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The enrichment process and morphological identification of anaerobic fungi isolated from buffalo rumen

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Abstract. *Agustina S, Wiryawan KG, Suharti S, Meryandini A. 2021. The enrichment process and morphological identification of anaerobic fungi isolated from buffalo rumen. Biodiversitas 23: 469-477.* Anaerobic fungi are one of the microbes that have an important role in rumen fiber degradation because they can produce cellulase enzymes and penetrate feed particles. Nevertheless, few studies were performed to test the potential of anaerobic fungi in Indonesia. Therefore, the present study was carried out to evaluate the impact of the enrichment process on pH value, the zoospores population, NH3 (ammonia) concentration, and VFA (Volatile Fatty Acid) proportion. In addition, this research was also performed to isolate anaerobic fungi from buffalo rumen and identify their morphological characteristics. The enrichment stage of anaerobic fungi was carried out using the Hungate method. Results showed that the population of fungi zoospores, pH value, ammonia concentration, the proportion of acetate, and total VFA were significantly affected by the incubation time ($P<0.01$). In addition, *Caecomyces*, *Neocallimastix*, and *Piromyces* were rumen anaerobic fungi isolated from buffalo rumen with different morphological characteristics. It can be concluded that the incubation time increased the zoospore population, the concentration of NH3, acetate proportion, and total VFA but decreased media's pH value.

Keywords: Anaerobic fungi, buffalo, enrichment, morphological identify, rumen

INTRODUCTION

Anaerobic fungi are microbes that have an important role in rumen processing. Ruminant performance was affected by rumen microorganisms such as fungi because it is needed in the rumen fermentation process (Suryapratama and Suhartati 2012; Suharti et al. 2015). Anaerobic fungi have a significant role in the fiber degradation process because they can produce fibrolytic enzymes (Guo et al. 2020). Anaerobic fungi also can produce enzymes that can degrade the lignified part of the feed cell wall (Puniya et al. 2015). Another benefit of rumen anaerobic fungi is that they can physically destroy plant cells wall using mycelium, so it can increasing energy sources for other rumen microbes (Yanuartono et al. 2019). The existence of several advantages possessed by anaerobic fungi makes these fungi one of the candidates for microbes that can be supplemented for ruminants to increase livestock production.

Puniya et al. (2015) stated that improved livestock performance could also be achieved by supplementing ruminants with microbes (direct-fed microbes). One of the microbes that can be supplemented to increase the digestibility of livestock is anaerobic fungi. A few studies showed that anaerobic fungi supplementation positively both *in vitro* and *in vivo*. The research conducted by Nagpal et al. (2011) showed that *in vitro* testing of rumen anaerobic fungi *Caecomyces* sp. significantly reduced the Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) content after 48 and 72 hours incubation. Sehgal et al. (2008) stated that calves supplemented by anaerobic fungi had higher feed efficiency, body weight gain and final weight than controls. Khejornsart et al. (2011) and Kittelman et al. (2012) stated that the types of livestock and feed could affect the diversity of fungi, so it is necessary to isolate fungal strains from ruminants. Nevertheless, there are not many investigations conducted to evaluate the benefit of anaerobic fungi in Indonesia. This study was aimed to evaluate the effect of the enrichment process on pH value, the zoospores population, NH_3 (ammonia) concentration, and VFA (Volatile Fatty Acid) proportion. In addition, the purpose of this research was to isolate anaerobic fungi from rumen buffalo and identify their morphological characteristics.

