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Molecular identification of anaerobic fungi isolated from buffalo rumen with their growth rate, cellulase enzyme activity, and fermentation products characteristics

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Abstract. *Agustina S, Wiryawan KG, Suharti S, Meryandini A. 2022. Molecular identification of anaerobic fungi isolated from buffalo rumen with their growth rate, cellulase enzyme activity, and fermentation products characteristics. Biodiversitas 23: 6448-6455.* Anaerobic fungi are essential microbes in the degradation process of feed fiber in the rumen with the ability to produce fibrolytic enzymes and rhizoids that can penetrate feed particles. The activity of cellulase enzymes in fungi is influenced by several factors such as the type of feed and livestock used as a source of isolate. Therefore, this research aimed to analyze the types of anaerobic fungi isolated from buffalo based on their DNA nitrogenous bases composition and also to evaluate the growth rate, cellulase enzyme activity, and fermentation product concentration of anaerobic fungi. The growth rate of fungi was examined by measuring the biomass formed, while cellulase enzyme activity was carried out using CMC, Avicel, and Filter Paper as substrates. The fungal fermentation products were analyzed using the HPLC and GC methods. The results showed that the fungi isolated from buffalo rumen were closely related to anaerobic fungi type *Piromyces* sp., *Caecomyces* sp., and *Neocallimastix frontalis*, with different growth rate, cellulase enzyme activity, and the fermentation products concentration in each type of anaerobic fungi. Therefore, it can be concluded that anaerobic fungi isolated from buffalo rumen can degrade cellulose. It was also discovered that fungi *Neocallimastix frontalis* had a higher growth rate, cellulase enzyme activity (CMCase, avicelase, and FPase), and fermentation products than *Piromyces* sp. and *Caecomyces* sp.

Keywords: Cellulase enzyme, *Caecomyces*, fermentation products, *Neocallimastix frontalis, Piromyces*

INTRODUCTION

Anaerobic fungi are microbes that belong to the phylum *Neocallimastigomycota*, order *Neocallimastigales*, and family *Neocallimastigaceae*, with the ability to degrade fiber effectively (Dollhofer et al. 2015; Edwards et al. 2017). In 2015, it was reported that there are 8 genera of fungi belonging to *Neocallimastigomycota*, which consist of 3 polycentric fungi, namely C*yllamyces, Anaeromyces*, and *Orpinomyces*, and 5 fungal monocentric which are *Buwchfawromyces, Caecomyces, Neocallimastix, Oontomyces*, and *Piromyces* (Dollhofer et al. 2015). The new type of fungus discovered also include *Pecoramyces ruminantum* (Hanafy et al. 2017), *Feramyces austinii* (Hanafy et al. 2018), and *Liebetanzomyces polymorphus* (Joshi et al. 2018). In 2020-2021, several new fungi were discovered, namely *Aklioshbomyces, Tahromyces, Khoyollomyces, Cappelomyces, Agriosomyces, Joblinomyces*, and *Ghazallomyces* (Hanafy et al., 2020). The new type of fungi also found by Stabel et al. (2020) were *Aestipascuomyces dupliciliberans* and the genus *Paucimyces polynucleatus* was identified by Hanafy et al. (2021). The genus of anaerobic fungi can be determined based on morphological characteristics such as the number of flagella in zoospores, morphology of the thallus (monocentric and polycentric), and rhizoid of filamentous or vegetative type (Nagpal et al. 2011).

However, difficulty in the determination of fungi through this method because each type of fungus has varied morphological characteristics (Paul et al. 2018). This makes it necessary to carry out molecular identification to determine the specific characteristics of each isolated fungus (Joshi et al. 2018). Anaerobic fungi diversity can be analyzed by using ITS 1 (Internal Transcribed Spacer) DNA as the target region (Paul et al. 2018; Edwards et al. 2019). In this research, molecular identification was also carried out to determine each type of anaerobic fungi isolated from the rumen of buffalo. Anaerobic fungi can be found in the digestive tract of herbivorous animals, especially in organs that play an important role in the feed degradation process such as rumen (Lillington et al. 2021). Xue et al. (2022) also stated that the population of anaerobic fungi in ruminants was higher in the rumen compared to other organs.

