In Vitro Gas and Methane Production from Fermented Rice Straw using *Trichoderma viride* and *Phanerochaete chrysosporium* Inoculant

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In Vitro Gas and Methane Production from Fermented Rice Straw using *Trichoderma viride* and *Phanerochaete chrysosporium* Inoculant

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Abstract. Livestock, as part of agriculture sector, has contribute to greenhouse gas (GHG) emission especially on methane (CH_4) compounds. Methane emission represent energy losses in form of gas reflecting low feed efficiency. Rice straw is a source commonly used as animal roughage. The quality of rice straw could be improved by fermentation treatment. This study investigated effects of fermentation using *Phanerochaete chrysosporium* and *Trichoderma viride* inoculant on *in vitro* gas and methane emission from rice straw. The rice straw was fermented in three weeks. This experiment consisted of four treatments and three replications being: (1) C (control, fermented rice straw with no inoculant), (2) TV (fermented rice straw using *T. viride* inoculant), (3) PC (fermented rice straw using *P. chrysosporium* inoculant), (4) TVPC (fermented rice straw using *T. viride* and *P. chrysosporium* inoculant). This research was arranged into a completely randomized block design. Results showed that TVPC treatment produced the lowest lignin content ($p<0.05$). TVPC treatment also produced the highest glucose compound by 1.70 mg/g ($p<0.05$). Meanwhile, there was no difference between all treatments in *in vitro* total gas and optimum gas (a+b) production. However, adding *T. viride* combination with *P. chrysosporium* in fermented process could decrease methane gas production ($P<0.01$). Results demonstrate that adding *T. viride* and *P. chrysosporium* inoculant in fermented process could increase the efficiency of rice straw as ruminant's feed. This was represent in the low methane production and could reduce a part of GHG emission from enteric fermentation.
Keywords: Methane production, *Phanerochaete chrysosporium*, Rice Straw, *Trichoderma viride*

1. Introduction

Ruminant's feed commonly used by farmers in Indonesia is agricultural byproduct in the form of rice straw. Wahyono et al. [1] reported that the constraints of rice straw as ruminant's feed are the high content of crude fiber, lignin and silica. This due to the rice straw is obtained from rice plants byproduct which are harvested in mature harvesting stage. The high fiber content also causes low digestibility. Furthermore, rice straw also contain a low crude protein content. High structural carbohydrate content will reduce the efficiency of energy conversion from plants to animal. High fiber content will affect the low digestibility of feed and affect the high percentage of enteric methane (CH_4) from fermented gas in the rumen [2]. Methane is produced from microbial fermentation of hydrolyzed dietary carbohydrate

(cellulose, hemicellulose, pectin and starch) in the rumen [3]. Methane from enteric ruminant's fermentation is one of the biggest contributors to greenhouse gas (GHG) emissions. As roughage, pre-treatment of rice straw needs to be done to increase the digestibility and reduce methane gas emission from the mechanism of rumen fermentation.

Pre-treatment using fungi in rice straw aims to increase the effectiveness of energy conversion. Fungi activity will increase the accessibility of delignification, cellulase and hemicellulase activities for hydrolyzing the fiber fraction on the substrate [4]. Several studies related to utilization of microbes inoculant for fermenter inoculant has been done to increase the nutrient profile and digestibility of ruminant's feed. Fermentation using *Aspergillus niger* could affected the fiber profile in the oil palm byproducts [1]. Nutrient profile, Organic matter (OM) and dry matter (DM) digestibility of fermented rice straw increased due to adding *A. niger* inoculant [1, 5, 6]. Fermentation using *Phanerochaete chrysosporium* combination with gamma irradiated and NaOH treatment could increase delignification efficiency in rice straw [4]. *Trichoderma viride* could be used for inoculant to increase the availability of nutrients in cocoa pod fermentation [7], while *P. chrysosporium* is a white root fungi (WRF) group that has a high ability to degrade lignin. There was not much information has reported about the utilization of *P. chrysosporium* and *T. viride* combination as inoculant for rice straw fermentation. Based on those findings, the purpose of this study is to investigated the effects of fermentation using *P. chrysosporium* and *T. viride* inoculant on *in vitro* total gas and methane emission from rice straw.

