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Yeast with surface displayed xylanase as a new dual purpose delivery vehicle of xylanase and yeast

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ABSTRACT

This study aimed to develop a yeast strain that surface displays a xylanase that was found from a rumen fosmid library and optimized by directed evolution (Orf6-un$_m$). The Orf6-un$_m$ enzyme was successfully surface-displayed using *Saccharomyces cerevisiae* EBY100 as host (referred to as EBY100-pYD1-*orf6-un$_m$*), yielding a specific xylanase activity of 137 U/g dry cells. The EBY100-pYD1-*orf6-un$_m$* had greater xylanolytic activity and produced more xylose from beechwood xylan than the purified Orf6-un$_m$ overexpressed in *Escherichia coli*. The EBY100-pYD1-*orf6-un$_m$* was evaluated for its effect on digestion of corn stover by in vitro rumen cultures. Both EBY100 and EBY100-pYD1-*orf6-un$_m$* increased volatile fatty acid production, dry matter degradation, and total bacteria population, while shortening the lag time of gas production. However, EBY100-pYD1-*orf6-un$_m$* increased both gas production and dry matter degradation, and shortened the lag time to greater magnitudes than EBY100. EBY100-pYD1-*orf6-un$_m$* may be used to deliver both xylanase and live yeast to feed animals.

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1. Introduction

Microbial degradation of plant cell wall materials is of great societal and agricultural importance. Dietary supplementation with exogenous fibrolytic enzymes, primarily xylanases and cellulases, has the potential to effectively improve digestion of feed, especially fibrous feed (Meale et al., 2014). Exogenous enzyme products are typically mixed into the rations of animals (poultry, swine, and cattle). However, direct supplementation of enzyme products to diets has several disadvantages (Meale et al., 2014). Firstly, the enzyme products need to be stored properly (at low temperature, for instance) to maintain their enzymatic activity. This may be difficult during transportation to and storage on farms. Secondly, the enzyme products need to be produced through a fermentation process and purified, adding cost to feeding operation. In addition, enzyme products in free form can be readily degraded by gastrointestinal microbes, especially when free enzymes are fed to ruminant animals. Such degradation of supplemented enzymes will decrease the enzyme activities, diminishing the efficacy of the enzyme supplementation. This is exemplified in the study by Hristov et al. (1998). Therefore, other modes of delivery of exogenous enzymes to animals are needed that can circumvent the above limitations.

Abbreviations: CMC, carboxymethyl cellulose; CMCase, carboxymethyl cellulase; DMD, dry matter degradation; DNS, 3,5-dinitrosalicylic acid; FBS, fetal bovine serum; GH, glycoside hydrolase; GP, gas production; HPLS, high performance liquid chromatography; PBS, phosphate buffered saline; RBB-xylan, remazol brilliant blue R-D-xylan; VFA, volatile fatty acid; YNB, yeast nitrogen base.

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Yeast (*Saccharomyces cerevisiae*) and its fermentation products have been commonly used to improve feed digestion and animal nutrition in a cost-effective manner (Oeztuerk and Sagmanligil, 2009; Poppy et al., 2012). Recent development

The activity of the EBY100-pYD1-*orf6-un$_m$* was further determined in three replicates using the 3,5-dinitrosalicylic acid (DNS) method (Bailey et al., 1992) with D-xylose as the standard. Briefly, an aliquot of the EBY100-pYD1-*orf6-un$_m$* cells was harvested by centrifugation (5000 × *g* for 10 min at 4 °C) and then washed once with PBS. The washed cells were freeze dried to determine the dry weight of cell mass. The dried yeast cells were resuspended in PBS, and the cell suspension (50 μl) was mixed with 50 μl 0.01 g/ml beechwood xylan (Sigma, Saint Louis, MO, USA) in McIlvaine's buffer (pH 5.0). After incubation at 50 °C for 20 min, 100 μl DNS was added to stop the reaction, and then the mixture was heated at 100 °C for 10 min. Optical density was determined at 540 nm on a SpectraMax M5 (Molecular Devices, Sunnyvale, CA). The amount of reducing sugar was determined from the D-xylose standard curve. The specific xylanase activity was defined as the amount (μmol) of xylose produced per min per gram dried cells of EBY100-pYD1-*orf6-un$_m$*.

