Kinetics of Bio-Hydrogen Production from Crude Glycerol by Locally Isolated Klebsiella pneumonia via Dark Fermentation

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Research Article

Keywords: Klebsiella pneumoniae, kinetic models, Gomperzt equation, bio-hydrogen, dark fermentation

Posted Date: October 23rd, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3460272/v1

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Additional Declarations: No competing interests reported.
Abstract

Bioconversion of crude glycerol (CG) to bio-hydrogen (Bio-H$_2$) is promising because the capital investment and operation is cheaper. It is an environmentally friendly approach for waste reduction and clean energy production. However, the selection of microbes that can efficiently metabolise CG under anaerobic conditions is limited. This results in the low production and yield of Bio-H$_2$. Hence, this study sought to examine a new indigenous bacteria (Klebsiella pneumoniae strain HS11286) that can consume CG and convert it into Bio-H$_2$ without the need for pre-treatment or acclimatization. Dark fermentation was employed as it offers advantages in terms of the substrates and producers used. The research focused on the kinetic study for growth and substrate utilisation by kinetic model. In the metabolism of CG by K. pneumoniae, it needed 6 hours for lag phase to adapt to start proliferating and releasing Bio-H$_2$, as well as the metabolites. Based on the HPLC results, three metabolites were produced during the metabolism of CG (i.e.: 1,3-propanediol, propionic acid, and ethanol). The kinetic analysis, and Bio-H$_2$ production were estimated using the kinetic Monod model and the modified Gompertz equation. The kinetic analysis revealed a growth rate ($\mu$) of 0.106 h$^{-1}$, glycerol consumption rate ($Q_{gly}$) of 1.572 g/L/h, and the yield coefficients $Y_{p/x}$, $Y_{p/s}$, and $Y_{x/s}$ of 30,758.51 mL/g cell, 479.26 mL/g substrate, and 0.016 g cell/g substrate, respectively. The modified Gompertz model predicted Bio-H$_2$ production of 10,155 mL at 620 mL/h with a 6 h lag period. In conclusion, K. pneumoniae strain HS11286 has the potential to produce almost 10 litre of hydrogen in a short period of time (less than 48 h) without substrate pre-treatment and bacteria acclimatisation or genetic engineering. This study highlighted that the K. pneumoniae strain HS11286 is a promising hydrogen-producer.

1. Introduction

Bio-hydrogen (Bio-H$_2$) is a renewable and sustainable alternative to fossil fuels (Hosseini, and Wahid, 2020). The combustion of Bio-H$_2$ as an energy carrier and fuel generates water as the only by-product, making it free from any emission of pollutants (Lanjekar et al., 2023; Rosen, and Koohi-Fayegh, 2016). Hence, Bio-H$_2$ has the potential to address the socio-economic and environmental challenges caused by the global warming and climate change (Acar, and Dincer, 2020; Valente et al., 2017). In addition, as a majority of fuel supplies are carbon-based, carbon dioxide (CO$_2$) emission is among the main cause of climate change and environmental deterioration (Mehra, and Paul, 2022). This further emphasize the relevance of Bio-H$_2$ as an alternative to carbon-based fuel. Subsequently, the production, storage, transport and utilization of Bio-H$_2$ have become a focal point of studies for the past decades as Bio-H$_2$ offers clean energy due to the fact that water is the only by-product during its combustion (Hosseini, and Wahid, 2020; Rosen, and Koohi-Fayegh, 2016). One of the most notable areas of research is the production of Bio-H$_2$ from various substrates through thermal, chemical, and biological processes. Until recently, production of Bio-H$_2$ from glycerol was mostly from chemical conversion technologies such as steam reforming (González et al., 2023; Tamošiunas et al., 2016), partial oxidation (Nda-Umar et al., 2019; Wang, 2010), dry reforming (Zakaria et al., 2015), auto-thermal reforming (Nda-Umar et al., 2019; Wang et
al., 2016), aqueous phase reforming (Raso et al., 2023; Fasolini et al., 2019), pyrolysis (Ng et al., 2017), and photocatalysis reforming (Reddy et al., 2018; Lucchetti et al., 2017). Despite the stability of some processes, the mentioned approaches require high reaction temperatures, which promotes the formation of encapsulated carbon that negatively reflects on catalyst stability (Chiodo et al., 2010). Subsequently, Bio-H$_2$ production from biochemical approaches has gained interest as green technology for the valorisation of industrial waste streams such as crude glycerol.

