Bioresource Technology 212 (2016) 334–337

Contents lists available at ScienceDirect

Bioresource Technology

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

Short Communication

On-site hydrolytic enzymes production from fungal co-cultivation of Bermuda grass and corn cob

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HIGHLIGHTS

- On-site enzymes were produced from fungal fermentation of Bermuda grass and corn cob.
- Fermented forage showed high cellulolytic, amylolytic and xylanolytic productivities.
- Fermented forage showed potential in ruminal digestibility improvement of animal feed.
- On-site enzymes production could represent economical availability of animal feed.

ARTICLE INFO

Article history:
Received 11 March 2016
Received in revised form 14 April 2016
Accepted 15 April 2016
Available online 19 April 2016

Keywords:
On-site enzymes production
Hydrolytic enzymes
Co-culture
Aspergillus niger
Trichoderma reesei

ABSTRACT

Solid state fermentation (SSF) is used to produce industrial enzymes. The objective of this study was to use a co-culture of *Aspergillus niger* GS1 and *Trichoderma reesei*, grown on a mixture of Bermuda grass and corn cob to obtain fermented forage (FF) rich in hydrolytic enzymes, as a value added ingredient for animal feed. FPase, amylase and xylanase productivities (dry matter, DM) were 8.8, 181.4, and 42.1 U g^{-1} h^{-1}, respectively (1 U = reducing sugars released min^{-1}), after 12–16 h of SSF with C/N = 60. Cellulose, hemicellulose and lignin decreased 1.6-, 2.7- and 1.9-fold (DM), respectively. *In vitro* ruminal and true digestibility of DM was improved 2.4- and 1.4-fold. Ruminal digestion of FF reduced 1.32-fold the acetate:propionate ratio, which may reduce the environmental impact of ruminants feeding. On-site hydrolytic enzymes productivity using SSF without enzymes extraction could be of economic potential for digestibility improvement in animal feed.

1. Introduction

Feed grains are widely used energy sources in ruminant diets, but their price keeps steadily increasing due to higher demand. Ruminal digestibility of forage cell walls ultimately limits nutrient availability due to harsh environmental conditions faced by microbial population, resulting in reduced fiber digestion (Sujani and Seresinhe, 2015; Tadele and Animut, 2015). The use of exogenous fibrolytic enzymes can increase forage utilization leading to animal weight gain, despite some controversial research reports (Sujani and Seresinhe, 2015). Additionally, on-site hydrolytic enzymes production by solid state fermentation (SSF) on forage as substrate, can be achieved by co-culture of fungi (Farinas, 2015). Bermuda grass (*Cynodon dactylon*), a typical warm-season perennial turf-grass mixed with corn cobs may be used to favor both gas and mass transfer due to their inherent structural porosity. Cellulases, xylanases and amylases are a group of enzymes which catalyze hydrolysis of hemicellulose material and starch mostly into glucose and xylose (Farinas, 2015; Sahnoun et al., 2015).

This work addresses the impact of using a co-culture of *Aspergillus niger* GS1 and *Trichoderma reesei*, by means of SSF on a mixture of Bermuda grass and corn cob, to obtain fermented forage rich in hydrolytic enzymes, as potential ingredient for animal feed.

2. Materials and methods

2.1. Biological material

A. niger GS1 (National Center for Biotechnology Information, NCBI No. GU395669) and *T. reesei* (NCBI No. AF510497) were used

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http://dx.doi.org/10.1016/j.biortech.2016.04.070
0960-8524/

as source of cellulases, amylases and xylanases. Each fungal strain was prepared on sterile granulated corn cob (50 g) supplemented with mineral solution (Díaz-Malváez et al., 2013) (1:1 weight ratio), inoculated with conidia suspension (10^7 conidia g^{-1}), and incubated for 7 d at 30 °C.

Bermuda grass (2.5 ± 0.3 cm long) and granulated corn cob (1.5 ± 0.2 mm) were used as solid support for SSF after drying at 80 °C (Memmert, Schwabach, Germany).

2.2. Effect of carbon/nitrogen ratio (C/N) on hydrolytic enzymes production during SSF

From preliminary experiments, the forage dry mixture (10 g) was incubated in wired stainless steel trays (Memmert GmbH, Schwabach, Germany) at 30 °C, 85% relative humidity and surface aerated (4–6 mL min^{-1}). The inoculum of each fungus was added at 10^7 conidia (g dry forage mixture)$^{-1}$. Bed thickness was 1 cm, and grass:corn cob ratio was 4:1 (w:w). Moisture content was adjusted to 80% (w v^{-1}) with a solution containing different C/N molar ratios: 3, 30, 60 and 120, to test the effect of supplementation over hydrolytic enzymes activity production. Ammonium sulfate supplementation was kept constant at 150 mM while sucrose was varied.

