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Isolation of indigenous microorganisms from the liquid produced by the bioprocess of corn straw as direct fed microbials

DIDIN SUPRIAT TASRIPIN[1], ENDAH YUNIARTI[2], BAMBANG KHOLIQ MUTAQIN[2,♥]

[1]Departement of Animal Production, Faculty of Animal Husbandry, Universitas Padjadjaran. Jl. Raya Bandung-Sumedang Km.21, Jatinangor 45363, West Java, Indonesia

[2]Department of Animal Nutrition and Feed Technology, Faculty of Animal Husbandry, Universitas Padjadjaran. Jl. Raya Bandung-Sumedang Km.21, Jatinangor 45363, West Java, Indonesia. Tel./Fax.: +62- 22-84288828, ♥email: kholiq@unpad.ac.id

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Abstract. *Tasripin DS, Yuniarti E, Mutaqin BK. 2022. Isolation of indigenous microorganisms from the liquid produced by the bioprocess of corn straw as direct fed microbials. Biodiversitas 23: 3452-3456.* Digestion of ruminants is highly dependent on microbial activity of rumen. Rumen microbes are responsible for degrading food nutrients into simpler components that can be easily utilized by the body. In order to improve the rumen ecosystem, the Direct Fed Microbials (DFM) was added. The addition of DFM to feed can be in the form of a mixture of several beneficial microbes (such as from the group of bacteria, yeast, and fungi) for the body of livestock. This group of microorganisms can be obtained from the results of the bioprocess of bioprocessed materials such as corn straw. In order to obtain the right microorganisms to be used as DFM, it is necessary to isolate the results of the bioprocess. The analysis was carried out by purification of indigenous microorganisms from the bioprocess of corn straw. Microbial purification can be done by isolation of microbes on agar media such as Nutrient Agar, MRS, and PDA by incubation to obtain a single cell. The results showed that isolation of the liquid from the bioprocess of corn straw was obtained in the form of Coccus and Bacilli which included gram-positive bacteria which were found to be dominant after incubation and microbial staining.

Keywords: Bioprocess, corn straw, direct fed microbial, indigenous microorganism, isolation

Abbreviations: DFM: Direct fed microbials, IMO: Indigenous microorganism

INTRODUCTION

A bioprocess is a physical, chemical, and biological process that simplifies complex chemical structures and makes digestion more efficient. Indigenous microorganisms are microbes that live and reproduce in media formed from a mixture of natural materials derived from various natural resources available and favored by these microorganisms. Indigenous microorganisms can be referred to as bio activators, consisting of a collection of Indigenous microorganisms that utilize the potential of local natural resources (Budiyani et al. 2016). Indigenous microorganisms are very easy to form, especially by utilizing vegetable waste in the environment, for example, plant remains such as corn straw (Mamilianti et al. 2012).

Indigenous microorganisms are useful for accelerating the process of decomposition of organic matter and improving the quality of feed which includes the fermentation process. Indigenous microorganisms includes different microbes that act as starters in liquid fertilizer or animal feed to accelerate the fiber degradation process (Mamilianti et al. 2012). Probiotics are live microbes that are given directly to livestock to improve the balance of microbes in the digestive system and reduce unwanted microbes. Probiotics given directly to ruminants are called "Direct Fed Microbials".

The mechanism of DFM action involves, regulating the balance of the rumen ecosystem so that it suppresses the production of lactic acid and aids the breakdown of cellulose as measured by the parameters of volatile fatty acids, ammonia and the ability to digest fiber, which in turn is greater livestock growth and productivity.

The DFM is a category of probiotic that is widely used in the feed industry (Fuller 1989; Mutaqin et al. 2018, 2020, 2021; Schrezenmeir and De Vrese 2001). The DFM must have some acid tolerance in the gastrointestinal tract (Chou and Weimer 1999; Tuomola et al. 2001). The digestive process involves an acidic environment and it is very important that DFM survives these conditions (Brashears et al. 2003). Microorganisms included in DFM are bacteria and yeast (fungi) (Mutaqin et al. 2017). The Lactobacillus group is a direct-fed microbe product from the most commonly used bacterial strains in livestock.

