Bioresource Technology 257 (2018) 76–83

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Characterization of *Enterococcus faecalis* JF85 and *Enterococcus faecium* Y83 isolated from Tibetan yak (*Bos grunniens*) for ensiling *Pennisetum sinese*

Junfeng Li[1], Xianjun Yuan[1], Seare T. Desta, Zhihao Dong, Wazha Mugabe, Tao Shao*

Institute of Ensiling and Processing of Grass, College of Prataculture Science, Nanjing Agricultural University, Nanjing 210095, China

ARTICLE INFO

Keywords:
Cellulolytic bacteria
Lactobacillus plantarum
Pennisetum sinese
Silage
Enzymatic hydrolysis

ABSTRACT

Two bacteria strains with cellulolytic potential isolated from Tibetan yak (*Bos grunniens*) rumen were identified as *Enterococcus faecalis* (JF85) and *Enterococcus faecium* (Y83). Isolates grow well within a range of temperature 15 to 55 °C and pH 3.0–7.0, respectively. Two strains were inoculated with or without *Lactobacillus plantarum* (Lp) to *Pennisetum sinese* silage for 90 days. All inoculants increased lactic acid content, decreased pH and lignocellulose contents compared with silage without additives (control). The lowest pH, highest lactic acid and largest reduction in lignocellulose contents were observed in JF85 + Lp and Y83 + Lp silages. Isolates alone or in combination with Lp significantly increased WSC, mono- and disaccharides contents as compared to the control. Combined addition efficiently improved enzymatic hydrolysis of *Pennisetum sinese* silage, indicated by higher glucose yield and cellulose convertibility. *Pennisetum sinese* ensiled with combined additives is a suitable storage and pretreatment method prior to sugars production from energy crop.

1. Introduction

The progressive depletion of fossil fuels, environmental pollution and climate change have triggered the global demand for renewable and sustainable energy. Lignocellulosic biomasses, including energy crops and agricultural residues offer potential substrates for the production of renewable energy through bioconversion (enzymatic hydrolysis and fermentation). Lignocellulosic biomass is the most abundant polymeric carbohydrates in the world and can be used as feedstock to generate fermentable sugars for sustainable biofuel production. *Pennisetum sinese*, a hybrid of *Pennisetum purpureum* and *Pennisetum americanum*, is a monocot C4 perennial grass and has been widely used as an energy crop (Li et al., 2014). *P. sinese* is well adapted to a wide variety of soils types, fertility levels, and weather conditions, and capable of yielding high biomass about 40 t DM per hectare per year (Lu et al., 2014). Thus, it has become an attractive renewable resource for the production of biofuel, feed, and chemical due to its low energy input, high yield potential, and wide availability in tropics and subtropics of Asia (Peng et al., 2017).

In the tropical and some subtropical regions, including south of China, the temperatures are suitable for *P. sinese* growth in early spring and later autumn. However, fresh grasses are not available during the winter months (Chou et al., 2009) hence constraining the supply patterns. The efficient conservation of fresh grasses could ensure yearly-round supply of carbohydrates for maintaining viable bioenergy supply chains. Ensiling is not only an appropriate method of storing feedstock for biofuel production with the potentially very low loss of carbohydrates but also a biological pretreatment method (Herrmann et al., 2011). Ensiling is widely used to preserve animal feed, and applicable to conserve lignocellulosic biomass such as *P. sinese* for biofuel production. Ensilage has proven to be better than fungal pretreatment for preserving giant reed harvested from August through December, since it could result in higher glucose and methane yields than untreated and fungal pretreated giant reed for all harvested times (Liu et al., 2016). Zheng et al. (2012) also reported that the ensiling process significantly improved the enzymatic digestibility of sugar beet pulp as compared to raw sugar beet pulp.

P. sinese demonstrates difficulty to ensiling due to its coarse and stemmy structures alongside low water soluble carbohydrate (WSC) and high fiber contents. Exogenous fibrolytic enzyme has been explored to induce direct conversion of structural carbohydrates into soluble sugars for LAB fermentation (Wang et al., 2002; Colombatto et al., 2004), but, high cost and instability of commercially enzymes limited their widespread application in silage. Microorganisms action altogether presents a combined storage and pretreatment benefits which require less energy and affords easy handling (Adekunle et al., 2016). Therefore, screening and isolating fibrolytic microorganism lineages with high substrate specific activities and stability is very crucial.

