Optimization of media composition for the production of biohydrogen from waste glycerol

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Abstract

Enterobacter aerogenes has a known ability to convert glycerol during a fermentative process to yield hydrogen and ethanol as the main products. A Box-Behnken design and response surface methodology were used to determine the optimal concentration of some media constituents and oxygen to maximize the yield of biohydrogen. Results indicated that the concentration of the salts studied: NH_4NO_3, $FeSO_4$, and Na_2HPO_4 and; the presence of oxygen in the pre-culture significantly influence the production of biohydrogen. Optimal conditions were determined to be 7.5% O_2 in the inoculum transfer step, ratio of inocula 18%, 8 g/L of Na_2HPO_4, 0.00625 g/L of $FeSO_4$ and 1.5 g/L of NH_4NO_3. These optimal conditions resulted in a measured yield of 0.85 mol H_2/mol glycerol at a substrate concentration of 15 g/L and a maximum predicted yield of 0.95 mol H_2/mol glycerol at a substrate concentration of 21 g/L. These results were obtained using lower concentrations of salts than in previous studies, corresponding to a 76% cost savings. These experimental results also demonstrated the importance of optimizing the amount of oxygen present in the biological system rather than maintaining complete anaerobic conditions.

Keywords: hydrogen, *Enterobacter aerogenes*, glycerol, Box-Behnken design

1 Introduction

Due to environmental concerns and decreasing fuel reserves, biodiesel is being increasingly used as a substitute for diesel oil. During biodiesel production, each mole of oil reacts with 3 moles of alcohols (such as methanol or ethanol) yielding 3 moles of biodiesel and 1 mole of glycerol. This increased availability of glycerol (GL) has resulted in a drastic decrease in its market value. In addition, the increased price of alcohol used in biodiesel production has had a negative impact on the cost-effectiveness of biodiesel production. As a result, there has

been an increase in research interest on ways to increase the value of glycerol-containing wastes over the past few years.

Anaerobic fermentative biohydrogen and bioethanol production from glycerol was evaluated by Yazdani et al. [1] who demonstrated that this approach would result in a decrease in bioethanol cost by about 37% when compared to conventional bioethanol production from corn. Several types of bacteria including: Klebsiella, Citrobacter, Clostridium, Escherichia, and Enterobacter species have been studied to determine their ability to convert glycerol into biohydrogen and bioethanol, as summarized in Table 1. Previous research by Ito et al. [2] showed that *E. aerogenes* is a strain leading to the highest H_2 and ethanol production (1.12 mole H_2/mole GL and 0.96 mole ethanol/mole GL [2]) and was therefore selected for this study. Despite the high yield of H_2 and ethanol obtained by Ito et al. [2], some challenges remain: the yield and rate of biohydrogen production must be further improved and the amount of costly compounds added to support the biological system must be minimized. As shown in Table 1, complex media composed of salts (phosphate, nitrate, sulfate, chloride, and others) and other nutrients such as organic and non-organic acids including yeast extract and tryptone have been used. Interestingly, nitrate salt has never been used in the studies summarized in Table 1 even though Zhou et.al. [3] showed that nitrate salts support bacterial growth. The presence of oxygen in the range of 0 to 72.7 mmol O_2/C-mol/h was also shown to influence the rate of production of hydrogen by *E. aerogenes* as well as the formation of by-products when using glucose as a substrate [4].

Accordingly, we proposed an integrated biodiesel/bioethanol/biohydrogen production scheme. In this integrated process, glycerol, the main by-product of biodiesel production, is mixed with media before being sent to a fermentation unit where glycerol is converted into ethanol, carbon dioxide, hydrogen and other minor by-products. This bioconversion would then not only produce hydrogen as clean energy to be used within the plant but would also generate ethanol that can be used as an alternative fuel or as a primary reactant in the biodiesel production process. The integrated process would improve the economical aspect of biodiesel production by using the ethanol produced as a substitute for methanol and by using or selling the hydrogen produced.

Considering the information reported in literature and with the objective of further improving the yield of hydrogen and ethanol produced from the conversion of glycerol by *Enterobacter aerogenes*, we designed an experimental plan to validate the following research hypotheses: 1. Replacing the commonly used ammonium sulfate by

ammonium nitrate will enhance cell growth and the conversion of glycerol into biohydrogen; 2. The concentration of salts such as $FeSO_4$ and Na_2HPO_4 can be reduced without sacrificing the biohydrogen production; 3. The availability of some oxygen and the inoculum volume influence the biohydrogen production; and lastly, 4. Results obtained with pure glycerol apply to crude glycerol conversion. Parametric studies and Box-Behnken experimental design models were used as tools to optimize the various factors studied (salts concentration, oxygen availability and volume of inoculum).

2 Materials and Methods

2.1 Microorganism and inocula preparation

Enterobacter aerogenes (ATCC 35029) was obtained from American Type Culture Collection (ATCC). The culture was started under aerobic condition in 100 mL of nutrient broth BD 234000 from Becton and Dickinson Company (12 g/L) and incubated at 37°C and 120 rpm for 20 to 24 hours to reach the stationary phase. Inocula were transferred into the nutrient broth contained in 250-mL serum bottles using a 5% inoculum ratio. These inoculum bottles were then incubated under semi-anaerobic conditions (initial dissolved oxygen concentration of 7.0 mg/L) at the same conditions as above until the stationary phase was reached. The initial level of dissolved oxygen was obtained by heating the broth at 50°C for 30 minutes and then cooling it over ice to 25°C before capping the bottles.

2.2 Media preparation and Biohydrogen production experiments

Otherwise specified, the glycerol-containing media was composed of the following constituents dissolved in deionised water: magnesium sulfate heptahydrate ($MgSO_4.7H_2O$: 0.2 g/L), calcium chloride ($CaCl_2$: 0.01 g/L), glycerol ($C_3H_8O_3$: 15 g/L) obtained from Sigma Aldrich, mono potassium phosphate (KH_2PO_4: 4 g/L), tetraethylenediamine disodium salts (Na_2EDTA: 0.014 g/L) obtained from Fisher Scientific, and variable concentration of selected salts to be optimized: ammonium nitrate (NH_4NO_3: 0 to 6 g), ferrous sulfate heptahydrate ($FeSO_4.7H_2O$: 0 to 0.02 g) obtained from Sigma Aldrich and disodium hydrogen phosphate (Na_2HPO_4: 0 to 8 g) obtained from Fisher Scientific. Pure glycerol was dissolved in deionized water in order to obtain a concentration of 40% w/w of glycerol. A 2-mL volume of the 40% glycerol solution was used for each

78 experiment, corresponding to a glycerol concentration of 15 g/L, which was selected based on preliminary
79 experiments. To reduce the amount of oxygen in the glycerol-containing media, the solution was boiled for 20
80 minutes, allowed to stand for 5 minutes, and then cooled on ice for 5 minutes with a continuous flushing of argon
81 in the headspace.

