OPTIMIZATION OF BIOMASS AND CONIDIA PRODUCTION OF *METARHIZIUM ANISOPLIAE* NATIVE ISOLATES

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Abstract

This study evaluated the production of mycelial biomass and conidia of native Metarhizium anisopliae isolates using both liquid and solid media, with a focus on the influence of vessel type on submerged fermentation. Two isolates (Ma ICDPP#1 and Ma ICDPP#2) were cultivated in liquid medium in conventional and baffled Erlenmeyer flasks to analyse the impact on biomass accumulation and blastospore concentration.

The results showed that Ma ICDPP#2 isolate produced significantly higher amounts of biomass and blastospores in conventional Erlenmeyer flasks compared to baffled flasks (7.65×10^5 blastospores/ml vs. 0.65×10^5 blastospores/ml, $p < 0.001$). For Ma ICDPP#1, the differences were less pronounced, and the flask type did not significantly influence biomass production ($p > 0.05$). Based on these findings, Ma ICDPP#2 was selected for further testing on solid substrates.

For solid-substrate production (barley seeds), Ma ICDPP#2 generated an average of 1.05×10^9 aerial conidia per gram of bioproduct after 14 days of incubation. The conclusions suggest that conventional flasks are more effective for biomass and blastospore production, while Ma ICDPP#2 exhibits superior potential for fungal bioproduct applications due to its higher yield.

Keywords: *Metarhizium anisopliae*, *biomass, conidia production, submerged fermentation*

1. INTRODUCTION

Entomopathogenic fungi (EPF) represent a promising ecological alternative for the biological control of agricultural insect pests (Fornelli et al., 2004; Kidanu & Hagos, 2020). Due to their host specificity and minimal impact on non-target species and the environment, EPF are increasingly used in plant protection strategies, particularly within Integrated Pest Management (IPM) programs (Skinner et al., 2014). Their effectiveness as biological control agents lies in their unique infection process which begins when asexual spores adhere to and penetrate the insect cuticle. These spores then develop within the hemocoel, ultimately causing host death through nutrient depletion or the toxic effects of fungal metabolites (Vega et al., 2012; Zhang & Zhang, 2025). In response to growing concerns over chemical residues, environmental pollution, and pest resistance to conventional insecticides, research has shifted toward the development of effective and sustainable microbial-based insecticides (Bamisile et al., 2021).

To produce a viable biological control agent, it is essential to isolate and characterize an effective strain, confirm its pathogenicity, and optimize a cost-efficient mass production process (Jaronski, 2013; Gutiérrez et al., 2024). Entomopathogenic fungi (EPF) can be mass-produced through liquid fermentation, yielding single-cell propagules, such as blastospores or submerged conidia, or multicellular structures, including mycelium or microsclerotia. Alternatively, solid-state fermentation allows for the production of aerial conidia, which are often more stable and effective in field applications.

Biphasic fermentation has emerged as a widely adopted strategy for producing entomopathogenic fungal biocontrol agents, combining the advantages of both submerged and solid-state cultivation systems. In this approach, the initial liquid (submerged) phase is used to generate a homogeneous and metabolically active fungal inoculum—typically consisting of blastospores or fragmented mycelium—which is then transferred onto a solid substrate for mass sporulation. The liquid phase enables rapid biomass accumulation under controlled conditions, while the subsequent solid-state phase supports high-yield

conidial production on substrates such as rice, barley, or wheat. This two-step system allows for improved control of inoculum quality, more uniform colonization of the solid substrate, and ultimately, greater consistency and viability of aerial conidia used in biopesticide formulations.

Building on this framework, the present study aimed to investigate specific aspects of entomopathogenic fungi (EPF) cultivation relevant to large-scale application. The main objective was to evaluate the conidial production capacity of a native *Metarhizium anisopliae* isolate on solid substrate using a biphasic fermentation system. A secondary objective was to compare the amount of mycelial biomass generated in two types of vessels during the submerged phase.

Two types of vessels were compared: a conventional Erlenmeyer flask and a baffled flask (with an internally grooved surface). The baffles, strategically positioned along the inner walls of the vessel, are designed to enhance oxygen transfer and ensure better mixing of the culture medium. By increasing the surface-to-volume ratio and promoting efficient agitation, they help reduce zones with low oxygen availability and create more favourable conditions for fungal growth.

This study contributes to the optimization of the early stages of entomopathogenic fungi mass production, providing experimental data relevant to the development of effective and sustainable bioinsecticides.

