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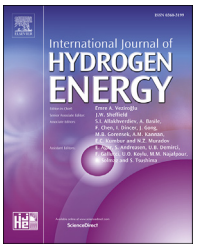

Identification of factors that accelerate hydrogen production by *Clostridium butyricum* RAK25832 using casamino acids as a nitrogen source

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ABSTRACT

The ability of *Clostridium butyricum* RAK25832 to use casamino acids as a nitrogen source was investigated. Strain RAK25832 showed the capacity to utilize different types of carbon sources. With glucose as a carbon source (10 g/L), the preferred final concentration of casamino acids was 26.67 g/L, with a cumulative hydrogen production, production rate, and yield of 2505 mL H_2/L, 160 mL/h, and 1.81 mol H_2/mol glucose, respectively. Eighteen metal elements were screened to identify the most important metals for biohydrogen production, and four elements were optimized. The optimal medium composition was $MgCl_2 \cdot 6H_2O$ (0.1 g/L), $K_2HPO_4 \cdot 3H_2O$ (6.67 g/L), $NaHCO_3$ (2.6 g/L), and $FeCl_2 \cdot 4H_2O$ (0.002 g/L). Vitamin supplementation of the medium showed no significant effect on hydrogen production. Under the optimized conditions, cumulative hydrogen production reached 3074 mL H_2/L. This is the first study to demonstrate the use of casamino acids as a nitrogen source by *C. butyricum*.

Abbreviations: HPB, Hydrogen-producing bacteria; PCR, Polymerase chain reaction; SMP, Soluble metabolite product; VFA, Volatile fatty acids; FID, Flame ionization detector; rRNA, Ribosomal ribonucleic acid; NCBI, National Center for Biotechnology Information; HAc, Acetic acid; HPr, Propionic acid; i-HBu, Isobutyric acid; n-HBu, N-butyric acid; EtOH, Ethanol; OD_{620}, Optical density at 620 nm; EDTA, Ethylenediaminetetraacetate; $V_{H,i}$, Cumulative hydrogen gas volume at the current (i) time; $V_{H,i-1}$, Cumulative hydrogen gas volume at the previous (i − 1) time; $V_{G,i}$, Total biogas volume at the current time interval; $V_{G,i-1}$, Total biogas volume at the previous time interval; $C_{H,i}$, Hydrogen gas fraction in the headspace at the current time interval; $C_{H,i-1}$, Hydrogen gas fraction in the headspace at the previous time interval; H, Cumulative hydrogen production (mL); λ, Lag time (hr); P, Hydrogen production potential (mL); R_m, Maximum hydrogen production rate (mL/hr); e, Constant, 2.718281828; Fd, Ferredoxin.

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Introduction

Since fossil fuels are the main source of greenhouse gases, there is increasing global interest for identifying feasible clean energy alternatives. One such alternative is hydrogen, as the final combustion results in the formation of water with almost no other emissions. Hydrogen also has a high calorific value of 242 kJ/mol. The conventional processes for hydrogen production such as gasification, water electrolysis, water gas shift reaction, and steam methane reforming are efficient, and contribute to most of the produced hydrogen worldwide [1,2]. Nevertheless, these processes require a large energy input from fossil fuels. Therefore, there has been recent attention paid to the possibility of biological hydrogen (biohydrogen) production from renewable resources such as waste biomass, as a promising process that combines energy recovery and waste minimization [3]. Biohydrogen production has already been investigated with various species of microorganisms [4,5] Fermentative hydrogen production has been shown to be a promising approach because it has the advantages of independence on the availability of light, higher hydrogen production rates, and a wide range of possible carbon sources such as low-cost wastes, organic compounds, and cellulosic substrates [6,7]. The isolation and identification of hydrogen-producing bacteria (HPB) with high yields and production rates are very important for promoting the commercial biohydrogen production process in a sustainable manner. Fermentative hydrogen production can be carried out through a wide range of microorganisms [3], including species of *Clostridium* [8], *Enterobacter* [9], and *Bacillus* [10].