2.4. Characterization of the EBY100-pYD1-orf6-un$_m$ xylanase

The effect of pH on the EBY100-pYD1-*orf6-un$_m$* activity was determined over a wide range of pH using two different buffer systems: pH 3.0–8.0 in McIlvaine's buffer and pH 9.0–10.0 in glycine–NaOH buffer (0.2 mM each) at 50 °C, which was the optimal temperature for Orf6-un$_m$ overexpressed in *E. coli*. The xylanase activity was determined as described above. To determine the pH stability, EBY100-pYD1-*orf6-un$_m$* cells were incubated in McIlvaine's buffers and glycine–NaOH buffer at various pH at 37 °C for 30 min, and the residual xylanase activity was determined as mentioned above. The optimal temperature for xylanase activity was determined in McIlvaine's buffer at the optimal pH (6.0) of Orf6-un$_m$ and at 30, 40, 50, 60, 70, 80, or 90 °C. The thermal stability of the EBY100-pYD1-*orf6-un$_m$* was evaluated by determining the residual xylanase activity after incubation for 30 min in McIlvaine's buffer (pH 6.0) at 50, 60, 70, 80, and 90 °C.

The hydrolysis products of the purified Orf6-un$_m$ xylanase overexpressed in *E. coli* (referred to as *E. coli*-expressed Orf6-un$_m$) and the EBY100-pYD1-*orf6-un$_m$* were determined using beechwood as the substrate. Briefly, 0.01 g/ml beechwood xylan solution in McIlvaine's buffer (pH 6.0) was incubated with Orf6-un$_m$ or the EBY100-pYD1-*orf6-un$_m$* (10 U/ml) at 50 °C. Samples were collected at 5 min, 0.5, 1, 2, 4, 6, 12, 24, and 36 h after start of the incubation and boiled for 10 min to inactivate the enzyme activity. The samples were analyzed, with xylose, xylobiose, xylotriose and xylotetraose as serving as reference standards, for released sugars using an Alliance HPLC system (Separations module e2695, Waters Corporation, Milford, MA, USA) equipped with a Sugar-Pak TM 1 column (300 mm × 6.5 mm) and refractive index detector (Waters 2414, Waters Corporation, Milford, MA, USA). Ethylenediaminetetraacetic acid calcium disodium salt hydrate (50 mg/l) was used as the mobile phase with a flow rate of 0.3 ml/min.

2.5. Evaluation of the EBY100-pYD1-orf6-un$_m$ using in vitro rumen fermentation

2.5.1. In vitro fermentation

The EBY100-pYD1-*orf6-un$_m$* was evaluated using *in vitro* ruminal fermentation of corn stover with a single factorial arrangement of three treatments: control without any yeast addition, yeast EBY100, and yeast EBY100-pYD1-*orf6-un$_m$*. Yeast was added at 2 g/l (dry cell mass), which is approximately equivalent to 120 g/d/cow for dairy cows (Mao et al., 2013). The *in vitro* fermentation experiment was conducted in four replicates per treatment. Fresh rumen fluid was collected from three donor sheep fed a mixed diet of lucerne hay and a concentrate mixture (50:50, wt/wt) twice daily. The ingredients of the concentrate mixture were detailed in Mao et al. (2013). The *in vitro* rumen fermentation was conducted using 120-ml serum bottles each containing 5 ml of fresh rumen fluid as the inoculum, 45 ml of buffered medium (Theodorou et al., 1994), and 500 mg of corn stover (ground to 1 mm particles). The corn stover was grown in Xingtai of Hebei Province, China and acquired in autumn. The acquired corn stover was dried at 65 °C for long-term preservation. Chemical composition (as DM %) of the corn stover was 5.8% crude protein, 70.8% neutral detergent fiber and 39.6% acid detergent fiber (the fibrous component contents were determined according to methods by Van Soest et al. (1991). No sulfite or/and heat stable amylase was included in analysis procedures, and were calculated without excluded of residual ash). Four extra bottles without corn stover or yeast preparation were included in parallel to provide a baseline for the fermentation of rumen fluid. All the setup procedures of the *in vitro* fermentation were performed in an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI, USA) with an atmosphere of carbon dioxide and hydrogen at the ratio of 95:5. The *in vitro* fermentation bottles were each sealed with a butyl rubber stopper and an aluminum crimp seal and incubated at 39 °C with horizontal shaking at 0.06 × *g*.