2. Experimental procedures

2.1. Sample Preparation

The research was conducted in industry and environment division and agricultural division, Center for Application of Isotope and Radiation (CIRA), BATAN, from July to September 2018. Sidenuk varety rice straw was obtained from agricultural division field. Rice straw was dried at 60^{o}C for three days then grinded to a size of 2 mm.

2.2. Fungal Culture Preparation

Cultures of *P. chrysosporium* and *T. viride* were obtained from selected culture collections in industry and environment division CIRA, BATAN. Each of 0.5 x 0.5 cm pieces *P. chrysosporium* and *T. viride* were cultivated in the potato dextrose broth (PDB) at 28-32 ^{o}C for 4 days in 100 rpm shaker waterbath. Liquid culture with 107 propagules/ml were obtained for solid substrate fermentation starter inoculant.

2.3. Fermentation of Rice Straw

The solid fermentation treatment following by: (1) C (control, fermented rice straw with no inoculant), (2) TV (fermented rice straw using *T. viride* inoculant), (3) PC (fermented rice straw using *P. chrysosporium* inoculant) and (4) TVPC (fermented rice straw using *T. viride* and *P. chrysosporium* inoculant). A total of 200 g of rice straw was put into 500 ml bottle fermentor and adding 20 ml of mineral nutrition solution (12g PDB, 1g $(NH_4)_2SO_4$, 0.5g KH_2PO_4 and 0.5g $MgSO_4.7H_2O$). Aquades was adding to initial the 60% moisture content. 1% NaOH and 1% H_2SO_4 were adding to initial the pH medium until 6. The fermentation medium was sterilized by autoclave at 121^{o}C for 30 minutes. 2 g inoculant were inoculated in sterile medium, then incubated for 21 days. Fermented rice straw then harvested, dried at 60^{o}C for two days then grinded to a size of 1 mm for the analysis of glucose [8], cellulose, hemicellulose, lignin [9], protein content [10] and *in vitro* gas test measurement.

2.4. In Vitro Gas Test

A total of 380 mg samples were transferred into 100 ml glass syringe (Fortuna® model, Germany). The rumen fluid was collected from fistulae buffalo before feeding in the morning. The buffalo diets were roughage and concentrates for 60:40 based on dry matter (DM). The rumen fluid was filtered using a four layer cotton cloth. The glass syringe containing the sample was incubated at 39 ^{o}C before rumen-buffer filled into the syringe.

The glass syringe containing the sample was added with 40 mL rumen-buffer following Menke et al [11] modification by Blümmel [12]. The incubation was carried out at 29 ^{o}C for 72h. The sample was replicated as many as three replications. Total gas production measurements were performed at 0, 2, 4, 6, 8, 10, 12, 24, 48 and 72 h incubation time. Methane production was measured after 72 h incubation time. Measurements of pH, ammonia (NH_3) and total volatile fatty acids (TVFA) were collected on samples-rumen-buffer fluid after 72 h incubation time.

2.5. Sample Analysis

Methane production was determined using MRU gas analyzer® (Germany) instrument after 72 h incubation time. Measurement of pH was determined using Hanna instrument digital pH meter. NH_3 measurement was measured using Conway microdifusion methods. TVFA measurement was conducted using steam destilation methods [10]. The gas kinetics variables were measured using using Ørskov and Mcdonald [13] exponential models using NEWAY® fit curve software as follows: $P = a+b(1-e^{-ct})$. P is the gas production at t time. "a" and "b" are soluble fraction and insoluble fraction but may also be degradable (ml/380 mg DM), respectively. While "c" is the gas production rate per t time unit (ml/h). Constanta of 't" is incubation time (h). The potential gas production was obtained by "a+b" calculation (ml/380 mg DM).