MATERIALS AND METHODS

Enrichment process

The enrichment stage of anaerobic fungi was carried out by using the Hungate method (Hungate 1969), and the media used in this stage was Orpin media which contained cellobiose and antibiotics (Chloramphenicol and Streptomycin) (Orpin 1977; Callaghan et al. 2015). The

medium prepared was composed of 150 mL L^{-1} mineral solution A (3 g L^{-1} K_2HPO_4), 150 mL L^{-1} mineral solution B (0.5 g L^{-1} $CaCl_2$, 6 g L^{-1} NaCl, 3 g L^{-1} KH_2PO_4, 0.6 g $MgSO_4.7H_2O$, and 6 g L^{-1} $(NH_4)_2SO_4$), 150 mL L^{-1} clarified rumen fluid, 2 mL L^{-1} hemin, 2 mL L^{-1}0.1% resazurin, 3 g L^{-1} yeast extract, 10 g L^{-1} peptone trypticase, 2 g L^{-1} cellobiose, 1 g L^{-1} L-cysteine-HCl and antibiotics (50 µg mL^{-1} chloramphenicol and 50 µg mL^{-1} streptomycin) (Orpin 1977; Callaghan et al. 2015).

The fresh buffalo rumen fluid was collected from Situ Gede Village, West Bogor District, Bogor, West Java, Indonesia, using stomach tube method. The rumen fluid (10 mL) was put into a strict anaerobe serum bottle that already contained 90 mL of liquid Orpin media with cellobiose and antibiotics. After that, the isolate was incubated at 39ºC for 0-5 days.

The measurement of pH value and zoospore population

The pH measurement of the isolates was carried out using a digital pH meter type pHep HI98107 (Hanna Instruments Indotama, North Jakarta, Indonesia). While the population of fungal zoospores was calculated using a Haemocytometer Neubauer under a microscope with a magnification of 40x (Sanjaya et al. 2010).

Quantification of NH_3 and partial VFA concentration

The concentration of NH3 in this study was measured using the Conway micro diffusion method (GLP 1966), whereas the measurement of VFA concentration of (acetate, propionate, butyrate, and valerate) was done using the Gas Chromatography (GC) method (Kristensen et al. 1996) using the Bruker Scion 436 GC instrument at the Laboratory of Animal Feed and Food Product Analysis, Ciawi Livestock Research Institute (BALITNAK), Ciawi, Bogor, West Java, Indonesia.

Isolation and morphological identification of anaerobic fungi

Isolation of anaerobic fungi was carried out using dot method (Ed-har et al. 2017). This method was done aseptically by inserting buffalo rumen fluid and agar media into a petri dish and homogenized. After that, the petri dish was put into an anaerobic jar with AnaeroGen. The rumen anaerobic fungi were incubated at 39ºC until the fungus colonies were visible in the media. The colonies of anaerobic fungi were moved into broth media, and this procedure was replicated three times until the pure fungal cultures were confirmed (Paul et al. 2010; Hooker et al. 2018).

After the pure fungal cultures were obtained, the morphological observations were carried out by looking at the growth rate (very slow, slow, fast, or very fast), color and diameter of the colony, hyphae state (branching and absence of bulkheads) (Sanjaya et al. 2010). In addition, morphological identification was also done by observing the nature of rhizoid growth (exogenous and endogenous) and observing the number of flagella in zoospores, zoospore shape, monocentric or polycentric thallus, and the type of rhizoid (vegetative cells or filamentous) microscopically to determine the genus of fungi (Ho and Barr 1995; Nagpal et al. 2011; Hess et al. 2020). The morphological properties of each isolate from buffalo rumen were observed under an Olympus CX31 microscope.

Statistical analysis

The results of morphological identification of buffalo rumen anaerobic fungi were analyzed descriptively, and the effect of incubation time on pH value, the population of zoospore, the concentration of NH_3, and VFA proportion were carried out using a Completely Randomized Design with four treatments (incubation zero day, one day, three days and five days) and four replicates (Steel and Torrie 1993). Data on the effect of incubation time were analyzed by one-way ANOVA (Analysis of Variance), and significantly different data ($P<0.05$ and $P<0.01$) were further tested with advanced Duncan advanced test (Steel and Torrie 1993).

RESULTS AND DISCUSSION

The enrichment stage

The enrichment method was observed as an effective method for increasing the population of targeted organism (Kamagata 2015). However, the enrichment process is affected by various factors, such as the length of incubation time and the substrate used. This was accordance with Aqil et al. (2015), which stated that incubation time and pH could affect the microbial enrichment process. The effect of enrichment incubation time on the zoospore population of anaerobic fungi, culture pH value, the concentration of NH_3, and the partial VFA proportion were presented in Figure 1-3 and Table 1.