In general, forage given to ruminants has high fiber content and low digestibility. Elghandour et al. (2015) stated that the digestibility of forage could be increased by using DFM (Direct-Fed Microbial) supplementation. It can increase rumen fermentation and feed efficiency (Elghandour et al. 2015; Puniya et al. 2015). One of microbes that can be used as DFM is an anaerobic fungus. Edwards et al. (2017) stated that anaerobic fungi have a major role in the degradation process of lignocellulose in feed. They can produce enzymes to degrade fibers such as cellulase, hemicellulase, as well as xylanase (Ho et al. 1996), and also penetrate feed particles using rhizoids (Ho and Barr 1995; Kittelmann et al. 2012; Solomon et al. 2016). Anaerobic fungi can produce xylanase, mannanase, galactanase, exoglucanase, endoglucanase and β-glucosidase wich plays an important role for feed fiber degradation (Chen et al. 2012; Youssef et al. 2013; Wang et al. 2014; Ma et al. 2022). These fungi also produce lignocellulase enzymes to degrade feed cell walls and generate acetate, formate, lactate, ethanol, H_2, and CO_2 as fermentation products (Gruninger et al. 2014).

Anaerobic fungi also produce rhizoids that can penetrate feed particles, so mechanical breakdown occurs (Dollhofer et al. 2018). Panahi et al. (2022) stated that the penetration process of rhizoids also could release lignin bonds from hemicellulose and cellulose. These several advantages of fungi indicate that anaerobic fungi can be used as microbes that are supplemented for ruminants to increase feed digestibility. Wang et al. (2019) reported that the addition of *Piromyces* sp. isolated from goat rumen could reduce NDF (Neutral Detergent Fiber) and ADF (Acid Detergent Fiber) content of corn silage. Nagpal et al. (2011) also reported that anaerobic fungi type *Caecomyces* sp. significantly reduces the NDF and ADF content in feed. Nagpal et al. (2009) reported that each type of fungus has different cellulase enzyme activities. Cellulase is an important enzyme for livestock-fed high-fiber feeds. Olukunle et al. (2021) stated that it is very useful for increasing feed digestibility. Cellulase is enzyme consisting of endoglucanase, exoglucanase and β-glucosidase enzymes that function to convert feed cellulose into glucose (Pukan et al. 2015; Nababan et al. 2019; Olukunle et al. 2021).

Therefore, this research was carried out to evaluate the growth rate, cellulase enzyme activity, and fermentation product concentration of anaerobic fungi isolated from buffalo rumen.

MATERIALS AND METHODS

Isolates and fungi preparation

This anaerobic fungus was isolated from local Indonesian buffalo type Badegur buffalo (*Bubalus bubalis*). From the morning until the afternoon, the buffalo were grazed and fed grass or legumes, while at night until the morning the buffalo were put in the cage and fed with rice straw. The anaerobic fungi used were isolates F1, F2, F3, F4, and F5 from buffalo rumen by Agustina et al. (2022). The isolates were maintained using Orpin media containing antibiotics and incubated at 39°C for 3 days before molecular identification (Jin et al. 2011).

Molecular identification

For molecular identification, genomic DNA of anaerobic fungi was isolated by using the methods given by Vaidya et al. (2018) with some modifications. The extracted DNA of rumen anaerobic fungi was carried out completely using the Internal Transcribed Spacer (ITS; partial 18S, complete ITS1, 5.8S, ITS 2, and partial 28S) with the use of PCR (Dagar et al. 2015). Subsequently, the DNA nitrogenous bases were BLAST using data registered in NCBI, and MEGA11 was used to align the DNA sequences and construct the phylogenetic tree of anaerobic fungi rumen.

