Original Article

ISOLATION OF A CELLULOLYTIC ROD AND ITS POTENTIAL APPLICATION IN BIO-ETHANOL PRODUCTION

ISOLAMENTO DE BASTÃO CELULÍTICO E SUA POTENCIAL APLICAÇÃO NA PRODUÇÃO DE BIO-ETANOL

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ABSTRACT: With the world's focus on bio-fuel, cellulolytic microorganisms are being exclusively explored. In this paper, a strain of rod, NBG, was obtained from the rumen of Inner Mongolia sheep by an enrichment method with Whatman No.1 filter paper as the selective substrate. The strain was found to effectively degrade filter paper in solution. It was identified as *Fibrobacter succinogenes* based on DNA G + C mol %, together with morphological, physiological and biochemical tests. The endo-glucanase, β-glucosidase and filter paper enzyme activity of NBG reached 62.5 ± 3.0 U•mL^{-1}, 169.0 ± 9.4 U•mL^{-1} and 30.8 ± 5.4 U•mL^{-1}, respectively, within 72 h of fermentation.

KEYWORDS: Isolation. Identification. Fibrobacter succinogene. Cellulolytic characteristics.

INTRODUCTION

The importance of cellulose hydrolysis in the context of conversion of plant biomass to fuels and chemicals is widely recognized (ARGYROS et al., 2011; FONTES; GILBERT, 2010; NAIK et al., 2012). Cellulolytic microorganisms play an important role in cellulose degradation and the carbon cycle in nature. There are a lot of kinds of microbes that can degrade cellulose in the rumen of ruminants. In the early 19th century, Hungate (HUNGATE, 1947) isolated and identified some rumen cellulolytic bacteria from the rumen of cattle. However, due to the limitations of the experimental methods and apparatuses, no systematic identification or enzyme assay analysis has been done on isolated strains. Moreover, there are few reports on the isolation and identification of rumen cellulolytic microbes and their cellulase from the ruminants in Inner Mongolia, China. The ruminants in this region have undergone long evolution towards crude feed tolerance. Therefore, the research on rumen cellulolytic microbes of Inner Mongolia sheep is promising in respect of enriching the collection of cellulolytic microbes and providing strains for cellulose hydrolysis. This work was undertaken to obtained a strain of rod, NBG from the rumen of Inner Mongolia sheep by an enrichment method with Whatman No.1 filter paper as the selective substrate.

MATERIAL AND METHODS

Animals

Three mature Mongolia sheep (aged between 1.5 and 2 years; between 25.0 kg and 32.0 kg in weight), each fitted with a permanent rumen cannula, were used to provide rumen fluid. The animals were housed in individual pens and had free access to water and mineral blocks. The experiment diet was composed of hay and concentrate (the forage to concentrate ratio was 70:30). The sheep were fed a diet composed of 70 % mixed grass, 11.20 % corn, 6.10 % wheat bran, 10.20 % soybean meal, 1.25 % stone powder, 0.25 % $CaHPO_4$, 0.50 % salt, 0.50 % compound additive. The animals were maintained on their diets for at least 1 month prior to sampling of rumen contents. The sheep were fed twice daily at 7:00 and 19:00.

Media

Medium A: The medium used for preliminary screening experiments was that of Gao et al., 2004, which contained 0.5 g of carboxymethyl cellulose sodium (CMC-Na, Sigma, Saint Louis, USA) as the sole carbon source and energy source.

Medium B: A modification of the medium of Joblin (JOBLIN, 1981) with both antibiotics and agar ignored.

Medium C: A modification of medium A, with filter paper replacing CMC-Na and without agar being added.

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Rumen fluid for the above media was prepared by a modified standard method (DEHORITY; TIRABASSO, 1989). All media were prepared according to Bryant's method (BRYANT, 1972).

