

Isolation and characterization of cellulolytic fungi from fresh solid waste from cattle farms in the pre-decomposition phase at cattle farm

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Abstract

Solid waste from cattle farming is a potential source of lignocellulose biomass for further utilization. The research conducted aims to isolate and identify cellulolytic fungi of fresh solid waste from cattle farms in the pre-decomposition phase. With qualitative descriptive methods (sampling, isolation, cellulolytic activity test, morphological, microscopic, physiological characterization, as well as pH and temperature variation tests on fungal growth), 4 cellulolytic fungi isolates were obtained that were able to hydrolyze cellulase, namely NFS1, NFS2, NFS3, and NFS4, with the highest cellulolytic index in NFS4 fungi which was 1.86 and the lowest, NFS1 0.65. Based on the morphological character and type of isolate spores NFS1 and NFS3 are suspected to be from the genus *Curvularia* sp., NFS2 comes from the genus *Penicillium* sp., and NFS4 comes from the genus *Aspergillus* sp. The 4 fungi are known to be tolerant to pH 3-7 and can. It grows well at 25°C, but is not capable of growing at 40°C

Keywords: Cattle Farm; Cellulolytic Fungi; Cellulase; Isolation; Solid Waste

1. Introduction

The livestock sector plays a crucial role in meeting the animal protein needs of the community, which has triggered the rapid growth of various business units in this field. A beef cattle farming company that has grown significantly in Lampung Province. The rapid growth of this industry is in line with data from the Central Statistics Agency (BPS) through the 2019 Data Collection of Beef Cattle, Dairy Cattle, and Buffaloes (PSPK), which recorded that the beef cattle population in Indonesia has reached 14.8 million heads. This figure shows a considerable increase when compared to the results of the 2011 Agricultural Census which at that time amounted to 10.2 million heads. Cumulatively, the beef cattle population during the 2011-2019 period experienced an average growth of 5.32% per year, or equivalent to an addition of around 653.1 thousand heads every year. However, this massive increase in population brings environmental challenges that are directly proportional to the amount of waste generated. The greater the number of beef cattle, the greater the volume of waste collected.

The cellulose present in cow manure can be broken down by microbes that produce the enzyme cellulase. One of the organisms that is able to play a role in the cellulose degradation process is the cellulolytic fungi. Subowo, (2015) stated that some species of microfungi are able to decompose lignin, cellulose and hemicellulose. Previous research has shown that the application of cellulolytic fungi can improve the water-holding ability of fungi-inoculated samples and balance the C:N ratio of cellulose waste (Hart *et al.*, 2002; Sivaramanan, 2016). Types of fungi that have the ability to degrade cellulose including *Trichoderma reesei*, *Aspergillus niger* and *Penicillium* (Elfiati *et al.*, 2019; Li *et al.*, 2021).

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Cattle waste contains abundant lignocellulose biomass, making it a very potential habitat for the growth of cellulose-breaking enzyme-producing cellulose-breaking fungi. Although it has enormous potential, the characterization and identification of the types of cellulolytic fungi found in the fresh solid waste has never been done until now. Therefore, considering the importance of the role of fungi in supporting the efficiency of organic solid waste treatment, this research is considered necessary to be conducted.

2. Methods

This research was carried out at the Botanical Laboratory and Microbiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Lampung.

2.1. Tools and Materials

The tools used in this study include Erlenmeyer's flask, test tubes, measuring cups, glass funnels, petri dishes, oyster needles, bunsen, knives, plastic *wrap*, ovens, analytical balances, autoclaves, microscopes, *laminar air flow*, aluminum foil, tissues, filter paper, incubators with *shakers*, pH meters, *vortexes*, micropipettes, and microtips, sterile spoons, hoes.

The ingredients used in this study include soil samples, 70% alcohol, *congo red*, aquaade, CMC, $MgSO_4$, KNO_3 , K_2HPO_4 , $CaCl_2$, yeast extract, agar powder, NaCl, HCl, NaOH, and spiritus.

2.2. Solid Waste Sampling

The sampling procedure was carried out three times at a depth of 5 to 10 cm from the surface taken from 1 location point. Sampling was carried out by applying the aseptic principle, namely by self-sterilizing using 70% alcohol and using gloves. The sampling location was initially dug with the help of a hoe, then the sample was taken using a tool in the form of a sterile spoon and put in sterile heat-resistant plastic to be then taken to the laboratory using a cool box.

