Simultaneous production of 2,3-butanediol, ethanol and hydrogen with a Klebsiella sp. strain isolated from sewage sludge

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ABSTRACT

A Klebsiella sp. HE1 strain isolated from hydrogen-producing sewage sludge was examined for its ability to produce H2 and other valuable soluble metabolites (e.g., ethanol and 2,3-butanediol) from sucrose-based medium. The effect of pH and carbon substrate concentration on the production of soluble and gaseous products was investigated. The major soluble metabolite produced from Klebsiella sp. HE1 was 2,3-butanediol, accounting for over 42–58% of soluble microbial products (SMP) and its production efficiency enhanced after increasing the initial culture pH to 7.3 (without pH control). The HE1 strain also produced ethanol (contributing to 29–42% of total SMP) and a small amount of lactic acid and acetic acid. The gaseous products consisted of H2 (25–36%) and CO2 (64–75%). The optimal cumulative hydrogen production (2.7 l) and hydrogen yield (0.92 mol H2 mol sucrose -1) were obtained at an initial sucrose concentration of 30 g COD l−1 (i.e., 26.7 g l−1), which also led to the highest production rate for H2 (3.26 mmol h−1 l−1), ethanol (6.75 mmol h−1 l−1) and 2,3-butanediol (7.14 mmol h−1 l−1). The highest yield for H2, ethanol and 2,3-butanediol was 0.92, 0.81 and 0.59 mol mol-sucrose−1, respectively. As for the overall energy production performance, the highest energy generation rate was 27.7 kJ h−1 l−1 and the best energy yield was 2.45 kJ mol sucrose−1, which was obtained at a sucrose concentration of 30 and 20 g COD l−1, respectively.

1. Introduction

Microbial production of 2,3-butanediol (2,3-BDO) by using Klebsiella pneumoniae was first investigated in 1906 (Harden and Walpole, 1906). Recently, there has been keen interest in the microbial production of 2,3-BDO because this product has a large number of industrial applications, as it acts as a feedstock for a variety of chemicals and liquid fuels. For instance, 2,3-butanediol can be used to produce 1,3-butadiene, which is used as an antifreeze agent (Magee and Kosaric, 1987). Other commercial applications of 2,3-BDO include the production of cosmetic products, explosives, plasticizers, pharmaceuticals, as well as a flavoring agent in food products (Afschar et al., 1993). In addition, 2,3-BDO is also a potentially valuable fuel additive with a heating value of 27.2 kJ g−1, which compares favorably with other liquid fuels, such as methanol (22.1 kJ g−1) and ethanol (29.1 kJ g−1) (Flickinger, 1980; Yu and Saddler, 1983). Due to high octane number, butane-

diol is considered as high grade aviation fuel; equimolar mixture of ethanol and diol can provide a significant heating value (27.7 kJ g−1) (Yu and Saddler, 1982).

A variety of obligate anaerobes (e.g., Clostridia) and facultative anaerobes (e.g., Klebsiella), under aerobic and anaerobic fermentation conditions, are able to convert carbohydrates (e.g., sucrose) to a number of soluble and gaseous products, such as 2,3-butanediol, ethanol, formic acid, acetic acid, acetone, H2 and CO2 (Syu, 2001; Gupta et al., 2005). In particular, Klebsiella species are capable of utilizing pentose as the carbon substrate, producing 2,3-butanediol, acetone, isopropanol, butanol, and H2 as soluble or gaseous metabolites (Rosenberg, 1980). K. pneumoniae is often used for the production of 2,3-butanediol from cellulolytic materials because of its broad substrate spectrum (e.g., major sugars and uronic acid of cellulosic hydrolysate) and culture adaptability (Jansen et al., 1984). Thus, bioconversion using Klebsiella sp. as biocatalysts is considered as an alternative approach in the conversion of biomass substrates to liquid fuels and chemical feedstocks (Jansen et al., 1984; Cao et al., 1997). Glycerol also known as one of the most effective carbon substrate for 2,3-BDO production by Klebsiella sp. (Deckwer, 1995; Biebl et al., 1998), Champluvier et al. (1989)
showed that *K. oxytoca* can convert lactose into 2,3-BDO by using permeabilized-cell system.