Sampling and strain screening

Equal amounts of rumen digesta were collected from the three sheep, 2 h after feeding at 07:00. The rumen digesta was mixed and then put into a thermos which was pre-warmed (39 °C) and filled with CO_2. After returning to the lab, the digesta was homogenized and then filtered through two layers of cheese cloth into a beaker which was continuously gassed with a heavy stream of CO_2. All subsequent operations were conducted under anaerobic conditions. The filtrate was then serially diluted, in 10-fold steps, in the anaerobic mineral solution of Bryant and Burkey (BRYANT; BURKEY, 1956). Quantities of dilutions sufficient to produce from 30 to 120 colonies per Hungate rolling tube (0.2 mL) were inoculated via syringe into tubes of reduced and melted medium A, and were maintained in a water bath at 46 °C to 48 °C. Within 15 min after inoculation, roll tubes were prepared by rapidly rotating inoculated tubes under a cold-water tap. The solidified tubes were incubated in an upright position at 39 °C and were regularly observed for the appearance of colonies. After 48 h, around 50 colonies appeared and 90 % of the colonies formed a transparent circle in the CMC agar. Thirty colonies were chosen for further purification based on the size of the CMC clearance zone. Individual colonies were picked and grown in the liquid form of medium A and was then re-isolated by repeating the process of rolling tube and colony selection on medium A until purified isolates were obtained. For further isolation, medium C was inoculated (20 %, v/v) with the purified isolates, then incubated at 39 °C. Then the filter paper was examined and when Gram-stained fibres were seen to be extensively colonized and degraded (2 to 4 days), the purified isolates were preserved at -20 °C and -70 °C in the presence of 10 % glycerol and used during the course of study.

Maintenance of cultures

For long-time storage, isolates were inoculated in medium C which was incubated for 48 h at 39 °C, sealed with 10 % of glycerol, vacuum freeze-dryed, and stored at -70 °C in an ultra-low temperature freezer.

Identification of strain

Biochemical properties: The identification was done by referring to Bergey's Manual of Deteminative Bacteriology (HOLT, 1994).

Morphological studies: A phase contrast microscope was used to observe cell morphology in the exponential growth phase.

DNA extraction: DNA was extracted from cultured colonies using the method of Zhang (ZHANG L., 2000).

Determination of DNA G + C mol %: The G + C mol % of the strain was determined with a UV-1700 UV-VIS spectrophotometer (Shimadzu). Escherichia coli K12 was used as a control strain. Briefly, the temperature of the genomic DNA in SSC (saline sodium citrate; 0.15 mol•L-1 NaCl with 0.015 mol•L-1 sodium citrate) buffer (25 mol•L-1) was increased slowly (0.5 °C/min) from 25 °C and the absorbance of the solution at 260 nm was monitored continuously against a blank containing SSC buffer only. The Tm of the DNA is defined as the temperature at 50 % hyperchromicity. The G + C mol % of the test strain and that of the control strain were determined. The G + C mol % of the genomic DNA of the test strain was calculated by Equation 1:

$$(G + C)\ mol\% = 51.2 + 2.08 \times [Tm\ (test\ strain) - Tm\ (control\ strain)] \quad (Eq.\ 1)$$

16Sr RNA sequence identification: To identify the bacterium more accurately, the 16Sr RNA sequence of the strain was amplified and sequenced. PCR amplification of 16Sr RNA was carried out using forward primer 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 1492R 5'-ACGGCTACCTTGTTACGACT-3'. The amplification procedure was as follows: 5 min at 94 ; 25 cycles of 30 s at 94 , 30 s at 55 , and 90 s at 72 ; after the cycles, an additional 5 min at 72 . The PCR product was sequenced by Tiangen Biotech (Beijing) Co. LTD. and the result was contrasted to the 16Sr RNA sequences available in the Genbank from the National Center for Biotechnology Information Database.

Enzyme assay

The enzyme activity of endoglucanase, β-glucosidase and filter paper enzyme were determined according to the method of Eveleigh (Eveleigh et al., 2009). The isolate was inoculated in medium B with inoculate size of 2 %, and incubated at 39 °C under anaerobic conditions for 72 h. One unit of enzyme activity is defined as the amount of enzyme that release 1μg of reducing sugar per minute.

Determination of cellulose degradation rate

The cellulose degradation rate was determined according to the method of Sluiter (2011).

