Preparation of a low-cost minimal medium for engineered *Escherichia coli* with high yield of human-like collagen II

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Abstract: In order to reduce the production cost of human-like collagen (HLC), a minimal medium was introduced. On the base of Design of experiments (DOE), especially Plackett-Burman design and central composite design, a modified minimal medium that could give a high yield of HLC was developed. The optimum minimal medium for engineered *E. coli* BL21 Δ*ptsG* contained 6.11g/L of glucose, 5.82g/L of $(NH_4)_2SO_4$, 1.80×10^{-4}g/L of thiamine and 3.00×10^{-2}L of trace element solution, the other ingredients were same to that in M9 medium. And the HLC production of *ptsG* mutant reached to 0.26g/L in this optimized minimal medium, which approached to 0.27g/L produced by the strain without deleting *ptsG* gene in an optimized complex medium.

Keywords: *E. coli*, a modified minimal medium, human-like collagen II, *ptsG* deletion.

INTRODUCTION

Escherichia coli have been commonly used as a host for exogenous protein expression due to its simple constituents and well-studied background (Bhandari *et al.*, 1997). Engineered *E. coli* BL21 was constructed for expressing human-like collagen (Luo *et al.*, 2005 and Fan 2005). Human-like collagen (HLC), which is a kind of recombinant collagen, is water-soluble (Luo *et al.*, 2005 and Fan 2005). Compared with the collagen extracted from the animal tissues with specific chemical solvents or enzymes, HLC possesses some remarkable advantages, such as low immunogenicity, easily modifiable and no virus risk, and it is regarded as a novel biomedical material for hemostatic materials, artificial bone scaffolds, skin materials and vascular scaffolds, etc (Zhu *et al.*, 2009).

Since HLC has good application prospects, the present study was focused on producing this special protein at a low cost. A modified *Luria-Bertani* (LB) medium, called the fermentation medium (FM), was developed by our groups and applied in the fermentation process. Although the production of HLC was high, the cost of this fermentation medium was so high that it could not be ignored owing to a great dosage of the expensive yeast extract. Moreover, the compositions of yeast extract were complicated and some trace ingredients could not be detected exactly, so this kind of organic nitrogen source could not supply a clear background for some quantitative metabolism research. Therefore, substituting the complex fermentation medium partly or wholly with a cheap and clear medium might save the cost. M9 medium, a minimal medium, was usually used to cultivate *E. coli* (Oleg *et al.*, 2007). Ammonium sulfate, an inorganic nitrogen source, is much cheaper than yeast extract. Although the M9 medium has such advantages, its effective conversion ratio was often low because the constitutions in M9 medium failed to fit specific strains well. A reasonable minimal medium that could provide a high level production of HLC wasn't given, and some medium optimization researches should be done. DOE has been proved to be an effective tool to optimize specific culture medium (Zhou *et al.*, 2011 and Zhang *et al.*, 2012).

Plackett-Burman (PB) design founded by Plackett RL and Burman JP was an efficient statistical tool to screen out the factors that had significant effects on the production of target products (Lu *et al.*, 2007 and Xu *et al.*, 2009). On base of the results of PB design, the steepest ascent experiment was used to find the neighbor of the optimum level of the significant influencing factors (Montgomeryd 1991); then the response surface methodology (RSM) was applied to ascertain the optimum level of the significant influencing factors, and the most popular RSM design was the central composite design (CCD). In fact, RSM has been used extensively in recent years. Chien-Yu, *et al.*, (2006) acquired a suitable medium for the production of *Antrodia cinnamomea* AC0623 by using RSM method. In order to obtain a reasonable modified minimal medium for this engineered *E. coli* strain and its corresponding Δ*ptsG* strain, all of these methods mentioned above would be applied comprehensively.

In this work, a modified minimal medium for engineered *E. coli* BL21 with/without *ptsG* gene was developed by DOE, and the growth and metabolism characters of these two strains in the modified minimal medium were also investigated so as to improve the yield of HLC.

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MATERIALS AND METHODS

Strains and Medium

Engineered *E. coli* BL21 carried plasmid pNWCP31 containing a resistance gene of kanamycin and temperature-sensitive promoter (Fan *et al.*, 2002). The *ptsG* mutant was reconstructed using one-step inactivation procedure proposed by Datsenko and Wanner (Datsenko *et al.*, 2000).

