Optimization of selected salts concentration for improved biohydrogen production from biodiesel-based glycerol using *Enterobacter aerogenes*

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ABSTRACT

Enterobacter aerogenes have a known ability to convert glycerol (GL) in a fermentative process to yield hydrogen and ethanol as the main by-products. The concentration of some media constituents was optimized to maximize biohydrogen yield and rate of production. *E. aerogenes* were cultured in aerobic conditions, and then transferred into anaerobic conditions before being cultured in a minimum mineral synthetic media (MMSM) containing 15 g/L GL. The concentration of selected salts were optimized in the following ranges: 0–300 mg/L MgSO_4 , 0–14 g/L Na_2EDTA , 0–10 mg/L CaCl_2 , 0–10 g/L Na_2HPO_4 , and 0–9.7 g/L KH_2PO_4 . The results of the full factorial design indicated that the production of biohydrogen required a minimal concentration of 3.5 mg/L EDTA, 200 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and no $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. A significant interaction between EDTA and MgSO_4 was also observed. Results from the phosphate salts optimization showed that Na_2HPO_4 gave better results than KH_2PO_4 . The optimal conditions determined using pure glycerol (commercial grade glycerol), were successfully applied to the fermentation of crude glycerol from biodiesel production. The results indicated promising yields of 0.79 and 0.84 mol/mol of glycerol for bioethanol and biohydrogen, respectively, and this at a faster rate than reported previously for *E. aerogenes*.

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1. Introduction

Due to environmental concerns and increasing needs for alternative fuels, biodiesel has been increasingly produced over the past two decades for use as a diesel substitute and additive. Although biodiesel is a good candidate as alternative fuel, production costs have been increasing because of several factors such as increased price of alcohol used as raw material and decreased value of glycerol. Since glycerol (GL) is produced at a ratio of 10 %wt of the biodiesel produced, the market for crude glycerol has been relatively depressed because of a larger supply of crude glycerol than the ability to turn it into refined product. As a result, the value of crude glycerol on the market has been decreasing, which significantly impacts biodiesel production economics. This has encouraged the use of glycerol-containing waste as a substrate in fermentation processes to produce biohydrogen and other chemicals such as 1,3 propanediol, ethanol, lactic acid, and acetic acid [1–14]. Several studies have been performed on the anaerobic fermentation of glycerol into biohydrogen and bioethanol using different microorganisms. Studies have been performed using pure

species [1,2,4–7,13–17] and mixed cultures [11], including *Klebsiella*, *Clostridium*, *Escherichia*, and *Enterobacter* species. *Klebsiella* [1,15], *Clostridium* [2,3,6,7,13], and *Escherichia* [16] which typically convert glycerol into 1,3 propanediol, whereas *Enterobacter aerogenes* [4,17] was reported by Ito et al. 2005 [9], and Jitrwung and Yargeau 2010 [4] to convert glycerol into two main products, hydrogen and ethanol.

One of the studies using *E. aerogenes* to produce biohydrogen from crude glycerol produced through biodiesel manufacturing, was based on a packed bed reactor (PBR) using porous ceramics as a support material and a synthetic media composed of salts, yeast extract, and tryptone to support bacterial growth. The maximum H_2 production rate and yield observed were 63 mmol/l/h and 0.85 mol/mol GL, respectively. Minor products such as 1,3 propanediol, pyruvic acid, lactic acid, acetic acid, and formic acid were also produced [4]. These experiments showed the potential of *E. aerogenes* grown in anaerobic conditions for the conversion of crude glycerol. However, this study and the others mentioned previously were using significant amount of costly additives such as salts, yeast extract and tryptone. Previous research has examined the effect of the concentration of some of the additives on bacterial growth, such as ammonium nitrate (NH_4NO_3), ammonium chloride (NH_4Cl), and sodium nitrate (NaNO_3). Results showed that NH_4Cl , NH_4NO_3 , and NaNO_3 supported *E. aerogenes* growth with optical