Anaerobic fungi growth curves

A fungal growth curve was made by measuring the biomass formed on liquid Orpin Media containing antibiotics and cellobiose as the carbon source after the specified incubation time. Each type of fungi had 5 replicates to measure the biomass of fungi. A total of 1 mL fresh fungal isolate was injected anaerobically into a Hungate tube containing 9 mL of liquid Orpin media, which was covered with rubber. Subsequently, every tube was incubated at 39ºC for different times ranging from 0 to 168 hours, the fungal biomass was measured every 12 hours for 7 days, and incubated isolates were centrifuged at 3,000 rpm for 10 minutes. The supernatant was discarded and the pellet obtained was dried at 105°C for 24 hours. The fungal biomass was obtained based on the difference between the initial and the final weight of biomass (Hakim et al. 2020).

Anaerobic fungal cellulase enzyme activity measurement (Miller 1959)

The enzyme activity in rumen anaerobic fungi was measured using the DNS method by observing the reducing sugar formed. Meanwhile, the cellulase enzyme measurements were carried out using CMC 1%, Avicel 1%, and filter paper as substrates. The enzyme activity studies were carried out using 5 replications. Each type of isolated fungi was put into a medium, containing substrate and incubated at 39ºC with different incubation times (0-5 days). Harvesting of crude extract enzymes was carried out by taking 5 mL of fungal isolates that had been incubated at different times and centrifuged at 6,000 rpm for 10 minutes and only the supernatant was taken. This was followed by the addition of 0.5 mL substrate and 0.5 mL supernatant into the tube, which was incubated for 60 minutes at 39ºC and pH 6.8 without shaking to measure CMCase, avicelase, and FPase enzyme activity (Nagpal et al. 2011; Sirohi et al. 2013; Wei et al. 2017). After incubation, 1 mL DNS (dinitrosalicylic acid) was added to the tube to stop the reaction and the absorbance was measured using a spectrophotometer at 540 nm wavelength.

Product fermentation measurement

Fungal isolates were grown anaerobically on liquid Orpin media containing cellobiose as an energy source and incubated for 7 days at 39ºC with four replications. Subsequently, the isolates were centrifuged at 5,000 rpm for 5 minutes, the supernatant obtained was taken and used to measure the fermentation products of anaerobic fungi.

The fermentation products of acetate, lactate, and formate were analyzed using the HPLC (High-Performance Liquid Chromatography) method (Li et al. 2017). The ethanol content produced by fungi was measured using GC (Gas Chromatography) (Li et al. 2017) and the estimated CO_2 and H_2 concentrations were calculated based on Lowe et al. (1987).

CO_2 = (acetate + ethanol)-formate

H_2 = ((acetate + ethanol)x2)-(formate + (ethanol x 2))

Statistical analysis

The results of molecular identification were analyzed descriptively and growth biomass, enzyme assay and fermentation products studies were analyzed by one-way ANOVA and followed by Duncan test (Steel and Torrie 1993).

RESULTS AND DISCUSSION

Molecular identification

Molecular identification is used to assign the type of microbes and the results of nitrogenous base sequence analysis were used to determine the phylotypes of fungi (Edwards et al. 2019). Based on Table 1 and Figure 1, the phylotypes and the phylogenetic tree of anaerobic fungi from buffalo rumen showed that F1 and F3 were closely related to *Piromyces* sp. PGL25, F2, and F5 were related to *Caecomyces* sp. CYR and F4 were closely related to *Neocallimastix frontalis* isolate SK. This was in accordance with the results of morphological analysis previously carried out by Agustina et al. (2022) that the isolates of buffalo rumen anaerobic fungi had the same morphological characteristics as *Piromyces* sp., *Caecomyces* sp., and *Neocallimastix*. It showed that molecular identification could be carried out to clarify and confirm the morphological identification results because according to Edwards et al. (2017), morphological identification alone is not enough to determine genera and species of anaerobic fungi. Joshi et al. (2018) also stated that the combination of morphological and molecular identification was an excellent way to determine the genus of the anaerobic rumen fungi that have been isolated.

