Malaysian Journal of Microbiology, Vol 17(1) 2021, pp. 97-102
DOI: http://dx.doi.org/10.21161/mjm.200749

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

SHORT COMMUNICATION

Effects of nutrient additives and incubation period on sporulation and viability of the entomopathogenic fungus, *Metarhizium anisopliae* (Hypocreales: Clavicipitaceae)

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Received 29 February 2020; Received in revised form 14 May 2020; Accepted 7 September 2020

ABSTRACT

Aims: *Metarhizium anisopliae* is an entomopathogenic fungus (EPF) that exists naturally in the environment and potentially be used as a biological control agent against many insect pests. This study aims to evaluate the effect of nutrient additives on the yield and viability of *M. anisopliae* spore and to determine the optimum incubation period for maximum spore production.
Methodology and results: In this study, *M. anisopliae* was cultivated by solid-state fermentation using rice as a growth medium. Three different nutrient additives were examined which aimed to maximize the production of *M. anisopliae* spores. Among the three nutrient additives evaluated, yeast (1.84 ± 0.04 g) supported better growth and spore production than molasses (0.58 ± 0.04 g) and palm oil (0.47 ± 0.09 g). The incubation period between 2-6 weeks produced higher spore yield (0.97 ± 0.02 g spores) at week 4 with a better spore viability (86.30 ± 0.45%) at week 2.
Conclusion, significance and impact of study: Hence, it is suggested that the optimum incubation period is between 2 and 6 weeks after inoculation, and *M. anisopliae* could be mass produced in large quantities on rice substrate with the addition of yeast as the nutrient additives.

Keywords: Entomopathogenic fungus, *Metarhizium anisopliae*, biocontrol, integrated pest management, mass production

INTRODUCTION

Growing public concerns on the overuse of pesticides in agriculture, and their impacts on the environment has prompted research into an alternative method of eco-friendly and safe technology to control pests and pathogens (Jyoti and Singh, 2017). The scenario of pest's treatment in current practise has been changed to integrated pest management (IPM) (Bich *et al*., 2018) which includes the use of biological, cultural, physical, mechanical and biotechnical methods, and more inventive microbial pesticides (Blanco-Metzler, 2004). The use of several potential biological agents and natural enemies such as pathogens, parasitoids, predators, and vertebrates for biological control has been recognized effective internationally (Muhammad, 2016). Among the natural enemies (pathogens), entomopathogenic fungi (EPF) has been widely studied because of its narrow host range, environmental friendliness, safety, and ease of mass production (Greenfield *et al*., 2015). EPF are fungal species that are pathogenic to insects. These fungal pathogens are common and play an important role in insect population dynamics, making it the earliest insect pests control agents (Maina *et al*., 2018) as they are

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(1) 2021, pp. 97-102
DOI: http://dx.doi.org/10.21161/mjm.200749

widespread in almost all classes of insects (Rai *et al*., 2014). Approximately 750 species of fungi from about 90 genera have been documented to be pathogenic, but only a few of these species are currently being developed as pathogens against insect pests (Fukatzu *et al*., 1997) and the most studied EPF for the biological control are *Metarhizium anisopliae, Beauveria bassiana*, *Lecanicillium lecanii*, and many more (Faria and Wraight, 2007).

Metarhizium anisopliae is commonly known as green muscardine fungi and have long been recognized for their biological control potential against insect pests (Bhanu Prakash *et al*., 2008). This species grows naturally in soils and most often found in disturbed habitats like agricultural fields as compared to forest ecosystems (Meyling and Eilenberg, 2007). Infections by *Metarhizium* species are easily recognized a few days after death when the white mould starting to grow on the cadaver that soon turns to olive green colour as the conidia form becomes matured (Tanada and Kaya, 1993). Recently, the fungus has commercially been widely developed as a microbial agent for pest management (Faria and Wraight, 2001) and the favourable results was obtained against aphids, whiteflies, mealybugs, and thrips in greenhouses and nurseries (Faria and Wraight, 2007). Although many insects have evolved their natural defences and successfully adapted in a dangerous environment that surrounds by EPF, they are frequently killed by specialist pathogens (Butt *et al*., 2016). This is because the fungal pathogen locked in an evolutionary battle to overcome these defences, which has contributed to a large number of isolates or strains that are adapted to specific groups of insects (Freimoser *et al*., 2003). Therefore, in this study, the MET-GRA4 strain of *M. anisopliae* was used as it was found to be infective towards red palm weevil and has the potential to be commercialized as biopesticides (Fong *et al*., 2018; Ishak *et al*., 2020).

