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The Potential of *Trichoderma AA1* and *AA2* as Local Indonesian Cellulolytic Inoculum in the Fermentation of *Gliricidia sepium* Leaf Meal

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The study aimed to test the cellulolytic ability of *Trichoderma* AA1 and AA2 inoculum as local Indonesian isolates and their application in the fermentation of *Gliricidia sepium* leaf meal. Measurement of cellulolytic ability includes qualitative cellulase activity (measurement of clear zone formation around fungal colonies) and quantitative cellulase activity (measurement of the amount of reducing sugar produced by broth enzymes from fungal cultures). Experiments of *Gliricidia sepium* fermentation using *Trichoderma* AA1 and AA2 were each designed using Completely Randomized Design. The treatments used consisted of fermentation duration of 0 (control), 2, 4, and 6 days—identification of *Trichoderma* AA1 and AA2 species using molecular identification method. The research results show that qualitative cellulase activities in *Trichoderma* AA2 were significantly higher compared to *Trichoderma* AA1. The CZD/CD ratio of *Trichoderma* AA1 and AA2 were 2.39±0.11 and 1.97±0.10, respectively. The CMC-ase activity of *Trichoderma* AA2 was 0.332±0.038 μmol/ml/min, and this was significantly higher compared to *Trichoderma*AA2, which was only 0.201±0.021 μmol/ml/min. *Gliricidia sepium* fermentation using AA1 and AA2 inoculum showed increased nutritional value, including soluble protein content, In-vitro digestibility of dry matter, and In-vitro digestibility of organic matter in 2 and 4 days of fermentation. There was a decrease in nutritional value in 6 days of fermentation. *Trichoderma* AA1 and AA2 were identified as *Trichoderma koningiopsis* and *Trichoderma asperellum,* respectively. The study concluded that *T. Koningiopsis* AA1 and *T. Asperellum* AA2 were proven to have cellulolytic abilities and could be used as inoculum in *Gliricidia sepium* fermentation to increase its nutritional value.
Keywords: *Trichoderma*, cellulase activity, molecular identification, in-vitro digestibility, fermentation.

INTRODUCTION

The study of cellulose-degrading microbes is becoming an increasingly important research focus in the context of improving the quality of animal feed (Sariri *et al.*, 2018; Sukaryani and Mulyono, 2019; Veloso *et al.*, 2023), including non-ruminant/poultry feed (Sun *et al.*, 2023). Cellulose is a glucose polymer with Β-(1,4) glucosidic bonds (Elsheekh *et al.*, 2022). Cellulose, together with hemicellulose and lignin, forms the structure of plant cell walls, and this biomass is an essential contributor to carbon residues on planet Earth (Onyia *et al.*, 2023).
The cellulase enzyme is a complex enzyme that synergizes with each other in hydrolyzing cellulose (Peláez *et al.*, 2022). The cellulase enzyme complex comprises endoglucanase, exoglucanase (Cellobiohydrolases, CBH), and β-glucosidase (Hewedy *et al.*, 2020). Hydrolysis of cellulose produces glucose (Awadalla *et al.*, 2017; Ismaiel *et al.*, 2022; Ramalingam and Revathi, 2022).
Legume leaves are a forage protein source with great potential as poultry feed. Unfortunately, the cell walls of legume leaves are composed of cellulose (Hewedy *et al.*, 2020; Onyia *et al.*, 2022). As is known, birds are unable to digest cell wall cellulose so that the nutrients in the cells are protected from attacks by poultry digestive enzymes (Mulyono *et al.*, 2021). Legume leaves' nutritive value is relatively low for poultry (Mulyono *et al.*, 2011).
Trichoderma is a microbe that has the potential to degrade legume leaf wall cellulose. Trichoderma is a genus of filamentous fungi known to have a high cellulolytic ability (Dong *et al.*, 2022; Onyia *et al.*, 2023; Silva *et al.*, 2023), which can degrade plant cell wall cellulose (Zayed *et al.*,

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2020; Undugoda and Kannangara, 2022), including legume leaves. The cellulolytic ability of *Trichoderma* is an exciting thing to study to increase the availability of legume leaf nutrients for poultry.

