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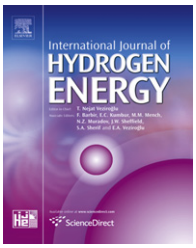

Hydrogen production by mixed bacteria through dark and photo fermentation

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ABSTRACT

Mixed bacteria were used to improve hydrogen yield from cassava starch in combination of dark and photo fermentation. In dark fermentation, mixed anaerobic bacteria (mainly *Clostridium* species) were used to produce hydrogen from cassava starch. Substrate concentration, fermentation temperature and pH were optimized as 10.4 g/l, 31 °C and 6.3 by response surface methodology (RSM). The maximum hydrogen yield and production rate in dark fermentation were 351 ml H_2/g starch (2.53 mol H_2/mol hexose) and 334.8 ml H_2/l/h, respectively. In photo fermentation, immobilized mixed photosynthetic bacteria (PSB, mainly *Rhodopseudomonas palustris* species) were used to produce hydrogen from soluble metabolite products (SMP, mainly acetate and butyrate) of dark fermentation. The maximum hydrogen yield in photo fermentation was 489 ml H_2/g starch (3.54 mol H_2/mol hexose). The total hydrogen yield was significantly increased from 402 to 840 ml H_2/g starch (from 2.91 to 6.07 mol H_2/mol hexose) by mixed bacteria and cell immobilization in combination of dark and photo fermentation.

1. Introduction

Biohydrogen production from biomass substrates is attracting more and more attention nowadays, since it can remove organic biomass wastes while simultaneously supplying clean hydrogen energy [1–4]. However, low yield and rate, and high cost are main obstacles in the development and application of biohydrogen technology. Cassava, which is grown extensively all over the world, is a kind of low-cost biomass and can grow well in barren and droughty areas. It mainly contains moisture (60–70%), starch (15–20%) and free sugars (4–6%). Because of no competition for lands with food crops and high productivity, cassava is considered as the ideal energy crop. According to Food and Agriculture Organization of the United Nations (FAO), the global and China's cassava production reached 228 and 4.4 million tons in 2007. At present, most cassava is used in chemical and textile industry, and few are used to produce fuel ethanol [5]. If cassava is used to produce hydrogen in biological fermentation, it will make great contribution to biomass energy.

Hydrogen production from starch was conducted in some researches, but hydrogen yields were only 100–200 ml H_2/g starch [3,4,6–12]. Conventional dark fermentation only can produce 2–4 mol hydrogen from 1 mol hexose with the production of acetate and butyrate [13], which can lead to environmental pollution. To solve this problem, many efforts were devoted to the utilization of organic acids solution produced from dark fermentation for further methane and hydrogen production in another process [14–16]. Our recent work showed that the combination of dark and photo fermentation could greatly improve hydrogen yield from 240 to 402 ml H_2/g starch [17]. Cassava starch was first hydrolyzed into reducing sugar by gelatinization or enzymatic hydrolysis, which was utilized by anaerobic bacteria in dark fermentation

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to produce hydrogen, carbon dioxide and volatile fatty acids (VFAs). Then the VFAs from dark fermentation were further fermented by pure photosynthetic bacteria *Rhodopseudomonas palustris* to produce hydrogen. However, the amylase and glucoamylase used for enzymatic hydrolysis may be too expensive for biohydrogen production. Condition optimization and bacterial strain improvement are required to obtain higher hydrogen yield at lower cost. Recently we successfully isolated mixed photosynthetic bacteria from activated sludge, which has higher hydrogen productivity and adaptability to environment. There are few reports about hydrogen production by mixed photosynthetic bacteria in the literatures [18].

In this study, response surface methodology (RSM) was conducted to explore the effects of substrate concentration, fermentation temperature and pH on hydrogen yield from gelatinized cassava starch in dark fermentation. Mixed photosynthetic bacteria and cell immobilization were used to improve hydrogen yield in the following photo fermentation.