Jin et al. (2011) isolated the *Piromyces* sp. from buffalo. Schulz et al. (2021) also stated that *Piromyces* sp. is found in buffalo in Africa, while Paul et al. (2018) reported that *Piromyces* sp. is a genus of anaerobic fungi commonly found in the digestive tract of herbivorous animals. According to Jin et al. (2011), *Piromyces* sp. is an anaerobic fungus with uniflagellate zoospores, monocentric thallus, spherical and oval sporangium, as well as filamentous rhizoids. Rabee et al. (2019) stated that *Neocallimastix* is the dominant genus of fungi found in the rumen in ruminants. Ho and Barr (1995) stated that the anaerobic fungi *Neocallimastix frontalis* and *Caecomyces* sp. can be isolated from the rumen and feces of buffalo. Paul et al. (2018) also reported that *Caecomyces* could be found in herbivorous animals from different geographical areas. Moreover, *Neocallimastix frontalis* is an anaerobic fungus that has zoospores with many flagella (polyflagella), monocentric thallus, and filamentous rhizoids (Jin et al. 2011), while *Caecomyces* sp. is a type of fungus that has rhizoid vegetative, bulbous thallus, uninflated zoospores, monocentric thallus, and spherical or ovoid zoospores (Wang et al. 2017).

Growth rate of buffalo rumen anaerobic fungi

The growth rate of anaerobic fungi was measured based on the fungal biomass formed during the incubation (Hakim et al. 2020). The results of the growth rates of 5 fungal isolates from buffalo rumen (Figure 2A) showed that each type of anaerobic fungi has a different growth rate, where *Neocallimastix frontalis* (F4) was higher than *Piromyces* sp. (F1 and F3) and *Caecomyces* sp. (F2 and F5). This was in line with Teunissen et al. (1991), who reported that *N. frontalis* produced a higher dry weight of fungal biomass than *Piromyces* sp. It also formed high biomass because of its ability to produce larger colonies and highly branched rhizoids (Agustina et al. 2022). The slow growth rate of *Caecomyces* sp. fungi was also caused by *Caecomyces* sp. does not produce thallus rhizoids but forms bulbous vegetative cells.

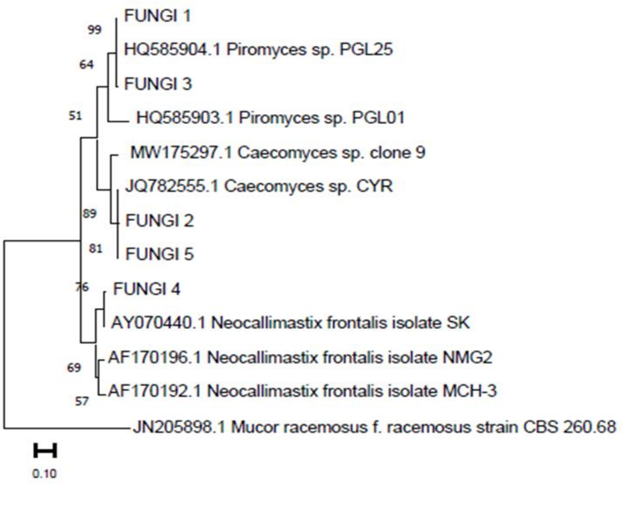

Figure 1. The phylogenetic tree of buffalo rumen anaerobic fungi using the maximum likelihood model was compared with data sequences from the NCBI. *Mucor racemosus* was used as an outgroup.

Table 1. The phylotypes of anaerobic fungi rumen isolated in buffalo using ITS1 and reference from NCBI Gene Bank

Fungi	Nearest taxa	Accession number	Query cover	E-Value	Similarity
F1	*Piromyces* sp. PGL25	HQ585904.1	99 %	0.00	99.77%
F2	*Caecomyces* sp. CYR	JQ782555.1	100 %	0.00	99.78%
F3	*Piromyces* sp. PGL25	HQ585904.1	99 %	0.00	99.53%
F4	*Neocallimastix frontalis isolate* SK	AY070440.1	99 %	0.00	99.56%
F5	*Caecomyces* sp. CYR	JQ782555.1	99 %	0.00	99.57%

The results also showed that the lag phase of the anaerobic fungi *N. frontalis* was shorter than other types. The lag phase for *N. frontalis* occurred at 0-24 hours, while *Caecomyces* sp. and *Piromyces* sp. occurred at 0-36 hours. This was agreed with the research by Nielsen et al. (2002), where the lag phase of anaerobic fungi *Neocallimastix* spp. isolated from Malaysian water buffalo reached the range of 16-24 hours, while *Piromyces* sp. and *Caecomyces* sp. occurred at 33.6 hours and 40 hours. This occurred because the fungi need time to produce motile zoospores. Dollhofer et al. (2015) stated that anaerobic fungi need 24-32 hours to complete their life cycle to produce new motile zoospores, while Jimenez et al. (2020) reported that fungi need 8-26 hours to produce motile zoospores.

