Fuel ethanol production using novel carbon sources and fermentation medium optimization with response surface methodology

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Abstract: In this study, ethanol production abilities of the novel carbon sources: sodium and calcium gluconate in different minimal and rich media were compared with glucose using *Escherichia coli* KO11. The strain produced higher ethanol yield in the rich medium Luria-Bertani (LB) than the other two minimal media: corn steep liquor (CSL) and M9 for two substrates (sodium and calcium gluconate). Additionally, higher ethanol yields were achieved when the strain was grown in LB and M9 medium with calcium gluconate than sodium gluconate, while the ethanol yields were similar when both sodium and calcium gluconate were added into CSL medium respectively. Response surface methodology was used to optimize the fermentation medium components for enhancing ethanol production using strain *E. coli* KO11 in CSL medium with calcium gluconate as the substrate in batch culture. The concentration of the potassium phosphate buffer is the only significant factor among five factors considered. A quadratic model was developed to describe the relationship between ethanol production and the factors. The optimal conditions predicted for five factors were 14.38 g/L CSL, 0.0398 g/L $FeCl_3$ $6H_2O$, 1.12 g/L $MgSO_4$ $6H_2O$, 15.41 g/L $(NH_4)_2SO_4$, and 1.58/1.26 g/L KH_2PO_4/K_2HPO_4 (2:1 molar ratio). The highest ethanol concentration under optimal conditions was 31.5 g/L, which was 5.6 g/L higher than that from the same fermentation concentration of calcium gluconate in LB media. The high correlation between the predicted and experimental values confirmed the validity of the model.

Keywords: gluconate salts, ethanol, response surface methodology, medium optimization, biofuel

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1 Introduction

Amid rising global energy demand and pressing environmental issues, there are growing interests in the production of fuels and chemicals from renewable resources. Ethanol remains the most actively pursued biofuel at the industrial level. However, the lack of low-cost technology to overcome the recalcitrance of cellulosic biomass impedes widespread of ethanol production from lignocellulosic biomass feedstocks[1,2]. An important strategy for lowering the overall process cost is process consolidation[3,4]. A novel biochemical route for fuels and chemicals production was proposed by Fan et al.[5], in which sugar acids were produced from cellulosic materials instead of sugars for subsequent conversion to fuels and chemicals. Advantage of the process is the consolidation of cellulase production and enzymatic hydrolysis steps, and potentially the pretreatment step. Sugar acids (majorly gluconate) produced from cellulosic biomass could potentially be cheaper than sugars produced from cellulosic biomass[5]. Gluconate was utilized via the *Entner-Doudoroff* pathway by *Escherichia coli* KO11 to produce ethanol and acetate as products, as shown in Figure 1[6]. Theoretically, 1.5 moles of ethanol, 0.5 mole of acetic acid, and 1.5 moles of ATP will be generated from per mole of gluconate[5]. The ethanol produced by *E. coli* strain KO11 reached

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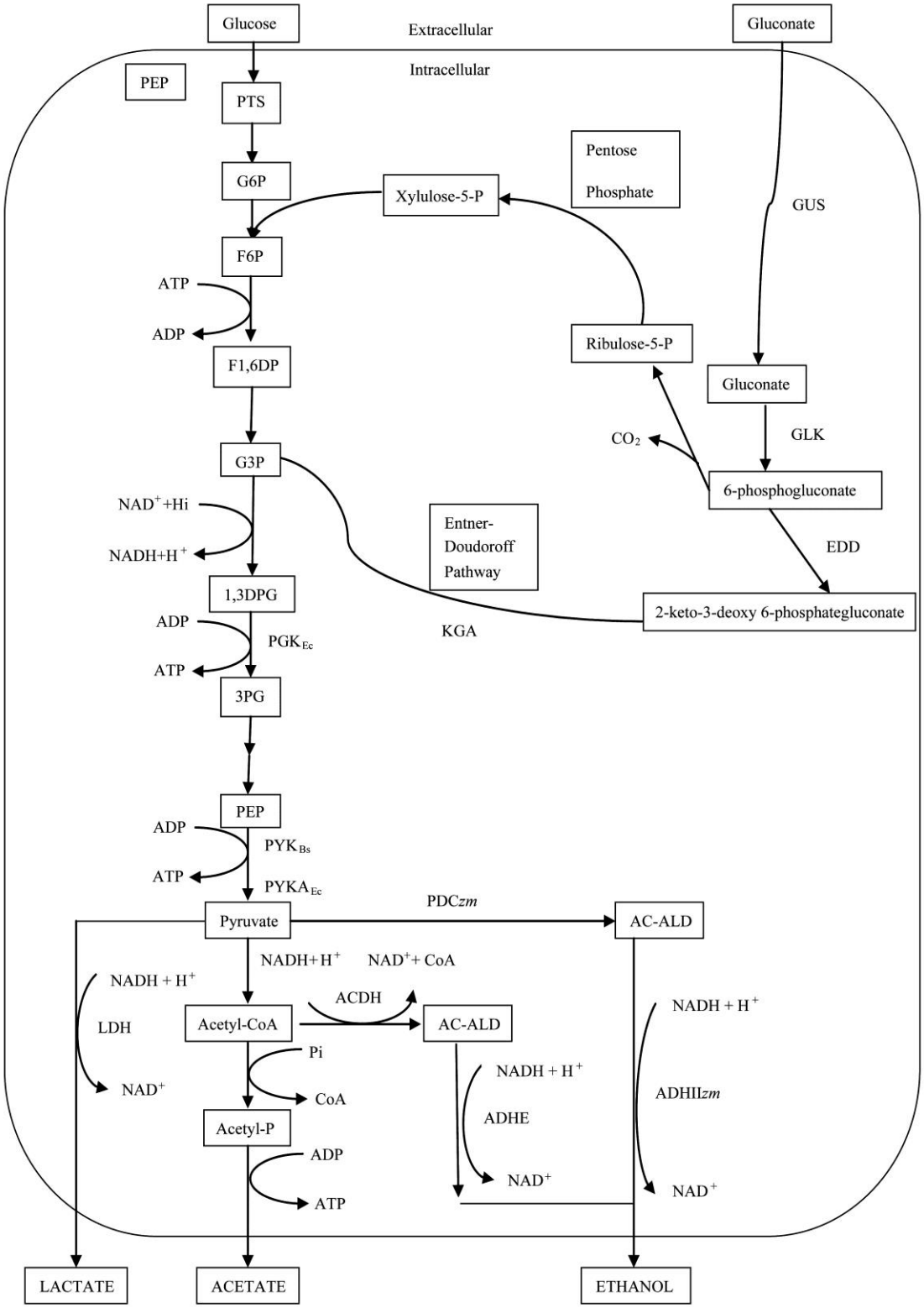
85% of the theoretical yield, while acetate production reached the theoretical yield when Luria-Bertani (LB) medium was used[5].



