

Fermentative Hydrogen Production by *Enterobacter* sp. KTSMBNL-01 Isolated from Municipal Sewage Sludge: Optimization Studies

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ABSTRACT

A fermentative hydrogen-producing strain KTSMBNL-01 was isolated from the sewage sludge and identified as *Enterobacter* sp. on the basis of the biochemical characteristics and 16S rRNA gene analysis. The optimization of batch conditions for the production of hydrogen by *Enterobacter* sp. was investigated. Various parameters namely initial pH, temperature and substrate concentrations were varied for hydrogen production. Maximum hydrogen yield (0.86 mol/mol G) and cell growth (1.59 g/L) was obtained at pH 7.0 and at 37 °C. The strain was capable of producing hydrogen in the presence glucose, fructose, xylose, maltose, lactose, cellulose and starch, where the best results revealed with glucose. This study showed that *Enterobacter* sp. can efficiently produce H₂ and it is a one more model microorganism for biohydrogen production.

General Terms

Hydrogen production, Optimization, Carbon source.

Keywords

Biohydrogen, *Enterobacter* sp., optimization, glucose.

1. INTRODUCTION

In modern practice, researchers have started looking for alternatives of fossil fuels not only to reduce the risk of overdependence on imported oil, gas supplies but also to solve our energy crisis. Hydrogen is an alternative candidate for the replacement of conventional fossil fuels [1,2]. The hydrogen technology is receiving extensive consideration owing to the exclusive properties such as clean, renewable and higher energy yield than other biofuels and also has zero CO₂ emissions [3]. Only 2% of hydrogen is currently being exploited and, the contribution of hydrogen to total energy market will be 8-10% in 2025 [4,5]. Hydrogen can be produced by two different methods such as physicochemical and biological methods. The physicochemical methods include electrolysis of water, steam reforming of hydrocarbons and autothermal process and these methods are energy intensive requiring high temperature (>850 °C) and electricity [6,7]. Consequently, there has been increasing interest in recent years on the biological production of

hydrogen using microorganisms and it is acknowledged to be environmentally benign. Biological methods mainly include photosynthetic and fermentative hydrogen productions. Among them, fermentative hydrogen production has higher efficiency, stability, high feasibility, easy control requirements and low operating costs [8]. With different metabolic pathways of hydrogen production, anaerobic fermentation is the highest in hydrogen production rates. Anaerobic fermentation of hydrogen is performed by many fermentative microorganisms, such as facultative anaerobes of the genus *Enterobacter* [9,10], anaerobes of the genus *Clostridium* [11,12], *Methanogens* [13] and *Citrobacter* species [14]. Researchers have reported that hydrogen gas production is greatly influenced by several physical and chemical factors such as pH, temperature, substrate concentration and nitrogen sources [15,16]. Previous studies have shown that culture conditions or operating parameters can also significantly affect cell growth and hydrogen production.

In this study, a hydrogen producing strain from municipal sewage sludge was isolated and identified for efficient hydrogen production at different pH, temperature and substrate concentrations. Single strain cultivation studies were carried out to investigate the efficiency of hydrogen production. This preliminary study assists an economic and environment friendly hydrogen gas production at a larger scale.

2. MATERIALS AND METHODS

2.1 Strain Isolation and Phenotypic Identification

Sewage sludge from a municipal waste water treatment plant (Tiruchirappalli, India) was used to isolate the hydrogen producing bacteria. The sewage sludge was serially diluted with sterile distilled water and spread on the sterile agar plates with synthetic medium containing 15 g/L tryptone; 0.5 g/L L-cystine hydrochloride; 5.5 g/L glucose; 5 g/L yeast extract; 2.5 g/L sodium chloride; 0.5 g/L sodium thioglycollate; 0.01 g/L resazurin and 0.75 g/L agar. The plates were incubated at 37 °C for 24 h in an anaerobic jar flushed with nitrogen gas to remove oxygen. After incubation, individual bacterial

colonies were picked and further purified by sub-culturing on same medium for more than three times to ensure the purity of the strains and used for subsequent studies.

