

## NUTRITIONAL COMPOSITION OF MAIZE HUSK SILAGE GENERATED FROM SOLID STATE FERMENTATION BY *TRICHODERMA VIRIDE* UP01

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### Abstract

Maize husk is an agricultural residue and may be an available substrate for nutrition improvement by cellulolytic fungi. To improve the nutrition values of maize husk for cattle and dairy feeding, cellulolytic fungus *Trichoderma viride* UP01 was isolated from maize husk dump and characterized through Solid State Fermentation (SSF). When *T. viride* UP01 was incubated in SS medium contained maize husk as the sole carbon source at 30°C for 7 days, the values of total cellulase, exoglucanase, endoglucanase,  $\beta$ -glucosidase, and xylanase were achieved at  $0.37 \pm 0.03$ ,  $0.08 \pm 0.02$ ,  $0.38 \pm 0.01$ ,  $0.16 \pm 0.01$ , and  $1.42 \pm 0.07$  U/mL, respectively. The optimal pH and temperature (°C) for the enzymatic activities were 50-60°C and pH 5.0-6.0. Maize husk supplementary with 1.0% (w/w) rice bran, 1.0% (w/w) molass, and 0.1% (w/w) of *T. viride* UP01 starter was fermented for 15 days at room temperature. The nutrition values of fermented maize husk silage resulted in increased protein (38.6 g/kg DM) fat (23.5 g/kg DM) and acetic acid (protein 21.1%, fat 16.4%, and acetic acid 13 g/kg DM) which was higher than the control without mold starter (protein fat and acetic acid). The isolates *T. viride* UP01 was the strain found to be the optimal starter at improving the nutrition values of maize husk silage for cattle and dairy feeding.

**Key words:** Maize husk, *Trichoderma viride*, Cellulase, SSF, Nutrition values.

### Introduction

Different kinds of agricultural residues including maize husk, stover, sugarcane bagasse, cassava and pumpkin are the alternative products to overcome the shortage of animal feed in Thailand. However, maize is a major crop for cattle and dairy feeding in Northern of Thailand with 60% of producing areas and  $2.8 \times 10^6$  ton of products (Anon., 2017). Previous studies in Phayao province, that showed maize residues (husk, stalk, and stover) without grain could be formulated and used as conventional animal feed in terms of availability roughage but the residues were limited by its high fiber, and could be changed into rotten plant when contamination by spoilage microorganisms (Danmek *et al.*, 2014). To resolve this problem, fermentation process by bacteria, fungi, and yeast has been applied for increasing the nutrition values of the substrates (Danmek *et al.*, 2014). Sharma & Arora (2011) reported the use of fungal inoculums for the fermentation of agricultural residues in order to improve the nutrition values of substrates. During solid state fermentation (SSF), the increased nutrition values were due to bioconversion of substrate that had been degraded by fungal enzymes. The microorganisms including yeast and fungi were prominent and replaced the previous microbial populations to improve protein content (Danmek *et al.*, 2014).

Cellulolytic microorganism could be used to improve nutrition values of roughage silage for ruminant feeding through fermentation or ensiling processes, involving the development of the digestibility of silage that contain large amounts of water insoluble carbohydrates or lignocellulosic biomass. Cellulolytic fungi that include *Trichoderma viride* is capable to degrade the lignocellulosic material selectively by firstly degrading cellulose component and secondly improve nutrition values of substrate. The fungus produce cellulase an important enzyme which is employed in industrial processing for hydrolysis of cellulosic materials into

reducing sugar constituents including glucose and fructose to lactic acid, acetic acid and other end products as bio-control agents to inhibit the activity of soil-borne pathogens (Harman *et al.*, 2004; Anwar *et al.*, 2014).