Ec = *E. coli*; Bs = *B. stearothermophilus*; Zm = *Z. mobilis*; PTS = phosphotransferase system; PGK_{Ec} = phosphoglycerate kinase; PYK_{Bs} = heterologous pyruvate kinase; PYKA = pyruvate kinase A; PYKF = pyruvate kinase F; LDH = lactate dehydrogenase; PTA = phosphotransacetylase; ACK = acetate kinase; ACDH = acetaldehyde dehydrogenase; ADHE = alcohol dehydrogenase; PDC_{Zm} = pyruvate decarboxylase; $ADHII_{Zm}$ = alcohol dehydrogenase; GUS = gluconate uptake system; GLK = gluconate kinase; EDD = 6-phosphogluconate dehydratase; KGA = phosphor-2-keto-3-deoxygluconate aldolase. Metabolites: G6P = glucose-6-phosphate; F6P = fructose-6-phosphate; F1, 6DP = fructose-1, 6-diphosphate; G3P = glyceraldehyde-3-phosphate; DHAP = dihydroxyacetone phosphate; 1,3 DPG = 1,3 – diphosphoglycerate; 3PG = 3-phosphoglycerate; PEP = phosphoenolpyruvate; AC-ALD = acetaldehyde

Figure 1 Central anaerobic metabolic pathway of glucose and gluconate in *E. coli* KO11[18-20]

Complex growth media, such as LB medium containing expensive laboratory nutrients (yeast extract and tryptone), are not feasible for the industrial production of ethanol. The development of inexpensive industrial media that retains high ethanol productivity and yield is essential for economical ethanol production from biomass feedstocks. Substantial efforts have been expended on formulating a minimal synthetic medium for ethanol production using *E. coli* KO11 as the ethanologen[7-10], and using glucose, xylose, or pretreated biomass as the substrate[11-15]. Gluconate salts are substantially different substrates from sugars. The minimal medium formulated using sugars as the substrates cannot be directly applied to sugar acids. In this study, the ethanol production from sodium and calcium gluconate using the reported synthetic minimal media[16,17] was investigated and compared with glucose. The subsequent optimization of the components of minimal media was studied by using response surface methodology (RSM). LB medium was used as a reference for comparing fermentation performance in terms of ethanol yield and productivity.

2 Materials and methods

2.1 Microorganism, medium, and culturing conditions

The engineered strain *E. coli* KO11 (ATCC29191) was purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA) and stored in 25% glycerol at negative 80℃. The strain was streaked on a fresh LB agar (Fisher, Pittsburgh, PA, USA) plate containing 0.034 g/L amphenicol chloride (Sigma, St. Louis, MO, USA) and incubated at 37℃ overnight. All chemicals used in the medium were purchased from Sigma (St. Louis, MO, USA) if they were not specified elsewhere.

Fermentations were carried out in the 250 mL serum bottle with a 200 mL working volume and purged with CO_2 gas to deplete the air. LB medium and two minimal media were used during the fermentation. Corn steep liquor (CSL) medium contained the following salts (per liter of distilled water): 10 g of CSL (~50% solids), 1 g of KH_2PO_4, 0.5 g of K_2HPO_4, 3.1 g of $(NH_4)_2SO_4$, 0.4 g of $MgCl_2$ $6H_2O$, and 0.020 g of $FeCl_3$ $6H_2O$. All the salt solutions for the medium were prepared as described previously[17]. M9 medium contained the following ingredients (per liter of distilled water): 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, and 0.5 g of NaCl. Three trace components were sterilized by filtration and then added into media at the following final concentrations: 0.002 M of $MgSO_4$ $7H_2O$, 0.0001 M of $CaCl_2$, and 0.001 g/L of thiamine-HCl. 150 mL seed cultures were grown in a 250 mL serum bottle at 37℃ at 220 r/min in LB medium containing 20 g/L glucose. To initiate the fermentation, 0.003 L of the liquid culture (OD_{600nm} =1.6) were inoculated into 0.2 L of fermentation medium. Samples were taken at various time intervals to monitor concentrations of ethanol, acetate, glucose, sodium and calcium gluconate.

2.2 Analytical method

The concentrations of glucose, sodium and calcium gluconate, ethanol, and acetate were analyzed using high-pressure liquid chromatography (Shimadzu, Japan) equipped with a refraction index detector and an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) at 60℃. The mobile phase was 0.005 M H_2SO_4 (Sigma, St. Louis, MO, USA) at the flow rate of 0.036 L/hour.

2.3 Experimental design and data analysis

A rotatable central composite design (CCD) with five factors and five levels (-2, –1, 0, 1, 2) was used to study response patterns, and JMP 8 software (SAS Institute Inc, NC, USA) was used to determine the optimal combination of variables. In this study, the CCD was a 2_V^{5-1} fractional factorial design with ten center points, and ten star points which are located at a distance of α = 2 from the center. The five independent variables were concentrations of CSL (designated variable X_1, expressed in g/L), $(NH_4)_2SO_4$ (X_2, g/L), KH_2PO_4/K_2HPO_4 (X_3, g/L), $MgSO_4$ $6H_2O$ (X_4, g/L), and $FeCl_3$ $6H_2O$ (X_5, g/L), while ethanol concentration (Yi, g/L) was the dependent output variable. The concentration of the substrate (calcium gluconate) was kept at optimal 80 g/L determined from the preliminary experiments. The range of variables is given in Table 1.