Crude glycerol (CG) is a major by-product of biodiesel production and accounts for 10% of the waste stream generated by the industry (Moklis et al., 2023). It is estimated that glycerol stocks will exceed 7.66 million tons owing to the projected increase in biodiesel demand worldwide (Monteiro et al., 2018). As CG is classified as Scheduled Waste S181 of the Environmental Regulations in Malaysia, it cannot be disposed freely (Ardi, Aroua, and Hashim, 2015). Hence, the development of processes that can utilise CG directly to produce value-added chemicals or energy carriers (e.g.: hydrogen) would be very beneficial. However, CG has impurities and needs to be pre-treated before it can be utilised as substrates. Among the biological approaches addressing the impurities of CG so that it can be utilised as a substrate, CG is considered a cheap carbon source for microbial conversion into Bio-H$_2$ production through fermentation.

Fermentation is one of the biological ways to produce Bio-H$_2$ from CG that has a high degree of reduction, which enables high yield of fuels, chemicals, and high energy contents. The process of fermentation is a biological route for the production of Bio-H$_2$, 1,3-propanediol, carotenoids, citric acid, succinic acid, polyhydroxyalkanoates (PHA), polyunsaturated fatty acids (PFA), and rhamnolipids from CG (Abad, and Turon, 2012). Nevertheless, the production of Bio-H$_2$ is more favourable as CG has a higher content of Bio-H$_2$ (8 atoms), thus it is capable of giving a high energy content of Bio-H$_2$ (up to 142.9 kJ/g) (Sarma et al., 2012). The production of Bio-H$_2$ from CG by using microorganisms as the producer has also been a point of interest by researchers as most of its impurities showed positive influences on microbial growth through its cell development (Chilakamarry et al., 2021). Ito et al. (2005) evaluated the production of Bio-H$_2$ and ethanol using pure and CG by Enterobacter aerogenes HU-101 strain. The strain could not tolerate the impurities in the CG, thus giving low Bio-H$_2$ yield compared to pure glycerol. Ngo and colleagues (2011) used hyperthermophilic eubacterium Thermotoga neapolitana DSM 4359 to produce Bio-H$_2$ from CG. Due to impurities in the CG, CG was pretreated first by heating at 45°C to remove methanol or ethanol, and solids from CG were removed by centrifugation. The Bio-H$_2$ yield from T. neapolitana DSM 4359 at 1.97 ± 0.09 mol H$_2$/mol glycerol was observed after 56 h of cultivation. Among the latest bioconversion of CG to Bio-H$_2$ was reported by Sarma et al. (2019) using an engineered strain of Clostridium pasteurianum. The hydA gene that was overexpressed encodes for hydrogenase, while a combination of dhaD1 and dhaK genes encoded for glycerol dehydrogenase and dihydroxyacetone kinase, respectively. The engineered hydA-overexpressed strain produced 1.1 mol H$_2$/mol glycerol, and 0.93 mol H$_2$/mol glycerol was produced by the dhaD1K-overexpressed strain. Nevertheless, although the yields were high in most of these studies, drawbacks such as the need for a higher reaction temperature or the pre-engineering of strains may be unsuitable for industrial applications. Alternatively, a pure culture
is much easier to manage and research, especially when describing the pathway, thus making it simpler to be optimised to produce a higher Bio-H\textsubscript{2} yield.

Fermentation can be conducted in the presence of light (photo fermentation) or without light (dark fermentation) (Dahiya et al., 2020; Laurinavichene et al., 2018). Dark fermentation (DF) was utilized in this study as it is the most prevalent approach utilized in regards to the production of Bio-H\textsubscript{2} from glycerol. Scientists regard DF as promising due to its versatilities of substrates and microbes as pure, mixed, engineered pure culture or a combination of cultures can be utilized (Rodrigues et al., 2019; Veeramalini et al., 2019). In addition, the system is stable with high production efficiency, requires no light in the process, and the hydrogen-involving enzyme is stable (Fuess et al., 2019). Furthermore, photo fermentation is limited to other types of substrates that usually do not include glycerol. Anaerobic fermentation is one of the most studied systems for DF and Bio-H\textsubscript{2} hydrogen is the key substrate in the metabolism of many anaerobic microorganisms (Kumar et al., 2018). Enterobacter sp., Clostridium sp., Bacillus sp., and Klebsiella sp. are among the commonly used microorganisms in dark- or anaerobic fermentation of glycerol. Though in the context of Bio-H\textsubscript{2} production, bacteria such as Clostridium sp. is not effective when paired with the utilization of CG due to its impurities (Cao, 2022). Consequently, the bacteria isolated in this study was chosen from the Klebsiella species as it is among the anaerobic bacteria that is predominantly used in Bio-H\textsubscript{2} production due to its rapid growth rate and ability to produce various valuable by-products (Cao, 2022). Such microorganisms are capable of using energy-rich hydrogen molecules and utilize the electrons from Bio-H\textsubscript{2} oxidation to produce energy. Generally, the rate and yield of Bio-H\textsubscript{2} obtained through dark- or anaerobic fermentation are typically high, ranging around 18–63 mmol/L/h and 0.53–1.12 mol H\textsubscript{2}/mol glycerol, respectively (Chookaew et al., 2014). With its simplicity and efficiency, glycerol fermentation of Bio-H\textsubscript{2} through the utilization of dark fermentation provides a high production rate and can be carried out using a variety of microorganisms that are easy to grow and maintain (Fuess et al., 2019). Moreover, when paired with the utilization of indigenous pure culture, a better understanding in the mechanism of the conversion process could be achieved. In addition, a pure culture allows for easier management in the production of Bio-H\textsubscript{2}. There is also the fact that its utilisation of waste leads to waste reduction, making it an economical energy production approach.