This showed that the low fungal biomass produced in the lag phase was caused by the low population and growth of rhizoids. Similarly, Lowe et al. (1987) stated that the nutrients used in the lag phase are preferred for zoospore enlargement. This is supported by the double time of zoosporangium growth of 0.44 m per hour compared to the development of the main rhizoid, which has a double time of 0.28 m per hour (Lowe et al. 1987)).

The curve (Figure 2A) showed that the anaerobic fungi *N. frontalis* (F4) log phase started at 24 hours, with the peak growth point occurring at 96 hours. The log phase of anaerobic fungi types *Caecomyces* sp. (F2 and F5) and *Piromyces* sp. (F1 and F3) started after 36 hours until the peak of growth occurred at 108 hours. The results also showed that after peak growth was reached, all fungi growth rates decreased. Vrabl et al. (2019) stated that the decrease in growth rate after the log phase was caused by the limited nutrients and accumulation of toxic substances in media cultures. At high concentrations, one of the substances toxic to fungi in the medium is hydrogen. This was in accordance with Saye et al. (2021) and Ma et al. (2022), who reported that the accumulation of hydrogen could inhibit the metabolism of rumen anaerobic fungi, while Li et al. (2021) also discovered that it hinders the performance of the hydrogenase enzyme. Hydrogenase is one of the important enzymes in the fungal hydrogenosome, which plays a role in the metabolism of carbohydrates to form energy (ATP) for fungi (Leger et al. 2013; Ma et al. 2022).

Moreover, another substance that can inhibit the growth of fungi was the accumulation of formic acid (Ma et al. 2022). According to Joblin and Naylor (1993), formic acid is a strong inhibitor for the growth of anaerobic fungi, which is produced through the fermentation process by anaerobic fungi together with acetate, lactate, ethanol, and CO_2 (Cheng et al. 2013; Hess et al. 2020; Panahi et al. 2022). Furthermore, formic acid can reduce the growth rate of fungi because it interferes with metabolic processes and fungal cell homeostasis (Hassan et al. 2015). Joblin and Naylor (1993) also stated that adding 20 mmol/L formic acids reduced the 58% cellulose degradation rate of the anaerobic fungus *Neocallimastix frontalis*.

Cellulase enzyme activity in anaerobic fungi isolated from buffalo rumen

Cellulase enzyme activity tests (CMCase, Avicelase and FPase) in this study were carried out at 39ºC and pH 6.8 to describe the activity of cellulase enzymes produced by anaerobic fungi in the rumen. Natsir et al. (2019) stated that the optimum pH condition for growth microbial and fermentation process in rumen was 6.8. The research conducted by Setyoko and Utami (2016) also showed that the optimum temperature for cellulase enzyme activity isolated from rumen fluid was 39ºC. Cellulase is an enzyme that can degrade cellulose into cellooligosaccharides, cellobiose, and glucose (Purkan et al. 2015). These compounds consist of endo-β-1,4-glucanase, exo-β-1,4-glucanase, and β-glucosidase with different action modes (Nababan et al. 2019).