2. Materials and methods

2.1. Bacterial strains and culture medium

The anaerobic activated sludge, which was collected in a methane plant, was boiled for 30 min to inactivate methanogens and acclimated for three times (each time for 72 h) to harvest mixed spore-forming hydrogen-producing bacteria. The mixed hydrogen-producing bacteria, primary bacterial strain of which was identified as *Clostridium* species (mainly *Clostridium butyricum*) after analysis of biochemical characteristics and 16S r DNA sequence (data not reported), were used as the inoculum in dark fermentation. The basal medium for hydrogen production contained (g/l): peptone, 4.0; L-cysteine, 0.5; NaCl, 3.0; $MgCl_2$, 0.1; $FeCl_2$, 0.1; K_2HPO_4, 2.5; vitamin liquid, 10 ml; trace element liquid, 10 ml. The vitamin liquid contained (g/l): glutamic acid, 0.01; ascorbic acid, 0.025; riboflavin, 0.025; citric acid monohydrate, 0.02; folic acid, 0.01; p-aminobenzoic acid, 0.01; creatine, 0.025. The trace element liquid contained (g/l): $MnCl_2$, 0.01; $ZnCl_2$, 0.05; H_3BO_3, 0.01; $CaCl_2$, 0.01; Na_2MoO_4, 0.01; $CoCl_2 \cdot 6H_2O$, 0.2; AlK $(SO_4)_2$, 0.01; $NiCl_2 \cdot 6H_2O$, 0.01 [13].

Mixed photosynthetic bacteria (PSB) isolated from active sludge, the primary bacterial strain of which was identified as *R. palustris* species after analysis of biochemical characteristics and 16S r DNA sequence in our previous study [19], were used as the inoculum in photo fermentation. The isolation method was as follows: 5 g active sludge was put in a conical flask mixed with 50 ml deionized water and then oscillated for 20 min at 120 r/min. 5 ml bacteria suspension stated above was added in a 100-ml bottle mixed with basal medium with acetate as carbon resource. The bottle was sealed with rubber stopper and placed in an illuminated incubator equipped with a microcomputer (Shanghai Boxun SPX-300I-G, China) at 30 ± 0.5 °C under a light intensity of 2000 lux. 7 days later, small quantities of red mixed bacteria were discovered on the bottle wall. The red bacteria were cultured again for 3 days in the same medium. The same procedure was operated for 3 times to enrich the mixed photosynthetic bacteria. The basal medium for photo fermentation contained (g/l): KH_2PO_4, 0.5; K_2HPO_4, 0.6; NaCl, 0.2; $MgSO_4$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.05; $NaHCO_3$, 2.0; sodium glutamate, 1.87; vitamin liquid, 1.0 ml; trace element liquid, 1.0 ml. The medium for hydrogen production was derived from the basal medium with additional carbon resource. The vitamin liquid contained (g/l): biotin, 0.1; nicotinic acid, 0.35; thiamine hydrochloride, 0.3; p-aminobenzoic acid, 0.2; pyridoxamine hydrochloride, 0.1; calcium pantothenate, 0.1; vitamin B_{12}, 0.05. The trace element liquid contained (g/l): EDTA–2Na, 2.0; $FeSO_4 \cdot 7H_2O$, 2.0; H_3BO_3, 0.1; $CoCl_2 \cdot 6H_2O$, 0.1; $ZnCl_2$, 0.1; Cu $(NO_3)_2 \cdot 5H_2O$, 0.05; $MnCl_2 \cdot 4H_2O$, 0.1; Na_2MoO_4, 0.75; $NiCl_2 \cdot 6H_2O$, 0.02; Na_2SeO_3, 0.001[17].

Table 1 – Central composite design (CCD) with three independent variables in dark fermentation.

Run	Starch (g/l)		Temperature (°C)		pH		Hydrogen yield (mol H_2/mol hexose)
	X_1	Code X_1	X_1	Code X_1	X_1	Code X_1	
1	1.6	−1.68	35	0	6.5	0	1.81
2	5	−1	25	−1	7.5	1	1.46
3	5	−1	25	−1	5.5	−1	1.62
4	5	−1	45	1	7.5	1	0.86
5	5	−1	45	1	5.5	−1	0.97
6	10	0	18.2	−1.68	6.5	0	1.60
7	10	0	35	0	4.8	−1.68	2.00
8	10	0	35	0	6.5	0	2.06
9	10	0	35	0	6.5	0	2.04
10	10	0	35	0	6.5	0	2.08
11	10	0	35	0	6.5	0	2.06
12	10	0	35	0	8.2	1.68	1.89
13	10	0	35	0	6.5	0	2.04
14	10	0	35	0	6.5	0	2.06
15	10	0	51.8	1.68	6.5	0	0.39
16	15	1	25	−1	7.5	1	1.60
17	15	1	25	−1	5.5	−1	1.71
18	15	1	45	1	5.5	−1	0.91
19	15	1	45	1	7.5	1	0.88
20	18.4	1.68	35	0	6.5	0	1.80