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# Quantification of Cellulolytic Bacteria Using In Vitro Culture Containing Treated or Untreated Cottonseed hulls Determined by Real-time Polymerase Chain Reaction

Mohsen Danesh Mesgaran, Ali Faramarzi-Garmrood, Alireza Vakili, and Hossein Jahani-Azizabadi

***Abstract*---**The objective of the present study was to quantify the cellulolytic bacteria population using in vitro culture containing sodium hydroxide treated or untreated cottonseed hulls (CH) determined by real-time polymerase chain reaction (RT-PCR). Cottonseed hulls were used as untreated or chemical treated using NaOH as 20 g/kg DM [a solution of NaOH (20%) was sprayed on CH and kept for 48 h at room temprature]. Forty-five ml of medium was supplied into a 100 ml bottle that approximately containing 0.45 g of the feed sample (4 replicates). Each bottle was inoculated under carbon dioxide with 5 ml of isolated rumen bacteria. The bottles were incubated for 96 h at 38.6° C. After the incubation, 1 ml of each bottle was sampled for DNA extraction. Then, quantification of cellulolytic bacteria was carried out using RT-PCR. Bacterial rDNA concentrations were measured relative to total bacteria amplification (ΔΔCt). Data were analyzed using the GLM procedure of SAS 9.1 and the means were compared by the Tukey test ($P< 0.05$). Chemical treatment had no significant effect ($P<0.05$) on the quantity of cellulolytic bacteria using present in vitro experimental conditions.

***Keyword*---**cellulolytic bacteria, cottonseed hulls, real-time polymerase chain reaction, sodium hydroxide,

**Mohsen Danesh Mesgaran, Ali Faramarzi-Garmrood, Alireza Vakili and Hossein Jahani-Azizabadi are with the Department of Animal Science, Ferdowsi University of Mashhad, Mashhad, Iran (corresponding author to provide phone: +98-511-8458015; fax: +98-511-8787430; e-mail: danesh@um.ac.ir).**

## I. Introduction

Treatment of lignocellulosic substances with an alkali solution removes lignin and decreases thecrystallinity of cellulose [1] thereby increasing the biodegradation of cell walls [2]. Improvement of low quality forages with NaOH has received a great deal of attention [3]. The close physical and chemical association between cellulose, plant cell wall matrix sugars (primarily hemicellulose) and lignin, as well as the crystalline arrangement of native cellulose polymers, prevent rapid and extensive degradation of plant structural carbohydrates by cellulolytic microorganisms in the ruminant gastrointestinal tract [4, 5, 6, 7] is a promising means of improving fiber digestibility allowing ruminants to more efficiently convert low quality fibrous feedstuffs to meat and milk [8]. Alkali has been associated with increased digestibility of cell wall monosaccharides [9] and increased bacterial colonization and adhesion to fiber particles [10]. Sodium hydroxide is involved with 1) the hydrolysis of polysaccharide ester bonds between lignin and hemicellulose and 2) the swelling of cellulose microfibrils to facilitate microbial entry [11]. A complex community of fibrolytic microorganisms catalyzes the degradation of fiber in the rumen. The major fibrolytic bacteria are the Gram-negative *Fibrobacter succinogenes*, and two species of Gram-positive bacteria, *Ruminococcus albus* and *Ruminococcus flavefaciens* [12]. The objective of the present experiment was to quantify the cellulolytic bacteria population using in vitro culture containing sodium hydroxide treated or untreated cottonseed hulls (CH) determined by real-time polymerase chain reaction (RT-PCR).

## I. Materials and method

*Feed Sample and treatment procedure*

Cottonseed hull samples were collected from industries located in North west Iran. Samples were used as untreated or chemical treated using NaOH as 20 g/kg DM [a solution of NaOH (20%) was sprayed on CH and kept for 48 h (CH2S48) at room temprature].

