Chapter 1

Mass Cultivation of Selected Entomopathogenic Microbes

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ABSTRACT

Entomopathogenic microbes are organisms that infect insects, mites, and ticks. They are commonly used as biopesticides to control insect populations. This study was designed to evaluate the growth of biocontrol microorganisms on organic biomass without nutrient supplementation, which are byproducts of both domestic and industrial processes. This is to ensure that microbes needed in large amounts, especially for biocontrol or bioremediation processes, can be cheaply cultured, thereby lowering the production costs of commercially available media. Entomopathogenic microbes used in this study include *Aspergillus niger, Beauveria bassiana, Metarhizium anisopliae, Bacillus subtilis* subspecies *spizizenii, Paenibacillus poppiliae, Serratia marcescens, Pseudomonas aeruginosa* and *Lysinibacillus sphaericus*. The biomass used includes rice water, spent mash grains, barley powder and maize shaft, which are occasionally used as animal feed supplements and sometimes even discarded as byproducts of domestic and industrial processes. Standard cells of microbes were inoculated into sterilized prepared formulations containing the organic byproducts. Incubation was done at a temperature of 37^{O}C for 24 hours and 25^{O}C for 72 hours, respectively, for bacteria and fungi. After the incubation process, *B. bassiana* grew most on the maize shaft, while *A. niger* and *B. subtilis* both had the most growth on spent mash grains. *M. anisopliae* recorded its lowest value of 2.67×10^{1} cfu/ml in maize shaft. The highest microbial count was noticed in *B. subtilis* (6.67 × 10^{9} cfu/ml) and *P. aeruginosa* (6.33 × 10^{9} cfu/ml) on spent mash grains and barley, respectively. *A. niger* had the highest overall growth across all the organic biomass substrate used while the lowest growth was recorded for *M. anisopliae*. Based on the data of this study, using organic byproducts for the mass production of biocontrol agents is feasible and could serve as a cost-effective solution that benefits both agriculture and the environment.

Keywords: Entomopathogen, Biomass, Spent grains, *Bacillus subtilis*.

INTRODUCTION

Microbial growth media are used for general, selective and differential cultivation of microorganisms, including bacteria, fungi and viruses. Microbial cultivation is important in biosciences as it makes it possible to isolate pure cultures from mixed populations, especially when studying microbial diversity and function in environmental and industrial contexts. In addition, it is used to identify pathogens responsible for certain infections in clinical settings. Furthermore, it is an important process in monitoring food and water safety by detecting microbial contaminants (Octavia and Wantini, 2017). It can be animate, like embryonated eggs for the cultivation of obligates like viruses and rickettsiae, or inanimate, like nutrient agar for the growth of bacteria (Gusmiaty *et al.,* 2020). Media may be solid or liquid. It can also be either animal cells or plant tissue cultures. It can equally be enriched for the growth of some fastidious organisms that will grow only if special nutrients are present in their culture medium. Any organic matter, including food items like yam or cassava, capable of being readily degraded by microbes can serve as a potential growth media (Aini & Rahayu, 2015). When a medium is being prepared for microbial growth, consideration must be given to the provision of carbon and energy sources and other growth factors such as amino acids, purines, pyrimidines and vitamins that are essential for the organisms (Wongjiratthiti & Yottakot, 2017). Media formulation for the growth of microbes is essential because of the direct need to study their morphology and biochemistry in diagnosis, biotechnology, research and development (Prescott *et al.*, 2002). Certain biological processes such as biocontrol, biofertilization and biodegradation often require large amounts of biomass and microbes (Nigam & Luke, 2016). Most of the conventional media that we have today are largely expensive, and it will not be economical to use those media to culture these microbes, thus defeating the purpose of having a safer and cost-effective method of insect approach in the first place (Panesar *et al.*, 2015). Therefore, materials that are primarily bioproducts of our domestic and industrial processes can be utilized, as most substances that can be degraded also tend to be used as a growth media for microbial culture (Rachel & Adebolu, 2014). A properly formulated media must also be capable of maintaining the morphological and biochemical properties of the organisms as it enhances, maintains and allows sporulation to take place in applicable cases (Elisabeth, 2015).

