATION OF GROUND MAIZE THROUGH MULTI-LEVEL EFFECTIVE MICROORGANISM APPLICATION: ENHANCING CRUDE PROTEIN AND REDUCING FIBER FRACTIONS VIA SOLID-STATE FERMENTATION

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ABSTRACT

Solid-state fermentation represents a strategic biotechnological approach to enhance the nutritional profile of cereal grains for animal feed applications. This investigation systematically evaluated the effects of varying effective microorganism (EM-4) inoculation levels, with and without urea supplementation, on crude protein and crude fiber content in ground yellow maize. A completely randomized design compared five treatments: control (T0), EM-4 alone (T1), and three urea-supplemented EM-4 levels (T2: 25 mL, T3: 35 mL, T4: 45 mL). Fermentation proceeded anaerobically for 72 hours at ambient temperature. Analysis of variance revealed highly significant treatment effects on both nutritional parameters ($P < 0.01$). Treatment T3 achieved optimal results with 10.51% crude protein (48.0% increase over control) and 2.47% crude fiber (8.9% reduction). Urea supplementation synergistically enhanced protein content through microbial single-cell protein accumulation and non-protein nitrogen conversion, while higher EM-4 levels promoted fiber degradation via cellulolytic enzyme activity. Conversely, lower EM-4 treatments showed preferential utilization of soluble carbohydrates, resulting in apparent fiber elevation. These findings establish intermediate EM-4 dosing with urea as an effective protocol for upgrading maize nutritional quality, offering practical implications for sustainable feed production systems.

***Keywords*:** Single-cell protein, Solid-state fermentation, Lignocellulose degradation, Non-protein nitrogen, Feed nutritional enhancement, Microbial consortia.

Article History (2026-027) || Received: 18 Jan 2026 || Revised: 10 Mar 2026 || Accepted: 12 Mar 2026 || Published Online: 2026

1. INTRODUCTION

Global livestock production faces unprecedented economic pressure from escalating feed costs, which consistently account for 60-80% of total production expenses in intensive farming systems (Alltech, 2025; AHDB, 2025). The competition between human food security demands and animal nutrition requirements has intensified raw material prices, creating urgent need for innovative feed enhancement strategies (Towards Food and Nutrition Business, 2025; Rhamya et al., 2025). Maize (*Zea mays* L.) remains a cornerstone energy source in animal nutrition worldwide, yet its utilization is constrained by suboptimal protein content and cost volatility (Singh et al., 2025). Contemporary research priorities emphasize sustainable approaches that elevate nutritional density while maintaining economic feasibility.

Biological processing technologies, particularly solid-state fermentation using beneficial microbial consortia, have emerged as promising interventions for improving feed quality characteristics (Bao et al., 2025). The application of effective microorganisms represents a strategic approach to restructure grain composition through enzymatic modification of structural carbohydrates and enhancement of nitrogenous compounds (Singh et al., 2025; Cangioli et al., 2024). These microbial preparations typically comprise synergistic combinations of lactic acid bacteria, photosynthetic bacteria, yeasts, and other beneficial taxa that collectively produce bioactive metabolites and degradative enzymes (Cangioli et al., 2024; Demir et al., 2024).

Recent advances in cereal grain fermentation have demonstrated substantial improvements in protein content through microbial biomass accumulation and metabolic nitrogen transformation (Wang et al., 2021; Zamudio-Sosa et al., 2026). The proliferation of microbial cells during fermentation contributes single-cell protein, while simultaneous production of proteolytic and cellulolytic enzymes facilitates matrix degradation and nutrient liberation (Sartori et al., 2015; Umokaso et al., 2022). Studies employing fungal and bacterial fermentation systems have reported crude protein increases ranging from 15-45% depending on substrate composition and fermentation parameters (Zhou et al., 2025).

Citation: Susanto JR, Hakim HR, Wardani S, Purnomo AW and Kurniawan HA, 2025. Bioaugmentation of ground maize through Multi-level effective microorganism application: enhancing crude protein and reducing fiber fractions via solid-state fermentation. Trends in Biotechnology and Plant Science, 4(1): 47-54. https://doi.org/10.62460/TBPS/2026.005

ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
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The integration of non-protein nitrogen sources, particularly urea, with fermentation processes presents synergistic potential for protein enhancement (Zamudio-Sosa et al., 2026). Urea serves as an economical nitrogen substrate that microorganisms convert into ammonia, subsequently assimilated into microbial amino acids and proteins (Zurak et al., 2023). Meta-analytical evidence indicates that controlled urea supplementation significantly elevates crude protein digestibility and nitrogen retention in fermented substrates, though optimal dosing requires careful calibration to prevent ammonia toxicity (Wang et al., 2021).