The DFM products can be in the form of yeast (fungi) consisting of *Saccharomyces, Pichia, Candida, Rhodotorula, Debaryomyces, Cryptococcus* (yeast) and *Aspergillus* (fungus) (Horincar and Bahrim 2017; Jimoh et al. 2012). In general, some fungal DFM are live cultures. The DFM criteria for yeast groups have the ability to utilize oxygen in the rumen to help create an anaerobic atmosphere (very little oxygen) and this can stimulate optimal rumen microbial growth (Wina 2005).

The *Lactobacillus* bacteria are a group of bacteria that belong to the direct fed microbes (DFM). *Lactobacillus* is a genus of gram-positive, facultative anaerobic or microaerophilic bacteria (Dellaglio et al. 2005). The use of

probiotics in ruminants can increase milk production, live weight and feed efficiency. The greater digestibility of crude fiber, automatically increases the consumption and supply of nutrients to the intestines. Ultimately, the overall response to production increases (Mutaqin et al. 2018; Yoon and Stern 1995).

Microbes belonging to DFM have been scientifically proven to be useful in maintaining the stability of the rumen ecosystem and can provide solutions to support gastrointestinal diseases. It has also been noted that some DFM can reduce methane production, reduce ammonia and have an effect on rumen fermentation (Callaway and Ricke 2012). The dose of DFM with *Lactobacillus acidophilus* for beef cattle is 1 x 10^9 CFU/day, for dairy cows it is 1 x 10^9 CFU/day (NP28) (AlZahal et al. 2014; Anindita and Soyi 2017; Blair 2011; Luan et al. 2015; Vipham et al. 2016).

Identification of microbial isolates resulted from the bioprocessing of corn straw, aimed to obtain direct fed microbe strains for ruminants to make it easier to characterize and classify them. This is important to facilitate their development. This research is expected to have the same potential as direct fed microbes, which has the ability to improve microbial balance in the digestive tract of ruminants.

MATERIALS AND METHODS

Study area

Sampling area for the collection of liquid samples from the bioprocess of corn straw was located in the silage banker of PT UPBS Pangalengan. Samples were taken as large as 3 liters from the waste liquid storage section of the corn straw bioprocess, the silage of which is used for dairy cow feed.

Experimental design

This research is a qualitative descriptive research conducted to explore and look for a potential content of feed resources to be researched. This research is to obtain types of microorganisms that are classified as DFM which are beneficial for livestock. This research uses an identification approach to the object of research which is then studied in detail for application in livestock farms.

This research was started by testing the indigenous microorganisms growth from the bioprocess of corn straw on the growth media (Nutrien Agar, de Man Rogosa Sharpe, Potato Dextrose Agar) by looking at the count of microbial colonies formed at time series. In each medium, 8 replications were maintained. Furthermore, it was calculated based on the total plate count method by looking at the total colonies with colony-forming unit on each sample.

Procedures

The research was conducted in the rumen microbiology room, the ruminant animal nutrition laboratory and animal feed chemistry, the faculty of animal husbandry and the central laboratory of Universitas Padjadjaran. The tools used were: incubator, autoclave, erlenmeyer, hotplate, bunsen lamp, petri dish, beaker glass, test tube, microscope, object glass, cover glass, ose needle, micropipettes, aluminum foil, cotton, digital camera and stationery. The materials used in this study were indigenous microbial liquids from the corn straw bioprocess, Nutrien Agar (NA), de Man Rogosa Sharpe (MRS), Potato Dextrose Agar (PDA), methyl red indicator (methyl red), Gram stain test material (purple crystal, lugol iodine, safranin, liquid alcohol concentration 95% and water distillate), NaCl, hydrogen peroxide (H_2O_2). The microorganisms were isolated as per the procedure (Mutaqin et al. 2017):

Equipment and materials for sterilization

All tools and materials to be used were autoclaved at 121ºC with steam pressure of 15 pa for 15 minutes.

