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Soluble Protein Concentrations during *Bacillus subtilis* Fermentation with *Moringa oleifera* Pod Powder

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ABSTRACT. The utilisation of agricultural residues as fermentation substrates may support microbial protein production while adding value to agricultural waste. This study evaluated soluble protein concentrations during submerged fermentation of *Moringa oleifera* pod powder with *Bacillus subtilis*, without intracellular protein extraction. Pod powder was added to the growth medium at concentrations of 0%, 1%, 2%, and 5% (w/v). Fermentation was conducted at 37 °C and 150 rpm for 48 h, with an initial pH of 7.00–7.20. The highest mean soluble protein concentration was 0.31 g/L at 5% supplementation, compared with 0.01 g/L in the control without pod powder. The 5% treatment had a significantly higher concentration than the control ($p < 0.05$), whereas differences among the 1%, 2%, and 5% treatments were not statistically significant. The pH decreased in all treatments, and fibrous material remained after fermentation, suggesting incomplete substrate conversion. These findings demonstrate an association between pod powder supplementation and soluble protein concentration under the conditions tested. However, the absence of matched uninoculated controls prevented differentiation between bacterial proteins and proteins originating from the pods or growth medium. Further controlled studies are needed to establish microbial protein production and assess nutritional composition, microbiological safety, bioavailability, and suitability for food or feed applications.

Keywords: soluble SCP, *Bacillus subtilis*, microbial protein, *Moringa oleifera*

1. INTRODUCTION

The increasing global demand for sustainable protein sources has intensified interest in microbial protein as an alternative to conventional livestock-derived proteins. The provision of adequate, safe, and nutritionally balanced food for all people on the planet is one of the major challenges in the extremely complex issue of food security, which is linked to global food production and supply systems (Hussain et al., 2025). Population growth, environmental degradation, climate change, and the inefficient utilisation of agricultural resources continue to challenge global food security. Conventional animal protein production requires extensive land, water, and feed resources while contributing substantially to greenhouse gas emissions. However, there is growing interest in developing environmentally sustainable protein production systems that utilise renewable biological resources and agricultural residues, therefore supporting the Sustainable Development Goals (SDGs), particularly SDG 2 (Zero Hunger), which emphasises the need for sustainable food production systems.

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Microbial proteins have emerged as one of the most promising alternatives to conventional protein sources. They are produced from microorganisms including bacteria, yeasts, fungi, and microalgae (Bajić et al., 2022), which rapidly convert inexpensive substrates into protein-rich biomass. Compared with conventional livestock production, microbial fermentation offers several advantages, including shorter production cycles, reduced land and water requirements, and independence from seasonal agricultural conditions. Furthermore, agricultural by-products can serve as inexpensive carbon sources, reducing production costs while contributing to circular bioeconomy initiatives through waste valorisation.

This study aimed to determine soluble protein concentrations, as few studies have investigated soluble microbial protein fractions. The downstream processing did not include a cell lysis step to extract intracellular microbial protein before determining the protein content, making the overall process more cost-effective. In addition, microbial cells, including Gram-positive bacterial cells, naturally contain relatively high levels of nucleic acids, which may increase the risk of uric acid accumulation when consumed by humans. Therefore, omitting the cell lysis step is expected to limit the release of intracellular nucleic acids into the soluble protein fraction obtained following fermentation.

Among bacterial species used for microbial protein production, *Bacillus subtilis* is considered particularly attractive because of its rapid growth, efficient protein secretion system, and Generally Recognised as Safe (GRAS) status. The pathogenicity of some Gram-negative bacterial strains, which possess lipopolysaccharides (LPS) and endotoxins in their outer membranes, is another important safety consideration (Alves et al., 2025). Unlike Gram-negative bacteria, *B. subtilis* does not produce lipopolysaccharide endotoxins, making it more suitable for food and feed applications. Previous studies have demonstrated the ability of *B. subtilis* to utilise various agricultural residues for microbial biomass production while simultaneously producing soluble proteins and industrially valuable enzymes.

