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The Effect of Nitrogen and Sulfur Addition on Bioethanol Solid Waste Fermented by the Consortium of *Trichoderma viride* and *Saccharomyces cerevisiae* towards Dry Materials, Organic Materials, Crude Protein and Non Nitrogen Protein

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Abstract

The aim of this study is to determine the effect of supplementation of nitrogen and sulfur in Bioethanol solid waste fermentation by a consortium of *Trichoderma viride* and *Saccharomyces cerevisiae* to dry matter, organic matter, crude protein and non-protein nitrogen. Experimental design used was a complete-random design factorial 3x3 patterns. The treatment consists of; first factor (N1 = 0%, N2 = 1.5%, N3 = 3%) and the second factor (S1 = 0%, S2 = 0.02%, S3 = 0.04%) in which each treatment was repeated three times. The results showed that there was an interaction in the Bioethanol solid waste which was fermented by a mixture of *Trichoderma viride* and *Saccharomyces cerevisiae on* dry matter and organic matter. The addition of nitrogen at a dose of 3% produced dry matter and organic matter within the average value of 65.56% and 63.81%. The addition of sulfur at a dose of 0.04% produced dry matter and organic matter within the average value of 59.67 and 57.58%. The addition of 1.50% nitrogen without giving sulfur can increase the crude protein content of the substrate ($P < 0.05$) significantly, whereas the non-protein nitrogen content of each treatment have different effects and not real. The results showed 1.5% urea to provide optimal results.

Keywords: Nitrogen, Sulfur, trichoderma viride, saccharomyces cerevisiae, dry matter, organic matter, crude protein, non-nitrogen protein

Introduction

According to Abdoil (Subandi, 2012b) biotechnologists have been succeeding in developing bio-fuel production. It is suggested not to convert edible vegetative to bioethanol. All of these vegetative are food for human consumption. Even the waste product of bioethanol must be further processed for animal feed. Bioethanol process produces solid waste and liquid waste. Solid waste which still has a fairly high potential for biomass can be used as animal feed. Based on the analysis in Ruminant Nutrition Laboratory of Food Chemistry and Faculty of Animal Husbandry, Padjadjaran University, solid waste for bioethanol

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production process still contains protein 2.47%, and 83.94% BETN which is potentially used as a concentrate (Suryani *et al.*, 2013). However, if the waste is fed singly addition to ruminants is still lack of protein; therefore, it needs to be done to improve the quality of fermentation process.

The great potential of microorganisms in improving the quality of the organic waste into food has been known for a long time, due to the aim of solving the feed shortage and preventing the environmental pollution (Apandi *et al*., 1984). Environmental care is in line with the objective of scientific development for moslem as said by Subandi (2012a) when they fail to find verse or sunnah for the source of their pursuit of knowledge ontologically or axiological Islamic, their academic works should axiologically Islamic. *Saccharomyces cerevisiae* is a mold which is often used to feed bioconversion *process.* This Utilization of *Saccharomyces cerevisiae* is based on the ability of the microbes to produce vitamins, enzymes, and nutrients (Dawson and Alison, 1993), those contain a glutamic acid that can improve feed palatability, assimilate and secrete proteins essential amino acids and contains vitamin B complex (Fardiaz, 1992).

Trichoderma viride is sometimes regarded as microorganisms which capable to break down the cellulose and has the ability to synthesize some essential factors to dissolve the cellulose that tied up by hydrogen bonds. *Trichoderma viride* is a potential fungus which potentially produces cellulose in large amounts to degrade cellulose (Mandels, 1970). The benefit of fungus in producing cellulose enzymes just because it can produce a complete cellulose which was required to hydrolyze crystalline cellulose and can produce fairly high protein (Volk, 2004).

Most of microbes that are used in the fermentation can use either inorganic compound or organic compound as a nitrogen source. Urea is one of the nitrogen non-protein sources which contain 45% nitrogen (Parakkasi, 1995). This compound has the function in forming microorganism protein that is part of the protein, nucleic acids and enzymes (Fardiaz, 1992).