* Corresponding author.
E-mail address: shaotaola@163.com (T. Shao).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.biortech.2018.02.070
Received 24 January 2018; Received in revised form 13 February 2018; Accepted 14 February 2018
Available online 17 February 2018
0960-8524/

Ensiling is a complex biochemical and microbiological process which progresses from aerobic conditions to anaerobic conditions. Thus, it is practical to screen and isolate facultative anaerobic cellulolytic bacteria as potential silage additives. It is established that ruminants have the unique ability to digest large amounts of cellulose-rich feedstuffs through rumen microbial fermentation (Nyonyo et al., 2014). Yaks (*Bos grunniens* or *Poephagus grunniens*) are regarded as one of the world's most remarkable domestic animals as they thrive in conditions of extreme harshness and deprivation. Since the areas which yaks reside have short growing seasons for herbage, yaks thrive on wilted herbages and straws during the long winter and early spring. Hence, it was hypothesized that higher activity of fibrolytic microorganisms could be isolated from yak rumen considering their long-term evolution and survival in harsh environmental conditions.

The objective of this study was to isolate and identify facultative anaerobic cellulolytic bacteria from yak rumen and further evaluate the effects of the isolates on the fermentation quality, structural carbohydrates degradability and enzymatic hydrolysis efficiency of *P. sinese* silage.

2. Materials and methods

2.1. Isolation, screening, and identification of cellulolytic bacteria

The modified CMC-amended agar medium was used as an isolation medium, composed of (L^{-1} distilled water): 10 g carboxymethylcellulose (CMC), 1.0 g peptone, 0.5 g yeast extract, 1.0 g L-cysteine hydrochloride, 2.0 g KH_2PO_4, 0.3 g $MgSO_4$, 1.4 g $(NH_4)_2SO_4$, 0.3 g $CaCl_2$, 2.0 g cellobiose, 20 g agar, 100 mL cell-free rumen fluid, and 1.0 mL trace nutrient solution (TNS). TNS contained (L^{-1} distilled water): 0.2 g $FeSO_4$, 0.15 g $MnSO_4$, 0.3 g $ZnCl_2$, 0.4 g $CoCl_2$. The basal culture medium (BC medium) used for cellulase production was prepared as followed (L^{-1} distilled water): 10 g CMC, 5 g peptone, 0.5 g yeast extract, 0.3 g $MgSO_4{\cdot}7H_2O$, 1.0 g NH_4NO_3, 1.0 g K_2HPO_4, 1.0 g KH_2PO_4, 0.05 g $FeCl_3{\cdot}6H_2O$, 0.02 g $CaCl_2$. The pHs of all the media were regulated to 6.5 before sterilization at 121 °C for 20 min.

Fresh rumen fluid was collected from five healthy Tibetan yaks aged 3–4 years with a body weight of 300 ± 30 kg at Nakchu (latitude 31.47° N, longitude 92.1° E, altitude 4500 m a.s.l. Tibet, China). Rumen contents were immediately inoculated into Hungate screw cap tubes containing Luria-Bertani (LB) medium supplemented with 1% (w/v) CMC. The inoculated medium was then agitated with an orbital shaker at 120 rpm, and 39 °C for 48 h. To isolate colonies, 10-fold serial dilution of log-phase cells of cultures were spread on CMC-amended agar plates and incubated at 39 °C for 48 h. The bacterial colonies were purified through subculturing on the same medium. At the end of the culture, CMC-amended agar plates were stained with 0.1% (w/v) Congo red, and only bacterial colonies showing clear halos indicating carboxymethylcellulose degradation were selected (Teather and Wood 1982). Cellulosic filter paper strips were added to the medium as the sole carbon source, and cultures were grown for 7 days at 25 °C to compare the cellulose degrading ability of isolates. Strain JF85 and Y83 exhibited the fastest cellulose decomposition among all isolated strains and were selected for the further study.

Physiological-biochemical and morphological properties of isolates were identified, evaluated, and compared using the method described in Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984) and commercially available identification systems (API 50CHL, bioMérieux, France). For phylogenetic identification of two isolates, the 16S rRNA gene fragment was amplified by polymerase chain reaction with a pair of universal primers, 27f and 1492r according to Assareh et al. (2012). The PCR products were purified using commercial DNA purification kit (Axygen, San Francisco, CA, USA) and analyzed for 16S rDNA using a 3730xl DNA Analyzer (ABI Applied Biosystems, San Francisco, CA, USA). The 16S rDNA sequences were aligned with the sequence of the reference microbes from GenBank to identify organisms using BLAST analysis. Phylogenetic analysis was determined as described by Gulfam et al. (2016).