Table1 Factors and coded levels in a rotatable central composite design (CCD)

Variables	Coded levels of the factors				
	-2	-1	0	1	2
Corn Steep Liquor (g/L), X_1	2	8	14	20	26
$(NH_4)_2SO_4$ (g/L), X_2	0.50	4.33	8.16	12	15.83
KH_2PO_4/K_2HPO_4 (g/L), X_3	0.68/0.44	2.72/1.76	4.76/3.08	6.80/4.40	8.84/5.72
$FeCl_3$ $6H_2O$ (g/L), X_4	0	0.027	0.053	0.080	0.107
$MgSO_4$ $6H_2O$ (g/L), X_5	0	0.533	1.066	1.600	2.133

Table 2 The rotatable central composite design (CCD) matrix for five independent variables (X_1~X_5)

Runs	X_1	X_2	X_3	X_4	X_5	Experimental ethanol/g·L^{-1}	Predicted ethanol/g·L^{-1}
1	1	1	1	1	-1	13.6	15.5
2	1	1	1	-1	1	11.9	14.3
3	1	1	-1	1	1	26.7	27.2
4	1	-1	1	1	1	18.3	20.2
5	1	1	-1	-1	-1	27.7	27.9
6	1	-1	1	-1	-1	9.3	10.8
7	1	-1	-1	1	-1	25.7	25.4
8	1	-1	-1	-1	1	26.8	27.0
9	-1	1	1	1	1	11.8	12.9
10	-1	-1	-1	-1	-1	24.9	23.5
11	-1	-1	1	1	-1	16.0	16.3
12	-1	1	1	-1	-1	17.0	17.8
13	-1	-1	1	-1	1	14.8	15.6
14	-1	-1	-1	1	1	25.6	24.5
15	-1	1	-1	-1	1	29.6	29.1
16	-1	1	-1	1	-1	29.5	28.5
17	2	0	0	0	0	19.3	16.1
18	0	2	0	0	0	26.9	25.2
19	0	0	2	0	0	11.9	7.4
20	0	0	0	2	0	29.6	28.8
21	0	0	0	0	2	26.0	24.3
22	-2	0	0	0	0	14.7	16.1
23	0	-2	0	0	0	22.7	22.7
24	0	0	-2	0	0	27.1	29.7
25	0	0	0	-2	0	28.8	27.7
26	0	0	0	0	-2	23.2	23.0
27	0	0	0	0	0	26.1	26.4
28	0	0	0	0	0	27.2	26.4
29	0	0	0	0	0	26.0	26.4
30	0	0	0	0	0	25.8	26.4
31	0	0	0	0	0	26.5	26.4
32	0	0	0	0	0	24.7	26.4
33	0	0	0	0	0	25.0	26.4
34	0	0	0	0	0	25.2	26.4
35	0	0	0	0	0	28.2	26.4
36	0	0	0	0	0	27.0	26.4
Optimal	0.0631	1.89	-1.56	-0.508	0.228	31.5	31.0

The relationships between the coded and the actual values were described according to Equation (1):

$$X_i = \frac{x_i - \overline{x}_i}{\Delta x_i} \quad (1)$$

where, X_i is the coded value of the independent variable i; x_i is the actual value of the independent variable i; $\overline{x}_i$ is the actual value on the center point of the independent variable i, and Δx_i is the step change value. The ranges of coded levels in this experiment were determined according to results of previous experiments and published data in the literatures[9,17,21,22]. Thirty-six experiments were carried out to optimize the medium components for fuel ethanol fermentation (Table 2).

The following quadratic model was developed to predict the optimal point:

$$Y_i = b_0 + \sum b_i X_i + \sum b_{ij} X_{ij} + \sum b_{ii} X_{ii}^2 \quad (2)$$

where, Y_i is the predicted response; b_0 is the offset term; and b_i, b_{ii}, and b_{ij} are linear effects, squared effects, and interaction terms, respectively. The statistical significance of the developed quadratic model was determined by an F-test; the proportion of variance obtained by the model was provided by the multiple coefficients of determination, R^2. The optimal values of the five factors were determined by response surface and predicted using the JMP 8 software, in which a sequential forward selection procedure was applied to locate more desirable values of the response.

3 Results and discussion

3.1 Comparison of fermentation in LB, CSL, and M9 media

In this study, sodium and calcium gluconate were applied as carbon sources in M9 and CSL media as well as LB media for the conversion of gluconate to ethanol. The ethanol fermentation performances of gluconate salts were compared with glucose in all three media.

3.1.1 Bioconversion of sodium gluconate into ethanol

Both sodium gluconate and calcium gluconate were successfully converted to ethanol in the un-modified M9 and CSL media (Figure 2a-i). When the two minimal media were used for both gluconate conversion, ethanol was produced at lower rates (0.097-0.140 g/(L ħ) ethanol, required longer fermentation times) in minimal medium, compared to them in the LB medium (0.26-0.27 g/(L ħ), Figure 2k-l). When sodium gluconate was used as the carbon source, the highest ethanol yield achieved (76.4% of the theoretical yield) was in LB medium, followed by CSL and M9 media, in which the ethanol yields were 75.3% and 68.3%, respectively. In aspect of ethanol productivity and sodium gluconate consumption (Figure 2k-l), the rate of ethanol production in LB medium was 0.27 g/(L ħ), which was 2.0 and 2.1 times faster than that for M9 and CSL media, respectively. The sodium gluconate consumption rate consisted with the ethanol yield and productivity. The highest up-taking rate of sodium gluconate was 1.66 g/(L ħ) in LB medium, as shown in Figure 2k and Figure 2l, which was 2.2 and 3.3 times faster than that of M9 (0.65 g/(L ħ)) and CSL (0.51 g/(L ħ)) media, respectively. The strain produced similar yields of ethanol to sodium gluconate in LB and CSL media (0.26 g ethanol/g sodium gluconate) while the yield was 9% lower than in the M9 medium, which was 0.24 g ethanol/g sodium gluconate. The differences in ethanol yields and production rates are likely due to LB medium, which provides the most easily accessible nutrients and trace elements among three medium, followed by CSL and M9 medium. M9 medium contains more salts than LB and CSL media, resulting in higher osmotic stress and ion strength that negatively affect cell growth and ethanol production during fermentation[21,22]. Additionally, the CSL and LB medium have better pH buffer capacity than that of M9 medium containing sodium gluconate, as shown in Figure 2j, which is another beneficial factor for ethanol fermentation.