Therefore, there is a dire need to formulate media for mass cultivation of microorganisms using cheap and locally sourced raw materials as growth

substrates to make them available, especially in developing countries such as Nigeria (Poopathi *et al.,* 2002).

RESEARCH METHODOLOGY

Biomass Collection and Preparation

Brewer's spent grains (spent mash grains) and barley grains, which are byproducts of brewing process, were collected from International Breweries Plc, Ilesha, and Osun State, Nigeria. The wet spent grains were sun-dried for 7 days to prevent enzyme denaturation and microbial degradation, as this process can reduce the amount of nutrients left within the biomass for the growth of the desired microbe that is to be mass-produced. The dried spent and barley grains were ground into powder using a high-speed blender. Maize shaft was collected from a pap vendor at Adekunle Ajasin University, Akungba-Akoko, as it contains some amounts of simple sugars which can be utilized for the growth of the desired microbe. The maize shaft was sun-dried for seven days to prevent enzyme denaturation and microbial spoilage and further blended after drying. All dried biomass were sieved after blending to obtain the finest particulate matter. Rice water was obtained by sieving the liquid from parboiled rice inside the laboratory.

Inoculated Microorganisms

Entomopathogenic fungi cultured were obtained from previous diseased insect studies in the Microbiology lab, while the cultured bacteria were obtained from the United States Department of Agriculture. Entomopathogenic fungi used include *Beauveria bassiana* isolated from diseased *Zonocerus variegatus, Metarhizium anisopliae* isolated from diseased *Coptotermes bellicosus*. Entomopathogenic bacteria used include *Paenibacillus popilliae* (NRRL B-4223), *Lysinibacillus sphaericus* (NRRL B-23338), *Serratia marcescens* (NRRL B-3401), *Bacillus subtilis* subspecies *spizizenii* (NRRL B-14472) and *Pseudomonas aeruginosa* isolated from the haemolymph of decomposing millipedes. All bacteria strains were sourced from the United States Department of Agriculture except *Pseudomonas aeruginosa,* which was isolated from millipede at the Microbiology Laboratory of Adekunle Ajasin University, Akungba-Akoko. The microbes were selected based on the fact that they are organisms with potential biocontrol properties. This makes them to be candidates for mass production and formulation. The bacteria were already identified using molecular biology method, while the fungi were identified on

specialized media using conventional method. Both microbes were resuscitated by cultivating in fresh broth and maintained on slants.

General Formulation Using Organic Biomass

Ten (10) g of dried spent mash grains, barley and maize shaft were measured into separate conical flasks and 100 ml of distilled water was poured into each conical flasks. The resulting mixture was agitated to allow the contents to dissolve. Floating debris was filtered out of the mixture using muslin cloth with the appropriate mesh size able to retain undissolved particles. The mixtures were then sterilized at 121 °C degrees for 15 min. After sterilizing, it was placed on the bench and cooled before being inoculated. For solidified biomass, 1.8 g of pure agar powder was measured into each conical flask containing 100 ml of the dissolved biomass. The agar acts as a solidifier. The resulting mixture was sterilized at 121 °C for 15 min. Media were allowed to cool before being dispensed into Petri dishes.

Preparation of Rice Water

Two hundred (200) g of white rice was cooked in 1000 ml of water for 15 minutes as specified by the instructions on the rice boiler. The rice was drained, and 200 ml of the resulting rice water was used as the broth. The mixture was divided into five (5) places inside a conical flask. This was sterilized at 121 °C for 15 min. After sterilization, the samples were placed on a bench and allowed to cool before inoculation. For solidified rice water, 1.8 g of pure agar powder was measured into each conical flask containing 100 ml of the rice water and sterilized.

Preparation of Standard Liquid Media for the Growth of Fungi and Bacteria

Thirteen 13 g of nutrient broth powder was dissolved in 1 L of water, while 30 g of Sabouraud dextrose agar was dissolved into 1 L of water as standard liquid media for culturing bacteria and fungi, respectively (Fawole & Oso, 2001). They were dispensed into smaller conicals, and the resulting media were sterilized at 121 °C for 15 min. Media were allowed to cool after sterilization before inoculation with the

selected microbe. For the standard solidified media, 28 g of Nutrient agar and 65 g of Sabouraud dextrose agar were dissolved in 1 L of water, sterilized and allowed to cool, respectively.