Fiber fraction modification constitutes another critical dimension of fermentation-mediated feed improvement (Wang et al., 2021). Lignocellulosic components—including cellulose, hemicellulose, and lignin—present digestive impediments that limit nutrient accessibility and metabolizable energy extraction (Østby et al., 2023). Microbial secretion of carbohydrate-active enzymes, notably cellulases, hemicellulases, xylanases, and ligninases, catalyzes depolymerization of these recalcitrant structures into simpler, more digestible forms (Østby et al., 2023; Sartori et al., 2015).

Contemporary investigations have explored dose-response relationships between microbial inoculant concentrations and nutritional outcomes (Terefe et al., 2021). Multi-strain consortia often exhibit superior performance compared to monocultures due to complementary enzymatic activities and metabolic cross-feeding (Cangioli et al., 2024; Singh et al., 2025). However, optimization remains substrate-specific, necessitating empirical determination of ideal inoculation rates, fermentation durations, moisture levels, and environmental conditions (Terefe et al., 2021).

The role of lactic acid bacteria in grain fermentation extends beyond protein synthesis to include organic acid production that reduces pH, inhibits spoilage organisms, and potentially enhances mineral bioavailability through phytate degradation (Terefe et al., 2021). Co-fermentation systems incorporating both lactic acid bacteria and yeasts have demonstrated enhanced volatile fatty acid profiles and improved palatability characteristics (Hu et al., 2025; Liszkowska et al., 2025). Recent work on high-moisture grain confirmed that specific bacterial strains significantly improved nutritional composition and aerobic stability (Terefe et al., 2021; Bao et al., 2025).

Despite growing recognition of fermentation biotechnology in feed processing, knowledge gaps persist regarding optimal microbial dosing strategies for ground cereal grains, particularly when combined with nitrogen supplementation (Umokaso et al., 2022). This investigation addresses these deficiencies by systematically evaluating multiple inoculation levels of effective microorganism preparations on fermented ground maize, with and without urea fortification. The primary objectives were to quantify changes in crude protein and crude fiber content across treatment regimens, thereby establishing evidence-based protocols for biological feed enhancement applicable to contemporary livestock production systems.

2. MATERIALS AND METHODS

Experimental Design and Treatments

This study utilized a completely randomized design (CRD) consisting of five treatment groups with four replications each, totaling 20 experimental units. The treatment configurations were as follows:

- T0 (Control): 2 kg ground yellow maize
- T1: 2 kg ground yellow maize + 25 mL EM-4 + 500 mL water
- T2: 2 kg ground yellow maize + 1% urea + 25 mL EM-4 + 500 mL water
- T3: 2 kg ground yellow maize + 1% urea + 35 mL EM-4 + 500 mL water
- T4: 2 kg ground yellow maize + 1% urea + 45 mL EM-4 + 500 mL water

Materials

The materials used in this experiment included ground yellow maize, effective microorganism preparation (EM-4), pharmaceutical-grade urea, molasses and distilled water. Equipment consisted of analytical balance, plastic buckets, adhesive tape, polyethylene bags, Kjeldahl apparatus, muffle furnace and standard laboratory glassware.

Fermentation Procedure

Prior to application, the EM-4 inoculant was activated by combining 10 mL concentrated EM-4 with 10 mL molasses and 1000 mL distilled water in a sterile container. This activation mixture was allowed to stand at ambient temperature for 24 hours to stimulate microbial activity.

Ground yellow maize (2 kg per experimental unit) was accurately weighed and transferred to sterile plastic fermentation containers. For treatments T2, T3, and T4, urea was first dissolved in the designated water volume to ensure uniform distribution. The activated EM-4 solution was then added according to treatment specifications, followed by the addition of water to achieve the required moisture level. Each container was manually mixed thoroughly for approximately 5 minutes to ensure homogeneous distribution of all components.

Following preparation, fermentation containers were sealed with perforated plastic covers secured with

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adhesive tape to maintain anaerobic conditions while allowing gas exchange. All containers were incubated at ambient temperature (27-30°C) for 72 hours (3 days). On day 4 post-inoculation, representative samples were collected from each experimental unit for proximate analysis.