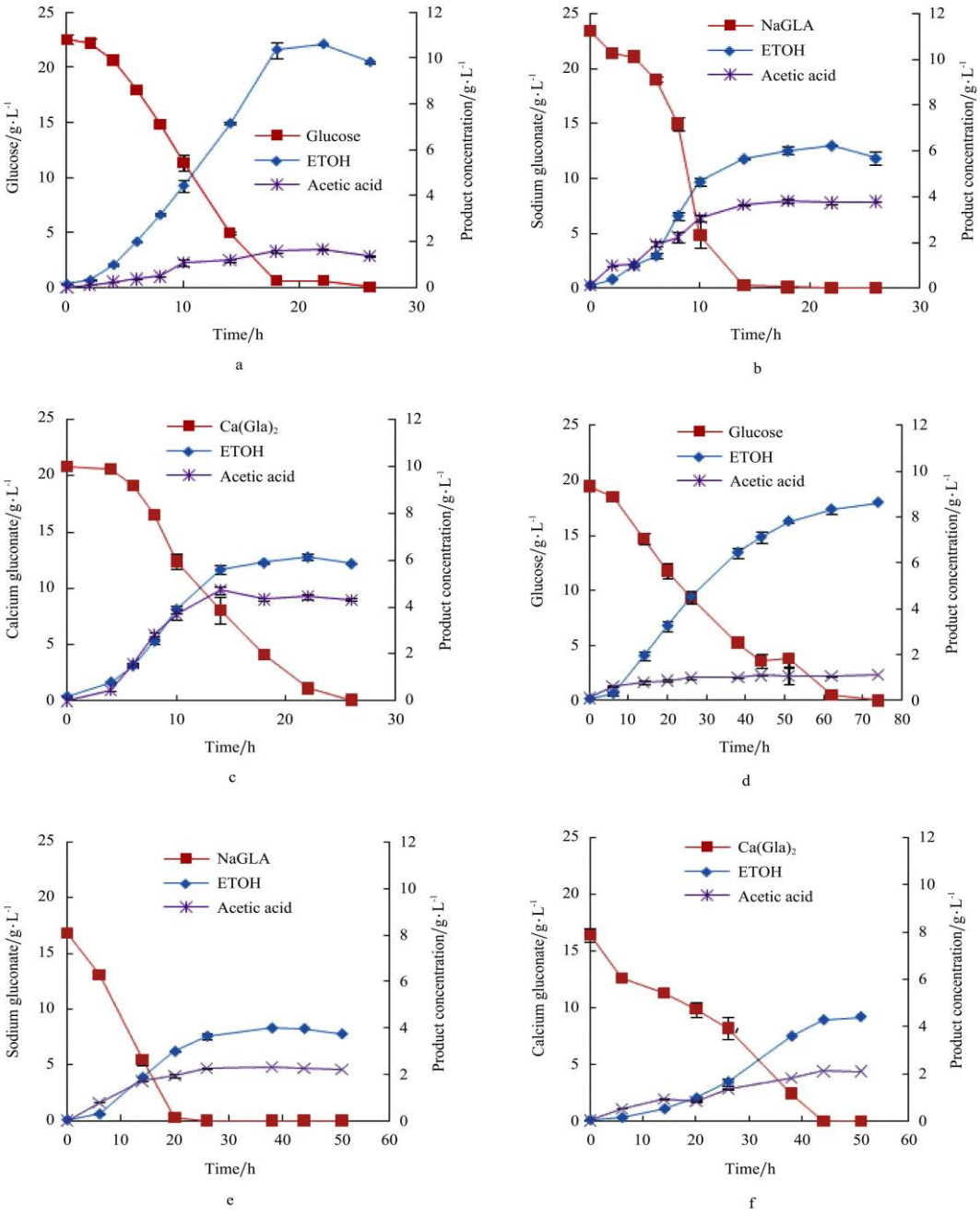
3.1.2 Bioconversion of calcium gluconate into ethanol

The ethanol yield from calcium gluconate in LB was 85% of theoretical yield, which is 10% higher than that of sodium gluconate (77%) achieved in LB medium, as shown in Figure 2k. However, the strain only produced slightly higher ethanol yields from calcium gluconate in CSL medium (76.5%), compared to 75.3% of theoretical ethanol yield from sodium gluconate in CSL medium. The yield of ethanol from calcium gluconate (76.7%) achieved in M9 medium was 1.12 times higher than that for sodium gluconate (68.3%), possibly due to the significant alleviation of osmotic pressure and ion strength resulting from a large amount of precipitation formed between calcium cation and phosphate group in M9 medium. A small amount of precipitations, mostly $CaCO_3$, were observed during ethanol fermentation in LB and CSL medium using calcium gluconate as carbon source. It is probably the reason of ion strength alleviation that the strain produced higher ethanol yields in LB and CSL medium containing calcium gluconate than sodium gluconate. The strain produced similar ethanol productivity in LB media for both sodium and calcium gluconate, as shown in Figure 2l. However, the lower ethanol productivities were detected in both CSL and M9 medium containing calcium gluconate due to the lower consumption rates of calcium gluconate than that of sodium gluconate in these two media. The yields of ethanol to calcium gluconate in all three media were higher than that of sodium gluconate, which suggested the better fermentation performance of strain KO11 using calcium gluconate than that of sodium gluconate. Moreover, the pH buffering abilities of LB and CSL medium containing sodium or calcium gluconate were better than that of M9 medium during the fermentation process, which is beneficial for cell growth and ethanol production, shown in Figure 2j.