2.6. Data Analysis

Data of fiber content, protein content, total gas, methane concentration and rumen fermentation products were analyzed using a completely randomized block design with five replication. Data were analyzed using analysis of variance (ANOVA) by SPSS 22.0 with the following statistical model of $Yij = \mu + \alpha i + \varepsilon ij$, where Y is the dependent variable, μ the overall mean, αi the inoculant effect and εij is the residual error. Differences among treatments were analyzed using Duncan Multiple Range Test [14].

3. Results and discussion

3.1. Fiber and Protein Content

The fiber and protein contents were shown in Table 1. There was no effects of fungi inoculant on hemicellulose content in fermented rice straw. Adding *P. chrysosporium* and *T. viride - P. chrysosporium* inoculants combination could increase cellulose and glucose content ($P<0.05$). Fermented rice straw by *P. chrysosporium* treatment also had lower lignin content than control ($P<0.05$). Interestingly, *T. viride - P. chrysosporium* inoculants treatment could increase protein content, better than *P. chrysosporium* single inoculant ($P<0.05$).

Table 1. Fiber and protein contents of fermented rice straw

Parameters	C	TV	PC	TVPC	SEM
Cellulose (%)	7.38^{a}	7.33^{a}	11.38^{b}	10.82^{b}	0.685
Hemicellulose (%)	45.59	44.58	42.02	43.48	0.657
Lignin (%)	19.31^{b}	18.54^{b}	14.98^{a}	17.44^{ab}	0.631
Glucose (mg/g)	0.98^{a}	0.94^{a}	1.32^{b}	1.70^{b}	0.096
Protein (%)	1.57^{ab}	1.58^{ab}	1.53^{a}	1.89^{b}	0.061

Standard error mean (SEM); Means with different superscripts within coloumn in same parameters were different ($P<0.05$); C (control, fermented rice straw with no inoculant); TV (fermented rice straw using *T. viride* inoculant); PC (fermented rice straw using *P. chrysosporium* inoculant); TVPC (fermented rice straw using *T. viride* and *P. chrysosporium* inoculant).

In present study, it could be seen that the changes in fiber contents were influenced by the type of inoculant. Lignin content in PC treatment is the lowest. This due to the *P. chrysosporium* characteristics is lignin degrading agents. Furthermore, cellulose levels also increase due to the breakdown of lignocellulose bonds after *T. viride - P. chrysosporium* inoculants combination treatment. On TV treatment, cellulose level did not change compared to control. This might be cellulose fraction was still

attached to lignin content. Low disestibility rates in rice straw due to the high lignification of cellulose[6]. Previous study stated that incubation rape straw with *P. chrysosporium* inoculant caused high losses in lignocelluloses, neutral detergent fiber (NDF) and acid detergent fiber (ADF) [15]. This statement is represented with present study due to lignin is a part of NDF and ADF content. Zakariah [7] reported that fungal inoculation could significantly increased crude fiber contents of the fermented substrates. The higher cellulose compounds also could be caused by increased cell wall of the fungal hyfa.

The high content of glucose in the TVPC treatment represents a continuous performance by combination of *P. chrysosporium* and *T. viride*, respectively. The role of *P. chrysosporium* in delignification process was continued by cellulose degradation by *T. viride*. This also represents that there was no negative effect between both inoculants. The mixed culture of lignocellulose fungal strains was found to be efficient in degrading greater amount of lignin and cellulose in rice straw than single strain [16]. In this study, the TVPC treatment was able to increase the protein content in rice straw. Similarly, Zakariah [7] stated that Inoculation with *T. viride* could increase crude protein level of cocoa pods. This due to the performance of inoculants that affect the differences of nutrient availability in fermented substrates. The increased of protein compound also might be caused by the growth of fungal in rice straw substrates.

