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Enhanced hydrogen production of *Enterobacter aerogenes* mutated by nuclear irradiation

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HIGHLIGHTS

- *Enterobacter aerogenes* cells were mutated by nuclear irradiation of ^{60}Co γ-rays.
- *E. aerogenes* mutants were screened with larger colour circles of acid by-products.
- Hydrogenase activity of the *E. aerogenes* ZJU1 mutant improved by 75.3%.
- Hydrogen yield (301 mL H_2/g glucose) with mutant was higher by 81.8% than the wild.

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ABSTRACT

Nuclear irradiation was used for the first time to generate efficient mutants of hydrogen-producing bacteria *Enterobacter aerogenes*, which were screened with larger colour circles of more fermentative acid by-products. *E. aerogenes* cells were mutated by nuclear irradiation of ^{60}Co γ-rays. The screened *E. aerogenes* ZJU1 mutant with larger colour circles enhanced the hydrogenase activity from 89.8 of the wild strain to 157.4 mL H_2/(g DW h). The hereditary stability of the *E. aerogenes* ZJU1 mutant was certified after over ten generations of cultivation. The hydrogen yield of 301 mL H_2/g glucose with the mutant was higher by 81.8% than that of 166 mL/g glucose with the wild strain. The peak hydrogen production rate of 27.2 mL/(L·h) with the mutant was higher by 40.9% compared with that of 19.3 mL/(L·h) with the wild strain. The mutant produced more acetate and butyrate but less ethanol compared with the wild strain during hydrogen fermentation.

1. Introduction

Renewable energy resources have attracted increasing attention because of the consumption of fossil fuels (Lin et al., 2008). From the perspective of economic development and ecological environment protection, hydrogen has been considered one of the most advantageous alternative source of energy because of its clean combustion product, namely, water (Nitsch et al., 1992). Microbial fermentation is a potentially important method for renewable hydrogen production that uses biomass as substrate. Firstly, the method is carbon neutral and requires less energy consumption as compared with other conventional hydrogen-producing methods, such as steam reforming, partial oxidation, gasification and water electrolysis (Jones, 2008; Angenent et al., 2004; Kothari et al., 2008; Holladay et al., 2009). Secondly, dark fermentation with biomass or carbohydrate-based substrates presents a more promising and feasible route for biological hydrogen production, thereby offering higher production efficiency, higher stability, simpler control requirements, lower operating costs and higher feasibility for industrialisation (Jayasinghearachchi et al., 2009). As a facultative anaerobe, *Enterobacter aerogenes* has high growth and hydrogen production rates, which have been widely studied in dark fermentation (Zhang et al., 2011). Nevertheless, for commercialisation in biological fermentative hydrogen production, its hydrogen yield needs to be further improved. The theoretical hydrogen yield of *E. aerogenes* is 4 mol/mol glucose (Zhang et al., 2011; Song et al., 2011). However, the hydrogen yield of *E. aerogenes* is approximately 1 mol/mol of glucose at pH of 6.0, which is considerably lower than the theoretical value (Tanisho, 1999; Yokoi et al., 1995; Tanisho et al., 1989; Converti and Perego, 2002; Zhang et al., 2011). Therefore, extensive research on *E. aerogenes* has been conducted to improve the hydrogen yield; thus far, a yield of 1.58 mol H_2/mol glucose has been experimentally

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reached mainly by optimising the cultivation conditions (Tanisho et al., 1998).

Several approaches, such as genetic engineering and metabolic engineering technologies for most promising micro-organisms, were recently conducted to enhance microbial hydrogen productivity (Rachman et al., 1997; Lu et al., 2008, 2011). However, the hydrogen yield of *E. aerogenes* remains markedly lower than the theoretical hydrogen yield of 4 mol/mol glucose. Complex gene analysis of various bacteria and the specific methods for different bacteria present considerable limitations for further research. Microbial mutation breeding could obtain stable genetic mutant strains with improved target properties; this approach has been widely used in industrial fermentation (Shi and Wu, 2013). The application of mutation techniques to improve the productivity of hydrogen-producing bacteria is a promising research direction. The *E. aerogenes* AY-2 mutant was obtained by allyl alcohol and proton suicide methods; its hydrogen yield of 1.17 mol H_2/mol glucose was higher by 109% than that of the wild strain HU-101 (Rachman et al., 1997). Lu et al. (2008) used He–Ne laser irradiation to improve the hydrogenase activity of the *E. aerogenes* HB-5 M mutant, which had a hydrogen yield of 0.95 mol H_2/mol glucose; the maximum specific rate of hydrogen production by the mutant was twice that of the wild strain W-23 (Lu et al., 2008). Numerous other positive mutants have been generated with atmospheric and room temperature plasma; the hydrogen yield of these mutants was approximately 1.134 mol/mol glucose, which was an increase of 26.4% (Lu et al., 2011). Evidently, the mutation breeding method can improve the hydrogen production of *E. aerogenes*. However, the enhancement of hydrogen yield needs further study.