The zoospores population of anaerobic fungi

Zoospores are the asexual stage in the life cycle of fungi, released from sporangium can move to fine substrates (Haitjema et al. 2014). According to the data, the zoospores population in liquid culture increased following the increase in the incubation time. The highest zoospore population reached after the anaerobic fungi were incubated for five days (3.580 ± 0.046 log cells/mL) followed then three days (3.510 ± 0.026 log cells/mL), one day (3.057 ± 0.031 log cells/mL) and zero day (3.030 ± 0.048 log cells/mL) (Figure 1). Day 0 to day 1 was observed as lag phase, day 1 to day 3 was log phase, and days 3 until day 5 was deceleration phase because the growth of zoospores has started to slow down. Dollhofer et al. (2015) said that anaerobic fungi need 24 until 32 hours to produce zoospores, so 0-24 hours was a lag phase in the production of fungal zoospores.

An anaerobic fungi's life cycle started when sporangium released motile zoospores (Orpin 1975). After that, zoospores moved to find a substrate source and formed colonies (Haijtema et al. 2014). Zoospores fungi move by responding to the presence of fresh feed particles in *in-vivo* studies and respond to phenolic acid, haemin, and soluble sugars in *in-vitro* studies (Saye et al. 2021). Fungi attach to the feed particle by extending the rhizoids

into the feed cells and forming colonies (Lillington et al. 2021). The sporangium and rhizoids continue to grow until the new motile zoospores are released from zoosporangium (Agustina et al. 2020; Lillington et al. 2021). Anaerobic fungi completed the life cycle after 24-32 hours (Dollhofer et al. 2015). The estimated life cycle time of anaerobic fungi ranged from 8-26 hours (Jimenez et al. 2020). The high growth rate of the zoospores population in the log phase was caused by the sporangium released motile zoospores after 24 hours incubation. Swift et al. (2021) stated that each fungi sporangium consists of many zoospores. In this phase, the nutrient content in the liquid culture was an important factor because it was needed to support the growth of fungal zoospores (Prihartantyo 2020).

According to the data, the growth rate of fungal zoospores decreased after 72 hours of incubation. Stanbury et al. (2017) stated that this phase was the deceleration phase because the availability of essential nutrients such as energy in the medium decreased. Vrabi et al. (2019) said that the limited amount of nutrients in the culture indicated that the end of the exponential growth has been reached. Stanbury et al. (2017) also said that in deceleration and death phase, the toxic fermentation products were accumulated and affected the growth of microorganisms. Formic acid, acetate, lactate, ethanol, and hydrogen were the fermentation products of anaerobic fungi (Gruninger et al. 2014; Kazda et al. 2014; Cheng et al. 2013; Li et al. 2017). Hassan et al. (2015) stated that the accumulation of formic acid could reduce 28.65% of the growth of fungi by affecting the fungal homeostasis cell and disturbing their metabolic process. The accumulation of another fermentation product that can interfere with the growth of anaerobic fungi was hydrogen (Joblin and Naylor 1993). The high concentration of hydrogen in the environment can interfere with the flow of H_2 through the hydrogenosomes, so the energy formation process in the cell is disrupted (Gordon and Phillips 1998). Marvin-Sikkema et al. (1993) stated that the high concentration of hydrogen interfered with the reduction process of NADP and interfered the activity of NAD(P)H: ferredoxin oxidoreductase enzyme. Saye et al. (2021) also stated that hydrogen accumulation limits the yield of dark fermentation in anaerobic fungi.