### *A. In vitro procedure*

The fermentation medium was prepared according to that described by Arroquy [13] including 400 ml cell-free ruminal fluid, cellobiose (0.05 g), $K_2HPO_4$ (0.45 g), $KH_2PO_4$ (0.45 g), NaCl (0.90 g), $(NH_4)_2SO_4$ (0.90 g), $MgSO_4 \cdot 7H_2O$ (0.09 g), $CaCl_2$ (0.09 g), resazurin (0.01 g), $NaHCO_3$ (4 g), and cysteine–HCl (0.5 g) per liter of medium. Rumen fluid was obtained from three sheep (49.5±2.5 kg body weight) fitted by rumen fistulae, before the morning feeding (The animals were fed 1 kg/d of DM alfalfa hay and 0.3 kg/d DM concentrate (165 g CP/kg DM)) and immediately strained through four layers of cheesecloth and centrifuged at 3000 RPM for 5 min. Then, the supernatant was centrifuged at 15000 RPM for 15 min. Forty-five ml of medium were distributed into a 100 ml bottle containing each experimental sample and autoclaved at 120 °C for 20 min. In order to isolate rumen fluid bacteria as inoculum, rumen fluid was immediately strained through four layers of cheesecloth. Then, the rumen fluid was centrifuged (3000 RPM, 10 min) and a solution of cycloheximide was added to protozoa free supernatant. Then, each bottle was inoculated with 5 ml of isolated rumen fluid bacteria and finely bubbled with $CO_2$, sealed and incubated. Incubations were on for 96 h at $39^{\circ C}$. After the incubation, 1 ml of the content of each bottle was sampled for DNA extraction.

### *B. DNA Extraction Procedure*

The extraction was done using Bioneer Accuprep Genomic DNA Extraction Kit. DNA concentrations were determined using Nanodrop® ND-1000 spectrophotometer. The 16s rRNA gene-targeted primer sets used in the present study are shown in TABLE I.

### *C. Real-time polymerase chain reaction design and assay conditions*

Total bacterial, *Fibrobacter succinogenes, Ruminococcus flavefaciens and Ruminococcus albus.* rDNA concentrations were measured by real time PCR. The 16s rRNA gene-targeted primer sets used in the present study are described in Table 1. DNA extract (0.5 µl ) was added to amplification reaction (25 µl ), containing 0.15 µl of each primer, 12.5 µl of SYBR® Green Jumpstart ™ Taq ReadyMix ™ (Sigma) containing 20 mM-tri (hydroxymethyl)-aminomethane-HCL (pH=8.3), 100 mM KCL, 7mM $MgCl_2$, 0.4 mM each dNTP, stabilizers, Taq DNA polymerase (0.05 unit/µl), JumpStart Taq, antibody and SYBR Green I. Cycling conditions were 95 °C for 5 min, forty cycles of 95 °C for 15 sec, 61°C for 15 sec and 72 °C for 30 sec, and a final extension of 81°C for 2 min. Fluorescence readings were taken after each extension step, and a final melting analysis was obtained by show heating with 0.2 °C/sec increment from 45 to 95.1 °C, with fluorescence collection at 0.2 °C at intervals. The threshold cycle (i.e. the amplification cycle in which product formation exceeds background fluorescence) of each standard dilution was determined during the exponential phase of amplification and regressed against the logarithm (base 10) of known total bacterial DNA standards that had been prepared. All post-run data analyses were performed using MJ Research Opticon Monitor Software (version 1.06). A bacterial rDNA standard curve was generated from DNA extracted from a mix (equal volumes) of 24 cultures of the following rumen bacterial strains all grown on Hobson's medium 2 (Stewart et al. 1997), from the Rowett Research Institute (Aberdeen, UK) culture collection.

### *D. Statistical Analysis*

Bacterial rDNA concentrations were measured relative to total bacteria amplification (ΔΔCt). Data were analyzed using the GLM procedure of SAS 9.1 and the means were compared by the Tukey test ($P < 0.05$).