2.3. Radiant Dilution

The waste sample was weighed 5 g and put into an erlenmeyer containing 45 mL of physiological NaCl (0.9%) sterile and then homogenized by shaking it as a 10⁻¹ dilution series. 1 mL is taken from 10⁻¹ dilution using a micropipette and then put into a test tube containing a 9 mL physiological solution, then homogenized using a *vortex* until a 10⁻² dilution is obtained. The working procedure is carried out up to a dilution level of 10⁻³ using the same method.

2.4. Cellulolytic Fungi Isolation

The insulation method used is *the pour plate* method. A total of 1 mL was taken from the 10⁻² and 10⁻³ dilution using a micropipette then each dilution series was inoculated into a petri dish and a medium was added so that *Carboxy Methyl Cellulose* (CMC) + *Mendels Media* (1 g CMC, 0.04 g $MgSO_4$, 0.15 g KNO_3 , 0.1 g K_2HPO_4 , 0.004 g $CaCl_2$, 0.4 g *Yeast Extract*, 3 g of agar, and 100 mL of aquades. then incubated for 5 × 24 hours in an incubator with a temperature of 25°C (Sutari, 2020).

2.5. Purification of Cellulolytic Fungi

Purification was carried out on each fungal colony that had been grown on CMC media for 7 × 24 hours. Then the colonies of fungi that are formed are selected and differentiated based on macroscopic morphology which can be seen from the appearance of color, shape, and distribution patterns of the colony. One colony from each of the selected fungi colonies was taken as many as 1 ose and scratched on the surface of the CMC media using the point method. Cultures that have been purely incubated at a room temperature of 27°C for 3 × 24 hours (Vasundhara *et al.*, 2019; Sibero *et al.*, 2018). The single cellulolytic fungi colony obtained was then inoculated as many as 1 ose in the slanted *Potato Dextrose Agar* (PDA) media as a stock.

2.6. Cellulolytic Enzyme Activity Test

Each fungal isolate that had been obtained was recultured in CMC media, then incubated at room temperature for 3 × 24 hours to determine the ability of fungi to degrade cellulose (Rudiansyah *et al.*, 2017). In order to make the formation of clear zones clearer, staining was carried out using a 0.1% *congo red* solution which was watered and left for 15 minutes, then discarded the remaining 0.1% *congo red* solution and then rinsed with 1 M NaCl. Observed the clear zones formed around the colony as a positive result, then the Cellulolytic Index (IS) was determined.

2.7. Determination of the Cellulolytic Index

The magnitude of enzymatic activity is determined by measuring the area of bacterial colonies and the area of clear zones formed using the gravimetric method according to Sumardi et al., 2018. The area of each colony and the area of the visible clear zone were calculated using the measurement of the colony diameter and the diameter of the clear zone and the gravimetric method.

The gravimetric method is carried out using the following formula:

$$\text{Luas Koloni} = \frac{\text{Bobot Replika Koloni}}{\text{Bobot Kertas } 1\text{cm} \times 1\text{cm}} \times 1\text{cm}^2$$

After obtaining the value of the colony area, the enzymatic index is then calculated. In the determination of the enzymatic index, it is calculated using the following formula

$$\text{Indeks Enzimatik} = \frac{\text{Luas Zona Jernih} - \text{Luas Koloni}}{\text{Luas Koloni}}$$

2.8. Characterization of Isolation

Isolation characterization is carried out in two ways, including

2.8.1. Macroscopic Characterization of Cellulolytic Fungi

Fungi isolate that have been aged 7 × 24 hours are directly observed in their morphology including the shape of the colony, the color of the colony on the surface (*above*), and the reverse of the colony, surface texture, type of mycelium, elevation and diameter of the colony (Gusmiaty, 2020).

2.8.2. Microscopic Characterization of Cellulolytic Fungi

Microscopic observation of cellulolytic mushroom isolates was carried out by slide culture method (Valencia and Meitiniarti, 2017). Slide culture is made by means of a sterile petri dish containing filter paper on which a flat glass object and an *object glass* holding rod have been placed. PDA media is aseptically dripped on a flat glass object then the fungal isolate is taken using an ose needle and cultured on CMC media. Glass cover is used to cover the mushroom culture media on the *object glass*. Sterile aqueducts are sufficiently dripped on filter paper to keep moisture at bay. The petri dish is incubated at room temperature for 3-5 days. Fungal cultures that have grown on *slide culture* are then observed with a microscope by means of a *glass cover on the slide culture* taken and placed on a flat glass object that has previously been dripped with a lactophenol *cotton blue solution*.