82 In order to perform the biohydrogen production experiments, inocula were transferred to glycerol-containing
83 media (18, 50 or 100 mL) placed in a 125-mL serum bottles, referred to as the experiment bottles. The transfer
84 was done using an aseptic syringe following the Hungate technique [17]. Inocula were taken from the inoculum
85 bottles, which were previously slightly over-pressurized with an argon/oxygen gas mixture of desired oxygen
86 composition (0, 50 and 100% oxygen to cover the entire range of concentrations). The inoculated experiment
87 bottles were placed in an incubator shaker at 37°C and 120 rpm until hydrogen production ceased. All results
88 reported here correspond to the yield of hydrogen obtained over the entire hydrogen production period.

89 *2.3 Preliminary optimization of oxygen levels and of inoculum volume*

90 The effect of oxygen concentration during pre-cultivation of the bacteria and during inoculation on the amount of
91 hydrogen produced was studied. The level of oxygen during pre-cultivation was varied by controlling the initial
92 amount of oxygen available in the inoculum bottle ($O_{2,B}$). The level of oxygen during inoculation was varied by
93 controlling the concentration of oxygen in the gas used during the transfer of the inoculum to the experiment
94 bottles ($O_{2,T}$). To vary the amount of oxygen available in the inoculum bottles, the ratio of the volume of media
95 added to the 125-mL serum bottle to the bottle volume was varied, resulting in media volumes of 18, 50 and 100
96 mL. The amount of oxygen in the inoculum transfer step was controlled by varying the concentration of oxygen in
97 the gas used to over-pressurize the inoculum bottles prior to the transfer of inocula to the experiment bottles. To
98 simultaneously study the effect of oxygen levels and the volume of inoculum on the production of hydrogen, a
99 Box-Behnken experimental design with three independent variables, described in Table 2, was used. Each
100 condition was performed in duplicate using the media described in section 2.2.

101 *2.4 Parametric studies for the determination of selected ranges of salts concentration*

102 To determine the range of concentration of the selected salts to be used in the second optimization study, the
103 media described in section 2.2 was prepared using different concentrations of ammonium nitrate (NH_4NO_3: 0 to 6

104 g), ferrous sulfate heptahydrate ($FeSO_4.7H_2O$: 0 to 0.02 g) and of disodium hydrogen Phosphate (Na_2HPO_4: 0 to
105 8 g).

106 *2.5 Optimization of oxygen concentration, volume of inoculum and selected salts concentration*

107 For the optimization of the concentration of the selected salts, the volume of the inocula and the effect of oxygen
108 concentration, a Box-Behnken experimental design with five independent variables, described in Table 3, were
109 used. Each condition was performed in duplicate.

110 For predicting the optimal conditions, the Software SAS 9.2 obtained from the company SAS was used to fit a
111 quadratic equation to correlate relationship between variables and response (i.e. volume of hydrogen). A
112 complete description of the process behaviour requires a quadratic or cubic model [18]. The quadratic correlation
113 was estimated as the following equation:

114 $$Y = b_0 + \Sigma b_i X_i + \sum b_{ii} X_i^2 + \Sigma b_{ij} X_i X_j \quad (1)$$

115 where Y is the response; X_i, and X_j are the uncoded independent variables, b_0, b_i, b_{ii}, and b_{ij} are the intercept,
116 linear, quadratic and interaction constant coefficients, respectively.

117 *2.6 Validation of results using crude glycerol*

118 The optimal conditions determined in the previous sections were used to evaluate the potential conversion of
119 crude glycerol under these conditions and to compare with the results obtained using pure glycerol. Crude
120 glycerol was first filtered (vacuum filtration using filter paper P8 and P4 purchased from Fisher Scientific) and then
121 dissolved in deionised water in order to obtain stock solutions with concentrations of 20, 40, and 60% w/w of
122 crude glycerol. Similarly, pure glycerol was dissolved in deionized water in order to obtain stock solutions of
123 concentrations of 20, 40, 60 and 80% w/w of pure glycerol. A 2-mL volume of each concentration was used for
124 experiments which tested the effect of glycerol concentration on hydrogen production, resulting in crude glycerol
125 concentrations of 12, 18, and 28 g/L and pure glycerol concentrations of 7, 15, 23 and 34 g/L.

2.7 Analytical Methods

The volume of biogas produced was measured using a hypodermic syringe every 24 hours. Samples of the biogas were analyzed by gas chromatography to determine the concentration of hydrogen. A Hewlett Packard GC model 5890 equipped with a 6' molecular sieve column 8A maintained at a temperature of 80°C was used. Argon was used as the carrier gas at a flow rate of 3.0 mL/min and a thermal conductivity detector (TCD) was used. Samples of liquid were collected to monitor cell growth and to characterize the final composition of the liquid when biohydrogen production ceased. Cell growth was monitored by optical density measurements performed at 600 nm using an Evolution 300 UV-visible Spectrometer. In order to determine the residual solution composition, the liquid samples were centrifuged at 10,000 rpm for 10 minutes. The supernatant was then mixed with silver powder and centrifuged again at 5,000 rpm in order to remove chloride ions. The supernatant was collected for liquid chromatography, ion chromatography and inductively coupled mass spectrometry (ICP-MS) analysis. A Hewlett Packard 1050 High-Performance Liquid Chromatograph (HPLC) equipped with a Rezex ROA Organic Acid H^+ 8% 150x7.80 mm column and a refractive index detector (RI) HP1047A was used to measure the concentration of glycerol, 1,3-propanediol, pyruvate, lactate, acetate, formate, and ethanol. The mobile phase was 0.035M H_2SO_4 at a flow rate of 0.8 mL/min and a run time of 12 minutes. The column temperature was 65°C and the temperature of detector was 50°C. Organic anions, namely formate and acetate, and inorganic ions such as nitrite, nitrate, sulfate, and phosphate were monitored using ion chromatography (IC). An ion chromatograph (Metrohm model 820 IC) equipped with a Metrosep A supp7 250/4.0 mm-5µm column maintained at 45°C and a conductivity detector was used with 3mM Na_2CO_3 as a mobile phase.