Maize husk is one of lignocellulosic materials contained high values of cellulose and hemicellulose that could be degraded by a combination of three main types of fungal cellulase including endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91), and  $\beta$ -glucosidase (EC 3.2.1.21) (Zhang *et al.*, 2013). However, the success of fermentation depends on optimum conditions based on fungal starter, substrate composition and incubation time that affect the nutrition values of fermentation product (Nuraini & Suslina, 2009). The most important mechanisms that should be present in the fermentation process is a source of agricultural products, nitrogen sources and other essential elements in the number and the appropriate balance (Carlile & Watkinson, 1995). Therefore, the main objective of this experiment was to study on the possibilities to improve the nutrition values of maize husk by SSF process with cellulolytic fungi isolated from maize husk dump.

### Materials and Methods

**Culture collection:** Standard fungus *T. viride* TISTR 3174 was obtained from Thailand Institute of Scientific and Technological Research (TISTR). The fungus was maintained in Potato Dextrose Agar (PDA) in the laboratory of Biotechnology program, School of Agriculture and Natural Resources, University of Phayao, Thailand. The fungus was maintained in PDA agar slants at 4°C until use.

**Substrate preparation:** Maize husk was collected from maize dumps near University of Phayao, Thailand (19°02'44.4"N, 99°52'38.2"E) in 2017. The sample was preserved in a sterile plastic bag, and then was transferred to the laboratory. Sample was dried at 50°C in a hot air

oven for 3 days and then ground, passed through 2.0 mm sieve before use. The sample was determined for lignocellulosic compositions (Goering & Van Soest, 1970) and used as substrate for enzyme production.

**Isolation and determination of cellulase producing strains:** The ground sample was used to isolate cellulolytic fungi by serial dilution and spread plate technique on SS agar (Danmek *et al.*, 2014) containing (g/L): CaCl<sub>2</sub> (0.1), MgSO<sub>4</sub> (0.5), KH<sub>2</sub>PO<sub>4</sub> (2.0), yeast extract (1.0), peptone (2.0), MnSO<sub>4</sub>·H<sub>2</sub>O (0.05), FeSO<sub>4</sub>·7H<sub>2</sub>O (0.05), ZnSO<sub>4</sub>·7H<sub>2</sub>O (0.005), and carboxymethylcellulose (CMC) (20), pH 5.5 at 30°C for 7 days. Cellulolytic fungi were detected by flooding the culture plate with 0.01% Congo red reagent to detect the zone of clearance. Cellulolytic fungi were transferred on PDA to prepare the homogenous spore suspension (10<sup>6</sup> spore/mL) by suspending in 0.85% (w/v) sodium chloride (NaCl).

**Solid state fermentation:** Maize husk (5.0 g) was weighed and put into a 250 mL Erlenmeyer flask containing 40 mL of the SS medium without glucose and moisture content was adjusted to 63% using Extech SDL550 Moisture Content Meter, Datalogger, USA (Danmek *et al.*, 2014). The culture was inoculated with 2.0 mL of each spore suspension (10<sup>6</sup> spore/mL) of isolates fungi and incubated at 30°C for 7 days. Crude enzyme was extracted by adding 50 mL of 50 mM sodium citrate buffer pH 5.0 containing 0.01% (w/v) Tween 80 and mixed on a rotary shaker at 150 rpm for 1.0 hour. The suspension containing the crude enzyme was centrifuged and clarified supernatant to make a total volume 100 mL for further studies.

**Characterization of fungal enzymes:** Crude enzymes were determined for activities of total cellulase (FPase), exoglucanase (Avicelase), endoglucanase (CMCase) and xylanase using the method described by Ghose (1987) and Ghose and Bisaria (1987). β-glucosidase activity was determined using the method described by Sternberg *et al.*, (1977). All reactions were determined by estimating the reducing sugar liberated using DNS reagent (Miller, 1959), and the activities of enzymes in the term of international units were calculated.

One unit of cellulase (FPase, Avicelase, CMCase, and β-glucosidase) is defined as the amount of enzyme that liberates 1.0 μmol of glucose per 1.0 min under the assay condition. In addition, one unit of xylanase is defined as the amount of enzyme that liberates 1.0 μmol of xylose per 1.0 min under the assay condition.