2.9. Selectivity Test of Acidity Level (pH) of Media and Storage Temperature on the Growth of Cellulolytic Mushroom Diameter

First, CMC media is prepared, homogenized on a *magnetic stirrer*, and the pH of the medium is measured using a pH meter. The pH of the medium is regulated by adding 0.5 M of HCL to lower the pH of the medium and adding 0.5 M NaOH to lower the pH of the medium. After obtaining the desired media pH, the CMC media is sterilized on an autoclave with a temperature of 121°C at a pressure of 1 atm for 15 minutes. Then the media is poured into a 9 cm diameter petri dish. Cellulolytic fungi isolate is taken using the help of an ose needle and inoculated in the center of the petri dish. After that, the petri dish is covered with *plastic wrap* to prevent contamination. Celelolytic fungi are incubated at a temperature of 25°C (room temperature) and a temperature of 40°C (inside an incubator). During storage, the growth of cellulolytic fungi diameter was observed (Syamsulhadi et al, 2023).

Observation of fungal growth was carried out by calculating the diameter of fungi on CMC media vertically and horizontally using a ruler. The formula for measuring the growth of fungal diameter is carried out by using the calculation method by Suseno et al. (2016) namely:

$$D = \frac{d1+d2}{2}$$

Description

- D. = diameter fungi
- d1. = vertical diameter of fungi

- d2. = diameter horizontal fungi

2.10. Data Analysis

Data obtained from isolation, characterization and physiological, macroscopic and microscopic identification of fungi were analyzed and elaborated by comparing the results of visualization with existing references. In the enzymatic activity test was carried out by observing the clear zones formed and determining the enzymatic index. Meanwhile, in the selectivity test, the acidity level (pH) and temperature of the medium on the growth of fungi were carried out by comparing the area of the diameter produced.

3. Results and discussion

Based on the results of isolation and selection of cellulolytic fungi from fresh solid waste from cattle farms, 4 fungal isolates were obtained that were able to produce clear zones in CMC media, each of which was coded NSF1, NSF2, NSF3, and NSF4 isolates. (Figure 1).

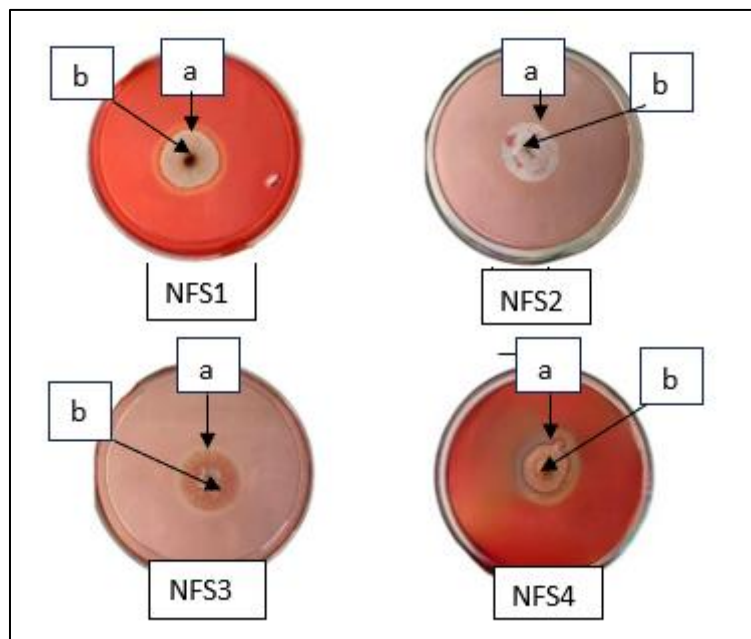


Figure 1 Results of Cellulolytic Fungi Isolation. a. clear zone; b. fungal colonies

The data from the calculation of the IS (cellulolytic index) of each isolate showed varying results. A total of 2 fungi isolates had moderate IS values, namely NSF2 and NSF4, while fungi isolate with isolate codes NSF1 and NSF3 were classified as low (Table 1).