3 Results and discussion

3.1 Preliminary optimization of level of oxygen in inoculum and of inoculum volume

Figure 1 presents the results of the 3^3 Box-Behnken design and Response Surface Methodology (RSM) used to optimize three independent variables: the level of oxygen in the inoculum bottles ($O_{2,B}$), the oxygen level used during the inoculum transfer ($O_{2,T}$), and the volume of inoculum (V_I). A comparison of the three graphs for oxygen concentration at 0, 50, and 100 percent indicated that the highest amount of hydrogen was obtained at 0% oxygen in the transfer step ($O_{2,T}$). Looking at the first graph on the left, based on a $O_{2,T}$ of 0%, the largest volume

of inoculum resulted in better results and the highest amount of oxygen in the inoculum bottle (ratio of 0) enhanced the hydrogen production.

Considering that the oxygen concentration range studied in the transfer step was large (0 to 100%) it was important to investigate whether the previous results predict an optimal production at strict anaerobic conditions or if semi-anaerobic conditions would suffice or may be better. To validate this hypothesis, repeated injections of oxygen-free argon gas into the inoculum bottle were used for multiple inoculum transfers from the same inoculum bottle, corresponding to a decreasing $O_{2,T}$ (20% and below as opposed to the range of 0 to 100% as presented in Figure 1). Figure 2a presents the amount of hydrogen produced in the experiment bottles inoculated from this inoculum bottle as a function of the cumulative amount of oxygen-free argon gas used in the transfer of inocula. The trend observed clearly indicates that the initial decrease in oxygen concentration was beneficial but that past a certain level, it had a detrimental effect. This indicates that a low concentration of oxygen is required but that oxygen-free conditions are not optimal. As a result, the concentration range used in the optimization study was 2.5 to 7.5% O_2 in the transfer step ($O_{2,T}$).

3.2 Determination of ranges of salts concentration for the optimization study

3.2.1 Ammonium salt

Many researchers have used $(NH_4)_2SO_4$ in the media used for biohydrogen production along with many other salts and nutrients as shown in Table 1. The objective of this parametric study was to identify the range of concentrations to use in the optimization study and to evaluate the possible replacement of $(NH_4)_2SO_4$ by NH_4NO_3. Figure 2b presents the results obtained using the same concentrations of both salts. The data clearly indicate that the use of NH_4NO_3 results in a higher hydrogen production when compared to the use of the same concentration of $(NH_4)_2SO_4$ and that an optimal point seems to be approximately 2 g/L. Based on these results, NH_4NO_3 was selected for the optimization study and the range of concentrations to study was set to 1.5 to 2.5 g/L.

3.2.2 Iron sulfate

Results obtained in the range of 0 g/L to 0.02 g/L of $FeSO_4$ indicate that the salt has a significant non-linear effect on biohydrogen production. Figure 2c, presents the results obtained both in the absence of oxygen and in the

presence of some oxygen (5%) in the inoculum transfer step and indicates that an optimal concentration of $FeSO_4$ is close to 0.005 g/L in both cases. These results also indicate a possible interaction between the two factors. From these results, the range concentration of $FeSO_4$ for the optimization study was set to 0.00375 to 0.00625 g/L.

3.2.3 Sodium phosphate

Results presented in Figure 2d indicated that Na_2HPO_4 is required to obtain a measurable amount of hydrogen and confirmed the existence of an optimal concentration within the range studied. Based on these results, a range of 4.0 to 8.0 g/L was selected for the optimization study.

3.3 Optimization of oxygen concentration, volume of inoculum and selected salts concentration

The five independent variables studied, consisting of % oxygen in the inoculum transfer ($O_{2,T}$), inoculum volume (V_I), amount of Na_2HPO_4 (PO_4), NH_4NO_3 (NO_3), and $FeSO_4$ (SO_4), were optimized for the yield of hydrogen and ethanol using a Box Behnken design and Response Surface Methodology (RSM). Figure 3, presenting the 95% prediction profile for each variable (grey area), summarizes the analysis of the 46 runs of the experimental design (performed in duplicate). Note that for each variable the middle line represents the average predicted values while the lower and upper lines represent the limit of range of the predicted values. The lower yield values reported here, as compared to some values on Figure 2, are explained by the fact that results for each variable are those obtained at the middle values of the range studied for each of the other variables, which don't correspond to the optimal conditions.

These results indicate that the percent of oxygen, Na_2HPO_4 and $FeSO_4$ significantly affect the amount of hydrogen and ethanol produced. The volume of inoculum has a slight influence and the concentration of NH_4NO_3 has a negligible effect on biohydrogen production, but a significant effect on ethanol production. For the most significant factor, the higher level of Na_2HPO_4, most probably acting as a buffer and balancing pH as acidic metabolites form, helped support growth and enhanced the production of hydrogen and ethanol. A higher concentration of $FeSO_4$ also enhanced the production of hydrogen and ethanol by providing iron and oxygen to the cells. A small amount of oxygen in the inoculum transfer step significantly enhanced the hydrogen and ethanol production while slightly more inhibited it. This confirms the trend observed in Figure 2a indicating that complete

anaerobic conditions are not desirable. We hypothesize that *E. aerogenes* uses this small amount of oxygen while adjusting to the new media in order to proliferate in the new environment. This hypothesis is supported by a study conducted by Tanisho et al. [19] reporting that *E. aerogenes* quickly respond to dissolved oxygen. *E. aerogenes* consume externally-supplied, gaseous oxygen before using oxygen from the decomposable salts (NO_3^- SO_4^{2-}, and PO_4^{3-}). This is also supported by results presented in Figure 2b which show that *E. aerogenes* utilize NO_3^- more readily than SO_4^{2-}, when provided as ammonium salts. These results also agree with a study performed by Zhou et al. [3] which showed that *E. aerogenes* utilize NO_3^- obtained from NH_4NO_3 (or $NaNO_3$) anaerobically more readily than aerobically. In addition, they found that NH_4^+ and NO_3^- consumption are heavily supported by metal ions such as Fe^{2+}. This was also observed in this study as presented in Figure 2c which indicates that without Fe^{2+}, obtained from $FeSO_4$, low hydrogen production is observed.

Results from the optimization clearly showed that a small amount of oxygen is required in the pre-culture in order to increase the hydrogen and ethanol production by *E. aerogenes*. The optimized conditions for pure glycerol (PG) were determined using the Box-Beknhen design and are: 8 g/L Na_2HPO_4, 0.00625 g/L $FeSO_4$, 1.5 g/L NH_4NO_3, an inoculum volume of 9.4 mL (18% vol inoculum) and 7.5% of oxygen in the gas used in the inoculum transfer step. The maximum yields obtained with the conditions tested (15 g/L of glycerol) were 0.78 mol ethanol/mol GL and 0.67 mol H_2/mol GL and can be compared with results from Ito et al [2] who report values of 0.67 mol ethanol/mol GL and 0.71 mol H_2/mol when using 10 g/L glycerol.

