

Shelf Life of *Trichoderma* Mutant Inoculum with Dried Mud and Glutinous Rice as Carrier Materials

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ABSTRAK

Mulyono AMW, Husein M, Ratriyanto A, Sukaryan S, Asmoro NW, Afriyanti. 2025. Umur simpan inoculum mutan *Trichoderma* dengan bahan pembawa lumpur kering dan beras ketan. JITV 30(3):178-184. DOI:<http://dx.doi.org/10.13443/jitv.v30i3.3443>.

Pembuatan inoculum berbentuk bubuk melalui proses pengeringan akan menyebabkan kerusakan sel spora *Trichoderma* dan menurunkan viabilitas spora. Penelitian ini mengkaji kapasitas penyimpanan inoculum mutan *Trichoderma* dengan bahan pembawa lumpur kering dan beras ketan. Spora mutan *Trichoderma* AA1 kering dicampur dengan media pembawa berupa campuran lumpur kering dan tepung ketan. Perbandingan lumpur kering dengan beras ketan sebanyak 6, yaitu: 0:10, 2:8, 4:6, 6:4, 8:2, dan 10:0. Campuran spora dan bahan pembawa disimpan dan dianalisis hingga 16 minggu. Ketersediaan inoculum pada seluruh kombinasi perlakuan diatas $3,19 \times 10^7$ CFU (Jumlah sel jamur). Fluktuasi ketersediaan terkecil ditunjukkan pada rasio 4:6 yang berkisar antara $3,54 \times 10^7$ dan $6,1 \times 10^7$ CFU. Aktivitas enzim selulase mengalami peningkatan selama penyimpanan 16 minggu dengan peningkatan sebesar 66,67%. Hasil penelitian menunjukkan bahwa *Trichoderma* dengan perbandingan lumpur kering:tepung ketan 4:6 mempunyai ketersediaan $3,54 \times 10^7$ CFU dan aktivitas pelepasan glukosa karboksimetil selulase (CMCase) 0,020 $\mu\text{mol}/\text{menit}$ setelah penyimpanan 16 minggu. Temuan ini mengindikasikan bahwa inoculum dengan formulasi tersebut berpotensi digunakan dalam fermentasi hijauan pakan untuk meningkatkan kecernaan pakan unggas.

Kata Kunci: Lumpur Kering, Beras Ketan, Inoculum, *Trichoderma* Mutan.

ABSTRACT

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Drying to produce powdered inoculum can damage *Trichoderma* spores and reduce spore viability, a factor commonly affected by carrier materials. This study investigated the storage capacity of *Trichoderma* mutant inoculum with dried mud and glutinous rice as carrier materials. Dried *Trichoderma* AA1 mutant spores were mixed with the carrier in the form of a mixture of dried mud and glutinous rice flour. There were 6 ratios of dried mud: glutinous rice, including 0:10, 2:8, 4:6, 6:4, 8:2, and 10:0. The spore-carrier mixtures were stored and analyzed for an additional 16 weeks. The availability of inoculum in all combinations of treatment was above 3.19×10^7 CFU (Colony Forming Units). The smallest availability fluctuations were observed at a ratio of 4:6, ranging from 3.54×10^7 to 6.1×10^7 CFU. Cellulase enzyme activity increased by 66.67% during 16 weeks of storage. The research suggests that *Trichoderma* with a ratio of dried mud: glutinous rice flour of 4:6 had an availability of 3.54×10^7 CFU and 0.020 $\mu\text{mol}/\text{minute}$ glucose-released carboxymethyl cellulase (CMCase) activity after 16 weeks of storage.

Key Words: Dried Mud, Glutinous Rice, Inoculum, *Trichoderma* Mutant

INTRODUCTION

The use of microbes in feed technology is very challenging, especially compared to chemical and physical treatments. However, the use of chemicals in feed technology is more complicated, as it must be neutralized to a pH of 7.0. In comparison, physical treatment might not provide the optimal results. The use of microbes is considered natural, environmentally friendly, and safe for livestock.