Table 1 Clear Zone Area, Colony Area, and Cellulolytic Index Values of Cellulolytic Fungi Isolate

Isolation Code	RLz(cm2)	RLk(cm2)	IS
NSF1	0.66	0.4	0.65
NSF2	1.4	0.63	1.20
NSF3	1	0.56	0.78
NSF4	0.86	0.3	1.86

3.1. Macroscopic and Microscopic Characteristics of Cellulolytic Fungi Isolate

Fungi characterization was carried out on the four cellulolytic fungi isolates, namely NSF1, NSF2, NSF3, and NSF4 by observing the morphology of the fungal colonies macroscopically. Based on the results of the characterization of the

four cellulolytic fungi isolates found in fresh solid waste of cattle farms have different morphological characteristics shown in Table 2 with reference to the book Illustrated Genera of Imperfect Fungi (Barnett and Hunter, 1998) and other supporting literature.

Table 2 Macroscopic and Microscopic Characterization of Cellulolytic Fungi Isolates

Character	Isolation Code			
	NFS1	NFS2	NFS3	NFS4
Karaker Macroscope				
Shape	Round	Irregular	Round	Irregular
Surface color	Chocolate	Putih	Chocolate	Greenish white
Colony back color	Dark brown	Yellow	Dark brown	Yellowish white
Margin	<i>Entire</i>	<i>Entire</i>	<i>Entire</i>	<i>Entire</i>
Texture	Cotton	Cotton	Kapas	Belundru
Elevasi	<i>Raised</i>	<i>Umbonate</i>	<i>Raised</i>	<i>Umbonate</i>
Thought	<i>Aerial</i>	<i>Immerse</i>	<i>Aerial</i>	<i>Aerial</i>
Microscopic Characters				
Hifa	Partition	Bersepta	Partition	Bersepta
Tipe Konidiofor/sporangiospor	Branching	Single	Branching	Single
Forms of codydia/sporangium	Elliptical and bulky	Round	Elliptical and bulky	Round

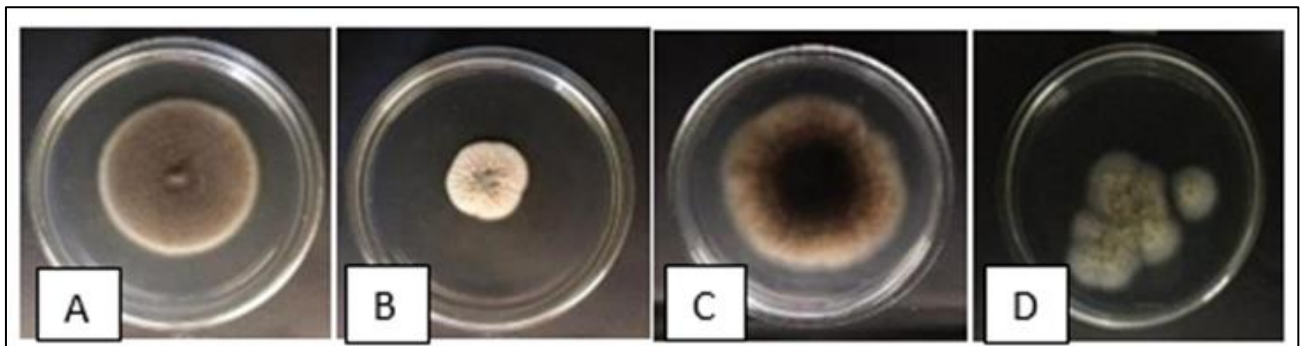
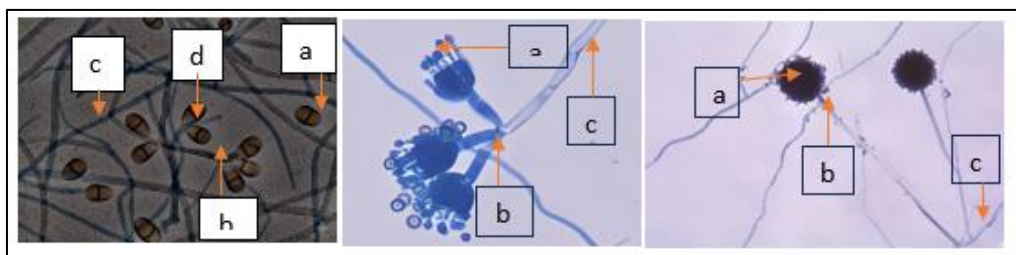


Figure 2 Macroscopic morphology of the upper surface of the isolate colony of cellulolytic fungi NFS1; B. NFS2; C. NFS3, D. NFS4



Description a : konidia; b : konidiofor; c : hifa; e : septa in conidia

Figure 3 Results of Microscopic Acidification of Cellulolytic Fungi Isolate with Magnification 400x: A. NFS1; B. NFS2, C. NFS4

Based on the results of the macroscopic and microscopic characterization of cellulolytic fungi isolates presented in Table 4., morphological variations between isolates reflect differences in colony character and microscopic structure. Such morphological variations are used as an initial basis for morphologically grouping fungi, referring to the key to identification described by Barnett and Hunter in *Illustrated Genera of Imperfect Fungi*.

