Bioresource Technology 101 (2010) 8784–8789

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Medium optimization for ethanol production with *Clostridium autoethanogenum* with carbon monoxide as sole carbon source

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ARTICLE INFO

Article history:
Received 7 January 2010
Received in revised form 25 May 2010
Accepted 11 June 2010
Available online 8 July 2010

Keywords:
Syngas fermentation
Clostridium autoethanogenum
Response surface methodology
Artificial neural network
Genetic algorithm

ABSTRACT

Plackett–Burman and central composite designs were applied to optimize the medium for ethanol production by *Clostridium autoethanogenum* with CO as sole carbon source, and a medium containing (g/L): NaCl 1.0, KH_2PO_4 0.1, $CaCl_2$ 0.02, yeast extract 0.15, $MgSO_4$ 0.116, NH_4Cl 1.694 and pH 4.74 was found optimal. The optimum ethanol yields predicted by response surface methodology (RSM) and an artificial neural network-genetic algorithm (ANN-GA) were 247.48 and 261.48 mg/L, respectively. These values are similar to those obtained experimentally under the optimal conditions suggested by the statistical methods (254.26 and 259.64 mg/L). The fitness of the ANN-GA model was higher than that of the RSM model. The yields obtained substantially exceed those previously reported (60–70 mg/L) with this organism.

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1. Introduction

Lignocellulosic biomass can be gasified into syngas and subsequently converted into ethanol by chemical or microbiological means (Huber et al., 2006). Some acetogens, such as *Clostridium ljungdahlii*, *Clostridium autoethanogenum* and *Clostridium carboxidivorans*, can convert H_2, CO and CO_2 to ethanol and acids (Abrini et al., 1994; Liou et al., 2005; Rajagopalan et al., 2002; Tanner et al., 1993). Microbial fermentation has high end-product specificity and does not require the application of high temperatures and pressures usually needed when chemical catalysts are employed (Ahmed and Lewis, 2007; Cotter et al., 2009a,b; Datar et al., 2004; Rajagopalan et al., 2002). A unique feature of *C. autoethanogenum* is its ability to grow on xylose. This characteristic may prove useful when hydrolysis and gasification are combined with fermentation. A majority of studies have concentrated on *C. ljungdahlii* or *C. carboxidivorans*, while few researchers have studied the fermentation capability of *C. autoethanogenum*. So, *C. autoethanogenum* was selected to be the candidate in our work.

The end products of autotrophs like *C. autoethanogenum* are highly depended on the pH of the medium. Lowering the pH increased ethanol production, while elevating the pH induced growth of the autotrophs (Datar et al., 2004; Gaddy and Clausen, 1992; Phillips et al., 1993). Phillips et al. (1993) designed a medium for culturing *C. ljungdahlii*, and a defined medium formulation was developed by Rajagopalan et al. (2002); however, it is not known if these media are ideal for ethanol production since ethanol is mostly generated under non-growth conditions (Klasson et al., 1992a,b).

Plackett–Burman (Plackett and Burman, 1946), artificial neural network (ANN)-genetic algorithm (GA) and central composite designs (CCD) (Box and Wilson, 1951; Hashimoto, 1997; Maier and Dandy, 2000; Mcculloch and Pitts, 1949; Morimoto et al., 1997a,b) have been proved to be useful for evaluating the relative significance of variables and optimization of the target metabolites production. ANN-GA used in CCD can address any required degree of accuracy and does not need to consider continuity or differentiability of the objective function (Nagata and Chu, 2003). It has been demonstrated that ANN-GA is more accurate than response surface methodology (RSM) (Duan et al., 2006; Erenturk and Erenturk, 2007; Garcia-Gimeno et al., 2005; Huang et al., 2007; Izadifar and Jahromi, 2007; Singh et al., 2009; Wang and Wan, 2009).

Therefore, in this study, Plackett–Burman, ANN-GA and CCD were used to screen the significant factors in the defined medium developed by Rajagopalan et al. (2002) for their influence on ethanol production. Plackett–Burman design was applied to screen the significant factors from the defined medium. Central composite design using RSM and ANN-GA was employed to access the optimum concentrations of the significant variables selected through Plackett–Burman design.

