Fuel 158 (2015) 471–478

Contents lists available at ScienceDirect

Fuel

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

Metabolically redirected biohydrogen pathway integrated with biomethanation for improved gaseous energy recovery

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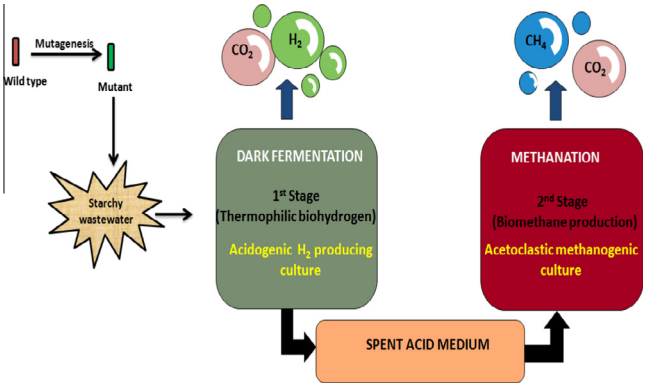
HIGHLIGHTS

- Development of strain using chemical mutagenesis for improvement of H_2 production.
- Establishing the relationship between NADH/NAD$^+$ ratio and rate of H_2 production.
- Proteomic study to assess the consequence of random mutagenesis.
- Continuous bioH_2 production with mutant using starchy wastewater.
- Biomethanation for improved energy recovery using steady state effluent.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 10 August 2014
Received in revised form 19 May 2015
Accepted 26 May 2015
Available online 3 June 2015

Keywords:
bioH_2
Metabolic redirection
NADH/NAD$^+$ ratio
Biomethanation
T. thermosaccharolyticum

ABSTRACT

Chemical mutagenesis approach was used for the improvement of H_2 yield using *Thermoanaerobacterium thermosaccharolyticum*. A H_2 yield of 3.12 mol mol^{-1} glucose was observed which was about 13.5% higher as compared to wild type. An inverse relation was observed between the NADH/NAD$^+$ ratio and the rate of H_2 production. Bioelectrochemical analysis showed an overall increase in reducing equivalent in mutants. Continuous H_2 production with mutant using starchy wastewater showed maximum H_2 production rate of 500 mL L^{-1} h^{-1} at a dilution rate of 0.25 h^{-1} and organic loading rate (OLR) of 8.75 g L^{-1} h^{-1}. The spent medium was mostly comprised of acetate (0.62 ± 0.3 g L^{-1}), butyrate (1.26 ± 0.34 g L^{-1}) and ethanol (0.10 ± 0.02 g L^{-1}). It was subsequently used as substrate for the biomethanation process to improve overall gaseous energy recovery. Highest methane production rate, methane yield and COD removal of 51.4 ± 0.4 mmol L^{-1} d^{-1}, 82.6 L kg^{-1} COD_{added}, and 66%, respectively were observed at an OLR of 14 g L^{-1} d^{-1} thereby improving overall gaseous energy recovery by 53.6%.

1. Introduction

The global energy requirements are vehemently dependent on fossil fuels whose reserves are limited [1]. The CO_2 and other emitted gases pose environmental pollution problem. Thus the need of the hour is to find out a clean and sustainable fuel. Hydrogen has gained importance throughout the world as an alternative and eco-friendly fuel. It has the highest energy density amongst all the gaseous fuels and it produces water as by product of combustion [1]. Biological H_2 production has drawn attention of researchers around the world. Apart from production of clean

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http://dx.doi.org/10.1016/j.fuel.2015.05.060
0016-2361/

energy in the form of H_2, this process can be coupled to waste management if industrial, food, domestic, agricultural and other wastes are considered as substrate. For the development of sustainable bioH_2 production, applications of thermophilic fermentative H_2 producing microorganisms has been explored due to their high H_2 yield and high production rate [2]. Fermentation at thermophilic temperatures has certain advantages over mesophilic dark fermentation viz. improved kinetics, stoichiometry of H_2 production and reduction of methanogenic contamination [3,4]. Moreover, industrial effluents which are often discharged at higher temperatures (such as distillery effluents or food processing wastewater), can be directly used as substrate for dark fermentation process [5]. *Thermoanaerobacterium thermosaccharolyticum* IIT BT-ST1 is a potent thermophilic bioH_2 producing microorganism [6]. It can utilize a wide range of carbon sources such as glucose, sucrose, fructose, etc. and produces ethanol and a mixture of fatty acids such as acetic acid, butyric acid, etc as principal metabolites.

During the fermentation process, the excess reducing equivalents could be disposed off via proton reduction mediated by H_2ase and electrons carrier leading to the formation of H_2 [7]. In anaerobic fermentation, NADH is usually generated by catabolism of glucose through glycolysis. The NADH pool is required for the formation of various metabolites like ethanol, butanediol, lactic acid and butyric acid. The NADH pool could be increased if the formation of these metabolites could be blocked by random mutagenesis or site directed mutagenesis [8].