In addition to formation of 2,3-BDO, gaseous (H₂) and liquid (ethanol) biofuels are also produced by *Klebsiella* sp. under suitable conditions (Menzel et al., 1996; Zeng et al., 1996). Ethanol considered as the most feasible alternative for gasoline to fuel the automobiles (Bayraktar, 2007; MacLean Heather and Lave Lester, 2003). Hydrogen is a clean recyclable and high-energy-content renewable energy carrier; it does not contribute to the greenhouse effect and can easily be used in fuel cells for efficient generation of electricity (Das and Veziroglu, 2001; Demirbas, 2007). Therefore, fermentation with *Klebsiella* sp. has the advantage of producing a variety of valuable industrial chemicals (e.g., 2,3-butanediol) and biofuels (e.g., ethanol and H₂). In the present study, an indigenous isolate *Klebsiella* sp. was used to produce 2,3-BDO, ethanol and hydrogen from sucrose substrate. The effects of pH control strategy and substrate concentration on formation of the desired products were investigated. This study is the preliminary step towards the development of effective fermentation systems for simultaneous production of commercially valuable products (e.g., 2,3-butanediol, ethanol and hydrogen).

2. Methods

2.1. Isolation and identification of the bacterial strain used in this study

The isolation of facultative bacteria was carried out by using EMB agar (BD Difco, Franklin Lakes, NJ, USA) and cultured at 37 °C under aerobic condition. One pure facultative culture expressing high H₂-producing activity was selected and designated as strain HE1. Morphological examination was performed with a light microscope (Zeiss Axioskop) identified as facultative strain HE1 as Gram-negative, rod shaped bacteria. Biochemical identification presented in the API 20E microtests (bioMérieux) was determined according to the recommendation of the manufacturers. The similarity of 16S rRNA gene sequence comparison indicates that facultative strain HE1 belonged to genus *Klebsiella*. The 16S rRNA gene sequences of the *Klebsiella* sp. HE1 isolate has been deposited in the NCBI nucleotide sequence databases under the accession number (AY540111).

2.2. Cultivation of Klebsiella sp. HE1

The *Klebsiella* sp. HE1 was stored and maintained at −70 °C on anaerobically incubated EMB agar plates and the temperature for batch growth was kept at 37 °C for 12 h. The composition of EMB agar medium was (g l⁻¹): bacto peptone 10; bacto lactose 5; bacto sucrose 5; dipotassium phosphate 2; bacto agar 13.5; Eosin Y 0.4; methylene blue 0.0065. The pre-culture was transferred to a 250 ml shake flask containing 100 ml substrate media to undergo batch growth, which was kept at 37 °C for 24 h. The substrate medium consisted of (g l⁻¹): sucrose, 17.8; NH₄HCO₃, 5.24; NaHCO₃, 6.72; K₂HPO₄, 0.125; MgCl₂ - 6H₂O, 0.1; MnSO₄ - 6H₂O, 0.015; FeSO₄ - 7H₂O, 0.025. The initial pH of the medium was control at 6.0–7.0 and the temperature for batch growth was kept at 37 °C. The cell concentration, pH, soluble product composition, and biogas production were monitored during the course of the experiments.