Subsequently, in this study, CG was used as the sole carbon source for Bio-H\textsubscript{2} production through DF using locally isolated bacteria, K. pneumoniae HS11286. A comprehensive kinetic analysis of the process was performed to elucidate the effects of operational parameters on substrate consumption, biomass growth, and product formation rate. Therefore, a better insight into the Bio-H\textsubscript{2} production of K. pneumoniae HS11286 without acclimatising it, or the pre-treat of the CG, as well as its dominant metabolic pathway were presented.

2. Materials and Methods

2.1 Materials and Chemicals
The CG used in this study was obtained from the biodiesel plant of Carotino Sdn. Bhd., Malaysia. The wastewater containing glycerol was obtained from the biodiesel plant of Vance Bioenergy Sdn Bhd, Malaysia. Pure glycerol was purchased from Sigma Aldrich, Malaysia. The minimal media used for DF contained crude/pure glycerol (20.0 g/L), KH$_2$PO$_4$ (1.3 g/L), K$_2$HPO$_4$$\cdot$3H$_2$O (3.4 g/L), (NH$_4$)$_2$SO$_4$ (2.0 g/L), MgSO$_4$$\cdot$7H$_2$O (0.2 g/L), CaCl$_2$$\cdot$2H$_2$O (0.02 g/L), FeSO$_4$$\cdot$7H$_2$O (0.005 g/L), and the yeast extract (0.5 g/L) (Varrone et al., 2013). The medium's final pH was adjusted to a pH of 6.8 before it was autoclaved at 121°C for 15 mins. Next, 20 g/L of agar was added to the medium to prepare plates for the culturing of the *K. pneumoniae* local strain.

### 2.2 Substrate and Inoculum Preparation

*K. pneumoniae* local strain isolated from biodiesel wastewater was used as the Bio-H$_2$ producer. One loop of *K. pneumoniae* colony was transferred into 200 mL of minimal media followed by incubation for 18–24 hours. The inoculum was used once the optical density (OD) reached, or exceeded, 0.8 at 600 nm wavelength.

### 2.3 Dark Fermentation

The biohydrogen was produced via dark fermentation (DF), where the minimal medium served as the fermentation medium. The set-up for the dark fermentation procedure is depicted in Fig. 1. Firstly, the *K. pneumoniae* inoculum was centrifuged and washed to remove any residual nutrients as to ensure that it would only consume the newly supplied glycerol as substrate. Before each experiment, the medium was sterilised at 121°C for 15 minutes. After the medium was cooled, the *K. pneumoniae* (10% of 24-hr inoculum) was added into the minimal medium (Liang et al., 2010). The inoculated medium was then flushed with nitrogen gas (N$_2$). N$_2$ containing less than 2 ppm O$_2$ was used as sparging gas at a flow rate of 110 ± 2.6 ml min$^{-1}$, for 10 minutes to create an anaerobic environment (Singhal, and Singh, 2014). The fermentation was carried out at an initial pH of 6.8, under room temperature and continuous stirring.