RESULTS AND DISCUSSION

Strain isolation

Thirty bacterial colonies which formed clear zones in the CMC agar were isolated and purified. After Gram staining and observing with a phase-contrast microscope, 5 rod strains were selected. By final screening using medium C, one rod strain (named as NBG), which made filter paper to be extensively colonized and degraded in 4 days, was selected.

Strain identification

After Gram staining, phase contrast microscopy at 1000 magnification of NBG cells in the exponential growth phase showed Gram-negative, non-helical, motile, mostly rod-shaped cells. The strain formed round, smooth, flat, irregular edge, milk white pigmented colonies with a diameter of 2 mm to 3 mm.

The biochemical characteristics and carbohydrate utilization are shown in Table 1. The G + C mol% of the DNA of the isolate was 47.2 %, which is the common value in *Fibrobacter succinogenes* (47 % to 49 %) according to Bergey's Manual of Deteminative Bacteriology (HOLT et al., 1994).

Table 1. Example of Tabular Results (12-point Arial here)

Characteristics	Results	Characteristics		Results
Acids from		Esculin		+
Glucose	+	Catalase		□ —·
Mannitol	—	Nitrate reduction		—
Lactose	+	H_2S synthesis		—
L-arabinose	—			
Raffinose	—	Temperature experiments	20 °C	++
Sorbitol	—		30 °C	++
Salicin	+		37 °C	#
Maltose	—		39 °C	+++
Melibiose	—		45 °C	++
Fructose	—	pH experiments	5.5	+
Glycerol	□ —·		6.3	+
Sodium lactate	□ —·		7.0	#
Galactose	—		7.8	+
D-xylose	—		8.0	—

* +: Positive reaction; —: Negative reaction; "+" to "+++": the cell concentration increases; #: the highest cell concentration

The 16Sr RNA gene sequence containing 1393 nucleotides of strain NBG is aligned with all related sequences in the NCBI database by the BLASTN program. The 16Sr RNA gene of a strain of *Fibrobacter succinogenes* S85 shared 98% identity with that NBG. The GenBank accession number for the 16Sr RNA gene sequence of isolate NBG is KC438280.

The physiological and biochemical characteristics and (G + C) mol % all revealed that NBG belonged to *Fibrobacter succinogenes*.

Fibrobacters are recognized as major bacterial degraders of lignocellulosic material in the herbivore gut (RANSOM-JONES et al., 2012). It is important to isolate different strains of *F. succinogenes* for the following reasons: 1. it has been suggested that within this group of organisms there is a "third" way of attacking cellulose except the cellulosome and free enzyme. 2. *F. succinogenes* can produce succinic acid, which is utilised in a variety of industries and chemical manufacturing processes (RANSOM-JONES et al., 2012). 3. *F. succinogenes* has significant potential for refining of lignocellulosic biomass in the generation of bio-ethanol fuel (LYND et al., 1991). However, there are few strains of *Fibrobacters* which have been isolated because *Fibrobacters* are difficult to be isolated and cultured (RANSOM-JONES et al., 2012). The present study isolated and identified a novel strain which will contribute to the research of *Fibrobacter* and supply a strain for producing succinic acid and bio-ethanol by consolidated bioprocessing (CBP) from cellulose.

There are 4 kinds of cellulolytic microbes. They are aerobic cellulolytic fungi, aerobic cellulolytic bacteria, anaerobic cellulolytic bacteria and anaerobic cellulolytic fungi (DOI, 2008). Now aerobic cellulolytic fungi are commonly applied in the cellulase production industry. For example, Trichoderma reesei, Aspergillus niger, Rhizopus stolonifer and Phanerochaete chrysosporium (DOI, 2008). However the high cost is a limiting factor for

cellulase production from aerobic fungi. Recently, with the concept of CBP being put forward, the research on anaerobic cellulolytic bacteria is highly valued. Anaerobic cellulolytic bacteria, such as *Clostridium thermocellum* and *Clostridium cellulolyticum*, are mainly used in bio-ethanol production (HASUNUMA et al., 2012). There are few studies on aerobic cellulytic bacteria and anaerobic cellulytic fungi.