Sewage sludge, food residue, manure, agricultural waste, and algae blooms are examples of waste biomass substrates that can be utilized in fermentative hydrogen production [11,12]. The principal organic components used in fermentation are carbohydrates and proteins. Carbohydrates, including starch and cellulose, are better organic substances that are utilized for fermentative biohydrogen production than proteins [13]. Carbohydrates can be readily hydrolyzed for reducing sugars such as xylose and glucose, which are also easily utilized by HPB for fermentative hydrogen production [14].

Although several microbial strains have been shown as feasible candidates for biohydrogen production, this always requires the use of a complex medium. Various nitrogen sources have been investigated in biotechnological studies to optimize the growth and metabolite production rates of microorganisms. Proteins are among the main components in waste biomass. For example, the protein content was reported to reach up to 72% of the dry weight in *Spirulina* biomass [15]. The most widely studied organic sources for this purpose are peptone, yeast extract, and casamino acids [16]. Yeast extract was found to be the favored nitrogen source for the growth of *Clostridium butyricum* W5 using glucose as the substrate [17]. The amino acids derived from proteins cannot easily be used by HPB to directly produce hydrogen [18,19]. The generally low yield of biohydrogen production has led researchers to focus on seeking high-yielding hydrogen-producing microorganisms, target genes for genetically modifying existing microorganisms, or fermentation process optimization [20,21]. Members of the class Clostridia have been confirmed as the main HPB in many hydrogen-production processes [22,23]. Urea and KNO_3 appeared to not be favored nitrogen sources by *Clostridia* spp [17]. However, there is little information available on the role of nitrogen source in the hydrogen production rate and formation of associated by-products in hydrogen fermentation. Therefore, detailed investigations for identifying suitable and new nitrogen sources, and to evaluate the optimal nitrogen concentrations in the fermentation broth for hydrogen fermentation by certain bacteria are clearly necessary.

Clostridium butyricum is well known as an anaerobic bacterium as well as hydrogen produce [24] and butyric acid producer [25]. Accordingly, several strains of *C. butyricum* have long been used for microbial industrial applications, especially for butyric acid production [24,26,27]. Glucose is metabolized to pyruvate via the Embden–Meyerhof–Parnas pathway and produces 2 mol of ATP and NADH, respectively. The butyrate-producing Clostridia produce butyrate concomitantly with acetate, H_2, CO_2, and trace lactate and other products [25] (Fig. 1). A theoretical investigation showed that the maximum yield of 4 mol H_2/mol glucose can be produced when acetic acid is the only volatile fatty acid (VFA), and a maximum yield of 2 mol H_2/mol glucose can be produced when butyric acid is the VFA product, using Eqs. (1) and (2) below [28]. However, a lower yield is usually obtained in practice as glucose is not only used for biohydrogen production but also to support microbial growth.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad \Delta G^\circ = -184\ \text{kJ} \tag{1}$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad \Delta G^\circ = -255\ \text{kJ} \tag{2}$$

Clostridium butyricum strain RAK25832 shows slight or no growth in media containing inorganic nitrogen as the sole nitrogen source but grows well in the presence of an organic nitrogen source such as yeast extract. However, the use of yeast extract as the nutrient source for bacterial culture has the main disadvantage of unpredictability, as it may contain several types of nutrients, vitamins, and amino acids such as glutamic acid, making it very difficult to define the media. Bacteria digest and break down proteins into simple compounds, i.e., amino acids and peptides. Casamino acids are mainly comprised of amino acids as they are derived from the acid hydrolysis of casein. Therefore, casamino acids might be a suitable, simpler alternative source of amino acids to yeast extract. Indeed, casamino acids are commonly used as a supplement in culture media or as an enrichment broth on its own, providing a suitable source of nutrients for enhancing the growth of certain bacteria [30–32].

In addition, vitamins and metal ions have been shown to be essential factors for biohydrogen production. Although a higher concentration of metals ions may inhibit the activity HPB, a trace level of metal ions is required for hydrogen production. Fe^{2+} is the most widely investigated metal ion for fermentative hydrogen production, which may be related to the fact that its presence is essential for hydrogenase activity

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