2.5.2. Sampling and measurement of fermentation parameters

The gas pressure inside each fermentation bottle was recorded at 3, 6, 9, 12, 24, 48 and 72 h of incubation using a digital pressure sensor (Ruyi, Shanghai, China). At the end of the 72 h incubation, the fermentation bottles were placed in ice water to stop fermentation. Then, three subsamples (1 ml each) were collected from each fermentation bottle after mixing and immediately stored at −80 °C for analysis of select microbial groups and determination of activity of cellulase, carboxymethyl cellulase (CMCase), and xylanase. Another 1-ml aliquot collected from each bottle was centrifuged at 6000 × *g* for 15 min, and the supernatant was stored at −80 °C for the analysis of volatile fatty acids (VFAs). The pH, ammonia N, and VFA concentrations of the fermentation samples were determined using the methods described by Hu et al. (2005). The content in each bottle was completely emptied into nylon bag (23 μm mesh) and the liquid was squeeze out. The bags were rinsed and then incubated in a hot air oven at 65 °C for 48 h to determine DM (Patra and Yu, 2014). Because most of the added corn

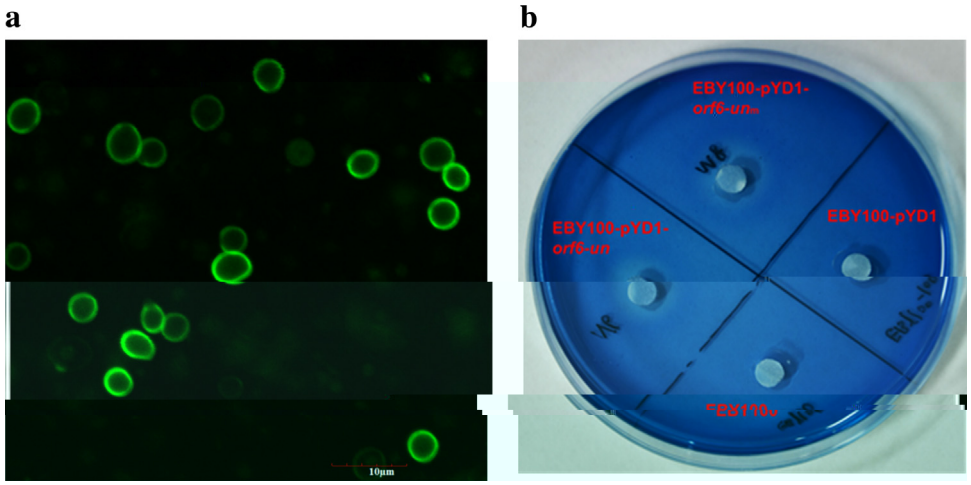

Fig. 1. Immunofluorescence of EBY100-pYD1-*orf6-un*$_m$ after staining with anti-V5-FITC antibodies (a) and xylanase activity test of the xylanase displayed on EBY100-pYD1-*orf6-un*$_m$ on remazol brilliant blue R-D-xylan agar plate (b).

3.2. Characterization of the EBY100-pYD1-orf6-un$_m$ xylanase

The optimal temperature of the EBY100-pYD1-*orf6-un*$_m$ was 50 °C (Fig. 2a). The EBY100-pYD1-*orf6-un*$_m$ was able to retain 80% of its xylanase activity after exposure to 50 °C for 30 min, but exposure to 60 °C for 30 min resulted in 63% loss of its activity (Fig. 2c). The optimal pH was 7 (Fig. 2b), and the EBY100-pYD1-*orf6-un*$_m$ was sensitive to pH (Fig. 2d). The EBY100-pYD1-*orf6-un*$_m$ had a specific xylanase activity about 137 U per gram of dry cells of EBY100-pYD1-*orf6-un*$_m$.

The hydrolysis products of beechwood xylan by EBY100-pYD1-*orf6-un*$_m$ were analyzed by HPLC (Fig. 3b). Beechwood xylan was rapidly hydrolyzed to xylotriose, xylobiose and xylose by EBY100-pYD1-*orf6-un*$_m$. As the hydrolysis reaction