Moringa oleifera is widely cultivated throughout tropical regions for its nutritional and medicinal properties. *M. oleifera* possesses several pharmacological properties, including antibacterial, anti-oxidant, anti-inflammatory, anti-diabetic, lipid-lowering, and anti-cancer activities (Cao et al., 2023). Although its leaves and seeds have been extensively investigated, mature pods are frequently discarded after seed removal despite containing fermentable carbohydrates, dietary fibre, and residual protein. These characteristics suggest that *M. oleifera* pod waste may represent an economical lignocellulosic substrate capable of supporting microbial growth during submerged fermentation while simultaneously reducing agricultural waste generation.

Although several agricultural residues have previously been evaluated for microbial protein production, little information is available regarding the soluble protein recovery during fermentation of *M. oleifera* pod waste with *B. subtilis*. Therefore, the present study investigated the suitability of *M. oleifera* pod powder as a fermentation substrate by evaluating its crude protein content, determining the influence of different substrate concentrations on

soluble protein production, and assessing its potential as a sustainable agricultural resource for microbial protein production under submerged fermentation conditions.

2. METHODOLOGY

2.1. Isolation of *Bacillus subtilis* and inoculum preparation

Bacillus subtilis was obtained from the Microbiology Laboratory culture collection, Faculty of Applied Sciences, Universiti Teknologi MARA (UiTM), Kuala Pilah, Malaysia. Single colonies of *B. subtilis* were selected from tryptic soy agar (TSA) plates and suspended in sterile saline solution to obtain a homogeneous bacterial suspension. The inoculum was prepared from a 24 h culture grown in nutrient broth and incubated under aerobic conditions, before being used for submerged fermentation ($OD_{600} = 0.53$).

2.2. Preparation of *Moringa oleifera* pod powder as fermentation substrate

Fresh *Moringa oleifera* pods were obtained from a local plantation in Gombak, Selangor, Malaysia. The pods had reached horticultural maturity, as indicated by their firm texture and light green flesh, consistent with the description provided by Goordeen & Mohammed (2021). Goordeen and Mohammed (2021) reported that pods reached horticultural maturity approximately 32 days after anthesis, with maturity indicated by firm, light greenish-cream flesh and well-formed seeds. The pods were washed thoroughly under running water to remove adhering impurities and cut into approximately 5 cm lengths. Blanching was performed in water maintained at 96 °C for 15 s as a pretreatment step according to Sudagar & Aruna (2020). Blanching was applied as a pretreatment to reduce surface microbial load and modify the physical characteristics of the pod prior to drying. The pretreated pods were dried in a hot-air oven at 60 °C until a constant weight was achieved. As mentioned by Farrahnor et al. (2025), 60 °C is the ideal drying temperature because it balances efficient moisture removal with maintaining the structural integrity of the composite. The dried material was ground into a fine powder using a grinder, sieved to obtain a uniform particle size (approximately 0.2 - 0.5 mm), and stored in airtight containers at -20 °C until fermentation. Fresh, pretreated, and dried pod samples were separately retained for crude protein analysis. Further moisture analysis was performed using a moisture analyser.

2.3. Fermentation with *Bacillus subtilis*

Submerged fermentation was carried out in 250 mL Erlenmeyer flasks containing a total of 100 mL nutrient medium supplemented with *M. oleifera* pod powder at different concentrations of 0% (control), 1%, 2%, and 5% (w/v). The selection of substrate concentration and fermentation parameters in this study was adopted and modified from previously published research on SCP production using plant-based substrates. The initial substrate concentration of cellobiose of 0.2% (w/v) has severely restricted the bacterial growth, while a better yield was demonstrated when substrate concentrations are between 0.5 and 5.0% (w/v) in different studies (Brenner & Johnson, 1984; Jaganmohan et al., 2013).

The growth medium consisted of 5 g/L yeast extract, 5 g/L peptone, 1 g/L KH_2PO_4 , 0.5 g/L $MgSO_4 \cdot 7H_2O$, 5 g/L NaCl, and 0.1 g/L $CaCl_2$, with the initial pH adjusted to 7.0–7.2 before sterilisation. The media were autoclaved at

121 °C for 15 min prior to inoculation. Each flask was inoculated with 10% (v/v) *B. subtilis* inoculum and incubated at 37 °C with agitation at 150 rpm for 48 h, under submerged fermentation conditions. These conditions were chosen and adjusted based on the literature approach by Helal et al. (2022). Three independent fermentation replicates were performed for each treatment. The maximum biomass yield (g/L) and crude protein (%) were obtained and further analysed. Following fermentation, the cultures were centrifuged at 16,000 rpm for 20 min to separate the pellet and supernatant fractions. The recovered pellets were washed twice with distilled water and dried at 50 °C for 24 h prior to further analysis.