Engineered *E. coli* were cultivated in an optimized complex medium called fermentation medium (FM) and an optimized minimal medium (MM) during batch phase. This optimized minimal medium was derived from M9 medium by using DOE. The fermentation medium with the carbon- nitrogen mole ratio 0.085:0.019 had been optimized by Guo, *et al.*, (2010) and proved to be fit for HLC expression in this engineered *E. coli*. All of media were prepared with deionized water. Concerning to the fermentation medium, 1 liter of medium contained Glucose (15.3g), Yeast extract (11.8g), K_2HPO_4 (8.8g), NaH_2PO_4 (4.25g), $(NH_4)_2SO_4$ (5.6g), $MgSO_4{\cdot}7H_2O$ (2.5 g), Kanamycin Kanamycin (5×10^{-3} g), and 1mL of Trace element solution. The minimal medium (per L) contained $Na_2HPO_4{\cdot}12H_2O$ (17.19g), $(NH_4)_2SO_4$ (3.97g), KH_2PO_4 (3.00g), NaCl (0.58g), $MgSO_4{\cdot}7H_2O$ (0.25g), $CaCl_2$ (1.11×10^{-5}g), Thiamin (1×10^{-4}g), Glucose (5g), Trace element solution (10mL), and Kanamycin (5×10^{-3}g). The trace element solution for the minimal medium was consistent to the previous report (Mai *et al.*, 2006).

Seed culture

The seeds in frozen glycerol stock were streaked on LB plates and grew overnight in an incubator under 34°C. One clone of strains was taken out and inoculated into two 250-mL flasks with 50mL of LB medium in, and then inoculums were incubated in a shaker at 34°C and 220 rpm for 12 hours. If the need of seeds was large, 12.5mL of the active seed cultures were inoculated into 50 mL of fresh LB medium and incubated for 10 hours under the conditions mentioned above (Guo *et al.*, 2010).

Flask culture

The two strains were cultivated respectively with the optimized complex medium and minimal medium at 34°C and 220 rpm. The temperature was up-shifted to 42°C after OD_{600} reaching 4.5~5.0 for inducing HLC expression, 3 hours later, the temperature was decreased to 39°C and hold on for about 3 hours. All experiments were done in triplicates.

Design of experiment

According to the results of Single-factor experiments, the factors which had distinct positive effects on the production of HLC were screened out through conducting PB design (Plackett *et al.*, 1946). PB statistical design was performed with Design-Expert software 7.0. All experiments were performed in triplicate. On the base of the results of PB design, the factors influencing the HLC production distinctively were chosen to continue the steepest ascent experiment. The steepest ascent experiment was used to judge the orientation and step change of ascent and find the top of "hill" (Montgomeryd 1991). According to the results of the steepest ascent experiment, the CCD was applied to optimize the levels of the influencing factors and determine the quantitative relationships between the variables (the influencing factors) and the responses (the HLC production) (Mead *et al.*, 1975 and Mathews *et al.*, 1981).

Analysis methods

A spectrophotometer (UNICO Model 2082PCS, USA) was applied to measure cell optical density at 600 nm. Dry cell mass (DCW) was used to describe cell concentration, all of samples were prepared from 50mL of the culture after centrifugation (10min, 10000rpm) and three times wash with distilled water, and dried to a constant weight by an oven at 105°C. Bio Profile analyzer 300A (NOVA biomedical, USA) was applied to measure the amount of glucose and acetate in broth. The method of hydroproline colorimetry was used to determine HLC concentration (Guo *et al.*, 2012).

RESULTS

Optimizing the modified medium for engineered E.coli BL21 by DOE

Based on the preliminary experiments, five factors that affected the HLC production greater than the other factors were chosen for performing single factor experiments. The five variables were the inoculation size and the dosages of glucose, thiamin, trace element and $(NH_4)_2SO_4$. The other compositions in the modified medium were same to that of the traditional M9 medium. When the influences of the dosages of glucose, thiamin, trace element and $(NH_4)_2SO_4$ were evaluated, the inoculation size was set at 6% (v/v). The results of the single-factor tests were shown in fig. 1.