The medium pH value

The pH value was one of the factors that can affect the growth of rumen microbes such as anaerobic fungi. Lund et al. (2020) said that the environmental conditions that significantly impact the growth and survival of microbes were pH value. Jin and Kirk (2018) also stated that the distribution of microbes was influenced by the pH value. The pH value of the media affects the metabolism process, growth rate, and microbial survival (Jin and Kirk 2018). The data in Figure 2 showed that with the increase in incubation time of anaerobic fungi significant ($P<0.01$) decrease in the pH value of the medium was observed. The lowest pH value occurred at five days incubation time with the pH value was 6.47 ± 0.06, then incubation time for three days (6.53 ± 0.06), one day (6.77 ± 0.06), and the highest pH value reached in control (zero day) which had the pH value 6.81 ± 0.05. This was caused by the product fermentation, such as increased organic acids, and affected the media's pH value. Anaerobic fungi produced formic acid, acetate, and lactate, affecting the media pH value (Kazda et al. 2014; Li et al. 2017). This was agreed with Peh et al. (2020) that the concentration of organic acid affected the pH value of media.

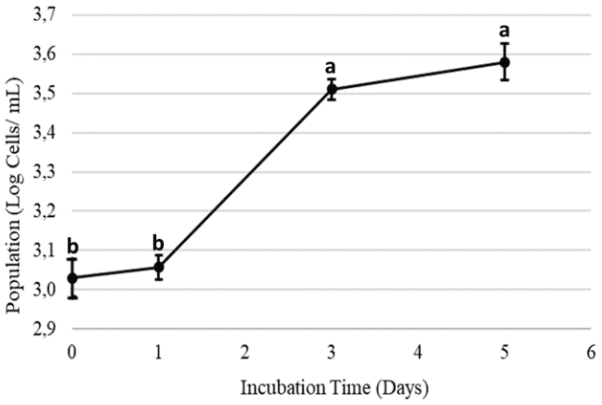

Figure 1. The effect of incubation time on fungal zoospore population.

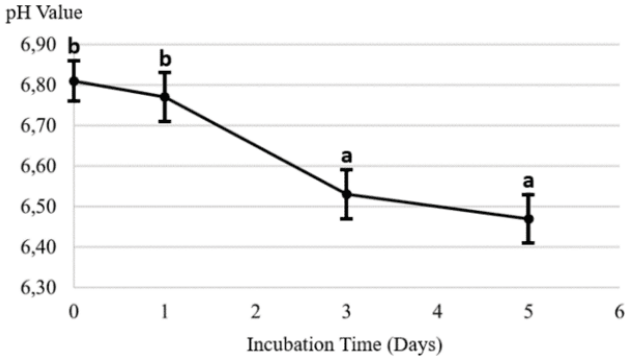

Figure 2. The effect of incubation time on the pH value of the media

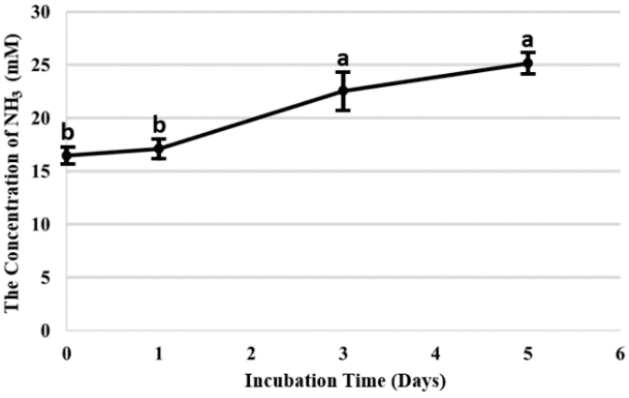

Figure 3. The effect of incubation time on NH_3 concentration

Table 1. The effect of incubation time on total VFA production and partial VFA proportion

Variable	Incubation Time (Days)			
	0	1	3	5
Total VFA (mM)	46.42±1.84d	51.52±2.08c	86.15±2.64b	105.80±0.62a
The Proportion of VFA (mM/100 mM)				
Acetate (C2)	26.86±0.81b	32.35±6.33b	57.31±0.57a	63.35 ± 2.46a
Propionate (C3)	48.01±3.15a	45.00±1.95a	27.08±0.62b	22.21±1.36c
Iso Butyrate + Butyrate (C4)	20.82±3.61b	18.52±3.80b	13.01±1.95a	11.59±2.07a
Iso Valerate + Valerate (C5)	4.31±1.00	4.13±1.95	2.85±1.07	2.59±0.76