2.2 Genotypic Identification

The chromosomal DNA was isolated by the Sambrook method [17]. The 16S rRNA gene was amplified by PCR with the primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTACGACTT-3'). Amplification was carried out in a thermal cycler (Bio rad T-100) with an initial denaturation at 94 °C for 2 min, followed by 28 cycles of denaturation at 95°C for 2 min, annealing at 52.3 °C for primer pair of 8F and 1492R for 1 min, and extension at 72 °C for 1 min. The thermal cycles were terminated by a final extension for 5 min at 72°C. The PCR amplified products were verified by agarose gel electrophoresis and used for the determination of 16S rRNA gene sequence. The PCR products were purified using Hiyield™ Gel/PCR DNA extraction kit according to the manufactures instructions. The resulting sequence was entered into the BLAST algorithm of NCBI database to obtain closely related phylogenetic sequences. And the phylogenetic relation was analyzed using Neighbor Joining method in Mega 4.0 software.

2.3 Optimization of Culture Conditions

All experiments were carried out in 120 ml serum bottle with a working volume of 70 ml of modified MYG medium containing glucose as carbon source. The above mentioned medium was used for the fermentation and it consisted of 10 g/L- D-glucose, 5 g/L- yeast extract and 10 g/L- malt extract. When the cells entered mid-stationary growth phase, the inoculants were injected into serum bottles. After inoculation, the serum bottles were flushed with pure nitrogen gas (99.999%) for 10 min and sealed with rubber plugs for anaerobic conditions. The bottles were kept under shaking condition for 120 rpm at 36 ± 1 °C for 24 h. After incubation the volume of gas produced was measured by a water displacement method. For optimization of hydrogen production on batch fermentation, effects of different carbon sources, pH and temperature were determined. Various carbon sources used are glucose, fructose, xylose, maltose, lactose, cellulose and starch was fixed at 5g/L. The initial culture pH value was controlled from pH 4.0 to 9.0 using 1 mol/L NaOH or HCl solution, the culture temperature was varied from 25 to 45 °C and the initial glucose concentrations were ranged from 5 to 40 g/L with 5 g/L increments.

2.4 Analytical Methods

The gas products were analyzed with gas chromatography (GC, Agilent 4890D) equipped with a thermal conductivity detector (TCD) and a porapak Q stainless column. Nitrogen gas was used as the carrier gas with a flow rate of 20 mL/min. The operational temperatures for the injection port, detector and column oven were maintained at 100 °C, 80 °C and 150 °C, respectively. The bacterial cell concentration was determined by UV-Visible spectrophotometer (Jenway 6320D) by measuring the absorbance at 600 nm [18]. The cell dry weight was determined by centrifuging culture broths (10,000 rpm for 5min), and the pellets was washed thrice with distilled water and dried at 100 °C. One OD600 unit corresponded to 0.5731 g/L dry cell weight. The concentration of glucose was analyzed using 3,5-dinitrosalicylic acid method (DNS). Metabolite products were analyzed using high performance liquid chromatography (Agilent 1100, USA) with the column of Aminex HPX-87H. The temperature of the

column was set at 65 °C, the mobile phase of H₂SO₄ (5 mmol/L) was used with a flow rate of 0.8 ml/min.

3. RESULTS AND DISCUSSION

The bacterial strain was successfully isolated from sewage sludge and screened for the production of hydrogen in MYG medium. Microscopic examination showed that the isolated bacterium was facultative anaerobic, gram negative, rod shape with no endospore formation. The 16S rRNA gene sequencing of 1015 bp was amplified from the genomic DNA of strain. The phylogenetic tree was constructed for the hydrogen producing strain (Figure. 1) and it exhibited 99% sequence similarity with *Enterobacter* sp. The 16S rRNA sequence was deposited in the NCBI database with the accession number JF263453.