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doi:10.1016/j.biortech.2010.06.072

2. Methods

2.1. Microorganism and medium

Clostridium autoethanogenum DSM 10061 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany) and revived in DSM medium 640 which contains 5 g/L of xylose. *C. autoethanogenum* was transferred to the enrichment medium developed by Rajagopalan et al.(2002) and incubated until the culture reached an OD_{600} of 1.2 (96–120 h) without shaking, at 37 °C. Cells were harvested by centrifugation at 12,000*g* for 10 min, resuspended and inoculated into the fermentation medium (as per experiment design Tables 1 and 2 10% of inoculum, v/v) and cultured at 37 °C with shaking at 150 rpm for 60 h to ensure all of the CO had been exhausted and maximum ethanol production was obtained (Barik et al., 1988; Sim et al., 2008).

For medium optimization, the pH was fixed between 4.5 (−1) and 5.0 (+1), NH_4Cl and KH_2PO_4 concentrations were set at 1.0 g (+1) and 0.1 g (+1) respectively, the $CaCl_2$ concentration of 0.02 was set as the minimum (−1), the amount of yeast extract was set between 0.15 (−1) and 0.3 (+1), and the $MgSO_4$ and NaCl concentrations were set at 0.3 g/L (+1), 0.15 g/L (−1) and 1.0 g/L (+1), 0.4 g/L (−1) respectively (Table 1) (Andersch et al., 1984; McNeil and Kristiansen, 1987). Concentrations varied according to the statistic matrix (Tables 1 and 2). Enrichment medium with xylose instead of CO was used to initiate growth of *C. autoethanogenum* before fermentation in serum bottles capped with rubber stoppers. Fermentation with CO as carbon source was carried out with 20 ml of medium in 100-ml infusion bags capped with rubber stoppers, CO/CO_2 (95/5, v/v), at a gauge pressure of 1 atm. The media were buffered with 10.0 g/L of morpholinoethanesulfonic acid (MES). The composition for trace metals and vitamins was previously described (Rajagopalan et al., 2002). 2 g/L of tryptase (Cotter et al., 2009a) was added to improve the ethanol production, 0.2 g/L cysteine-HCl (Sim et al., 2008) was used as reducing agent, and 0.5 mg/L resazurin was added as redox indicator. The final pH of the medium was adjusted with 1 M KOH or 1 M HCl. Before sterilization (20 min, 121 °C), the media were placed in an anaerobic chamber for 24–36 h. Vitamin solution and cysteine, sterilized by filtration, were added to the medium after autoclaving. All experiments were carried out in triplicate.

2.2. Extraction of ethanol and analytical methods

Cell free culture supernatant was obtained by centrifugation at 12,000*g* for 10 min at 4 °C and then frozen at 4 °C until analysis. Ethanol concentration was measured with a gas chromatograph (Agilent 6820) equipped with a flame ionization detector (FID) and a fused-silica capillary column (DB-FFAP, 30 m × 0.25 mm × 0.25 μm). Nitrogen was used as carrier gas at a flow rate of 30 mL/min and split ratio of 1/50.The injector and detector temperature were 250 and 300 °C, respectively. The initial oven temperature was 40 °C. After 5 min, the temperature was increased at a rate of 20 °C /min until it reached 140 °C, and then increased at a rate of 40 °C/min until it reached 250 °C.

2.3. Plackett–Burman design

The Plackett–Burman design was firstly employed to identify the significant variables on ethanol production from the main inorganic components, yeast extract and pH. According to Plackett–Burman design (Plackett and Burman, 1946), each variable is represented at two levels, high (+1) and low (−1). In this study, seven assigned variables together with four dummy variables were tested in 12 experiments. The effect of variables was firstly computed as followed:

$$E_{Vi} = \frac{\sum y_{Vi(+)} - \sum y_{Vi(-)}}{N/2} \tag{1}$$

where E_{Vi} represents the effect of variable *i*, $y_{Vi(+)}$ and $y_{Vi(-)}$ are the response of the high and low levels of variable *i*, respectively, *N* stands for number of trials.

