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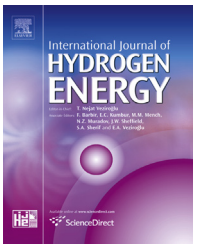

# Biohydrogen production from crude glycerol by immobilized *Klebsiella* sp. TR17 in a UASB reactor and bacterial quantification under non-sterile conditions

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ABSTRACT

Biohydrogen production from crude glycerol by immobilized *Klebsiella* sp. TR17 was investigated in an up-flow anaerobic sludge blanket (UASB) reactor. The reactor was operated under non-sterile conditions at 40◦C and initial pH 8.0 at different hydraulic retention times (HRTs) (2–12 h) and glycerol concentrations (10–30 g/L). Decreasing the HRT led to an increase in hydrogen production rate (HPR) and hydrogen yield (HY). The highest HPR of 242.15 mmol $H_2$/L/d and HY of 44.27 mmol $H_2$/g glycerol consumed were achieved at 4 h HRT and glycerol concentrations of 30 and 10 g/L, respectively. The main soluble metabolite was 1,3-propanediol, which implies that *Klebsiella* sp. was dominant among other microorganisms. Fluorescence *in situ* hybridization (FISH) revealed that the microbial community was dominated by *Klebsiella* sp. with 56.96, 59.45, and 63.47% of total DAPI binding cells, at glycerol concentrations of 10, 20, and 30 g/L, respectively.



## Introduction

Hydrogen has potential as a fuel for the future because it is clean and has a high energy yield compared with hydrocarbon fuels [1]. Among the biological methods of hydrogen production, dark fermentation has various advantages such as its ability to use a wide range of substrates and no requirement for a light source. Thus, this method is relatively energy saving and environmentally friendly [2,3].

Crude glycerol is a by-product obtained from biodiesel production. An increase in biodiesel production would inevitably result in an increase in crude glycerol production [4]. Crude glycerol has high levels of impurities and its disposal is costly and energy intensive [5]. In order to make biodiesel production more sustainable, the conversion of crude glycerol

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to a variety of value-added products, such as hydrogen [6], 1,3-propanediol [7,8], 2,3-butanediol [9], and ethanol [10] has been studied. Conversion of crude glycerol to hydrogen is an attractive approach.

Investigations on hydrogen production from dark fermentation have been focused on using pure cultures [11], in which the genus *Clostridium* has been most studied for various waste materials such as food wastes [12], palm oil mill effluent [13], and molasses [14]. However, *Clostridium* is an obligate anaerobe, requiring a strictly anaerobic condition which makes it difficult to use for industrial production [15]. Thus, using facultative bacteria for the conversion of crude glycerol to hydrogen by dark fermentation is more appropriate.

*Klebsiella* sp. is able to convert crude glycerol to hydrogen at a high rate and yield [16,17]. It is also easy to grow and will produce various valuable by-products, such as 1,3-propanediol, 2,3-butanediol [18], and ethanol [19]. To make it more attractive for industrial applications, hydrogen should be produced under non-sterile conditions to minimize production costs. Under these conditions, the microorganisms present in the reactor during operation should be quantified to determine the dominant strains.

Up-flow anaerobic sludge blanket (UASB) reactor is an effective process in wastewater treatment systems as it exhibits high organic removal efficiency [20–22]. In addition, it has also been employed for hydrogen production from various substrates such as starch-wastewater [23], desugared molasses [24], coffee drink manufacturing wastewater [1], and cheese whey [25]. However, it has not been reported for hydrogen production from crude glycerol.

The objective of this work is to investigate the hydrogen production from crude glycerol in a UASB reactor using *Klebsiella* sp. TR17 immobilized on heat-pretreated methanogenic granules under non-sterile conditions. Subsequently, the microbial communities in the UASB reactor were determined by fluorescence *in situ* hybridization (FISH) in order to evaluate the role of immobilized *Klebsiella* sp. TR17 in the fermentation system.