Microbes are widely used to improve the nutritional quality of feed ingredients. *Trichoderma* is one of the fungi with high cellulolytic activity (Zayed et al. 2020; Iannaccone et al. 2022), and it has been shown to improve the nutritive quality of cellulose-rich feed ingredients (Jayasekara & Ratnayake 2019; Taye & Etefa 2020). *Trichoderma* is characterized by rapid growth, mostly bright-green conidia, and a repeatedly branched conidiophore structure (Singh et al. 2020; Siti et al. 2021; Iqbal et al. 2022). Previous studies showed

that *Trichoderma* produces cellulolytic enzymes that degrade cell wall substrates into glucose, which is readily absorbed by microbes as an energy source (Grujić et al. 2019). The degradation of substrate cell walls releases the substrate cell contents (Jha 2021), which can be digested by endogenous enzymes of animals, including poultry (Dimitrova 2020). Besides, Adebami & Adebayo-tayo (2020) and Gooruee et al. (2024) observed that *Trichoderma* produces complex cellulase enzymes, including cellobiohydrolase (1,4- β -glucan cellobiohydrolase, EC 3.2.1.91), endoglucanase (1,4- β -glucanohydrolase, EC 3.2.1.4), and cellobiose (β -glucosidase, EC 3.2.1.21).

The application of *Trichoderma* in feed biotechnology requires an appropriate inoculum. For that, the inoculum carrier must be able to carry sufficient quantities of *Trichoderma* spores under appropriate physiological conditions, ready for inoculation into the fermentation medium. Inoculum carrier quality can be determined by its ability to maintain the spore availability during storage. The shelf life and availability of inoculum are affected by storage temperature and nutrient content of carrier medium (Zope et al. 2019; Rimkus et al. 2023). Khan & Mohiddin (2018); Rajput et al. (2014) observed that *Trichoderma pseudokoningii* inoculum in substrates containing sorghum and rice showed higher availability than in wheat straw and rice hull.

Furthermore, dried mud has the potential to serve as an inoculum carrier for *Trichoderma* spores due to its naturally abundant availability (Munir et al. 2013; Pandya & Albert 2014; Singh et al. 2021). Moreover, adding glutinous rice to the inoculum might improve the nutrient content required by the microbes. Therefore, the study on the shelf life of *Trichoderma* inoculum with different carrier materials is essential as a preliminary step before its application in forage fermentation to improve feed quality. Thus, the objective of this study was to investigate the storage capacity of *Trichoderma* inoculum with a mixture of dried mud and glutinous rice as carrier materials.

MATERIALS AND METHODS

Experimental design

Carrier media consisted of dried mud and glutinous rice mixtures in the ratios 0:10, 2:8, 4:6, 6:4, 8:2, and 10:0. Each ratio was analyzed in five replicates. Inoculum availability was determined at weeks 0, 4, 8, 12, and 16 of storage.

Preparation of *Trichoderma* spores, medium, and carrier

The mutant *Trichoderma* AA1 used in this study was initially isolated from oil palm bunches. The

isolate was subjected to chemicals: EMS (Ethyl Methanesulfonate) and ETBr (Ethidium Bromide) mutagenesis as previously described by Mulyono (2008), and the selected mutant was maintained in the laboratory culture collection. The mutant *Trichoderma* AA1 was used in this study (Mulyono 2008). 5 mL of 0.05% Triton X-100 solution was mixed with the mutant *Trichoderma* AA1 spore culture in the agar medium, and the mixture was stirred until the spores dissolved, ready for inoculation. Potato dextrose agar (PDA) is used as a medium, and 100 ml of PDA solution is sterilized in an autoclave at 15 psi for 15 min. When the PDA solution reached about 50°C, it was then poured onto plates, 10 ml per plate. The medium was stored at room temperature for 24 hours until it hardened into solid materials and was ready for inoculation (Sarhan 2015; Oeng et al. 2021).

Carrier media were prepared from dried mud and glutinous rice in ratios of 0:10, 2:8, 4:6, 6:4, 8:2, and 10:0. The dried mud was obtained from Gadjah Mungkur Reservoir in Wonogiri Regency, Indonesia. Each mixture was prepared to a maximum of 100 g, sterilized in the oven at 170°C for 2 hours, and cooled to room temperature thereafter.