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densities (OD_{660}) of 7.0, 3.7, and 2.3 respectively. This growth was associated with a consumption of NH_4^+ with no residual NO_3^- . Effect of iron ion (Fe^{2+}) concentration on hydrogen production from a starch solution (15 g/L) was studied and optimal conditions of pH 7.0 to 8.0, Fe^{2+} concentration of 10 mg/L and NH_4CO_3 concentration of 5.64 g/L were determined [18]. The addition of a chelating agent such as EDTA (Ethylenediaminetetraacetic acid) or NTA (nitrilotriacetic acid), in combination with Fe^{2+} , showed that EDTA enhanced H_2 photoproduction by *Rhodospirillum rubrum* by inhibiting biosynthesis of hydrogen uptake hydrogenase and mobilization of iron [19]. Until now, most studies have used minimum mineral synthetic media (MMSM) containing 7 common salts (Na_2HPO_4 , NH_4NO_3 , $FeSO_4 \cdot 7H_2O$, KH_2PO_4 , $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, and Na_2EDTA). In our previous work using *E. aerogenes* [14] we reported optimized concentrations of three of these salts (6 g/L Na_2HPO_4 , 1.5 g/L NH_4NO_3 , 6.25 mg/L $FeSO_4$) along with an optimal inoculum volume ratio of 18% and oxygen concentration in the inoculation step of 7.5% O_2 . These conditions used with commonly used concentrations of the other salts (4 g/L KH_2PO_4 , 200 mg/L $MgSO_4$, 10 mg/L $CaCl_2$, 14 mg/L Na_2EDTA and 15 g/L of glycerol), resulted in hydrogen yields of 0.68 and 0.85 mol H_2 /mol glycerol for pure and crude glycerol, respectively [14].

The objective of the study presented here was to optimize the concentration of the other four salts commonly used (KH_2PO_4 , $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, and Na_2EDTA) and the research hypotheses were: 1) EDTA acts as an inhibitor at high concentration and using excess EDTA results in a lower production rate; 2) concentration of salts such as $CaCl_2$, $MgSO_4$ and Na_2HPO_4/KH_2PO_4 can be optimized to improve hydrogen production and lower operating costs, and 3) conditions optimized for pure glycerol conversion apply to crude glycerol fermentation. The experiments described in the following sections were designed to confirm or refute these hypotheses.

2. Materials and methods

2.1. Microorganism and inoculums preparation

E. aerogenes (ATCC 35029), obtained from the American Type Culture Collection (ATCC) was started under aerobic conditions in 100 mL of nutrient broth BD 234000 from Becton and Dickinson Company (12 g/L). Cultures were incubated for a period of 20–24 h, at 37 °C and 120 rpm, to reach the stationary phase, 10 mL were used as inoculum. 190 mL of the same nutrient broth was placed in 250-mL serum bottles which were then heated at 50 °C to ensure dissolution of the compounds, and then cooling down to 25 °C before capping of the bottles to create a static oxygen condition. 10-mL inoculums were then transferred into the 250-mL serum bottles containing 190 mL of the same nutrient broth. Following the inoculation, the serum bottles were incubated at 37 °C, 120 rpm for 20–24 h until stationary phase was reached.

2.2. Minimum mineral synthetic media (MMSM) and glycerol concentration

The following compounds were added per litre of deionised water to prepare the MMSM at a pH of 6.8: ammonium nitrate (NH_4NO_3 : 1.5 g), ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$: 0.00625 g), mono potassium phosphate (KH_2PO_4 : 0–9.749 g), disodium hydrogen phosphate (Na_2HPO_4 : 0–12.2 g), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$: 0–0.300 g), calcium chloride ($CaCl_2$: 0–0.015 g) obtained from Sigma Aldrich, tetraethylenediamine disodium salts (Na_2EDTA : 0–0.014 g) obtained from Fisher Scientific and either 15 g/L of pure glycerol (PG) obtained from Sigma Aldrich or 18.5 g/L crude glycerol (CG)

obtained from Rothsay Biodiesel, Canada. The media was boiled in 125-mL serum bottles for 20 min, warmed for 5 min, and then cooled down on ice for 5 min with a continuous flushing of argon in the headspace to remove oxygen prior to capping the bottles (referred to as the experiment bottles).