2.4. Determination of crude protein content of *M. oleifera* pod

The crude protein content of fresh, pretreated, and dried *M. oleifera* pod samples was determined using the Kjeldahl method (AOAC International, 2023). Approximately 1 g of each sample was digested with concentrated sulphuric acid in the presence of potassium sulphate and selenium catalyst tablets. The digested samples were distilled with sodium hydroxide, and the liberated ammonia was collected in boric acid solution before titration with 0.1 M hydrochloric acid. Nitrogen content was converted to crude protein using a nitrogen conversion factor of 6.25. All analyses were conducted in triplicate.

2.5. Determination of total soluble protein by *B. subtilis*

The fermentation products were centrifuged at 16000 rpm for 20 minutes to fully separate the pellet and supernatant. The soluble protein produced by *B. subtilis* fermentation was determined using Bradford protein assay. The Bradford assay estimates protein concentration through the binding of Coomassie Brilliant Blue dye to proteins (Kielkopf et al., 2020). Bovine serum albumin (BSA) standards at concentrations of 0, 0.1, 0.3, 0.5, 0.7, 0.9, and 1.0 g/L (Canvax, Spain) were prepared using phosphate-buffered saline (PBS) (R&M Chemicals, Malaysia). Each fermentation fraction was collected and diluted with PBS (1:50) prior to the analysis. A total of 40 µL aliquot of each diluted sample was mixed with 2 mL of Bradford reagent and incubated at room temperature for 5 mins. The absorbance was measured at 595 nm using a UV–visible spectrophotometer (PG Instruments Ltd, UK). Protein concentrations were determined using a bovine serum albumin (BSA) calibration curve generated by linear regression ($R^2 = 0.993$). The regression equation was ($A = 0.612C - 0.00604$), where (A) represents absorbance at 595 nm and (C) represents protein concentration. Protein concentration was calculated as ($C = (A + 0.00604)/0.612$).

The total protein yield was calculated using this equation:

$$\text{Total protein yield (g/L)} = (\text{Supernatant (g)} \times \text{Protein fraction}) / \text{Fermentation volume (L)}$$

2.6. Analysis of fermented protein powder

The whole fermentation product was transferred to a drying oven (Shinsaeng Scientific, South Korea) and spread evenly as a thin layer on a clean drying tray to promote uniform moisture removal. The samples were dried at 50 °C for 24 hours, or until a constant weight was reached, indicating that the moisture had been completely removed. After drying, the samples were cooled to room temperature in a desiccator to minimize moisture absorption from the surrounding air. The dried material was ground into fine powder using a laboratory grinder (Mill Powder Tech,

Taiwan) at 19000 rpm and stored in airtight containers under dry conditions until further analysis. The moisture and protein content of the fermented product was analysed. The protein yield was calculated and compared with the total soluble protein yield.

2.7. Determination of moisture content

The moisture contents of fresh and dried *M. oleifera* pod samples and the final soluble protein powder were determined using a moisture analyser (BEL Engineering, Italy). Approximately 1 g of each sample was dried to constant mass. Moisture content was expressed as the percentage mass loss relative to the initial sample mass.

2.8. Determination of pH value

The pH of the fermentation medium was measured before fermentation and immediately after the completion of the 48 h fermentation period using a calibrated pH meter (Trans Instruments, Singapore). Measurements were performed in triplicate for each treatment.

2.9. Statistical analysis

All experiments were conducted in triplicate, and the results are presented as mean $\pm$ standard deviation (SD). Statistical analysis was conducted using SPSS version 29.0. One-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test, was used to determine significant differences among treatments.