Fermentation

The solid medium was inoculated with 0.1 ml of mutant *Trichoderma* AA1 spore solution in a petri dish, then incubated for 7 days. Microbial growth was observed daily. When the spores covered the entire surface of the medium, the fermentation process was terminated, and the spores were harvested.

Spore harvest was performed by drying the spore culture with media in the oven at 50°C for 24 hours. Dried cultures were then mixed with 100 g of carrier substrate and kept in the oven at 50°C for 24 hours until the moisture content was less than 10%. Dried cultures with carrier substrate were packed in aluminum foil, tightly sealed, and stored at room temperature for 16 weeks (Rajput et al. 2014).

Determination of inoculum availability

One gram of dried culture was placed in a reaction tube and mixed with sterile physiological saline until the volume reached 10 ml. This culture solution was vortexed for 1 minute to form a spore suspension. The spore suspension was diluted 10^{-2} , 10^{-3} , and 10^{-4} , and 0.1 ml of each dilution was inoculated onto a petri dish containing solid potato dextrose agar (PDA). The cultures were incubated for 48 hours, and several colonies per ml of spore suspension were counted to determine the number of colonies-forming units (CFU) (Patagundi et al. 2014).

Determination of cellulase activity

Cellulase activity was determined qualitatively by the Congo Red staining method (Grata 2020) and quantitatively by measuring carboxymethyl cellulase (CMCase) activity. The 1% CMC substrate was mixed with culture supernatant in a 50 mM citrate buffer at pH 4.8 for 60 minutes. The reducing sugar released was measured using the dinitrosalicylic acid method (Miller 1959).

Data analysis

Statistical data were analyzed in Excel, and mean values were displayed in tables and graphs. Data on culture Availability and cellulase activity during storage were presented descriptively (Steel et al. 1996).

RESULTS AND DISCUSSION

Inoculum availability

The range of initial concentration of inoculum was between 5.18×10^7 and 6.68×10^7 colony-forming units (CFU). After 16 weeks of storage, the inoculum concentrations mixed with up to 60% dried mud as a carrier remained above 3.61×10^7 CFU (Table 1). This finding indicated that a combination of dried mud and glutinous rice can be utilized as a *Trichoderma* inoculum carrier with high spore availability. Modifying and manipulating media nutrients may increase spore availability (Abuhena et al. 2022). The presence of dried mud as a carrier material was similar to the natural habitat of *Trichoderma* spp., namely soil (Pandya & Albert 2014; Martinez et al. 2023). The inoculum grows in a suitable medium (Iqbal et al. 2017).

The CFU concentration after 16 weeks of storage showed that the treatment with dried mud at a proportion less than 60% resulted in a lower decrease in CFU value than 80% or 100% dried mud. The dried mud cannot be used in high proportions. The lowest decrease in CFU value (31.66%) was observed with a 4:6 ratio of dried mud:glutinous rice, indicating that this combination better maintains *Trichoderma* spore availability. Furthermore, spore availability fluctuates when the proportion of dried mud exceeds 40%. This result indicates that the shelf life of *Trichoderma* inoculum varied significantly across substrate media (Kaushal & Chandel 2017). A previous study showed that the shelf-life of *Trichoderma pseudokoningii* in different substrates attained the peak at 60-75 days and declined gradually thereafter (Rajput et al. 2014).

Substrates rich in nutrients promote a better shelf life than low-nutrient substrates. A combination of dried mud and glutinous rice as a carrier medium yielded better spore availability, likely due to the presence of dried mud as a nitrogen source and glutinous rice as a carbon source. Both nitrogen and carbon in the carrier medium of the inoculum promote conidial production, leading to more spore production. Following this result, Rajput et al. (2014) reported that storage ability and growth of *T. pseudokoningii* inoculum were affected by the presence of carbon and nitrogen in the carrier medium, and Rodrigo et al. (2020) reported the same in *Trichoderma asperellum*.

A 4:6 ratio of dried sludge to glutinous rice produced the most stable inoculum (3.54×10^7 CFU) after 16 weeks of storage, serving as an effective dose to initiate microbial activity in the fermentation system. This finding indicates that the carrier combination maintains spore viability and ensures sufficient spores are available for potential use in feed fermentation.