Sulfur is a substance for the synthesis of amino acids (Hendrick, 1961). The addition of sulfur to the substrate plays a role in metabolic sulfur and nitrogen. Nitrogen retention would be optimal if the sulfur content is also optimal. The low level sulfur content will make use of nitrogen became also in a low level.

During the fermentation process, the increasing water content of organic matter overhaul by enzymes produced microbes (Winarno *et al*., 1980). Changes in organic matter followed by change or loss / depreciation of the dry ingredients for the dry ingredients a food ingredient consisting of organic and inorganic materials (McDonald *et al*., 1981).

The utilization of nitrogen and sulfur by any type of mold will be in a different metabolism. Consortium by the two types of fungi *(Saccharomyces cerevisiae* and *Trichoderma viride)* is expected to have competition. This utilization will conduct an interaction in the utilization of the nutrients that will lead to optimal proportions in certain doses. Thus this will effect on the fermentation process and will changes dry matter, organic matter, crude protein and nitrogen non-protein substrates.

Materials and methods

Materials used in this study consist of solid waste, urea, sulfur, a consortium of *Trichoderma viride* and *Saccharomyces cerevisiae* (4% of the *substrate).* This study was conducted with an experimental method and experimental design. It used a complete randomized design (CRD) 3 x 3 factorial with three replications (Gaspersz, 1995). The second factor is the level of urea supplementation with doses 0%, 1.5%, 3% and a sulfur level of supplementation with doses 0%, 0.02%, 0.04%.

The process of making inoculums *Trichoderma viride* and *Saccharomyces cerevisiae* can be described as follows: Add 90 g rice flour, 10 g bioethanol solid waste and 100 ml of water into a plastic bag. Then tie up and steam for 1 hour, then store it to a temperature of 27-30°C. Then each of the fungus material put into different plastic as much as 1 ml and put into the steamed materials. Stir it until smooth; the plastic perforated using a needle. The inoculums were

incubated for 7 days in a temperature of 30-35°C. After that the inoculum was dried at a temperature of 40-50°C and milled.

The process of Bioethanol solid waste fermentation can be described as follows: put 100 g of Bioethanol solid waste and 100 ml of distilled water into a plastic bag. Then tie up the plastic and steam for 15 minutes, store it for 30 minutes until the moisture out and the waste is cold. Next, the source of nitrogen (urea) and sulfur (Na sulfate) was added according to treatment and then stir until smooth, a consortium of *Trichoderma viride* and *Saccharomyces cerevisiae* added to the substrate, then stir until it all well blended and put them into plastic. The substrate is kept at room temperature for 7 days (Oboh, 2006).

Finally, the fermentation products were dried in the oven, milled for analysis of crude protein and nitrogen non-protein by using kjedahl method.

Data analysis techniques

This research was conducted with the experimental method that is the experiment using complete randomized design (CRD) factorial 3 x 3 patterns with three replications (Steel and Torrie, 1980, Gaspersz, 1995). The treatment consists of the addition of urea (N1 = 0%, N2 = 1.5%, N3 = 3%) and the addition of sulfur (S1 = 0%, S2 = 0.02%, S3 = 0.04%). Then performed a print-variance analysis, which was followed by Duncan's multiple range test.

Results and discussion

The average value of measured dry of Bioethanol solid waste from each treatment and its repetition are presented in Table 1.

Table 1: Mean ingredients fermentation product dried solid waste bioethanol manufacturing

Nitrogen	Replay	Sulfur ((%)			Average (%)
		S1	S2	S3	
N1	1	57.11	62.15	64.28	
	2	47.38	64.77	65.50	
	3	59.85	62.56	65.31	
Average		54.78	63.16	65.03	60.99
N2	1	67.58	79.72	55.76	
	2	64.24	75.25	59.86	
	3	61.02	78.35	51.57	
Average		64.28	77.77	55.73	65.93
N3	1	75.69	68.03	60.73	
	2	74.49	57.62	59.21	
	3	78.95	60.45	54.89	
Average		76.38	62.03	58.28	65.56

Notes: N1: Supplementation level of 0%, N2: Supplementation level of 1.5%, N3: Supplementation level of 3%, S1: Supplementation level of 0%, S2: Supplementation level of 0.02%, S3: Supplementation level of 0.04%.