The optimal pH for enzyme activities was determined by changing the assay reaction mixture using several buffers including 50 mM of sodium acetate pH 3.0-5.0, sodium phosphate pH 6.0-8.0. The optimum temperature for the enzyme activities was determined by measuring the activity at different temperatures (40–80°C) in the optimal buffer. The effects of metal ions which included Mg (MgSO<sub>4</sub>), Ca (CaCl<sub>2</sub>), Mn (MnSO<sub>4</sub>), Fe (FeSO<sub>4</sub>), Co (CoCl<sub>2</sub>), Cu (CuSO<sub>4</sub>), Zn (ZnSO<sub>4</sub>), and ethylenediaminetetraacetic acid (Na-EDTA) were investigated by incorporating them to 0.1 M final concentration mixture prior to determination of enzyme residual activities.

**Maize husk silage by solid state fermentation:** Spore suspension (10<sup>6</sup> spore/mL) of the isolate demonstrating the best level of cellulase and xylanase activities was prepared in 0.85% normal saline (NaCl) by adjusting spore amount to 10<sup>6</sup> spore/mL. 300 mL of suspension was added into 1 kg glutinous rice flour, then dried at 40°C and powdered for the mold starter (Danmek *et al.*, 2014). Maize husk was chopped by a conventional forage harvester to 0.5 cm. Ten-kilogram of the substrate was transferred into 30 kg plastic bag and supplemented with 1.0% (w/w) rice bran, 1.0% (w/w) molass, and 0.1% (w/w) of *T. viride* UP01 starter, covered with cheesecloth and stored at ambient temperature (average 30°C) for 15 days. The Dry matter (DM) recovery of the fermented maize husk silage was determined by subtracting the initial weights from the final weights of the bag, considering the DM content of the fermented material. Physical characteristics and nutrition values of fermented silage were determined by the standard of quality assessment of Anon., (2006). In addition, in all experiments crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF) contents, pH values, and volatile fatty acid (lactic acid, acetic acid, butyric acid) were determined by standard of quality assessment of Anon., (2006). The number of yeasts, fungi, and bacteria were observed on PDA by spread-plate technique. The plates were incubated at 30 °C the counting of yeasts and bacteria of incubated plates was done for 2 days but yeast for 3 days. All the microbiological data were shown in the term of log-transformed.

### Statistical analysis

The analysis of variance (ANOVA) using completely randomized design (CRD) was carried out and then compared the difference of each average treatment with independent sample.

For determining the differences between experimental groups F-test was conducted by using the statistical program (SPSS Version 22). The significance of testing was set at  $p < 0.05$ .

### Results and Discussion

On the basis of cultural, morphological and biochemical characterization, the isolated microorganisms were also identified using the cultural characteristics on PDA plate. The conidia, and hyphae structures were also identified. The fungal isolates were obtained from maize husk sample by serial dilution on SS agar (Danmek *et al.*, 2014). Up on colony morphological study and microscopic analysis, the isolates were found to be *Aspergillus flavus* (21.43%), *A. parasiticus* (10.71%), *A. fumigatus* (10.71%), *A. niger* (10.71%), *A. oryzae* (7.14%), *Mucor* sp. (14.29%), *T. viride* (10.71%) *N. crassa* (3.57%) and *Penicillium* sp. (10.71%) (Table 1). This confirms the findings of Odhiambo *et al.*, (2013) and Abe *et al.*, (2015) that isolated fungi from maize and soil samples normally included *A. flavus*, *A. parasiticus*, *A. fumigatus* and *Trichoderma* spp. In this study, the diameters of the zones of clearance on CMC plate of the cellulolytic isolates ranged between 5.16±0.42 to 8.15±0.26 cm while all isolates *T. viride* showed the highest zone of clearance with the range of 7.23±0.88 to 9.12±0.13 cm. The pure cultures of *T. viride* were preserved on PDA slants at 4°C for further studies on cellulase production.