3. RESULTS AND DISCUSSION

3.1. Crude protein content of *Moringa oleifera* pods

The crude protein content of fresh, pre-treated, and dried *M. oleifera* pod samples is presented in Table 1. Drying significantly increased the measured crude protein content compared with both fresh and pre-treated samples ($p < 0.05$).

Table 1. Crude protein content of *M. oleifera* pod samples.

Type of sample	Protein content (%)
Fresh pod	2.40 $\pm$ 0.41 ^b
Pre-treated pod	1.77 $\pm$ 0.13 ^b
Dry pod	11.90 $\pm$ 0.07 ^a

Values are expressed as mean $\pm$ SD of three independent replicates. Means sharing the same superscript letter are not significantly different ($p > 0.05$).

The dried pod powder exhibited the highest crude protein content (11.90%), followed by fresh pods (2.40%), whereas the pre-treated pods showed the lowest protein content (1.77%). No significant difference was observed between the fresh and pre-treated pod samples ($p > 0.05$).

The present study demonstrated that drying significantly increased the measured crude protein content of *M. oleifera* pods. The crude protein content increased from 2.40% in the fresh pods to 11.90% in the dried pods, whereas the

pre-treated pods exhibited the lowest protein content (1.77%). The higher protein content observed after drying was expected because the removal of moisture concentrated the remaining dry matter, thereby increasing the proportion of protein expressed on a dry-weight basis rather than increasing the absolute amount of protein. The blanching treatment applied during pretreatment appeared to have little influence on protein retention under the conditions used in this study, as no significant difference was observed between the fresh and pre-treated samples. These findings indicate that oven drying at 60 °C effectively reduces moisture while preserving the nutritional quality of the substrate for subsequent fermentation. Furthermore, consistent with the findings of Shin et al. (2025), the maximum crude protein yield is obtained at 60 °C for 24 hours.

The protein content obtained in the present study was comparable to values reported in previous studies. Joshi and Jain (2011) reported protein contents of 10.18% on a dry-weight basis and 1.48% on a fresh-weight basis, which were slightly lower than those obtained in the present study. Such variations may be attributed to differences in cultivar, pod maturity, environmental conditions, or processing methods. Similarly, Cauto et al. (2024) reported that mature *M. oleifera* pods contained approximately 10.1 g protein per 100 g dry weight, which is consistent with the crude protein content determined in the present study. Previous studies have also demonstrated that moderate drying temperatures effectively preserve protein quality while reducing moisture content (Alahmad et al., 2021; Shin et al., 2025). Therefore, the drying conditions employed in this study were considered suitable for producing dried *M. oleifera* pod powder with relatively high protein content while maintaining its suitability as a fermentation substrate.

3.2. Total soluble protein yield

The total soluble protein yields obtained after submerged fermentation are presented in Table 2. Soluble protein was detected in all treatments, confirming protein production by *Bacillus subtilis* under the selected fermentation conditions.

Table 2. Total protein yield determination of soluble protein in the supernatant of crude protein extract

<i>M. oleifera</i> pod content (w/v)	Total protein yield (g L ⁻¹)
0%	0.01 ± 0.00 ^b
1%	0.04 ± 0.02 ^{ab}
2%	0.24 ± 0.17 ^{ab}
5%	0.31 ± 0.14 ^a

Values are expressed as mean ± SD of three independent replicates. Means sharing the same superscript letter are not significantly different ($p > 0.05$).

The highest total soluble protein yield was obtained from the 5% (w/v) *M. oleifera* pod treatment (0.31 g L⁻¹), followed by the 2% treatment (0.24 g L⁻¹), the 1% treatment (0.04 g L⁻¹), and the control treatment (0.01 g L⁻¹). Statistical analysis indicated that the 5% treatment produced a significantly higher protein yield than the 0%

treatment ($p < 0.05$), whereas no significant differences were observed between the 5%, 2%, and 1% treatments or between the 2%, 1%, and 0% treatments ($p > 0.05$).

Following submerged fermentation, soluble protein was detected in all treatments, confirming that *Bacillus subtilis* secreted proteins into the surrounding fermentation medium under the selected conditions. Among the treatments, supplementation with 5% (w/v) *M. oleifera* pod powder produced the highest total soluble protein yield, whereas the control treatment produced the lowest yield.

