Biological conversion of carbon monoxide to ethanol: Effect of pH, gas pressure, reducing agent and yeast extract

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Abstract

A two-level full factorial design was carried out in order to investigate the effect of four factors on the bioconversion of carbon monoxide to ethanol and acetic acid by *Clostridium autoethanogenum*: initial pH (4.75–5.75), initial total pressure (0.8–1.6 bar), cysteine–HCl·H_2O concentration (0.5–1.2 g/L) and yeast extract concentration (0.6–1.6 g/L). The maximum ethanol production was enhanced up to 200% when lowering the pH and amount yeast extract from 5.75 to 4.75 g/L and 1.6 to 0.6 g/L, respectively. The regression coefficient, regression model and analysis of variance (ANOVA) were obtained using MINITAB 16 software for ethanol, acetic acid and biomass. For ethanol, it was observed that all the main effects and the interaction effects were found statistically significant ($p < 0.05$). The comparison between the experimental and the predicted values was found to be very satisfactory, indicating the suitability of the predicted model.

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Keywords

CO–bioconversion; *Clostridium autoethanogenum*; Factorial design; Medium optimization; Waste gas.

1. Introduction

Biological conversion of waste gases containing carbon monoxide (CO) using acetogens offers a possibility through which waste can be efficiently utilized for generating valuable fuels like ethanol, butanol and hydrogen (Mohammadi et al., 2011 and Munasinghe and Khanal, 2010). Different bioreactors can be used for (waste) gas treatment or bioconversion (Abubackar et al., 2011a and Kennes et al., 2009). However, one major bottleneck for the commercialization of this technique is the poor aqueous solubility of carbon monoxide gas. Hence, for systems containing CO as sole substrate, the bioconversion process is limited by the CO gas–liquid mass transfer at high cell concentration. Besides, the process is kinetically limited when either the cell concentration or the CO consumption rate is too low (Abubackar et al., 2011a). These rate-limiting conditions would decrease the process yield and CO–bioconversion process and are often encountered at some point in the bioconversion.

Homoacetogens able to produce ethanol from carbon monoxide include *Clostridium ljungdahlii*, *Clostridium carboxidivorans* $P7^T$, *Clostridium ragsdalei*, *Alkalibaculum*

bacchi, *C. autoethanogenum*, *Clostridium drakei*, and *Butyribacterium methylotrophicum*, among others (Liu et al., 2012 ; Mohammadi et al., 2011 and Mohammadi et al., 2012). These unicarbonotrophic bacteria follow the acetyl-CoA biochemical pathway or Wood-Ljungdahl pathway for cell growth and product formation (Abubackar et al., 2011a). Apart from ethanol, acetic acid is one of the prominent metabolites found during CO conversion using these microorganisms. In most of the previous studies, low ethanol to acetic acid ratios were generally obtained. However, by optimizing the medium composition and operating conditions, this ratio can be increased (Kundiyana et al., 2011a and Kundiyana et al., 2011b). In the present research, a microcosm study was performed using *C. autoethanogenum* as the biocatalyst.

C. autoethanogenum is a strictly anaerobic gram positive rod shaped (0.5 × 3.2 μm) bacterium, originally isolated from rabbit faeces using CO as the sole carbon and energy source. (Abrini et al., 1994). In one study, the authors used Plackett–Burman design to screen significant ethanol enhancing factors from the defined medium developed for *C. carboxidivorans*. Optimal levels of these significant factors were evaluated by central composite design (CCD) using a response surface methodology (RSM) and an artificial neural network-genetic algorithm (ANN-GA). It was concluded that an optimal medium containing (g/L) NaCl 1.0, KH_2PO_4 0.1, $CaCl_2$ 0.02, yeast extract 0.15, $MgSO_4$ 0.116 and NH_4Cl 1.694, at pH 4.74 could yield an ethanol concentration of around 0.25 g/L (Guo et al., 2010). Another research reported a concentration of 0.06–0.07 g/L with a 1:13 ethanol to acetate ratio in liquid-batch continuous syngas fermentation using a xylose adapted *C. autoethanogenum* culture (Cotter et al., 2009). These studies reveal the importance of medium composition in increasing the overall ethanol production. Hence, the different operating conditions still have to be optimized in order to enhance ethanol production and save on operating costs.