And then the standard deviation (*SD*) of dummies was calculated by Eq. (2):

$$SD = \sqrt{\frac{\sum (E_d)^2}{n}} \tag{2}$$

where *SD* represents the effect of dummy variables, and *n* is the number of the dummy variables. *T*-test was finally performed as follows to determine the significance of these factors.

$$t_{Vi} = \frac{E_{Vi}}{SD} \tag{3}$$

Four dummy variables were studied in 12 experiments to calculate the standard error. R^2 (the coefficient of determination) was used to examine the fitness of the Plackett–Burman design. Average value of ethanol production was taken as the response. Variables with confidence levels above 90% were considered to have significant effect on ethanol production and thus were used for further optimization.

Table 1
The Plackett–Burman design for screening variables in ethanol production by using *Clostridium autoethanogenum*.

Trail	Levels	Factors NaCl (g/L)	NH_4Cl (g/L)	KH_2PO_4 (g/L)	$MgSO_4$ (g/L)	$CaCl_2$ (g/L)	Yeast extract (g/L)	pH	Ethanol (mg/L)
	1	1.000	1.000	0.100	0.300	0.040	0.300	5.00	
	−1	0.400	0.600	0.040	0.150	0.020	0.150	4.50	
1		−1	−1	−1	1	1	1	−1	38.1 ± 1.52
2		−1	1	−1	−1	−1	1	1	97.8 ± 4.31
3		−1	−1	1	1	1	−1	1	43.4 ± 1.35
4		1	−1	1	1	−1	1	−1	69.6 ± 3.12
5		1	−1	1	−1	−1	−1	1	96.0 ± 4.24
6		1	1	1	−1	1	1	−1	164.94 ± 5.67
7		1	1	−1	1	−1	−1	−1	143.10 ± 5.12
8		−1	1	1	−1	1	−1	−1	144.81 ± 5.01
9		1	−1	−1	−1	1	1	1	79.84 ± 4.31
10		−1	−1	−1	−1	−1	−1	−1	116.45 ± 3.62
11		−1	1	1	1	−1	1	1	36.62 ± 1.67
12		1	1	−1	1	1	−1	1	45.27 ± 2.14

Table 2
The experimental results together with RSM and ANN predictions of CCD design in the terms of ethanol yield.

Trail	Levels	Factors			Ethanol (mg/L)		
		NH_4Cl X_1 (g/L)	$MgSO_4$ X_2 (g/L)	pH X_3	RSM	ANN	Experimental
	1.68179	2.3409	0.1841	5.00			
	1.00000	2.0000	0.1500	4.80			
	0	1.5000	0.1000	4.50			
	−1.00000	1.0000	0.0500	4.20			
	−1.68179	0.6591	0.0159	4.00			
1		−1.00000	−1.00000	−1.00000	158.26	150.42	152.68 ± 4.12
2		1.00000	−1.00000	−1.00000	150.41	144.22	145.85 ± 5.07
3		−1.00000	1.00000	−1.00000	173.27	168.86	168.00 ± 4.19
4		1.00000	1.00000	−1.00000	234.88	241.08	242.62 ± 5.52
5		−1.00000	−1.00000	1.00000	210.04	200.15	202.29 ± 5.15
6		1.00000	−1.00000	1.00000	180.23	180.83	185.49 ± 4.01
7		−1.00000	1.00000	1.00000	199.85	205.45	204.40 ± 5.21
8		1.00000	1.00000	1.00000	239.49	237.29	245.07 ± 5.54
9		−1.68179	0.00000	0.00000	181.02	190.53	189.37 ± 4.75
10		1.68179	0.00000	0.00000	207.75	205.36	199.41 ± 3.35
11		0.00000	−1.68179	0.00000	140.06	151.58	147.57 ± 4.96
12		0.00000	1.68179	0.00000	202.51	197.24	195.01 ± 5.65
13		0.00000	0.00000	−1.68179	197.78	203.86	202.34 ± 4.87
14		0.00000	0.00000	1.68179	245.20	246.42	240.65 ± 5.09
15		0.00000	0.00000	0.00000	235.57	233.54	235.91 ± 3.72
16		0.00000	0.00000	0.00000	235.57	233.54	235.83 ± 3.65
17		0.00000	0.00000	0.00000	235.57	233.54	235.62 ± 3.85
18		0.00000	0.00000	0.00000	235.57	233.54	235.46 ± 4.21
19		0.00000	0.00000	0.00000	235.57	233.54	235.31 ± 3.97
20		0.00000	0.00000	0.00000	235.57	233.54	235.29 ± 4.43

2.4. Central composite design

A five-variable central composite design was used to optimize important valuables selected by the Plackett–Burman design. Central composite design (Box and Wilson, 1951) consists of a 2^k full factorial design, $2k$ axial designs at a distance α from the origin, and m center points, where k is the number of valuables and m is more than 1. Eq. (4):

$$X_i = \frac{(A_i - A_0)}{\Delta A} \tag{4}$$

describes the relationship between the coded values and actual values, where X_i represents coded value, A_i is the actual value, A_0 is the actual value of the variables at the centre point, and ΔA is the step change.