A possible explanation is that increasing the concentration of *M. oleifera* pod powder supplied a greater amount of fermentable nutrients, allowing *B. subtilis* to sustain cellular growth and protein secretion for a longer period during fermentation. *M. oleifera* pods contain residual protein, fermentable carbohydrates and amino acids that may contribute to microbial metabolism during fermentation. Sánchez-Machado et al. (2010) reported that immature pods contain appreciable amounts of arginine and lysine, while Owon et al. (2021) demonstrated that *M. oleifera* pods contain relatively high concentrations of both essential and non-essential amino acids. These nutrients may have supported bacterial growth and contributed to the increased soluble protein yield observed at higher substrate concentrations. The increased protein yield may therefore reflect enhanced microbial physiological activity supported by improved substrate availability rather than an increase in protein secretion by individual cells alone.

Despite the improvement in protein yield, the recovered pellets remained predominantly fibrous after fermentation, indicating that the lignocellulosic substrate was only partially bioconverted by *B. subtilis*. A possible explanation is that the complex structure of cellulose, hemicellulose and lignin limited substrate accessibility, thereby restricting complete degradation by *B. subtilis* under the selected fermentation conditions. The bacterium likely utilised the more readily available nutrients present in the substrate for growth and protein synthesis, whereas much of the structural fibre remained in the recovered biomass because of the nature of lignocellulosic materials. Similar observations have been reported for other agricultural residues, where cellulose, hemicellulose and lignin are not completely degraded during microbial fermentation unless appropriate pretreatment is applied (Singh et al., 2023; Asim et al., 2025). Therefore, the fermentation process in the present study should be regarded as a partial bioconversion process that converted accessible nutrients into valuable microbial products while leaving a considerable proportion of the structural biomass intact.

This finding supports the concept of agricultural waste valorisation, whereby low-value agricultural residues are transformed into higher-value products through microbial fermentation rather than being completely degraded (Montefrio et al., 2025; Papaioannou et al., 2022).

3.3 Moisture content

The moisture contents of fresh and dried *M. oleifera* pods are presented in Table 3.

Table 3. Moisture content of fresh and dried *M. oleifera* pods.

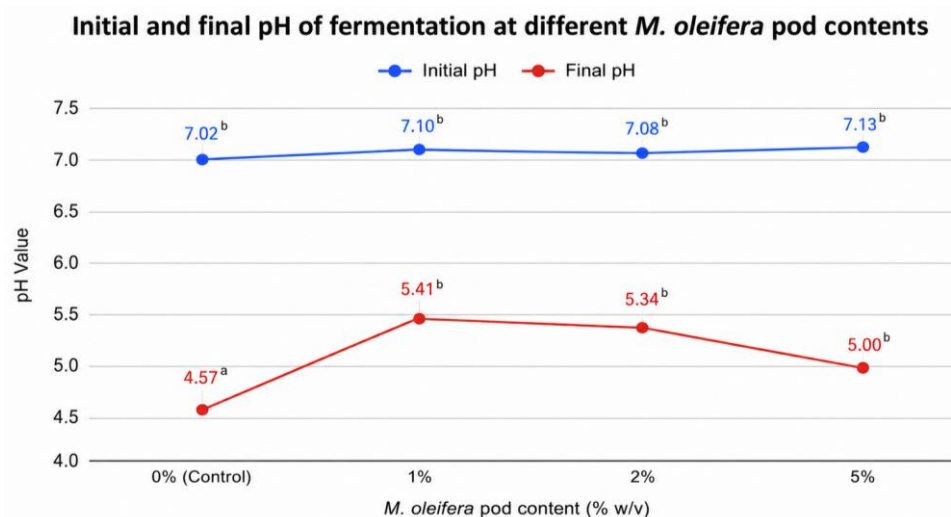
Type of sample	Moisture content (%)
Fresh pod	86.28 ± 1.54^a
Dry pod	12.49 ± 0.72^b

Values are expressed as mean $\pm$ SD of three independent replicates. Means sharing the same superscript letter are not significantly different ($p > 0.05$).