Figure 2 also showed that the changes in pH value during incubation time was still in the normal range for fungi to support the growth of fungi because rumen anaerobic fungi can grow in environment with pH range from 6 to 7, and the population rapidly decline if the environmental pH was outside this range (Lee at al. 2015). The low decrease in this pH value was because acetate was more produced by anaerobic fungi than lactate. This may be because acetate has a higher constant value (4.78 pKa) than lactic acid (3.89 pKa) so acetic acid released H^+ ions to media lower than lactic acid (Sutrisna et al. 2015; Muchsiri et al. 2016; Asih et al. 2017). This was agreed with Cheng et al. (2013) research results that showed anaerobic fungi produced 204.2 ppm acetic acid and 186.2 ppm lactic acid as the final product.

The concentration of NH_3

The production of ammonia (NH_3) in the rumen was also an important parameter because NH_3 is a compound produced from the protein degradation process in the rumen (Suryani et al. 2020). Suryapratama and Suhartati (2012) stated that NH_3 in the rumen was needed as a nitrogen source for rumen microbes. The result presented in Figure 3 indicated that the incubation time significantly ($P<0.01$) increased the concentration of NH_3 produced by anaerobic fungi in the media. The lowest NH_3 concentration reached during the incubation period for one day and zero-day value 17.11 ± 0.90 mM and 16.47 ± 0.75 mM respectively. The highest concentration of NH_3 was reached when anaerobic fungi were incubated for five days (25.15 ± 0.92 mM) and followed by three days incubation (22.55 ± 1.69 mM). This was caused by the dead bacterial cell proteins was breakdown into ammonia in the media. Ji et al. (2018) said that the addition of antibiotics to the media would inhibit the growth of rumen bacteria and cause cells bacterial die. Shen et al. (2018) stated that the protein synthesis process in bacteria was inhibited by antibiotics. Hartinger et al. (2018) said that dead bacteria degraded by protease enzymes owned by anaerobic fungi and produced ammonia (NH_3).

In contrast to cellulolytic bacteria, anaerobic fungi besides having cellulase activity also have the ability to degrade protein in feed particles (Hess et al. 2020). Yildirim et al. (2017) also stated that anaerobic fungi could produce proteolytic enzymes. Hess et al. (2020) said that *Neocallimastix frontalis* could produce protease. Anaerobic fungi produced extracellular protease enzymes to provide amino acids and facilitate the feed cells penetration process (Gruninger et al. 2014).

The proportion of partial VFA

Storm et al. (2012) stated that VFA (Volatile Fatty Acids) is an important nutrient for ruminant. VFA was short chain fatty acids produced from the fermentation carbohydrates by microbes such as rumen anaerobic fungi (Nisa et al. 2017; Syamsi et al. 2018; Filasari et al. 2019). VFA was major energy source for ruminants and carbon skeleton for rumen microbial protein formation (Sairullah et al. 2016; Wole et al. 2018; Dhia et al. 2019). The effect of incubation time on the proportion of VFA partial and VFA total is presented in Table 1. The results presented in Table 1 revealed that the incubation time significantly ($P<0.01$) increased the total VFA amount and the proportion of acetic acid, but the incubation time also significantly ($P<0.01$) decreased the proportion of butyrate and propionate. It was decreased because when the acetate production increased significantly, propionate and butyrate production did not increase. This was in line with Gordon and Phillips (1998) that the main product of carbohydrate fermentation process by anaerobic fungi was acetate without propionate and butyrate production. It was also agreed with Edwards et al. (2017) which stated that anaerobic fungi did not produce propionate and butyrate as fermentation products. It was happened because anaerobic fungi could not produce propionate and butyrate (Peng et al. 2021).