2.3. Biohydrogen production experiments

Following the preparation of the glycerol-containing MMSM (Section 2.2) and inoculums bottles (Section 2.1), experiments were started. Prior to taking inoculum from the inoculum bottles, an argon-oxygen gas mixture (7.5% O_2) was used to over-pressurize the inoculum bottles while maintaining the semi anaerobic conditions identified in previous work [14] has being beneficial to the production of hydrogen by *E. aerogenes*. 9.4 mL of inoculum was then transferred from an inoculum bottle into the experiment bottles containing 50 mL of glycerol-containing MMSM. The transfer was done following the Hungate technique [20], using an aseptic syringe. The experiment bottles were incubated at 37 °C and 120 rpm until hydrogen production ceased. Each conditions tested was run using two replicates and only the averages are reported.

The experiments were performed in three different phases including pre-screening experiments and a full factorial design to determine the optimal concentration of three selected salts, $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, and Na_2EDTA (Optimization I, see Section 2.3.1), an optimization of the ratio of Na_2HPO_4 (PH) and KH_2PO_4 (KP) while maintaining the amount of phosphate (PO_4^{3-}) constant (Optimization II, see Section 2.3.2) and lastly, the application of the optimized conditions to the crude glycerol which consisted in the comparison of the results obtained using pure and crude glycerol (Application to crude glycerol, see Section 2.3.3).

2.3.1. Optimization I: concentration of MG, CA, and ED

Preliminary experiments were performed to determine the optimal ranges of concentration to be used. The screening of salts was done by varying the concentration of MG and CA by 50% above and below the concentrations reported in literature (MG 200 mg/L, CA 10 mg/L [14]), and including a concentration of 0 mg/L. The concentrations studied were 0, 100, 200, 300 mg/L for MG and 0, 5, 10, 15 mg/L for CA. ED concentration was varied in order to obtain four concentrations below the value reported in literature (ED 14 mg/L [8]): 0, 3.5, 7.0, 10.5, and 14 mg/L.

A full factorial design was then used to determine the optimal concentrations of MG, CA and ED in the most favourable ranges identified in the screening step. A two levels factorial design including a middle point was set up. The three salts were tested at the low concentration of 0 mg/L, middle points of 1.75, 100 and 5 mg/L and high concentrations of at 3.50, 200, and 10 mg/L for ED, MG, and CA respectively.

2.3.2. Optimization II: ratio of Na_2HPO_4 to KH_2PO_4

In order to study the effect of two phosphate salts commonly used as buffer, potassium and sodium salts, experiments were performed by maintaining a constant molar amount of phosphate equivalent to the level used in previous studies (8.0 g/L Na_2HPO_4 and 4.0 g/L KH_2PO_4 [14]), while varying the ratio of Na_2HPO_4 (PH) to KH_2PO_4 (KP). The levels of PH and KP were adjusted to obtain five different mass ratios PH/KP: 0 (PH only), 0.43, 0.67, 0.84 and 1 (KP only). For each ratio tested, the pH was adjusted to 6.8 using either 10% potassium hydroxide or 10% phosphoric acid.

2.3.3. Application of the optimized conditions to crude glycerol

The optimized conditions obtained from previous work [14] and through the optimization studies described in the earlier sections (Sections 2.3.1 and 2.3.2) were used to study the conversion of

Fig. 1. Hydrogen production using various concentrations of Na₂EDTA under fixed levels of 200 mg/L MgSO₄ and 100 mg/L CaCl₂.

crude glycerol obtained from a biodiesel production company. Crude glycerol was vacuum filtered using filter paper P8 and P4 purchased from Fisher Scientific before being mixed with deionized water in order to obtain a concentration of 18.5 g/L of crude glycerol (corresponding to 15 g/L of glycerol). The pH was adjusted to 6.8 using 10% phosphoric acid.

2.4. Analytical methods

The volume of biogas produced was measured every 24 h using a gas tight syringe. Biogas samples were analysed by gas chromatography to determine hydrogen concentration. A Hewlett Packard 5890 gas chromatograph equipped with a molecular sieve column 8A maintained at a temperature of 80 °C, using 3.0 mL/min argon as a carrier gas, and a thermal conductivity detector (TCD) was used. When hydrogen production ceased, samples of liquid were collected to characterize the residual solution composition. The liquid samples were centrifuged at 10,000 rpm for 10 min, and the supernatant was collected for liquid chromatography and ion chromatography analysis. A Hewlett Packard 1050 High-Performance Liquid Chromatograph (HPLC) equipped with a Rezex ROA-Organic Acid H⁺ 8% 150 × 7.80 mm column and a refractive index detector (RI) HP1047A was used to measure the concentration of glycerol, 1,3 propanediol, 1-propanol, methanol, pyruvate, lactate, acetate, formate, and ethanol. The mobile phase was 0.035 M H₂SO₄ at a flow rate of 0.8 mL/min. The column temperature was 65 °C and the temperature of the RI was 50 °C. Inorganic ions such as nitrite, nitrate, sulfate, and phosphate were monitored using an Ion Chromatograph (IC) from Metrohm, model 820 IC, equipped with a Metrosep A supp7 250/4.0 mm–5 μm column maintained at 45 °C and a conductivity detector and using 3 mM Na₂CO₃ as a mobile phase.