Drying significantly reduced the moisture content of *M. oleifera* pods from $86.28 \pm 1.54\%$ to $12.49 \pm 0.72\%$ ($p < 0.05$). The reduction in moisture content improved the storage stability of the substrate by limiting microbial deterioration prior to fermentation while facilitating grinding into a finer and more homogeneous powder. A more uniform particle size improves mixing with the fermentation medium and promotes better contact between the substrate and the bacterial cells during submerged fermentation. This observation may be attributed to improved substrate dispersion within the fermentation medium, allowing greater contact between bacterial cells and the available nutrients. Lekjing et al. (2024) reported a higher crude protein content in dried soursop leaves than in fresh leaves, suggesting a concentration effect associated with moisture loss. Consequently, drying reduced the moisture content and increased the measured crude protein percentage on an as-is basis, while also producing a drier and more easily ground substrate for subsequent fermentation.

3.4. Changes in pH during fermentation

The pH values of the fermentation media before and after fermentation are presented in Figure 1. Prior to inoculation, all treatments exhibited similar initial pH values ranging from 7.02 to 7.13, with no significant differences observed among treatments ($p > 0.05$).

**Figure 1.** pH value of culture broth samples before and after fermentation.

Following 48 h of submerged fermentation, the pH decreased in all treatments. The greatest reduction was observed in the control treatment, which reached a final pH of 4.57, whereas the media supplemented with *M. oleifera* pod powder maintained comparatively higher final pH values ranging from 5.00 to 5.41. The final pH of the control treatment differed significantly from those of the supplemented treatments ($p < 0.05$), while no significant differences were observed among the 1%, 2%, and 5% treatments ($p > 0.05$).

Fermentation resulted in a reduction in pH in all treatments, indicating active microbial metabolism throughout the fermentation period. The control treatment exhibited the greatest reduction in pH, whereas media supplemented with *M. oleifera* pod powder maintained comparatively higher final pH values. A possible explanation is that metabolic utilisation of available nutrients resulted in the production of acidic fermentation by-products, leading to a decrease in the pH of the culture medium after 48 h. According to Ainaa et al. (2016), decreasing pH is commonly associated with active microbial growth because organic acids accumulate as metabolic by-products during fermentation. However, supplementation with *M. oleifera* pod powder may have moderated the pH reduction through its natural buffering capacity or by altering the rate at which nutrients were metabolised. Although bacterial growth was not monitored directly throughout fermentation, these findings suggest that differences in substrate composition influenced fermentation kinetics and metabolic activity, resulting in different final pH values among treatments. Previous studies by Wilks et al., (2009) demonstrated that *B. subtilis* possesses acid adaptation mechanisms that enable continued metabolic activity under moderately acidic conditions, suggesting that the observed pH reduction did not necessarily inhibit bacterial growth.

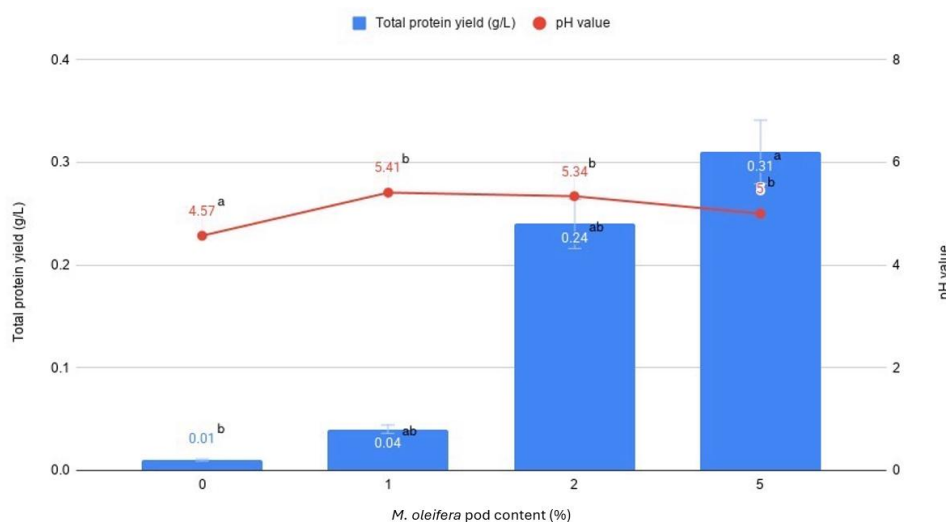


Figure 2. Comparison of total protein yield and final pH value for each treatment.