3.2. In Vitro Total Gas and Methane Concentration

Data on *in vitro* total gas production and methane concentration were presented in Table 2 and Figure 1 respectively. Total gas production reflect the substrates degradability in *in vitro* incubation. There was no effect of fungi inoculant on total gas production at 4-48 h incubation. The differences were only seen at the beginning and the end of incubation ($P<0.05$). The highest potential gas production (a+b) was showed by control treatment ($P<0.05$). however, TVPC treatment produced the highest gas production rate (c) per t time unit ($P<0.05$). Adding *P. chrysosporium* and *T. viride* - *P. chrysosporium* inoculants combination could reduce the methane concentration that produced by fermented rice straw ($P<0.05$).

Table 2. *In vitro* total gas production and gas kinetics of fermented rice straw (ml/380 mg DM)

Parameters	Incubation time (h)									Gas kinetics	
	2	**4**	**6**	**8**	**10**	**12**	**24**	**48**	**72**	**a+b**	**c**
C	2.58^{ab}	3.85	4.50	5.79	6.59	7.40	13.17	23.77	26.67^{b}	36.86^{b}	0.019^{a}
TV	3.01^{b}	4.59	5.70	6.49	7.44	8.40	13.77	22.01	25.16^{ab}	31.62^{a}	0.022^{b}
PC	2.40^{ab}	3.76	4.65	5.55	6.45	7.65	13.04	21.60	23.55^{a}	28.92^{a}	0.025^{bc}
TVPC	2.12^{a}	3.77	4.70	5.87	6.57	8.00	14.23	23.19	25.53^{ab}	31.00^{a}	0.026^{c}
SEM	0.145	0.189	0.207	0.176	0.180	0.199	0.231	0.394	0.436	1.166	0.001

Standard error mean (SEM); Means with different superscripts within coloumn in same parameters were different($P<0.05$); C (control, fermented rice straw with no inoculant); TV (fermented rice straw using *T. viride* inoculant); PC (fermented rice straw using *P. chrysosporium* inoculant); TVPC (fermented rice straw using *T. viride* and *P. chrysosporium* inoculant); a+b (potential gas production, mg/380 mg DM); c (gas production rate per t time unit; mL/h).

Changes in fiber fraction generally did not significantly affect total gas production. This could be due to the composition of hemicellulose did not change even though adding the inoculants (Table 1). Previous study stated that hemicelluloses seemed easier to degrade than cellulose and lignin [15]. Hemicellulose was more easily attacked by rumen microbes, which may be due to the lower degree of

polymerization than cellulose. Changes may be seen after an incubation periods > 72 h. However, a significant difference was shown by parameters that showed the efficiency digestibility of substrate, namely the rate of gas production (c) and methane concentration.

The efficiency of substrates utilization was not only a result from total gas production, therefore the concentration of methane need to be measured to determine the effectiveness of feed material [5]. The high availability of glucose is an important factor that affect the lower methane concentration PC and TVPC treatments. The high availability of glucose will help the rumen microbes in obtaining energy for their existence. This could reduce the proportion of cellulose digestion that found in cell wall fractions. Widiawati et al. [2] reported that a substrate containing many cell walls would produce a greater proportion of acetic acid which would have an impact on increase in methane concentration. Methane was a fermentation product that was not utilized and contributed to GHG emission [5].