Solid state fermentation technology is commonly used to produce *Metarhizium* by using natural grains such as rice (Mendonca, 1991). Rao (1989) reported that rice grain is suitable for multiplication of *Metarhizium anisopliae*, and it remains as a preferred substrate because it is cheap and locally available (Jaronski and Jackson, 2012). *M. anisopliae* can grow on natural insect host and on non-living media under unnatural conditions (Rachappa *et al*., 2005). However, the viability of these fungal spores will diminish with time mainly depending on favourable nutritional and environmental conditions (Moore *et al*., 2000) such as temperature, light and involves cellular signaling and metabolic responses. As the spore matured, by preparing for dormancy their wall became thicken, energy reserves are accumulated and the decreasing of their metabolism (Moore-Landecker, 2011). Therefore, it is essential to determine the effective media culture for spore production and their viability. Nutritional factors such as nitrogen sources, carbon sources, with the common trace elements and growth factors are required to promote rapid growth of a given isolate with aimed for mass production (Jenkins *et al*., 1998). Thus, this study was conducted to evaluate the effect of nutrient additives on the spore yield and viability, and to determine the optimum incubation period for maximum spores' production.

MATERIALS AND METHODS

Maintenance of fungal cultures

Metarhizium anisopliae (strain MET-GRA4) was isolated from clay loam soil in Felda Tenang (05°20'N,102°57'E), Kuala Terengganu (Grace *et al*., 2017). This strain was maintained and multiplied by sub-culturing it on potato dextrose agar (PDA), incubated at 28 °C for 14 days (Ramle *et al*., 2005).

Preparation of spore suspension

The spore suspension was prepared by scraping off the spore by adding sterilized distilled water containing 0.02% Tween 80 into a sporulated agar plate. The suspension was filtered through a muslin cloth and then vortexed regularly to obtain homogeneous mixtures. The concentration of the stock suspension was determined by using an improved Neubauer haemocytometer which was calibrated to 1×10^8 spores/mL. These suspensions represented the primary stock suspensions for the subsequence experiments in this study (Gindin *et al*., 2006).

Production of spores on the solid substrate

Rice (Jati, Serba Wangi Sdn. Bhd.) was used as the solid substrate on different parameters such as nutrient additives and incubation periods that influence the growth of the fungus during fermentation. The treatments involved three nutrient additives namely yeast (Sigma-Aldrich), molasses (Sigma-Aldrich) and palm oil (Saji, Delima Oil Products Sdn. Bhd.). Four different incubation periods were tested which are 2, 4, 6, and 8 weeks. Each treatment was replicated three times and repeated twice.

Effect of different nutrient additives on spore production and their viability

The methods were carried out based on Insyirah (2018) with minor modifications where, three nutrient additives, namely yeast, molasses, and palm oil were evaluated in the production of *M. anisopliae*. A total of 15 g rice in three Erlenmeyer flasks were soaked in distilled water, and 0.15 g yeast was added as nutrient additives in one of the treatments, followed by 0.15 mL molasses and 0.15 mL palm oil, for treatment 2 and 3, respectively. The flasks containing rice were then soaked for 18 h. The ratios used were 15 g rice: 15 mL water: 0.15 g/mL nutrient additives. Subsequently, all the flasks were cooked using a microwave for about 10 min and autoclaved at 103.42 kPa for 40 min. After cooling, each flask was inoculated with 75 µL of 10^8 spores/mL of *M. anisopliae.* The flasks were incubated at 28 °C for 4 weeks (28 days). There were three replications for each

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(1) 2021, pp. 97-102
DOI: http://dx.doi.org/10.21161/mjm.200749

treatment. The most effective nutrient additives that yielded the highest number of spore and viability (%) was chosen for the next objective, which was the effect of different incubation period on spore production and their viability.

Effect of different incubation period on spore production and their viability

Four incubation periods (2, 4, 6, and 8 weeks) were tested as a different treatment in the production of *M. anisopliae*. A total of 15 g rice in four Erlenmeyer flasks were soaked overnight in distilled water and 0.15 g yeast was added as nutrient additives. Yeast was selected as the best nutrient additives on the maximum spore production based on the first objective result. The flasks containing rice and yeast were soaked for 18 h. Subsequently, all the flasks were cooked using a microwave and autoclaved at 103.42 kPa for 40 min. After cooling, rice was inoculated with fungus by pipetting 75 uL of 10^8 spores/mL of *M. anisopliae* into each flask. The flasks were incubated at 28 °C for 2, 4, 6 and 8 weeks. There were three replications for each treatment.

Spore harvesting and drying

The spores were harvested at four different time intervals, 2, 4, 6 and 8 weeks. At its maximal production, rice covered with the spore mass was collected from the flasks and dried at room temperature for 1 week. The drying process was run to reduce moisture content and allow the spores to separate from the rice (Posada-Flórez, 2008). Harvesting then was done manually around 20 min. Briefly, the rice was shaken vigorously until the spore powder filtered out from the muslin cloth. The collected spore powder was weighed and placed into separate sterile vials. The spore powder was used for further assessments include the determination of spore yields and spore viability (Posada-Flórez, 2008). The spore yields was determined by measured the spores collected using laboratory analytical balance. The weight of the spores then were recorded.