2.3. Crude enzymes extract and determination of hydrolytic enzymes activity

The crude enzymatic extract from fermented forage was obtained according to Díaz-Malváez et al. (2013) and used to quantify enzyme activity. Crude extract was incubated 24 h at 4 °C to maximize enzymatic activity recovery (Szabo et al., 2015), followed by centrifugation and vacuum filtration at 4 °C using Whatman No. 1 paper (Sigma–Aldrich). Exo-1,4-β-D-glucanase (FPase) was determined (Ghose, 1987), and it was found that not significant activity was retained (<7%). Potato soluble starch and birch wood xylan (1%, w v^{-1}) were substrates for alpha amylase and endo-1,4-β-D-xylanase activity during 10 min. The released reducing sugars were quantified according to Miller (1959). One activity unit (U) was defined as the amount of enzyme that releases 1 μmol of glucose or xylose equivalents per min at 50 °C, pH 5.5. Enzymatic activity was expressed as U g^{-1} of fermented DM.

2.4. Production of hydrolytic enzymes during SSF, and effect of single or mixed cultures on their expression

The production of hydrolytic enzymes activity in fermented forage (FF) was studied at the C/N ratio producing maximum titers, according to Section 2.2 during 24 h. The degree of synergy (DS) between both strains was calculated (Andersen et al., 2008).

2.5. Chemical characterization and ruminal digestibility of forage mixture

The forage mixture subjected to SSF conditions producing the highest amount of hydrolytic enzymes (Section 2.4), was analyzed before and after fermentation. Crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF), lignin, hemicellulose, cellulose, energy index of non-fibrous and non-structural carbohydrates were determined by near infrared reflectance spectroscopy, NIR (Foss, Hillerød, Denmark) according to Undersander et al. (1993).

Dry matter of forage mixture was used to determine *in vitro* ruminal digestibility (RD) and *in vitro* true digestibility (TD) (ANKOM Technology, 2015), for 96 h.

2.6. Analysis of volatile fatty acids (VFA)

The effect of FF on ruminal fluid metabolism was determined by VFA quantification by gas chromatography (Agilent 7890, Agilent Technologies, CA, USA) equipped with a flame-ionization detector. Filtered (0.22 μm, Merck Millipore, MA, USA) samples of ruminal fluid were collected from each digestibility treatment at 3 and 96 h.

2.7. Statistical analysis

Experiments were done in triplicate and data were subjected to a one-way analysis of variance (ANOVA) for each experiment to determine significant differences among mean values using Tuckey or Dunett tests with Minitab software 16.2.4 (PA, USA). Statistical significance was defined as $p < 0.05$.

3. Results and discussion

3.1. Effect of C/N ratio on production of hydrolytic enzymes by SSF

Supplementation of different C/N ratios for SSF resulted in optimal activity titers of FPase, amylase and xylanase (Fig. 1a). FPase showed a maximum activity of 23.2 ± 1.8 U g^{-1} at C/N = 30, whereas no significant activity difference was observed for SSF performed at C/N = 60 or 120. Only 37.3% of the highest FPase activity was achieved at C/N ratio = 3.

High amylase activity (525.5 ± 50.9 U g^{-1}) was observed at C/N = 30 and 60, without significant difference, whereas the lowest activity was obtained at C/N = 3 or 120. For xylanase, a maximum of 159.0 ± 12.5 U g^{-1} was obtained at C/N ratio of 60, while activity was 56.5% lower at C/N ratio of 120.

Cooperative transcriptional regulation in response to small inducer molecules released from hemicellulosic biomass, constitutes a reasonable means to harmonize expression levels of hydrolytic genes to produce an optimal enzyme composition (Tani et al., 2014). The effect of C/N ratios and SSF variables on the improvement of hydrolytic enzymes production has been reported (Sahnoun et al., 2015; Yoon et al., 2014). As FF is intended for animal feed, palatability is an important issue (Tadele and Animut, 2015), and it may be more easily accepted by ruminants if it provides enough sweetness (C/N = 60) without compromising high levels of hydrolytic enzymes activity.

3.2. Production of hydrolytic enzymes during SSF, and effect of single or mixed cultures on their expression

Production of hydrolytic enzymes as a function of fermentation time was evaluated at C/N = 60 (Fig. 1b). The enzymes activity profile revealed that maximum activity of FPase was 141.5 ± 12.8 U g^{-1} after 16 h, whereas highest amylase and xylanase activities were 2176.5 ± 179.8 U g^{-1} and 505.2 ± 39.8 U g^{-1} after 12 h. Maximum productivities of FPase, amylase, and xylanase were 8.8 U g^{-1} h^{-1}, 181.4 U g^{-1} h^{-1} and 42.1 U g^{-1} h^{-1}. Most studies on SSF report longer incubation times (48–168 h), and therefore obtain lower productivities than in this study (Farinas, 2015; Yoon et al., 2014).

During the first 4 h fungal strains colonize the substrate surface, followed by activation of xylanolytic enzymes synthetic pathways, whereas this phenomenom is 4 h slower for FPase and amylolytic activities.

Using *A. niger* only, the amounts of amylase and FPase activities were significantly lower than those produced by the co-culture. On the other hand, using *T. reesei* only, FPase activity was significantly lower than that produced by the co-culture.