Inoculation of Formulated Media with Microorganisms

Five different bacteria and two fungi were inoculated into all the prepared standard media and the biomass. A loopful of each bacterial colony was picked from the pure 24-hour old culture using an inoculating loop and introduced into the solid and liquid formulation of both the standard media and the biomass. The colony was streaked on plates containing solidified growth media using quadrant streaking for solid media preparation. The fungal mycelial was inoculated into all media preparations using a wire loop. For the solid media preparation, the mycelium was introduced into the centre of the plates. The bacteria plates were incubated for 24 hours at 37 °C, while the fungi were kept for 7 days at 25 °C in a shaker flask.

Harvesting of Microbes from Biomass and Growth Media

Microbial suspensions were harvested by separating the cells from the biomass and standard liquid media. Bacteria broth cultures were centrifuged to separate and obtain their cells from the broth. The cells were washed twice by reconstituting with 10 ml of 0.9 percent sterile saline and centrifuging again. The suspension was serially diluted, and the appropriate diluent was cultured to enumerate the viable bacterial cells present. Fungal cells and conidia were dislodged from the mycelial mass within the broth cultures using a magnetic stirrer. The mycelial mass was removed from the broth and spores by filtering through a sterile muslin clothe. Spores were washed and concentrated by centrifugation and resuspended in distilled water. The solution was serially diluted, and the suitable diluent was cultured by pour-plating to determine the concentration of viable cells within the suspension.

RESULTS

Table 1 shows the pattern of microbial growth on the biomass and standard media. On the solidified media, *B. bassiana* had the most growth on maize shaft, while *A. niger* and *B. subtilis* both had the most growth on spent mash grains. No growth was recorded for *B. subtilis* on Sabouraud dextrose agar. The growth of *B. subtilis* (6.67×10^9 cfu/ml) on Spent grain formulation compete favourably with the standard media (7.00×10^8 cfu/ml) (Table 2).

In contrast, *M. anisopliae* could not utilize most of the formulated organic biomass. The standard solid media supported the growth of all the microbes. The standard media had the highest growth concentration compared to the organic biomass except for *B. subtilis* and *P. aeruginosa* on spent mash grains and powdered barley, respectively.

Table 3 shows the fungi growth on biomass, with *A. niger* growing better on the biomass spent grain (6.67×10^9 sfu/ml) compared to the standard media (8.67×10^8 sfu/ml). However, the lowest count of *S. marcescens* growth on spent mash grains was recorded. Based on the microbial yield, spent mash grains were observed to have supported the growth of more microbes, while the rice water yielded the lowest amount of growth. Figure 1 shows the growth of two of the fungi (*B. bassiana* and *A. niger*) on different biomass on standard media, with *A. niger* showing moderate growth on rice biomass.

Table 1: Growth of microorganisms on biomass and standard solid media

Organism	Barley	Maize	SMG	Rice	SDA	NA	-ve Control
Beauvaria bassiana	+	+ +	+	+	++	ND	-
Aspergillus niger	+	+	+ +	+	++	ND	-
Metarhizium anisopliae	-	-	+	+	++	ND	-
Bacillus subtilis subspecies *spizizenii*	-	+	+ +	+	ND	++	-
Paenibacillus popilliae	+	+	+	+	ND	++	-
Lysinibacillus sphaericus	+	-	+	+	ND	++	-
Pseudomonas aeruginosa	+ +	+	+	+	ND	++	-
Serratia marcescens	-	-	-	+	ND	++	-

Key: ++ = Luxuriant growth, + = Minimal growth, – = No growth, ND = Not Determined
SDA = Sabouraud dextrose agar, NA = Nutrient agar, SMG = Spent mash grains