2. MATERIALS AND METHODS

Two *Metarhizium anisopliae* isolates (Ma ICDPP#1 and Ma ICDPP#2) were used in this study. These isolates are maintained in the entomopathogenic microorganism collection of ICDPP. For each fungal isolate, a freshly subcultured colony grown on PDA medium was used. To obtain a conidial suspension, conidia were transferred using a microbiological loop into a container with 50 ml of sterile distilled water supplemented with 0.01% Tween 80 as a surfactant. To facilitate spore disaggregation, the suspension was subjected to ultrasonic treatment for 3 minutes at 40 Hz.

Fungal biomass quantity and the concentration of propagules produced by *M. anisopliae* were assessed through cultivation in Goral liquid medium (Goral, 1971). The original composition of this medium was slightly modified by omitting the addition of antibiotics.

Ingredients	Goral
KH_2PO_4	5
Mg $SO_4 \cdot 7H_2O$	2
$NaNO_3$	5
Zaharose	20
Corn syrup	0,8

Table 1. Composition of liquid media (g l^{-1})

For each experimental variant, four replicates were established. A volume of 250 ml of liquid culture medium was distributed into 500 ml flasks, followed by sterilization and cooling. The liquid media were inoculated with a conidial suspension of *M. anisopliae* to achieve a final concentration of 1×10^6 conidia/ml. After inoculation, the flasks were incubated on a shaking incubator (Model LSI-3016R, Daihan LabTech Co., Ltd, Korea) at 150 rpm and 23°C for 5 days.

After incubation, three sub-samples of 10 ml each were taken from every flask. The liquid culture was vacuum-filtered using membrane filters (Sartorius; 0.2 µm). The membranes were weighed before and after filtration. They were then dried in a drying oven until a constant weight was achieved. The

difference between the weight of the empty membranes and that of the membranes with dried fungal biomass represented the amount of biomass produced.

To determine the concentration of blastospores (propagules formed in liquid culture), 1.5 ml samples were aseptically collected from each isolate. The samples were appropriately diluted and counted microscopically at 400× magnification using a Bürker counting chamber. For solid substrate inoculation, 25 ml of each liquid culture was used.

To evaluate differences in mycelial biomass production between the two types of culture vessels (conventional Erlenmeyer and baffled Erlenmeyer flasks), the data were analysed using an independent t-test. Biomass values were expressed in mg/ml.

Following the submerged cultivation phase, the liquid culture of Metarhizium anisopliae isolate Ma ICDPP#2 obtained in conventional Erlenmeyer flasks—characterized by high biomass and blastospore yields—was used as a pre-inoculum for solid-state fermentation. This approach was adopted to capitalize on the high physiological activity and viability of the propagules obtained in liquid medium, which are suitable for rapid and homogeneous colonization of the solid substrate.

A volume of 600 ml of barley seeds was placed into autoclavable polypropylene bags (50 × 30 cm), to which 500 ml of distilled water was added. The bags were sterilized at 120 °C for 1 hour and allowed to cool to room temperature. Each bag was inoculated with 20 ml of liquid pre-inoculum prepared as described above. The viability of the inoculum was verified by plating 0.5 ml on PDA medium in Petri dishes.

To enable gas exchange during incubation, each bag was equipped with a cotton plug. After inoculation, the substrate was mixed thoroughly to ensure uniform distribution of the fungal biomass. Bags were incubated at 25 °C, and after one week, they were manually remixed to prevent compaction of the colonized substrate and to promote homogeneous fungal growth.

After 14 days of incubation, conidial production was quantified. A 5 g sample of the colonized substrate was suspended in 50 ml of sterile distilled water containing 0.01% Tween 80 and glass beads. The suspension was sonicated for 3 minutes to facilitate the detachment of conidia from the substrate. Serial decimal dilutions were then prepared, and conidia were counted using a hemocytometer, as previously described.

3. RESULTS

The results showed that, for the MaICDPP#1 isolate, the mean biomass in the conventional Erlenmeyer flask was 1.667 mg/ml, while in the baffled flask it was 1.278 mg/ml (Fig. 1). The t-test yielded a t-value of 0.844 and a p-value of 0.435, indicating no statistically significant difference in biomass production between the two types of vessels ($p > 0.05$). Thus, the vessel type did not significantly influence mycelial biomass production for this isolate under the tested conditions.

In contrast, for the MaICDPP#2 isolate, the mean biomass in the conventional Erlenmeyer flask was 1.875 mg/ml, compared to 0.9426 mg/ml in the baffled flask. The t-test revealed a t-value of 4.54 and a p-value of 0.0046, indicating a statistically significant difference in biomass production between the two vessel types ($p < 0.05$). Therefore, the conventional Erlenmeyer flask supported significantly higher biomass production compared to the baffled flask for this isolate.