2.3. Batch H₂ fermentation

The pre-culture of *Klebsiella* sp. (30 ml) was inoculated into a 2.5 l fermenter (Model MDL6C, Mazubishi Inc.; Tokyo, Japan), containing 1.5 l substrate medium. To investigate the effect of pH, the batch culture was conducted under an initial pH of 7.3, 6.4, 5.5 (not controlled) and a controlled pH of 7.5, 6.25, 5.5 by automatic addition of 3 N NaOH. The initial sucrose concentration used was 10 g COD l⁻¹ (8.9 g l⁻¹), 20 g COD l⁻¹ (17.8 g l⁻¹) and 30 g COD l⁻¹ (26.7 g l⁻¹), while three Fe²⁺ added concentrations (i.e., 0.0125, 0.025 and 0.0375 g l⁻¹) were examined. In all cases, the culture temperature was 37 °C under a fixed agitation rate of 100 rpm. The composition of gaseous (mainly H₂ and CO₂) and soluble products (mainly 2,3-butanediol, ethanol, lactic acid, and acetic acid) was monitored with respect to time. The gas produced from the culture was measured with a gas meter (Type TG1; Ritter Inc., Germany). The gas volumes presented in this work were calibrated to 25 °C and 760 mmHg.

2.4. Analytical methods

The gas products were analyzed by gas chromatography (GC) using a thermal conductivity detector (TCD). The 2,3-butanediol, ethanol, lactic acid and acetic acid were also detected by GC using a flame ionization detector (FID). The conditions and columns used for GC analysis were identical to those reported previously (Lee et al., 2003; Lee et al., 2004a; Lee et al., 2004b). Standard methods (APHA 1995) were used to determine biomass concentration (in terms of volatile suspended solid; VSS). The carbohydrate concentration in the effluent was also measured using Standard methods (via phenol-sulfuric acid method) (APHA 1995).

3. Results and discussion

3.1. Typical soluble metabolites production by Klebsiella sp. HE1 from sucrose

Using sucrose as the sole carbon source, *Klebsiella* sp. HE1 could convert sucrose into a variety of soluble microbial products (Table 1) along with the biogas products (mainly H₂ and CO₂) (Table 2). In soluble microbial products (SMP), regardless of the initial sucrose concentration used, the major products were 2,3-butanediol and ethanol, which accounted for nearly 87% of the total production (Table 2). In soluble microbial products (SMP), regardless of the initial sucrose concentration used, the major products were 2,3-butanediol and ethanol, which accounted for nearly 87% of the total SMP (Table 1). Meanwhile, a small quantity of lactic acid and acetic acid was also observed, contributing to nearly 10–11% and 3–5% of total SMP, respectively. Comparison between hydrogen production (Table 2) and soluble metabolites production (Table 1) shows that lower hydrogen production was accompanied

| Table 1: Composition of soluble metabolites and H₂ content with batch fermentation under different sucrose concentration (Temperature = 37 °C, initial pH 7.3 (pH not controlled)) |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Sucrose concentration (g COD l⁻¹) | H₂ content (%) | TAIOH (mg COD l⁻¹) | SMP (mg COD l⁻¹) | HAC/SMP (%) | HLA/SMP (%) | 2,3-BuOH/SMP (%) | ETOH/SMP (%) | TAIOH/SMP (%) |
| 10 | 25 | 4774 ± 342 | 5030 ± 387 | 5.0 ± 0.5 | 11 ± 0.5 | 42 ± 4 | 42 ± 4 | 84 ± 8 |
| 20 | 30 | 9349 ± 673 | 9860 ± 745 | 4.0 ± 0.4 | 11 ± 0.9 | 56 ± 5 | 29 ± 3 | 85 ± 8 |
| 30 | 36 | 10,718 ± 671 | 11,208 ± 729 | 3.0 ± 0.2 | 10 ± 0.4 | 58 ± 4 | 29 ± 3 | 87 ± 7 |

HAC: acetic acid; HLA: lactic acid; 2,3-BuOH: 2,3-butanediol; ETOH: ethanol. TAIOH: total alcohol (TAIOH = 2,3-BuOH + ETOH). SMP: soluble microbial products (SMP = HAC + HLA + 2,3-BuOH + ETOH).
by a higher total alcohol content (2,3-butanediol plus ethanol) and a higher total alcohol to SMP ratio (i.e., TAIOH/SMP).