Table 1
ANOVA results.

Term	Sum of squares	Degree of freedom	F-value	P-value Prob > F
CaCl ₂	0.00004	1	0.04	0.8414
MgSO ₄	0.01248	1	13.77	0.004
EDTA	0.00074	1	0.81	0.388
CaCl ₂ · MgSO ₄	0.00112	1	1.23	0.293
CaCl ₂ · EDTA	0	0	0	0.9972
MgSO ₄ · EDTA	0.00802	1	8.85	0.0139
Error	0.00906	10		
Total	0.03145	17		

2.5. Statistical methods

The optimization of the concentration of the three salts was performed in a full factorial design with middle point. Each condition was performed in duplicate (2 Blocks), and the middle point was added to verify the performance of the model. For predicting the optimal concentrations based on the main and interaction effects, the Software SAS 9.2 obtained from the company SAS was used to fit the first-order regression model as follows:

$$Y = b_0 + \sum b_i X_i + \sum b_{ij} X_i X_j \quad (1)$$

where Y = hydrogen yield; X_i ($i = 1, 2, 3$) are the coded variables that represent the concentrations of ED, MG, and CA respectively; b_j ($j = 1, 2, 3$) are the regression coefficients; b_{ij} are the interaction terms.

3. Results and discussion

3.1. Preliminary experiments to determine range of salts concentration

Results showed that in the range of 0 mg/L–300 mg/L, MgSO₄ (MG) did not have any significant effects on lag phase, production rate, and hydrogen yield (data not shown). For CaCl₂ (CA), over the range of 0 mg/L to 15 mg/L studied, no significant changes in the lag phase, production rate, and hydrogen yield were observed (data not shown). Results indicated that in the range studied of 0 mg/L to 14 mg/L, Na₂EDTA (ED) had a significant effect on hydrogen production rate and yield, as can be seen in Fig. 1. The average production rates, calculated over the exponential phase, decreased from 0.088 mol/mol GL/day at 0 mg ED/L to 0.042 mol/mol GL/day at 14.0 mg/L. The maximum rate of production and hydrogen yield were observed at a concentration of ED of 3.5 mg/L. Kern et al. 1992 [10] showed that the addition of 0.5 mM (146 mg/L) EDTA supported the growth of *R. rubrum* and increased the H₂ production by three folds. The optimal concentration observed here is much lower, indicating that the optimal amount of Na₂EDTA might be strain dependent.

The results of the preliminary experiments seem to indicate that only ED would affect hydrogen production over the ranges studied. However, when considering the inhibition effect of ED observed at the high concentration of ED used when studying the individual effects of MG and CA on hydrogen production, we hypothesized that the inhibition effect of ED may have interfered with the MG and CA results. Ranges of 0–200 mg/L for MG, 0–10 mg/L for CA

Fig. 2. Comparison of results of the full factorial design obtained at the various combinations of the low (L), medium (M) and high (H) levels of ED-MG-CA.

Fig. 3. Comparison the effect of the amounts of Na_2HPO_4 (PH) and KH_2PO_4 (KP) in pure glycerol tested at various mass ratios PH/KP.

and 0–3.5 mg/L for ED were thus selected to perform the full factorial design allowing to study the main effects and the interaction effects of these three salts, as presented in the following section.

3.2. Optimization of concentration of salts

The results of the 9 runs with 2 replicates (blocks) of the factorial design were analysed using the software SAS 9.2 for the main effects and interaction effects of the three salts. The results of the two-way ANOVA are presented in Table 1. Amongst the three trace salts studied, only MG had a P -value less than 0.1 indicating a significant effect on hydrogen production. The results for MG are similar to those reported by Alshiyab et al. 2008 which indicated that $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ are all required for the production of biohydrogen by *Clostridium acetobutylicum* (NCIMB 13357) growing on glucose (5 g/L) [1]. On the contrary, the concentration of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ had no significant effect on hydrogen yield indicating again that the optimal concentrations seem to be highly dependent on the biological system used.