In the present research, *C. autoethanogenum* was used to convert bottled carbon monoxide gas into a valuable fuel product such as ethanol, and to investigate the effect of various process parameters on the bioconversion process, such as the initial pH, initial total pressure, cysteine–HCl·H_2O concentration and yeast extract concentration, and to obtain a reduced regression model that describes the process for products and biomass using a 2^4 full factorial design. In this manuscript, the authors simply called initial total pressure, cysteine–HCl·H_2O and yeast extract as “pressure”, “cysteine–HCl” and “YE”, respectively and in the tables and figures, initial pH as simply “pH”.

2. Methods

2.1. Microorganism and medium composition

C. autoethanogenum DSM 10061 was acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany), and was grown and maintained on DSMZ medium 640 with 0.5% xylose. The medium was prepared by boiling for a few minutes, while being degassed, and then cooled continuously under N_2 for 15 min to remove oxygen. Cysteine–HCl was added, and the pH of·the medium was adjusted to 6.0, by adding a small volume of either 2 M HCl or 2 M NaOH solutions.

2.2. Bioconversion studies

For batch experiments, serum vials with a total volume of 200 mL were used, with 75 mL working volume. The experimental set-up and the method used for media preparation are described elsewhere (Abubackar et al., 2011b). The culture was maintained under anaerobic conditions and agitated at 150 rpm on an orbital shaker, inside an incubation chamber at 30 °C. 10% of actively growing culture, which was grown with CO as sole substrate, was used as the inoculum and was aseptically transferred to each experimental vial. Headspace samples of 0.2 mL were used for CO measurements, and 1 mL of liquid sample was periodically withdrawn from the vials (once every 24 h) in order to measure the optical density ($OD_{\lambda=600\ nm}$) related to biomass concentration. The same 1 ml sample was then centrifuged for 10 min (25 °C, 7000×*g*) and the supernatant was used to check both ethanol and acetic acid concentrations.

2.3. Analytical equipment and measurement protocols

Gas-phase CO concentrations were measured using an HP 6890 gas chromatograph equipped with a thermal conductivity detector. The GC was fitted with a 15 m HP-PLOT Molecular Sieve 5A column (ID: 0.53 mm, film thickness: 50 μm). The oven temperature was initially kept constant at 50 °C, for 5 min, and then raised by 20 °C min^{-1} for 2 min, to reach a final temperature of 90 °C. The temperature of the injection port and the detector were maintained constant at 150 °C. Helium was used as the carrier gas. The water-soluble products, acetic acid and ethanol, in the culture broth were analyzed using a HP-5890 Series II GC equipped with a flame ionization detector and a 0.25 mm (ID) × 30 m HP-INNOWax capillary column (Agilent Technologies, Forster, CA, USA). Helium was used as the carrier gas. The oven temperature was held at 80 °C for 2 min, then heated to 160 °C at a rate of 10 °C min^{-1}, and maintained thereafter at 160 °C for 1 min. The injector and detector temperatures were kept constant, at 220 and 260 °C, respectively. Cell mass was estimated by measuring sample absorbance at a wavelength of 600 nm using a UV–visible spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain). The measured absorbance was then compared to the previously generated calibration curve, to calculate the corresponding biomass concentration (mg/L).

2.4. Experimental design and statistical analysis

A two level four factor (2^4) full factorial experimental design was used to study the combined effects of initial pH (low 4.75 and high 5.75), initial total pressure (low 0.8 bar and high 1.6 bar), cysteine–$HCl{\cdot}H_2O$ concentration (low 0.5 g/L and high 1.2 g/L) and yeast extract concentration (low 0.6 g/L and high1.6 g/L) on products formation (ethanol and acetic acid) and culture stability during the carbon monoxide bioconversion process by *C. autoethanogenum*. Of particular interest for optimizing ethanol production as a biofuel; this study was focused on estimating the optimum range of these parameters that enhances ethanol production.

The software package Minitab 16 (Minitab Inc. State College, PA, USA) was used to design the experiments and for data analysis in the form of analysis of variance (ANOVA). The response variables (*Y*) that were analyzed were the maximum products concentrations (g/L) and biomass concentration (mg/L) obtained from the different experimental trials.