From fig. 1, the inoculation size and the concentrations of glucose, thiamin, trace element and $(NH_4)_2SO_4$ were found to have distinct effects on the production of HLC. Then, the dosages of these five factors were optimized by PB design, and the experimental protocol of PB design was listed in table 1. In table 1, -1 and +1 represented the low level and the high level of the coded factors respectively, the corresponding real values were 8g/L and 10g/L of glucose, 1.5×10^{-4}g/L and 2.5×10^{-4}g/L of thiamin, 15mL and 25mL of trace element solution, 5.29g/L and 7.93g/L of $(NH_4)_2SO_4$, and 4% and 8% of inoculation size respectively. The variable with *p*-value less than 0.05 was considered to be significant. The fitting equation of the first-degree polynomial was shown below:

$Y_1 = 0.15\text{-}0.026\ X_1 - 7.27\ E - 003\ X_4$

Table 1: The protocol of PB design and the corresponding results

Run	Glucose	Thiamin	Trace element	$(NH_4)_2SO_4$	Inoculums size	HLC
	X_1	X_2	X_3	X_4	X_5	(g/L ± s.d.)
1	1.00	1.00	1.00	1.00	-1.00	0.13±0.03
2	-1.00	1.00	-1.00	-1.00	-1.00	0.19±0.07
3	-1.00	-1.00	1.00	-1.00	1.00	0.21±0.05
4	-1.00	-1.00	-1.00	-1.00	-1.00	0.18±0.08
5	1.00	-1.00	1.00	1.00	-1.00	0.14±0.03
6	1.00	-1.00	-1.00	1.00	-1.00	0.13±0.05
7	-1.00	1.00	-1.00	1.00	1.00	0.16±0.05
8	-1.00	1.00	1.00	1.00	-1.00	0.16±0.04
9	1.00	1.00	-1.00	1.00	1.00	0.12±0.02
10	1.00	-1.00	-1.00	-1.00	1.00	0.13±0.04
11	-1.00	-1.00	1.00	1.00	1.00	0.17±0.06
12	1.00	1.00	1.00	-1.00	1.00	0.12±0.04

Table 2: The experimental protocol and results of CCD

Run	Glucose		$(NH_4)_2SO_4$		$OD_{600\,nm}$ at 12^{th} (± s.d.)	HLC (g/L±s.d.)
	Code X_1	g/L	Code X_2	g/L		
1	0.00	7.50	1.41	5.55	4.86±0.11	0.17±0.04
2	0.00	7.50	0.00	4.63	5.11±0.18	0.23±0.06
3	0.00	7.50	-1.41	3.70	4.76±0.16	0.16±0.04
4	0.00	7.50	0.00	4.63	5.20±0.16	0.22±0.05
5	-1.00	7.00	-1.00	3.96	4.91±0.15	0.16±0.04
6	0.00	7.50	0.00	3.96	5.10±0.13	0.20±0.05
7	-1.00	7.00	1.00	5.29	4.91±0.17	0.17±0.04
8	1.00	8.00	-1.00	3.96	4.96±0.10	0.18±0.03
9	-1.41	6.80	0.00	4.63	4.82±0.11	0.15±0.04
10	0.00	7.50	0.00	4.63	5.20±0.18	0.22±0.05
11	1.00	8.00	1.00	5.29	5.02±0.18	0.19±0.04
12	0.00	7.50	0.00	4.63	5.15±0.19	0.22±0.05

Table 3: The optimum minimal medium for the *ptsG* mutant and its parent strain

	Parent strain (± s.d.)	*ptsG* mutant (± s.d.)
Glucose (g/L)	7.64	6.11
Thiamin (g/L)	1.50×10^{-4}	1.80×10^{-4}
Trace element (mL)	25	30
$(NH_4)_2SO_4$ (g/L)	4.68	5.82
Inoculums size (%)	8%	8%
HLC (g/L)	0.23 ± 0.02	0.26 ± 0.01
Biomass (OD_{600})	5.11 ± 0.19	4.98 ± 0.18

And the R^2-adj, R^2 and R^2-pred of the fitting equation (Eq. 2) were 95.24%, 97.19% and 93.13% respectively. All these parameters indicated that Eq. 2 could truly describe the practical experiments and the dosages of glucose and $(NH_4)_2SO_4$ affected the production of HLC significantly. In Eq. 2, the regression coefficients of glucose concentration and $(NH_4)_2SO_4$ concentration were negative for the HLC production, which meant that their dosages were too high. The steepest ascent experiments were conducted and the results were displayed in fig. 2. Because Run 4 reached the highest production of HLC, the dosages used in Run 4 were chosen for the further optimization by CCD.

The experimental protocol of CCD were designed with Design-Expert software and shown in table 2, and HLC production and biomass were served as the key responses. A quadratic polynomial model involving the HLC production and OD_{600} were obtained by the multiple regression analysis.