3.1.3 Comparison of fermentation ability of glucose with gluconate salts

The bioconversion of glucose to ethanol was investigated in all three media as well as for the comparison of ethanol fermentation performance with sodium and calcium gluconate. The highest ethanol yield achieved was 96.8% in the LB media, followed by CSL and M9 medium, in which the ethanol yields were 92.2% and 85.5%, as shown in Figure 2k, respectively. The ethanol yield of glucose in LB, M9, and CSL media were 14%, 12%, and 21% higher than that of calcium

gluconate in the corresponding media, respectively, as well as 27%, 25%, and 22% higher than that of sodium gluconate in LB, M9, and CSL media. The ethanol productivity of glucose in the LB medium was 0.48 g/(L ħ) (Figure 2l), which is 66% and 82% higher than that of calcium and sodium gluconate in LB medium. However, the strain produced lower ethanol productivity of glucose in M9 and CSL media than that of sodium gluconate in both medium, as well as that of calcium gluconate in CSL medium. The ethanol productivities of gluconate salts in M9 and CSL media were consisted with substrate consumption rates. Both gluconate salts were consumed faster than glucose in M9 and CSL media. Particularly, the strain consumed the gluconate salts three times faster than glucose in CSL medium. The higher ethanol productivities and substrate up-taking rates of gluconate salts suggested that they might be good potentially alternative substrates for fuel ethanol production. In addition, as shown in Figure 2j, the pH values of culture media containing gluconate were relatively constant during the fermentation while the pH values decreased in the media containing glucose as the culture continued. The high pH buffering ability of gluconate salts in the media will render a great beneficiary in the pH value control during the ethanol fermentation at industrial scale.