3. Results and discussion

Some of the main parameters that affect the CO–bioconversion process are pH, mass transfer, reducing agent concentration and YE concentration (Mohammadi et al., 2011). The design matrix in uncoded values and the observed and predicted values of the responses are presented in Table 1. Three experiments were performed at central points in replication for an estimation of the variance (experimental error) of an effect. Using the least square technique with Minitab, the individual and interaction effects of the parameters can be approximated to a linear regression model. For 95% confidence level, the *p*-value, the probability value that is used to determine the statistical significance of the effects in the model should be less than or equal to 0.05 for the effect to be statistically significant.

Table 1.

2^4 Factorial design of experiments for ethanol, acetic acid and biomass production in the study.

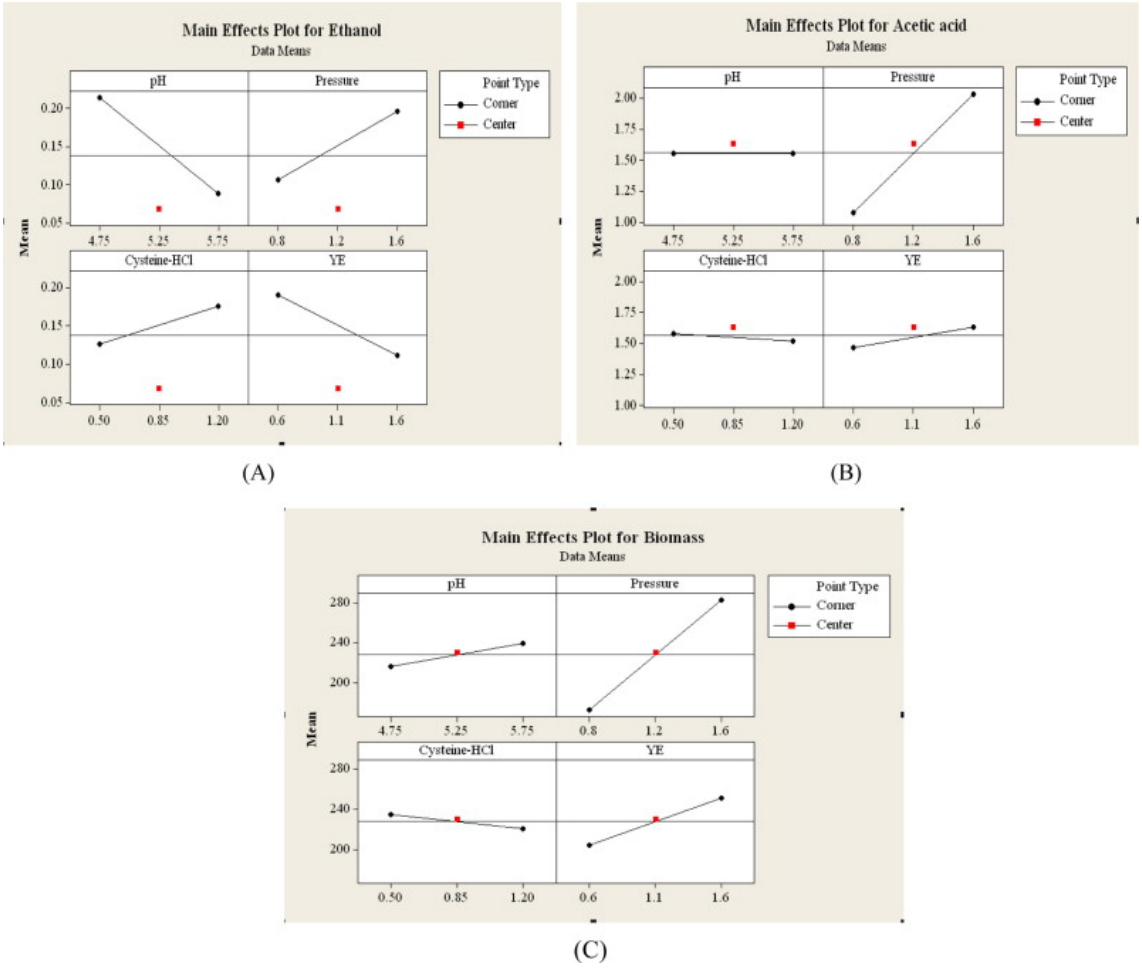
Run No.	pH	Pressure (Bar)	Cysteine–HCl (g/L)	YE (g/L)	Ethanol production (g/L)		Acetic acid production (g/L)		Biomass production (mg/L)	
					Observed	Predicted	Observed	Predicted	Observed	Predicted
1	4.75	0.8	0.5	0.6	0.115677	0.11568	0.930341	0.9428	141.9	145.99
2	5.75	0.8	0.5	0.6	0.072725	0.07272	0.933748	0.9462	159.02	144.69
3	4.75	1.6	0.5	0.6	0.278010	0.27802	1.950899	1.9558	259.63	253.25
4	5.75	1.6	0.5	0.6	0.080230	0.08026	2.145072	2.15	291.13	302.27
5	4.75	0.8	1.2	0.6	0.141760	0.14176	0.848527	0.8434	161.47	154.47
6	5.75	0.8	1.2	0.6	0.095745	0.09576	1.238078	1.2328	172.17	153.17
7	4.75	1.6	1.2	0.6	0.649213	0.64922	1.66778	1.6552	187.78	216.77
8	5.75	1.6	1.2	0.6	0.090824	0.09082	2.040535	2.0282	263.30	265.79
9	4.75	0.8	0.5	1.6	0.106121	0.10612	0.999329	0.987	175.84	189.71
10	5.75	0.8	0.5	1.6	0.089211	0.0892	1.132418	1.12	221.10	238.73
11	4.75	1.6	0.5	1.6	0.192213	0.19222	2.220619	2.2156	326.00	302.33
12	5.75	1.6	0.5	1.6	0.077787	0.07778	2.330991	2.3258	303.36	301.03
13	4.75	0.8	1.2	1.6	0.155645	0.15564	1.270428	1.2756	153.52	153.23
14	5.75	0.8	1.2	1.6	0.070136	0.07016	1.231282	1.2362	197.25	202.25
15	4.75	1.6	1.2	1.6	0.070065	0.07006	2.521777	2.5342	320.49	310.81
16	5.75	1.6	1.2	1.6	0.130568	0.13058	1.354848	1.3672	310.09	309.51