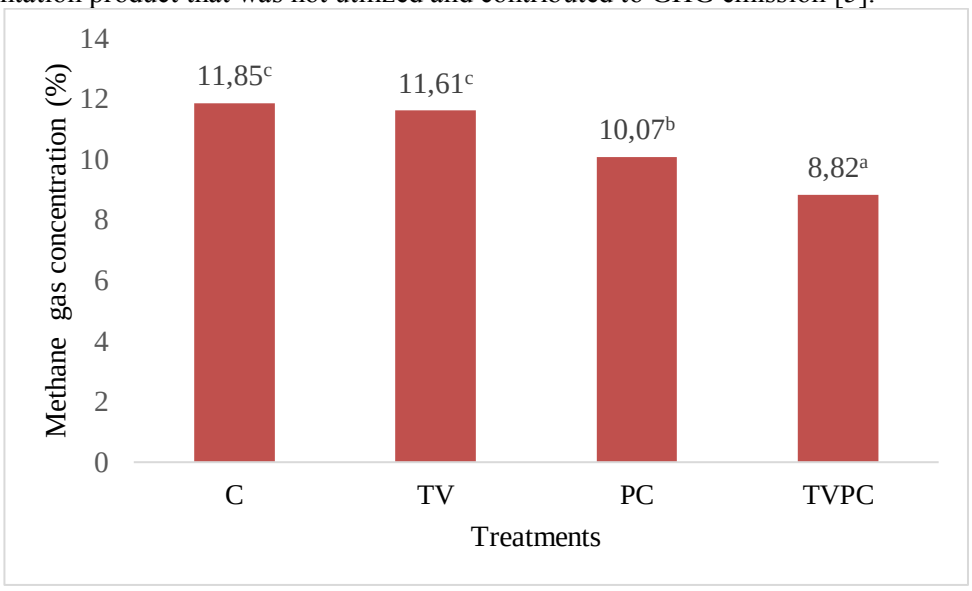

Figure 1. Methane gas concentration of fermented rice straw. C (control, fermented rice straw with no inoculant); TV (fermented rice straw using *T. viride* inoculant); PC (fermented rice straw using *P. chrysosporium* inoculant); TVPC (fermented rice straw using *T. viride* and *P. chrysosporium* inoculant)

3.3. Rumen Fermentation Products

Table 3 shows the rumen fermentation product of fermented rice straw. There was no significant different between all treatments in NH_3 and TVFA. Adding *T. viride* - *P. chrysosporium* inoculants combination could reduce the pH condition by 4.76% compared to control ($P<0.05$).

Table 3. Rumen fermentation products of fermented rice straw

Parameters	C	TV	PC	TVPC	SEM
pH	6.72^{b}	6.51^{ab}	6.49^{ab}	6.4^{a}	0.050
NH_3 (mg/100 ml)	6.67	7.39	8.98	9.51	0.490
TVFA (mM)	70.41	66.67	55.00	58.33	10.710

Standard error mean (SEM); Means with different superscripts within coloumn in same parameters were different ($P<0.05$); C (control, fermented rice straw with no inoculant); TV (fermented rice straw using *T. viride* inoculant); PC (fermented rice straw using *P. chrysosporium* inoculant); TVPC (fermented rice straw using *T. viride* and *P. chrysosporium* inoculant).

The pH value of the control treatment reflect the higher cellulolytic bacterial activity than other treatments. Cellulolytic bacteria optimally live in near neutral pH. This due to the low availability of glucose in the control treatment so that cellulolytic bacteria will be more developed in the *in vitro* ecosystem. Numerically, TVPC treatment produced the highest NH_3 due to the high protein content in

the treatment (Table 1). Numerically, TVFA produced by the control treatment is the highest. This is a representation of the high total gas production in the same treatment (Table 2). TVFA values are positively correlated with total gas production *in vitro* [17].

4. Conclusion

The combination of *T. viride* and *P. chrysosporium* inoculant could increase the availability of glucose compound on fermented rice straw. This could be increase the efficiency percentage of rice straw fermentation in the rumen. This statement was represented by the low methane concentration that produced by TVPC treatment. Addition of fungi innoculants in the rice straw fermentation has been shown to reduce methane emission from ruminant's enteric fermentation. However, adding *T. viride* and *P. chrysosporium* inoculant did not affect the *in vitro* total gas production. Further study is needed to determine the chemical structure change in rice straw after fermentation process.

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