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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.

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 in yeast genetic engineering enables new specific metabolic activities to be introduced to yeast, including expression of exogenous enzymes on cell surface through cell-surface display. By this technology, exogenous enzymes or peptides are expressed and displayed (also anchored) on the cell surface of yeast cells by fusing them with an anchoring motif of an indigenous yeast protein (Kondo and Ueda, 2004; Yeasmin et al., 2011). Because the introduced new enzymes are immobilized on the cell surface of yeast, the stability of the enzymes is enhanced and their activities are improved (Han et al., 2009). Yeast cell surface display has been successfully used in a number of studies to arm yeast with activities of exogenous cellulases (mostly as minicellulosomes) (Tsai et al., 2010; Kim et al., 2013) or xylanases (Yeasmin et al., 2011; Duquesne et al., 2014). All these studies aimed to introduce cellulase and xylanase activities to yeast so that yeast can be used to produce ethanol directly from plant cell wall materials. We hypothesized that yeast cell surface display can be used to deliver exogenous enzymes to farm animals to improve the efficacy of the exogenous enzymes. To test this hypothesis, a xylanase, which was discovered from a fosmid library of rumen microbiome and optimized through directed evolution (Du et al., 2014), was displayed on the cell surface of *S. cerevisiae* and evaluated using in vitro fermentation.

2. Materials and methods

2.1. Construction of a shuttle plasmid carrying the xylanase gene orf6-un_m

The *orf6-un*$_m$ gene was initially discovered in a fosmid library constructed from the rumen microbiome of sheep, and it encodes a xylanase belonging to glycoside hydrolase (GH) family 11 (Du et al., 2014). Its xylanase activity was enhanced using directed evolution (Du et al., 2014). The *orf6-un*$_m$ gene was amplified by PCR using the forward primer 5′-TGACGGATCCGATTTTTGTCAAACTGCCGC-3′ (the 5′ extension containing a *Bam*HI restriction site, underlined) and the reverse primer 5′-CACCTCGAGCGCCCCCTCGATATAGACCT-3′ (the 5′ extension containing an *Xho*I restriction site, underlined) (Du et al., 2014). The PCR cycling conditions consisted of an initial step of 4 min at 94 °C; followed by 30 cycles of 0.5 min at 94 °C, 0.5 min at 58 °C, and 1 min at 72 °C; and a final extension step of 10 min at 72 °C. The amplified products were purified using an E.Z.N.A.® Cycle Pure Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) and digested with both *Bam*HI and *Xho*I (TaKaRa, Dalian, China). The purified *orf6-un*$_m$ gene was then ligated into the *Bam*HI-*Xho*I restriction site of the yeast surface display vector pYD1 (Invitrogen, Shanghai, China) using T4 DNA ligase. The resultant recombinant plasmid, referred to as pYD1-*orf6-un*$_m$, was transformed into *Escherichia coli* DH5α (Transgen, Beijing, China) using the heat-shock method. The cloned *orf6-un*$_m$ gene was confirmed by PCR using the above PCR primers and then by sequencing. The plasmid pYD1-*orf6-un*$_m$ was prepared from transformed *E. coli* DH5α using a Plasmid Maxi Kit (Omega Bio-Tek, Inc., Shanghai, China). The cloned Orf6-un$_m$ xylanase was overexpressed in *E. coli* as described by Du et al. (2014).

2.2. Transformation of S. cerevisiae EBY100, and expression, and surface display of Orf6-un_m

The pYD1-*orf6-un*$_m$ plasmid was transformed into *S. cerevisiae* EBY100 using a small-scale yeast transformation protocol per the instruction of the pYD1 Yeast Display Vector Kit (Invitrogen, Shanghai, China). The Minimal Dextrose tryptophan-free Agar Plates contained 6.7 g/l yeast nitrogen base without amino acids (YNB), 20 g/l glucose, 0.1 g/l leucine, and 15 g/l agar, and they were used to screen for positive clones containing the recombinant plasmid. For expression and surface display of the Orf6-un$_m$ enzyme, recombinant *S. cerevisiae* EBY100 harboring the pYD1-*orf6-un*$_m$ plasmid (referred to as EBY100-pYD1-*orf6-un*$_m$) was pre-cultivated in 10 ml YNB-CAA (6.7 g/l YNB, 5 g/l casamino acids) medium containing 20 g/l glucose at 30 °C overnight with shaking until OD_{600} reaching between 2 and 5. The yeast cells were harvested by centrifugation (5.000 × *g* for 10 min at room temperature), resuspended in YNB-CAA medium containing 20 g/l galactose to an OD_{600} between 0.5 and 1, and cultivated at room temperature (20–25 °C) for 48 h with shaking. The cells were then harvested by centrifugation (5000 × *g* for 10 min at 4 °C) and resuspended in 1× phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) for immunofluorescence test and for xylanase activity assay.

2.3. Visualization of yeast surface displayed xylanase and xylanase activity assay

The yeast surface-displayed xylanase Orf6-un$_m$ was visualized following immunostaining using fluorescent antibody and microscopy. The cells of EBY100-pYD1-*orf6-un*$_m$ were washed twice with 2 ml PBS, resuspended in 2 ml PBS containing 0.1 g/ml fetal bovine serum (FBS), and incubated on ice for 20 min. After washing once again with PBS, the yeast cells were combined with the fluorescent antibody Anti-V5-FITC (Invitrogen Corporation, Shanghai, China), which was diluted 300 fold with PBS containing 0.1 g/ml FBS. After incubation on ice for 1 h in dark, the yeast cells were washed with PBS (without FBS) three times, and the binding of Anti-V5-FITC to the yeast cell surface was visualized using a fluorescence microscope.

The activity of the Orf6-un$_m$ xylanase displayed on the yeast cell surface, EBY100-pYD1-*orf6-un*$_m$, was assessed using remazol brilliant blue R-D-xylan (RBB-xylan) plates (Sigma, Saint Louis, MO, USA), and xylanase activity was indicated by hydrolysis of the xylan, and thus halos, surrounding yeast colonies (Biely et al., 1985). Cells of EBY100 and EBY100-pYD1, both of which did not have surface displayed xylanase, were included for comparison.