The addition of acetic acid proportion on five days, three days, and one day incubation was 6.04 mM/100 mM total VFA, 24.96 mM/100 mM total VFA, and 5.49 mM/100 mM total VFA respectively. The acetic acid produced on day zero and day one was low due to the small anaerobic fungi population. Knight et al. (2018) stated that the population of microbes affected the total concentration of fermentation products. Table 1 also showed that the main VFA product produced by anaerobic fungi isolated from buffalo rumen was acetic acid. This was agreed with Nagaraja (2016) and Li et al. (2021), who stated that acetate was the main fermentation product of anaerobic fungi. The breakdown of glucose into acetate produces more ATP than lactate because the acetate conversion process reduces pyridine nucleotides and produces ATP (Marvin-Sikkema et al. 1990; Wilken et al. 2021). Graaf et al. (2011) and Leggieri et al. (2021) showed that pyruvate converted to Acetyl CoA first and finally converted to acetate. Every transformation of Acetyl CoA to acetate by ASCT (Acetate: Succinyl CoA Transferase) and SCS (Succinate CoA Synthase) enzyme in hydrogenosomes produce ATP (Graaf et al. 2011; Makiuchi and Nozaki 2014; Gawryluk et al. 2016).

Table 2. The morphological identification results of five anaerobic fungi isolated from buffalo rumen

Variable	Anaerobic Fungi Isolated from Buffalo Rumen				
	F1	**F2**	**F3**	**F4**	**F5**
Growth Rate	Very Slow	Very Slow	Very Slow	Very Slow	Very Slow
Color	White	White	White	White	White
Diameter (mm)	2.63±0.75	2.02±1.32	2.57±0.92	10.91 ± 1.67	2.09±0.80
Hyphae	B & HB	MS	B & S	HB & NS	MS
Rhizoid	Endo & Exo	Endo	Endo & Exo	Exo	Endo
Rhizoid Types	Filamentous	Vegetative	Filamentous	Filamentous	Vegetative
Thallus	Monocentric	Monocentric	Monocentric	Monocentric	Monocentric
Zoospores Shape	Globose	Globose	Globose	Globose	Globose
Flagella	Monoflagellate	Monoflagellate	Monoflagellate	Polyflagellate	Monoflagellate
Genus	*Piromyces*	*Caecomyces*	*Piromyces*	*Neocallimastix*	*Caecomyces*

Note: B: Branched, HB: Hightly Branched, S: Segmented, NS: Not Segmented, MS: Mono Spherical

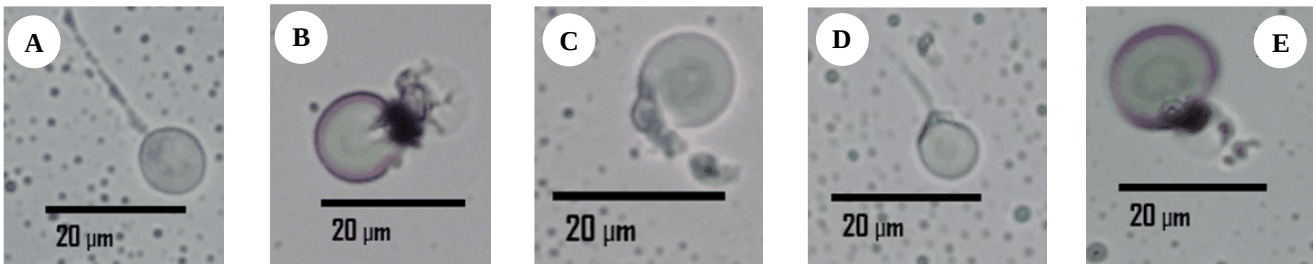

Figure 4. The morphology of rumen anaerobic fungi isolated from buffalo. (A, D) *Piromyces* zoospores (F1, F3), (B) *Neocallimastix* zoospores (F4), (C, E) The sporangium of *Caecomyces* (F2, F5)