### 2.4 Analytical Methods

The total biogas volume (i.e.: carbon dioxide, CO$_2$ and biohydrogen, Bio-H$_2$), was collected every 3 h for 48 h and measured using the water displacement method. Afterwards, the potassium hydroxide (KOH) displacement method was used to ensure that only hydrogen remained after the gas scrubbing. The Bio-H$_2$ yield was calculated via Eq. 1. The number of moles of hydrogen gas was determined using Eq. 2, based on the ideal gas law at standard temperature and pressure (STP), in which $P$ is pressure, $V$ is hydrogen volume (L), $n$ is the mole of hydrogen gas, $T$ is the temperature (K), and $R$ is the ideal gas constant (0.082 atm.L.mol$^{-1}$.K$^{-1}$).

\[
\text{Hydrogen yield} = \frac{\text{Mole of hydrogen generated}}{\text{Mole of glycerol used}} \quad (1)
\]

\[
PV = nRT \quad (2)
\]
The Bio-H₂ produced was analysed using gas chromatography (GC) equipped with a thermal conductivity detector (TCD). The GC-TCD is operated with the gas column HP-PLOT Molesieve 5A with capillary dimensions: 30 mm × 530 µm × 25 µm nominal using the carrier gas argon (Ar) at 99.99% purity. The column pressure was set at 3.58 psi with 3.0 mL/min flow. The oven was set at 350°C and ramped at 24°C/min for a runtime of 25.46 min. The TCD was set at 250°C, 5 mL reference flow, and 7 mL/min helium make-up flow. The volume of gas injected into the GC-TCD column was 20 µL (Ahmad, 2016).

### 2.5 Product Analysis using High-Pressure Liquid Chromatography (HPLC)

The collected supernatant of the samples was further analysed using HPLC fitted with a RID detector (Agilent Technologies, AMINEX® Ion Exclusion HPX-87H, 300×7.8 mm). The analytical process generated two results, namely: substrate consumption and metabolite produced. The procedure used for this analysis was adopted from Drożdżyńska et al. (2014). In the analysis, 900 µL of the supernatant was pipetted into a clean tube before adding 100 µL of 0.005 mM H₂SO₄. Next, the mixture was filtered into a GC vial through a 0.2 µm membrane filter. The initial and final concentrations of glycerol were quantified by referencing the developed glycerol standard curve. The percentage of glycerol utilised was calculated using Eq. 3, in which \( C_{gi} \) denotes the initial concentration of glycerol and \( C_{gf} \) as the final concentration of the glycerol. The metabolite products comprising of; acetic acid, formic acid, propionic acid, ethanol, and 1,3-propanediol, were determined using the same procedure.

\[
\% \text{Glycerol utilized} = \frac{C_{gi} - C_{gf}}{C_{gi}} \times 100 \quad (3)
\]

### 2.6 Determination of Kinetic Coefficients

The kinetics of bacterial growth, product formation, and substrate consumption were determined using the growth kinetic theory and the modified Gompertz model. In a batch growth, the specific growth rate (\( \mu \)) vary along the log phase. This is due to the accumulation of biomass, the decreasing of substrate, the accumulation of products and the decreasing of nutrients over time, which may occur in a fermentation process where there is an absence in addition and removal of nutrients. The specific growth rate is expressed in Eq. 4.

\[
X_f = X_i \times e^{\mu(t_f - t_i)} \quad (4)
\]

In which, Eq. 4 can be rearranged into Eq. 5 to determine \( \mu \) (the specific growth rate), where \( X_i \) is the initial cell mass concentration (g/L), \( X_f \) is the final cell mass concentration (g/L), \( t_i \) is the initial hour (h) and \( t_f \) is time at \( f \)th hour.

\[
\mu = \frac{ln \frac{X_f}{X_i}}{(t_f - t_i)} \quad (5)
\]
The growth, biogas, and Bio-H$_2$ productions of each sample were subsequently analysed using optical density (OD). Liquid samples were centrifuged to separate the cells from the medium. The supernatant was analysed using HPLC to assess the glycerol consumption and metabolite production. Alternatively, the Bio-H$_2$ potential was evaluated using the modified Gompertz equations. The modified Gompertz equation is more advantageous than the growth kinetic model for calculating the rate of Bio-H$_2$ production due to the numerous steps involved in the anaerobic degradation of organic materials (Keskin et al., 2019). The modified Gompertz equations (Eq. 6 and Eq. 7) were used to fit the cumulative Bio-H$_2$ production curves for batch reactor to obtain the Bio-H$_2$ production potential, Bio-H$_2$ production rate, and lag phase (Zhang et al., 2019). In the equations, $Q_{H2}$ is the biohydrogen production rate (mL/h), $H_{max}$ is the maximum hydrogen production potential (mL), $R_{max}$ is the maximum rate of hydrogen production (mL/h), $\lambda$ is the lag phase (h) or the time to exponential hydrogen production, $H_t$ is the cumulative hydrogen production as a function of time (mL), and $t$ is time (h). Other calculations were made based on the plotted graph of the collected data.