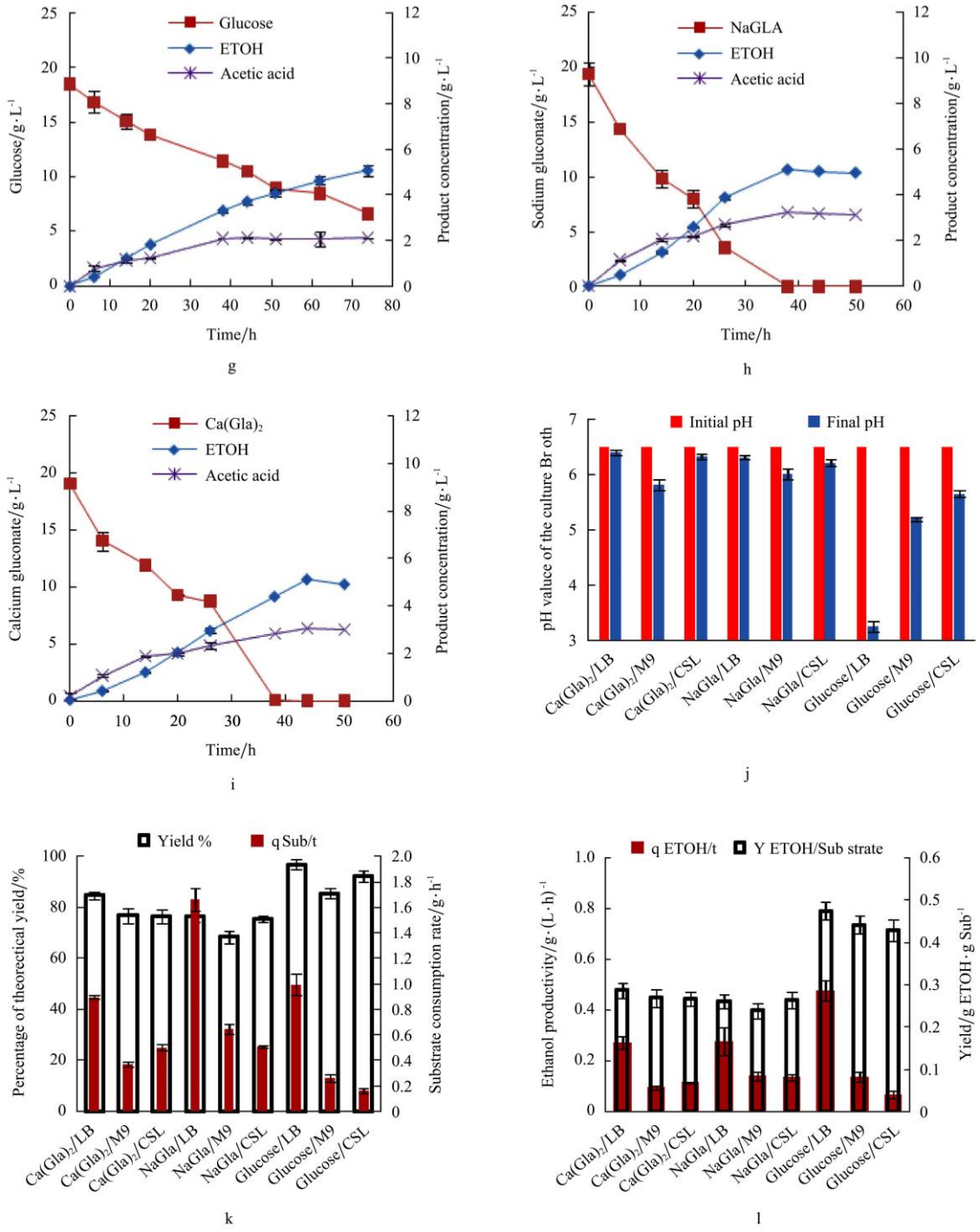


Figure 2 Comparison of glucose, sodium and calcium gluconate ethanolic fermentation in LB, CSL, and M9 medium. (a)-(c): glucose, sodium and calcium gluconate in LB medium, respectively; (d)-(f): glucose, sodium and calcium gluconate in M9 medium, respectively; (g)-(i): glucose, sodium and calcium gluconate in CSL medium, respectively; j: the starting and final pH value of the culture broth; k: percentage of ethanol theoretical yields from different media and the substrate consumption rate (g substrate/hour); l: the ethanol productivity and yield. ($Y_{ETOH/Substrate}$ is the yield of ethanol produced to substrate consumed (g/g): percentage of theoretical yield is the ethanol yield vs. the theoretical yield; $q_{ETOH/t}$ is ethanol productivity (g/(L ħ)); $q_{Sub/t}$ is substrate consumption rate (g/(L ħ)); ETOH stands for ethanol; NaGla stands for sodium gluconate; $Ca(Gla)_2$ stands for calcium gluconate). Product concentration of Y axis label in Figure 2 stands for the concentration of ethanol and acetic acid.

3.2 Response surface analysis of medium constituents

Considering the higher ethanol yield and productivity, better pH buffering capacity of the substrate in the medium and the simplicity and cheapness of medium, the calcium gluconate and CSL medium were chosen for further medium component optimization using RSM.

Experimental results were analyzed by JMP 8 software using multiple regression analysis. The corresponding quadratic regression model was constructed as shown in Equation (3).

$$y = 26.376 - 0.0003X_1 + 0.623X_2 - 5.599X_3 + 0.281X_4 + 0.314X_5 - 0.43X_1X_2 - 0.277X_1X_3 + 0.749X_1X_4 + 0.811X_1X_5 + 0.918X_2X_3 - 0.902X_2X_4 - 1.091X_2X_5 + 0.511X_3X_4 + 0.003X_3X_5 - 0.419X_4X_5 - 2.577X_1^2 - 0.612X_2^2 - 1.957X_3^2 + 0.476X_4^2 - 0.68X_5^2 \quad (3)$$

The actual concentrations of ethanol produced in the experiments and the predicted values based on the quadratic regression model are presented in Table 2. Regression analysis of the data yielded a coefficient of determination (R^2) of 0.937; this means that 93.7% of the variability in ethanol production could be explained by the proposed quadratic model, suggesting a satisfactory fitness of the model. The adjusted R^2 value was 0.852, which also implied satisfactory fitness. The difference between the predicted and experimentally produced ethanol concentrations verified both positive and negative dispersion of the observed values.

Table 3 ANOVA of full quadratic model

Source	Degree of freedom (DF)	Sum of squares (SS)	Mean square (MS)	F-value	p-value
Regression	20	1210.8	60.5	11.11	
Error	15	81.8	5.45		<0.0001
Total error	35	1292.6			

An analysis of variance (ANOVA, Table 3) for the response surface quadratic model showed that the fitted second-order regression model is highly significant, with a Fisher's test (F-test) value of 11.11 (P<0.0001). The student test (T-test) was used to determine the significance of the regression coefficients of the variables, in which a smaller p-value indicates a higher level of significance. If a variable had a p-value below 0.05, the test parameter is significant at the 95% level of confidence.

As shown in Table 4, concentration of phosphate buffer (X_3) had the most significant effects on ethanol production, with p <0.0001, suggesting a strongly linear effect on the response. By contrast, the other four factors, concentrations of CSL (X_1), ammonia sulfate (X_2), ferric chloride (X_4), and magnesium sulfate (X_5), had negligible linear effects on the response (P>0.1). Based on regression coefficients, F-values, and p-values, the phosphate buffer (X_3), the quadratic term of curvature CSL (X_1^2), and the quadratic term of the curvature phosphate buffer (X_3^2) had the most significant effects on ethanol production. The two-factor interaction between ammonia sulfate and magnesium sulfate (X_2X_4) had medium significance on ethanol yield since its p-value (0.0812) is above 0.05 but below 0.1.

Table 4 Regression coefficients and their significance for quadratic model

Term	Estimate	Standard error	F-value	t-value	p-value
Intercept	26.376	0.728	*	36.23	<0.0001
X_1	–0.0003	0.477	0	0	1.0
X_2	0.623	0.477	1.710	1.31	0.211
X_3	–5.599	0.477	138.026	–11.75	<0.0001
X_4	0.281	0.477	0.347	0.59	0.564
X_5	0.314	0.477	0.435	0.66	0.519
X_1X_2	–0.430	0.584	0.542	–0.74	0.473
X_1X_3	–0.227	0.584	0.152	–0.39	0.702
X_2X_3	–0.918	0.584	2.475	–1.57	0.137
X_1X_4	0.749	0.584	1.645	1.28	0.219
X_2X_4	–0.902	0.584	2.389	–1.55	0.143
X_3X_4	0.511	0.584	0.766	0.88	0.395
X_1X_5	0.811	0.584	1.932	1.39	0.185
X_2X_5	-1.091	0.584	3.496	–1.87	0.0812
X_3X_5	0.0028	0.584	0	0	0.996
X_4X_5	–0.419	0.584	0.514	–0.72	0.484
X_1^2	–2.577	0.413	38.985	–6.24	<0.0001
X_2^2	–0.612	0.413	2.197	–1.48	0.159
X_3^2	–1.957	0.413	22.480	–4.74	0.0003
X_4^2	0.476	0.413	1.330	1.15	0.267
X_5^2	–0.680	0.413	2.718	–1.65	0.12

Note: R^2 = 0.937, adjusted R^2 = 0.852.

Since some factors, the two-factor interactions, and quadratic terms of curvature were found to be non-significant, the full quadratic model (Equation (3)) was simplified to Equation (4), which only includes significant linear terms and high order terms.

$$y = 26.376 - 5.599X_3 - 1.091X_2X_5 - 2.577X_1^2 - 1.957X_3^2 \quad (4)$$

The simplified regression model yielded a coefficient of determination (R^2) of 0.856, indicating that 85.6% of the variability in ethanol production could be explained

by the simplified model, suggesting a satisfactory fitness of the model. The adjusted R^2 value was 0.832, which implied satisfactory fitness as well. An analysis of variance (ANOVA, Table 5) for the response surface simplified quadratic model showed highly significance of the simplified model as well, with a Fisher's test (F-test) value of 35.66 (P<0.0001).

