Malaysian Cocoa Journal 2026, Vol. 18

SHORT COMMUNICATION

PRELIMINARY STUDY: SCREENING OF PROTEASE-PRODUCING ENDOPHYTIC BACTERIA TO BE DEVELOPED FOR NANO-FORMULATED BIOFERTILIZER FOR COCOA PLANT GROWTH.

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Malaysian Cocoa J. 18: 76-80 (2026)

ABSTRACT – *The production of extracellular protease enzymes by cocoa endophytic bacteria was evaluated using the skim milk agar hydrolysis assay. The formation of clear hydrolysis zones indicated the secretion of proteases capable of degrading casein, the major protein component in skim milk. Among the five bacterial isolates tested, four isolates (PA, PD, BK, and UL) exhibited proteolytic activity, while isolate BL showed no detectable hydrolysis zone under the experimental conditions. Isolate PA demonstrated the highest protease activity with an average hydrolysis zone of 11.1 mm, followed by BK (9.2 mm), PD (9.0 mm), and UL (6.7 mm). The variation in hydrolytic activity among isolates indicates that extracellular protease production is strain-dependent and influenced by bacterial metabolic capacity and environmental conditions. The high proteolytic activity exhibited by isolate PA suggests its potential for improving nutrient mineralization and supporting plant growth-promoting functions. The findings indicate that isolate PA is a promising endophytic bacterial candidate for further physiological, molecular, and greenhouse studies toward the development of sustainable microbial biofertilizer formulations for cocoa cultivation.*

Keywords: Endophytic bacteria, protease enzyme, cocoa, extracellular enzyme, biofertilizer

INTRODUCTION

Endophytic bacteria colonize internal structures of plant host without causing disease or negative effects. They often produce bioactive substances such as plant growth hormones to increase plant growth (Khan *et al*., 2014), creating a substantial attention due to increasing demand in bringing down the use of chemical fertilizers and pesticides, as effort in promoting environmental protection (Vale *et al*., 2010). In many cases, the symbiosis between plant and endophyte occurs in such a way that the plant protects and feeds the endophyte, while the endophyte, in return, produces plant growth hormones to enhance plant growth. Agricultural-based problems caused by the long-term use of chemical fertilizer products, not only pollutes the environment, but also causes an imbalance in the proportions of various nutrients, the destruction of organic matter in the soil and a decrease in the structural integrity and properties of aggregates, leading to soil compaction, salinization and others (Wang *et al*, 2020). These bacteria can produce various extracellular enzymes, phytohormones, and metabolites that enhance nutrient availability and improve plant–microbe interactions (Hardoim *et al*., 2015). Among these enzymes, proteases are particularly important because they hydrolyze complex proteins into peptides and amino acids, which can subsequently be mineralized into inorganic nitrogen forms available for plant uptake (Burns *et al.*, 2013).

Recent studies have highlighted the importance of plant growth-promoting microorganisms, including bacteria and fungi, due to their ability to improve nutrient cycling, produce growth regulators, and enhance plant resilience under environmental stress conditions (Muthusamy *et al*., 2023; Purnima & Singh, 2023). Bacterial enzymes such as proteases, cellulases, amylases, and

phosphatases contribute to organic matter degradation, nutrient mineralization, and soil biochemical activity. Protease-producing microorganisms, for example, hydrolyze protein compounds into peptides and amino acids, which are further converted into inorganic nitrogen forms essential for plant nutrition. Therefore, microbial enzyme activity can enhance nutrient availability while supporting sustainable nutrient management systems (Chia *et al*., 2024).

Endophytic bacteria are particularly promising candidates for enzyme-based biofertilizer development because they naturally colonize plant tissues and possess multiple plant growth-promoting characteristics. These bacteria may produce extracellular enzymes, phytohormones such as indole-3-acetic acid (IAA), siderophores, and other metabolites that contribute to plant growth enhancement and stress adaptation. Recent advances in microbial biofertilizer technology have focused on selecting multifunctional bacterial strains and developing microbial consortia to improve consistency and field performance (Muthusamy *et al*., 2023; Singh *et al*., 2023). Technologies such as encapsulation, carrier-based formulation, and drying approaches are being investigated to maintain microbial viability and functional enzyme activity during storage and application (Fadiji *et al*., 2024).

Microbial protease production varies significantly among bacterial species and strains due to differences in genetic capability, metabolic activity, and environmental conditions such as pH, temperature, nutrient availability, and cultivation period (Gupta *et al*., 2002; Rao *et al*., 1998).

For cocoa cultivation, the selection of efficient enzyme-producing bacteria may support sustainable crop production. Therefore, this study aimed to evaluate extracellular protease production among cocoa endophytic bacterial isolates using the skim milk agar hydrolysis assay and identify promising bacterial candidates for future biofertilizer development.