Regarding the comparison between the two isolates, for MaICDPP#1 the biomass production in the conventional flask was slightly below 2.0 mg/ml, while in the baffled flask it was around 1.2 mg/ml. For MaICDPP#2, biomass production in the conventional flask was slightly above 2.0 mg/ml, whereas in the baffled flask it was around 1.0 mg/ml. Overall, MaICDPP#2 produced more biomass than MaICDPP#1, but both followed the same trend of higher biomass accumulation in the conventional Erlenmeyer flasks. In both cases, the baffled flasks appeared to reduce mycelial biomass production compared to the conventional ones. This may suggest that the increased aeration and grooved interior of the baffled flasks were not optimal for biomass accumulation under the experimental conditions.

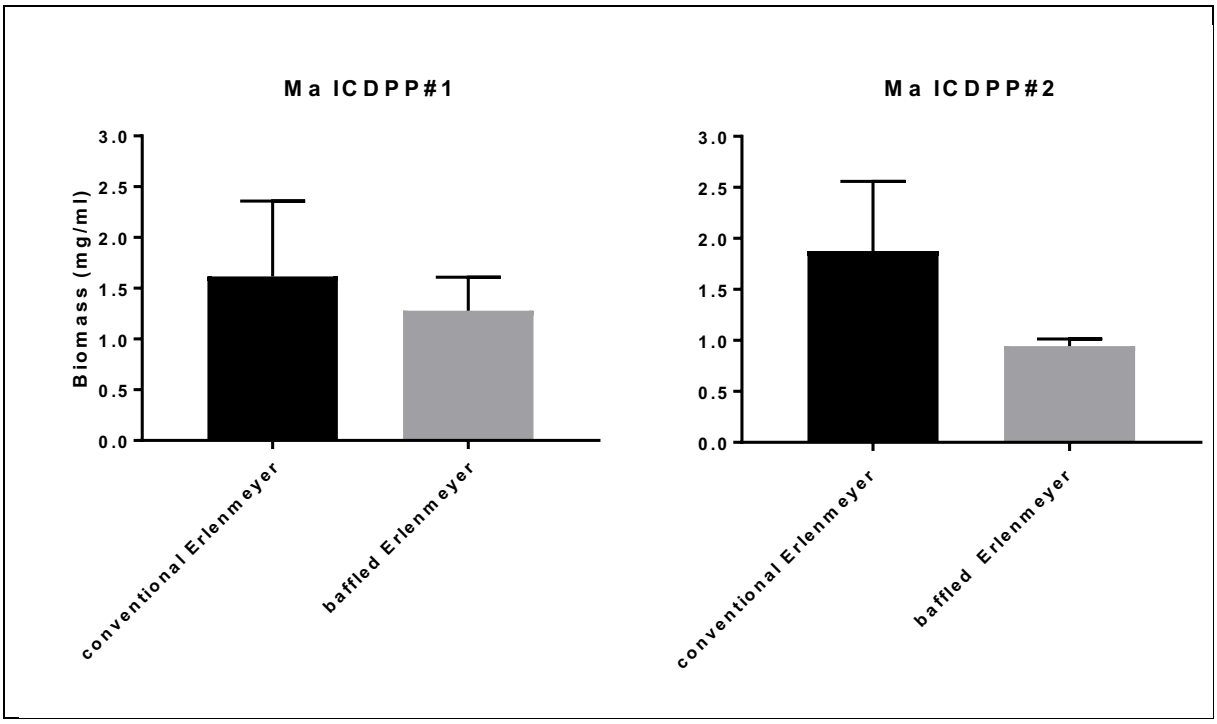

Figure 1. Comparison of mycelial biomass production of *Metarhizium anisopliae* ICDPP#1 and ICDPP#2 isolates in conventional and baffled Erlenmeyer flasks

Blastospores production of the MaICDPP#1 isolate after 4 days of fermentation was higher in the conventional Erlenmeyer flask compared to the baffled vessel (Fig. 2). The mean concentration of blastospores in the conventional Erlenmeyer was 4.625×10^5 blastospores/ml with a standard deviation of 1.63, while in the baffled Erlenmeyer it was 2.2×10^5 blastospores/ml with a standard deviation of 0.99. The t-test yielded a value of 2.54 and a p-value of 0.052, suggesting that, at a significance level of 0.05, the difference is not statistically significant. However, the result is close to the threshold for significance, indicating a potential trend toward higher blastospore production in the conventional flask.