Sampling

Liquid samples were obtained from the indigenous microorganism liquid resulting from the bioprocess of the corn straw. Samples were grown on medium. After growth, the bacteria were isolated to see the shape of the microbe's appearance.

Isolation of bacteria.

A serial dilution of the collected the sample was performed by taking 1 mL of sample in a test tube containing 9 mL of distilled water in order to obtain a 10^{-1} dilution. A 10^{-2} dilution is obtained by taking 1 mL of 10^{-1} dilution and putting it in a test tube containing 9 mL. Distilled water and the serial dilution was maintained at 10^{-9}. 1 mL of the 10^{-8} and 10^{-9} dilutions were withdrawn and then placed in a petri dish containing agar medium. The mixture was flattened and incubated with the cup upside down for 24-48 hours at 30ºC (Mutaqin et al. 2017).

The morphology of bacterial cells included the gram stain test as well as the physiological properties test with the catalase and the oxidase test were carried out as per given below:

Gram staining.

The slide was cleaned with alcohol and passed over a Bunsen flame several times, then the bacterial isolate was taken aseptically with a needle and smeared on the slide. The bacterial isolates were then stained with purple-violet drops and left for 1 minute, after which the isolates were washed with running water and air-dried. The bacterial isolates were then dripped with Iodine drops and left for 1 minute, washed with running water and air-dried.

In addition, the bacterial isolates were given 95% alcohol drops for 30 seconds, then drained with water and dried in the wind. The bacterial isolates were then scratched with safranin for 30 seconds, and washed with water, dried with paper towels and air-dried, and observed under a microscope. Gram-positive bacteria marked with purple color indicated that the bacteria were able to bind the crystal violet color, while gram-negative bacteria marked with pink indicated that the bacteria were unable to bind the crystal violet color and stained only with safranin (Mutaqin et al. 2017).

Cellular form

The growing bacteria were then observed under a microscope for the shape of the cells so that the shape (cocci or bacillus) can be known.

Physiological properties

Catalase test

Two drops of H_2O_2 were placed on a clean slide. Bacterial isolates were collected using an ose needle, then transferred to a slide and mixed. A positive test is indicated by the formation of oxygen bubbles, which indicated that the organism in question produces the enzyme catalase, which converts hydrogen peroxide into water and oxygen.

Oxidase test

In a sterile glass, the oxidase paper strip of the object was preserved, then the bacteria were taken from the tilted NA culture using a sterile loop and rubbed over the oxidase paper strip on the slide and streaked with physiological NaCl. Then observed for the color change on the oxidase paper strip. When it turns blue, it indicated oxidizing properties (Mutaqin et al. 2017).

Data analysis

The analysis was carried out by the purification of indigenous microorganisms from the corn straw bioprocess obtained from the silage banker of PT UPBS Pangalengan. Microbial purification can be done by the microbial isolation method on agar media such as Nutrient Agar (NA), de Man Rogosa Sharpe (MRS), and Potato Dextrose Agar (PDA) with incubation to obtain single cells. Those are further categorized according to the form of the microbe, whether it is in the form of a coccus or a bacillus using the gram staining method to determine whether it is gram-positive or gram-negative.

RESULTS AND DISCUSSION

Comparison of colonies on growing media

Calculation of the colonies count per unit time with the total plate count method can show the most effective media to see the microorganisms that grow and develop. Calculation of the number of microorganisms per colony can see the development of growth series, so you can see a trend of the growth of these microorganisms. Comparison of colony calculations shown on Table 1.

Table 1 shows the growth of indigenous microorganisms that grew a lot based on the colony count on nutrient agar (NA) media, followed by MRS and PDA. However, within 24 hours of colony-based growth, the value was 1 x 10^9 CFU. Thus, this number included the number of colonies that grew rapidly. Colony increases per hours with different media shown in Figure 1.