In this study, the highest 2,3-butanediol (2,3-BDO) production of 6281 mg COD l$^{-1}$ occurred when the culture medium contained an initial sucrose concentration of 30 g COD l$^{-1}$. The 2,3-BDO production in this work appears to be more than that observed in regular dark H$_2$ fermentation work using similar carbon source (Wu and Chang, 2007; Wang et al., 2006), indicating that the growth of Klebsiella sp. HE1 on sucrose was indeed an alcohol-rich dark fermentation.

### 3.2. Effect of pH and sucrose concentration on H$_2$ production by Klebsiella sp. HE1

Klebsiella sp. is known as a hydrogen producer (Wu and Chang, 2007) but there is little information regarding biohydrogen production performance of a pure strain of Klebsiella sp. in a quantitative fashion. Moreover, the transient relationship between hydrogen production and cell growth of Klebsiella sp. has not yet been revealed in detail. We demonstrate in Fig. 1 the profiles of cell growth,
cumulative hydrogen production, and hydrogen production rate during batch fermentation of Klebsiella sp. In a typical growth curve of Klebsiella sp., as indicated in Fig. 1, we observed that the evolution of H\textsubscript{2} appeared to start after the middle of exponential growth stage (ca. 6 h) with the maximal hydrogen production rate (62 ml h\textsuperscript{-1} l\textsuperscript{-1}) occurring at 14 h, where cell growth had entered early stationary phase. This seems to imply that generation of hydrogen gas was not a preferable event during assimilation of carbon substrate for the gain of biomass. This may be due to a predominant metabolic electron flow towards biosynthesis, decreasing the availability of free electrons towards hydrogenase for H\textsubscript{2} production. In contrast, when cell growth started to slow down, H\textsubscript{2} production became more efficient. The hydrogen production increased sharply since the onset of hydrogen evolution and reached a maximal value of 650 ml after cultivation for 15 h (Fig. 1).

Table 2 also shows the dependence of pH level and the way of pH control on H\textsubscript{2} production from sucrose by Klebsiella sp. HE1. At an initial pH of 7.3 (pH not controlled) and initial sucrose concentration of 20 g COD l\textsuperscript{-1}, the cumulative hydrogen production (1.31) and hydrogen production rate (54 ml h\textsuperscript{-1} l\textsuperscript{-1}) were both maximum. When the pH was controlled at 7.5, the cell growth was fast (specific growth rate was 0.53 h\textsuperscript{-1}), but hydrogen production was not observed. Under pH control, the hydrogen production rate decreased from its maximal value (67.2 ml h\textsuperscript{-1} g\textsuperscript{-1} cell\textsuperscript{-1}) to zero when the controlled pH was raised from 5.5 to 7.5. However, when pH was not controlled, an elevation of initial pH from 5.5 to 7.3 allowed the culture to maintain a higher hydrogen production rate (74–93 ml h\textsuperscript{-1} g\textsuperscript{-1} cell\textsuperscript{-1}). The results indicate that the favorable pH for cell growth and for H\textsubscript{2} and/or soluble metabolites production was different. Hence, controlling of culture pH at a fixed value may not be necessarily beneficial. In contrast, we found that without controlling pH at a constant value but setting an appropriate initial level seemed to improve the efficiency of hydrogen production.

Fermentative hydrogen production by the HE1 strain was strongly affected by the initial substrate concentration. Both cumulative H\textsubscript{2} production and H\textsubscript{2} content in gas increased as the substrate concentration increases from 10 to 30 g COD l\textsuperscript{-1} (Table 2). In addition, volumetric H\textsubscript{2} production rate, specific H\textsubscript{2} production rate and hydrogen yield all reached maximum when sucrose concentration was 30 g COD l\textsuperscript{-1}, indicating that over the substrate concentration range examined, H\textsubscript{2} production performance increased with an increase in substrate concentration. The results also suggest that there is no visible substrate inhibition to H\textsubscript{2} fermentation with the Klebsiella sp. strain within the sucrose concentration range of 0–30 g COD l\textsuperscript{-1}. Over that sucrose concentration range, higher sucrose concentration seemed to favor H\textsubscript{2} production both kinetically (i.e., higher rate) and thermodynamically (i.e., higher yield) (Table 2).