**Table 1. Fungal isolates from maize husk dump and the percentage of occurrence.**

Fungal	Number of isolates	% Occurrence
<i>A. flavus</i>	6	21.43
<i>A. parasiticus</i>	3	10.71
<i>A. niger</i>	3	10.71
<i>A. oryzae</i>	2	7.14
<i>A. fumigatus</i>	3	10.71
<i>Mucor</i> sp.	4	14.29
<i>T. viride</i>	3	10.71
<i>Penicillium</i> sp.	3	10.71
<i>N. crassa</i>	1	3.57

**Table 2. Composition of fermented maize husk silage in the experiments.**

Parameters (g/kg)	without starter	with 0.1% starter
Dry matter	451.3 <sup>a</sup>	432.8 <sup>b</sup>
Crude Protein	21.1 <sup>b</sup>	38.6 <sup>a</sup>
Ether extract	16.4 <sup>b</sup>	23.5 <sup>a</sup>
NDF	582 <sup>a</sup>	599 <sup>a</sup>
ADF	433 <sup>a</sup>	426 <sup>a</sup>
Lactic acid	64 <sup>a</sup>	62 <sup>a</sup>
Acetic acid	13 <sup>b</sup>	18 <sup>a</sup>
Butyric acid	0.5 <sup>a</sup>	1.4 <sup>a</sup>
pH	3.7 <sup>a</sup>	3.7 <sup>a</sup>
Yeast (log <sub>10</sub> CFU/g silage)	3.3 <sup>a</sup>	2.5 <sup>b</sup>
Fungi (log <sub>10</sub> CFU/g silage)	2.1 <sup>a</sup>	2.2 <sup>a</sup>
Bacteria (log <sub>10</sub> CFU/g silage)	3.1 <sup>a</sup>	3.0 <sup>a</sup>

Maize husk is an agricultural residue, it contains the high values of cellulose (33.15% DM) and hemicellulose (44.67% DM) but low values of lignin (4.91% DM) (Danmek *et al.*, 2014). To improve the nutrition values of maize husk for cattle and dairy feeding, isolates *T. viride* were characterized through solid state fermentation (SSF) contained maize husk as the sole carbon source at 30°C for 7 days. The fungus of *T. viride* UP01 (Fig. 1) was

found to be the best cellulase and xylanase producer under acidic conditions with pH 5.5. When testing the crude enzyme at 50°C, this strain gave 0.37±0.03, 0.08±0.02, 0.38±0.01 0.16±0.01 and 1.42±0.07 U/mL of total cellulase, exoglucanase, endoglucanase, β-glucosidase and xylanase, respectively (Fig. 2). Gomes *et al.*, (2006) and Gautam *et al.*, (2010) reported that *Trichoderma* species including both *T. reesei* and *T. viride* produced the high values of cellulase activities in the sixth to nine days of incubation by Solid State Fermentation (SSF) when using wheat bran or municipal solid waste as substrates.

Among the physical characteristic's determination, pH of the growth medium plays an important role by inducing morphological changes in microbes and in the enzyme secretion. The effect of pH on the enzyme activities indicates that the total cellulase, exoglucanase, endoglucanase, β-glucosidase, and xylanase activities are active in the pH range 4-8 with the highest activity at pH 5-6 (Fig. 3). When the effect of temperatures on enzymatic activities were studied, *T. viride* UP01 showed the highest activities at 60°C and were found to be 0.44±0.03, 0.18±0.03, 0.42±0.01, 0.22±0.01 and 1.63±0.02 U/mL of total cellulase, exoglucanase, endoglucanase, β-glucosidase and xylanase, respectively. A decrease in enzyme activities was found when temperature was over than 60°C (Fig. 4). The results of the interaction between the temperature and pH revealed that increasing both temperatures (>60°C) and pH (>6.0), led to a decrease of enzyme activity. Several reports indicated that cellulase and xylanase activities were favored in the acidic range of pH 4 to 6 and temperature range 50-70°C (Romero *et al.*, 1999; Shafique *et al.*, 2009; Brijwani *et al.*, 2010; Tishkov *et al.*, 2013; Onofre *et al.*, 2014). Supporting of this result, Gomes *et al.*, (2006) reported both *T. reesei* and *T. viride* showed the best activities of cellulase at pH range between 4.5 to 5.5 and the temperature range at 50 to 55°C. In addition, Shankar *et al.*, (2014) reported the enzyme from *Trichoderma* sp. was functional in the pH range between 4.0 to 6.5 with optimum activity at pH 5.0 for crude enzyme and 5.0 for partially purified enzyme at temperature range of 30-70°C.