MATERIALS AND METHODS

Culture preparations

Nutrient broth (Oxoid, USA) was used as the basic culture medium for the cultivation of the endophytic bacterial isolates. The prepared medium was heated with continuous stirring until completely dissolved and then sterilized by autoclaving at 121°C for 15 minutes. After sterilization, the medium was cooled to approximately 45–50°C and aseptically poured into sterile Petri dishes. The plates were allowed to solidify before use. Cocoa endophytic bacterial isolates were cultured by aseptically transferring a single colony from preserved bacterial stocks onto nutrient agar plates using a sterile inoculating loop. The inoculated plates were incubated under suitable growth conditions until visible bacterial colonies developed. Pure cultures were obtained by repeated sub-culturing of individual colonies to ensure uniform growth and absence of contamination. Freshly grown cultures were subsequently used for protease enzyme screening and other bacterial characterization assays.

Skim milk agar preparation

Skim milk agar (SMA) was prepared by dissolving skim milk powder and agar in distilled water according to the modified method described by Gupta *et al*. (2002) and Ouertani *et al*. (2018). A typical SMA medium consisted of 10% (w/v) skim milk and 2% (w/v) agar. The skim milk solution and agar solution were prepared separately to prevent protein degradation during sterilization. The agar solution was autoclaved at 121°C for 15 minutes, while the skim milk solution was sterilized separately by filtration through a 0.22 µm membrane filter. After cooling to approximately 45–50°C, the sterile skim milk solution was aseptically mixed with the agar solution and poured into sterile Petri dishes.

Protease Screening Assay

Five endophytic bacterial isolates (PA, PD, BK, UL, and BL) were tested for protease screening assay. Isolates were inoculated onto SMA plates by spot inoculation. The inoculated plates were incubated at the optimum growth temperature for the bacterial isolates. After incubation, the formation of transparent hydrolysis zones surrounding bacterial colonies was observed, indicating casein degradation by extracellular proteases. The diameter of the clear zones was measured in millimetres (mm), and the average hydrolysis zone was calculated from triplicate measurements. Larger hydrolysis zones were considered indicative of higher extracellular protease production.

RESULTS AND DISCUSSIONS

Protease production by cocoa endophytic bacterial isolates was evaluated using the skim milk agar (SMA) hydrolysis assay. The formation of clear hydrolysis zones surrounding bacterial colonies indicated the secretion of extracellular proteases capable of degrading casein, the major protein component in skim

milk. Among the five isolates tested, four isolates (PA, PD, BK, and UL) exhibited detectable proteolytic activity, whereas isolate BL did not produce a visible hydrolysis zone under the experimental conditions (Figure 1). Protease activity was determined based on the diameter of the clear zone formed around each bacterial colony after incubation.

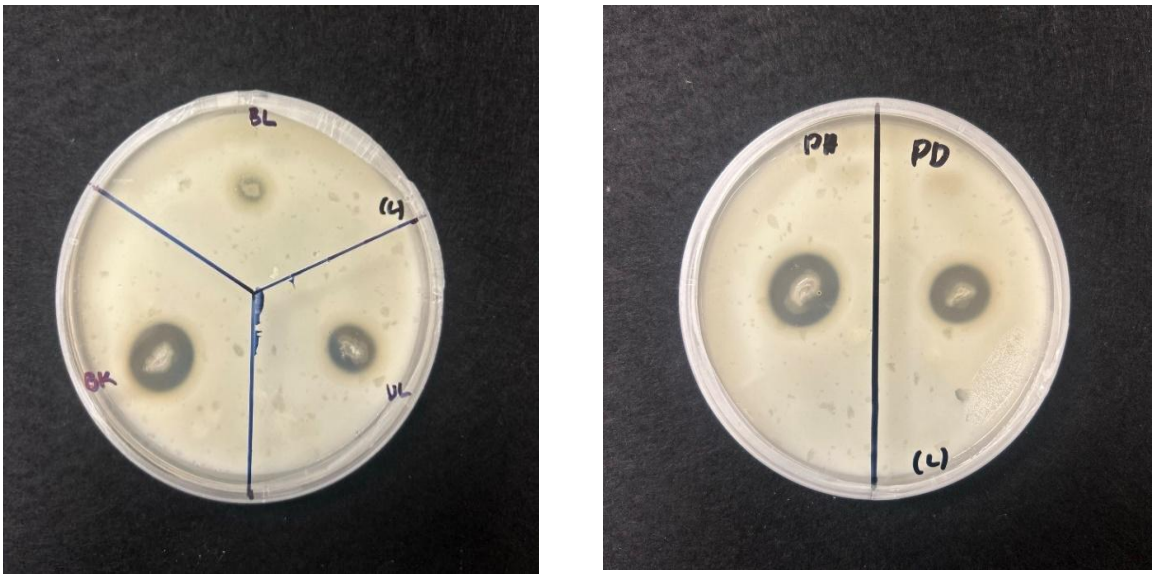


Fig 1. Screening of endophytic bacterial isolates for protease production on skim milk agar.