Proteomics study could be used as a powerful tool for investigating dynamic physiological responses in microbial systems with respect to stress conditions like mutation or exposure to radiation. A slight change in the environment, such as exposure to chemical or physical mutagens can cause wide variations in the proteome of microorganisms. Thus proteome study could provide a better understanding of the physiological changes in mutant [9]. BioH_2 production can be produced from different pure substrates (glucose, fructose, cellobiose, etc.) and also from various wastes (organic fraction of municipal waste, starchy wastewater, lignocellulosic wastes, etc.). To make dark fermentative H_2 production a sustainable process, the feedstock should be cheap like wastewaters [10]. Starchy wastewaters discharged from canteens or food processing industries are rich in carbohydrates and have high chemical oxygen demand (COD). It might cause environmental pollution if it is discharged untreated into the water bodies. Moreover, they are discharged at very high temperatures. Thermophilic bioH_2 production process using such wastewater would certainly help in waste management along with energy generation [11]. H_2 production from organic waste is usually coupled with the production of volatile fatty acids which are also suitable feedstock for biomethanation process. So, CH_4 production could be considered for treatment of spent media generated from fermentative H_2 production leading to maximization of gaseous energy recovery. Combination of acidogenic hydrogenesis and methanogenesis has gained greater attention in under the eponym of Biohythane [12–14].

The present study was aimed to (a) develop an alcohol production pathway defective mutant using chemical mutagenesis, (b) explore the role of reducing equivalent content ($NADH/NAD^+$) in mutants and wild type towards H_2 production, (c) investigate the physiological changes in both mutant and wild type using proteomic studies, and (d) maximize the gaseous energy recovery by treating the spent medium from dark fermentation process by the 2nd stage biomethanation process. To best of our knowledge, this is the first report showing the variation in rate of hydrogen production, proteome profile and $NADH/NAD^+$ ratio in response to random mutagenesis in *T. thermosaccharolyticum*.

2. Materials and method

2.1. Culture condition

Media used for isolation of mutants consisted of Na_2HPO_4 (4.2 g L^{-1}), KH_2PO_4 (1.5 g L^{-1}), NH_4Cl (1.95 g L^{-1}), $MgCl_2$ (0.18 g L^{-1}), yeast extract (2.0 g L^{-1}), glucose (10 g L^{-1}), cysteine HCl (1 g L^{-1}), vitamins solution (DSMZ medium No141, German Collection of Microorganisms and Cell Cultures).

2.2. Strategy of random mutagenesis and selection of mutants having defective alcohol production

The late log phase culture of *T. thermosaccharolyticum* IITBT ST1 was exposed to 2% (v/v) ethyl methane sulfonate for 30 min at 60 °C without shaking. After exposure to mutagen, 1 mL of culture was centrifuged at 10,000*g* for 10 min. The cell pellet was then washed with 0.2 M phosphate buffer pH 7, resuspended in 5 mL sterile media and incubated for 1 h at 60 °C. The mutant cells were screened on solid screening media supplemented with Bromocresol Green (0.04 g L^{-1}) and allyl alcohol (filter-sterilized) [15]. For selection of allyl alcohol resistant mutants (Adh enzymes convert allyl alcohol to acrolein), only green colonies (pH > 4) were taken into consideration instead of the yellow one (pH < 4) to find the low metabolite producers. All the mutants were grown anaerobically using a 3.5 L anaerobic jar (Hi-Anaerobic system Mark II, Hi-Media). Hydrogen production characteristics of the mutants and wild type were studied using media composition mentioned in Section 2.1.

2.2.1. Two dimension gel electrophoresis study for differential protein expression in mutant

A 17 cm-long immobilized pH gradient of 3–10 IPG strips (GE life USA) was used for isoelectric focusing (IEF). IEF was performed at a current limit of 50 μA/strip at 20 °C using Protean IEF cell (Bio-Rad). The IEF was done as follows: active rehydration at 250 V for 12 h; 250 V (linear) for 15 min; 10 kV (linear) for 3 h; and 10 kV (rapid) until a total 80 kV h for a combined total of approximately 92 kV h. After IEF, each IPG strip was equilibrated in 6 ml of equilibration base buffer for 20 min. For second dimension electrophoresis, a 12.0% w/v SDS–polyacrylamide was used [16]. Silver nitrate staining was used for visualizing proteins. All 2D experiments were carried out in triplicate.

2.2.2. Procedure for protein identification by mass spectrometry: peptide mass fingerprinting (PMF)

The protein spots were excised from the gel, digested with sequencing grade trypsin (Promega, Madison, WI). The trypsin digested spots were lyophilized and were eluted with acyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA and analyzed by MALDI-TOF analysis (Microflex LRF 20, Bruker Daltonics) [17]. The search program MASCOT was used for protein identification by peptide mass fingerprinting. The database search was performed considering following parameters: trypsin as the cleaving enzyme with a maximum of one missed cleavage, iodoacetamide (Cys) as a complete modification, oxidation (Met) as a partial modification, monoisotopic masses, and a mass tolerance of ±0.1 Da.

2.3. Continuous production of H_2 in continuous stirred tank reactor (CSTR)

Continuous H_2 production was carried out in 600 mL double jacketed continuous stirred tank reactor with working volume of 500 mL. Agitation inside the reactor was done by a magnetic