3.4 Comparison of yields using crude glycerol

A comparison between the results obtained with pure glycerol (PG) and crude glycerol (CG) using the optimized salts concentrations and oxygen concentrations was performed. The results are shown in Figure 4 and indicate that over the 30 days of the experiment, two production phases occurred. Overall, CG (18 g/L) yielded hydrogen at 0.85 mole/mole GL, which is higher than the value obtained with PG (15 g/L), 0.67 mole/mole GL. Similar initial lag phases (λ, measured in days) were obtained but a longer second lag-phase time was observed for pure glycerol. Using the modified Gompertz equation [20] to model the hydrogen production, the second lag-phase was 7 and 9 days for CG and PG, respectively. There was also a significant difference in the rates of production (R_m, measured in mole/ mole GL/day). Again using the Gompertz equation, it was determined that CG had primary and secondary production rates of 0.2037 and 0.0550 respectively, while PG had rates of 0.1334 and 0.0112,

231 respectively. The CG rates were therefore 1.5 and 5 times higher than PG rates. The shorter lag-phase and faster
232 reaction rate might be explained by the presence of other organic compounds such as free fatty acids and non-
233 glycerol organic matter which may support the growth of bacteria during the second lag phase. Similarly, Seifert et
234 al. [14] obtained a higher biohydrogen production using crude glycerol from biodiesel production as opposed to Ito
235 et al. [2] who reported that residual compounds in crude glycerol inhibited microbial production of hydrogen. The
236 addition of yeast extract and tryptone by Ito et al. may have resulted in an over-supply of organic compounds in
237 the system, as suggested by Thompson et al. [21], and may explain the different results. Analysis of the liquid
238 phase for the various products resulting from the glycerol conversion: pyruvate, lactate, acetate, ethanol,
239 methanol and H_2 (Figure 5) indicate similar production levels and provide no insight on explaining the increased
240 yields obtained using crude glycerol. However, again it indicates that there is a significant production of ethanol
241 which is worth investigating further.

242 **3.4.1 Effects of Glycerol concentration**

243 The effect of glycerol concentration on the yield of hydrogen was also studied for both pure and crude glycerol.
244 The results reported in Figure 6 indicate that concentrations above 12 g/L crude glycerol gives better results and
245 that different optimal concentrations exist for each type of glycerol; approximately 17 g/L for pure glycerol and 21
246 g/L for crude glycerol (no measured values available at these concentrations). Using the Gompertz equation to
247 model the hydrogen production from crude glycerol presented in Figure 6, the predicted maximum hydrogen yield
248 would be 0.95 mole/mole GL at a glycerol concentration of 21 g/L. Fabiano and Perego [20] observed a similar
249 effect of glycerol concentration on the amount of hydrogen produced although Ito et al [2] reported a decrease in
250 hydrogen production with an increase glycerol concentration. These results support the hypothesis that other
251 compounds present in crude glycerol better supported the microbial system and might have contributed to the
252 hydrogen production. However, an excess of these organic compounds has a sharper inhibition effect on
253 biohydrogen production than glycerol alone as revealed by the steeper decreasing slope past the optimal point
254 (Figure 6).

255 Importantly, the optimization performed here was conducted using a less complex media that the one used in
256 most previous research. By comparison with Ito et al. [2] who reported the highest yield for pure and crude
257 glycerol converted by *E. aerogenes* (Table 1), the simplified media composition used in this study resulted in an

approximate media cost saving in $CAD/L of 76% without sacrificing the hydrogen and ethanol yields. The data used to estimate these costs are summarized in Table 4.

4 Concluding remarks

In this study, the concentration of three salts and the volume of inoculum were optimized and the importance of maintaining some oxygen in the pre-culture and inoculum transfer, rather than the conventionally used anaerobic conditions was demonstrated, in order to maximize the yield of hydrogen. We were successful in increasing the hydrogen yield from the previously reported maximum value of 0.71 [2] to a predicted value of 0.95 mol H_2/mol GL and 0.79 ethanol/mol GL for a glycerol concentration of 21 g/L in crude glycerol, while at the same time decreasing the cost of salts addition by 76%. In order to continue to improve the viability of biohydrogen processes, the rate of hydrogen production must be further increased. Other possible ways of increasing the rate of hydrogen production include optimizing the concentration of other salts and nutrients such as yeast extract, tryptone, or peptone, which have been customarily used by many researchers as summarized in Table 1 as well as optimizing the cell density. Another alternative would be to improve the hydrogen-capability of the strain used in such systems.

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Figure captions

Figure 1: Three-dimensional response plots for the hydrogen production at various levels of inoculum volumes and levels of oxygen in the inoculum transfer and inoculum volume

Figure 2: **(a)** H_2 production as a function of the cumulative volume of argon gas injected in the inoculum bottle prior to transfer of inoculums; **(b)** Comparison of hydrogen production using 0 g/L to 6 g/L of $(NH_4)_2SO_4$ (◆) and NH_4NO_3 (■); **(c)** Comparison of hydrogen production using 0 g/L to 0.02 g/L of $FeSO_4$ for two concentrations of oxygen in the inoculum transfer: 5% O_2 in Argon gas (■) and pure Argon (◆); **(d)** Hydrogen production using concentrations of Na_2HPO_4 in the 0 g/L to 8 g/L range (Error bar: standard deviation of three replicates)

Figure 3: 95% Prediction Intervals for the five factors studied (% oxygen ($O_{2,T}$), inoculum volume (V_I), concentration of Na_2HPO_4 (PO_4), NH_4NO_3 (NO_3) and $FeSO_4$ (SO_4))

Figure 4: Comparison of the cumulative hydrogen production (mole/mole GL) using pure glycerol (◆) and crude glycerol (■) at the same glycerol concentration of 15 g/L (Error bar: standard deviation of three replicates)

Figure 5: Comparison of metabolites produced using pure glycerol and crude glycerol obtained from biodiesel process at the same glycerol concentration, 15 g/L

Figure 6: Comparison of the hydrogen production using various concentrations of pure glycerol (PG) and crude glycerol (CG) obtained from biodiesel process

