An approach for enhancing the production of human-like collagen II by enlarging the metabolic flux at pyruvate node

MA Xiaoxuan[1, 2], ZHU Chenhui[1, 2], Shang Zifang[1, 2], ZHANG Gaoping[1, 2] and FAN Daidi[1, 2]**

[1]Shaanxi R&D Center of Biomaterials and Fermentation Engineering, School of Chemical Engineering, Northwest University, Xi'an, China

[2]Shaanxi Key Laboratory of Degradable Biomedical Materials, School of Chemical Engineering, Northwest University, Xi'an, China

Abstract: As a key metabolite of glycolysis, Pyruvate affects cell growth and metabolism directly. In order to enhance the production of human-like collagen (HLC) II, pyruvate was added into fermentation broth, and response surface methodology (RSM) to optimize the addition time and dosage of pyruvate and the concentration of phosphate buffer. And the results of PB-design showed that only two variables- the dosage of pyruvate and the concentration of buffer-had significant effects on the production of human-like collagen. Quadratic polynomial models were established after analyzing the results of full factorial central composite design. The optimal dosage of pyruvate and the concentration of buffer were 8.23 mmol·L^{-1} and 0.17 mol·L^{-1} respectively. Under the optimal cultivation condition, Cell OD_{600} was increased 28.72%**;** the maximal production of human-like collagen could be up to 0.29 g·L^{-1} which was 11.54% higher than that of the control group 0.26 g·L^{-1} (the highest production of the former study).

Keywords: Pyruvate, human-like collagen, optimization, response surface methodology.

INTRODUCTION

Human-like collagen (HLC), expressed by recombinant *Escherichia coli* BL21 containing human-like collagen cDNA transcribed reversely from human collagen mRNA, was a water-soluble protein and has a tri-helix structure (1). The advantages such as low immunogenicity, easily modifiable and no virus risk (2) have made this collagen to be a novel biomedical material used as hemostatic material (3), a novel scaffolding biomaterial for artificial bones (4, 5), skin tissues (6), and vascular tissues etc. (7, 8).

On account of the widespread use of HLC, the increment of production was what we wanted most. Metabolism engineering has proved to be an effective approach to enhance the recombinant protein production in the genetically engineered bacteria, especially recombinant *Escherichia coli* (9, 10, 11) One of the main goals of metabolic engineering is to improve the particular biosynthetic capacity through engineering of the target pathway based on rational assumptions for its improvement.

According to metabolic network of *E. coli* growing in the medium with glucose served as the sole carbon source (12), twenty kinds of amino acids that ultimately combine to form the protein product, ten of them are biochemically derived from TCA cycle precursors, ten of them are derived from glycolysis precursors and four of them are derived from the pentose phosphate pathway. Moreover, pyruvate and the intermediate metabolites (α-ketoglutarate and oxaloacetate) of TCA cycle serve as the precursors for the thirteen kinds of amino acids synthesis, as disclosed by the analysis of the composition of total proteins needed for producing 1g biomass, the requirement for amino acids from pyruvate and TCA cycle could account for 73.9% (13). Actually, when the synthesis of recombinant protein stared, the onset of "protein burden'', which could leads to a decrease in glycolytic flux and growth rate. Because of the large number of requirement of pyruvate and TCA cycle intermediate metabolites to synthesis amino acids and protein burden, if the precursors of pyruvate and TCA cycle intermediate metabolites diminish continuously, an imbalance flux may arise. Consequently, it may result in limitation in cell growth and recombinant protein synthesis. It is clearly that, replenishing the precursors for amino acids synthesis should provide a feasible way to improve recombinant protein production.

Up to till, it is hardly to find some reports on this strategy, the reason for this phenomenon is that pyruvate is a kind of organic acid and may affect the cell growth and metabolism by changing the medium pH. On the base of the complicated metabolic mechanism of *E. coli*, there may be a point where a suitable dosage of pyruvate will result in a positive effect rather than a negative effect. In this study, we have thought over adding pyruvate into medium to enlarge the metabolic flux flowing into the key pathway of synthesizing human-like collagen. The effects of pyruvate on cell growth, human-like collagen synthesis and acetic acid accumulation were all considered.