Table 5 ANOVA of the simplified quadratic model

Source	Degree of freedom (DF)	Sum of squares (SS)	Mean square (MS)	F-value	p-value
Regression	5	1106.4	221.3	35.66	
Error	30	186.2	2.368		< 0.0001
Total error	35	1292.6			

Note: R^2 = 0.856, adjusted R^2 = 0.832.

3.3 Interactions between significant factor (KH_2PO_4/K_2HPO_4 (g/L), x_3) and other factors

Since X_3 is the only significant factor, the interactions between factor X_3 and other factors were investigated in this study. The three-dimensional response surface graphs and contour plots based on the final model are depicted in Figure 3a-d. They were obtained by holding the other three variables at zero (coded value) while varying the two variables of interest within their experimental range. The coded model was used to generate response surfaces and contour curves for the analysis of the variables' effects on ethanol production. The statistically optimal values of variables were obtained when moving along the major and minor axes of the contour. The response at the central point corresponded to the maximal degree of achievable ethanol concentration for that set of variables.

Figure 3a shows the response surface plot and the contour plot as a function of the concentrations of the phosphate buffer and CSL and indicates the effects of their interaction on ethanol production. As shown in Figure 3a, the ethanol yield increased as the concentration of the phosphate buffer (KH_2PO_4/K_2HPO_4 (2:1)) decreased from 8.84/5.72 g/L (coded level 2) to 1.578/1.261 g/L (coded level 1.56). Ethanol yield then reached its highest value at about 30 g/L and decreased with the reduction of the phosphate buffer in the medium. It is likely due to the higher osmotic pressure associated with higher concentration of potassium cation, which will inhibit cell growth at higher concentration. While the relatively low concentration of phosphate buffer could also inhibit cell growth due to short of phosphate as a trace nutrient[23]. The concentration of CSL affected the ethanol yield in a similar way except that the optimal ethanol yield was reached when the CSL concentration was 14.4 g/L (coded level 0.063). The real mechanism of CSL affecting ethanol production has not been elucidated well.

Effects of the concentrations of $(NH_4)_2SO_4$ and phosphate buffer on ethanol production are shown in Figure 3b. Ethanol yields increased with increased ammonia sulfate concentration since it functioned as a nitrogen source (Figure 3b). Meanwhile, the anion sulfate group precipitated calcium cation out from the medium, which benefited the cell growth and ethanol production. The effect of phosphate buffer on ethanol production varies in the same way as it did in the CSL and phosphate buffer interaction. When the concentration of $(NH_4)_2SO_4$ reached 15.4 g/L (coded level 1.89), the highest ethanol production achieved was about 30 g/L as well. Figure 3c and Figure 3d illustrate the response surface and contour plot as a function of ferric sulfate and magnesium sulfate concentrations. Both ferric chloride and magnesium sulfate had very similar effects on ethanol yield. The optimal concentrations of ferric chloride and magnesium sulfate for ethanol production were 0.0397 g/L and 1.19 g/L, respectively. As shown in Figure 3a-d, the concentration of phosphate buffer has a significantly negative correlation with ethanol production, while ammonia sulfate has a positive correlation with ethanol yield.

3.4 Evaluation of the optimum concentrations of media components

According to the main observations of the interactions, maximum ethanol production would be obtained by keeping the following medium composition, CSL: 14.38 g/L; $(NH_4)_2SO_4$: 15.41 g/L; KH_2PO_4/K_2HPO_4 (2:1, molar ratio): 1.58/1.26 g/L; $FeCl_3$ $6H_2O$: 0.0398 g/L; and $MgSO_4$ $6H_2O$: 1.19 g/L. The predicted ethanol concentration was experimentally verified by fermenting KO11 in the medium at the previously listed concentrations of components. A control of KO11

