Effect of Solid-State Fermentation Pretreatment Using Bacillus spp. on Protein Hydrolysates Produced from Defatted Black Soldier Fly Larvae (Hermetia illucens L.)

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Abstract

Solid-state fermentation with *Bacillus subtilis, B. cereus,* and *B. stearothermophilus* (45 °C, 0-4 days) was evaluated as a pretreatment for black soldier fly larvae (BSFL, *Hermetia illucens*) protein hydrolysates, followed by alkaline extraction, isoelectric precipitation, and bromelain hydrolysis. The highest protein recovery (66.57 ± 3.15%) occurred after two days of fermentation with *B. cereus*. The degree of hydrolysis ranged from 25.67 ± 2.07% to 62.67 ± 1.58%, indicating progressive protein degradation during fermentation. SDS-PAGE showed that the hydrolysates were dominated by peptides below 10 kDa, and fermentation shifted the amino acid profile, with glutamic acid remaining the most abundant in all treatments. Antioxidant activity increased after fermentation, and *B. subtilis* gave the strongest effect. No antibacterial activity against *S. aureus* or *E. coli* was detected, possibly because extraction, precipitation, and hydrolysis reduced or removed antimicrobial peptides. Overall, *Bacillus*-assisted solid-state fermentation is a promising biological pretreatment for improving the nutritional and functional properties of BSFL hydrolysates, with *B. cereus* being best for protein recovery and *B. subtilis* for antioxidant potential.

Keyword: Protein hydrolysate, black soldier fly larvae, *Bacillus* spp., solid-state fermentation, bromelain, bioactive peptides

Introduction

Black soldier fly larvae (BSFL, *Hermetia illucens L.*) are a promising alternative protein source because they efficiently convert organic waste into nutrient-rich biomass. Depending on rearing substrate and processing, BSFL contains about 27-55% protein on a dry matter basis, with a balanced amino acid profile suitable for food and feed (Zulkifli et al., 2022, Lu et al., 2022). BSFL protein hydrolysates made with commercial proteases have shown antioxidant and antimicrobial activities (Su et al., 2020). However, studies on different *Bacillus* species as biological pretreatment during solid-state fermentation of BSFL remain limited, particularly regarding microbial growth, protein enrichment, hydrolysis, molecular weight distribution, and bioactivity.

This study therefore examined solid-state fermentation of BSFL using *Bacillus subtilis, B. cereus,* and *B. stearothermophilus* to determine the best strain and fermentation duration for improving protein quality. Effects were assessed through microbial growth kinetics, crude protein content, degree of hydrolysis, amino acid composition, SDS-PAGE molecular weight distribution, and antioxidant and antibacterial activities. The findings offer insight into Bacillus-assisted fermentation as a sustainable strategy for enhancing the nutritional and functional value of BSFL proteins for food and feed applications.

Research Method

Black soldier fly larvae (BSFL) meal was characterized by proximate analysis (AOAC, 2012; Kjeldahl, nitrogen-to-protein factor 4.76) and defatted by Soxhlet extraction with n-hexane (70°C, 6 h). The defatted meal underwent solid-state fermentation with *Bacillus subtilis, B. cereus,* or *B. stearothermophilus* (10% w/w inoculum at mid-exponential phase, 45°C, 0-4 days). Proteins were extracted with 0.25 M NaOH (1:5 w/v, 60°C, 1 h, three cycles), quantified by the Bradford assay (595 nm, BSA standard), freeze-dried, and hydrolyzed with bromelain (3% w/w of protein, pH 8.0, 50°C, 24 h).The hydrolysates were characterized for degree of hydrolysis (formol titration relative to Kjeldahl total nitrogen), molecular weight distribution (12% SDS-PAGE, 10-180 kDa marker), and amino acid composition (HPLC, UPLC, and LC-MS/MS). Antioxidant activity was determined by the DPPH radical scavenging assay (517 nm, IC_{50}), and antibacterial activity against Staphylococcus aureus and Escherichia coli by Kirby-Bauer disk diffusion (20,000 ppm, ampicillin as positive control, triplicate).