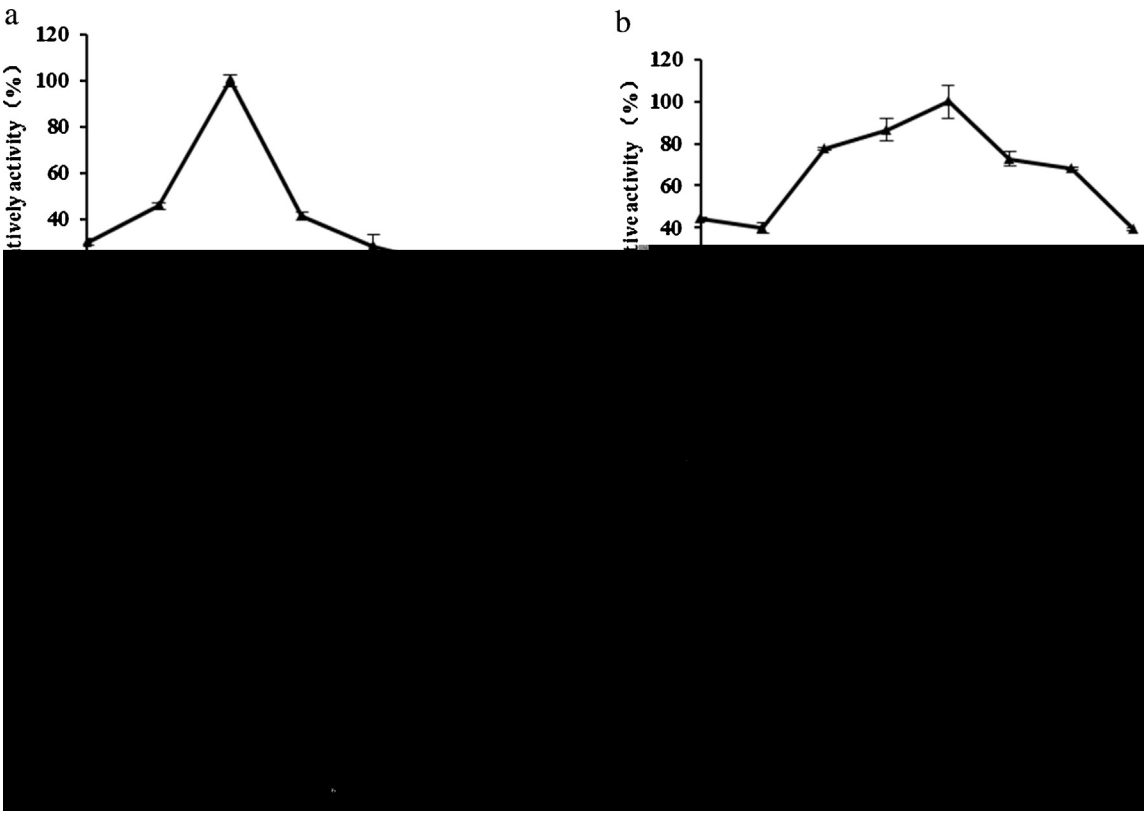

Fig. 2. Effects of temperature (a) and pH (b) on the EBY100-pYD1-*orf6-un*$_m$, and its thermal stability (c) and pH stability (d) following 30 min incubation.

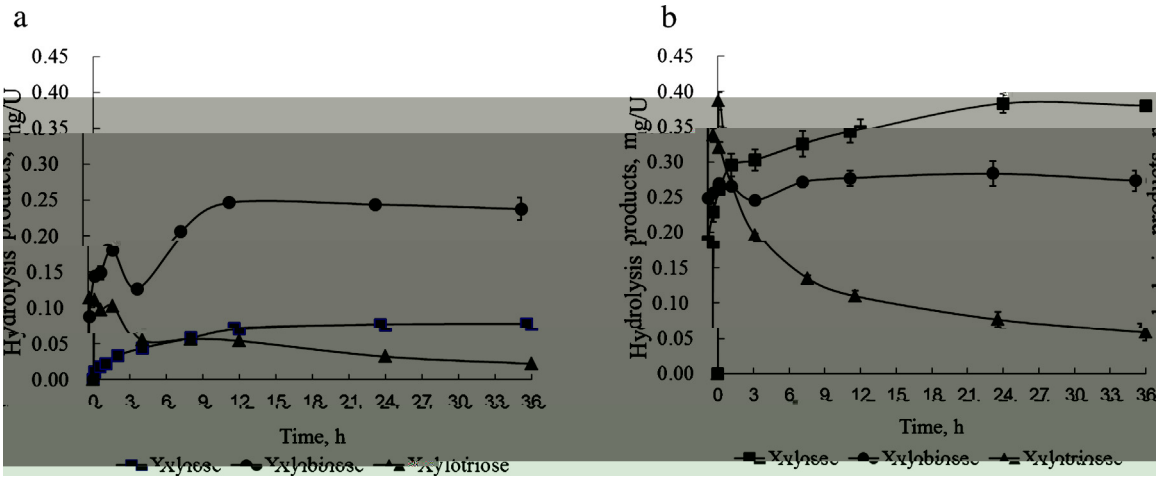

Fig. 3. Hydrolysis products of beechwood xylan by purified Orf6-un$_m$ overexpressed in *E. coli* (a) or EBY100-pYD1-*orf6-un*$_m$, yeast EBY100 with surface displayed Orf6-un$_m$ (b) at 50 °C and pH 6.0.