2.4.1. Response surface methodology

The statistical technique, Response Surface Methodology, was employed to optimize the screened variables, and the relationship between variables and responses was expressed by a second order polynomial Eq. (5):

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_i\beta_j X_i X_j \tag{5}$$

where Y is the predictive response; β_0 is offset term; X_i, and X_j stand for the independent variables; β_i, β_{ii} and β_{ij} represent regression coefficients of the model.

2.4.2. Artificial neural network and genetic algorithm

ANN model together with GA was also employed to access the optimum concentration. The variables selected through Plackett–Burman design were included as input variables, and the yield of ethanol as output, and they were scaled by Eq. (6):

$$X^* = 2\frac{X - X_{\min}}{X_{\max} - X_{\min}} - 1 \qquad Y^* = \frac{Y - 0}{500 - 0} \tag{6}$$

And these values were rescaled by Eq. (7):

$$X = \frac{(X_{\max} - X_{\min})(X^* + 1)}{2} + X_{\min} \qquad Y = 500 \times Y^* \tag{7}$$

2.5. Statistical analysis

Experimental designs and corresponding data were conducted with the help of Minitab 15.0 (Minitab Inc., Pennsylvania, USA).The ANN models and GA described in this study were implemented with Matlab v6.5 software.

Table 3
Effects of Plackett–Burman design for ethanol production.

	Symbol	Effect	t-value	P-value
NaCl (g/L)	x_1	20.26	1.59	0.187
NH_4Cl (g/L)	x_2	31.50	2.47	0.069
KH_2PO_4 (g/L)	x_3	5.81	0.46	0.672
$MgSO_4$ (g/L)	x_4	−53.94	−4.23	0.013
$CaCl_2$ (g/L)	x_5	−7.20	−0.56	0.602
Yeast extract (g/L)	x_6	−17.00	−1.33	0.253
pH	x_7	−46.36	−3.64	0.022

SD = 22.09, R^2 = 91.31%, R^2 (adj)=76.11%.

Table 4
Analysis of variance for the quadratic response surface model.

Term	Coefficient	Standard error	t-value	P-value
Constant	235.573	3.087	76.320	<0.0001
X_1	7.948	2.048	3.881	0.003
X_2	18.567	2.048	9.066	<0.0001
X_3	14.097	2.048	6.884	0.001
X_1^2	−14.563	1.994	−7.305	<0.0001
X_2^2	−22.728	1.994	−11.401	<0.0001
X_3^2	−4.978	1.994	−2.497	0.032
$X_1 X_2$	17.364	2.676	6.489	<0.0001
$X_1 X_3$	−5.491	2.676	−2.052	0.067
$X_2 X_3$	−6.302	2.676	−2.355	0.040