2.2. Enzyme assay

Two isolates were cultured in BC medium, agitated at 150 rpm and 39 °C. The dynamic activities of CMCase and FPase of isolates were assayed at 12 h intervals during the 4 days of incubation. The cultures were centrifuged at 10,000*g* for 15 min at 4 °C, and the supernatants were collected as crude enzyme for enzyme assay. FPase activity was measured employing filter paper (Whatmann NO. 1) as a substrate (Ghosh, 1994). The release of reducing sugars in 50 mM citrate-phosphate buffer (pH 4.8) for 1 h at 50 °C was measured by 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). CMCase activity was determined by measuring reducing sugars release from CMC. The reaction mixture consisting of 1% (w/v) CMC in 50 mM citrate-phosphate buffer (pH 4.8) was incubated with the enzyme at 50 °C for 30 min in a total volume of 2.0 mL. Then, the reactions were terminated by adding 3 mL of DNS reagent, and all the mixtures were heated for 10 min in a boiling water bath. One unit (U) of enzyme activity was defined as the amount of enzyme needed release 1 μmol of glucose per minute.

2.3. Ensilage of P. sinese

P. sinese was cultivated in the experimental field of Nanjing Agricultural University, Jiangsu, China (latitude 32.07° N, longitude 118.78° E, altitude 17 m a.s.l., Jiangsu, China), and harvested at maturity growth stage on 9 November 2016. The grass was wilted for 6 h and then chopped into lengths of 1–2 cm with manual forage chopper (Sh-2000, Shanghai Donxe Industrial Co., Ltd., Shanghai, China). The *P. sinese* used for ensiling had a dry matter (DM) content of 225.67 ± 3.5 g/kg fresh matter and the WSC concentration of 57.32 ± 2.1 g/kg DM. The composition of structural carbohydrate was 604 g of neutral detergent fiber (NDF)/kg of DM, 352 g of acid detergent fiber (ADF)/kg of DM and 28 g of acid detergent lignin (ADL)/kg of DM. *Lactobacillus plantarum* (Lp, Biogrowing Co., Ltd., Shanghai, China) was used after culturing in deMan Rogosa Sharpe (MRS) medium following the procedures of Zheng et al. (2012). Cellulolytic bacteria were cultivated for 1–2 days in LB-CMC media until the OD of the culture reached 1.5 at 590 nm. The cells were harvested by centrifugation at 8000*g* for 5 min at 4 °C, washed three times in sodium phosphate buffer (1 M, pH 7.0) to remove residual media. Cell pellets were then resuspended in sterilized deionized water to an OD_{590} value of 0.5.

P. sinese grass were treated as follows: (1) C, control, without additives, (2) Lp, applied at 1×10^6 colony forming units (cfu)/g FW (fresh weight), (3) JF85, *E. faecalis* JF85 applied at 1.1×10^6 cfu/g FW, (4) Y83, *E. faecium* Y83 applied at 1.5×10^6 cfu/g FW, (5) JF85 + Lp, *E. faecalis* JF85 combined with *L. plantarum*, and (6) Y83 + Lp, *E. faecium* Y83 combined with *L. plantarum*. Additives were sprayed and thoroughly mixed with the chopped grass (760 g). Subsequently the mixtures were packed into 1-L plastic laboratory silo (diameter 9.5 cm, height 18.7 cm; Lantian Biological Experimental Instrument Co., Ltd, Jiangsu, China), followed by sealing with two screw tops (internal and external). A total of 180 laboratory silos (6 ensiling days × 6 treatments × 5 replications) were made and then stored at ambient temperature (25 ± 3 °C).

2.4. Analytical methods

Five randomly selected silos for each treatment were opened after 3, 7, 14, 30, 60 and 90 days of ensiling, respectively. At each sampling interval, *P. sinese* silage for each silo was emptied, mixed thoroughly and placed into an ethanol-disinfected plastic container. A sub-sample of silages was blended with distilled water at water-to-sample material (fresh weight) ratio of 3:1 for 24 h and filtered through two layers of