Spore viability

Spore germination for each treatment was performed according to Hussain *et al*. (2015) with few modifications. A total volume of 100 µL of spore suspension in 0.02% Tween 80 (10^7 spores/mL) was streaked on Petri dishes containing PDA. All plates (three replicates) were sealed with parafilm and incubated at 28 °C for 12-18 h. After that, the spore viability was determined by counting germinated, and ungerminated spores from each replicate under compound microscope with an image analyzer with magnification of 400× (Figure 1). The spore viability was calculated using the formula below:

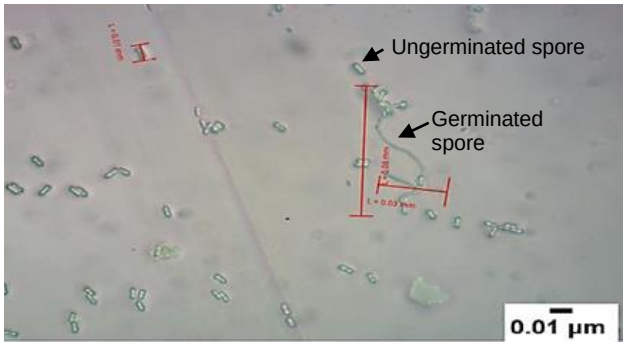

Figure 1: Germinated and ungerminated spores of *Metarhizium anisopliae* (Magnification: 400×).

Spore viability (%)=

$$\frac{Germinated\ spores}{Germinated\ spores + Ungerminated\ spores} \times 100$$

Statistical analysis

Data obtained were analyzed using ANOVA (One-Way Analysis of Variance) (α = 0.05) of SPSS (Statistical packaging for Social Sciences) (software version 21.0). If ANOVA significant then a multiple comparison was performed using Tukey's mean at α = 0.05 as a significant level.

RESULTS AND DISCUSSION

Effect of different nutrient additives on spore production and their viability

The effect of three nutrient additives on spore production is shown in Table 1. Among the nutrient additives evaluated, the spore yield for rice + yeast (1.84 ± 0.04 g) was significantly higher (p<0.05) compared to all treatments and control. For spore viability, the result indicated that all treatments, rice + yeast (86.06 ± 3.32 %), rice + molasses (62.95 ± 14.81%) and rice + palm oil (42.42 ± 7.71%) shows no significant differences (p>0.05) with rice (control) (69.88 ± 1.55%).

Table 1: Effect of different nutrient additives on spore yield and spore viability in 4 weeks incubation period.

Medium + nutrient additives	Spore yield (g)*	Spore viability (%)*
Rice (Control)	0.69 ± 0.05^{b}	69.88 ± 1.55^{a}
Rice + yeast	1.84 ± 0.04^{a}	86.06 ± 3.32^{a}
Rice + palm oil	0.47 ± 0.09^{c}	42.42 ± 7.71^{b}
Rice + molasses	0.58 ± 0.04^{ac}	62.95 ± 14.81^{ab}

*The results are mean and standard deviation of three replicates. Data with different letter indicates a significant difference at p<0.05 according to Tukey's Post-hoc test within the same treatment.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 17(1) 2021, pp. 97-102
DOI: http://dx.doi.org/10.21161/mjm.200749

In the present study, rice was used as the substrate for this solid fermentation process. According to Dorta and Arcas (1998), rice is a good medium to mass produce *M. anisopliae* as it provides a large surface area for aeration and physically supports the fungus to produce conidia and, in the same time supplies nutrients for fungal growth (Jenkins *et al*., 1998). For the nutrient additives, the highest spore yielded and spore viability was recorded from yeast followed by molasses and palm oil, respectively. It showed that yeast as the nitrogen sources support the rapid growth and produce high spore yields of the biocontrol agent *M. anisopliae*. Mendonca (1991) also reported that the superiority of rice with the addition of 1% yeast produced a better spore yield of *M. anisopliae*. In the previous study, according to Costa *et al*. (2002), nitrogen sources such as yeast extract also had supported the rapid growth and high cell yields of the biocontrol agent *Pantoea agglomerans*. This is probably because yeast extract is a good substrate for many microorganisms (Jackson *et al*., 1998) as it contains amino acids and peptides, carbohydrates and water-soluble vitamins (Peppler, 1982). Even though it is well documented that combination of rice and yeast produced high spore yield, however, most of these studies were conducted outside Malaysia. Hence, in this study, we tested the effect of yeast on the spore yield and the obtained yield was more than 1 g, with the viability of spore reached more than 80% indicating that rice supplemented with yeast produced better spore production and quality compared to other nutrient additives.