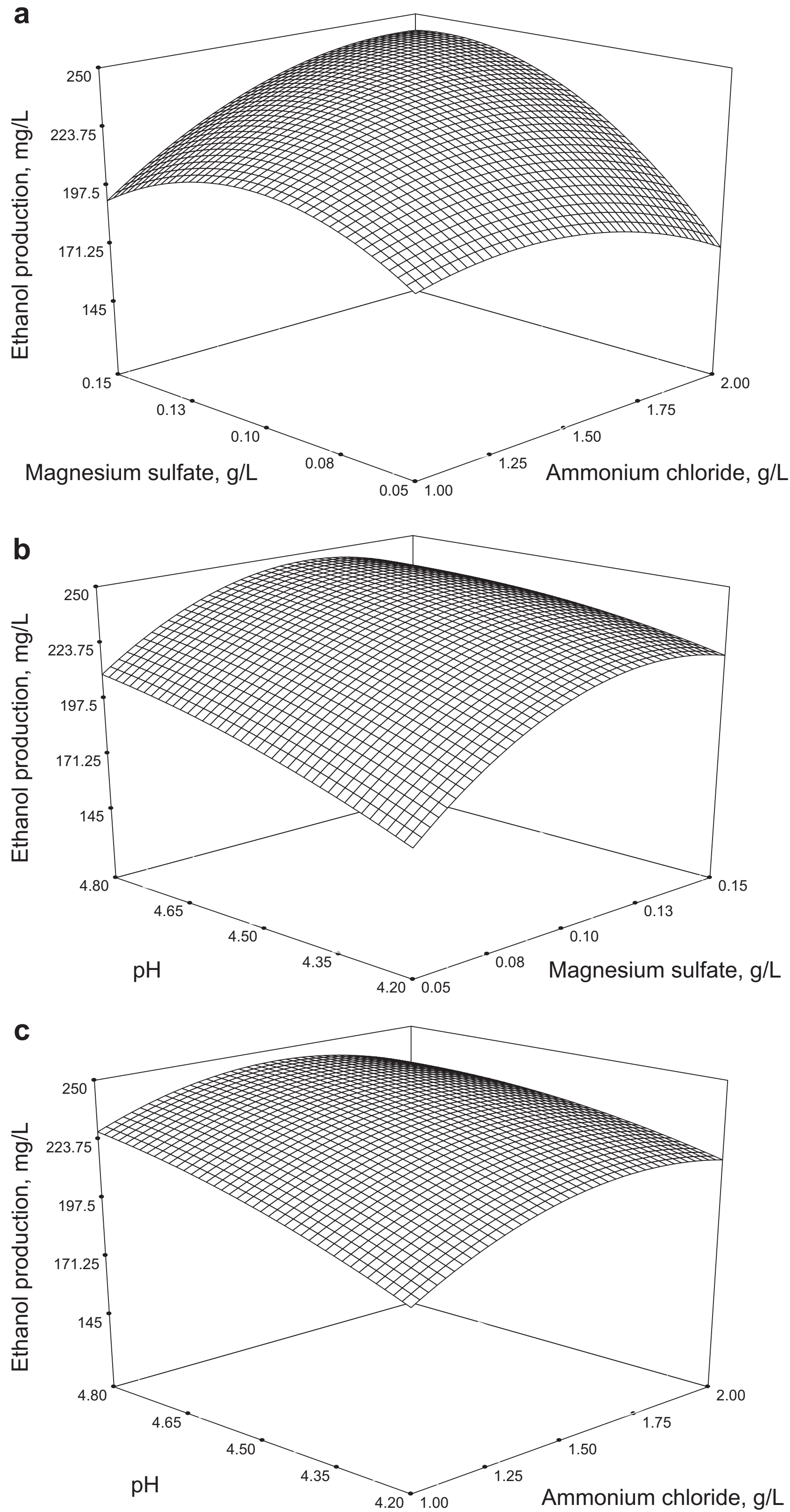

Fig. 1. Contour plot for ethanol production by *Clostridium autoethanogenum*, (a) effects of magnesium sulfate and ammonium chloride, (b) effects of pH and magnesium sulfate, (c) effects of pH and ammonium chloride.

3. Results and discussion

3.1. Plackett–Burman design

Plackett–Burman design was adopted to screen the important fermentation parameters which have significant effects on ethanol production with *C. autoethanogenum*. NaCl, NH_4Cl, KH_2PO_4, $MgSO_4$, $CaCl_2$, yeast extract and pH were selected through 12 runs with the Plackett–Burman design. The design matrix and its corresponding yield are illustrated in Table 1. Table 3 shows the main effects of each medium constituent, its associated *t*-values and *p*-values. According to the Plackett–Burman design, NaCl, NH_4Cl, and KH_2PO_4 had positive effects and thus a +1 level would be expected to improve the production of ethanol. $MgSO_4$, $CaCl_2$, Yeast extract, and pH showed negative effects, and a −1 level would be helpful for the high production of ethanol. As a result, a medium containing (g/L), NaCl 1, KH_2PO_4 0.1, $CaCl_2$ 0.02, yeast extract 0.15 was chosen. Dummy variables were used to calculate standard deviation (*SD*), *t*-values and *p*-values. The *SD* of 22.09 indicated interactions among the factors. $MgSO_4$, NH_4Cl and pH, which had a *p*-value less than 0.1, were significant compositions that affected the yield of ethanol production. In the results listed in Table 3, R^2 was 0.9131, which means that model could explain 91.31% of the total variations in the system. The optimum value of $MgSO_4$, NH_4Cl and pH, were further investigated by CCD.

