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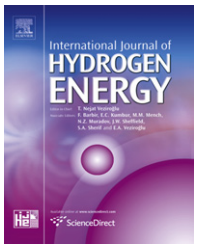

Co-culture of *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* for enhancing hydrogen production via thermophilic fermentation of cornstalk waste

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ABSTRACT

A strategic method utilizing the co-culture of *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* has been developed to improve hydrogen production via the thermophilic fermentation of cornstalk waste. The hydrogen yield in the co-culture fermentation process reached 68.2 mL/g-cornstalk which was 94.1% higher than that in the mono-culture. The hydrogen fermentation process was successfully scaled-up from 125 mL anaerobic bottles to an 8 L continuous stirred tank reactor, and the hydrogen production from cornstalk waste was significantly improved in the bioreactor system due to efficient mixing and mass transfer. The hydrogen yield in the bioreactor reached 74.9 mL/g-cornstalk which was 9.8% higher than that in the 125 mL anaerobic bottle. The present work indicates that the direct microbial conversion of lignocellulosic waste by co-culturing *C. thermocellum* and *C. thermosaccharolyticum* is a promising avenue for enhancing hydrogen production.

1. Introduction

Hydrogen is one of the most promising energy carriers due to its efficient conversion to usable power, high energy density and the fact that it is environmentally friendly. Microbial fermentation is a potential method for biohydrogen production with clear advantages such as low energy input and high production rate [1,2].

Recently, biological hydrogen production from renewable lignocellulosic waste has attracted significant attention [3–9]. *Clostridium thermocellum*, a cellulose-degrading bacterium, has demonstrated the potential for the direct production of hydrogen from lignocellulosic waste without the need for an extensive pretreatment process [10–13]. However, the biohydrogen production yield of these cellulose-degrading *C. thermocellum* strains is low [14–17]. A combination of cellulose-hydrolyzing bacteria and highly efficient hydrogen-producing bacteria has been developed to improve hydrogen production from cellulosic waste under thermophilic or mesophilic fermentation conditions [3,17–20].

In order to achieve highly efficient hydrogen production, a synergistic co-culture process must be developed that optimizes the combination of cellulolytic and hydrogen-producing microbial strains, allows for the scale-up of the fermentation process, and includes low-cost pretreatment methods for the lignocellulosic waste. The objective of the present study was to evaluate the hydrogen production resulting from the co-culture of the cellulolytic hydrogen-producing strain, *C. thermocellum*,

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in combination with a non-cellulolytic hydrogen-producing stain, *C. thermosaccharolyticum*. The co-culture conditions were optimized, and the co-culture process was scaled-up in a continuous stirred tank reactor (CSTR).

2. Materials and methods

2.1. Microorganisms and media

C. thermocellum DSM 7072 and *C. thermosaccharolyticum* DSM 869 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Both stains were cultivated in CM4 medium as follows (g/L): 1.5 KH_2PO_4, 3.8 $K_2HPO_4 \cdot 3H_2O$, 1.3 $(NH_4)_2SO_4$, 1.6 $MgCl_2 \cdot 6H_2O$, 0.013 $CaCl_2$, 5.0 yeast extract, 1.25×10^{-3} $FeSO_4 \cdot 7H_2O$, 1.0×10^{-3} resazurin, and 0.5 cysteine-HCl. The *C. thermocellum* culture was subcultured every 3 days into fresh CM4 medium with 10 g/L microcrystalline cellulose (Sinopharm Chemical Reagent Co., Ltd., China), and the *C. thermosaccharolyticum* culture was subcultured every 24 h into fresh CM4 medium with 10 g/L xylose. The final pH values of these media were adjusted to 7.2 with sodium bicarbonate.

2.2. Co-culture experiments in anaerobic bottles

Cornstalks, obtained from the Daxing district, in Beijing, China, were dried at 60 °C. Cornstalks used in all experiments were milled to 1 mm powder with a plant mill. The main chemical components of the cornstalks were as follows (%, w/w): cellulose 31.5, hemicellulose 29.8, lignin 10.8 and total soluble sugar 12.7.

All batch experiments were carried out in 125 mL anaerobic bottles with a working volume of 50 mL at 55 °C. Each bottle was sealed with a butyl rubber stopper and a screw cap, and was gassed and degassed with 100% nitrogen gas before autoclaving at 115 °C for 20 min. The cornstalk concentration was 10 g/L, and the inoculum volume of *C. thermocellum* (OD_{600} = 1.18–1.23) in mono- or co-cultures was 10% of the liquid culture medium volume. For co-culture experiments, 0.025, 2.5, 250, 1250, 2500 or 5000 μL of *C. thermosaccharolyticum* 24 h cultures (OD_{600} = 1.09–1.17) was inoculated into the liquid medium along with *C. thermocellum* to give a *C. thermosaccharolyticum* to *C. thermocellum* inoculation ratio of 0.000005:1, 0.0005:1, 0.05:1, 0.25:1, 0.5:1 or 1:1. To optimize the *C. thermosaccharolyticum* inoculation time, *C. thermosaccharolyticum* was inoculated into the liquid culture medium after 12, 24, 36 and 48 h of *C. thermocellum* inoculation, respectively. Three bottles containing inoculums and CM4 medium without cornstalk were incubated at the same time as a control. These bottles were manually mixed once each day. The batch experiments were carried out in triplicate.

2.3. Co-culture experiments in a continuous stirred tank reactor

A continuous stirred tank reactor (CSTR) with a working volume of 8 L was constructed to scale-up the co-culture process (Fig. 1). The reactor temperature was controlled using a Pt100 sensor and a proportional-integral-derivative (PID) temperature controller (Beijing Huibang XMT614, China). A magnetic stirrer was utilized continuously to ensure sufficiently mixing in the CSTR. CM4 medium (4 L) with 10 g/L cornstalk was added to the CSTR prior to autoclaving at 115 °C for 30 min. The autoclaved CSTR was flushed with nitrogen gas for 30 min prior to inoculation with 10% (v/v) of exponentially growing *C. thermocellum* cultures. After 24 h, 100 mL of *C. thermosaccharolyticum* cultures was inoculated into the *C. thermocellum* mono-culture to initiate the co-culture process.

2.4. Analysis

The optical density of the cultivated cells was measured at 600 nm using an ultraviolet (UV) spectrophotometer (Unico UV-2100 spectrophotometer, Dayton, NJ). Cell cultures with solid substrate were vibrated and centrifuged at 500 rpm for 10 min, and the resulting supernatant containing cells was used for the measurement of the optical density [10].

The gas composition was measured using gas chromatography (GC) equipped with a thermal conductivity detector and two columns (Plot Q polymer column and a molecular sieve column) separated by a switch valve (Agilent GC 7890, Santa Clara, CA). Helium was used as the carrier gas at a flow rate of 23 mL/min. The oven, injector, and detector temperatures were set at 50, 150, and 250 °C, respectively. The gas produced in the anaerobic bottle was collected in an airtight bag for GC analysis. The gas volume produced in the anaerobic bottle was measured using a glass syringe, and the gas volume produced in the CSTR was measured using a wet gas meter [10]. The cumulative hydrogen production data were fitted to a modified Gompertz equation [10], a suitable model to describe the cumulative hydrogen fermentation process in a batch experiment

$$H(t) = P\exp\left\{-\exp\left[\frac{R_m e}{P}(\lambda - t) + 1\right]\right\}$$

Fig. 1 – Photograph of continuous stirred tank reactor (CSTR) used in the current study.