3.1. Main effects plot

Fig. 1 shows the main effects plot for the responses. From the main effects plot for ethanol, it is observed that increasing the initial pH and higher YE concentrations had a negative effect on ethanol production, whereas increasing initial pressure and cysteine–HCl concentration had a positive effect. These fermentation results are consistent with the trend observed in some other CO–bioconversion studies suggesting that lowering the pH and YE concentration results in the production of more reduced compounds such as ethanol (Barik et al., 1988 and Phillips et al., 1993). The product spectrum shifted

from acidogenic to solventogenic phase when lowering the medium's pH. This was proposed to be due to the following reason: the product, acetic acid, is a lipophilic weak acid and thus permeates through the cell membranes, resulting in a decrease in internal pH due to the conduction of H^+ ions from inside. At low internal pH, the external pH plays a major role in keeping the cell under non-stressed condition (Mohammadi et al., 2011). Hence, at both low external and internal pH, the cells under stress condition overcome the situation by producing solvents. Eliminating YE was found to enhance the ethanol production using *C. ljungdahlii* (Barik et al., 1988). However, for this organism to provide structural integrity, a minimum concentration of 0.01% is said to be necessary (Abubackar et al., 2011a). One potential bottleneck of CO–bioconversion is the mass transfer limitation due to the sparingly soluble nature of that substrate. Hence, one way to overcome this limitation is by increasing the pressure. In batch fermentation, different CO pressures mean different gaseous substrate concentrations which are directly proportional to the metabolite production and cell density. It was also observed that addition of reducing agents, thereby providing more electrons into the culture medium, will shift the microbial metabolism towards solventogenesis. This occurs due to availability of more reducing equivalents for the conversion of acetyl-CoA to products.

Fig. 1.



Main effects plot for (A) Ethanol, (B) Acetic acid and (C) Biomass.