Proximate Analysis

Crude Protein Determination

Crude protein content was determined using the Kjeldahl method. Sample material (1.0 g) was accurately weighed and transferred to a 250 mL Kjeldahl digestion flask. To each flask was added 10 g potassium sulfate, 0.7 g mercuric oxide, and 20 mL concentrated sulfuric acid. The flasks were placed at 45° angles in a digestion block and heated gradually to boiling until the solution became clear, indicating complete organic matter oxidation. Heating continued for an additional 30 minutes after clarification.

After cooling, the digest was quantitatively transferred to a distillation apparatus with 100 mL distilled water. Sodium hydroxide solution (50 mL, 40% w/v) was added to liberate ammonia, which was then steam-distilled into 50 mL boric acid solution (4% w/v) containing mixed indicator. The distillate was collected until approximately 150 mL volume was obtained, then titrated with standardized 0.1 N hydrochloric acid. Crude protein percentage was calculated by multiplying total nitrogen by the conversion factor 6.25.

Crude Fiber Determination

Crude fiber content was determined using the sequential acid-alkaline digestion method. Sample material (2.0 g) was first defatted by Soxhlet extraction with petroleum ether for 6 hours. The defatted residue was transferred to a 600 mL beaker containing 200 mL boiling 1.25% sulfuric acid solution and refluxed for 30 minutes with continuous stirring. The mixture was filtered hot through pre-weighed sintered glass crucibles and washed with hot distilled water until neutral.

The residue was then returned to a beaker with 200 mL boiling 1.25% sodium hydroxide solution and refluxed for 30 minutes. After hot filtration and washing with distilled water, the residue was rinsed with 25 mL acetone and transferred to pre-weighed crucibles. The crucibles were dried at 105°C for 3 hours, cooled in a desiccator, and weighed. Subsequently, the crucibles were incinerated in a muffle furnace at 550°C for 4 hours to obtain ash weight. Crude fiber percentage was calculated as the difference between oven-dried weight and ash weight, expressed on a dry matter basis.

Statistical Analysis

All analytical measurements were performed in duplicate. Data obtained were subjected to analysis of variance (ANOVA) using SPSS software version 26.0. Treatment effects were considered statistically significant at $P < 0.01$. When significant differences were detected, Duncan's Multiple Range Test was employed for pairwise comparisons at the 0.05 significance level to determine homogeneous subsets among treatment means.

3. RESULTS

Crude Protein Content

The crude protein content of ground yellow maize subjected to fermentation with EM-4 at varying inoculation levels is presented in Fig. 1. Analysis of variance revealed highly significant treatment effects on crude protein concentration ($P < 0.01$), indicating that fermentation parameters substantially influenced protein accumulation.

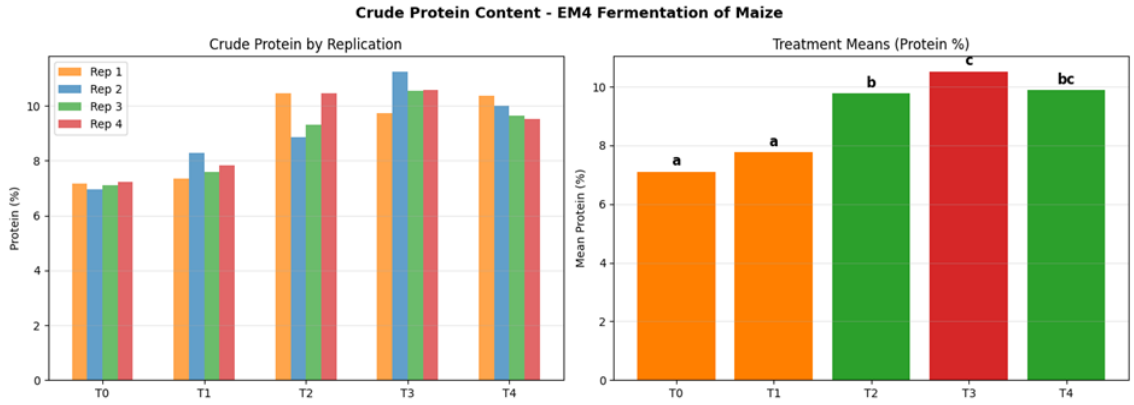

Fig. 1: Crude Protein Content (%) of Ground Yellow Maize Fermented with EM-4 at Different Levels.