Macroscopic characterization was carried out through observation of colony shape, surface color and colony back color, margin, texture, elevation, and mycelium growth type. The results of the observations showed that NFS1 and NFS3 isolates had similar morphological characters to fungi of the genus *Curvularia*. The two isolates form a round-shaped colony with a surface color of brown to blackish-brown, cotton-like texture, *entire-type* margins, raised elevation, and aerial-type mycelium. The color of the colony appears black-brown.

Microscopically, NFS1 and NFS3 isolates show septated hyphae, branched conidiophores, and elliptical and blocked conidia. This character corresponds to the description of *Curvularia* according to Barnett and Hunter which is characterized by insulated, pigmented, elliptical to curved conidia, as well as simple conidiophores that can branch. The similarity of these characters is also supported by the research of Wahidah *et al.* (2021) which states that *Curvularia* sp. has blackish-gray to dark brown colonies with a cotton-like texture and insulated conidia. *Curvularia* sp. is known to have the potential to produce antimicrobial, antioxidant, and acetylcholinesterase inhibitor compounds.

NFS2 isolates exhibit macroscopic characteristics in the form of circular colonies with a white surface color and grayish-white colony reverse color. The colony has a velvet-like texture, *margin entire*, umbonate *elevation*, and immersed-type mycelium. The colony growth is relatively slow to moderate with a diameter of 2–3 cm after 7 days of incubation, and there is a radial line without the formation of zoning or exudate droplets. Microscopic observations of NFS2 isolates showed the presence of septated hyphae, a single, smooth-walled, hyaline-colored conidiophore, and semicircular to cylindrical conidia arranged like long chains with uniseriate phylids. The character shows a resemblance to fungi of the genus *Penicillium*. This is in line with previous research by Yanti *et al.* (2019) which reported that *Penicillium* has white colonies with a circular shape. Microscopically, this genus is characterized by septated hyphae, hyaluronic conidiophores, metula and phylids cylindrical, as well as globose-shaped or elliptical conidia (Valencia and Meitiniarti, 2017; Yanti *et al.*, 2019).

NFS4 isolates show the morphological characteristics of colonies that are close to fungi of the genus *Aspergillus*. Macroscopically, the colony is irregularly shaped with a velvety texture, aerial-type mycelium, *umbonate elevation*, and filamentous-type colony edges. The color of the colony's surface appears white to greenish-white, while the reverse color of the colony is yellowish. This character corresponds to the description of Saif *et al.* (2020) which states that *Aspergillus* colonies generally have a velvety texture and colony color variations that depend on the species. Microscopically, NFS4 isolates have septated hyphae, smooth-walled single conidiophors, and rounded to semicircular conidia. This character corresponds to the microscopic characteristics of *Aspergillus* reported by Nyongesa *et al.* (2015), namely having uniseriate or biseriate fialids, globose to pyriform vesicles, and radiate or columnar conidia arrangements. Chen *et al.* (2017) also reported that *Aspergillus* has hyaline to light brown conidiophores with rounded to semirounded vesicles.

Based on the compatibility of macroscopic and microscopic characters with Barnett and Hunter identification keys and supported by supporting literature, NFS1 and NFS3 isolates are indicated to belong to the genus *Curvularia*, NFS2 isolates have similarities to the genus *Penicillium*, and NFS4 isolates exhibit similar characters to fungi of the genus *Aspergillus*.

3.2. Selectivity Test of Acidity Level (pH) of Media and Storage Temperature on Growth of Cellulolytic Fungi Diameter

Table 3 Growth of cellulolytic fungal colony diameter at pH 3, 5, and 7

pH	Growth Diameter (cm)			
	NFS1	NFS2	NFS3	NFS4
pH 3	5.00	6.10	3.45	5.90
pH 5	9.00	8.60	4.67	8.12
pH 7	8.97	7.92	3.45	6.60

The effect of the pH of the medium and storage temperature showed different results in each treatment. Observations were made over 7 days, the growth of the diameter of the cellulolytic fungi at various acidity levels (pH) of the media on the media was shown in (Table 2) and (Table 3) for the growth of the diameter of the cellulolytic fungi at various storage temperatures.