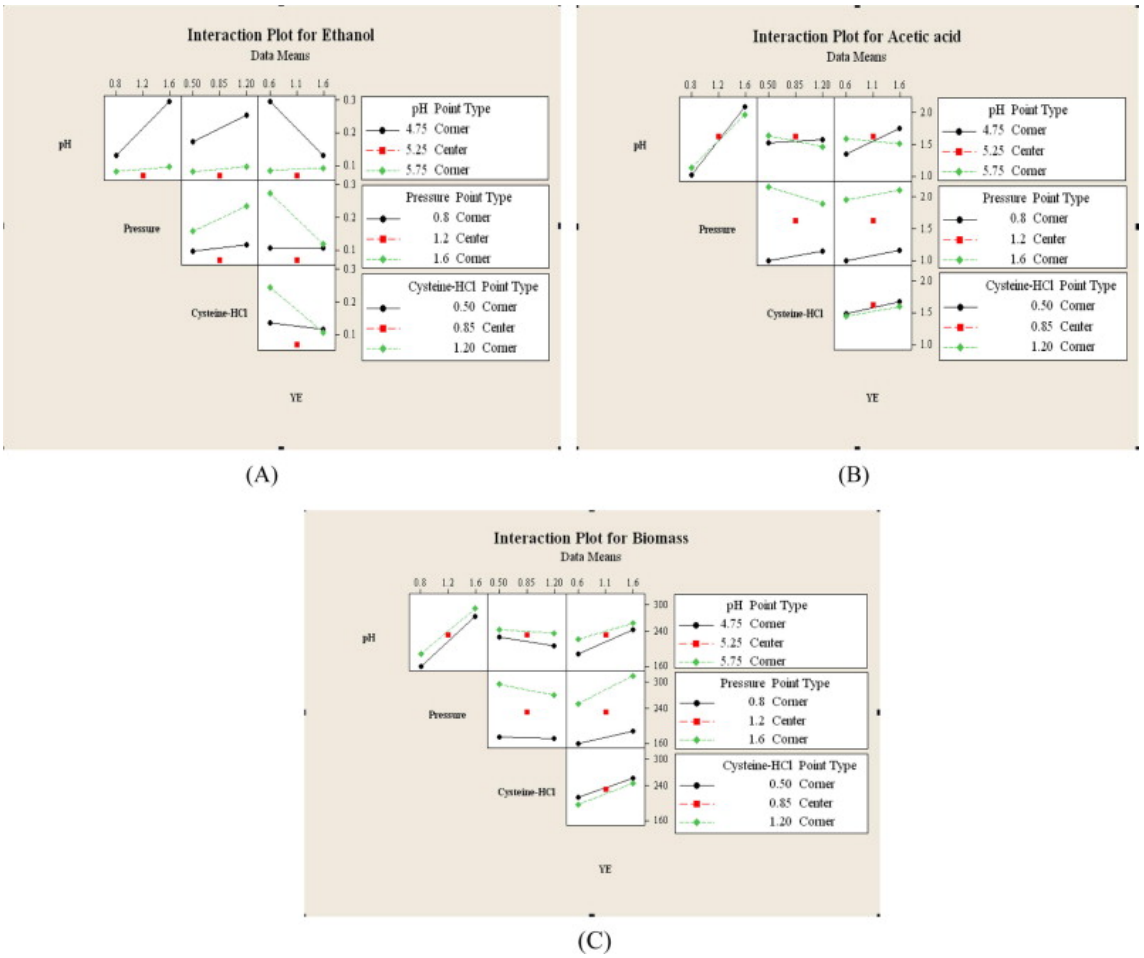
For acetic acid, it is evident that pH does not exert any effect on acetic acid production. Cysteine–HCl showed only a slight change in response across the studied level. This result is fairly consistent with the observation of Sim and Kamaruddin (2008), who studied the effect of cysteine–HCl on acetic acid production with *Clostridium aceticum* in a range of 0.1–0.5 g/L and found that the cysteine–HCl concentration was less significant. YE had a slightly positive effect on acetic acid production at high concentration. This may be due to the high cell growth achieved at increasing concentrations of yeast extract. Moreover, it has been reported that acetic acid is a growth-related product (Barik et al., 1988).

From the main effect plot for biomass, it is obvious that out of the four parameters studied, only increases in cysteine–HCl showed a slightly negative effect on biomass growth, whereas, increasing the other three factors had a strong positive influence on biomass. Since any organism shows its highest metabolic activity at its optimum pH, stepping down or stepping up in pH has a negative impact on cell growth. The optimum pH for growth of *C. autoethanogenum* is between 5.8 and 6.0 (Abrini et al., 1994). Hence, cell density increases proportionally when the pH is increased from 4.75 to 5.75. The reducing agent, cysteine–HCl, is essential for lowering the redox potential of the growth medium by scavenging the oxygen. However, a high amount of reducing agent is detrimental for cell growth and leads to a lower cell concentration (Sim and Kamaruddin, 2008). As YE provides nutrients for cell metabolism, an increase in the amount YE therefore increases the cell concentration.