3.2. Central composite design

$MgSO_4$, NH_4Cl and pH screened by the Plackett–Burman design were further studied by central composite design to establish their optimum levels. The central composite design consisted of a 2^3 full factorial design, 2∗3 axial designs and 6 center points, that is, a total number of 20 experiments. The design matrix of CCD, the variables and corresponding results are presented in Table 2.

3.2.1. Response surface methodology

The relationship among $MgSO_4$, NH_4Cl and pH were identified by RSM. Table 4 presents an analysis of variance (ANOVA) for the quadratic response surface model. According to the regression analysis of the experimental design, the interactive model term NH_4Cl*pH, with a *p*-value of more than 0.05, was insignificant, while all the other model terms ($p < 0.05$) were significant. The fitness of the polynomial model equation was judged by R^2. A R^2 value of 97.34% and an adjusted R^2 value of 94.94% confirmed good agreement. Therefore, the results in terms of the production of ethanol can be illustrated by the following quadratic regression equations:

$$Y = 235.573 + 7.984X_1 + 18.567X_2 + 14.097X_3 - 14.563X_1^2 - 22.784X_2^2 - 4.978X_3^2 + 17.364X_1X_2 - 6.302X_2X_3$$

where the production of ethanol as *Y* is a multiple function of the $MgSO_4$ and NH_4Cl concentrations and pH.

The RSM predicted model is presented as three-dimensional graphs in Fig. 1a–c to indicate the interaction among the variables that influenced the production of ethanol. The optimal process parameters of RSM model were established by the central point of the contour plot. The predicted highest ethanol production of 247.48 mg/L could be obtained at a concentration 0.120 g/L $MgSO_4$ and 1.661 g/L NH_4Cl at a pH 4.80. Experimental ethanol production of 254.26 mg/L was obtained under these optimum conditions.

3.2.2. Artificial neural network and genetic algorithm

Fig. 2 compares the ethanol yields predicted by the ANN and RSM with the corresponding experimental yields. The standard deviation *S* was introduced to analyze residual of ANN and RSM.

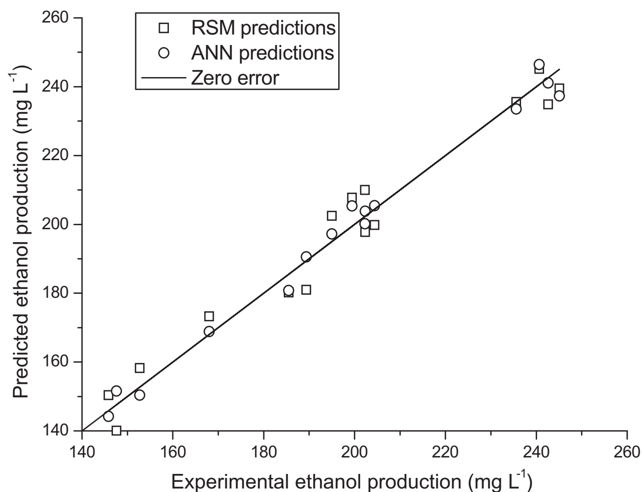

Fig. 2. RSM and ANN predictions versus experimental values.

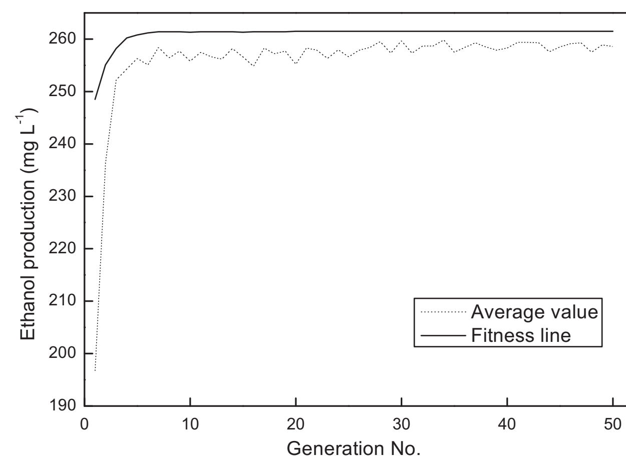

Fig. 3. Evolution of the fitness and average value (ethanol production) over the 50 generations in the genetic algorithm.