For the MaICDPP#2 isolate, blastospore production after 4 days of fermentation was also higher in the conventional Erlenmeyer flask. The mean concentration of blastospores was 7.65×10^5 blastospores/ml with a standard deviation of 0.48, while in the baffled flask it was 0.65×10^5 blastospores/ml with a standard deviation of 0.30. The t-test indicated a value of 12.22 and a p-value < 0.001, showing that the difference between the two vessel types is highly statistically significant at the 0.05 level.

The main differences in blastospore production between the two strains, MaICDPP#1 and MaICDPP#2, reflect their distinct responses to culture conditions: MaICDPP#2 shows a higher growth capacity under conventional conditions but is more sensitive to the presence of baffle; MaICDPP#1, on the other hand, displays more moderate growth in the conventional flasks and is less affected by the baffled flask.

These observations may indicate differences in the physiological requirements or environmental interactions of the two strains, particularly in terms of aeration and agitation dynamics within the culture vessels.

Based on the obtained results, MaICDPP#2 was selected as the most suitable isolate for solid substrate cultivation, for the following reasons:

-higher overall biomass and blastospore production under conventional conditions: MaICDPP#2 showed significantly greater biomass and blastospore yields in conventional Erlenmeyer flasks compared to MaICDPP#1, indicating a stronger growth capacity under standard fermentation conditions.

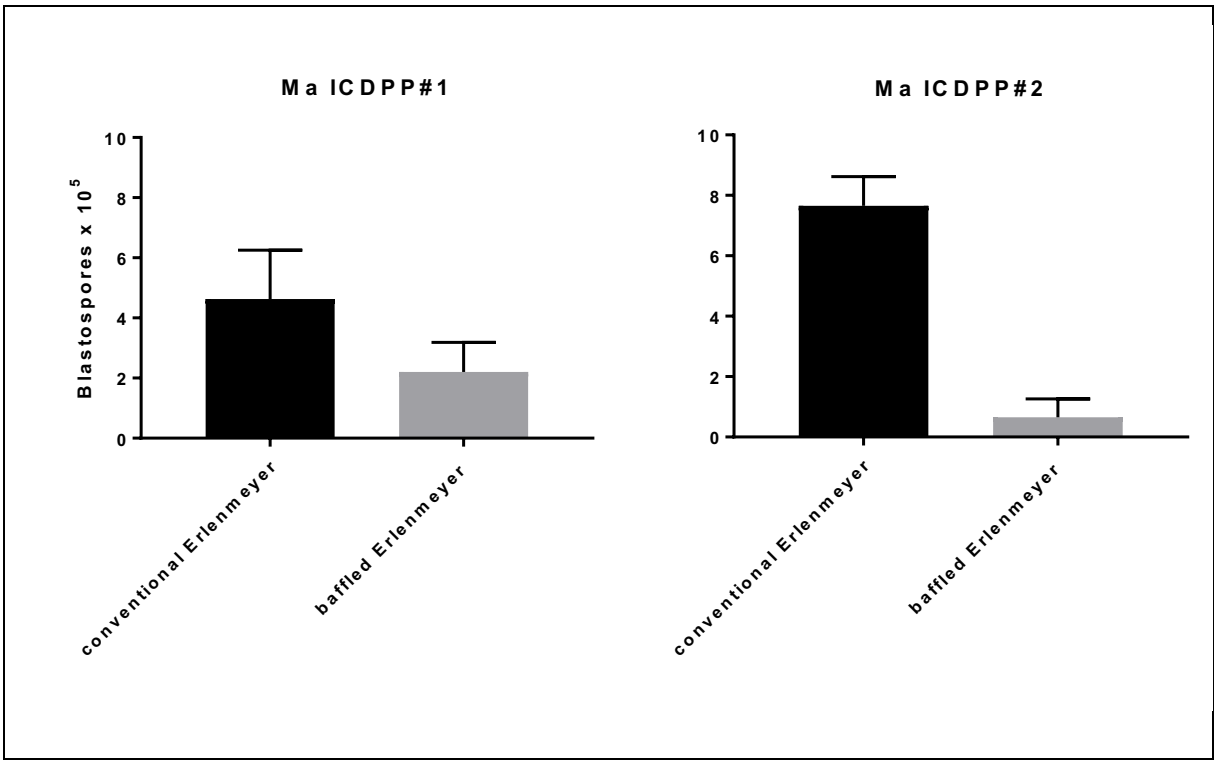

Figure 2. Comparison of blastospore production of *Metarhizium anisopliae* ICDPP#1 and #2 in conventional and baffled Erlenmeyer flasks

-higher blastospore production: MaICDPP#2 demonstrated significantly greater blastospore production in the conventional Erlenmeyer flask (7.65×10^5 blastospores/ml) compared to MaICDPP#1 (4.625×10^5 blastospores/ml), with the difference being statistically significant.