Figure 1

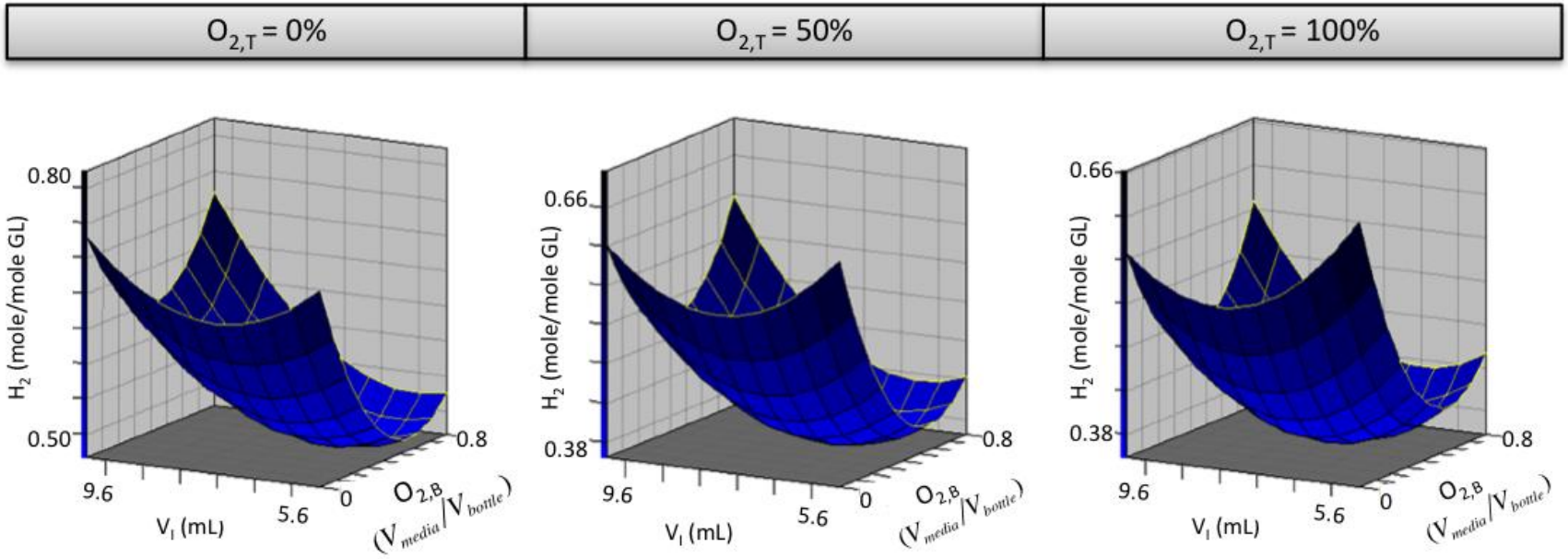
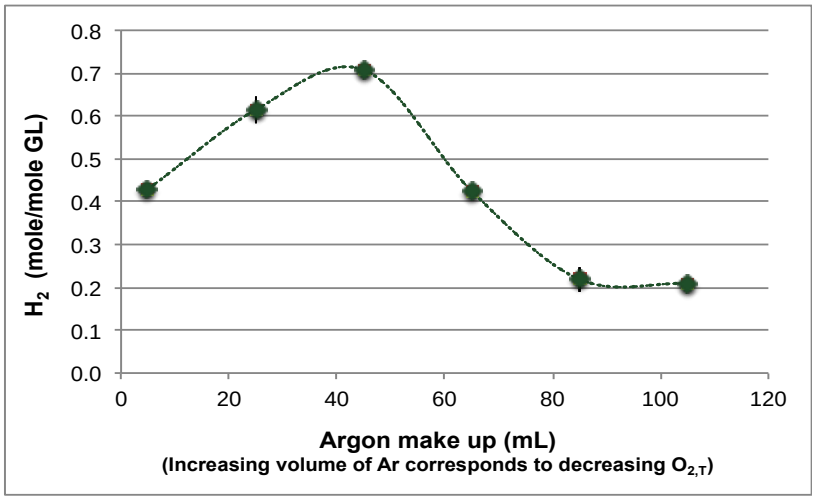
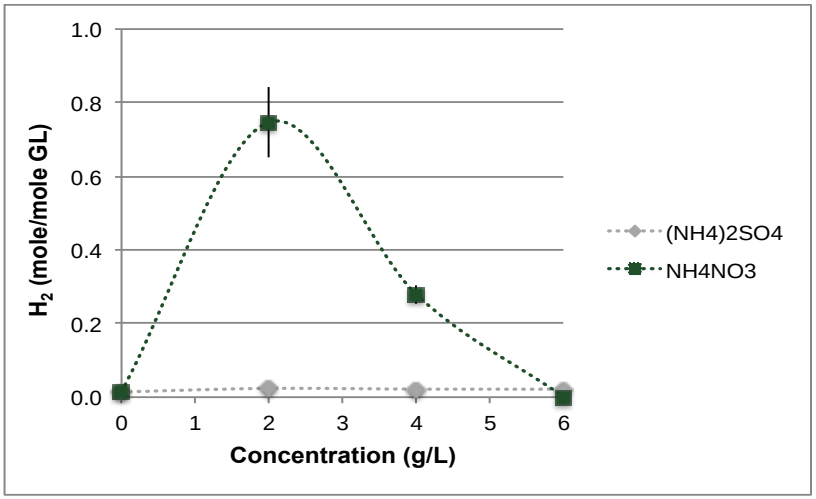

$O_{2,T}$ = 0%
$O_{2,T}$ = 50%
$O_{2,T}$ = 100%
H_2 (mole/mole GL)
0.80
0.50
0.66
0.38
0.66
0.38
9.6
5.6
V_l (mL)
0
0.8
$O_{2,B}$
(V_{media}/V_{bottle})