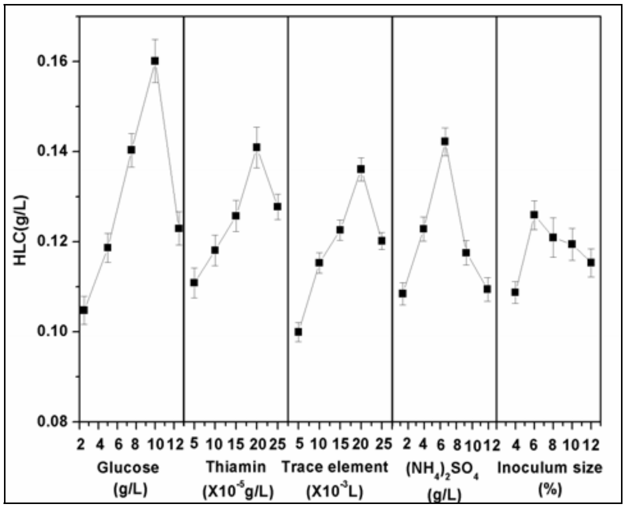

Fig. 1: The production of HLC with changing a single factor in M9 medium; *error bars* indicated the standard deviation.

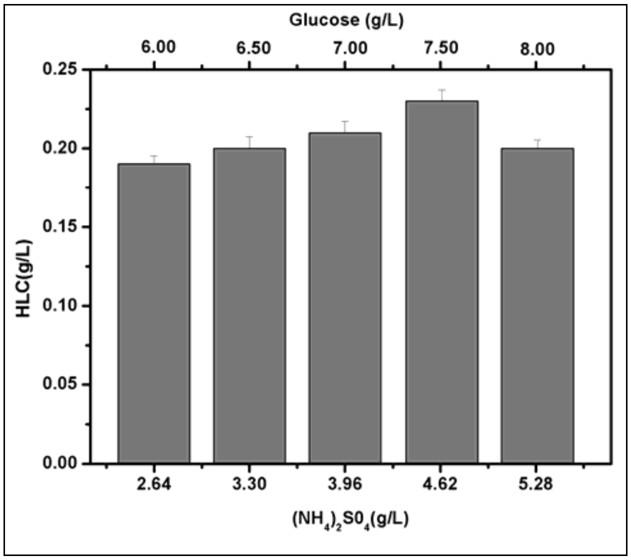

Fig. 2: The results of the steepest ascent; error bars indicated the standard deviation.

Y_2 represented the HLC concentration; R^2 value of the fitting model was 94.29%, which suggested that the model could describe the practical experiments well. F-value of the model was 23.12, which implied the model was significant and the noise interference probability was 0.03%. The *p*-value for lack of fit was 0.61 that was bigger than 0.05, which meant that the theoretic model could fit the experimental data well. In view of the deducing partial derivative results, the maximal HLC concentration reached 0.23g/L finally when the concentrations of glucose and $(NH_4)_2SO_4$ were 7.64g/L and 4.68 g/L respectively. Y_3 represented OD_{600} at the end of cultivation, the maximal OD_{600} was 5.16 when the concentrations of glucose and $(NH_4)_2SO_4$ were 7.64g/L and 4.68 g/L respectively too. Interestingly, higher HLC productivity came from higher biomass (OD_{600}).

$Y_2 = 0.22+0.011\ X_1 +4.22\ E-003\ X_2 +4.21\ E-005\ X_1X_2 - 0.02\ X_{2/1} -0.025\ X_{2/2}$

$Y_3 = 5.15 +0.057\ X_1 +0.025\ X_2 +0.015\ X_1X_2 -0.093\ X_{1/2} -0.15X_{2/2}$

From Plot A and B in fig. 3, the maximal HLC productivity and biomass (OD_{600}) located near to the center point. Carbon and nitrogen were important nutritional substances for the cell growth and the expression of target proteins in engineered *E. coli* (Li *et al.*, 2002), especially when glucose and $(NH4)_2SO_4$ were respectively acted as the sole source of carbon and nitrogen in the modified minimal medium.

The optimum recipe for a high production of HLC and biomass of engineered *E. coli* BL21 was: 7.64g/L of glucose, 4.68g/L of $(NH_4)_2SO_4$, 25mL of trace element solution, 1.5×10^{-4} g/L of thiamin and 8% of inoculation size. The other components were same to that of M9 medium.

Optimizing the minimal medium for ptsG mutant by DOE

On the base of the optimization results of the modified minimal medium for engineered *E. coli* BL21, the minimal medium for *ptsG* mutant was further optimized by DOE with Design-Expert software so as to yield a greater amount of HLC and biomass. Finally, the maximal production of HLC and biomass (OD_{600}), 0.26g/L and 4.98, were obtained in the optimized minimal medium listed in table 3.