$$H_t = H_{max} \times \exp \left\{ -\exp \left[ \frac{R_{max} \times e}{H_{max}} (\lambda - t) + 1 \right] \right\} \quad (6)$$

$$Q_{H2} = \frac{H_{max}}{\lambda + \frac{H_{max}}{R_{max}}} \quad (7)$$

3. Results and Discussion

3.1 Hydrogen Production from Crude Glycerol

The growth of bacteria during batch fermentation depends on the culture conditions. *K. pneumoniae* would produce hydrogen efficiently and increase the hydrogen yield if the substrates could be fully utilized, hence resulting in an increase of biomass with time. Glycerol serves as an excellent substrate for fermentation process (Viana et al., 2012). The anaerobic fermentation of glycerol typically produces alcohols (e.g.: ethanol, $n$-butanol), organic acids (e.g.: propionic acid, formic acid, acetic acid, lactic acid), as well as products such as Bio-H$_2$, and diols (e.g.: 1,3-propanediol) (Kongjan et al., 2021; Yen et al., 2014; Metsoviti et al., 2012; Wu et al., 2011). However, in energy generation, the microbes selected for the anaerobic process are required to produce products such as Bio-H$_2$, acetate, methanoate ($\text{HCO}_2^-$), and CO$_2$ in the form of bicarbonate (Viana et al., 2012). Subsequently, the production of Bio-H$_2$ and biomass growth by *K. pneumoniae* strain HS11286 was observed in this study after a lag phase of 6 h, marking the exponential phase of the DF process (Fig. 2). This stage was followed by the accelerated production of Bio-H$_2$ until 30 h, and the stabilization phase was achieved after 30 h.

This may have been due to the declining medium pH from 6.0 to 5.0. A similar phenomenon was reported by Ngo et al. (2011), who observed a decline in pH after 24 hours of cultivation using glycerol as substrate and *Thermotoga neapolitana* as the microbe. In the 48-hours fermentation period, 10,000 mL of
Bio-H₂ was collected. Furthermore, there was less than 5 g/L glycerol left in the culture. This implied that *K. pneumoniae* can efficiently degrade the selected substrate as it was able to convert almost 97% of glycerol during the fermentation process, with the maximum biomass concentration (*X*<sub>max</sub>) reaching 0.485 g/L.

Three metabolites were produced, namely 1,3-propanediol, propionic acid, and ethanol (Fig. 3). The metabolites and their corresponding profiles were different from others reported in past literatures. Most studies involving glycerol fermentation reported a significant production of 1,3-propanediol. Alternatively, the quantity of 1,3-propanediol in this study was considered insignificant due to its low production. Based on previous literatures, high concentrations of 1,3-propanediol cannot be produced under strictly anaerobic conditions (Sun et al., 2022; Zhang, and Xiu, 2009). Subsequently, the low concentration of 1,3-propanediol detected in this study could be caused by nitrogen sparging that resulted in strict anaerobic conditions for the functioning of the *Klebsiella* species (Paranhos, and Silva, 2020; Kavitha et al., 2019). This claimed was supported by Szymanowska-Powałowska (2014) who reported that sparging of fermentation medium with nitrogen during fermentation of glycerol by *Klebsiella oxytoca* shifted the metabolism towards ethanol production. Overall, the strict anaerobic conditions may have instigated the restricted production of 1,3-propanediol by *K. pneumoniae*. Additionally, excess NADH₂ from acid production may have also contributed to the low concentration of 1,3-propanediol (Drożdżyńska et al., 2014). Hence, as the acid produced in this study was very low, the 1,3-propanediol produced was also low in concentration.