Figure 1 shows an increasing trend in each growing media in time series. Linear equation on media NA y=10x-5,5 (R^2=0,99), MRS y=6,5x-2,63 (R^2=0,92), PDA y=3,6x-4,81 (R^2=0,79). The linear equation indirectly shows an increase which gradually decreases with a marked minus constant value in each equation.

Isolation of indigenous microorganisms

Physiological testing at an advanced stage is necessary to identify a bacterium. Physiological tests performed in this study included catalase and oxidase tests. Catalase test is used to determine the presence of catalase enzyme in bacterial isolates. Catalase is an enzyme that can catalyze the decomposition of hydrogen peroxide (H_2O_2) into water and O_2. Hydrogen peroxide (H_2O_2) is toxic to the bacterial cells because this material is able to deactivate enzymes in cells and is very harmful to the bacterial cell itself. This test is very important to know the nature of a bacterium to the need for oxygen.

Isolation of indigenous microorganisms from the bioprocess of corn straw using different growth media, namely Nutrient Agar (NA), de Man Rogosa Sharpe (MRS), and Potato Dextrose Agar (PDA) on a petri dish for 48 hours showed in Figure 2.

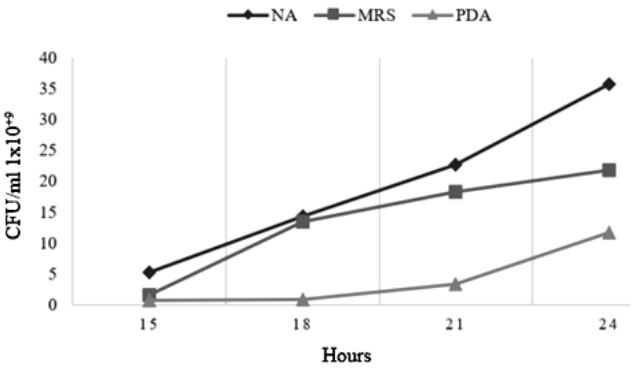
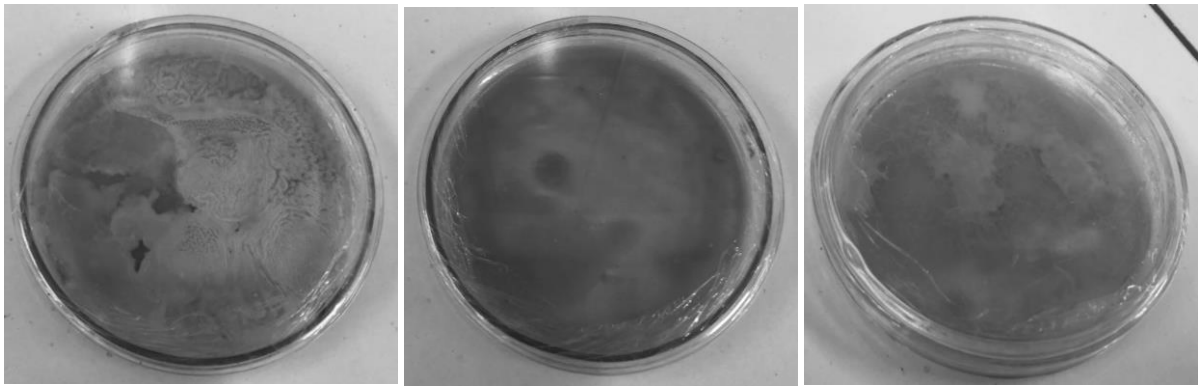