MATERIALS AND METHODS

Microorganisms and medium

Recombinant *Escherichia coli* BL21, the strain producing human-like collagen, carries a plasmid containing a

**Corresponding author:* e-mail: fandaidi@nwu.edu.cn

kanamycin resistance gene and allows high temperature induction (14). The culture medium, in 1L broth, the carbon-nitrogen mole ratios were 0.065: 0.017, which have been optimized by GUO Jiaqing, has proved to fit for human-like collagen expression in *E. coli* BL21 (15). Phosphate Buffer (K_2HPO_4 - NaH_2PO_4) was adopted with its initial pH value 7.0. Other components in the medium were as follows: 1.8g·L^{-1} $MgSO_4 \cdot 7H_2O$, 0.8g·L^{-1} EDTA, 0.6 ml trace element (16) and 0.003g kanamycin.

Seed culture

Primary seed cultures were established by inoculating Recombinant *Escherichia coli* BL21 cells from plate to a 300 ml flask containing 50 ml *Luria-Bertani* medium and cultivating for 12 hours at 34°C and 220 rpm. Secondary seed cultures were prepared by sub-culturing about 12.5 ml of primary seed cultures into 50 ml *Luria-Bertani* and cultivating for 10 hours.

Batch culture

All the test samples were cultured under the same growth conditions: 34°C, 190 rpm, and 5h cultivation. When OD_{600} reached 5.5 ~ 7.0, the temperature was increased to 42°C to induce recombinant *Escherichia coli* BL21 cells synthetizing collagen for 6 hours. All experiments were performed in triplicate.

Analysis methods

Cell OD was measured turbid imetrically at 600 nm with a spectrophotometer (UNICO Model 2082PCS, USA). The acetate was measured using a biochemistry analyzer (Bioprofile 300A, Nova biochemical corporation). Human-like collagen was determined by hydroproline colorimetry.

Response surface methodology

Human-like collagen (HLC) concentration was set as the response value. The variables are the factors that influence the human-like collagen production, which were the addtion time of pyruvate, the dosage of pyruvate, and the concentration of phosphate buffer.

Planckett-burman design

The adding time of pyruvate (X_1), the dosage of pyruvate (X_2) and the concentration of phosphate buffer (X_3), were investigated. All experiments were performed in triplicate protocols. And the *Plackett-Burman* statistical design was conducted with Design-Expert software to determine the significant factors.

The steepest ascent design

The significant factors (the dosage of pyruvate and the concentration of phosphate buffer) which influence the human-like collagen production obviously were determined according to the results of *Planckett-Burman* experiments. In order to determine the optimal regions of these significant factors, the steepest ascent experiments were conducted. The non-significant factors were kept at corresponding optimized levels according to the single factor experiments (the adding time of pyruvate was point of temperature induction). The orientation and step change of ascent were deduced from the sign of coefficient given by Design-Expert software and the experience of experiments, respectively.

Full factorial central composite design

The optimal region that determined by steepest ascent was applied to full factorial central composite design (CCD). A 2^2 factorial center composite design with four axis points (α=1.414) and six central points leading to a total 14 experiments were employed to optimize the human-like collagen production. The variables are the significant factors that determined in the *Planckett-Burman* design. The code conversion equation is shown as below:

$$x_i = (X_i - m_i) / l \quad (1)$$

Where m_i is the optimal value of the significant factors that determined by steepest ascent; x_i is code value; X_i is conversion value; l is step change. The second-degree polynomial equation is

$$Y_i = \alpha_0 + \sum \alpha_i x_i + \sum \alpha_{ii} x_i^2 + \sum \alpha_{ij} x_i y_j \quad (2)$$

Where Y_i is the predicted response of human-like collagen concentration, x_i and y_j are the variables; α_0 is the offset term; α_i is the ith linear coefficient; α_{ii} is the ith quadratic coefficient; and α_{ij} is the ijth interaction coefficient. The Full Factorial Central Composite Design design and results were also conducted with Design-Expert software 7.0.

Verification

According to the results of RSM, optimal cultivate condition were obtained. The experimental group was carried out under the optimized conditions, while the control group was accomplished under non-optimized conditions.