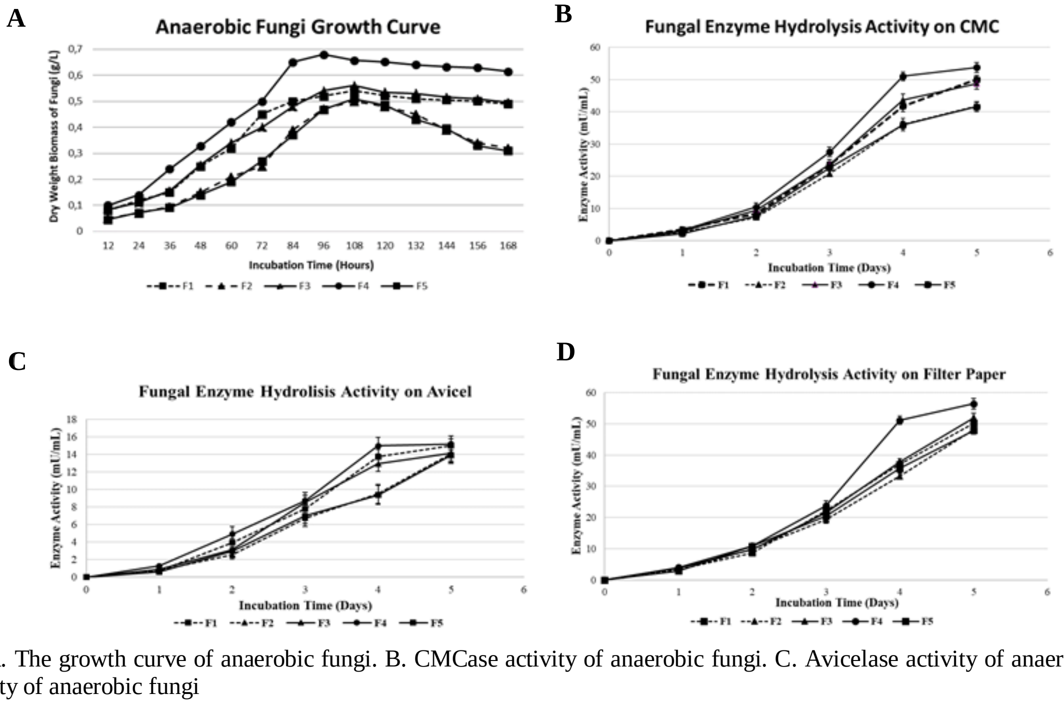

Figure 2. A. The growth curve of anaerobic fungi. B. CMCase activity of anaerobic fungi. C. Avicelase activity of anaerobic fungi. D. Fpase activity of anaerobic fungi

Table 2. The fermentation products of anaerobic fungi from buffalo rumen

Variable	**Concentration (mM)**				
	F1	**F2**	**F3**	**F4**	**F5**
Acetate	57.31±1.27b	55.01±0.98c	58.96±1.38b	64.35±1.07a	54.73±0.99c
Lactate	33.55±1.02b	27.76±1.13c	32.08±0.85b	39.32±1.43a	26.52±1.61c
Formate	71.01±0.85b	63.27±1.27c	69.45±1.04b	75.21±0.97a	62.53±1.23c
Ethanol	24.28±1.04b	22.42±0.96c	24.71±1.29b	27.23±1.16a	21.96±0.56c
CO_2	10.58±0.91c	14.16±0.81b	14.22±1.27b	16.37±1.30a	14.16±0.31b
H_2	43.61±1.69c	46.75±0.71b	48.47±1.72b	53.49±1.24a	46.93±0.74b

Note: F1 and F3: *Piromyces* sp., F2 and F5: *Caecomyces* sp. and F4: *Neocallimastix frontalis*. a, b, c: significant difference ($p<0.05$)