Discussion & Result

Effect of *Bacillus* spp. Fermentation on BSFL

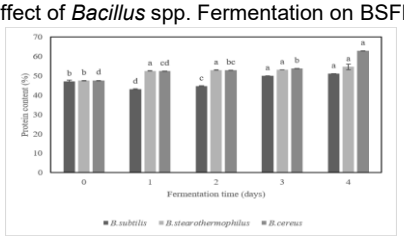


Figure 1. Changes in crude protein content of BSFL during solid-state fermentation

B. subtilis gave the lowest crude protein increase, reaching 51.07% after four days (Figure 1), in line with its lowest specific growth rate. This is likely because it devotes much of its resources to secreting extracellular alkaline proteases that degrade proteins into peptides and amino acids before they are assimilated into biomass (Su et al., 2020, Mäder & Antelmann, 2022). *B. stearothermophilus* reached 54.58%, consistent with its sustained growth, as its thermostable enzymes stay active at 45 °C and support prolonged nutrient assimilation (Nazina et al., 2001). *B. cereus* gave the highest crude protein (62.85%, $p < 0.05$), with the highest specific growth rate in the exponential phase and a broad range of extracellular hydrolytic enzymes, including proteases, that enable efficient use of proteinaceous substrates (Stenfors Arnesen et al., 2008).

Structural Changes of Hydrolysate Protein During Fermentation

Fermentation altered the amino acid profile of BSFL in a strain-dependent manner. In the control, leucine, lysine, valine, and phenylalanine were the most abundant essential amino acids, while glutamic acid, aspartic acid, and alanine dominated the non-essential ones. *B. stearothermophilus* gave the greatest increases in branched-chain amino acids, lysine, glycine, and proline but decreased methionine and cysteine. *B. subtilis* yielded the highest glutamic acid (59.07 g/kg), likely due to its extracellular protease activity and the GS-GOGAT pathway, whereas *B. cereus* showed small but balanced increases in essential amino acids. Histidine decreased in all strains, consistent with earlier SSF studies (Chotinu-Kul et al., 2025; Dai et al., 2021), whereas the decreases in methionine and serine differed from Dai et al. (2021), which may reflect substrate, strain, and degradation efficiency. SDS-PAGE showed smears near 10 kDa, indicating hydrolysates consisted mainly of peptides below 10 kDa, consistent with An et al. (2023).

Antioxidant and Antibacterial Activity of Fermented BSFL Protein Hydrolysate

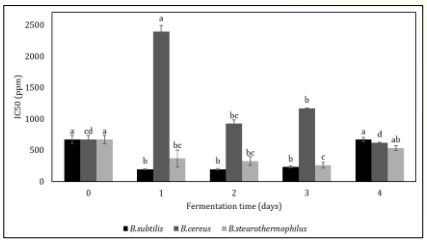


Figure 2. Effect of fermentation time on the antioxidant activity (IC_{50}) of BSFL protein hydrolysates

B. subtilis reduced IC_{50} (Figure 2) from 671.20 ppm (control) to 194 ppm on day 1, which rose to 232.29 ppm on day 3 and 669.59 ppm on day 4, likely because the peptides were further metabolized by the bacteria. *B. cereus* had the highest IC_{50} on day 1 (2393.35 ppm) and the lowest on day 4 (618.17 ppm), possibly related to hydrophobic amino acid production. *B. stearothermophilus* showed moderate activity, with IC_{50} falling to 261.78 ppm on day 3 and rising to 533.66 ppm on day 4, still below the control. No antibacterial activity against *S. aureus* and *E. coli* was detected in any hydrolysate, although ampicillin inhibited S. *aureus* but not *E. coli,* possibly due to resistance. The absence of activity may reflect low AMP concentration and sample preparation, particularly isoelectric precipitation at pH 4.3-4.5, which may have removed AMPs (pI 8.9-10.7).

Conclusion

Solid-state fermentation is a promising pre-treatment for improving the nutritional quality and bioactivity of BSFL proteins. *B. cereus* showed the fastest growth and the highest crude protein after four days at 45°C, while protein recovery peaked on day 2 for B. subtilis and *B. cereus* and on day 3 for *B. stearothermophilus.* Subsequent enzymatic hydrolysis gave DH values of 25.67-62.67% and enriched several amino acids, notably glutamic acid, aspartic acid, proline, leucine, and valine, with peptides predominantly below 10 kDa. No antibacterial activity was detected, but the *B. subtilis* fermented hydrolysate showed the strongest antioxidant activity on days 1-3. Protease activity and optimization of extraction and hydrolysis were not examined, so further characterization is needed to define optimal conditions for producing BSFL hydrolysates with enhanced bioactivity.