Based on Table1 showed that the lowest dry matter content is obtained at 0% nitrogen addition treatment (N1) and sulfur 0% (S1) that is 54.78%, while the highest value obtained in 1,5% nitrogen addition treatment (N2) and sulfur 0.02 % (S2) with a mean value of 77.77%. Based on print-variance analysis, showed the interaction between nitrogen and sulfur. Both nitrogen and sulfur treatments provide significant differences ($P < 0.05$) on a dry matter content.

The interaction between nitrogen and sulfur occurred because the two molds were competed each other in using nitrogen and sulfur for their growth. These will effect on the substrate dry matter fermentation. The dry matter changes may occur due to mold growth, substrate

decomposition and moisture changes. The changes in water levels are caused by evaporation, substrate hydrolysis or metabolic water production (Gervais, 2008).

The Duncan's multiple range tests is carried out to know the differences between each treatment. The addition of nitrogen treatment is presented in Table 2. The table shows that the N1 treatment (nitrogen 0%) markedly lower (P <0.05) than N3 (nitrogen 3%) and N2 (nitrogen 1.5%). While N3 and N2 treatments showed no significant differences (P <0,05).

Table 2: Duncan's multiple range test of treatment effect of nitrogen on dry ingredients.

Treatment	Mean (%)	Significance (0.05)
N1	60.99	a
N3	65.56	b
N2	65.93	b

Description: The same letter in significance column indicates no significant difference (P> .05).
N1: The level of supplementation of 0%, N2: supplementation level of 1,5%, N3: supplementation level of 3%.

The increasing of dry matter content in the solid waste is thought utilized by the body molds for the synthesis of proteins that are part of the dry, so that the increasing protein content substrates can change the dry ingredients. Increased protein occurs during the process of fermentation is caused by the presence of microbial activity due to the addition of proteins and the origin of mass due to growth of microbial cells (Tangendjaja and Pattyusra, 1993).

Based on the research Dhalika, *et al*. (2003), nitrogen supplementation with a dose of 1.50% and 0.04% sulfur on the substrate of onggok fermentation by yeast has increased crude protein up to 6.44%. Based on previous research, Bioethanol solid waste which was fermented by a mixture of *Saccharomyces cerevisiae* and *Trichoderma viride* at doses 2%, for 8 days can increase the crude protein from 2.8% to 3.8 without giving minerals.

The addition of sulfur also causes changes in dry ingredients, but the results are inversely related to treatment with the addition of nitrogen where high sulfur produces a low dry matter compared with no addition of sulfur (P <0.05), as shown in Table 3.

Table 3: Duncan's multiple range test of treatment effect of sulfur on dry ingredients

Treatment	Mean (%)	Significance (0.05)
S3	59.67	a
S1	65.14	b
S2	67.65	b

Description: the same letter in column indicates no significance significantly different (P> 0.05)
S1: Supplementation level of 0%, S2: Supplementation level of 0.02%, S3: Supplementation level of 0.04%

As a result, a lot of dry ingredients are converted into fermented products such as CO_2 and water, thus there is a decline in water levels of dry matter. Energy metabolism which affected on the substrate utilization had changed the dry matter content. The mold growth is strongly influenced by the availability of sources of energy, sources of several minerals, N source, especially S and P. In its activity, the mold used carbohydrates as a source of carbon. The breakdown of carbohydrates will be followed by the release of energy, carbon dioxide and water. The heat released causes the substrate temperature increases. The more sulfur (0,04%), the more energy is used for mold's growth so that the resulting of dry material would be lower.

The average value of the measurement result of Bioethanol organic solid waste materials are presented in Table 4. Based on the table it can be seen that the average organic matter content has a fluctuating change, the average content of organic matter derived from the lowest nitrogen

treatment with the addition of 0% (N1) and sulfur 0% (S1), whereas the mean value of the highest content of organic matter is derived from treatment with the addition of 1.5% nitrogen (N2) and 0.02% sulfur (S2).