Mutagenesis by γ-ray irradiation can cause morphological and biochemical changes in cells (Feng et al., 2015). A large number of free radicals generated by γ-rays in the cells attack the key functional genes and cause gene breakage and recombination because γ-rays interact with molecules and atoms, especially with water molecules (Kovacs and Keresztes, 2002). γ-rays possess better mutagenic ability and strong penetrability compared with other forms of radiation. Cheng et al. (2014) significantly increased the growth rate and lipid productivity of microalgae using γ-rays irradiation. To the best of our knowledge, studies concerning hydrogen-producing bacteria mutated by γ-ray irradiation have yet to be reported. A large number of samples are randomly selected to test the hydrogen yield in a batch experiment after irradiation to screen the positive mutant strains (Ren et al., 2006). It's quite difficult to test each strain's hydrogen yield or producing rate to screen the positive mutant strains because of tremendous bacteria strains. Although positive mutant strains could be obtained by large-scale experiments on selection and fermentation, this traditional method is time-consuming, inefficient and difficult to perform.

To our best knowledge, no studies on *E. aerogenes* mutated by ^{60}Co γ-rays to enhance hydrogen production have been reported in literature to date. γ-rays possess better mutagenic ability and strong penetrability compared with other forms of radiation. The mutation process of γ-ray irradiation has the advantages of high efficiency and low cost. The screened *E. aerogenes* mutant with high hydrogen productivity has the potential to be utilized on the biological hydrogen production through the anaerobic fermentation of biomass wastes, such as molasses wastewater and food waste in future industrial applications. This paper is intended to fill in this gap in the state of the art. Thus, the objectives of the present study are to:

(1) Screen *E. aerogenes* positive mutants with improved hydrogen production using colour circles of acid by-products.
(2) Improve hydrogenase activity and hydrogen production of *E. aerogenes*, and compare metabolic by-products of the mutant and wild strains.
(3) Examine the hereditary stability of the screened *E. aerogenes* ZJU1 mutant.

2. Materials and methods

2.1. Bacterial strains and medium

Enterobacter aerogenes ATCC13408 was purchased from China General Microbiological Culture Collection Centre. The Luria Bertani (LB) culture medium contained 5 g/L yeast extract, 10 g/L peptone and 10 g/L NaCl. The solid LB medium also contained 20 g/L agar.

2.2. Mutagenesis and selection of E. aerogenes

1 mL of the overnight seed culture of *E. aerogenes* ATCC13408 was inoculated into 100 mL of the LB medium. Batch cultivations were performed aerobically in a shaker incubator at 37 °C and 180 rpm for 6–8 h until the cells reached the mid-log phase. The bacterial cell culture was centrifuged to prepare a bacterial suspension with normal saline. Six centrifuge tubes (5 mL) with 1 mL of bacterial suspension were immersed in ice water and irradiated with 0, 200, 400, 600, 800 and 1000 Gy of ^{60}Co γ-rays, respectively (Zhejiang Institute of Agriculture and Nuclear Application Technology, Zhejiang, China). All samples were immersed in ice water for 1–2 h after irradiation to inhibit the activity of intracellular repair enzymes.

A 0.1 mL aliquot of each sample was diluted with equal amounts of LB medium. The diluted samples were evenly applied on the solid medium in an incubator (37 °C) for 24 h. The lethality percentage of *E. aerogenes* irradiated by different doses of ^{60}Co γ-rays was calculated based on the following equation:

$$\textit{Lethality percentage} = [(A - I)/A] \times 100\%, \quad (1)$$

where A is the total colony count of the sample without irradiation and I is the total colony count irradiated by ^{60}Co γ-rays. All colony numbers were obtained by the CFU (colony-forming unit) method on solid medium.

The irradiated samples of each bacterial suspension were respectively diluted and evenly applied on the screening medium. The screening medium contained 20 g/L of glucose, 5 g/L of yeast extract, 10 g/L of peptone, 10 g/L of NaCl, 20 g/L of agar and 0.4 g/L of bromcresol purple. Yeast, peptone and NaCl were same with the culture medium to help bacterial cells grow and reproduce. The screening medium was used for selecting positive mutants by hydrogen production. Glucose substrate was essential to provide suitable carbon source for hydrogen and metabolite production in dark fermentation. The colour of bromcresol purple will change from purple to yellow in acid condition. The size of the yellow circle visually shows the quantity of acid by fermentation, and then indirectly evaluates hydrogen productivity of strains. If one mutant is surrounded by larger yellow circle, it has a larger yield of acid by-products. It's probable that the yield of acetic acid or butyric acid is positively correlated with the hydrogen yield of the mutant. Positive mutants can be screened based on sizes of yellow circles by the pH indicator. Several well-grown strains with larger yellow circles were selected from the screening medium in an incubator (37 °C) after 12–24 h. Sole cells of the mutant strains were selected with an inoculation loop and used to inoculate 100 mL LB medium; these cells were cultured until the mid-log phase. The hydrogen yields of the selected mutants with larger yellow circles were verified by fermentative experiments to further screen the mutant with markedly improved hydrogen productiv-