Characterization of the ability of *Trichoderma* inoculum to produce cellulase enzymes needs to be done before the inoculum is applied (Hewedy *et al.*, 2020; Elsheekh *et al.*, 2022; Ismaiel *et al.*, 2022; Dehghan *et al.*, 2023; Onyia *et al.*, 2023). It was done to ensure the effectiveness of *Trichoderma* inoculum application on the fermentation of cellulose-rich substrates (Koubová *et al.*, 2023; Veloso *et al.*, 2023), including *Gliricidia sepium* leaf meal (GSLM).

This study aims to test the cellulolytic ability of *Trichoderma* AA1 and AA2 inoculum as local Indonesian isolates and their application in the fermentation of *Gliricidia sepium* leaf meal (GSLM). Assessing local *Trichoderma* isolates becomes essential when the next isolate application is done in the same place. It is related to Trichoderma's adaptability to the local environment (Onyia *et al.*, 2023). The results of this research will provide the basis for innovative solutions regarding the potential of cellulolytic *Trichoderma* inoculum to increase the availability of legume leaf nutrients for poultry.

The subsequent impact will be to reduce dependence on imported feed ingredients. Apart from that, this research also has broader potential, namely to reduce the environmental effects through studying the processing of cellulose-rich biomass waste into a renewable energy source, bioethanol (Onyia *et al.*, 2022; Saini *et al.*, 2023) or decomposition of lignocellulosic biomass to reduce environmental pollution (Sahrawat and Garg, 2023; Saini *et al.*, 2023). In the field of plant cultivation, *Trichoderma* also has the potential to improve plant health, namely as a mycoparasitic biocontrol agent, biopesticide, and biofertilizer on plant roots (Hewedy *et al.*, 2020; Suyanto *et al.*, 2023).

MATERIALS AND METHODS

***Material preparation*:** The microbes used were two *Trichoderma* inoculum (AA1 and AA2). The inoculum was made by inoculating *Trichoderma* isolate spores on a PDA medium for seven days. The spores formed are separated from the PDA medium and mixed with a carrier material: 60% glutinous rice flour and 40% zeolite flour.

***Qualitative cellulase activities*:** Qualitative cellulase activity is determined by the ability of *Trichoderma* spores to grow and form a clear zone around the colony (Vázquez-Montoya *et al.*, 2020). *Trichoderma* AA1 and AA2 inoculum were each inoculated on a medium of 1% CMC agar (Rawway *et al.*, 2018). The composition of the medium includes Carboxy methyl cellulose (CMC) 10 g/l as the sole carbon source, Peptone 1.4 g/L, (NH4)2SO4 1.4 g/L, Urea 0.3 g/L, KH2PO4 2 g/L, CaCl2 0.3 g/L, MgSO4.7H2O 0.3 g/L, MnSO4.H2O 0.016 g/L, ZnSO4.H2O 0.014 g/L, CoCl2 0.014 g/L, FeSO4.7H2O 0.005 g/L, Triton X-100 0.1%, and agar 15 g/L (Mulyono, 2008). The culture was incubated at 30ºC for four days and then at 45ºC for 24 hours. After incubation, the fungal colonies in Petri dishes were flooded with 1% Congo Red solution for 15 minutes and then rinsed for 5 minutes using 1N NaCl (Castrillo, 2020). Cellulase activity was determined by measuring the apparent zone diameter (CZD) and colony diameter (CD) and then calculating the CZD / CD ratio (Talantan *et al.*, 2018).

***Quantitative cellulase activity*:** Quantitative cellulase activity was determined by measuring Carboxy methyl cellulase (CMC-ase) activity. *Trichoderma* AA1 and AA2 inoculum were each inoculated in a liquid medium containing 1% CMC as the sole carbon source. The composition of the medium includes Carboxy methyl cellulose (CMC) 10 g/l as the sole carbon source, Peptone 1.4 g/L, (NH4)2SO4 1.4 g/L, Urea 0.3 g/L, KH2PO4 2 g/L, CaCl2 0.3 g/L, MgSO4.7H2O 0.3 g/L, MnSO4.H2O 0.016 g/L, ZnSO4.H2O 0.014 g/L, CoCl2 0.014 g/L, FeSO4.7H2O 0.005 g/L (Mulyono, 2008). The culture was incubated using a rotary shaker at 30ºC for four days (Onyia *et al.*, 2022). The culture was centrifuged at 5,000 rpm, 4ºC for 15 minutes. The resulting supernatant (crude enzyme) was then used for CMC-ase measurements. CMC-ase activity was assayed by measuring the release of reducing sugars in a reaction mixture containing 1 mL of broth enzyme, 1 mL of 1% CMC as substrate, and 1 mL of cytric buffer 50 mM (pH 4.8) 1 at 45°C for 60 min. Reducing sugars released were assayed by the dinitrosalicyclic acid (DNS) method (Miller, 1959).