Based on its superior performance in submerged fermentation, isolate MaICDPP#2 was selected for further testing on a solid barley substrate. After 14 days of incubation, the mean conidial yield reached 1.05×10^9 conidia/g of bioproduct, confirming the isolate's strong capacity to sporulate effectively under solid-state conditions.

Figure 3. Biopreparation based on *Metarhizium anisopliae (MaICDPP#2)*

4. DISCUSSIONS

The results of this study have direct implications for the development of *Metarhizium anisopliae*-based bioinsecticides, providing practical insights into isolate selection and cultivation system optimization. The superior performance of the *MaICDPP#2* isolate in conventional Erlenmeyer flasks, both in terms

of biomass production and blastospore concentration, indicates a high level of adaptability to simple technological conditions—an essential factor for industrial-scale production.

Although baffled flasks are typically designed to enhance oxygen transfer and mixing efficiency, in this case, their use significantly reduced production, particularly for the *MaICDPP#2* isolate. This behavior suggests that, under submerged fermentation conditions for this fungal species, excessive aeration or turbulence may inhibit proper mycelial organization and propagule formation—a phenomenon also reported by Machado Magalhães & Dias (1994). Similarly, Park et al. (2002), in studies on *Cordyceps militaris*, observed that agitation speed affects both mycelial morphology (e.g., compaction, size) and exopolysaccharide production—higher speeds led to morphological changes that negatively impacted yields.

On the other hand, the use of baffled flasks is a common strategy in optimizing submerged fermentation for certain fungal strains, where increased aeration is beneficial. For instance, in a recent study, *M. anisopliae* and other entomopathogenic fungi were successfully cultivated in baffled Erlenmeyer flasks (250 ml), containing 90 ml of culture medium and 10 ml of pre-inoculum, incubated at 28 °C and 300 rpm. After 8 days of cultivation, microsclerotia and blastospore formation were observed (Rivas-Franco et al., 2020). Sar et al. (2022) also successfully cultivated *Rhizopus oryzae* in 250 ml baffled Erlenmeyer flasks, achieving high biomass accumulation in a potato protein liquid (PPL) medium. *Isaria fumosorosea* was cultivated in baffled flasks of the same volume, where increased aeration promoted rapid blastospore production, with more than 55% of total biomass formed after just three days of incubation (Jackson, M.A., 2012).

From an applied perspective, isolate *MaICDPP#2* yielded 1.05×10^9 aerial conidia per gram of bioproduct on barley—a value exceeding the average reported for other native isolates under comparable conditions. For example, Roshandel et al. (2016) investigated different grain-based substrates and observed that *M. anisopliae* produced up to 3.1×10^8 conidia/g in rice flour and 3.0×10^8 conidia/g in Sabouraud Dextrose Agar + 1% yeast extract, 14 days post- inoculation. Merino-Peñafiel et al. (2019) reported that conidial production by *M. anisopliae* reached 9.33×10^8 conidia/g on rice and 9.67×10^8 conidia/g on wheat under unsupplemented conditions. When carbon–nitrogen sources were added, the yield increased up to 2.08×10^9 conidia/g on rice. In a recent study, a different species within the *Metarhizium* genus—*M. novozealandicum* strain AgR-F177—was evaluated for its sporulation capacity on various substrates. The isolate produced 7.41×10^8 conidia/g on an oat-based semisolid medium, and a higher yield of 1.68×10^9 conidia/g was obtained under solid-state fermentation on cooked rice (Villamizar et al., 2021). These results emphasize not only the importance of substrate type, but also the variability in sporulation potential between *Metarhizium* species.

From an operational perspective, the use of conventional flasks reduces costs associated with specialized equipment and facilitates the transition to semi-industrial or small-scale production units—an essential consideration for applied research institutions and local bioinsecticide producers.

Compared to other strains reported in the scientific literature, MaICDPP#2 stands out not only for its high conidial production but also for its ability to rapidly generate viable blastospores in simple liquid media. This dual potential—under both submerged and solid-state fermentation—represents a competitive advantage in the development of versatile bioformulations suitable for both foliar treatments and soil applications.

5. CONCLUSIONS

The sporulation level of the *M. anisopliae* MaICDPP#2 isolate confirms its high potential for solid formulations intended for direct field application.

The results validate MaICDPP#2 as a promising candidate for the development of effective bioproducts, while the use of conventional flasks during the initial cultivation stages ensures a favorable cost-efficiency ratio, supporting the practical feasibility of the production technology.

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