The hydrolytic activity of enzymes in Figures 2B, 2C, and 2D, which was produced by fungi from buffalo rumen showed that all types of fungi could hydrolyze CMC, avicel, and Filter Paper, including *Neocallimastix frontalis* (F4), *Caecomyces* sp. (F2 and F5) and *Piromyces* sp. (F1 and F3). This was in line with the research by Hess et al. (2020), which stated that anaerobic fungi could produce fiber-degrading enzymes. Gruninger et al. (2014) also reported that anaerobic fungi could produce cellulase enzymes that can degrade fiber content in the feed. CMCase describes the activity of the endoglucanase enzyme. (Kirn et al. 2018; Olukunle et al. 2021), avicel explained the activity of exoglucanase (Sirohi et al. 2013), and FPase described total cellulase enzyme activity (Eida et al. 2016). FPase was the total activity of cellulase enzymes, which consists of endo-1,4-β-D-glucanase, 1,4-β-D-glucan-cellobiohydrolase, and β-glucosidase (Reyes et al. 2019). The results showed that the highest enzyme activity in all types of fungi was FPase (47.89-56.45 mU/mL), followed by CMCase (41.56-53.74 mU/mL), and avicelase (13.90-15.17 mU/mL). Nagpal et al. (2011) also stated that the anaerobic fungi types *Neocallimastix, Caecomyces,* and *Piromyces* isolated from ruminants and herbivores had higher FPase enzyme activity than CMCase. Similarly, Nagpal et al. (2009) reported that the FPase enzyme in *Caecomyces* sp. was higher activity than CMCase, while Ho et al. (1996) stated that Filter Paper was pure cellulose which can induce anaerobic fungi to produce enzymes, especially cellulase. The results also showed that all anaerobic fungi isolated from buffalo rumen had the lowest Avicel hydrolysis activity compared to other substrates. This was agreed with the research by Sirohi et al. (2013) who stated that anaerobic fungi had lower avicelase enzyme activity compared to CMCase.

The results in Figure 2 showed that all anaerobic fungi isolated from the rumen of buffalo have increased activity of cellulase (CMCase, Avicelase, and FPase) after passing through the growth lag phase (24 hours). The results of a study conducted by Ho et al. (1996) showed that the CMCase and FPase activity of anaerobic fungi types *N. variabilis*, *P. spiralis*, *P. minutus*, and *C. communis* also increased rapidly after 24 hours. The results also showed that all anaerobic fungi reached optimum conditions at 5 days because the peak of fungal growth occurred at 96-108 hours (4-4.5 days) and enzyme activity was measured every 24 hours. Therefore, the measured enzyme was the accumulation of enzymes produced by the fungus at maximum growth.

The results of Hodrova et al. (1998) also showed that the optimum conditions for the β-glucosidase enzyme in the anaerobic fungi types *Orpinomyces Joyonii* and *Caecomyces communis* were reached on day 5. From Figure 2, it is discovered that the anaerobic fungus *N. frontalis* (F4) has higher CMCase, Avicelase and FPase activity (53.74±1.57 mU/mL, 15.17±0.95 mU/mL, and 56.45±1.73 mU/mL), followed by F1 (49.99±1.31 mU/mL, 15.02±0.77 mU/mL, and 50.20±1.07 mU/mL), F3 (48.74±1.79 mU/mL, 14.14±1.04 mU/mL, and 51.97±1.45 mU/mL), F2 (41.79±1.24 mU/mL, 14.10±1.15 mU/mL, and 48.13±1.16 mU/mL) and F5 which has the lowest concentration of CMCase, Avicelase and FPase (41.56±1.52 mU/mL, 13.90±0.62 mU/mL, and 47.89±1.14 mU/mL). This was caused by *N. frontalis* having a better rhizoid growth rate compared to other types of fungi (Figure 2A). Gruninger et al. (2014) stated that when fungi penetrate the substrate with rhizoids, enzymes are produced to degrade cells in feed. The rhizoids are morphological structures of fungi that can secrete metabolic enzymes and help fungi attach to substrates (Lange et al. 2019). Lange et al. (2019) stated that in both monocentric and polycentric fungi, rhizoids were the main part of fungi that secrete metabolic enzymes. Lillington et al. (2021) reported that the cellulase enzyme was generally located in fungal rhizoid cells and zoospore bodies. Meanwhile, Jimenez et al. (2020) stated that rhizoids in fungi were the key to structural enzymatic hydrolysis of plants because rhizoid growth was related to fibrolytic enzyme activity.

In the rumen, all types of anaerobic fungi synergistically degrade feed fiber enzymatically by producing cellulase and mechanically by producing rhizoids which can penetrate feed cells. Matthews et al. (2019) stated that anaerobic fungi in the rumen would initiate the feed degradation process and produce cellulolytic enzymes. Edwards et al. (2019) also said that anaerobic fungi could increase the degradation of feed fiber and produce high cellulase enzymes.