Ha et al. (2011) stated that the sources of carbohydrates in the medium, such as cellobiose converted into glucose by β-glucosidase enzyme, and then the glucose is converted into several fermentation products. After that, rumen anaerobic fungi catabolize glucose by carrying out a glycolysis process (Embden-Meyerhof-Parnas pathway) to produce pyruvate (Hagen et al. 2020; Wilken et al. 2021). Marvin-Sikkema et al. (1993) said that the formation of acetic acid could be through 2 formations: firstly, it is through phosphoenolpyruvate (PEP) which was reduced to oxaloacetate or it is through the PEP pathway which was reduced directly to pyruvate. In the first formation pathway, phosphoenolpyruvate is converted into oxaloacetate by PEP carboxylase or carboxykinase (Marvin-Sikkema et al. 1993). After that, according to Marvin-Sikkema et al. (1993) malate dehydrogenase enzyme reduce oxaloacetate to malate, and malate entered into hydrogenosome. In the hydrogenosome, malate is decarboxylated by malic enzymes to formed pyruvate and then reduced to AcetylCoA and finally formed acetic acid (Hackstein et al. 2019). The reduction of pyruvate to Acetyl-CoA in hydrogenosomes carried out by the enzyme Pyruvate: Ferredoxin Oxidoreductase (PFO) (Marvin-Sikkema et al. 1993; Stairs et al. 2015). The PFO enzyme reduces ferredoxin and catalyzes pyruvate decarboxylation process to form Acetyl-CoA and CO_2 (Atteia et al. 2013; Leger et al. 2016; Zimorski et al. 2019; Rotterova et al. 2020).

The second acetate formation pathway, PEP was broken down into pyruvate using the enzyme pyruvate kinase (Marvin-Sikkema et al. 1993). After that, the pyruvate converted into Acetyl-CoA the same as in the first formation of acetate pathway, but the enzyme used to reduce it was pyruvate formate lyase enzyme (Marvin-Sikkema et al. 1993). Then Acetyl-CoA was transferred to the hydrogenosomes. The last step to form acetic acid either in the first pathway or in the second pathway, Acetyl-CoA will be reduced to acetic acid involving the ASCT (Acetate: Succinate CoA Transferase) enzyme (Marvin-Sikkema et al. 1993). Muller et al. (2012) and Wilken et al. (2021) stated that acetate formation in hydrogenosome was more active via PFL (Pyruvate Formate Lyase) than via PFO (Pyruvate Ferredoxin Oxidoreductase).

The morphological properties of anaerobic fungi

Morphological identification was one of the methods that can be used to determine the type of anaerobic fungi (Nagpal et al. 2011; Hess et al. 2020; Kar et al. 2021). The morphological identification of anaerobic fungi isolated from buffalo rumen is presented in Table 2. Table 2 showed that every fungus isolated from buffalo rumen had a very slow growth with white colonies and different diameter sizes. This was in accordance with the research results conducted by Joshi et al. (2018) that rumen anaerobic fungi have a very slow growth rate of more than 15 days. Colony diameter measurement results showed that each type of fungus had a different diameter. Table 2 showed that the type of fungi that has the largest diameter was *Neocallimastix* (10.91 ± 1.67 mm) then *Piromyces* (2.63 ± 0.75 and 2.57 ± 0.92 mm), and *Caecomyces* which has the smallest colony diameter (2.02 ± 1.32 mm and 2.09

± 0.80 mm). The study results conducted by Paul et al. (2011) presented that *Neocallimastix* formed colonies with a large diameter (12 mm) on agar media containing cellobiose. On the other hand, Paul et al. (2010) stated that the anaerobic fungi type *Piromyces* only had a small diameter (2 mm) colony. This was caused by *Neocallimastix* fungi having highly branched rhizoids which formed larger colony sizes compared to other types of anaerobic fungi (Gordon and Phillips 1998).