RESULTS

Modeling for human-like collagen production

Screening experiments

The variable with p-value less than 0.05 was considered to be significant. According to p-value, the dosage of pyruvate and the concentration of phosphate buffer affected the production of HLC significantly. The fitting equation of the first-degree polynomial was shown bellow:

$$Y = 22.96 - 0.52 X_2 + 0.32 X_3 \quad (3)$$

R^2 value of the fitting analysis was 94.28%. The *Lack of Fit* F-value of 1.53 implies the *Lack of Fit* is not significant. Not significant *Lack of Fit* F-value implies that the model could fit the actual well. In order to obtain the maximal production of HLC, these two significant

factors (the dosage of pyruvate and the concentration of phosphate buffer) were further optimized to approach the optimal region in RSM so as to establish an efficient equation. The effect of the dosage of pyruvate (X_2) to the human-like collagen production was negative according to the *Planckett-Burman* design, and the effect of the concentration of phosphate buffer (X_3) to the HLC production was positive. The step changes of X_2 and X_3 were 2.64 and 0.02, respectively. The path of steepest ascent and results were shown in table 3. It was found that run 2 was the most closest to the optimal region. Therefore, run 2 was selected for the extended optimization using a full factorial central composite design.

Based on the results of *Planckett-Burman* and the Steepest Ascent design, the values of the dosage of pyruvate and phosphate buffer were further optimized using a full factorial central composite design. Experimental design and results were listed in table 4.

With Design-Expert 7.0 software, a quadratic polynomial model for HLC production was obtained by multiple regression analysis of the results of full factorial central composite experiments (table 5).

$$Y_1 = 28.14 + 0.68X_2 + 0.30X_3 + 0.43X_2X_3 - 3.04X_2^2 - 2.85X_3^2 \quad (4)$$

Y_1 represents the HLC concentration. R^2 was 97.16% and it meant that the model could explain up to 97.16% of the total variation in response. The *p*-value of 0.4963 for *Lack of Fit* is much larger than 0.05, which means the model fit well. According to the deducing partial derivative results, the maximal HLC concentration of 28.19×10^{-2} g·L^{-1} could be realized when x_2 was 0.12 and x_3 was 0.00. According to Eq. (1), the conversion values of the dosage of pyruvate and the concentration of buffer were 8.23 mmol·L^{-1} and 0.17 mol·L^{-1}, respectively.

Effects of the dosage of pyruvate and the concentration of buffer on biomass

The method of RSM was used for optimizing the addition of pyruvate and buffer on biomass. According to the results of *Planckett-Burman* and the Steepest Ascent design, a full factorial CCD was adopted. Experimental design and results were listed in table 6.

Two quadratic polynomial models describing biomass were obtained by multiple regression analysis of the results of full factorial central composite experiments.

$$Y_2 = 7.72 + 0.25X_2 - 0.012X_3 - 0.055X_2X_3 - 0.46X_2^2 - 0.59X_3^2 \quad (5)$$

$$Y_3 = 8.32 + 0.19X_2 - 0.037X_3 - 0.020X_2X_3 - 0.063X_2^2 - 0.075X_3^2 \quad (6)$$

Where Y_2 is the biomass (OD_{600}) at 5th hour, Y_3 is the biomass (OD_{600}) at the end of the fermentation, X_2 and X_3 was the dosage of pyruvate and the concentration of buffer respectively.

R^2 of model Y_2 and Y_3 was 97.94% and 96.45% individually. As shown in table 7, in the light of the results of *p*-value for *Model* and *Lack of Fit*, we could know that models of Y_2 and Y_3 could fit the actual well. Based on the deducing partial derivative results, the maximal biomass (OD_{600}) at 5th hour and the final biomass (OD_{600}) (at 11th hour) was 7.75 and 8.33, respectively. Correspondingly, the dosage of pyruvate and buffer concentration was 8.60 mmol·L^{-1}, 0.17 mol·L^{-1} and 8.31 mmol·L^{-1}, 0.17 mol·L^{-1}, individually.