Meanwhile, propionic acid was observed to be among the predominant acid detected during the glycerol fermentation process with 5.9 g/L as its final volume produced in the study (Fig. 3). Acids, such as propionic acid, that are produced from glycerol fermentation have been reported in previous studies (Boonyawanich et al., 2021; Dams et al., 2018; Rivero et al., 2014). In a study by Rivero et al. (2014), propionic acid was reported as the major acid produced during the co-digestion of glycerol and sewage sludge for improved production of Bio-H₂. It was noted that the concentration of propionic acid reported was lower than the current study with a reading of 2.8–3.1 g/L. Another predominant product of this study was ethanol (Fig. 3). Its production started during the deceleration phase of *K. pneumoniae* growth, and during the peak period of Bio-H₂ production, which was 12 hours onward (Fig. 2). Ethanol was continuously produced even as the production of Bio-H₂ stopped, thus proving ethanol as a major metabolite (Kannah et al., 2019; Rodrigues et al., 2019). These findings were in agreement with previous studies that have also reported ethanol as one of the major soluble metabolites produced in batch dark hydrogen fermentation for Bio-H₂ production (Shao et al., 2020; Show et al., 2019). Studies by Show et al. (2019) and Khan et al. (2016) reported the production of ethanol and acetate as among the main metabolites produced during acidogenesis. Overall, ethanol was the predominant product over acid (propionic acid) and diol (1,3-propanediol) that was produced during the fermentation of glycerol.

Metabolites are important indicators of Bio-H₂ performance since the yield is determined from metabolite-based equations of reaction, and the metabolic pathway can be identified based on the metabolite
produced (Park et al., 2019). Hence, the metabolic reaction of *K. pneumoniae* was considered based on the formation of propionic acid and ethanol as these metabolites were the most predominant. The production of propionic acid occurred during the pH decline to 5.0 at 15 hours incubation time (Fig. 2 and Fig. 3). This may indicate the important function of pH as a study by Rivero et al. (2014) reported the improvement of acid concentrations and total acidity during acidogenic stage. In glycerol metabolic pathway, propionic acid is derived from succinate that is produced from phosphoenolpyruvate (PEP) through the reductive branch of the tricarboxylic acid (TCA) cycle (Cheng et al., 2013). However, the results indicated that the production of propionic acid did not produce Bio-H$_2$. Nevertheless, the acid production process is necessary as it generates adenosine triphosphate (ATP) that is required for product synthesis and cell maintenance (Zeng et al., 1993). Figure 4 shows the simplified version of propionic acid metabolism after glycerol enters the cell.

Generally, ethanol is considered as a metabolite that does not produce Bio-H$_2$. However, this statement was based on the fermentation of glucose substrate by *Clostridium*, whereas *Klebsiella* is considered as a powerful workhorse for glycerol conversion (Kumar, and Park, 2018; Wong et al., 2011). While *Clostridium* is unable to grow effectively in glycerol and requires strict anaerobic conditions, *Klebsiella* is able to tolerate high glycerol concentrations even with impurities (Kumar, and Park, 2018; Wong et al., 2011). *K. pneumoniae* converts glycerol to ethanol and produces ATP, formate and water. Subsequently, formate was then converted to carbon dioxide (CO$_2$) and hydrogen gas (H$_2$) (Eq. 8 and Eq. 9) (Zeng et al., 1993).

During the formation of formate in *K. pneumoniae*, the equations involve showed the production of one mol of hydrogen during the conversion of glycerol to ethanol.

$$Glycerol \rightarrow Ethanol + ATP + Formate + H_2O$$

$$C_3H_8O_3 \rightarrow C_2H_5OH + ATP + HCO_2^- + H_2O \quad (8)$$

$$Formate \rightarrow Carbondioxide + hydrogengas$$

$$HCO_2^- \rightarrow CO_2 + H_2 \quad (9)$$

The conversion of glycerol was initialised by the oxidation of glycerol to dihydroxyacetone (DHA) by glycerol dehydrogenase enzyme (Eq. 10). The DHA was then phosphorylated by the DHA kinase enzyme into dihydroxyacetone phosphate (DHA-P) (Eq. 11). Next, the DHA-P was oxidized to phosphoenolpyruvate (PEP), which was then followed by its conversion to pyruvate and succinate.

$$Glycerol + NAD^+ \rightarrow Dihydroxyacetone + NADH + H^+$$

$$C_3H_8O_3 + NAD^+ \rightarrow C_3H_6O_3 + NADH + H^+ \quad (10)$$

$$Dihydroxyacetone + ATP \rightarrow Dihydroacetonephosphate + ADP$$

$$C_3H_6O_3 + ATP \rightarrow C_3H_7O_6P + ADP \quad (11)$$
The pyruvate was then broken down to acetyl-CoA by ferredoxin reduction (Fig. 5). This ferredoxin reduction is catalysed by pyruvate ferredoxin oxidoreductase. The reduced ferredoxin (Fd) is then oxidized by a hydrogenase that reproduces oxidized Fd and $H_2$ gas (Maru et al., 2013). Lastly, ethanol was obtained from the reduction of acetaldehyde from acetyl-CoA. The reductive pathways serve as an electron sink for oxidation, consuming reducing equivalents generated during the stepwise oxidation of glycerol to achieve redox equilibrium. During the reductive pathway, some of the electrons can be diverted to merge with protons, which are subsequently released as $H_2$ gas under anaerobic conditions and low partial pressures (Selembo et al., 2009). It is also suggested that an acetaldehyde or alcohol dehydrogenase may carry out an important role in glycerol fermentation to recover redox equilibrium (Murarka et al., 2008).