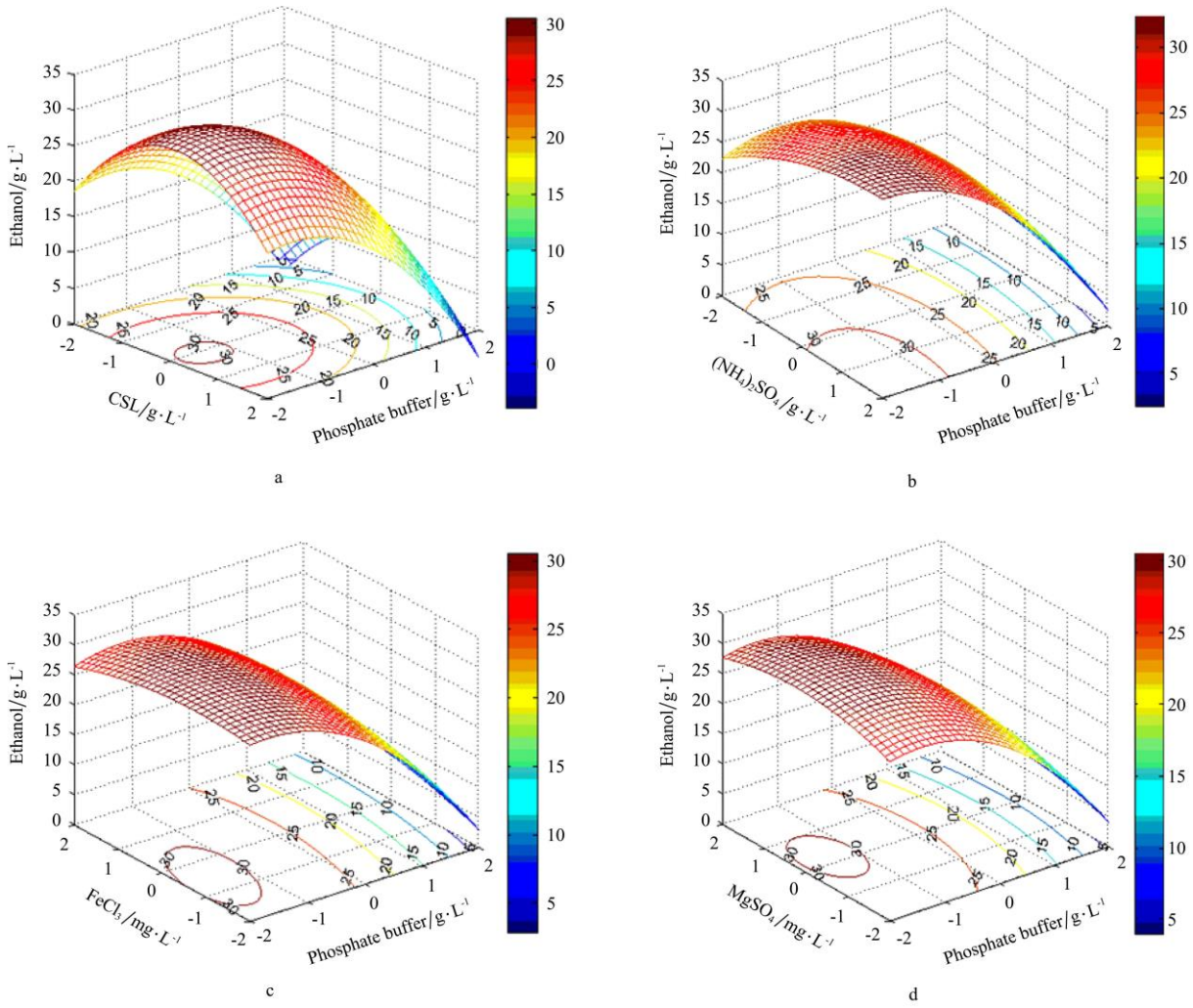


Figure 3 Response surface and contour plot of ethanol production by strain KO11. (a): phosphate buffer vs. corn steep liquor (CSL) with the constant level of: $(NH_4)_2SO_4$ 8.16 g/L, $FeCl_3$ $6H_2O$ 0.0267 g/L, $MgSO_4$ $6H_2O$ 0.533 g/L; (b): phosphate buffer vs. $(NH_4)_2SO_4$ with the constant level of: CSL 14 g/L, $FeCl_3$ $6H_2O$ 0.0267 g/L, $MgSO_4$ $6H_2O$ 0.533 g/L; (c): phosphate buffer vs. $FeCl_3$ $6H_2O$ with the constant level of: CSL 14 g/L, $(NH_4)_2SO_4$ 8.16 g/L, $MgSO_4$ $6H_2O$ 0.533 g/L; phosphate buffer vs. $MgSO_4$ $6H_2O$ with the constant level of: CSL 14 g/L, $(NH_4)_2SO_4$ 8.16 g/L, $FeCl_3$ $6H_2O$ 0.0267 g/L.

cultured in the rich medium LB was also set up for comparison of ethanol yield. The engineered strain KO11 produced 31.5 g/L of ethanol in the optimized CSL minimal medium, which was 95.8% of the predicted ethanol concentration. It is about 21.5% higher than that of ethanol from KO11 cultured in LB medium (25.9 g/L) under the same operation conditions and the concentration of calcium gluconate (80 g/L). Moreover, the cost of optimized synthetic medium is US $2.383 per litre, which is $0.29 per litre less than the cost of rich medium LB, as shown in Table 6, according to prices provided by Sigma. The cost of optimized CSL medium reduced 12.2% than that of LB medium.

Table 6 Cost of the media

Optimized CSL medium					
Component	Concentration /g·L^{-1}	Cata. No. (Sigma)	Price /$	Size /kg	Cost /dollar·L^{-1}
CSL	14.38	C4648	100	2.5	0.58
$(NH4)_2SO4$	15.41	A4418	82.9	1	1.28
KH_2PO4	1.58	P9791	125.5	1	0.20
K_2HPO4	1.26	P9666	157.5	1	0.20
$FeCl_3$	0.0398	157740	40.7	1	0.00
$MgSO_4$	1.19	M7506	111	1	0.13
				Total cost	2.38
Rich medium: LB					
Component	Concentration /g·L^{-1}	Cata. No. (Sigma)	Price /$	Size /kg	Cost /dollar·L^{-1}
Yeast extract	5	92114	75.4	0.5	0.75
Tryptone	10	T7293	192	1	1.92
				Total cost	2.67

4 Conclusions

The research showed that the strain KO11 produced lower ethanol yields and productivity in both unmodified minimal M9 and CSL media than that of rich medium LB. A more than 10% higher ethanol yields were detected when calcium gluconate was used as a carbon source in LB and M9 media than that of sodium gluconate in the media. The strain produced similar ethanol yield when both sodium and calcium gluconate were used as carbon source in the un-optimized CSL medium. The higher substrate up-taking rates of the strain were detected in the M9 and CSL medium containing sodium and calcium gluconate than that containing glucose. Additionally, the media containing sodium and calcium gluconate yielded better pH buffering capacity than that of glucose in all three media.

Response surface methodology was used for optimizing the CSL medium components for ethanol production by *E. coli* KO11 using calcium gluconate as the substrate. A maximum ethanol titer of 31.5 g/L was achieved under the predicted optimum component levels. The concentration of ethanol was increased by 21.5% as compared to ethanol titer produced in the unformulated CSL medium with same starting concentration of sodium gluconate under the same culture conditions. The ethanol concentration produced in the validation experiment (31.5 g/L) was similar to the predicted ethanol concentration (30.0 g/L) by the simplified quadratic model. Furthermore, the ethanol concentration achieved in the optimized synthetic medium is even 5.57 g/L higher than that from rich LB medium containing the same level of calcium gluconate as the substrate.

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