Figure 1. Colony increases per hour with different media

Table 1. Comparison of colony calculations

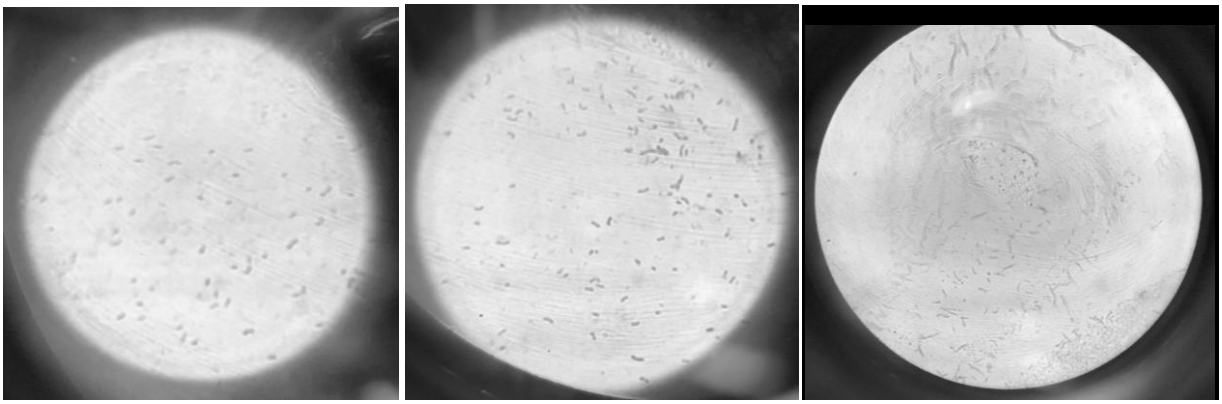
Media	Hours				
	15	18	21	24	27
	(CFU/mL 1 x 10^9)				
NA (A)	5,25±3,694^c	14,25±5,600^b	22,75±12,127^b	35,75±9,161^a	TNTC
MRS (B)	1,63±0,916^d	13,38±1,768^c	18,25±3,284^b	21,88±3,227^a	TNTC
PDA (C)	0,63±0,518^c	0,88±0,641^b	3,38±2,774^b	11,75±1,909^a	TNTC

Note: 1) Different superscripts in the direction of the column indicate significantly different (p<0,05), 2) too numerous to count (TNTC), 3) Capital letters A, B, C indicate most sequential colony time series.



A **B** **C**

Figure 2. Liquid culture of corn straw bioprocess on media: A. NA, B. MRS, C. PDA



A **B** **C**

Figure 3. Observing the shape of microbes using a microscope in different media: A. NA, B. MRS, C. PDA

Figure 2 shows the appearance of the growing media conditions that are densely packed with microbes grown by pouring. The results after 48 hours showed the density of colonies that gathered into an area formed by growing microorganisms. Liquid culture on media NA is shown on appearance of thick white microorganism growth. The color is more faded in MRS and PDA media. This shows that NA media is very suitable for the growth of indigenous microorganisms from the bioprocess of corn straw.

The next step was to purify the microbes grown on agar media by isolating the microbes, which were then incubated in an incubator. Incubation of isolates with different agar media, each of three isolation tubes, in an incubator machine at 37^{o}C for 12-48 hours.

The next step was to observe the microbial isolates that had been incubated using a microscope. Observations with a microscope were carried out to determine the shape of the microbes that had been isolated from the liquid produced from the bioprocess of corn straw shown in Figure 3.

The results of observing the form of microorganisms in the form of coccus, which is quite numerous and spread Figure 3 (a). Figure 3 (b) shows the results of observations of the form of microorganisms in the form of bacilli, which are quite numerous and spread evenly. Figure 3 (c) shows the results of observations of the shape of microorganisms in the form of hyphae or elongated threads that are different from the results of observations on other media.

Based on the results of observations from the isolation of the liquid from the bioprocess of corn straw, it was obtained that the form of coccus and bacillus, which included gram-positive bacteria, was found to be dominant after microbial staining was carried out. A direct fed microbes have good characteristics according to having good probiotic requirements (Fuller 1989): (i) Strains should be non-pathogenic and non-toxic. (ii) The strain should be able to survive and metabolize in the digestive environment. (iii) The strain should be stable and able to stay for an extended period of time under storage conditions.

These observations, it can be indicated that local microbes resulting from the bioprocess of corn starch in the form of coccus and bacillus can have the potential as direct fed microbes to be used as an addition to ruminant animal feed that requires additional microbes to improve the rumen ecosystem.

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