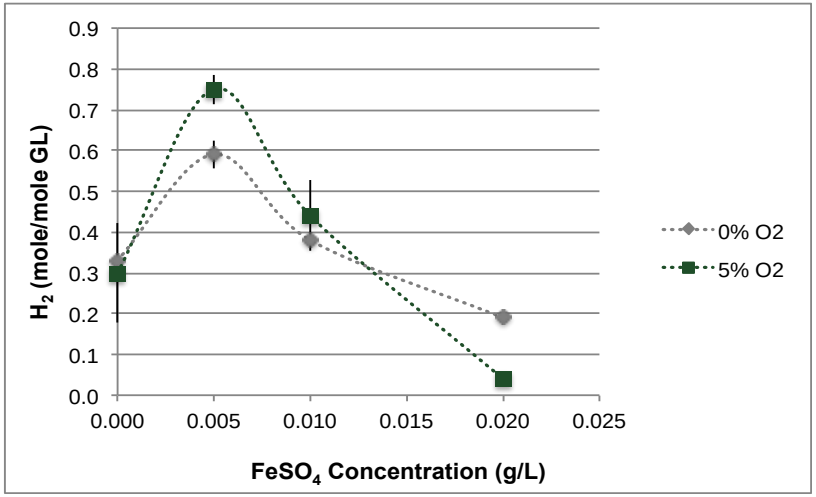
Figure 2



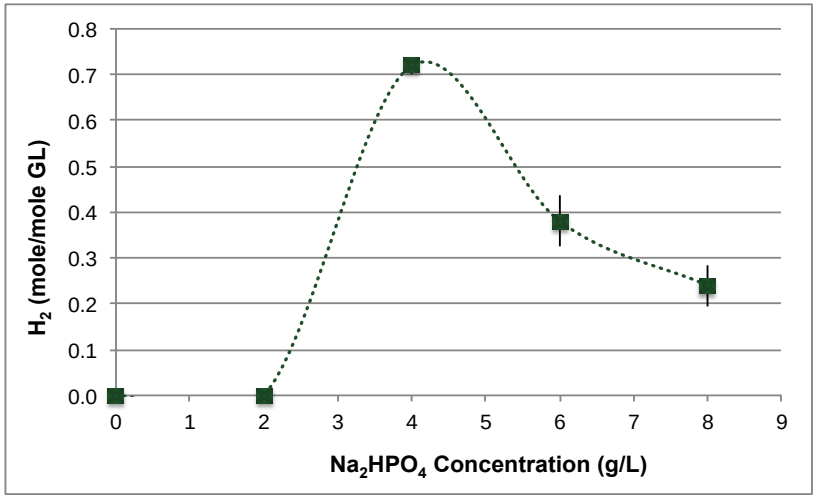
(a)



(b)



(c)



(d)

Figure 3

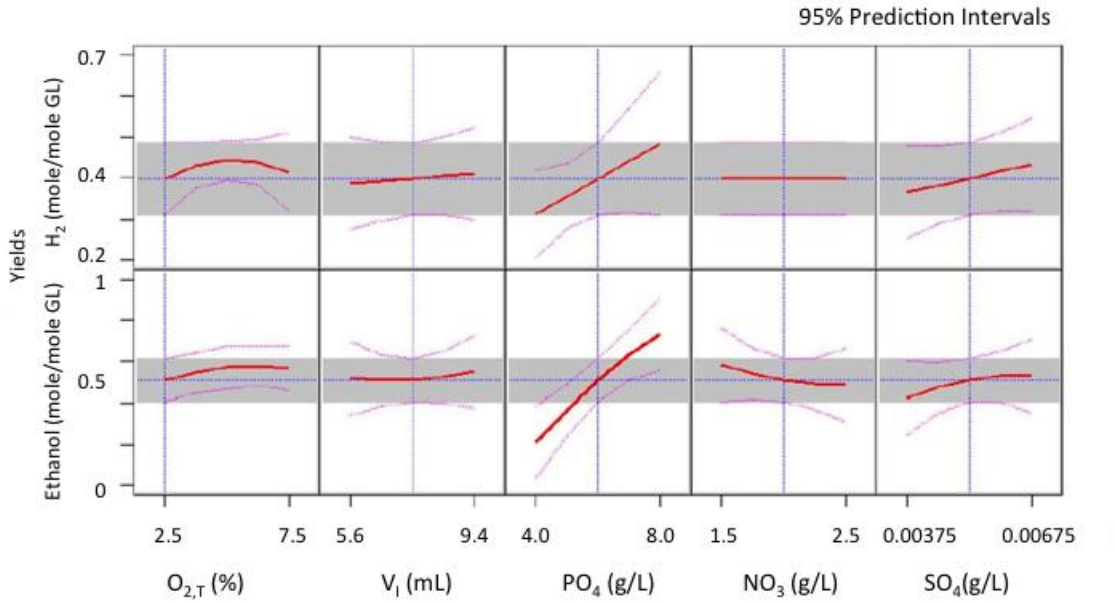

95% Prediction Intervals
Yields
H2 (mole/mole GL)
Ethanol (mole/mole GL)
0.7
0.4
0.2
1
0.5
0
2.5
7.5
5.6
9.4
4.0
8.0
1.5
2.5
0.00375
0.00675
O2,T (%)
Vi (mL)
PO4 (g/L)
NO3 (g/L)
SO4(g/L)

Figure 4

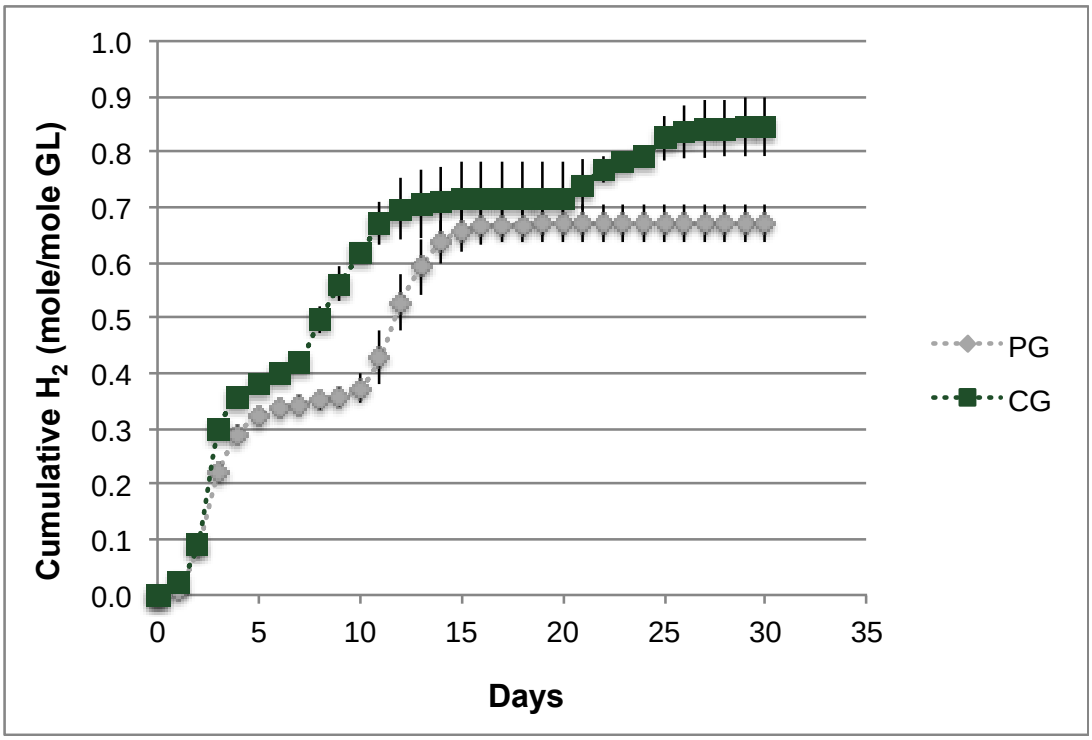

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Cumulative H_2 (mole/mole GL)
0
5
10
15
20
25
30
35
Days
PG
CG