Table 2: Growth of bacteria on biomass and standard liquid media

Organism	Barley (cfu/ml)	Maize (cfu/ml)	Spent mash grains (cfu/ml)	Rice (cfu/ml)	Nutrient broth (cfu/ml)
Bacillus subtilis subspecies *spizizenii*	7.67×10^{1}	3.67×10^{4}	6.67×10^{9}	4.67×10^{4}	7.00×10^{8}
Paenibacillus popilliae	5.00×10^{3}	3.33×10^{3}	5.33×10^{2}	2.00×10^{3}	6.67×10^{6}
Lysinibacillus. sphaericus	2.33×10^{5}	1.67×10^{4}	3.67×10^{4}	1.67×10^{2}	4.33×10^{7}
Pseudomonas aeruginosa	6.33×10^{9}	5.33×10^{4}	5.00×10^{3}	2.33×10^{3}	6.00×10^{8}
Serratia marcescens	5.33×10^{1}	5.00×10^{1}	8.00×10^{1}	6.00×10^{3}	7.33×10^{7}

Each value represents the mean of triplicate data obtained from the same diluent

Table 3: Growth of fungi on biomass and standard liquid media

Organism	**Barley (sfu/ml)**	**Maize (sfu/ml)**	**Spent mash grains (sfu/ml)**	**Rice (sfu/ml)**	**SDA (sfu/ml)**
Beauvaria bassiana	4.67×10^{3}	8.33×10^{7}	5.00×10^{6}	8.00×10^{3}	6.00×10^{7}
Aspergillus niger	7.00×10^{5}	4.33×10^{4}	6.67×10^{9}	4.33×10^{5}	8.67×10^{8}
Metarhizium anisopliae	1.33×10^{2}	2.67×10^{1}	5.33×10^{3}	3.00×10^{3}	5.00×10^{6}

Each value represents the mean of triplicate data obtained from the same diluent

Plate 1: *B. bassiana* growth on maize agar

Plate 2: *B. bassiana* growth on maize broth

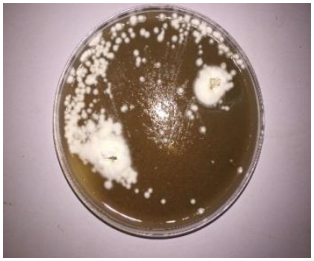

Plate 3: *B bassiana* growth on spent mash

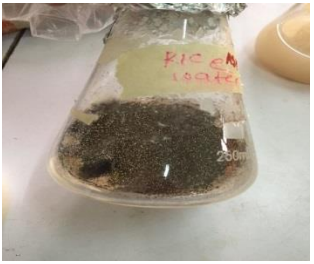

Plate 4: Growth of *A. niger* on rice water

DISCUSSION OF THE RESULTS

Many entomopathogenic fungi-based bioinsecticides like BotaniGard and Mycotrol WP have been formulated and commercially manufactured using different organic byproducts (Hafiza *et al.*, 2014). The practicality of the process is also demonstrated in the data collected in this study. Laleye (1990) earlier reported a modification of potato medium supplemented with growth factors, yielding an excellent result. This study reveals that *B. bassiana* has a luxuriant growth in maize broth and maize agar biomass formulation compared to the other formulated media, thus making the maize a potential candidate for the commercial production of the fungi. This observation and the higher conidial count of the organism in maize broth compared to the Sabouraud dextrose broth is related to Salkkone's report (2004). In his findings, Salkkone (2004) stated that *B. bassiana* is naturally present in internal plant tissues like maize. The plant uses the organism as an adaptive protection against herbivores and insects. Since they can support the organism on-field, this may be why they can support the organism's growth when used as a substrate. It is regarded as an economical alternative for large-scale production of fungi,

especially when combined with enzymatic hydrolysis to improve nutrient availability (El-Hag *et al.,* 2017). Adesemoye and Adedire (2005) studied the feasibility of developing alternative media to Sabouraud dextrose agar (SDA) using local cereal species as the basal media. They observed that all the fungal species grew to some extent better on the formulated media in relation to the standard set-up.

Rice water used in this study is able to support microbial growth. However, microbial growth is lower than that of other biomass, possibly because these other biomasses contain more growth factors. Rice water's starch content has been reported to support the growth of bacteria such as *Bacillus thuringiensis*, which is widely used in microbial insecticides. Studies by Wang *et al.* (2001) demonstrated that rice water-based fermentation media enhanced the production of bacterial spores. Dorta and Arcas (1998) also reported that rice is a suitable medium to mass multiply *M. anisopliae* because it provides nutrients and a large surface area on which conidia can be produced.