### 3.3. Effect of Fe\textsuperscript{2+} concentration on batch fermentation of Klebsiella sp. HE1

For facultative Klebsiella species, such as K. pneumoniae, hydrogen gas can be produced via the enzymatic reactions mediated by hydrogenase, formate dehydrogenase, or nitrogenase (Kovacs et al., 2006). For hydrogenase-driven H\textsubscript{2} production, the supply of ferrous ions (Fe\textsuperscript{2+}) in the medium might be important since Fe\textsuperscript{2+} is usually a cofactor to hydrogenases (Yvain et al., 2002). Hence, in this study, we examined the effect of Fe\textsuperscript{2+} ion on hydrogen fermentation. As indicated in Table 4, addition of Fe\textsuperscript{2+} ion at a concentration of 0.0125–0.0375 g l\textsuperscript{-1} did not have significant influence on hydrogen production rate and yield, whereas the cell growth rate seemed to be higher when more Fe\textsuperscript{2+} was added (data not shown). As the Fe\textsuperscript{2+} content did not significantly affect the H\textsubscript{2} production with Klebsiella sp. HE1, it is likely that the hydrogen production by the HE1 strain essentially followed the pathways catalyzed by nitrogenase or formate dehydrogenase. Indeed, the literature pointed out that hydrogen production by K. pneumoniae is associated mainly with the activity of nitrogenase (Vignais et al., 2001). This was consistent with what we observed from our Fe\textsuperscript{2+} addition experiments.

### 3.4. Overall energy production efficiency

Since our dark fermentation systems produced a significant amount of gaseous and liquid biofuels (i.e., H\textsubscript{2}, ethanol and 2,3-butanediol), the process performance in terms of energy generation derived from the combination of the three biofuels was calculated according to their combustion heat values (DOE, 2001a, 2001b). As depicted in Table 5, the maximum energy generation rate was 27.7 kJ h\textsuperscript{-1} l\textsuperscript{-1}, taking place at a sucrose concentration of 30 g COD l\textsuperscript{-1} and an initial pH of 7.3. Despite a slightly lower

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**Table 4**

<table>
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<tr>
<th>Fe\textsuperscript{2+} ion concentration (g l\textsuperscript{-1})</th>
<th>Initial pH</th>
<th>Final pH</th>
<th>H\textsubscript{2} content (%)</th>
<th>Cumulative H\textsubscript{2} production (l)</th>
<th>Specific H\textsubscript{2} production rate (ml h\textsuperscript{-1} g\textsuperscript{-1} cell\textsuperscript{-1})</th>
<th>Volumetric H\textsubscript{2} production rate (ml h\textsuperscript{-1} l\textsuperscript{-1})</th>
<th>H\textsubscript{2} yield (mol H\textsubscript{2} mol sucrose\textsuperscript{-1})</th>
<th>Sucrose conversion (%)</th>
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**Table 5**