Based on Figure 2, the total protein yield increased progressively with increasing *M. oleifera* pod concentration, from 0.01 g/L in the 0% treatment to 0.31 g/L in the 5% treatment. Statistical analysis showed that the 5% treatment produced a significantly higher total protein yield than the 0% treatment ($p < 0.05$), whereas the 1% and 2% treatments were not significantly different from either the 0% or 5% treatments. These findings indicate that increasing the availability of *M. oleifera* pod as a substrate generally promoted soluble protein concentration by *B. subtilis*, although the improvement became statistically evident only at the highest substrate concentration.

The treatment with the highest total protein yield (5%) did not correspond to the lowest pH. This suggests that soluble protein production was not directly correlated with medium acidification. Instead, protein production appeared to depend primarily on substrate availability, which supported protein secretion despite a relatively higher final pH. Therefore, pH changes alone cannot be used as an indicator of protein yield under the present fermentation conditions. The absence of significant differences in final pH among the 1%, 2%, and 5% treatments, despite the increasing protein yield, further supports the hypothesis that the addition of *M. oleifera* pod substrate may have contributed to the increased soluble protein concentration by providing additional nutrients, rather than through changes in fermentation pH. These findings demonstrate that *M. oleifera* pod concentration had a greater influence on soluble protein yield than on the final acidity of the culture broth.

The findings demonstrate that *M. oleifera* pod powder has potential as a sustainable agricultural substrate for soluble protein production by *B. subtilis*. Increasing the substrate concentration was associated with higher measured soluble protein recovery under the conditions tested. Nutrient availability is an important factor influencing *B. subtilis* growth and metabolism, as demonstrated in previous studies involving nutrient optimisation during fermentation (Su et al., 2020). However, bacterial growth was not directly measured in the present study. Thus, the observed increase in soluble protein recovery cannot be attributed conclusively to enhanced microbial growth.

4. CONCLUSION

This study provides preliminary evidence for the use of *Moringa oleifera* pod powder in fermentation with *Bacillus subtilis* to recover soluble protein. Dried pods had a higher measured crude protein content (11.90%) than fresh (2.40%) and pretreated (1.77%) pods, although these differences should be interpreted in relation to moisture content and the reporting basis. The highest mean soluble protein concentration was observed at 5% (w/v) supplementation (0.31 g/L), followed by 2% (0.24 g/L), 1% (0.04 g/L), and the control without pod powder (0.01 g/L). Only the 5% treatment differed significantly from the control, while differences among the supplemented treatments were not significant. Thus, a clear concentration-dependent response was not established. The measured concentrations could not be attributed specifically to bacterial protein production because the Bradford assay did not distinguish proteins originating from the bacterium, pod powder, or growth medium. The absence of concentration-matched uninoculated controls and direct bacterial biomass measurements also limited interpretation of the contribution of fermentation. Future studies should incorporate these controls, validated biomass measurements, and protein characterisation to determine the microbial contribution to the recovered protein. These investigations would help establish whether moringa pod waste can support protein production and agricultural waste valorisation.

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AUTHOR CONTRIBUTIONS

Puteri Insyiraah: Methodology, Formal analysis, Investigation, Resources, Data curation, Writing - Original draft;
Safiyah Shahimi: Conceptualization, Validation, Writing - Review & Editing, Supervision, Visualization, Project administration.

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DECLARATION OF GENERATIVE AI IN THE WRITING PROCESS

During the preparation of this manuscript, the authors used ChatGPT (OpenAI) to assist with language editing and improve grammar and clarity. The authors reviewed and revised the content as necessary and take full responsibility for the final version of the manuscript.

DATA AVAILABILITY

Data is available on reasonable request.

COMPETING INTEREST

The authors declare that there are no competing interests.

COMPLIANCE OF ETHICAL STANDARDS

Not applicable.

SUPPLEMENTARY MATERIAL

No supplementary material is associated with this article.

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