***Molecular identification of Trichoderma*:** Identification of *Trichoderma* AA1 and AA2 species refers to (Larasati et al., 2021). Genomic DNA extraction with Quick-DNA Magbead Plus Kit (Zymo Research, D4082). PCR amplification with MyTaq HS Red Mix, 2X (Bioline, BIO-25048). DNA sequencing uses the Bi-directional Sequencing technique. The sequences obtained were then matched to the GenBank database using the Basic Local Alignment Search Tool (BLAST) at https://blast.ncbi.nlm.nih.gov/Blast.cgi. Phylogenetic trees are created by aligning all sequences and their comparison sequences using the Neighbor-Joining approach by the NCBI Blast Tree Method.

***Trichoderma inoculum test on Gliricidia sepium leaf meal fermentation*:** Experimental design. The experiment was GSLM fermentation, with the treatments used being fermentation durations of 0 (control), 2, 4, and 6 days. The experiment was designed using a Completely Randomized Design (CRD). Each treatment used three replications of the experimental unit. The microbial inoculum used in the fermentation was *Trichoderma* AA1 and *Trichoderma* AA2, which were tested separately.

Inoculum preparation. Four ml of molasses was dissolved in 120 ml of distilled water in a 500 ml Erlenmeyer flask, sterilized in an autoclave at a pressure of 15 PSI for 15 minutes, then cooled to room temperature. Add 4 g of

Trichoderma inoculum to the Erlenmeyer flask, then incubate aerobically for 24 hours on an incubator shaker.

Fermentation of *Gliricidia sepium* leaves. Four hundred g of *Gliricidia sepium* leaf meal (GSLM) flour was sterilized in an autoclave at a pressure of 15 PSI for 15 minutes. After cooling, mix 400 g of GSLM evenly with 120 ml of inoculum solution and 400 ml of sterile distilled water, then put it into the bioreactor from a sterile stainless steel tray. The culture was incubated aerobically for six days. At 0, 2, 4, and 6 days of fermentation, around 30 g of fermented GSLM biomass was sampled. The samples were stored in a vacuum pack and then tested for the content of soluble protein, crude fibre, In-vitro digestibility of dry matter (IVDDM), and In-vitro digestibility of organic matter (IVDOM). The measurement method for each test parameter includes soluble protein (Sudarmadji *et al.*, 1997), crude fibre (AOAC, 2005), IVDDM and IVDOM (Tilley and Terry, 1963)

RESULTS

***Cellulase activity*:** Qualitative and quantitative cellulase activity of *Trichoderma* AA1 and AA2 isolates showed significant differences $p<0.05$, presented in Table 1.

Clear Zone Diameter (CZD) produced by *Trichoderma* AA2 26.5±0.45 mm is more extensive than *Trichoderma* AA1 23.4±0.76 mm. Likewise, the CZD/CD Ratio and CMC-ase Activity of *Trichoderma* AA2 are higher than *Trichoderma* AA1. The CZD/CD ratio of AA1 is 1.85±0.10, and AA2 is 2.39±0.11 then CMC-as AA1 is 0.201±0.021, and AA2 is 0.332±0.038. However, the colony diameter of AA2 is larger than that of AA1.

Table 1. Cellulase activity variables from *Trichoderma* AA1 and AA2 isolates.

Cellulase activity variables	Isolates		P *
	AA1	AA2	
Qualitative activity:			
Clear zone diameter (CZD, mm)	23.4±0.76	26.5±0.45	0.003
Colony diameter (CD, mm)	13.1±0.77	11.3±0.58	0.132
CZD/CD ratio	1.85±0.10	2.39±0.11	0.002
Quantitative activity:			
CMC-ase (μmol/ml/min)	0.201±0.021	0.332±0.038	0.002

* $P < 0.05$ indicates a significant difference.

***Molecular identification*:** Based on the results of the Internal Transcribed Spacer (ITS) Analysis in Table 2 and Fig. 1. *Trichoderma* AA1 was identified as *Trichoderma koningiopsis* with a similarity range value (99.83%- 100%), and *Trichoderma* AA2 was identified as *Trichoderma Asperellum* with a similarity value of 100%.