The result of the organic matter in the analysis of using print-variance shows the interaction on a combination of nitrogen and sulfur treatments and each treatment gave significant differences (P <0,05). Duncan's multiple range tests was conducted to determine each treatment presented in Tables 5 and 6. Table 5 shows that the addition of nitrogen increases the organic matter content where N2 and N3 was significantly higher (P <0.05) than N1, while Table 6 shows the provision of a higher sulfur (S3) at a dose of 0.04% produces the decline of organic materials.

Table 4: Mean ingredients of organic content on bioethanol solid waste fermentation

Nitrogen	Replay	Sulfur (%)			Average
		S1	S2	S3	
N1	1	55.87	60.87	61.47	
	2	44.41	61.21	62.33	
	3	58.71	59.68	63.92	
Average		53.00	60.59	62.57	58.72
N2	1	64.81	78.88	53.89	
	2	63.17	72.52	57.05	
	3	58.9 0	75.86	50.26	
Average		62.29	75.75	53.73	63.93
N3	1	74.76	66.71	58.42	
	2	72.76	55.37	58.09	
	3	77.35	58, 00	52.84	
Average		74.96	60.03	56.45	63.81

Notes: N1**:** supplementation level of 0%, N2: supplementation level of 1.5%, N3: supplementation level of 3%, S1: Supplementation level of 0%, S2: Supplementation level of 0.02%, S3: Supplementation level of 0.04%.

Similar with dry ingredients, the addition of nitrogen and sulfur treatments produced the same pattern on organic matter, because organic materials are part of the dry matter which was reduced by mineral. Based on the analysis, minerals contained in Bioethanol solid waste has a small value, so it does not have a major impact on overhaul of organic matter compared to the dry ingredients. Therefore, the interaction is the same as the data of dry matter from the compet-

-etion due to the fungus in the use of both nitrogen and sulfur and organic matter substrates for their growth.

The same case occurs in the addition of nitrogen in increasing the organic matter content (Table 5). This is due to the nitrogen that is used by both the fungus to be a body protein, while the protein is part of the organic material. Therefore, an increasing of protein will also increase the organic matter content.

Table 5: Duncan's multiple range test of treatment effect of nitrogen on organic ingredients

Treatment	Mean (%)	Significance (0.05)
N1	58.72	a
N3	63.81	b
N2	63.92	b

Description: The same letter significance column indicates no significant difference (P> 0.05)
N1: The level of supplementation of 0%, N2: supplementation level of 1.5%, N3: supplementation level of 3%

The analysis of sulfur also the same with the data on dry matter. The addition of sulfur can decrease the organic matter content. As explained in the dry ingredients, sulfur is used in the process of energy metabolism, so the addition of it can improve substrate fermentation which will effect on the decreasing of organic matter.

Table 6: Duncan's multiple range test of treatment effect of sulfur on organic ingredients

Treatment	Mean (%)	Significance (0.05)
S3	57.58	a
S1	63.41	b
S2	65.45	b

Description: the same letter in significance column indicates no significant difference (P> 0.05)
S1: Supplementation level of 0%, S2: Supplementation level of 0.02%, S3: Supplementation level of 0.04%.

The average value of the analysis of the crude protein content of Bioethanol solid waste fermented by a consortium of *Saccharomyces cerevisiae* and *Trichoderma viride* and its repetition are presented in Table 7.

Table 7: Mean protein content of coarse bioethanol solid waste fermentation

Factor N	Replay	Factor S			Average
		S1	S2	S3	
N1	1	4.97	3.36	4.73	4.35
	2	4.82	4.67	4.64	4.71
	3	3.96	4.96	4.27	4.40
Average		4.58	4.33	4.55	4.49
N2	1	12.97	7.93	7.04	9.31
	2	13.14	12.39	13.79	13.11
	3	12.97	11.27	9.08	11.11
Average		13.11	10.53	9.97	11.18
N3	1	7.89	11.74	10.77	10.13
	2	7.81	7.81	10.74	8.79
	3	8.31	11.78	12.19	10.76
Average		8.00	10.44	11.23	9.89
Total flats		8.54	8.43	8.58	

Description: N1: 0% supplementation level S1: 0% supplementation level, N2: 1.5% supplementation level S2: 0.02% supplementation level, N3: 3% supplementation level S3: 0.04% supplementation level