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Duncan's Multiple Range Test demonstrated that T0 differed significantly from T2, T3, and T4 ($P < 0.01$) but showed no significant difference compared to T1. Treatment T1 exhibited highly significant differences relative to T2, T3, and T4 ($P < 0.01$). Treatment T2 demonstrated significant divergence from T3 ($P < 0.05$) but maintained statistical similarity with T4. No significant difference was observed between T3 and T4.

The control treatment (T0) yielded the lowest crude protein content at 7.10%, while T3 achieved the highest concentration at 10.51%, representing a 48.0% relative increase. Treatment T1, containing EM-4 without urea supplementation, produced 7.76% crude protein, demonstrating marginal elevation above the control. The incorporation of 1% urea in T2, T3, and T4 consistently enhanced protein content, with values of 9.76%, 10.51%, and 9.88%, respectively. These findings indicate that urea supplementation synergistically amplifies the protein-enhancing effects of microbial fermentation.

Crude Fiber Content

Crude fiber concentrations following fermentation treatments are summarized in Fig. 2. Statistical analysis confirmed highly significant treatment effects on crude fiber levels ($P < 0.01$), demonstrating that microbial inoculation intensity and urea supplementation substantially modified fiber fractions.

Duncan's post-hoc analysis revealed that T0 did not significantly differ from T1, T2, T3, or T4 ($P > 0.05$). Treatment T1 showed no significant difference relative to T2 ($P > 0.05$) but differed highly significantly from T3 and T4 ($P < 0.01$). Similarly, T2 demonstrated highly significant differences compared to T3 and T4 ($P < 0.01$). No significant difference was detected between T3 and T4 ($P > 0.05$).

Treatments T1 and T2 exhibited elevated crude fiber content at 3.93% and 3.47%, respectively, exceeding the control value of 2.71%. Conversely, T3 and T4 demonstrated substantial fiber reduction, with values of 2.47% and 2.59%, respectively. Treatment T3 achieved the lowest crude fiber concentration, representing an 8.9% reduction relative to the control and a 37.2% decrease compared to T1. These contrasting patterns suggest differential microbial enzymatic activities depending on inoculation level and substrate nitrogen availability.

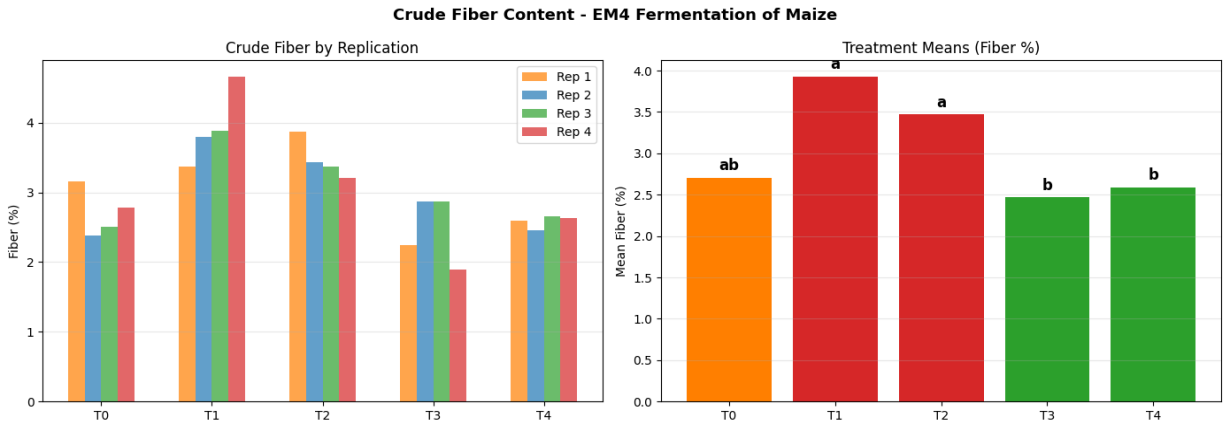

Fig. 2: Crude Fiber Content (%) of Ground Yellow Maize Fermented with EM-4 at Different Levels.

4. DISCUSSION

Crude Protein Enhancement through Fermentation

The substantial elevation in crude protein content observed across fermented treatments demonstrates the efficacy of microbial bioprocessing for nutritional enhancement of cereal grains. Treatment T3 achieved a 48.0% protein increase relative to the control, a finding consistent with contemporary research documenting significant crude protein improvements following solid-state fermentation of agricultural substrates (Terefe et al., 2021). This protein augmentation derives primarily from microbial single-cell protein accumulation during fermentation, wherein rapidly proliferating microorganisms contribute proteinaceous biomass to the substrate matrix (Bao et al., 2025; Zhuang et al., 2024).