Quantitative CMCase

Quantitative CMCase is used to measure the activity of the cellulase enzyme, with activity expressed as μmol glucose released per minute (Vimal et al. 2017). The cellulase activity of *Trichoderma* inoculum with dried mud proportions up to 60% increased after 16 weeks of storage, although the increase varied depending on the dried mud-to-glutinous rice ratio. However, cellulase activity remained constant or decreased when proportions of dried mud were 20% or more (Table 2). This finding indicates that the *Trichoderma* inoculum requires nutrients supplied by the carrier medium. The high proportion of dried mud led to lower cellulase activity than the high proportion of glutinous rice. This result was in line with the finding on inoculum availability, where the 4:6 ratio of dried mud:glutinous rice yielded the best availability. *Trichoderma* is a cellulolytic organism that produces cellulolytic enzymes to hydrolyze cellulose (Singh et al. 2017; Strakowska et al. 2014). Cellulase enzymes are produced due to microbial activity in the growth medium. In this study, higher cellulase activity was observed in the medium containing a higher proportion of glutinous rice, consistent with the number of *Trichoderma* colonies.

The cellulase activity of *Trichoderma* inoculum with dried mud: glutinous rice at 4:6 was consistent ($0.020 \mu\text{mol/minute}$) after 16 weeks. Although this study did not directly test the inoculum on forage materials, maintaining active cellulase production during storage indicates that the inoculum can hydrolyse plant cell wall polysaccharides into glucose when applied in feed fermentation. Such enzymatic activity is essential to improve the digestibility of forages rich in cellulose.

Qualitative CMC-ase

The formation of a clear zone around colonies grown on a plate containing a single carbon source derived from cellulose indicates the secretion of cellulase by the growing colonies. The ratio of the apparent zone diameter to the colony diameter is an indicator of qualitative cellulase activity (Patagundi 2014; Dewiyanti et al. 2021). The changes in precise zone ratios after 16 weeks of inoculum storage were relatively small in all treatments. The use of single-carrier materials, both dried mud and glutinous rice, resulted in a decrease in the clear zone ratio. Whereas the use of a combination of carrier materials with the ratio of dried mud: glutinous rice 2:8, 4:6, and 8:2 increased the clear zone ratio (Table 3). The ratios of dried mud:glutinous rice 0:10 and 4:6 resulted in higher

clear-zone averages than other ratios. The qualitative cellulase activity fluctuated in weeks 2 and 16, but was relatively constant in other weeks, with precise zone ratios of about 4-5. Extremely high values were observed in the treatment group for the 0:10 ratio at week 2 and the 8:2 ratio at week 16. In general, based on the comprehensive results of measuring inoculum availability and quantitative and qualitative cellulase activity, the use of dried mud:glutinous rice at a ratio of 4:6 yielded the best results.

The formation of a clear zone indicates the secretion of the cellulase enzyme from the *Trichoderma* inoculum, demonstrating the inoculum's consistent cellulolytic potential for breaking down cellulose. This stability is essential to ensure predictable performance when the inoculum is applied to forage fermentation systems.

Table 1. Number of colony-forming units (CFU, $\times 10^7$) per ml of inoculum spore suspension in various combination ratios of dry mud (DM) and glutinous rice (GR) as carrier medium during storage

Ratio DM: GR	Storage Time (Weeks)					Changes (%)
	0	4	8	12	16	
0:10	6.68	5.80	3.96	3.82	3.37	-49.55
2:8	6.66	2.66	3.48	4.46	2.74	-58.86
4:6	5.18	4.56	4.54	4.06	3.54	-31.66
6:4	7.62	1.16	1.62	1.72	3.61	-52.62
8:2	6.24	2.36	4.10	1.98	1.20	-80.77
10:0	6.10	1.33	3.39	3.26	0.51	-91.64