This study indicated that the formulation of spent mash grains was able to support most of the organisms inoculated onto it, with the exception of *S. marcescens*. This might be because spent mash grains, which are byproducts of brewing process, are rich in crude protein, crude fibre, ether extract, vitamins and minerals, which support microbial growth (Dhiman *et al.,* 2003). Spent mash grains are also able to support the growth of *M. anisopliae* according to findings from this study. Research has also shown that spent mash grains can significantly support the growth and sporulation of fungi like *Metarhizium anisopliae* due to its rich nutrient profile. For example, Obeng-Ofori and Sackey (2015) found that using brewers' spent mash grains led to high spore yields and increased virulence against insect pests. *M. anisopliae* was also mass-produced by Metchnikoff in 1888 on sterilized brewer's mash with sand granules for spreading on field crops in the biocontrol of pests, and later became a Nobel Prize winner (Taliyan *et al.,* 2020).

Aspergillus niger and *P aeruginosa* show marked growth on all media used. This can be attributed to the fact that both organisms are ubiquitous. Their presence everywhere may be attributed to the fact that many substrates are able to support their growth, thus making them grow on all the media formulated. The growth of *Lysinibacillus sphaericus* on the grains used can be attributed to the fact that the microbe is closely associated with rice and similar grains of agricultural importance due to their potential to control mosquito infestation. Research has evaluated the efficacy of *B. sphaericus* formulations against *Psorophora columbiae* larvae in small rice plots, indicating its application in such environments (Bowles *et al.,* 1990).

Paenibacillus popillae shows nominal growth on all the media used, this further emphasizes that the organism is in close association with plants, conferring protection on the plants against the invasion of insect pests.

Another notable observation of this study is the inability of *S. marcescens* to grow well on most of the formulated biomass except rice media. This might mean that the strain of *S. marcescens* used in this study might be relatively more fastidious compared to the other microbes. However, the rice water specifically contains the nutrients the microbes require for growth. The light reddish colouration obtained from the growth of *Serratia* inside rice broth can be attributed to the production of prodigiosin, which is the reddish pigment produced by many strains of the bacterium (Paul *et al.*, 2024).

CONCLUSION

Utilizing organic byproducts such as the brewers' mash, which significantly supported the growth of microbes in this study in the mass production of microbes used in biocontrol, offers a sustainable and cost-effective solution that benefits both agriculture and the environment. These byproducts are often abundant, cheap, and rich in nutrients, making them ideal substrates for cultivating microbes.

In addition, using these byproducts also addresses issues regarding waste management challenges by reusing materials that would otherwise contribute to environmental pollution. Maize shafts and rice water which are often discarded, can be reused as a feedstock for the mass cultivation of these microbes. Waste is minimized, and resources are reused in closed-loop systems. It must however be noted that organic biomass often has inconsistent chemical and nutrient profiles, which can sometimes result in unpredictable microbial growth and product yield. Availability may also change with seasons, thus impacting its reliability as a feedstock. Poorly processed biomass is prone to spoilage and can harbour competing microorganisms or pathogens that may outcompete or harm the target microbes.

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enriched the scope of this study, allowing for deeper insights into the biological control of insect pests.

CONFLICT OF INTEREST

The authors wish to state that there are no conflicts of interest during this study.

REFERENCES

Adesemoye, A. O., & Adedire, C. O. (2005). Use of cereals as basal medium for the formulation of alternative culture medium for fungi. *World Journal of Microbiology and Biotechnology*, 21(3), 329-336.

Aini, N., & Rahayu, T., (2015). Alternatif Media for Fungal Growth Using a Different Source of Carbohidrats Semin. Nas. XII *Pendidik. Biol.* FKIO 861–6.

Bowles, D. E., Meisch, M. V., Weathersbee, A. A., Jones, J. W., Efird, P., & Bassi, D. (1990). Efficacy of *Bacillus sphaericus* formulations against *Psorophora columbiae* larvae in small rice plots. *J Am Mosq Control Assoc.*;6(4):631-4. PMID: 1983018.