proceeded, xylose increased, while xylotriose decreased. After 36 h of hydrolysis, the major product was xylose and xylobiose with a yield of 0.38 and 0.27 mg/U, respectively, while only negligible amounts of xylotriose (0.06 mg/U) were produced.

3.3. Effect of yeast with surface-displayed xylanase on ruminal fermentation in vitro

Compared with the control, the addition of yeast EBY100 resulted in increased ($P < 0.05$) cumulative gas production after 6 h *in vitro* incubation (Fig. 4). However, the addition of EBY100-pYD1-*orf6-un*$_m$ corresponded with greater ($P < 0.05$) cumulative gas production than in the control during the entire incubation. No significant difference in cumulative gas production was noted within the first 9 h of incubation between EBY100 and EBY100-pYD1-*orf6-un*$_m$, but thereafter the latter led to greater ($P < 0.05$) gas production. EBY100-pYD1-*orf6-un*$_m$ also resulted in significantly ($P < 0.05$) increased potential gas production and gas production rate, while shortening the lag time of gas production. The EBY100 had similar effect on gas production but to a smaller magnitude than EBY100-pYD1-*orf6-un*$_m$ (Table 2).

Supplementation with EBY100 or EBY100-pYD1-*orf6-un*$_m$ affected other fermentation parameters (Table 2). Both EBY100 and EBY100-pYD1-*orf6-un*$_m$ reduced ($P < 0.05$) the culture pH and the acetate to propionate ratio ($P < 0.05$), and increased ($P < 0.05$) the total VFA and ammonia nitrogen concentration. The above effects were greater ($P < 0.05$) for EBY100-pYD1-*orf6-un*$_m$ than for EBY100, except for similar ($P > 0.05$) effect on the pH, butyrate concentration, and acetate to propionate ratio. EBY100-pYD1-*orf6-un*$_m$ resulted in greater degradation of corn stover than EBY100 at 24 h, but not at 72 h, of incubation.

Both EBY100 and EBY100-pYD1-*orf6-un*$_m$ significantly increased ($P < 0.05$) populations of total bacteria, *F. succinogenes*, and *R. albus* (Table 3). Supplementing EBY100 increased ($P < 0.05$) the activity of endogenous cellulase, CMCase, and xylanase present in the liquid fraction of the *in vitro* cultures collected at the end of the incubation, while supplementation with

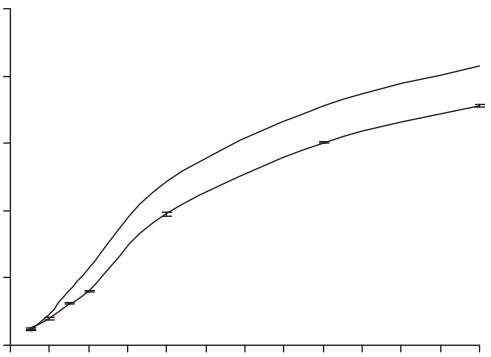

Table 2
Effects of yeast with surface-displayed xylanase on pH, gas production (GP), ammonia N concentration, volatile fatty acid (VFA) concentration, and dry matter degradation (DMD) of *in vitro* ruminal cultures fed corn stover.

	Control	EBY100	EBY100-pYD1-*orf6-un$_m$*	SEM
pH	6.69[a]	6.55[c]	6.59[b]	0.009
GP parameters				
Potential GP, ml/g	168.2[c]	195.3[b]	210.3[a]	2.50
Rate of GP, ml/h	5.3[c]	6.6[b]	7.0[a]	0.04
Lag time, h	5.9[a]	5.2[a]	3.2[b]	0.27
Total VFA, mmol/l	54.7[c]	66.1[b]	74.6[a]	1.69
VFA molar proportion, mol/100 mol				
Acetate	68.5[a]	66.7[b]	66.8[b]	0.38
Propionate	27.4	28.2	28.1	0.23
Butyrate	4.0[b]	5.2[a]	5.1[a]	0.23
Acetate: propionate	2.50[a]	2.37[b]	2.38[b]	0.032
Ammonia N, mg/dl	15.6[c]	21.7[b]	26.5[a]	1.21
DMD, g/kg				
24 h	487[b]	501[b]	525[a]	6.0
72 h	737	719	725	9.6

[a,b,c]Means with different superscripts within a row differ ($P < 0.05$).