Palm oil can be used as carbon sources for biocontrol agents which can act as the nutrient that influences maximum spore production (Jaronski and Mascarin, 2017). However, in this experiment, the result of the rice supplemented with palm oil showed that the final growth obtained for *M. anisopliae* was lower than 1 g for spore yield and less than 50% for spore viability as the optimal viability needed is above 50% (Ramle *et al*., 2005).

Meanwhile, molasses was selected as one of the nutrient additives in this study because it can provides energy source and encourages the growth of microorganisms as it contains sugar, vitamins, minerals, carbohydrates and various types of nutrients (Jimenez *et al*., 2004). However, the production of *M. anisopliae* was significantly lower (p<0.05) than rice supplemented with yeast. Therefore, it can be inferred that molasses and palm oil probably contain only the nutrients that satisfy no more than the minimum requirement for spore growth. Therefore, the quality and quantity of spore obtained were unable to reach the desired level.

Effect of different incubation period on spore production and their viability

Table 2 shows that spore yield at week 4 (0.97 ± 0.02 g) was significantly higher (p<0.05) than the three other weeks. In turn, the three other weeks did not differ significantly from each other (p>0.05). Whilst, the spore viability per flask was gradually decreased from week 2 (86.30 ± 0.45%) to week 4 (79.82 ± 2.15%) and maintained the viability at week 6. Afterward, the germination of spores was reduced to below 70%. Thus, the incubation period obviously influenced the yield and viability of the spores. Therefore, the recommended incubation period to harvest spores was between 2-6 weeks after inoculations.

Table 2: Effect of different incubation periods on spore yield and spore viability.

Medium	Incubation period (weeks)	Spore yield (g)*	Spore viability (%)*
Rice+ yeast	2	0.75 ± 0.03^{b}	86.30 ± 0.45^{a}
Rice+ yeast	4	0.97 ± 0.02^{a}	79.82 ± 2.15^{ab}
Rice+ yeast	6	0.80 ± 0.05^{b}	80.37 ± 5.62^{ab}
Rice+ yeast	8	0.77 ± 0.04^{b}	68.30 ± 7.02^{b}

*The results are mean and standard deviation of three replicates. Data with different letter indicates a significant difference at p<0.05 according to Tukey's Post-hoc test within the same treatment.

Generally, the incubation periods of *M. anisopliae* reached a peak for spore yields at week 4, and one possible explanation for the declining trend in germination after 4 weeks incubation is that these conidia produced were probably a mix of older conidia from the first cycle and with the young conidia from the second cycle of production, which affected the overall conidium germination (Daryaei *et al*., 2016). These results are similar to a study conducted by Darby and Mandels (1955) against bioherbicide agents, *Myrothecium verrucaria,* where very young conidia were not mature enough to germinate quickly, while old conidia failed to germinate because of additional nutrient requirements for germination or a second dormancy.

Meanwhile, for spore viability, it was slightly fluctuated at weeks 2, 4, and 6 but decreased at week 8, which is less than 70%. Hence, we concluded that the incubation period on week 2 until week 6 is sufficient enough in the production of *M. anisopliae* spores because the fungus consuming nitrogen and carbon sources on the medium actively during this period. While at week 8, the spore viability declined probably because at this time there were lack of additional nutrients flow in the medium (Gohel *et al*., 2013). Furthermore, in the other study stated by Hallsworth and Magan (1996), vegetative hyphae that grow under nutrient deficiency conditions could have produced immature and weak conidia after 20 days. Therefore, an increased incubation length sometimes not good for spore quantity and quality. Future research

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

should be focused on optimizing the growth conditions such as the ratio of yeast and size of containers to obtain higher biomass desirable for the industrial development of the biocontrol product.

CONCLUSION

Result of this study showed that yeast was the best nutrient additive in the solid state fermentation and incubation period between 2-6 weeks was required for mass production of *M. anisopliae.* The parameters evaluated in this study will be a promising strategy for medium-scale production of spores with low costs and substrates along with additional nutrient that are easily available. Furthermore, the results obtained provide information for a greater understanding of key culture conditions and nutritional requirements that can enhance the large scale production of *M. anisopliae* spores.

ACKNOWLEDGEMENTS

This study was supported by Sime Darby Research-UKM-UMT Grant (VOT: 53235). The authors also wish to thank Plant Biosecurity Division, Department of Agriculture of Terengganu for the continuous support and advices; Faculty of Science and Marine Environment and Centre of Research and Field Service, Universiti Malaysia Terengganu for allowing the use of their facilities.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538