Figure 6 depicts the metabolic pathway of glycerol by *K. pneumoniae* based on the results of this study. Firstly, as glycerol was consumed by the *K. pneumoniae*, it was actively transported into the cell by transporter proteins. This intracellular transport of glycerol followed the oxidative pathway before conversion into DHA by the enzyme glycerol dehydrogenase that yielded nicotinamide adenine nucleotide (NADH$_2$), an electron carrier. Next, the DHA was phosphorylated into DHA-P by consuming ATP, where the process was mediated by glycerol kinase enzyme. Subsequently, the DHA-P was converted to PEP through oxidation, followed by its conversion to pyruvate and succinate. The formation of formate, which resulted in the release of CO$_2$ and Bio-$H_2$, was initiated during the conversion of pyruvate to ethanol. Alternatively, propionic acid was produced from the conversion of succinate. Throughout this pathway, NADH and ATP were produced. Excess NADH was regenerated into NAD$^+$ and produced 1,3-propanediol, while ATP was used for biomass synthesis and maintenance. The regeneration of NAD$^+$ also produced one hydrogen. Overall, the *K. pneumoniae* HS11286 was considered as an excellent hydrogen-producer due to its ability to produce metabolites that are essential in Bio-$H_2$ production from its consumption of CG.

### 3.2 Kinetic Analysis of Hydrogen Production by *Klebsiella pneumoniae*

Based on the cumulative results of the hydrogen production curves, as well as the time profile of biomass, glycerol uptake, pH and ethanol production (Fig. 2), it was deduced that the production of hydrogen is a growth-associated product, as its production started in parallel with growth entering the exponential phase. Moreover, the small period of lag phase (less than 6 hours) observed during the dark fermentation process (Fig. 2) indicated that the environment condition was suitable for favourable microbial metabolism and early onset of exponential phase (Jain et al., 2022; Gadhe et al., 2013). Subsequently, the graph of time profiles for growth, products, and substrate were utilized to estimate the kinetic parameters. Table 1 shows the computed kinetic parameters from this study. From the results, it could be inferred that as the cells grew, the rate of change in cell mass concentration ($X$) defines growth rate ($\mu$). In addition, substrate to biomass ($S/X$) ratio is significantly as it affects the metabolic and
kinetic characteristics of microorganisms, thus affecting the efficiency of their Bio-H$_2$ production (Preethi et al., 2019).

Table 1
Computed kinetic parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum hydrogen production, H$_{2\text{max}}$ [mL]</td>
<td>9935</td>
</tr>
<tr>
<td>Maximum cell dry weight, X$_{\text{max}}$ [g/L]</td>
<td>0.485</td>
</tr>
<tr>
<td>Glycerol consumption rate, Q$_{\text{glycerol}}$ [g/L/h]</td>
<td>1.572</td>
</tr>
<tr>
<td>Specific growth rate, µ [h$^{-1}$]</td>
<td>0.106</td>
</tr>
<tr>
<td>Maximum hydrogen production per unit of cell, Y$_{p/x}$ [mL/g]</td>
<td>30758</td>
</tr>
<tr>
<td>Maximum hydrogen production per unit of substrate, Y$_{p/s}$ [mL/g]</td>
<td>479.26</td>
</tr>
<tr>
<td>Overall yield coefficient, Y$_{x/s}$ (g cells/g substrate)</td>
<td>0.016</td>
</tr>
</tbody>
</table>

The cumulative H$_2$ production from crude glycerol by *K. pneumoniae* was fitted to the modified Gompertz equation (Eq. 6 and Eq. 7), and the coefficients were compared to previous studies with similar background (Table 2) so as to review the differences in growth kinetics of various hydrogen-producers. Overall, the differences between the studies were relatively apparent as *K. pneumoniae* recorded the highest H$_{\text{max}}$ (mL) and R$_{\text{max}}$ (mL/h) compared to the other studies with reading of 10155 mL and 620 mL/h, respectively. Additionally, its 6 h lag period was among the shortest. This indicated an excellent performance of H$_2$ production by *K. pneumoniae* HS11286. Variations between the studies were probably due to the difference in the hydrogen-producers used, the origin of the CG and the selected approach taken in each study.
Table 2
Summary of growth kinetics of hydrogen producing bacteria