Based on Table 7, it can be seen that the average of crude protein content of bioethanol processing solid waste by a consortium of *Trichoderma viride* and *Saccharomyces cerevisiae* is 4.33% - 13.11%. The lowest crude protein content is obtained fr) of 4.33% while increasing om the treatment without the addition of nitrogen (N1) and 0.02% sulfur (S2 the value of the crude protein content is obtained from the treatment of 1.5% addition of nitrogen (N2) and sulfur 0% (S1) amounted to 13.11%. Based on the calculation of print-variance, there is an interaction between nitrogen and sulfur (P <0.05) as well as the influence of nitrogen dose (P <0.05) crude protein content, but the sulfur dose had no significant effect (P> .05).

The interaction between nitrogen and sulfur indicates that in a certain level, both of the two components affect the growth of the two fungi *(Trichoderma viride* and *Saccharomyces cerevisiae).* These interactions occurred in the form of a combination between sulfur and nitrogen which produce a fluctuated crude protein values. The highest crude protein is obtained from treatment of N_2S_1 (1.5% urea and

0% sulfur). This condition happened because nitrogen gave more affects the growth of fungi *(Trichoderma viride* and *Saccharomyces cerevisiae)* than sulfur did, whereas sulfur produces a not significantly crude protein or it may that sulfur which is in the substrate have enough content for the both mold's growth, so the addition of sulfur did not needed anymore.

The difference between treatments was showed by Duncan test (Table 8). Based on the test, it pointed that the higher the addition of urea, the higher the content of the crude protein. The increasing of the crude protein content value of Bioethanol solid waste fermented by *Trichoderma viride* and *Saccharomyces cerevisiae* is caused by the conversion of the adding urea into protein by the fungus.

Table 8: Duncan's multiple range test of treatment effect of nitrogen on the crude protein content

Treatment	Mean Protein Content of Coarse	0.05 significance
N1	4.49	a
N3	9.89	b
N2	11.18	b

Note: Different letters in signify column towards addressing significantly different (P <0.05)

The process of starch bioconversion as the carbon source and the presence of non-protein nitrogen sources such as urea which is added to the media causing mold metabolic processes go hand in hand in supporting the growth of fungi that increase the substrate crude protein content. The presence of nitrogen supplementation on the substrate also affects the crude protein content of Bioethanol solid waste fermented by *Trichoderma viride* and *Saccharomyces cerevisiae.* The growth and the development of fungi required the element carbon and also nitrogen. Nitrogen can be obtained from the degradation of nitrogen-containing compounds in the substrate (Nuswantara *et al.,* 2001).

Trichoderma viride and *Saccharomyces cerevisiae* can grow well because of the availability of the materials needed by the fungus, as proposed by Pratitis (2010) that the growth of mold greatly influenced by the availability of energy sources, source N and some minerals, especially S and P.

The increase of the crude protein content value of Bioethanol solid waste by *Trichoderma viride* and *Saccharomyces cerevisiae* occurs as a result of cooperation between *Trichoderma viride* and *Saccharomyces cerevisiae* in solid waste bioethanol fermentation. *Trichoderma viride* was able to increase the crude protein (Phillipe, 2001) and *Saccharomyces cerevisiae* can produce single cell protein content of crude protein in the range of 47-53% (Suriawiria, 1985).

The raising Starch content in Bioethanol solid waste and the presence of inorganic nitrogen sources urea which is added to the substrates caused the conversion of starch into cell protein lasts balanced in improving the quality of Bioethanol solid waste. The amount of starch utilization for protein synthesis is affected by a process of fermentation that takes place in the system itself, i.e., the decomposition of starch into simple sugar monomers, the decomposition of urea into NH_3 as the nitrogen source of ready-made and the process of formation of the protein through a carbon-ammonia ($R\text{-}NH_{3)}$. The above three processes must occur simultaneously in order to obtain synergy and maximum results (Wang *et al.,* 1979).