<table>
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<th>Sucrose Concentration (g COD l\textsuperscript{-1})</th>
<th>2,3-ButOH production rate (mol h\textsuperscript{-1} l\textsuperscript{-1})</th>
<th>ETOH production rate (mol h\textsuperscript{-1} l\textsuperscript{-1})</th>
<th>H\textsubscript{2} production rate (mol h\textsuperscript{-1} l\textsuperscript{-1})</th>
<th>2,3-ButOH yield (mol 2,3-ButOH mol sucrose\textsuperscript{-1})</th>
<th>ETOH yield (mol ETOH mol sucrose\textsuperscript{-1})</th>
<th>H\textsubscript{2} yield (mol H\textsubscript{2} mol sucrose\textsuperscript{-1})</th>
<th>Energy generation rate\textsuperscript{a} (kJ h\textsuperscript{-1} l\textsuperscript{-1})</th>
<th>Energy yield\textsuperscript{b} (kJ mol sucrose\textsuperscript{-1})</th>
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<td>0.92</td>
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<td>1.58</td>
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\textsuperscript{a} Energy generation rate = 2,3-ButOH production rate (mol h\textsuperscript{-1} l\textsuperscript{-1}) × 2461 kJ mol 2,3-ButOH\textsuperscript{-1} + ETOH production rate (mol h\textsuperscript{-1} l\textsuperscript{-1}) × 1366 kJ mol ETOH\textsuperscript{-1} + H\textsubscript{2} production rate (mol h\textsuperscript{-1} l\textsuperscript{-1}) × 286 kJ mol H\textsubscript{2}\textsuperscript{-1} (DOE, 2001a, 2001b).

\textsuperscript{b} Energy yield = [mol 2,3-ButOH produced × 2461 kJ mol 2,3-ButOH\textsuperscript{-1} × mol ETOH produced × 1366 kJ mol C\textsubscript{2}H\textsubscript{5}OH\textsuperscript{-1} × mol H\textsubscript{2} produced × 286 kJ mol H\textsubscript{2}\textsuperscript{-1}] ÷ mol sucrose consumed (DOE, 2001a, 2001b).
energy generation rate (23.6 kJ h⁻¹ l⁻¹) when compared to using 30 g COD l⁻¹ of sucrose, using a sucrose concentration of 20 g COD l⁻¹ gave the best energy yield of 2.45 kJ mol hexose⁻¹ (Table 5). The foregoing difference in energy production efficiency could be attributed to the variation in bacterial population structure in the batch reaction systems under different conditions (e.g., difference in sucrose concentration). Moreover, since H₂ is in gas phase and the liquid fuels (ethanol and 2,3-butanediol) have distinct boiling points (78 and 180 °C, respectively), separation of the three biofuels produced from the HE1 strain would be relatively easy, leading to additional economical benefits arising from simple downstream processing. Therefore, using Klebsiella sp. HE1 to produce three types of biofuels (H₂, ethanol and 2,3-butanediol) via fermentation process seems to be feasible in future applications.

4. Conclusions

This work demonstrates feasibility of using an indigenous Klebsiella sp. HE1 strain for simultaneous production of H₂, ethanol and 2,3-butanediol as biofuels or industrially applicable biochemical products. The H₂-producing HE1 strain successfully isolated from aerobic and anaerobic sludge exhibited the ability to produce 2,3-butanediol, ethanol and hydrogen gas from sucrose substrate. The highest production rate for H₂ (3.26 mmol h⁻¹ l⁻¹) and ethanol (6.75 mmol h⁻¹ l⁻¹) and 2,3-butanediol (7.14 mmol h⁻¹ l⁻¹) was obtained from using 30 g COD l⁻¹ of sucrose substrate. However, the highest yield for H₂, ethanol and 2,3-butanediol were 0.82, 0.81 and 0.59 mol (mol sucrose)⁻¹, respectively. All those values were obtained from using an initial pH of 7.3 (pH not controlled). The overall energy generation performance was also dependent on initial sucrose concentration. Using 30 g COD l⁻¹ of sucrose as substrate, the best energy generation rate of 27.7 kJ h⁻¹ l⁻¹ was obtained, while using 20 g COD l⁻¹ of sucrose exhibited the best energy yield (2.45 kJ mol sucrose⁻¹). This study shows that the efficiency of 2,3-butanediol, ethanol and hydrogen production was higher when the sucrose concentration was 30 g COD l⁻¹ and the initial pH was 7.3 (not controlled).

Acknowledgements

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