Single-cell protein production represents a sustainable biotechnological approach to address global protein deficiencies, with microbial biomass offering balanced amino acid profiles comparable to conventional protein sources (Chamodi et al., 2025; Ayele et al., 2025). The effective microorganism consortium employed in this investigation contains diverse microbial taxa including lactic acid bacteria, yeasts, and photosynthetic bacteria that collectively synthesize substantial protein during logarithmic growth phases (Guo et al., 2025). Research on grass-based substrates reported achieving 16.62 g/L biomass yield with Kluyveromyces marxianus, demonstrating the

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substantial protein production capacity of microbial fermentation systems (Guo et al., 2025). Studies utilizing agricultural waste for single-cell protein production documented protein yields ranging from 20-50 g/L depending on substrate composition and fermentation optimization (Irfan et al., 2025; Abedfar et al., 2025).

The conversion of non-protein nitrogen from urea into microbial protein represents a critical mechanism underlying the enhanced crude protein observed in T2, T3, and T4 treatments. Microorganisms hydrolyze urea into ammonia through urease enzyme activity, subsequently assimilating this nitrogen into amino acids and cellular proteins (Bao et al., 2025; Wang et al., 2021). Research on hydrogen-oxidizing bacteria demonstrated that nitrogen-fixing microbial systems achieve protein contents exceeding 62%, highlighting the substantial protein production potential of diazotrophic microorganisms (Hu et al., 2020; Jinpeng et al., 2025).

Role of Urea Supplementation and Microbial Synergism

The pronounced difference between T1 (7.76% protein) and urea-supplemented treatments T2, T3, T4 (9.76-10.51% protein) underscores the synergistic relationship between non-protein nitrogen availability and microbial protein synthesis. Urea supplementation provides readily accessible nitrogen substrate that accelerates microbial proliferation and enhances protein biosynthesis efficiency (Bao et al., 2025; Hu et al., 2025). Contemporary investigations on silage fermentation confirmed that urea addition significantly elevates crude protein content while modifying microbial community composition toward protein-producing taxa (Bao et al., 2025).

The optimal performance of T3 (35 mL EM-4 + 1% urea) compared to higher inoculation T4 (45 mL EM-4 + 1% urea) suggests substrate limitation or potential competitive inhibition at elevated microbial densities. Excessive microbial populations may deplete available carbon sources prematurely, leading to stationary phase entry before maximum protein accumulation occurs (Zhuang et al., 2024; Mattedi et al., 2023). Additionally, ammonia toxicity from excessive urea hydrolysis at higher microbial densities could inhibit protein synthesis and cellular metabolism (Bao et al., 2025). Research on fermentation optimization emphasized the critical importance of balancing inoculum concentration, substrate availability, and nitrogen supplementation for maximizing single-cell protein yield (Irfan et al., 2025).

The nitrogen originally present in urea supplements undergoes transformation into multiple nitrogenous fractions including microbial protein, free amino acids, ammonia, and other non-protein nitrogen compounds (Wang et al., 2021; Zurak et al., 2023). This complexity explains why crude protein measurements in fermented materials reflect both authentic protein and residual non-protein nitrogen, potentially overestimating true protein content (Abedfar et al., 2025). Despite this analytical limitation, fermentation-enhanced materials demonstrate improved digestibility and amino acid availability compared to unfermented substrates (Jatkauskas et al., 2024).

Enzymatic Degradation of Fiber Fractions

The contrasting crude fiber responses among treatments illuminate differential microbial enzymatic activities and substrate preferences. Treatments T3 and T4 achieved substantial fiber reduction (2.47% and 2.59%, respectively), while T1 and T2 exhibited elevated fiber content (3.93% and 3.47%, respectively). These divergent patterns reflect complex interactions between microbial metabolism, enzyme production, and available nutrient pools.

The fiber reduction in T3 and T4 indicates robust production of cellulolytic and hemicellulolytic enzymes by the effective microorganism consortium at higher inoculation rates combined with adequate nitrogen availability. Cellulase enzymes hydrolyze β-1,4-glycosidic bonds in cellulose polymers, releasing glucose monomers that microorganisms subsequently metabolize for energy and growth (Perim et al., 2024; Østby et al., 2023). Hemicellulases including xylanases and mannanases degrade hemicellulose components, further liberating fermentable sugars and reducing structural fiber content (Østby et al., 2023; Sartori et al., 2015). Research on Trichoderma reesei cellulase supplementation in poultry diets demonstrated significant improvements in nutrient digestibility through cellulose bond cleavage (Perim et al., 2024; Yu et al., 2025).