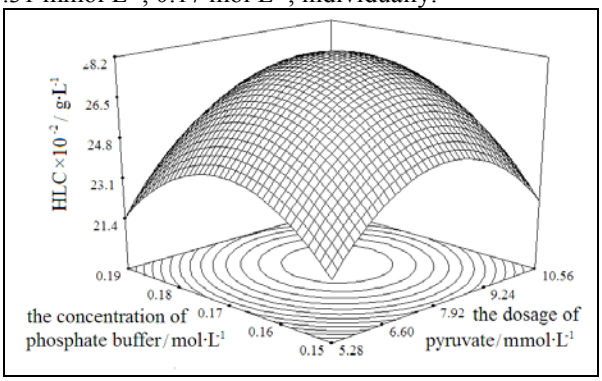


Fig. 1: Response surface plot for the production of human-like collagen

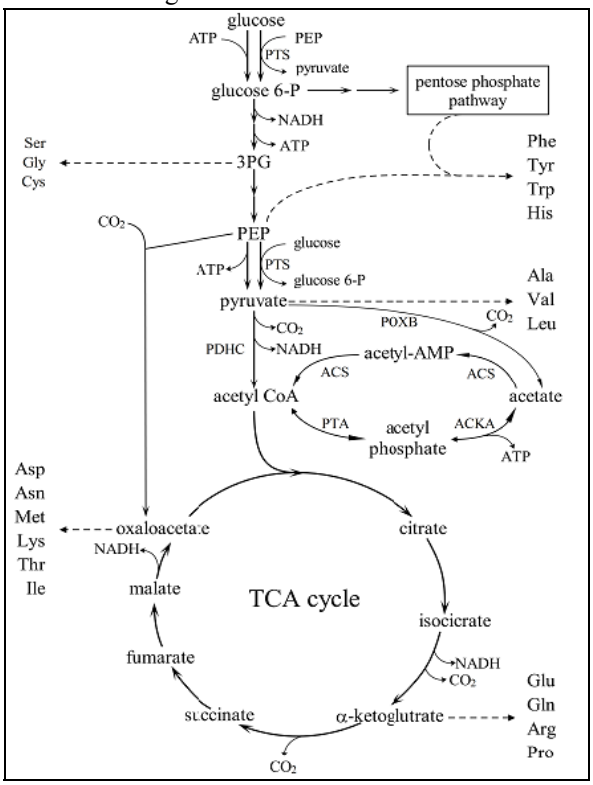
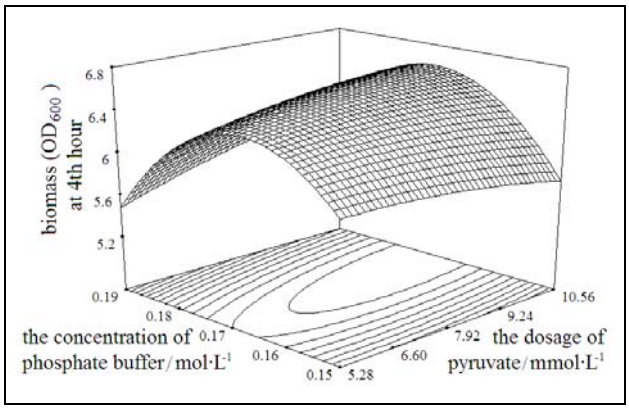
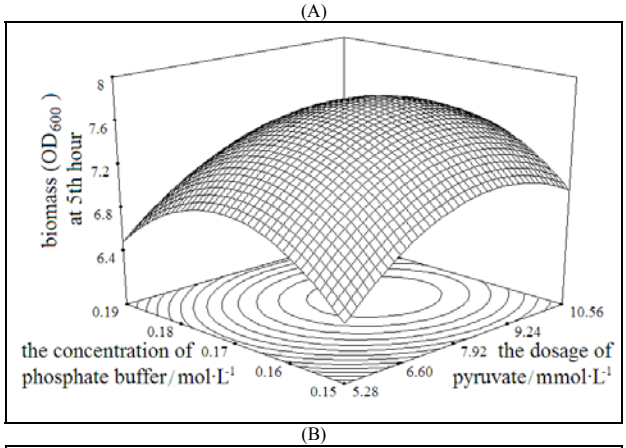


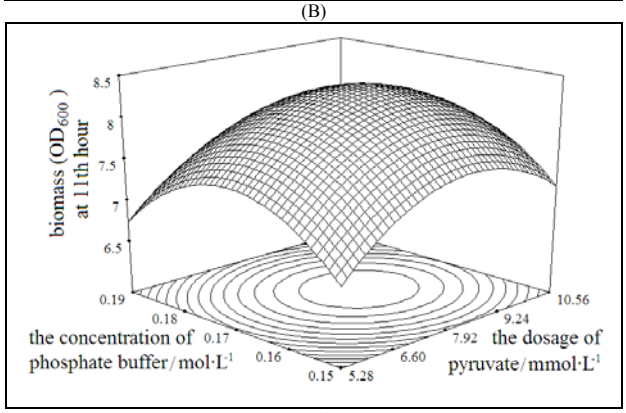
Fig. 2: Overview of the central metabolic pathway in *E. coli*. The biosynthetic pathways leading to the synthesis of amino acids are indicated by dotted lines. PTS, glucose-phosphotransferase system; PTA, phosphotransacetylase; ACKA, acetate kinase; ACS, acetyl-CoA synthetase; POXB, pyruvate oxidase; PDHC, pyruvate dehydrogenase complex; TCA, tricarboxylic acid cycle.