***Gliricidia sepium leaf flour fermentation*:** The fermentation test in Table 3. showed a significant change $p<0.05$ in pH, soluble protein content, and crude fibre. The fermentation pH decreased consecutively from 4.00 to 3.00 on the 6th day in AA1 and AA2. The soluble protein content increased consecutively in AA1 from 7.49% on day 0 to 8.50% on day six, while in AA2, 7.46% on day 0 to 8.44% on day 6.

Table 2. Test results for similarity of *Trichoderma* AA1 (left) and AA2 (right) sequences with other fungal isolates based on Internal Transcribed Spacer (ITS).

No.	Comparative fungal isolates	Query Coverage (%)	Percentage Identity (%)	No.	Comparative fungal isolates	Query Coverage (%)	Percentage Identity (%)
1	*Trichoderma koningiopsis* isolate Tk905	100	100.0	1	*Trichoderma sp*. isolate SDAS203443	100	100
2	*Trichoderma koningiopsis* strain 323N1	100	100.0	2	*Trichoderma asperellum* strain IIPRCPT-94	100	100
3	*Trichoderma sp*. strain ZQMRS9	100	99.83	3	*Trichoderma asperellum* US strains	100	100
4	*Trichoderma sp*. isolate yi1440_1	100	99.83	4	*Trichoderma asperellum* isolate TR24	100	100
5	*Trichoderma sp*. ovalisporum isolate CTCCSJ-W-QT22094	100	99.83	5	*Trichoderma asperellum* strain ACCC32915	100	100
6	*Trichoderma sp*. OTU003 AN-2016	100	99.83	6	*Trichoderma asperellum* strain ACCC32913	100	100
7	*Trichoderma sp*. Isolate F17T2IIIA	100	99.83	7	*Trichoderma asperellum* strain ACCC32912	100	100
8	*Trichoderma koningiopsis* isolate XXTF5	100	99.83	8	*Trichoderma asperellum* strain ACCC32892	100	100
9	*Trichoderma koningiopsis* U strain	100	99.83	9	*Trichoderma sp*. isolate yi1255_1	100	100
10	*Trichoderma koningiopsis* strain NECC30330	99	99.83	10	*Trichoderma asperellum* isolate T337	100	100

Table 3. Results of inoculum testing of *Trichoderma koningiopsis* AA1 and *Trichoderma asperellum* AA2 in fermentation of Gliricidia sepium leaf meal.

Test variables	Inoculum	Fermentation time			
		0 day (unfermented)	2 days	4 days	6 days
pH of fermentation	AA1	4.00^{b}	3.67^{ab}	3.67^{ab}	3.00^{a}
	AA2	4.00^{b}	4.00^{b}	3.33^{a}	3.00^{a}
Soluble protein (%)	AA1	7.49^{a}	7.55^{a}	7.75^{a}	8.50^{b}
	AA2	7.46^{a}	7.36^{a}	8.43^{b}	8.44^{b}
Crude fiber (%)	AA1	23.04^{a}	23.77^{ab}	26.69^{c}	24.80^{b}
	AA2	22.79^{a}	22.85^{a}	26.08^{b}	26.31^{b}
IVDDM (%)	AA1	64.27^{b}	65.63^{c}	56.40^{a}	50.76^{a}
	AA2	54.45^{b}	63.65^{c}	57.86^{a}	56.20^{a}
IVDOM (%)	AA1	59.87^{b}	61.15^{c}	50.35^{a}	50.76^{a}
	AA2	49.42^{a}	59.32^{c}	51.69^{b}	49.28^{a}

^{ab} on the same line shows a significant difference (P<0,05)

AA1= *Trichoderma koningiopsis* AA1, AA2= *Trichoderma asperellum* AA2, IVDDM= *In-vitro* digestibility of dry matter, IVDOM= *In-vitro* digestibility of organic matter.