The fermentation products of fungi

The measurement of fermented products was done for up to 7 days to measure all fermented products produced by rumen anaerobic fungi until the end phase of growth (death phase). The results showed that all fungi isolated from buffalo rumen could produce acetate, lactate, formate, ethanol, CO_2, and H_2 as fermentation products, as presented in Table 2. This was in accordance with the statements of Lowe et al. (1987), Cheng et al. (2013), and Gruninger et al. (2014), where the end products of anaerobic fungi fermentation were acetate, formate, lactate, ethanol, hydrogen, and carbon dioxide. It was also discovered that the most abundant fermentation product produced by all types of fungi isolated from buffalo rumen was formate (62.53-75.21 mM), followed by acetate (54.73-64.35 mM), H_2 (43.61-53.49 mM), lactate (26.52-39.32 mM), and CO_2 (10.58-16.37 mM). The absence of methanogens in this isolate caused high formate produced in the culture. This was agreed with the research by Li et al. (2016), stating that formate acid was the main fermentation product in pure fungal cultures, where approximately 41.68% of carbon in mono-culture will be converted into formate and acetate in hydrogenosomes. According to Li et al. (2017), the addition of a methanogen type *Methanobrevibacter thaueri* as co-culture on *Piromyces* sp. can increase acetate production and decrease formate concentration. Hook et al. (2010) also reported that adding methanogens can reduce formate concentrations because methanogens can use H_2, CO_2, and formate as substrates to produce methane. Furthermore, Boxma et al. (2004) and Cheng et al. (2013) discovered that fungi produce high formate because of its formation in cytosol and hydrogenosomes.

The data also showed that the anaerobic fungi *Neocallimastix frontalis* produced higher fermentation products than others. According to Saye et al. (2021), the anaerobic fungi *Neocallimastix*, given cellulose substrate produced higher ethanol (2.310-3.750 µmol glucose/L) compared to *Piromyces* sp., which only produced 157 µmol glucose/L ethanol. The high fermentation product produced by the anaerobic fungi *Neocallimastix frontalis* was because the fungus has a higher growth rate and cellulase enzyme activity than others, as presented in Figure 2. Anaerobic fungi from buffalo rumen will degrade cellobiose in culture media to produce the final fermentation product. Li et al. (2017) also stated that the energy source is brought into the cell before being used, and the cellobiose will be carried into fungal cells via the cellodextrin transporter (CDT) and converted into glucose by the β-glucosidase. The glucose in fungal cells will pass through the glycolysis stage to produce ATP and other fermentation products (Boxma et al. 2004) when it is introduced into the cytoplasm by glucose transporters (Ma et al. 2022).

In the early stages of glycolysis, glucose is converted into PEP (Pospoenol pyruvate) followed by its conversion into oxaloacetate and pyruvate in the cytosol (Cheng et al. 2013). Boxma et al. (2004) stated that some of the pyruvate formed in the cytosol are inserted into hydrogenosomes or converted to lactate, formate, and Acetyl Co-A to produce ethanol. In the hydrogenosomes, the oxaloacetate formed in the cytosol is converted into malate (Boxma et al. 2004; Cheng et al. 2013). Subsequently, the malate and pyruvate formed incorporated into hydrogenosomes to produce H_2, CO_2, formate, as well as acetate and release energy in form of ATP (Ma et al. 2022). When the pyruvate in the hydrogenosomes is converted into acetate, the hydrolysis process produces ATP. Meanwhile, when it is converted into formate, no ATP is formed (Boxma et al. 2004; Cheng et al. 2013).

Based on the results, the anaerobic fungi isolated from the rumen of buffalo were belong to the genus *Piromyces* sp. (F1 and F3), *Caecomyces* sp. (F2 and F5), and *Neocallimastix frontalis* (F4). Anaerobic fungi *Neocallimastix frontalis* had a higher growth rate, cellulase enzyme activity (CMCase, avicelase, and FPase), and fermentation products than *Piromyces* sp. and *Caecomyces* sp.

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