(A)



(B)

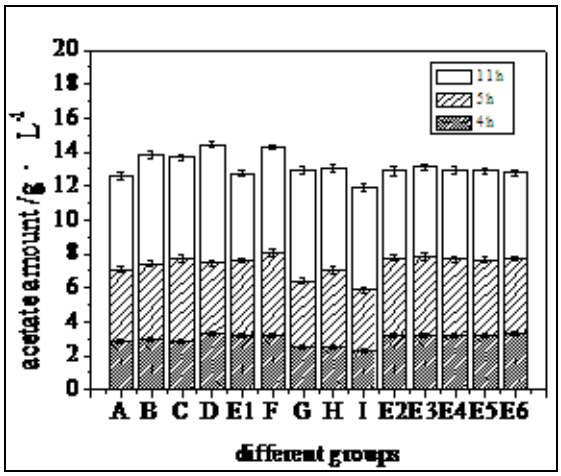


(C)

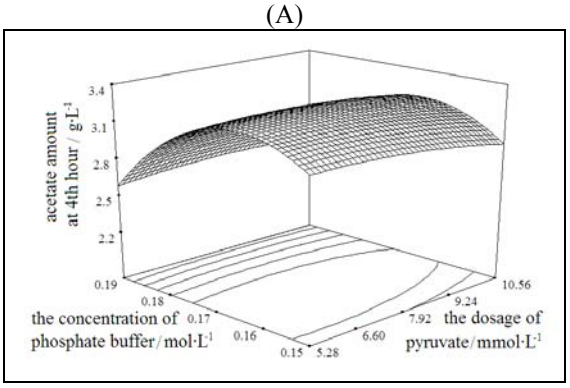
Fig. 3: Response surface plots describing the effects of the concentration of pyruvate and buffer on biomass

Acetic acid

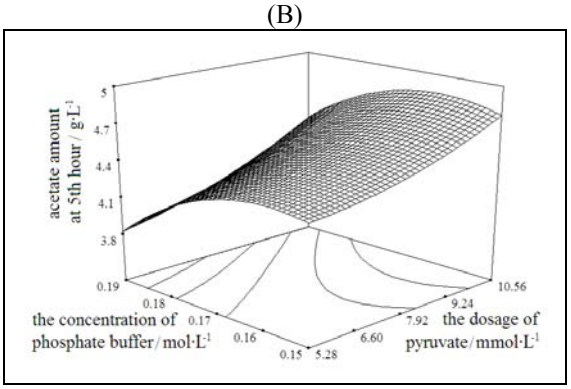
Acetate, a major by-product of *Escherichia coli* metabolism, was found to be given several hazards to cell growth and protein synthesis. Acetate could reduce the rate of RNA, DNA and lipid synthesis (17). Moreover, acetate production represents a diversion of carbon that might be used for cell growth or the protein synthesis (18). Additionally, acetate interferes with methionine biosynthesis, which causes the inhibitor homocysteine to be accumulated, and finally acetate brings a bigger inhibitory to recombinant protein-producing cells than to wild-type cells (19).



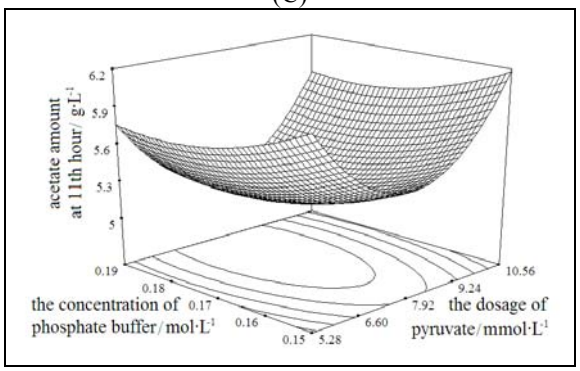
(A)



(B)



(C)



(D)