Enzyme assay

Without optimization the culture medium and culture conditions, the degradation ratio of filter paper of the wild type strain reached 74.5 % ± 7.4 % and the enzyme activity of endoglucanase, β-glucosidase and filter paper degradation reached 62.5 ± 3.0 U•mL^{-1}, 169.0 ± 9.4 U•mL^{-1} and 30.8 ± 5.4 U•mL^{-1}, respectively, within 72 h of fermentation.

For different cellulolytic microorganisms, the cellulose degradation rate and cellulase activity are different. However, it is not very consistent to compare the cellulose degradation rates and cellulase activities of different strains because of different kinds of cellulose sources, different determination methods, etc. At the same time, anaerobic cellulolytic microorganisms usually have lower cellulase activity than aerobic cellulolytic microorganisms though their cellulose degradation rates are higher than that of aerobic cellulolytic microorganisms because they have a different cellulose degradation model. Aerobic cellulolytic microorganisms usually degrade cellulose by free enzyme. Anaerobic cellulolytic microorganisms usually degrade cellulose by cellulosomes (HASUNUMA et al., 2012). That is why it is difficult to compare the cellulase activities from different kinds of microorganisms.

Arobic cellulolytic microorganisms usually have higher cellulase activities because they secrete free cellulase. The enzyme activities of CMCase, Fpase and β-glucosidase for *Aspergillus niger* NS-2 were 310 U•g^{-1} dry substrate, 17 U•g^{-1} dry substrate and 33 U•g^{-1} dry substrate respectively (BANSAL et al., 2012). FPase activity, CMCase activity and β-glucosidase activity of A. niger NRRL 567 were (401 ± 23.8) IU•g^{-1} dry weight, (544.7 ± 24.5) IU•g^{-1} dry weight, (285.4 ± 11.7) IU•g^{-1} dry weight, respectively (DELABONA et al., 2012). *Streptomyces griseoaurantiacus* ZQBC691 generated 34.13 IU•mL^{-1} of endoglucanase (CHU et al., 2012). *Trichoderma harzianum* produced 121 FPU•g^{-1} cellulase and 1730 IU•g^{-1} β-glucosidase (DELABONA et al., 2012). *Acremonium cellulolyticus* secreted (12.8 ± 0.65) U•mL^{-1} FPU enzyme, (40.80 ± 0.02) U•mL^{-1} CMCase and (87.56 ± 0.09) U•mL^{-1} β-Glucosidase (Dwiarti et al., 2012). Anaerobic cellulolytic microorganisms usually have lower cellulase activities because they degrade cellulose via cellulosomes, although the cellulose degradation rates are higher. For example, *C. thermocellum* hydrolyzed cellulose at rates approaching 2.5 g•L^{-1}•h^{-1} (ARGYROS et al., 2011). The emzyme activities of *Clostridium* sp. TCW1 were 1.08 g•L^{-1} exoglucanase and 1.80 g•L^{-1} CMCase.

CONCLUSIONS

A cellulolytic rod was isolated from the rumen of Inner Mongolia sheep.

It was identified as *Fibrobacter succinogenes*.

Three kinds of cellulase activities and the degradation ratio of filter paper were determined and the results suggested that the rod could be applied in the degradation of cellulose and have a potential in producing bio-ethanol by CBP from cellulose.

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RESUMO: Com o foco mundial em biocombustíveis, microrganismos celuloliticos estão sendo exclusivamente explorados. Neste trabalho, uma cepa de bastão, NBG foi obtida do rumen de ovelhas do interior DA Mongolia com o método de enriquecimento e o papel de filtro No.1 como substrato seletivo. A cepa foi encontrada para degradar efetivamente o papel de filtro na solução. Foi identificado como Fibrobacter succinogenes baseado em DNA + c mol% juntamente com testes morofológicos, fisiológico e bioquímicos. A endo-glucanase e o papel de filtro chegaram com relação a atividade de enzima NBG 62.5 ± 3.0 U.ml-1, 169±9.4U.ml-1 e 30.8±5.4U.ml-1, respectivamente, com 72 horas de fermentação.

PALAVRAS-CHAVE: Isolamento. Identificação. Fibrobacter succinogene. Características celulíticas.

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