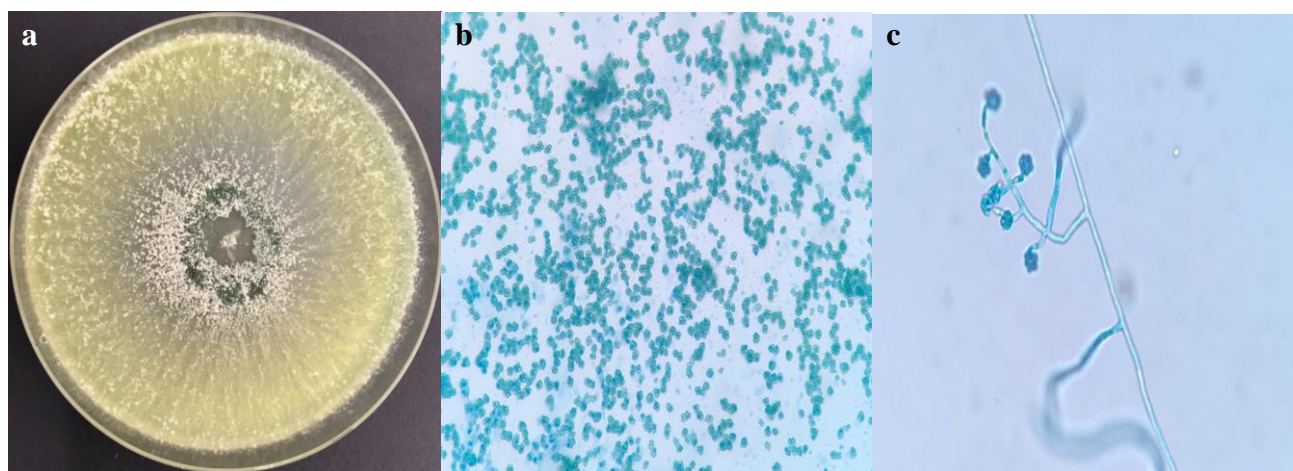


Fig. 1. Morphological characteristics of *T. viride* UP01 on PDA plate at 30°C for 7 days; (a) culture on PDA, (b) conidia, (c) conidiophore.

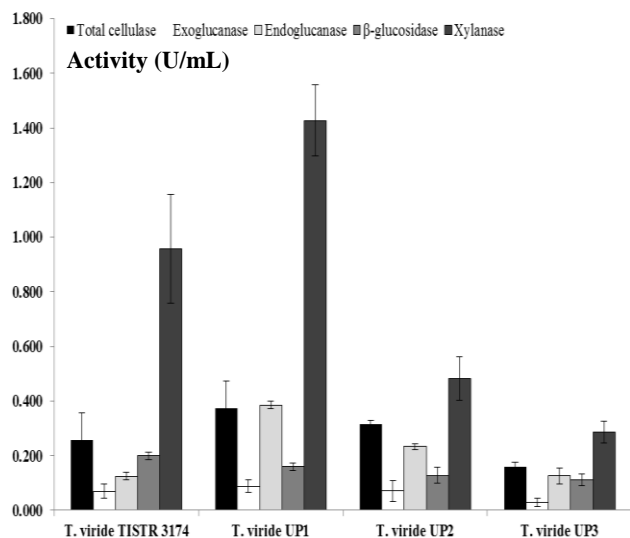


Fig. 2. Cellulase and xylanase activities of isolated cellulolytic strains of *T. viride* under SSF in SS medium at 30°C for 7 days.