Figure 5

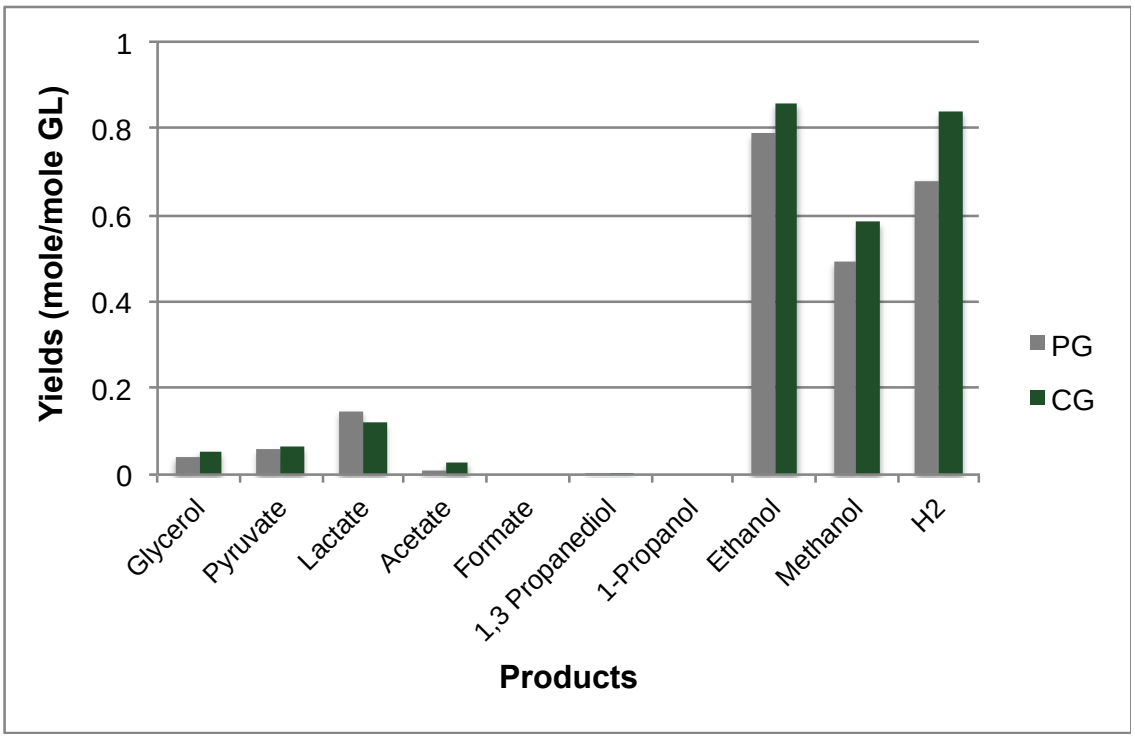

Yields (mole/mole GL)
1
0.8
0.6
0.4
0.2
0
Glycerol
Pyruvate
Lactate
Acetate
Formate
1,3 Propanediol
1-Propanol
Ethanol
Methanol
H2
Products
PG
CG

Figure 6

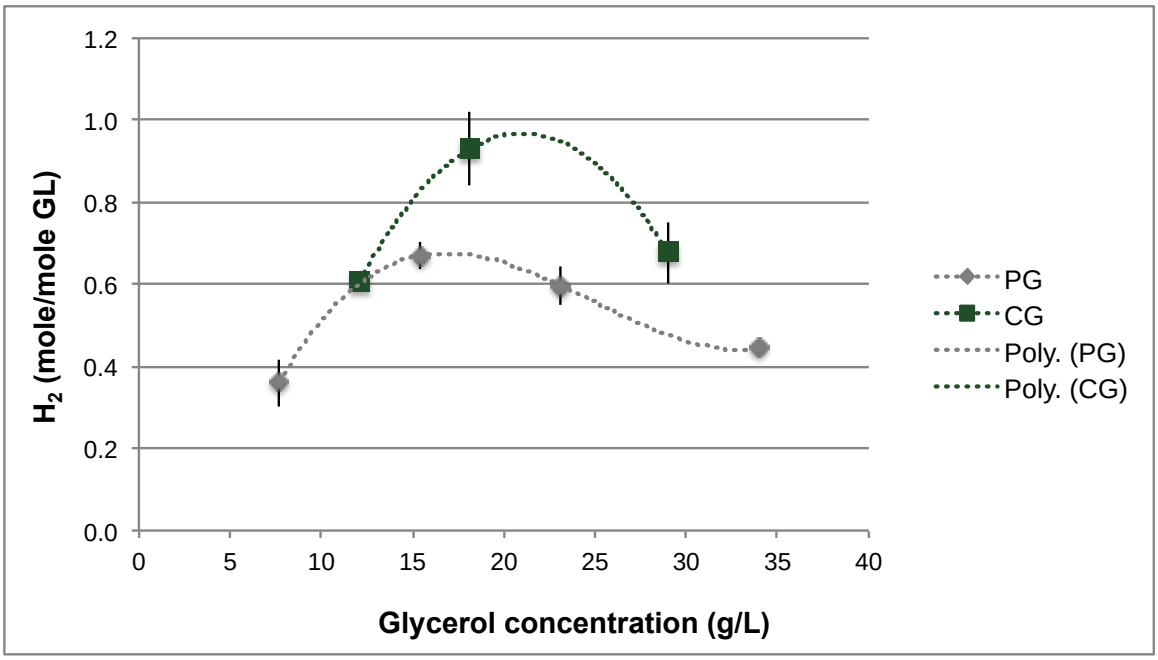

1.2
1.0
0.8
0.6
0.4
0.2
0.0
H_2 (mole/mole GL)
0
5
10
15
20
25
30
35
40
Glycerol concentration (g/L)
PG
CG
Poly. (PG)
Poly. (CG)

Table 1

Table 1: Comparison of media composition and hydrogen and ethanol yields for the conversion of pure glycerol and crude glycerol using various types of bacteria