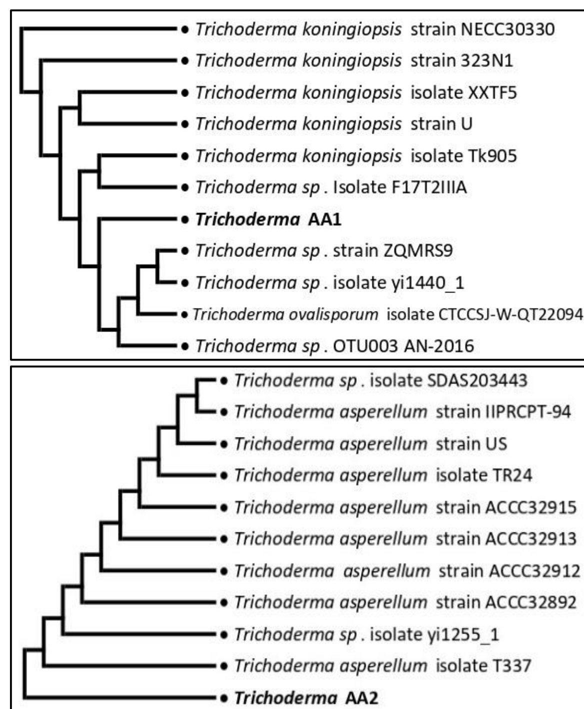

Figure 1. *Trichoderma* AA1 (left) and AA2 (right) phylogenetic tree based on Internal Transcribed Spacer (ITS) analysis.

The crude fibre content increased with increasing fermentation time; AA1, initially 23.04%, increased on the 2nd day to 23.77% and the 4th day to 26.69%, then decreased on the 6th day to 24.80%. In contrast to AA2, crude fibre increased consecutively from 22.79% to 26.31% on the 6th day. The increase in in-vitro dry matter digestibility and in-vitro organic matter digestibility occurred in fermentation for two days but decreased in fermentation for 4 and 6 days.

DISCUSSION

***Cellulase activity*:** Cellulase activity shows how much the cellulase enzyme can hydrolyze cellulose compounds into reducing sugar. Both qualitative and quantitative cellulase activity values for the cellulase enzyme produced by *Trichoderma* AA1 and AA2 isolates showed significant differences (Table 1).

The qualitative and quantitative cellulase enzyme activity measurement results have the same trend. The Trichoderma AA2 isolate had cellulase enzyme activity significantly higher than *Trichoderma* AA2. It shows that *Trichoderma* AA2 has a higher cellulolytic ability than *Trichoderma* AA1. It should be noted that *Trichoderma* AA1 was isolated from the soil around the roots of pine trees, while *Trichoderma* AA2 was from the soil around the bamboo roots. Pine and bamboo trees have different fibre compositions. Pine cellulose is associated with lignin (Wu *et al.*, 2020; Yang *et al.*, 2023), while bamboo cellulose is associated with silica (Runge and Paul, 2015). Lignin makes the structure of the wood cell walls in trees solid and stiff (Funda *et al.*, 2020). Bamboo cell walls contain lower lignin than pine trees but have a higher silica content, making them more flexible. This difference in cell wall structure may cause differences in the cellulase activity of *Trichoderma* AA1 and *Trichoderma* AA2.

Trichoderma is a filamentous fungi with great potential in cellulolytic abilities (Dong *et al.*, 2022; Onyia *et al.*, 2023; Silva *et al.*, 2023). The cellulase enzyme complex from Trichoderma can degrade cellulose, the main component of plant cell walls (Elsheekh *et al.*, 2022). The cellulase enzyme complex comprises endoglucanase, exoglucanase (Cellobiohydrolases, CBH), and β-glucosidase (Hewedy *et al.*, 2020). Hydrolysis of cellulose produces glucose (Awadalla *et al.*, 2017; Ismaiel *et al.*, 2022; Ramalingam and Revathi, 2022). Cellulose is an antinutrient for poultry

(Mulyono *et al.*, 2023), so hydrolysis of forage cellulose is a way to increase the nutritive value of forage for poultry.

Trichoderma, locally sourced from tropical regions, has been widely studied and shown encouraging results as a biocontrol agent, compost decomposer, and plant growth promoter (Hewedy *et al.*, 2020). However, research on the cellulolytic ability of local tropical *Trichoderma* as an inoculum to improve the quality of forage for livestock, especially the leaves, is still very limited. For this reason, the results of this research will make a major contribution to the development of forage for livestock in the future.