## Materials and methods

### *Microorganism and culture medium*

*Klebsiella* sp. TR17 (accession number in Genbank AB647144) was isolated from crude glycerol-contaminated soil. The optimum conditions for hydrogen production for this strain were pH 8.0 and temperature at 40◦C [19]. The culture medium contained: 11.14 g/L glycerol, 3.4 g/L $K_2HPO_4$, 2.47 g/L $KH_2PO_4$, 6.03 g/L $NH_4Cl$, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 2.0 g/L yeast extract, 2.0 g/L $CaCO_3$, 5.0 mg/L $FeSO_4 \cdot 7H_2O$, 2.0 mg/L $CaCl_2$, and 2.0 mL/L trace element solution [26]. The crude glycerol with 50% purity was obtained from the Biodiesel Pilot Plant at Prince of Songkla University.

### *Experimental set-up and operation of UASB reactors*

The 1.3 L UASB reactor (6 cm diameter × 47 cm height) was made from glass with 1.0 L working volume and operated at 40◦C with water internal jacket recirculation. Fresh medium was fed from the bottom by a peristaltic pump while the evolved gas and effluent were discharged from the top of the reactor. The methanogenic granules were obtained from a UASB reactor of a seafood wastewater treatment system (Chotiwat Manufacturing Co., Ltd., Songkhla Province, Thailand). The methanogenic granules were autoclaved at 121 °C for 30 min to kill methanogenic activity before being used as carriers for immobilization of *Klebsiella* sp. TR17. For the set-up, 440 mL of the heat-pretreated methanogenic granules were transferred to each UASB reactors with 560 mL of the inoculum ($OD_{660}$ = 0.5) [27]. After inoculation, the reactors were operated in batch mode for 24 h and fed with 10 g/L pure glycerol, then the culture medium was re-circulated for 7 days at 12 h HRT (flow rate of 1.38 mL/min) in order to enhance bacterial immobilization on the granules before changing to crude glycerol. After reaching steady state, the reactors were operated at the HRTs of 12, 10, 8, 6, 4, and 2 h, respectively. The steady state of each HRT was established when the value of the hydrogen production rate was less than 5% difference, and the final pH of the effluent was constant [28]. The culture media containing glycerol concentrations of 10, 20, and 30 g/L were fed to each UASB reactor. The reactors were monitored by examining the effluent every three days for volatile suspended solids (VSS) concentration, and measuring twice a day for soluble metabolic products and glycerol residuals. Gas production and pH were measured daily.

### *Fluorescence in situ hybridization (FISH)*

The FISH technique was selected for detection and quantification of *Klebsiella* sp. TR17 immobilized on heat-pretreated methanogenic granules. The samples were taken from each UASB reactor with different glycerol concentrations (10, 20, 30 g/L) at the end of the operation experiments. Table 1 shows the list of the specific oligonucleotide probes and hybridization conditions used in this study. Probes labeled with the sulfoindocyanine dyes Cy3, EUB338 [29] and Enterbact D [30], were used for the hybridization to target all bacteria and *Klebsiella* sp., respectively. Fixation of samples started by adding 375 mL of sludge samples to 1125 mL of 4% (v/v) paraformaldehyde (pH 7.2). Then, the samples were mixed

**Table 1 – Oligonucleotide probes used for FISH technique.**

| Probe | Specificity | Sequence (5′ to 3′) | FA (%)[a] | NaCl (M)[b] | Ref. |
|---|---|---|---|---|---|
| EUB338 | All bacteria | GCTGCCTCCCGTAGGAGT | 35 | 0.08 | [29] |
| Enterbact D | *Klebsiella* sp. | TGCTCTCGCGAGGTCGCTTCTCTT | 0 | 0.90 | [30] |

[a] Formamide concentration in the hybridization buffer.
[b] Sodium chloride concentration in the washing buffer.

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