3.2. Interaction effects plot

The interaction effects plots are shown in Fig. 2 and represent the mean response at all possible combinations of each two factors studied. If the two lines are non-parallel, it is an indication of interaction between the two factors.

Fig. 2.



Interaction effects plots for (A) Ethanol, (B) Acetic acid and (C) Biomass.

The interaction plot for ethanol showed that there is a strong interaction between each two factors. Whereas for acetic acid, only minor interactions were observed for YE with pressure and with cysteine–HCl. Also, no remarkable interactions between the pairs of factors were seen for biomass production. When the initial medium pH was 5.75, the maximum ethanol production was close to 0.1 g/L, same at low and high level of each other factors, describing the importance of low initial medium pH for increasing ethanol production. It is possible that higher amounts of carbon substrate are channeled towards the cell mass at high (+) level of pH. A higher amount of ethanol was observed at a pressure of 1.6 bar, for both concentrations of cysteine–HCl and YE, than at a pressure of 0.8 bar. A high amount of ethanol was also found to be produced for a higher cysteine–HCl concentration of 1.2 g/L at both levels of each other factors. In fact a slight reduction in ethanol production was observed at a YE concentration of 1.6 g/L compared to ethanol produced for a cysteine–HCl concentration of 0.5 g/L at 1.6 g/L of yeast extract.

At high (+) YE concentration level, an increase in pressure from 0.8 to 1.6 bars leads only to a very minor improvement in ethanol production and increases significantly acetic acid and biomass concentrations, showing the importance of lowering the YE concentration for improving ethanol production. Even though growth ceases at low pH and low YE concentration, it can easily be observed from the interaction plot that there

is around 200% improvement in ethanol production under such condition. Interaction between total pressure and cysteine–HCl, at their highest concentrations, has a positive influence on ethanol production and a negative effect on both acetic acid and biomass formation. Also, at low pressure, an increase in cysteine–HCl concentration does not make any major difference in their production. This can easily be interpreted by the fact that at a higher pressure, resulting in more supply of carbon substrate, an increment in reducing agent allows the microbes to use the additional carbon for producing highly reduced products.

3.3. Regression analysis and prediction of regression model

The statistical software was used to evaluate the observed experimental results to derive a regression function by using an ordinary least square method. Regression results determine the statistical significance, direction and magnitude of the relationship between an effect and the response. The sign of each regression coefficient indicates the direction of the relationship. Only the effects with low *p*-values are said to be statistically significant and can be meaningfully utilized in obtaining the regression function or model (Montgomery, 2005). A comparison between experimental values and the predicted values obtained using the regression equation is performed and satisfactory correlation was found between these values ($R^2 > 0.9$).

The regression models proposed are as follows:

Maximum ethanol production = $0.15100 - 0.06259\ A + 0.04512\ B + 0.02450\ C - 0.03953\ D - 0.03867\ AB - 0.01608\ AC + 0.04305\ AD + 0.01455\ BC - 0.03893\ BD - 0.02936\ CD - 0.00713\ ABC + 0.04473\ ABD + 0.02938\ ACD - 0.02703\ BCD + 0.03757\ ABCD.$

Maximum acetic acid production = $1.5510 - 0.0002\ A + 0.4780\ B - 0.0294\ C + 0.0817\ D - 0.0610\ AB - 0.0553\ AC - 0.1202\ AD - 0.1034\ BC - 0.0820\ ABC - 0.0828\ ABD - 0.1259\ ACD - 0.0272\ BCD - 0.0561\ ABCD.$

Maximum biomass production = $227.75 + 11.93\ A + 54.97\ B - 7.00\ \mathrm{C} + 23.20\ \mathrm{D} - 12.58\ ABD + 11.24\ BCD.$

These regression models are confined for each variable within the following range: (*A*) initial pH = 4.75–5.75, (*B*) pressure = 0.8–1.6 bars, (*C*) cysteine–HCl = 0.5–1.2 g/L and (*D*) YE = 0.6–1.6 g/L.