Table 3
Effects of EBY100-pYD1-*orf6-un$_m$* on select rumen microbial populations and activities of cellulose, carboxymethyl cellulase (CMCase) and xylanase present in the supernatant of *in vitro* ruminal cultures after 72 h incubation.

	Control	EBY100	EBY100-pYD1-*orf6-un$_m$*	SEM
Rumen microbial population, marker gene copies/ml rumen fermentation culture				
Total bacteria, 10^{10}	3.9[b]	7.8[a]	7.2[a]	0.38
Fungi, 10^6	1.4	1.9	2.2	0.23
Fibrobacter succinogenes, 10^{10}	0.6[b]	1.1[a]	0.9[a]	0.06
Ruminococcus flavefaciens, 10^5	2.9	2.4	2.6	0.21
Ruminococcus albus, 10^8	0.2[c]	2.7[a]	2.2[b]	0.14
Endogenous enzyme activity, U/ml rumen fermentation culture				
Cellulase	0.50[b]	0.60[a]	0.57[a]	0.020
CMCase	0.38[b]	0.41[a]	0.40[ab]	0.008
Xylanase	1.87[b]	2.34[a]	1.96[b]	0.116

[a,b]Means with different superscripts within a row differ ($P < 0.05$).

EBY100-pYD1-*orf6-un$_m$* only increased the activity of endogenous cellulase ($P < 0.05$), though it also numerically increased the activity of xylanase and CMCase (Table 3).

4. Discussion

Using a xylanase gene (*orf6-un$_m$*) of rumen origin and yeast, we developed a yeast strain that successfully expressed and displayed the xylanase on its cell surface. The EBY100-pYD1-*orf6-un$_m$* was more thermostable but more sensitive to pH than *E. coli*-expressed Orf6-un$_m$. Activity of the EBY100-pYD1-*orf6-un$_m$* was at least the same as, if not greater than, that of the *E. coli*-expressed Orf6-un$_m$. However, the optimal conditions for enzyme activity of EBY100-pYD1-*orf6-un$_m$* were rather different from those found in the alimentary track of animals. Therefore, enhancing the growth or the enzyme activity of EBY100-pYD1-*orf6-un$_m$* under the conditions of alimentary track warrants further investigation. The surface display of this xylanase did not alter the major products of xylan hydrolysis except significantly increased xylose yield (Fig. 3). When evaluated using in vitro rumen cultures with corn stover as substrate, both EBY100 and EBY100-pYD1-*orf6-un$_m$* increased gas production, which agrees with the meta-analysis of Desnoyers et al. (2009), but EBY100-pYD1-*orf6-un$_m$* also shortened lag time and increased DMD at 24 h. These results suggest that EBY100-pYD1-*orf6-un$_m$* might have enhanced DMD and fermentation at the early stage of the *in vitro* fermentation. It remains to be determined if EBY100-pYD1-*orf6-un$_m$* contributed to early release of fermentable sugars (i.e., xylose and xylooligosaccharides) from the corn stover. Another interesting finding is that EBY100-pYD1-*orf6-un$_m$* increased ammonia concentration to a greater magnitude than EBY100. This is contradictory to the finding of other studies that showed no significant effect of yeast supplementation on ammonia concentration in the rumen (Hristov et al., 2010; Monnerat et al., 2013). However, in those studies, different feeds were used. Future studies are needed to confirm the effect of yeast and EBY100-pYD1-*orf6-un$_m$* on ammonia production from corn stover and ammonia utilization by rumen cultures. Although increased ammonia concentration in the rumen is often linked to increased proteolysis and poor dietary N utilization, ammonia is the preferred and major N source for microbial protein synthesis (Bach et al., 2005). The increases in population of total bacteria and fungi observed in the *in vitro* cultures that received EBY100 or EBY100-pYD1-*orf6-un$_m$* suggests increased microbial protein synthesis.

Both EBY100 and EBY100-pYD1-*orf6-un$_m$* resulted in increased VFA concentrations. The mechanisms underpinning increase in VFA production and fiber degradation in animals fed yeast are not fully understood. Wallace and Newbold (1992)

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