From table 3, the two modified minimal media were different distinctly in the concentrations of key substrates. The *ptsG* mutant produced more HLC than its parent strain, but harvested less biomass. This diversity might be engendered by the deletion of *ptsG* gene. The *ptsG* mutant could produce 0.26g/L of HLC in this optimized minimal medium, which was as high as the maximum production of HLC in the optimized complex medium reported by Guo, *et al.* (2010). So it was worthy to compare the production of HLC in the optimized minimal medium and the fermentation medium.

Identifying the high production of HLC by engineered E.coli in this optimized minimal medium

Since the optimum concentrations of four key substrates were different obviously, the comparison experiments of these two strains were preformed in their own optimum medium respectively at the same temperature and rotary rate of the shaker. In the fermentation medium, *ptsG* mutant strain produced more biomass and HLC than its parent strain; whereas, in the optimized minimal medium, it yielded more HLC but less biomass (fig. 4).

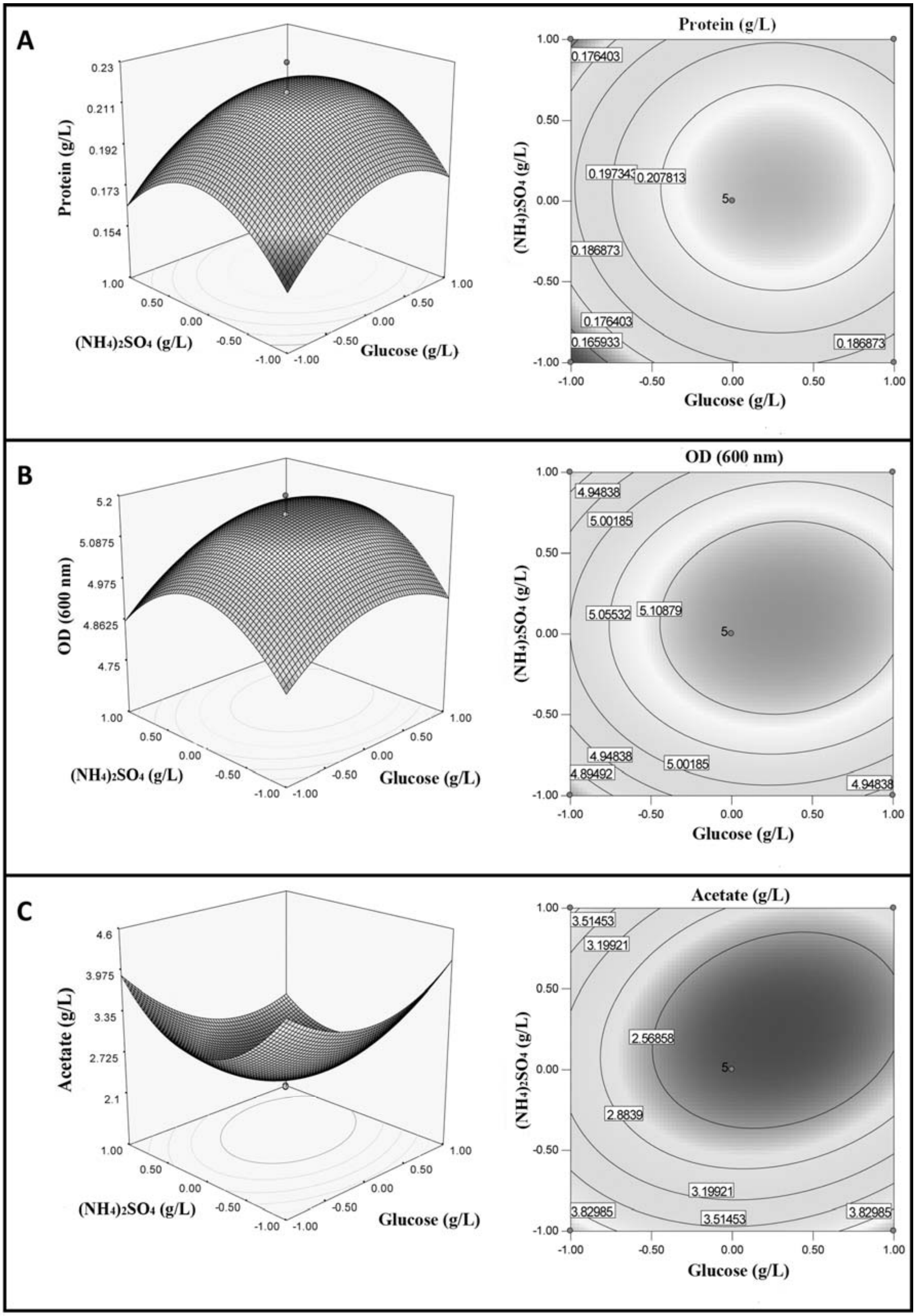

Fig. 3: Response surface plots and contour plots. A, B, and C were for the HLC production, biomass and acetate accumulation respectively.