Based on table 3, the highest growth was found in NFS1 isolates with pH 5 treatment with a diameter area of 9.00 cm, the 2nd highest value was pH 7 treatment on the same isolate with a diameter area of 8.97 cm and the lowest growth was found in NFS3 isolates with pH 7 treatment with a diameter of 3.45 cm.

The results of the study showed that *Culvularia* sp. NFS1 and NFS3 isolated from cattle farm's fresh solid waste can grow at a pH range of 4-8. This does not contradict the research of Rao *et al.* (2020) which states that *the Culvularia spicifera* fungi that have been studied are able to live in the pH range of 4-10. Agarwal (1958) stated that *Culvularia penniseti* is able to live in the pH range of 4.4-7.8. Previous research conducted by Matur *et al.* (2016) reported that *Culvularia* grows optimally at pH 4.4-7.8. From the results of the analysis, it was found that *Penicillium* sp. NFS2 isolated from cattle farm's fresh solid waste can grow in the pH range of 5 to 7. This does not contradict the research of Hakim *et al.* (2020) who stated that the fungus *P. crustosum*. can grow at a pH range of 5-7 and *P. islandicum* which cannot grow at a pH above 8. Li *et al.* (2025) reported that *Penicillium* spp. isolated from Yellow Stone National Park, Anaconda, Montana, can grow optimally at pH 5. The results of the study found that the fungus *Aspergillus* sp. NFS4 isolated from fresh solid waste from cattle farms can grow at a pH range of 5 to pH 7 This does not contradict the research of Hakim *et al.* (2020) which states that *Aspergillus* fungi can grow in the range of pH 5 to pH 7. Abubakar *et al.* (2013) stated that *Aspergillus parasiticus* grows in the pH range of 5 to pH 7.

Table 4 Growth of cellulolytic fungi diameter at temperatures of 25°C and 40°C

Temperature	Growth Diameter (cm)			
	NFS1	NFS2	NFS3	NFS4
25°C	8.94	8.86	3.76	7.10
40°C	-	-	-	-

Description: (-) There is no fungal growth on the media

Table 4 shows that NFS1 isolate has the highest growth value at 25°C of 8.94 cm followed by NFS2 isolate of 8.86 cm, while the lowest growth value is found in NFS3 isolate with a diameter area of 3.76 cm. At high temperature treatment (40°C) there was no growth in all four cellulolytic fungi isolates.

Based on the results of the research, the fungi *Culvularia* sp. NFS1, *Penicillium* sp. NFS2, *Culvularia* sp. NFS3, and *Aspergillus* sp. NFS4 grows well at 25°C, with a growth diameter area of 8.94 cm, *Aspergillus* sp. NFS4 7.1 cm, and *Penicillium* sp NFS2 3.76. The four fungi are not able to grow at extreme temperatures of 45°C, but this study could not identify the optimal temperature for the growth of the two fungi isolates because the temperature ranges used are too small, namely 25°C and 45°C, further research with more pH variations is needed to determine the optimal temperature for the growth of the two fungi isolates. The results of this study are in line with the results of previous research by Kurmawar *et al.* (2016) which states that the growth temperature of the *genus Aspergillus* sp. is around 25-40°C. some *Aspergillus* genera such as, *A. mulifidum*, *A. niger*, and *A. asperellum*, their growth stops at 50°C. Temperature factors have diverse physiological roles, including in protecting cells from high temperatures or thermal stress (Walker and White, 2018). Although thermotolerant conditions for fungal growth are possible, the majority of fungi generally grow optimally at temperatures around 25°C (Wahidah et al., 2022).

4. Conclusion

Based on the results of this study, 4 fungi isolates that have the ability to degrade cellulite were obtained, namely *Culvularia* sp. NFS1, *Penicillium* sp. NFS2, *Culvularia* sp. NFS3, and *Aspergillus* sp. NFS4, with the highest cellulolytic index in the fungi *Aspergillus* sp. NFS4 is 1.86 and the lowest is *Culvularia* sp, NFS1 is 0.65. The 4 fungi are known to be tolerant to pH 3-7 and can. It grows well at 25°C, but is not capable of growing at 40°C.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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