Fig. 4: Changes in the amount of acetate at full factorial central composite experiment. (a) the dosage of pyruvate and the concentration of phosphate buffer in each group

was: A 4.19 mmol·L^{-1}, 0.17 mol·L^{-1}; B 5.28 mmol·L^{-1}, 0.15 mol·L^{-1}; C 5.28 mmol·L^{-1}, 0.19 mol·L^{-1}; D 7.92 mmol·L^{-1}, 0.14 mol·L^{-1}; E1, E2, E3, E4, E5 and E6 7.92 mmol·L^{-1}, 0.17 mol·L^{-1}; F 7.92 mmol·L^{-1}, 0.20 mol·L^{-1}; G 10.56 mmol·L^{-1}, 0.15 mol·L^{-1}; H 10.56 mmol·L^{-1}, 0.19 mol·L^{-1}; I 11.65 mmol·L^{-1}, 0.17 mol·L^{-1}.

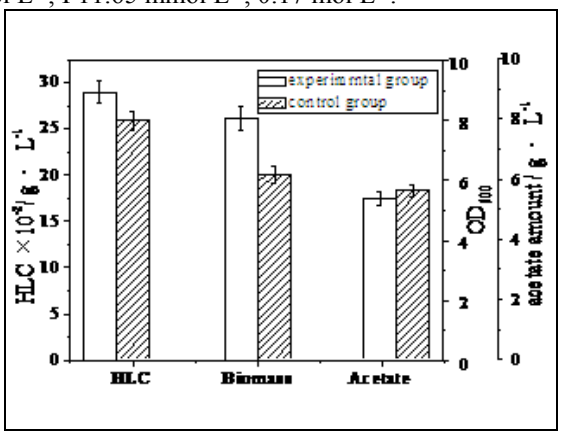


Fig. 5: Results of verification experiment.

According to fig. 2, acetate is formed from pyruvate by pyruvate oxidase and phosphotransacetylase/acetate kinase. The additional pyruvate could also leads to an increasing metabolic flux of acetate. The effect of adding pyruvate on acetate accumulation was investigated too.

Based on the multiple regression analysis of full factorial central composite experiments on acetate amounts, which came from biomass CCD experiment, a quadratic polynomial model was obtained.

$$Y_4 = 5.15 + 0.057X_2 - 0.15X_3 + 0.011X_2X_3 + 0.68X_2^2 + 0.14X_3^2 \quad (7)$$

The R^2 of model Y_4 is 98.34%, and the F value of *Lack of Fit* for model Y_4 is 3.58, which mean that the model could fit the actually experimental data well. Based on the deducing partial derivative results, the minimum acetate amount at 11th hour was 5.15g·L^{-1}, and the corresponding concentration of pyruvate and buffer was 7.86 mmol·L^{-1}, 0.18 mol·L^{-1} which were similar to that results optimized by the production of HLC and biomass.

Table 1: Design of *Plackett-Burman* experiment and results

Run	The adding time of pyruvate	The dosage of pyruvate	The concentration of phosphate buffer	HLC
	X_1	X_2	X_3	×10^{-2} g·L^{-1}
1	1	-1	-1	23.04±0.03
2	-1	1	1	22.78±0.05
3	1	1	1	22.94±0.09
4	-1	1	1	22.68±0.07
5	-1	-1	-1	23.22±0.04
6	-1	-1	1	23.94±0.03
7	1	-1	1	23.72±0.06
8	1	-1	1	23.58±0.10
9	1	1	-1	22.08±0.04
10	-1	1	-1	21.86±0.03
11	-1	-1	-1	23.38±0.11
12	1	1	-1	22.24±0.05

Table 2: The results of regression analysis of the *Planckett-Burman* design

Term	Level		Coefficient	F value	p-value Prob>F	Significant level
	-1	1				
Intercepts			22.96	74.23	< 0.0001	***
X_1 (h)	0	1	-0.52	103.50	< 0.0001	***
X_2 (mmol·L^{-1})	10.56	21.12	0.32	44.95	0.0001	**
X_3 (mol·L^{-1})	0.1	0.15				
Lack of Fit				1.53	0.3496	not significant

Table 3: Design of the path of the Steepest Ascent and results

Run	The dosage of pyruvate	The concentration of phosphate buffer	HLC
	X_2 (mmol·L^{-1})	X_3 (mol·L^{-1})	×10^{-2} g·L^{-1}
1	10.56	0.15	24.98±0.06
2	7.92	0.17	27.27±0.07
3	5.28	0.19	23.48±0.04
4	2.64	0.21	21.12±0.04