DM= Dry Matter, GR= Glutinous Rice

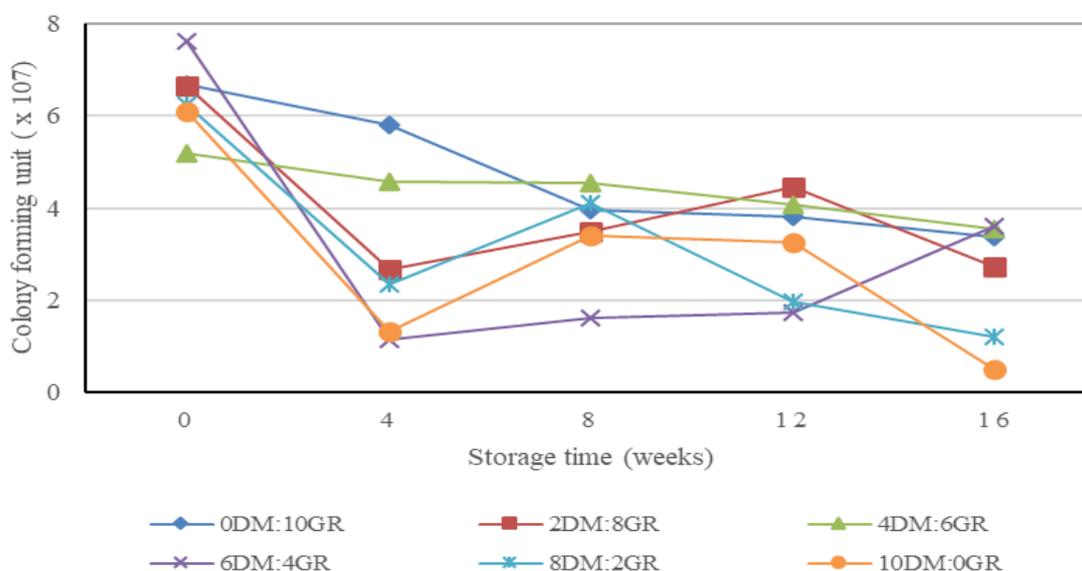


Figure 1. Availability of *Trichoderma* inocula in various combination ratios of dried mud (M) and glutinous rice (G) during storage

Table 2. Carboxymethyl cellulase activity (CMCase, μmol minute per minute glucose released) of *Trichoderma* inoculum in various combination ratios of dry mud (DM) and glutinous rice (GR) as carrier medium during storage

Ratio DM: GR	Storage Time (Weeks)							Changes (%)
	0	2	4	6	8	12	16	
0:10	0.014	0.047	0.038	0.014	0.040	0.003	0.040	185.71
2:8	0.015	0.013	0.031	0.034	0.016	0.014	0.026	73.33
4:6	0.012	0.020	0.018	0.014	0.029	0.025	0.020	66.67
6:4	0.025	0.030	0.027	0.019	0.033	0.018	0.045	80.00
8:2	0.021	0.031	0.031	0.028	0.041	0.031	0.036	71.42
10:0	0.015	0.017	0.016	0.004	0.017	0.012	0.011	-26.67

DM= Dry Matter, GR= Glutinous Rice

Table 3. The qualitative activity of carboxymethyl cellulase (ratio of clear zone: colony diameters) of *Trichoderma* inoculum in various combination ratios of dry mud (DM) and glutinous rice (GR) as carrier medium during storage

Ratio DM: GR	Storage Time (Weeks)					Average	Changes (%)
	0	2	4	6	16		
0:10	4.65	11.0	3.35	3.94	3.39	5.07	-12
2:8	4.74	6.39	3.44	3.76	3.71	4.79	42
4:6	5.07	6.43	3.46	4.62	3.58	4.93	26
6:4	4.93	4.35	3.21	4.16	3.50	4.14	-5
8:2	4.56	3.68	3.77	4.20	3.93	4.88	101
10:0	4.24	4.28	3.08	4.96	3.24	3.90	-16

DM= Dry Matter, GR= Glutinous Rice

CONCLUSION

The study concluded that the *Trichoderma* inoculum, using a combination of glutinous rice sludge as a carrier material, had a life force of 4.6, resulting in a glucose-releasing carboxymethyl cellulase (CMCase) activity of 0.020 μmol minute⁻¹ after 16 weeks of storage. Although this study focused on the inoculum's shelf life and enzyme activity, the findings indicate that an inoculum with a 4:6 ratio of dried mud to glutinous rice can be used for forage fermentation. The combination of stable spore viability and consistent cellulase activity provides a strong basis for developing *Trichoderma*-based inoculum carriers in feed biotechnology to improve forage quality and animal nutrition.

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