Conversely, the elevated fiber in T1 and T2 treatments likely results from preferential microbial utilization of nitrogen-free extract components including simple sugars and starch, while leaving recalcitrant fiber fractions relatively intact. Microorganisms generally metabolize readily available carbohydrates before investing metabolic resources into producing extracellular lignocellulolytic enzymes (Zhuang et al., 2024; Mattedi et al., 2023). The nitrogen-free extract fraction, comprising soluble carbohydrates, provides immediate energy for microbial growth and maintenance, whereas cellulose and hemicellulose require enzymatic pre-digestion before assimilation (Wang et al., 2021; Terefe et al., 2021).

As microorganisms consume nitrogen-free extract during fermentation, the relative proportion of fiber in the dry matter increases even if absolute fiber mass remains constant, explaining apparent fiber elevation in some treatments (Terefe et al., 2021). This proportional shift phenomenon has been documented in multiple fermentation studies where extensive degradation of soluble carbohydrates results in compensatory increases in fiber percentage despite improved overall digestibility (Zhuang et al., 2024; Mattedi et al., 2023).

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Optimization and Practical Implications

Treatment T3 emerges as the optimal fermentation protocol, balancing maximal protein enhancement (10.51%) with substantial fiber reduction (2.47%). This combination represents ideal nutritional modification for livestock feed applications, increasing protein density while reducing anti-nutritive fiber components. The superior performance of T3 compared to higher-dose T4 emphasizes the importance of optimizing microbial inoculation rates rather than assuming proportional dose-response relationships (Zhuang et al., 2024; Hou et al., 2025).

The findings validate solid-state fermentation with effective microorganisms as a viable strategy for upgrading low-protein cereal grains into enhanced feed ingredients. This biotechnological approach offers economic and environmental advantages over chemical processing methods, utilizing renewable microbial catalysts and generating minimal waste streams (Bao et al., 2025; Ayele et al., 2025). Implementation at industrial scale could reduce feed costs while maintaining livestock performance, contributing to sustainable intensification of animal production systems (Alltech, 2025; AHDB, 2025).

5. CONCLUSION

This investigation establishes that controlled solid-state fermentation of ground yellow maize using intermediate effective microorganism dosing (35 mL EM-4) combined with 1% urea supplementation optimally enhances nutritional quality for livestock feed applications. The T3 treatment achieved 10.51% crude protein—a 48.0% improvement over unfermented maize—while simultaneously reducing crude fiber to 2.47%, representing substantial gains in both protein density and carbohydrate accessibility. These dual nutritional improvements address critical limitations of maize as a standalone energy source, offering a practical biotechnological solution for feed formulation challenges. The synergistic interaction between microbial consortia and non-protein nitrogen sources demonstrates predictable dose-response relationships that can be scaled for commercial implementation. Implementation of this fermentation protocol enables cost-effective upgrading of conventional grains without reliance on expensive protein supplements, while maintaining environmental sustainability through biological processing. The established optimal parameters provide a robust foundation for industrial adoption, potentially reducing feed costs across intensive livestock production systems while improving dietary protein efficiency and overall animal performance.

Declarations

Funding: This research article did not receive any financial support from any agency/institution.

Acknowledgement: NA

Conflicts of Interest: All authors declare that they have no conflicts of interest.

Data Availability: The data collected for this article are included in the article.

Ethics Statement: No prior study was conducted on live animals/humans; thus, it did not require any ethical approval.

Author's Contributions: JRS, HRK and HAK designed and conducted the experiment jointly. SW and AWP collected and analysed the data. HAK wrote the initial draft. Review and editing were performed by JRS. All authors approved the final version of the manuscript.

Generative AI Statements: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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Citation: Susanto JR, Hakim HR, Wardani S, Purnomo AW and Kurniawan HA, 2025. Bioaugmentation of ground maize through Multi-level effective microorganism application: enhancing crude protein and reducing fiber fractions via solid-state fermentation. Trends in Biotechnology and Plant Science, 4(1): 47-54. https://doi.org/10.62460/TBPS/2026.005