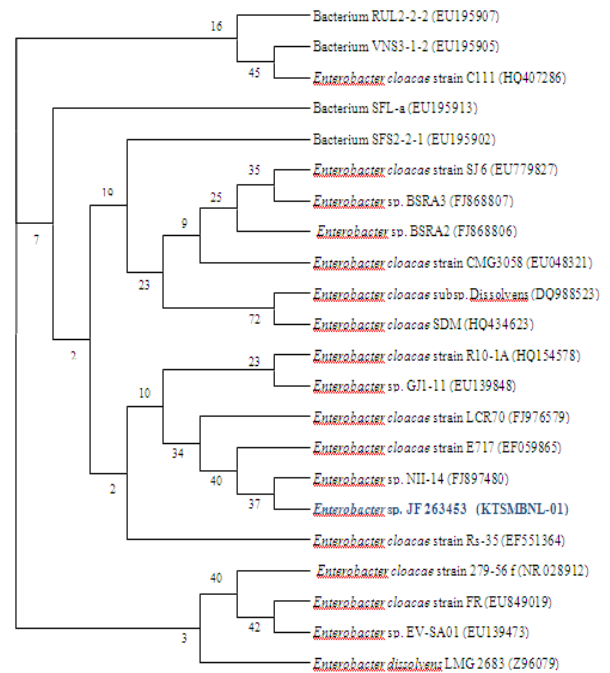


Fig 1: Neighbor-joining tree showing phylogenetic relationship between strain KTSMBNL-01 and other known strains based 16S rRNA sequences.

The pH of the solution plays a vital role in controlling various factors during anaerobic biohydrogen production. The effect of initial pH (4.0 to 9.0) of the medium on the cell growth and the hydrogen production yield by *Enterobacter* sp. is illustrated in Table 1. Maximum yield of hydrogen production was attained at pH 7.0 and further increase in pH drastically lowered the hydrogen content because the alkaline pH destroyed the cell's capability to maintain internal pH [18,19]. There was no hydrogen production and cell growth at pH 4.0. The results of this study showed that higher cell growth was at pH 9.0 and maximum hydrogen production at pH 7.0. At initial pH 9.0 the maximum cell growth of 1.59 g/L was observed but hydrogen yield was decreased to 0.17 mol/mol G.

Table 1. Effect of initial pH on hydrogen production by *Enterobacter* sp. KTSMBNL-01

Initial pH	Final pH	Cell growth (g/L)	H ₂ yield (mol/mol G)
4.0	3.8	0.08	0
5.0	4.1	0.56	0.28
6.0	5.0	1.13	0.53
6.5	5.7	1.21	0.73
7.0	5.8	1.26	0.86
7.5	6.1	1.32	0.77
8.0	6.1	1.41	0.41
9.0	6.3	1.59	0.17

Temperature is a very important parameter in bio hydrogen production and also influences the cell growth. The effect of temperature on hydrogen production yield was investigated at different temperatures ranging from 25 °C to 45 °C and maximum was 37 °C (Figure 2(a)). Cell growth and hydrogen production was decreased when temperature was increased from 38 °C to 45 °C and there was no hydrogen production at 45 °C. This may be due to denaturation of the key enzymes or inactivation in the metabolic pathway at high temperature [20]. Maximum hydrogen production was at 37 °C at an initial pH of 7.0.

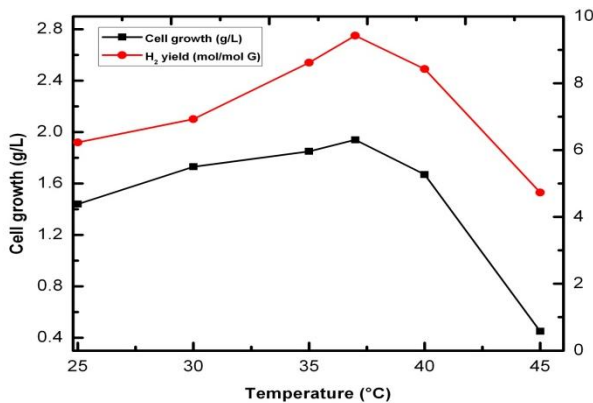


Fig 2(a): Effect of temperature on hydrogen production by *Enterobacter* sp. KTSMBNL-01