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Substrate</th>
<th>Process</th>
<th>Coefficients</th>
<th>R²</th>
<th>References</th>
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<td></td>
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<td>R_{max} (mL/h)</td>
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<td>Dark fermentation</td>
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<td>93.1</td>
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<td>Anaerobic digestion</td>
<td>18.1</td>
<td>1093</td>
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<td>4.20</td>
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<td>N.S. Navaol</td>
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<td>Crude glycerol</td>
<td>Dark fermentation</td>
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<td>10155</td>
<td>620</td>
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4. Conclusion

*Klebsiella* is a species that has been known to have potential in Bio-H\textsubscript{2} production. Subsequently, by utilizing a strain from the *Klebsiella pneumoniae* species, specifically strain HS11286, the kinetic models for CG conversion into Bio-H\textsubscript{2} have been successfully elaborated. The growth and metabolism of the *K. pneumoniae* during the bioconversion of CG into Bio-H\textsubscript{2} were also successfully analysed. Overall, *K. pneumoniae* HS11286 needed 6 hours for lag phase to adapt before it started to proliferate and release Bio-H\textsubscript{2}, as well as the metabolites. The kinetic parameters gave specific growth rate (μ) of 0.106 h\textsuperscript{-1}, glycerol consumption rate (Q_{gly}) of 1.572 g/L/h, and yield coefficients Y_{p/x}, Y_{p/s} and Y_{x/s} at 30758.51 mL/g cell, 479.26 mL/g substrate and 0.016 g cell/g substrate, respectively. Meanwhile, the modified
Gompertz model gave a prediction of 10155 mL of Bio-H$_2$ at 620 mL/h, with 6 h lag period. Based on the kinetic analysis, the metabolism of glycerol fermentation by *K. pneumoniae* HS11286 follows the oxidative pathway. The conversions of CG to products such as Bio-H$_2$, ethanol, and propionic acids yielded in high amount of NADH that regenerated into NAD+ (oxidized form of electron carrier), which subsequently resulted in the release of Bio-H$_2$. Overall, it is evident that Bio-H$_2$ can be produced without the need for acclimatisation of bacteria nor to pre-treat the CG. In this study, the indigenous bacteria *K. pneumoniae* HS11286 was recorded to have efficiently produced Bio-H$_2$ as it recorded up to 10000 mL of Bio-H$_2$ within a short period using simple processing. The fact that this indigenous bacterium can directly consume CG implies that the process is cheaper compared to the genetically engineered bacteria, or those that need to acclimatise the bacteria to CG. Therefore, the *K. pneumoniae* HS11286 functioned as an excellent hydrogen-producer.

**Declarations**

**Funding**

This work was supported by the Ministry of Higher Education, Malaysia (MOHE) and Universiti Teknologi Malaysia (UTM) under the Fundamental Research Grant Scheme (FRGS/2/2013/SG05/UTM/02/10).

**Competing Interests**

The authors have no relevant financial or non-financial interests to disclose.

**Author Contributions**

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Roslindawati Haron, with brain-storming and guidance of Roshanida A. Rahman, Ramli Mat and Tuan Amran Tuan Abdullah. The first draft of the manuscript was written by Roslindawati Haron and Nur Aizura Mat Alewi. All authors commented and contributed on previous versions of the manuscript. All authors read and approved the final manuscript.

**Data Availability**

The datasets generated and/or analysed during the current study are publicly available in the Library of Universiti Teknologi Malaysia repository, http://eprints.utm.my/id/eprint/102644/1/RoslindawatiHaronPSChE2020.pdf.pdf

**References**


**Figures**
Figure 1

Dark fermentation arrangement: (a) Schematic diagram (b) Experimental setup
Figure 2

Time profiles of *K. pneumoniae* strain HS11286 dark fermentation
Figure 3

Production of metabolites
Figure 4
Simplified version of propionic acid production

Figure 5
The mechanism of pyruvate to ethanol
Figure 6

Summarised pathway of bioconversion of glycerol to Bio-H$_2$

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Reductive Dehydration, mediated by co-enzyme glycerol dehydrase

Oxidative Dehydrogenation, by enzyme glycerol dehydrogenase

Phosphorylation: Dehydrogenation, by enzyme glycerol dehydrogenase
Figure 7

Unnumbered image in the Results and Discussion section.

Supplementary Files

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