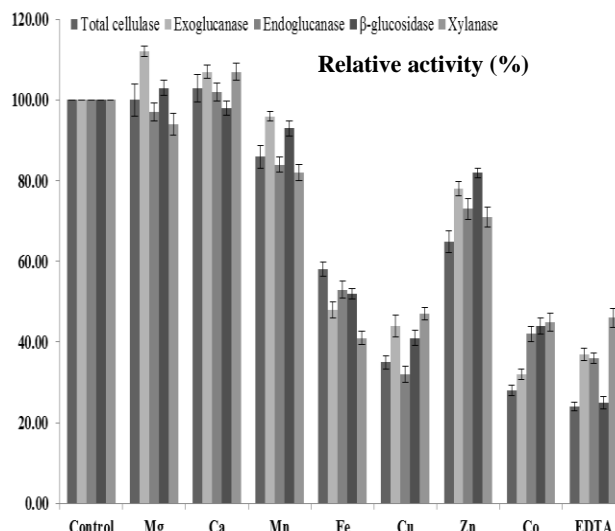


Fig. 5. Effect of metal ions and substances on crude enzyme activities of *T. viride* UP01.

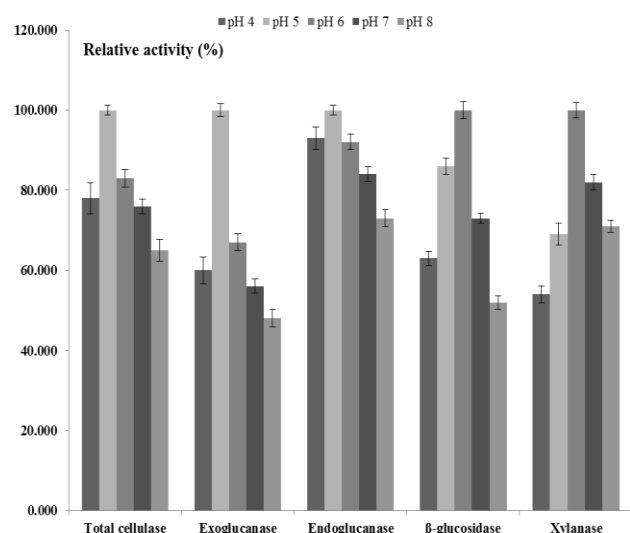


Fig. 3. Effect of pH on crude enzyme activities of *T. viride* UP01

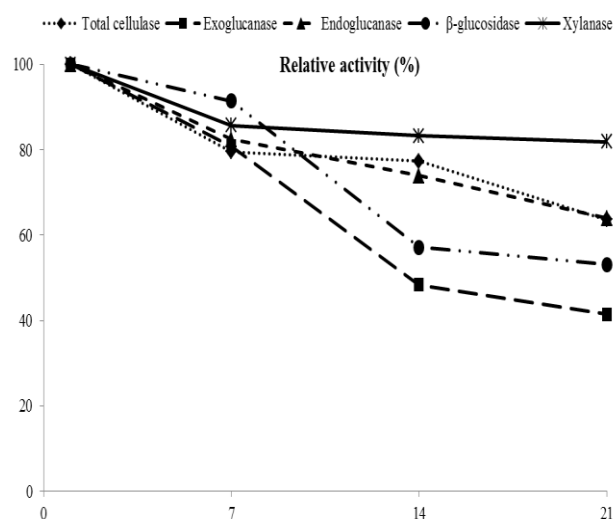


Fig. 6. Stability of crude enzyme from *T. viride* UP01 at 30°C for 21 days.

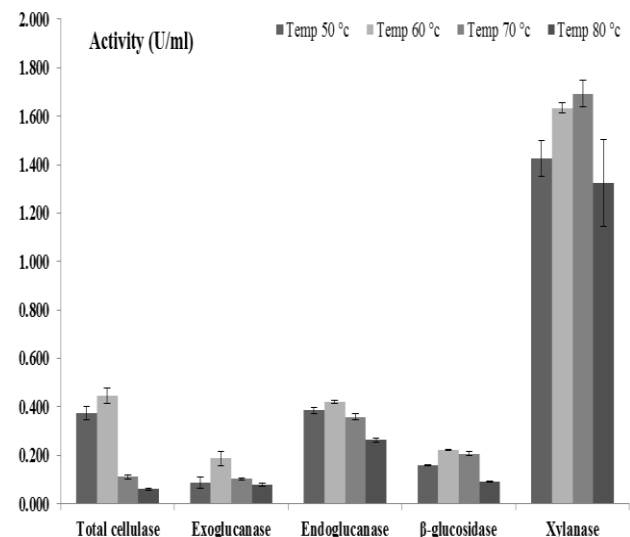


Fig. 4. Effect of temperature on crude enzyme activities of *T. viride* UP01.