The results showed that the five isolated fungi had different morphologies (Table 2 and Figure 4). These differences indicated that isolated fungal genera were different too. There were three types of fungi identified from the buffalo rumen which were *Piromyces* (F1 and F3), *Caecomyces* (F2 and F5) and *Neocallimastix* (F4). This was in accordance with Jin et al. (2011) which stated that the anaerobic fungus *Piromyces* could be isolated from buffalo rumen. Ho et al. (1995) also reported the isolation of *Neocallimastix, Piromyces,* and *Caecomyces* fungi from rumen of buffalo. Based on the identification results in Table 2, it was observed that the isolates of fungi F1 and F3 were rumen anaerobic fungi, including the *Piromyces* genus. This was agreed with the results of research by Nagpal et al. (2011), and Wang et al. (2017) that *Piromyces* was a type of rumen anaerobic fungus that has a thallus with one center (monocentric) and has zoospores with 1 flagellum (monoflagellate). Wang et al. (2019) said that the fungal genus *Piromyces* has filamentous and extensive rhizoids. *Piromyces* sp. also has zoospores with globus to irregular shapes with different diameter sizes varied from 4.5 µm to 9.5 µm (Barr et al. 1989). Hess et al. (2020) also said that *Piromyces* sp. was monocentric anaerobic fungi with have exogenous and endogenous thallus development. Barr et al. (1989) stated that *Piromyces* sp. was a fungus with two development stages. In the first stage, the rhizoid develops endogenously and at the end develop exogenously because the tubular part of the fungus come out as sporangiophores and the primary nucleus released from zoospores (Barr et al. 1989).

Table 2 and Figure 4 showed that anaerobic fungi F2 and F5 had different morphologies from F1, F3, and F4. Anaerobic fungi F2 and F5 were types of fungi with the genus *Caecomyces* because this type of fungus had a bulbous rhizoid type. This was in line with Wang et al. (2017) and Li et al. (2021) where *Caecomyces* was isolated as a genera of rumen anaerobic fungi that produced thalli consisting of bulbous vegetative cells and sporangia. Table 2 also showed that F2 and F5 fungi have zoospores with globose shape and have 1 flagellum (monoflagellates). Cheng et al. (2007) stated that *Caecomyces* had globose and ovoid zoospores with diameter 5.0 ± 1.1 µm and it had single flagellum and Henske et al. (2017) said that the size of *Caecomyces* sporangium was varied from 20 µm until 50 µm. After the zoospores are attached to the substrate, it is attached to inactive and formed holdflas (Cheng et al. 2007). After that, one or two bulbous rhizoids extend from the holdflas and developed into vegetative thallus with an average diameter of holdflash, rhizoid, and young sporangia was 14.1 ± 3.7 µm, 7.7 ± 1.4 µm, and 6.8 ± 1.3 µm, respectively (Chen et al. 2007).

The last type of fungus that was isolated from the rumen of buffalo was F4. The results in Table 2 and Figure 4 also showed that the F4 fungi have a monocentric thallus, multiflagellated ovoid zoospores, and extensive mycelium development. According to Comlekcioglu et al. (2010), multiflagellates fungi with monocentric reproductive development, large sporangia with extensive rhizomycelium in their zoospores were the characteristic of anaerobic fungi type *Neocallimastix* sp. Gruninger et al. (2014) and Dagar et al. (2015) stated that *Neocallimastix* was a type of rumen anaerobic fungi with a variable number of flagella ranging from 7 to 30. Wang et al. (2017) stated that *Neocallimastix* sp. had ovoid to globose zoospores with zoospore diameters size 7-22 µm. *Neocallimastix* was monocentric anaerobic fungus with highly branched exogenous rhizoid development (Gordon and Phillips 1998). According to Ho and Barr (1995) *Neocallimastix* spp. had ellipsoidal, ovoid, globose, and irregular shapes sporangia with sporangia sizes varying from 8.5 µm to 170 µm.

We can conclude from the present study that the incubation time raises the zoospore population, the concentration of NH_3, acetate proportion and total VFA but decreases the pH value of media. *Caecomyces*, *Neocallimastix* and *Piromyces* are rumen anaerobic fungi isolated from buffalo which have different morphological characteristics in each type.

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