4. Conclusion

In this experimental range, higher ethanol production was favored by a lower pH and YE concentration and a higher pressure and cysteine–HCl concentration. A maximum ethanol concentration of 0.65 g/L was obtained under the following conditions: pH = 4.75 (the lowest value tested), pressure = 1.6 bar (the highest value tested), cysteine–HCl = 1.2 g/L (the highest value tested), and YE concentration = 0.6 g/L (the lowest value tested). Such maximum ethanol concentration is considerably higher than that achieved (0.06 and 0.25 g/L) with *C. autoethanogenum* in previous studies (Cotter et al., 2009 and Guo et al., 2010).

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References

Abrini et al., 1994
J. Abrini, H. Naveau, E.J. Nyns
Clostridium autoethanogenum, sp-nov, an anaerobic bacterium that produces ethanol from carbon-monoxide
Arch. Microbiol., 161 (1994), pp. 345–351

H.N. Abubackar, M.C. Veiga, C. Kennes
Biological conversion of carbon monoxide: rich syngas or waste gases to bioethanol
Biofuels Bioprod. Biorefin., 5 (2011), pp. 93–114

H.N. Abubackar, M.C. Veiga, C. Kennes
Bioconversion of carbon monoxide to bioethanol: an optimization study
C. Kennes, E.R. Rene, M.C. Veiga (Eds.), Biotechniques for Air Pollution Control IV, La Coruña, Spain (2011), pp. 347–351

S. Barik, S. Prieto, S.B. Harrison, E.C. Clausen, J.L. Gaddy
Biological production of alcohols from coal through indirect liquefaction
Appl. Biochem. Biotechnol., 18 (1988), pp. 363–378

J.L. Cotter, M.S. Chinn, A.M. Grunden
Influence of process parameters on growth of *Clostridium ljungdahlii* and *Clostridium autoethanogenum* on synthesis gas
Enzyme Microb. Technol., 44 (2009), pp. 281–288

Y. Guo, J. Xu, Y. Zhang, H. Xu, Z. Yuan, D. Li
Medium optimization for ethanol production with *Clostridium autoethanogenum* with carbon monoxide as sole carbon source
Bioresour. Technol., 101 (2010), pp. 8784–8789

C. Kennes, E.R. Rene, M.C. Veiga
Bioprocesses for air pollution control
J. Chem. Technol. Biotechnol., 84 (2009), pp. 1419–1436

K. Liu, H.K. Atiyeh, R.S. Tanner, M.R. Wilkins, R.L. Huhnke
Fermentative production of ethanol from syngas using novel moderately alkaliphilic strains of *Alkalibaculum bacchi*
Bioresour. Technol., 104 (2012), pp. 336–341

D.K. Kundiyana, M.R. Wilkins, P.B. Maddipati, R.L. Huhnke

Effect of temperature, pH and buffer on syngas fermentation using *Clostridium* strain P11
Bioresour. Technol., 102 (2011), pp. 5794–5799

D.K. Kundiyana, R.L. Huhnke, M.R. Wilkins
Effect of nutrient limitation and two-stage continuous fermentor design on productivities during ‘‘*Clostridium ragsdalei*’’ syngas fermentation
Bioresour. Technol., 102 (2011), pp. 6058–6064

M. Mohammadi, G.D. Najafpour, H. Younesi, P. Lahijani, M.H. Uzir, A.R. Mohamed
Bioconversion of synthesis gas to second generation biofuels: A review
Renew. Sus. Energy Rev., 15 (2011), pp. 4255–4273

Mohammadi, M., Younesi, H., Najafpour, G.D., Mohamed, A.R., 2012. Sustainable ethanol fermentation from synthesis gas by *Clostridium ljungdahlii* in a continuous stirred tank bioreactor. J. Chem. Technol. Biotechnol. 87, in press, http://dx.doi.org/10.1002/jctb.3712.

D.C. Montgomery
Design and analysis of experiments
(sixth ed.)Wiley and Sons, New York (2005)

P.C. Munasinghe, S.K. Khanal
Biomass-derived syngas fermentation into biofuels: opportunities and challenges
Bioresour. Technol., 101 (2010), pp. 5013–5022

J.R. Phillips, K.T. Klasson, E.C. Clausen, J.L. Gaddy
Biological production of ethanol from coal synthesis gas-medium development studies
Appl. Biochem. Biotechnol., 39 (1993), pp. 559–571

J.H. Sim, A.H. Kamaruddin
Optimization of acetic acid production from synthesis gas by chemolithotrophic bacterium – *Clostridium aceticum* using statistical approach
Bioresour. Technol., 99 (2008), pp. 2724–2735