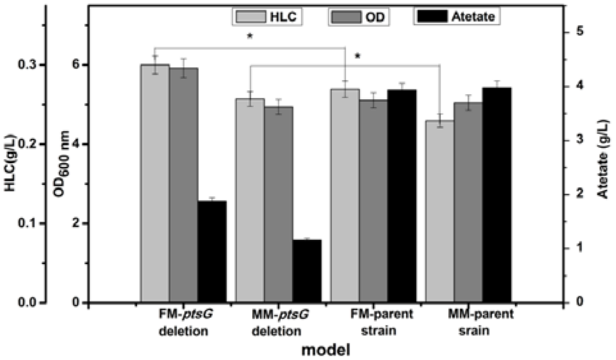

Fig. 4: Comparing the key characters of two strains in two kinds of media.

*" represented a statistically significant difference at the 5% level ($P < 0.05$).

DISCUSSION

According to the analysis results of response surface plots, too high or too low concentrations of glucose and $(NH_4)\ _2SO_4$ would impair the production of HLC (O'Kennedy *et al.*, 2000 and Chen *et al.*, 2003). It was well known that too low concentration of nutrients reduced cell growth rate and the production of target products, but too high concentration of nutrients in medium usually resulted in a large amount of growth inhibitors, especially acetate (Kleman *et al.*, 1994). Concerning acetate accumulation, it was well known that the deficiency of dissolved oxygen usually leaded to the formation and diffusion of acetate. Currently, it was found that acetate was also formed with a high level of dissolved oxygen, and metabolic overflow was considered to activate acetate formation (Marjan *et al.*, 2007). When the uptake rate of glucose was higher than the useful metabolism rate for synthesizing biomass and the target products, metabolic overflow occurred and acetyl-CoA was diverted from TCA cycle toward acetate formation (Koh *et al.*, 1992). In fig. 3C, the changing trend of acetate amount was almost wholly opposite to that of HLC production. Acetate could interfere with methionine biosynthesis, and further inhibit recombinant protein expression (Chen *et al.*, 2003). The minimum amount of acetate appeared close to the center point where the HLC production was at the maximum. The relationship between acetate and biomass was similar to that between acetate and HLC.

Generally, the *ptsG* mutant strain possessed more powerful ability to synthesize HLC both in the optimized minimal medium and complex medium. Additionally, the HLC production of the *ptsG* mutant strain in the optimized minimal medium was very close to the value 0.27g/L of HLC produced by its parent strain consuming the fermentation medium. The biomass (OD_{600}) of the *ptsG* mutant strain in the optimized complex medium was 5.92, which was the highest in all of experiments, and the other biomasses were at the similar level. Because all cultivation time was set at 12h, the higher biomass was correspond to the higher growth rate, that is to say, *ptsG* mutant strain grew at a slower rate as compared to its parent strain in glucose defined medium, but the deletion of *ptsG* gene did not slower cell growth in complex medium. These results were agreed to that of Chih-Hsiung, *et al.* (1994). In the minimal medium, glucose should provide the correct carbon precursors and the energy required to form new biomass (Picon *et al.*, 2008). However, in the complex medium, because organic nitrogen could provide some carbon source, the role of glucose supplying the precursors for synthesizing biomass was decreased; most glucose was served as an energy supplier (Chih-Hsiung, *et al.*, 1994).

Furthermore, the acetate accumulation of the *ptsG* mutant strain was far less than that of its parent in these two types of media. Deleting the *ptsG* gene leaded to a decreased cell growth rate owing to slower glucose uptake rate, which was beneficial for converting glucose to HLC.

CONCLUSIONS

In this study, a cheap modified minimal medium with a clear background for producing HLC was gained by a series of optimum designs. The HLC expression levels of the *ptsG* mutant strain in optimized defined medium almost approached to the production of its parent in the complex culture, and the mutant strain displayed more powerful ability to produce HLC but weaker ability to accumulate acetate than its parent strain.

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