***Qualitative cellulase activity*:** The clear zone diameter (CZD) value is an expression of the ability of the microbial colony to form a clear zone, which is the area of cellulose in the agar medium that has been degraded by the cellulase enzyme (Elsheekh *et al.*, 2022). The longer the diameter of the clear zone, the higher the microbial cellulase activity. This research shows that the CZD formed by *Trichoderma* AA1 is significantly lower (P=0.003) than the CZD formed by Trichoderma AA2 (Table 1). This indicates that the *Trichoderma* AA2 isolate produces cellulase enzymes with more significant activity than Trichoderma AA1. Trichoderma's ability to produce cellulase is not only determined by the CZD variable. Another variable that needs to be considered is the size of the *Trichoderma* colony that produces the cellulose. Variable colony diameter (CD) is the ability of *Trichoderma* to reproduce and form colonies. The longer the colony diameter means, the higher the ability of *Trichoderma* to reproduce. This study showed that the CD formed by Trichoderma AA1 and Trichoderma AA2 was not significantly different (P=0.132) (Table 1). It shows that the number of *Trichoderma* AA1 and *Trichoderma* AA2 fungal cells producing the cellulase enzyme is relatively the same.

The CZD/CD ratio variable will be more appropriate for determining the magnitude of the cellulase complex enzyme activity (Mulyono, 2008). This variable expresses cellulase activity per unit of fungal isolate. The higher the CZD/CD ratio, the higher the cellulase activity. The results of this study show that the CZD/CD ratio of *Trichoderma* AA2 forms is very significantly (P=0.002) higher than that of *Trichoderma* AA1 with mean CZD/CD ratio values of 2.39 ± 0.11 mm and 1.85 ± 0.10 mm respectively. (Table 1). It shows that *Trichoderma* AA2 can produce cellulase enzymes with higher activity.

***Quantitative cellulase activity*:** The results shown by the CZD/CD ratio variable can only detect cellulase activity qualitatively. For this reason, variables that can display data on how much cellulase enzyme activity can degrade cellulose quantitatively are needed. One variable that can be used is Carboxy Methyl Cellulase (CMC-ase) activity. This CMC-ase variable expresses the amount of reducing sugar (glucose equivalent) produced from the activity of the cellulase enzyme (Elsheekh *et al.*, 2022; Sahrawat and Garg, 2023). The average CMC-ase activity of Isolate *Trichoderma* AA2 was 0.33 µmol/ml/min, significantly higher than Trichoderma AA2, which was only 0.20 µmol (Table 1).

***Molecular identification of Trichoderma*:** Based on molecular identification using the Internal Transcribed Spacer (ITS), a phylogenetic tree was formed, as shown in Figure 1. This tree depicts the evolutionary relationships and kinship relationships between *Trichoderma* AA1 and AA2 and various other species or isolates.

Figure 1 shows that *Trichoderma* AA1 is related to 10 comparative fungal isolates from the NCBI database. Of the ten comparative fungal isolates, there were 5 *Trichoderma koningiopsis*, 4 *Trichoderma* sp., and one species of *Trichoderma ovalisporum*. *Trichoderma* AA2 is related to 10 comparative fungal isolates from the NCBI database. Of the ten comparative fungal isolates, there were 8 *Trichoderma asperellum* and two species *Trichoderma* sp.

Based on the DNA sequence similarity test of *Trichoderma* AA1 and AA2 with ten comparative fungal isolates can be seen in Table 2. Query coverage is the percentage of the length of the query sequence (*Trichoderma* AA1) with the target sequence (comparison fungus) (Larasati *et al.*, 2021). If the query sequence covers all target sequences (from the NCBI database), then the query coverage is 100%. From (Table 2) it can be seen that the query coverage value for *Trichoderma* AA1 sequences against nine comparison fungi is 100%. Meanwhile, the query coverage value for the *Trichoderma* AA1 sequence against the remaining comparison fungus was 99%. It shows that the *Trichoderma* AA1 sequence is very similar to the sequences of 10 comparison fungi. Percentage identity shows the similarity between the query sequence and target sequence. For microorganisms, it uses 16srRNA primers, similar to species level (above 97.5%) and genus level (above 95%) (Goebel and Stackebrandt, 1994). From Table 2, it can be seen that the percentage identity of the *Trichoderma* AA1 sequence to the sequences of the two comparison fungi is 100%, namely *Trichoderma koningiopsis* isolate Tk905 and *Trichoderma koningiopsis* strain 323N1. Meanwhile, the percentage identity of the *Trichoderma* AA1 sequence to the sequences of 8 other comparison fungi was 99.83%. It shows that *Trichoderma* AA1 has a very high degree of similarity (to 8 comparison fungi) and even has perfect similarity to 2 other fungi.