## III. Results and Discussion

Quantity of the major species of cellulolytic bacteria existing under in vitro culture condition relative to total bacteria population is shown in TABLE II. Chemical treatment had no significant effect on the quantity of cellulolytic bacteria using present in vitro experimental conditions. Results of the present study indicate that the in vitro relative quantity of the major species of cellulolytic bacteria was not influenced by sodium hydroxide treatment of CH. Therefore, it was concluded that the treatment of CH with NaOH solution, as done in the present study might not alter the fibrolytic bacteria population. It was previously indicated that the digestibility of fibrous materials is generally related to rumen bacterial populations which are capable of producing wide range of fibrolytic enzymes [12]. The cellulolytic bacteria Ruminococcus flavefaciens readily attach to unlignified cell walls in perennial ryegrass leaves [14] And recent observations suggest that the attachment depends on physical and chemical conditions prevailing in the rumen as well as on the nature of the plant substrate [13]. Results of the present study are not in agreement with others. Odenyo [15] used wheat straw or alkaline hydrogen peroxide treated wheat straw in a medium culture containing complex or defined species of *Ruminococcus flavefaciens* and *Ruminococcus albus* with or without addition of phenylpropanoic acid or phenylacetic acid. They indicated that both bacteria extended the degradation of alkaline hydrogen peroxide treated wheat straw comparative to the untreated wheat straw. Latham [10] treated wheat straw with sodium hydroxide to enhance the digestion of the cell wall. They suggested that treatment with sodium hydroxide increases the number of adhesion sites available to the cellulolytic bacteria but that this alone does not permit them to digest many of the quantitatively important types of plant cell wall. It is concluded that although it is expected that sodium hydroxide treatment of cellulosic feedstuffs improves the digestion potential in the rumen, but data from the present study reveals that sodium hydroxide

treatment does not necessarily alter the concentration of cellulolytic bacteria.

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TABLE I. 16s rRNA Gene-Targeted Primer Sets

| Target species | Length | Primer | Sequence |
|---|---|---|---|
| Total Bacteria | 120 | Forward | 5′-GTGSTGCAYGGYTGTCGTCA- 3′ |
| | 120 | Reverse | 5′-ACGTCRTCCMCACCTTCCTC- 3′ |
| *Fibrobacter succinogenes* | 175 | Forward | 5′-GTTCGGAATTACTGGGCGTAAA- 3′ |
| | 175 | Reverse | 5′-CGCCTGCCCCTGAACTATC- 3′ |
| *Ruminococcus albus* | 122 | Forward | 5′-CCCTAAAAGCAGTCTTAAGTTCG- 3′ |
| | 122 | Reverse | 5′-CCTCCTTGCGGTTAGAACA- 3′ |
| *Ruminococcus flavefaciens* | 155 | Forward | 5′-CGAACGGAGATAATTTGAGTTTACTTAGG- 3′ |
| | 155 | Reverse | 5′-CGGTCTCTGTATGTTATGAGGTATTACC- 3′ |

TABLE II. Quantity of The Major Species of Cellulolytic Bacteria Existing in The In vitro Culture Relative to Total Bacteria Population

| Cellulolytic Bacteria | Samples | | s.e.m | P |
|---|---|---|---|---|
| | Untreated Cottonseed hulls | NaOH-treated Cottonseed hulls* | | |
| *Fibrobacter succinogenes* × ($10^{-4}$) | 12 | 13 | 0.002 | > 0.05 |
| *Ruminococcus flavefaciens* ×($10^{-7}$) | 950 | 560 | 0.003 | > 0.05 |
| *Ruminococcus albus* × ($10^{-4}$) | 17 | 14 | 0.004 | > 0.05 |

* A solution of NaOH (20%) was sprayed on cottonseed hulls and kept for 48 h at room temprature