Figure 2(b) shows the variations in hydrogen fermentation with different carbon sources such as glucose, fructose, xylose, maltose, lactose, cellulose and starch by *Enterobacter* sp. KTSMBNL-01. The initial concentration of each carbon source was fixed at 5 g/L with initial pH of 7.0 at 37 °C. The results clearly indicate that glucose exhibited the best hydrogen production when compared to other carbon sources. Prakasham and al 2009 and Temudo and al 2009 [21,22] have reported that the variation in hydrogen production by different carbon sources is due to different substrate transport level or metabolism. From the result (Figure 2(c)) it was observed that cell growth and hydrogen production rate were increased when the glucose concentration was increased from 5.0 to 20 g/L. When the initial glucose concentration was 20 g/L the maximum hydrogen production rate and yield was 410 ml H₂/L/h and 0.65 mol/mol G, respectively. With further increase in glucose concentration the cell growth and hydrogen production rate decreased drastically.

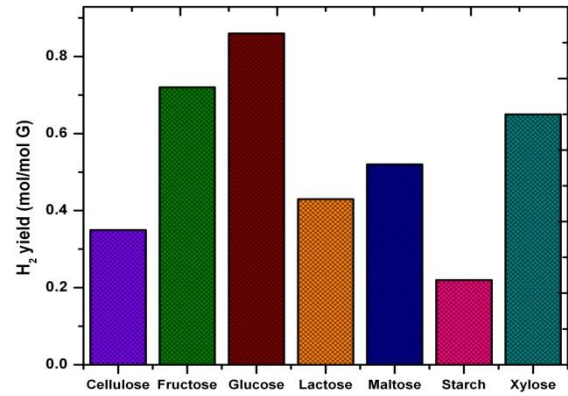


Fig 2(b): Effect of different carbon sources on hydrogen production by *Enterobacter* sp. KTSMBNL-01

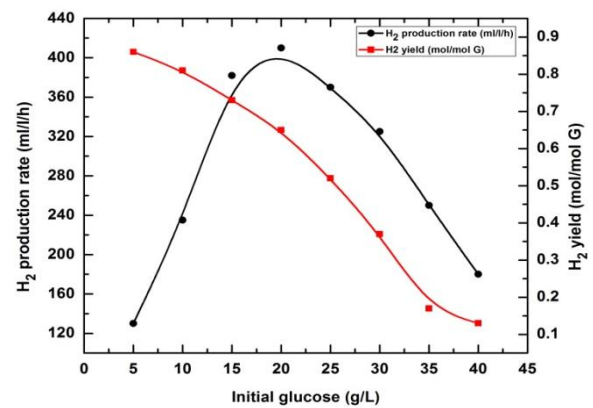


Fig 2(c): Effect of initial glucose concentration on hydrogen production by *Enterobacter* sp. KTSMBNL-01

4. CONCLUSIONS

A hydrogen producing strain was isolated from municipal sewage sludge and identified as *Enterobacter* sp. KTSMBNL-01. The result showed a maximum hydrogen production rate of 410 ml H₂/L/h and H₂ yield of 0.86 mol/mol G at an initial glucose concentration of 20 g/L and 5 g/L, at 37 °C and an initial pH 7.0 respectively. Ethanol was generated during hydrogen production as metabolites. Finally it can be concluded that *Enterobacter* sp. could be an ideal alternative and effective hydrogen producer from municipal sewage sludge.

5. ACKNOWLEDGMENTS

The authors would like to acknowledge the financial support of UGC-University Grants Commission, New Delhi, India for providing this project. And the authors also gratefully and whole heartedly thank Dr. Hima Bindu, Associate professor, Jawaharlal Nehru Technology University, Hyderabad for providing lab facilities.

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