Inoculums	Reference	Yields (mole/mole GL)		Glycerol (g/L)	Concentration (mg/L)*																			
					Organic acids				Organic compounds			Phosphate				Sulfate				Chloride					
		H2	ET		Nicotinic acid	Na_2EDTA	p-aminobenzoic acid	acetic acid	Yeast extract	Tryptone	Biotin	K_2HPO_4	KH_2PO_4	Na_2HPO_4	NaH_2PO_4	$(NH_4)_2SO_4$	$MgSO_4.7H_2O$	$FeSO_4.7H_2O$	$ZnSO_4.7H_2O$	$CaCl_2.2H_2O$	$NiCl_2$	NH_4CL	$MgCl_2.6H_2O$	$CaC_{l2}.2H_2O$	$CoCl_2.6H_2O$
PURE GLYCEROL																								
Enterobacter aerogenes	[2] Ito et al. (2005)	1.05	1.00	5-25	0.002				5	5		7	5.5			1	0.3			0.21	2.E-05				
Escherichia coli	[5] Murarka et al. (2008)	0.94	0.92	10										0.19				0.422				0.51	0.11	0.008	
Enterobacter aerogenes	**This Study**					0.004								12.2			0.2	0.006							
Mixed (municipal waste)	[6] Seifert et al. (2009)	0.41	NR	30					5			0.25	0.25				0.3					0.5		0.05	0.02
Mixed (wheat soil)	[7] Selembo et al. (2009)	0.28	0.06	3					5			4.58	2.45			2	0.2	0.005				13.8		1.5	
Klebsiella pneumoniae	[8] Solomon et al. (1994)	0.10	NR	15										1.38								5.35	0.26	2.9	
Mixed (waste water)	[9] Temudo et al. (2008a)	0.05	0.67	4.5		0.05							0.78					0.003	0			1.34	0.12	6.E-04	6.E-04
Clostridium acetobutylicum	[10] Gonzalez-Pajuelo et al. (2004)	0	NR	59			0.008	2	2		4.E-05	0.5	0.5				0.2	0.038				1.5			0.01
Clostridium pasteurianum	[11] Taconi et al. (2009)	NR	0.36	5					1			0.5	0.5			3	0.2	0.005						0.02	
Enterobacter agglomerans	[12] Barbirato et al. (1996)	NR	0.15	20					2			5	3			2	0.4							0.1	0.004
Klebsiella pneumoniae	[12] Barbirato et al. (1996)	NR	0.03	20					2			5	3			2	0.4							0.1	0.004
Clostridium freundii	[12] Barbirato et al. (1996)	NR	0.01	20					2			5	3			2	0.4							0.1	0.004
Clostridium butyricum	[12] Barbirato et al. (1996)	NR	0	20								3.4	1.3			2	0.2							0.02	
Clostridium pasteurianum	[13] Biebl (2001)	NR	0.02	30					1			0.5	0.5			3	0.2	0.005						0.02	
Clostridium butyricum	[14] Biebl (1991)	NR	NR	20.5					1			3.4	1.3			2	0.2	0.005						0.02	
Clostridium butyricum	[15] Saint-Amans et al. (1994)	NR	NR	25			0.008				4.E-02	0.55	0.55				0.2	0.010				1.65			0.01
CRUDE GLYCEROL																									
Enterobacter aerogenes	[2] Ito et al. (2005)	1.12	0.96	1.7	0.002				5	5		7	5.5			1	0.3				2.E-05			0.02	
Enterobacter aerogenes	**This Study**					0.004								12.2			0.2	0.006							
Mixed (municipal waste)	[6] Seifert et al. (2009)	0.71	NR	10					5			0.25	0.25				0.3					0.5		0.05	0.02
Klebsiella pneumoniae	[16] Liu and Fang (2007)	0.53	NR	20.4					3			3.4	1.3			2	0.2	0.005							
Mixed (wheat soil)	[7] Selembo et al. (2009)	0.31	NR	3					3					4.58	2.45	2	0.2	0.005				13.8		1.5	
Clostridium pasteurianum	[11] Taconi et al. (2009)	NR	0.23	5					1			0.5	0.5			2	0.2	0.005						0.02	
Clostridium acetobutylicum	[10] Gonzalez-Pajuelo et al. (2004)	NR	NR	59			0.008	2	2		4.E-05	0.5	0.5				0.2	0.038				1.5			

*Few other compounds not listed here were used in some studies

Table 2: Factors and levels used in the 3^3 Box-Behnken experimental design

FACTORS	LABEL	LOW LEVEL	CENTER POINT	HIGH LEVEL
Initial O_2 concentration in the inoculum bottle expressed as a volume ratio, media/bottle	$O_{2,B}$	0.15	0.4	0.8
Volume of inoculum (mL)	V_I	5.6	7.5	9.4
O_2 level in the gas used to transfer inoculum (%)	$O_{2,T}$	0	50	100

Table 3

Table 3: Factors and levels used in the 3^5 Box-Behnken experimental design

FACTORS	LABEL	LOW LEVEL	CENTER POINT	HIGH LEVEL
O_2 level in the gas used to transfer inoculum (%)	$O_{2,T}$	2.5	5	7.5
Volume of inoculum (mL)	V_I	5.6	7.5	9.4
Na_2HPO_4 (g/L)	PO_4	4	6	8
NH_4NO_3 (g/L)	NO_3	1.5	2.0	2.5
$FeSO_4$ (g/L)	SO_4	0.00375	0.005	0.00625

Table 4

Table 4 Estimation of media cost for comparison between this study and Ito et al. [2]

Additives	Na2HPO4	K2HPO4	KH2PO4	(NH4)2SO4	NH4NO3	NH4Cl	K2SO4	FeSO4.7H2O	MgSO4.7H2O	MgCl2.6H2O	CaCl2.2H2O	NaCl	Na2EDTA	Na2MoO4.2H2O	Na2SeO3	NiCl2	Nicotinic a.	MOPS	Tricine	Yeast Extract	Tryptone	MnCl2.4H2O	CuCl2.2H2O	H3BO3	AlK(SO4)2	Na2EDTA	Cost of additives (CAD $/L of media)
$CAD/Kg*	$65	$160	$161	$79	$64	$83	$95	$149	$100	$242	$103	$43	$140	$276	$742	$638	$74	$1,190	$480	$61	$247	$184	$371	$51	$496	$140	
Ito et al. (2005) (g/L used)		7	5.5	1					0.25		0.02			0.12	0.0002	0.00002	0.002			5	5	0.5	0.001	0.1	0.01	0.5	$3.85
This study (g/L used)	12.2				1.5			0.01	0.2				0.004														$0.91

*Prices obtained from Sigma Aldrich Canada in 2010