The average value of the analysis of non-protein nitrogen content of Bioethanol solid waste fermentation along with the repetition presented in Table 9. Based on Table 9, it can be seen that the average of nitrogen non-protein content of Bioethanol solid waste fermentation product by *Trichoderma viride* and *Saccharomyces cerevisiae* varies, ranging from 0.16% -0.58%. The optimal nitrogen non-protein content of Bioethanol solid waste fermentation by *Trichoderma viride* and *Saccharomyces cerevisiae* is about 0.16%. It is obtained from the addition of nitrogen treatments 0% (N1) and 0.04% sulfur (S3), while the value of nitrogen

non protein content of the highest of 0.58% is obtained from the addition of nitrogen treatment 3% (N3) and sulfur 0% (S1). The presence of non-protein nitrogen values came from bioethanol solid waste and the addition of urea.

Table 9: Non-Protein nitrogen content mean of bioethanol solid waste fermentation

Factor N	Replay	Factor S (%)			Average
		S1	S2	S3	
N1	1	0.13	0.48	0.16	0.26
	2	0.27	0.67	0.16	0.37
	3	0.63	16	0.15	0.31
Average		0.34	0.44	0.16	0.31
N2	1	0.38	0.53	0.08	0.33
	2	0.62	0.70	0.60	0.64
	3	0.16	0.39	0.41	0.32
Average		0.39	0.54	0.36	0.43
N3	1	0.51	0.56	0.39	0.49
	2	0.72	0.28	0.33	0.44
	3	0.51	0.31	0.33	0.38
Average		0.58	0.38	0.35	0.44
Total flats		0.36	0.45	0.29	

Description: N1: 0% supplementation level S1: 0% supplementation level, N2: 1.5% supplementation level S2: 0.02% supplementation level, N3: 3% supplementation level S3: 0.04% supplementation level

Based on the results of print-variance, there is no interaction between nitrogen and sulfur ($P > 0.05$) or the dose of nitrogen and sulfur. The lack of effect of the addition of sulfur and nitrogen content of non-protein nitrogen urea is added allegedly utilized by fungi or partially lost during the process of fermentation, because urea is dissolved easily lost this was confirmed by the results of average content of non-protein nitrogen in the nitrogen addition treatment in below 1% and the same as the solid waste that is not given nitrogen. At the optimal fermentation process on the substrate nitrogen is converted by the fungus (combination between *Trichoderma viride* and *Saccharomyces cerevisiae*) into proteins and little is left to be non-protein nitrogen, otherwise if urea is not optimally utilized by the fungus, so it will be many remaining nitrogen in the form of non- protein nitrogen.

The higher the level of nitrogen addition, the higher the content of pure protein. Nitrogen which is derived from urea is used by microorganisms in the fermentation process is then broken down into ammonia compounds during the fermentation process. Microorganisms combine with ammonia to form the products of carbohydrate metabolism and amino acid form of pure protein (Stanton and Whittier, 2010). Microbes in the fermentation process can produce enzymes that can degrade complex compounds into simpler and synthesize proteins which are protein enrichment process (Darmawan, 2006). Based on this, it can be seen that most of nitrogen non-protein compounds in the form of pure nitrogen is converted into proteins in the form of microbial protein (Dhalika *et al.*, 2003).

Conclusion

The addition of nitrogen and sulfur in Bioethanol solid waste fermented by *Trichoderma viride* and *Saccharomyces cerevisiae* generate the interaction on the content of dry matter and organic matter.

The addition of nitrogen in Bioethanol solid waste fermented by *Trichoderma viride* and

Saccharomyces cerevisiae gave significant differences (P <0.05), the level of 3% produced dry matter and the highest organic matter, that is 65.56% and 63.81%.

The addition of Sulfur into Bioethanol solid waste fermented by *Trichoderma viride* and *Saccharomyces cerevisiae* produce dry matter and real organic (P <0,05). The decreasing of dry matter and organic matter are obtained from S3 treatment (0.04%) that is each of the mean value 59.67% and 57.58%.

There is an interaction between nitrogen and sulfur supplementation on crude protein but not in the nitrogen non-protein.

The supplementation of nitrogen at the level of 1.50% without the addition of sulfur in the fermentation of bioethanol solid waste by a consortium of *Trichoderma viride* and *Saccharomyces cerevisiae* can produce optimal crude protein for about 13.03%.

The supplementation of sulfur up to 0.04% had no significant effect on the results of crude protein and nitrogen non-protein.

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