Test the similarity of *Trichoderma* AA1 to 10 comparison fungi, with an expectation value (E value) of 0.0. E value is a number that describes how many times you would expect a match by chance in a database of that size. The lower the Evalue is, the more significant the match. By considering the query parameters coverage, percentage identity, and E value, it can be indicated that *Trichoderma* AA1 is a species of *Trichoderma koningiopsis*.

From (Table 2) it can be seen that the query coverage value and percentage identity of the Trichoderma AA2 sequence for the ten comparison fungi is 100%. It shows that *Trichoderma*

AA2 has a perfect degree of similarity to the ten comparison fungi. This result is supported by an E value of 0.0. By considering the query parameters coverage (all 100%), percentage identity (all 100%), and E value (all 0.0) and the types of comparative fungal species (8 species of *Trichoderma asperellum* and two species of *Trichoderma* sp.), it can be indicated that Trichoderma AA2 is a species of *Trichoderma asperellum.*

***Test of Trichoderma Inoculum on Gliricidia sepium leaf meal fermentation*:** The results of the AA1 and AA2 inoculum tests on *Gliricidia sepium* leaf meal (GSLM) fermentation showed significant changes (P<0.05) in all test variables (Table 3). The pH variable decreased while the soluble protein and crude fibre content increased. The IVDDM and IVDOM variables showed increased fermentation for two days, then decreased when the fermentation period was increased to 4 and 6 days.

The decrease in pH value of GLSM fermentation using AA2 inoculum was faster than that using AA1 inoculum. The use of AA1 inoculum resulted in a significant reduction in pH in 6 days of fermentation while using AA2 inoculum resulted in 4 days of fermentation. The decrease in pH occurred due to the formation of organic acids in the fermentation process (Tu *et al.*, 2024). This decrease in pH was also found (Mulyono *et al.*, 2018), who carried out fermentation on cassava bagasse.

The increase in soluble protein content occurred faster in GLSM fermentation using AA2 inoculum (4-day fermentation) compared to AA1 inoculum (6-day fermentation). As is known, *Trichoderma* fermentation on cellulose-rich materials, including GLSM, will degrade cell wall cellulose so that proteins that were previously protected in the cells are freed (Mulyono *et al.*, 2023). This phenomenon can be a reference for why GLSM fermentation using Trichoderma can increase soluble protein content.

Changes in crude fibre content in GLSM fermentation showed different patterns between AA1 and AA2 inoculum use. The use of AA1 and AA2 increased crude fibre content in 4-day fermentation. However, in 6-day fermentation, there was a difference in the pattern; namely, the use of AA2 inoculum decreased crude fibre, while in AA2, there was no decrease. The increase in crude fibre occurs due to the formation of microbial cell wall components (Garcia-Rubio *et al.*, 2020). The decrease in crude fibre that occurs when the fermentation period is increased to 6 days is due to the increasing number of GLSM cell wall components that experience degradation (Zexer *et al.*, 2024).

The pattern of changes in IVDDM and IVDOM values in GLSM fermentation using AA1 and AA2 inoculums showed similarities. IVDDM and IVDOM values increased in 2-day fermentation and decreased in 4- and 6-day fermentation.

Looking at the pattern of value changes in all variables, GLSM fermentation using AA1 and AA2 inoculums showed increased nutritive quality in 2 and 4 days of fermentation. Fermentation for six days indicates that fermentation is too long, marked by decreased nutritive value in all variables. The best fermentation time is 2 or 4 days, depending on the purpose of applying fermented GLSM. If GLSM fermentation is intended as feed, then two days of fermentation is more appropriate because there is an increase in the digestibility of fermented GLSM.

***Conclusion*:** The study concluded that *T. koningiopsis* AA1 and *T. asperellum* AA2 were proven to have cellulolytic abilities and could be used as inoculum in GSLM fermentation to increase its nutritive value. In addition, it can be a decomposer agent for processing cellulose-rich biomass into renewable energy sources.

***Authors' contributions*:** A. M. W. Mulyono, M. Husein designed, completed the experiments; S. Sukaryani, A. K. Sariri, E. A. Yakin prepared the draft; L. Windyasmara, N. H. Qui reviewed and finalized the draft.

***Conflict of Interest*:** The authors declare no conflict of interest.

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