Dorta, B., & Arcas, Y. J. (1998) La esporulación de *Metarhizium* en fermentación en estado sólido con aireación forzada, *Enzyme and Microbial Technology*, **23**: 501-505.

El-Hag, E. A., Kord, A.Y., & Kanan, H. O. (2017). Evaluation of agricultural byproducts for mass production of entomopathogenic fungi. *Journal of Insect Science*, 17(1), 27.

Elisabeth, D. A. A. (2015). Added value improvement of taro and sweet potato commodities by doing snack processing activity. *Procedia Food Science, 3, 262-273*

Fawole, M. O. and Oso, B. A. (2001). Laboratory manual in Microbiology. 3rd Edition. Farmhouse Publishers, Nigeria.

Gusmiaty, S. H., Restu, M., & Amal, F., (2020). The ability of rhizosphere fungi to isolate of mahogany [*Swietenia mahagoni* (L.) Jacq.] in dissolving phosphate *Plant Cell Biotechnol. Mol. Biol. 21*

Hafiza, T. G., Shafqat, S., and Fawad, Z. A. K. (2014). Entomopathogenic fungi as effective insect pest management tactic: A Review, *Applied Sciences and Business Economics*, 1(**1**): 10-18.

Laleye, S. A., (1990). Modification of potato extract medium for the growth of some selected organisms. B.Sc. Thesis. University of Ado-Ekiti, Ado-Ekiti Nigeria.

Nigam, P. S., & Luke, J. S. (2016). Food additives: production of microbial pigments and their antioxidant properties. *Curr. Opin. Food Sci.* 7, 93–100.

Obeng-Ofori, D., & Sackey, E. (2015). Utilization of brewers mash for enhancing the production of Metarhizium anisopliae spores for the biocontrol of insect pests. *Journal of Applied Microbiology*, 119(1), 123-130.

Octavia, A., & Wantini, S., (2017). Perbandingan Pertumbuhan Jamur *Aspergillus flavus* Pada Media PDA (Potato Dextrose Agar) dan Media Alternatif dari Singkong (*Manihot esculenta Crantz*) *J. Anal. Kesehat.* 6 625–31

Panesar, R., Kaur, S., & Panesar, P. S. (2015). Production of microbial pigments utilizing agro-industrial waste: a review. *Curr. Opin. Food Sci.* 1, 70–76.

Paul, T., Mondal, A., Bandyopadhyay, T. K., & Bhunia, B. (2024). Prodigiosin production and recovery from *Serratia marcescens*: process development and cost–benefit analysis. *Biomass Conv. Bioref.* 14, 4091–4110.

Poopathi, S., Kumar, K. A., Kabilan, L. & Sekar, V. (2002). Development of low-cost media for the culture of mosquito larvicides, *Bacillus sphaericus* and *Bacillus thuringiensis serovar. Israelensis. World Journal of Microbiology and Biotechnology 18, 209–216*

Prescott, L. M., Harley, J. P., & Klein, D. A. (2002). Microbiology, 5th ed. pp. 105–106. London: McGraw Hill Publishers. ISBN 0-07-232041-9.

Rachael, O. T., & Adebolu, T. T. (2014). Effect of formulated culture media on growth of some fungal species. *International Journal of Botany and Research, 4(5), 29-34.*

Saikkonen, K., Wali, P., Helander, M. and Faeth, S. H. (2004). Evolution of endophyte-plant symbioses. *Trend Plant Science,* 9, 275–280.

Taliyan, A., Kumar, A., Singh, R., Rana, R., Rana, S. (2020). Mass multiplication of *Metarhizium anisopliae* on different media, *International Journal of Agricultural Invention,* 5(**1**): 138-144.

Wang, D., Sakoda, A., & Suzuki, M. (2001). Biological efficiency and nutritional value of Pleurotus ostreatus cultivated on spent beer grain. *Bioresource Technology* 78(3): 293–300.

Wongjiratthiti, A., & Yottakot, S. (2017). Utilisation of local crops as alternative media for fungal growth. *Pertanika J. Trop. Agric. Sci. 40 (2): 295 – 304.*

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