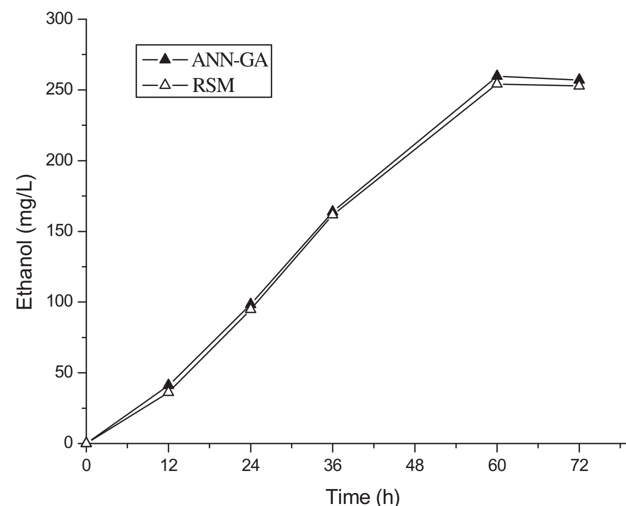

Fig. 4. Ethanol production using the optimal medium (describe medium) suggested by response surface methodology and artificial neural network-genetic algorithm.

$$S_{RSM/ANN} = \sqrt{\frac{\sum (\text{Prediction}_{RSM/ANN} - \text{Zero error})^2}{20}}$$

Since the S_{RSM} of 5.35 was higher than the S_{ANN} of 3.29, the fitness and prediction accuracy of the ANN model was higher than that of the RSM. For the GA-derived optimal conditions, optimum values of ethanol production were obtained over 50 generations (Fig. 3). At pH 4.74 and $MgSO_4$ and NH_4Cl concentrations of 0.116 and 1.694 g/L, respectively, the maximum achievable ethanol production was 261.48 mg/L according to the ANN-GA model. The experimental ethanol yield under the above conditions was 259.64 mg/L, which was near 254.26 mg/L, the ethanol yield obtained under the condition recommended by RSM model. Therefore, the ANN-GA model performed better than RSM model in the optimization studies. Fig. 4 shows ethanol production over time using the optimal media suggested by RSM and ANN-GA. The maximum ethanol production was obtained at 60 h. After 60 h, production decreased slightly, which is attributable to the depletion of CO.

It is noteworthy that the end-products of autotrophs also depend on the mass transfer of substrate to the cell and the redox potential environment (Phillips et al., 1993; Sim et al., 2008). In the infusion bag system, the effects of CO and reducing environment were negligible. The pressure in the infusion bag was stable at 1.0 atm and thus the different amounts of CO, in the bag, had the same chance to dissolve. Reducing environments were the same for all samples at the beginning (0.2 g/L cysteine-HCl). In this study, the effect of key CO-utilizing enzymes during fermentation was attributed to the different composition of the media which also play a significant role in the redox environment. The pH was adjusted with KOH or HCl, and different pHs resulted in different concentrations of Cl^- and K^+. However, this concentration was relatively low compared with the existing Cl^- (existing as KCl, NaCl, $CaCl_2$ and NH_4Cl) and K^+ (existing as KCl and KH_2PO_4). Our optimal ethanol concentration, around 260 mg/L was still low compared with ethanol yields obtained with enzymes or chemical catalysis which can be as high as 50 g/L in less than three days (Munasinghe and Khanal, 2010). Therefore further studies are required to improve the yield of ethanol with this bacterium cultured on syngas.

4. Conclusion

Plackett–Burman and central composite designs were used to optimize ethanol production from CO by *C. autoethanogenum*. The predicted and experimentally achieved yields were in good agreement, but the yield has to be improved further for this bacterium to be useful for large-scale ethanol production from syngas.

Acknowledgements

This work was funded by the National High Technology Research and Development Program of China (863 program, No. 2007AA05Z406) and the Knowledge Innovation Program of the Chinese Academy of Sciences (No. KGCX2-YW-335, KSCX-YW-11-A3 and KSCX2-YW-G-075-09).

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