The effect of metal ions and substances were also summarized in Fig. 5. None of the metal ions and substances induced both cellulase and xylanase activities. Approximately 50% of relative activities were found in the present of Cu ion (35±1.6% in total cellulase, 44±2.8% in exoglucanase, 32±2.0% in endoglucanase, 41±1.9% in β-glucosidase and 47±1.5% in xylanase respectively). The inhibition of enzyme activities might be affected by the high ion and substance concentration (0.1 M final concentration). According to Tejirian & Xu (2010) the high values inhibitions of cellulase were found in the presence of Cu and EDTA. Shankar *et al.*, (2014) showed that Fe<sup>3+</sup> was found to be extremely inhibiting chemical substance for cellulase activities, whereas Cu<sup>2+</sup> and Co<sup>2+</sup> were inhibitory to a small extent. In addition, Zeng *et al.*, (2016) reported that Zn<sup>2+</sup>, Ca<sup>2+</sup> and Mn<sup>2+</sup> could remarkably induce the enzyme activity while Cu<sup>2+</sup> and Co<sup>2+</sup> could inhibit the cellulase activity. When the crude enzyme of *T. viride* UP01 was preserved at room temperature, approximately 50% relative activities were found after 14 days of preservation (Fig. 6).

The possibilities to improve the nutritional composition of maize husk silage generated from SSF by *T. viride* UP01 were determined in the laboratory. Maize husk was fermented with 1.0% (w/w) rice bran, 1.0% (w/w) molass, and 0.1% (w/w) of *T. viride* UP01 starter for 15 day at room temperature. At the end of the fermentation period, the DM content of fermented maize husk silage with the mold starter was 432 g/kg. The NDF and ADF content were 632 and 526 g/kg DM, respectively. In contrast, loss of hemicellulose occurred in the fermentation process could be due to a combination of enzymatic activities from *T. viride* UP01 and acid hydrolysis under fermentation process when compared with the control. The protein and fat contents (38.6 and 23.5 g/kg DM) verified in the fermented maize husk silage in the treatment with the starter were higher than the control without the starter (21.1 and 16.4 g/kg DM). In addition, the pH value of the fermented maize husk silage was 3.7. This result could be due to the characteristics of fungal starter, microbial community and fermentation process. The number of yeasts, fungi and bacteria on fermented maize husk silage were found to be 2.5, 2.2, and 3.0 Log<sub>10</sub> CFU/g respectively. Moreover, the concentrations of lactic and butyric acid in fermented maize husk silage were not affected by the starter (Table 2) except acetic acid ( $p < 0.05$ ) because an aerobic solid-state fermentation. The chemical and microbiological communities were changed when silages were exposed to air (Ranjit & Kung, 2000). Avila *et al.*, (2009) also observed an increase in yeast and mold count in all silages of Mombasa grass after opening the silos (during aerobic exposure). Overall of this study, fermented maize husk silage contained a starter of *T. viride* UP01 help improves the nutrition contents of maize husk and a more stable at low pH.

## Conclusions

The experiment of this study investigated the possibility of using low cost agricultural product including maize husk as the substrate for cattle and dairy feeding. The nutrition values of the sample were improved by fermenting with *T. viride* UP01 through SSF. This process increased nutrition values including the protein level to 38.6 g/kg DM which was 1.83-fold greater than the control without the starter (21.1 g/kg DM). The isolates *T. viride* UP01 was the strain found to be the optimal starter for improving the nutrition values of maize husk silage for cattle and dairy feeding.

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