The results showed variation in protease activity among the tested isolates (Table 1). Isolate PA demonstrated the highest proteolytic activity, producing the largest average hydrolysis zone of 11.1 mm, followed by BK (9.2 mm), PD (9.0 mm), and UL (6.7 mm). The larger hydrolysis zone produced by isolate PA suggests a higher level of extracellular protease secretion and/or greater enzyme diffusion through the agar medium compared with other isolates. In contrast, the absence of a clear zone around isolate BL indicates that this isolate either lacks protease-producing capability or that enzyme production was not induced under the tested culture conditions. These findings demonstrate that extracellular protease production is isolate-dependent, even among bacterial isolates obtained from the same cocoa host plant.

Table 1: Zone of Diameter for Protease Enzyme Hydrolysis Test

Phenotypic name	Diameter 1 (mm)	Diameter 2 (mm)	Diameter 3 (mm)	Average (mm)	Clear zone (+/-)
PA	10.4	11.7	11.2	11.1	+
PD	9.9	8.1	9	9.0	+
BK	9.2	9.2	9.2	9.2	+
BL	-	-	-	-	-
UL	7.8	5.5	6.9	6.7	+

The differences in protease production among bacterial isolates may be attributed to variations in genetic characteristics, metabolic capacity, and environmental responses. Extracellular enzyme synthesis is regulated by bacterial genotype and influenced by cultivation factors such as nutrient availability, pH, temperature, and incubation duration (Gupta *et al*., 2002; Rao *et al*., 1998). Therefore, differences in protease activity among isolates may reflect their distinct physiological adaptations and enzyme secretion capabilities.

Proteases are important extracellular enzymes involved in the degradation of proteinaceous organic matter into peptides and amino acids. These degradation products can subsequently undergo microbial mineralization, releasing inorganic nitrogen compounds that are available for plant uptake. Consequently, protease-producing microorganisms contribute significantly to nutrient cycling, organic matter decomposition, and improvement of soil fertility (Burns *et al*., 2013). In agricultural systems, the application of protease-producing bacteria may enhance nutrient availability and support sustainable nutrient management by reducing dependence on synthetic fertilizers.

The observed variation in protease activity is consistent with previous studies demonstrating that bacterial strains differ considerably in their ability to produce extracellular enzymes. Endophytic bacteria acquire functional traits that support their survival and interaction within plant tissues, including enzyme production, nutrient mobilization, and plant growth promotion (Hardoim *et al*., 2015). Therefore, the high protease activity exhibited by isolate PA suggests that it may possess greater potential as a beneficial endophytic bacterium for improving nutrient mineralization and supporting plant growth.

Beyond nutrient cycling, protease-producing bacteria may have additional agricultural applications, including enhancement of organic residue decomposition and indirect suppression of certain plant pathogens through degradation of protein-based extracellular compounds involved in pathogen survival and infection processes (Gupta *et al*., 2002). However, protease activity alone is insufficient to confirm biofertilizer effectiveness; therefore, further evaluation of other plant growth-promoting characteristics, including indole-3-acetic acid (IAA) production, phosphate solubilization, nitrogen fixation, siderophore production, biofilm formation, stress tolerance, and greenhouse performance, is required.

For the development of an encapsulated or nano-formulated microbial biofertilizer, the selected isolate should also be evaluated for compatibility with encapsulation materials, maintenance of bacterial viability, and retention of functional enzyme activity following formulation and storage. Overall, the superior protease-producing ability of isolate PA highlights its potential as a promising candidate for further physiological, molecular, and greenhouse studies toward the development of a sustainable microbial biofertilizer formulation for cocoa cultivation.

CONCLUSIONS

The present study demonstrated that cocoa endophytic bacterial isolates possess varying abilities to produce extracellular protease enzymes, as evaluated through the skim milk agar hydrolysis assay. Four isolates (PA, PD, BK, and UL) exhibited proteolytic activity, while isolate BL showed no detectable protease production under the tested conditions. Among the evaluated isolates, PA showed the highest protease activity with an average hydrolysis zone of 11.1 mm, indicating superior extracellular enzyme secretion and potential capability for protein degradation compared with the other isolates. The findings confirm that protease production is isolate-dependent and influenced by the physiological and metabolic characteristics of individual bacterial strains. Therefore, isolate PA represents a promising candidate for further evaluation as a beneficial endophytic bacterium for microbial nano-formulated biofertilizer development for improved cocoa production.

ACKNOWLEDGEMENTS

The author also would like to thank the Director-General, Deputy Director-General (R&D) and Director of Biotechnology Cocoa, Malaysian Cocoa Board (MCB) for permission to publish and reviewing this paper. The project was funding under the 13th Malaysian Development Fund

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