As hypothesized, a significant interaction effect ($P < 0.05$) was found between ED and MG, as shown in Table 1. The interaction of ED and MG can be explained by the different mechanisms of action of each compound in the metabolic conversion of glycerol by *E. aerogenes*. It can be hypothesized that MG is needed to supply Mg^{2+} (Magnesium ion) for growth rather than for hydrogen production. This hypothesis is supported by results reported by Alshiyab et al.

Fig. 5. Comparison of products formed using the reference conditions and the two sets of optimized conditions for the conversion of pure glycerol (PG) and crude glycerol (CG).

2008 [1] which indicated that an increase in the concentration of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ did not significantly increase hydrogen production but increased glucose consumption from 87% to 98%. Mg^{2+} may in the same way help in decreasing the lag phase, as observed in Fig. 2, which shows that the 4 combinations using high level of MG yielded a shorter lag phase than the 4 combinations without MG.

3.3. Optimization of phosphate buffer

Results presented in Fig. 3 showed that KP was not essential to *E. aerogenes* for hydrogen production while the amount of PH greatly affected the lag phase, production rate and yield. Results showed that increasing Na_2HPO_4 (PH) reduced the lag phase duration time from 3 to 2 days and increased rate of hydrogen production from 0.020 to 0.166 mol H_2 /mol GL/day.

3.4. Application of optimized conditions to crude glycerol

The reference conditions reported in our previous work [14] and the two sets of optimized conditions obtained in the previous sections using pure glycerol were tested for the conversion of crude glycerol (18.5 g/L, equivalent to 15 g/L GL) into hydrogen. To facilitate comparison, results obtained for pure and crude glycerol are presented in Fig. 4a and b, respectively. By comparing results

Fig. 4. Comparison of the results obtained using the reference conditions in literature [14] and the two set of optimized conditions for the conversion of (a) pure glycerol and (b) crude glycerol.

obtained in *Optimization I* (squares) to the reference conditions (diamonds), it is noted that optimizing the concentration of Na₂EDTA and MgSO₄ significantly reduced the second lag phase from 7 days to 4 days for both PG and CG. When comparing *Optimization II* (triangles) with *Optimization I* (squares), the results confirmed that while KH₂PO₄ was not needed, Na₂HPO₄ significantly increased the rate of production by preventing the second lag phase. The disappearance of the diauxic growth pattern following the optimization of the salts concentrations is still unexplained and a more thorough study of this phenomenon would be interesting. The results also indicated that CG (Fig. 4b) yielded better hydrogen yield and rate of production than pure glycerol (Fig. 4a) suggesting that other compounds present in crude glycerol supported the conversion of glycerol and the production of biohydrogen.

Fig. 5 presents results of the gas and liquid analysis and confirm that the main products formed are hydrogen and ethanol, as reported in Jitrwung and Yargeau [14], Seifert et al. [15], and Ito et al. [4]. These also indicate an almost complete conversion glycerol (residual concentration below the limit of detection) when using the conditions determined in *Optimization II*, for both PG and CG.

4. Conclusion

The three hypotheses of the studies were confirmed and the optimization of salts concentrations not only enhanced the hydrogen production but also decreased the salts requirements, leading to lower operating costs. The yields of biohydrogen and bioethanol, which were obtained using the optimized conditions for CG were approximately 0.84 and 0.79 mol/mol GL, respectively. Comparing with Seifert et al., 2009 [21] reporting 0.41 mol H₂/mol GL for a range of glycerol concentration of 5–30 g/L, Jitrwung and Yargeau 2011 [14] reporting the production of 0.85 mol/mol GL over 14 days after 2 days of lag phase, this optimization resulted in an equal or higher conversion, shorter lag phases and higher production rate. This study further demonstrates the potential of producing biohydrogen and bioethanol from crude glycerol produced through biodiesel manufacturing by *E. aerogenes*. However, challenges remain to scale-up and transfer these results to a continuous mode of operation required for industrial application.

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