Table 4: Design and results of full factorial central composite experiment for HLC

Run	The dosage of pyruvate		The concentration of phosphate buffer		HLC
	Code x_2	X_2 (mmol·L^{-1})	Code x_3	X_3 (mol·L^{-1})	×10^{-2} g·L^{-1}
1	0	7.92	-1.41	0.14	21.71±0.06
2	0	7.92	0	0.17	28.73
3	-1.41	4.19	0	0.17	21.17±0.03
4	1	10.56	1	0.19	24.05±0.07
5	0	7.92	1.41	0.20	22.7±0.09
6	0	7.92	0	0.17	27.27
7	0	7.92	0	0.17	29.08
8	0	7.92	0	0.17	28.13
9	1.41	11.65	0	0.17	22.50±0.10
10	-1	5.28	-1	0.15	21.75±0.08
11	-1	5.28	1	0.19	21.39±0.07
12	0	7.92	0	0.17	27.82
13	0	7.92	0	0.17	28.30
14	1	10.56	-1	0.15	22.68±0.04

Table 5: Regression analysis of the HLC central composite experiment

Term	Coefficient	Coefficient standard error	*F* value	*p*-value Prob>F
Intercept	28.14	0.21	90.07	< 0.0001
X_2	0.68	0.18	13.53	0.0062
X_3	0.30	0.18	2.63	0.1437
$X_2 * X_3$	0.43	0.26	2.71	0.1385
$X_2 * X_2$	-3.04	0.19	246.86	< 0.0001
$X_3 * X_3$	-2.85	0.19	217.73	< 0.0001
Lack of Fit			0.92	0.4963

Table 6: the Design and results of CCD experiments for biomass

Run	The dosage of pyruvate		The concentration of phosphate buffer		Biomass (OD_{600}) at 5th hour	Biomass (OD_{600}) at 11th hour
	Code x_2	X_2 (mmol·L^{-1})	Code x_3	X_3 (mol·L^{-1})		
1	0	7.92	-1.41	0.14	6.52±0.23	7.04±0.26
2	0	7.92	0	0.17	7.78±0.24	8.52±0.27
3	-1.41	4.19	0	0.17	6.43±0.20	6.96±0.26
4	1	10.56	1	0.19	6.92±0.23	7.24±0.24
5	0	7.92	1.41	0.2	6.44±0.19	6.66±0.26
6	0	7.92	0	0.17	7.76±0.34	8.24±0.38
7	0	7.92	0	0.17	7.83±0.32	8.20±0.39
8	0	7.92	0	0.17	7.78±0.37	8.38±0.40
9	1.41	11.65	0	0.17	7.07±0.32	7.22±0.33
10	-1	5.28	-1	0.15	6.42±0.31	6.56±0.32
11	-1	5.28	1	0.19	6.54±0.30	6.72±0.29

Verification

By virtue of the optimum concentration of pyruvate and buffer for enhancing the production of HLC and biomass and reducing the accumulation of acetic acid, the verification experiment conditions were: the dosage of pyruvate and buffer were 8.23 mmol·L^{-1} and 0.17mol·L^{-1}, respectively. Under the optimum operation conditions, the maximal concentration of HLC could reach up to 28.96×10^{-2} g·L^{-1} which increased by 11.72% in comparison to the control group 25.92×10^{-2} g·L^{-1} and increased by 10.53% in comparison to the GUO Jiaqing's optimization 26.2×10^{-2} g·L^{-1} (16). According to Eq. (4),

Table 7: Regression analysis of the central composite experiment for biomass

		F Value	*p*-value Prob>F	Significant level
Y_2	Model	69.20	<0.0001	Significant
	X_2	39.31	0.0002	Significant
	X_3	0.92	0.4963	Not significant
	Lack of Fit	0.71	0.5844	Not significant
Y_3	Model	43.48	<0.0001	Significant
	X_2	8.81	0.0179	Significant
	X_3	0.35	0.5694	Not significant
	Lack of Fit	2.52	0.1719	Not significant

the predict value of HLC production under the optimal condition was 28.19×10^{-2} g·L^{-1} with 2.02% tolerance to the actual HLC concentration production value, which proved that the regression equation could anticipate the actual experiments well. Biomass (OD_{600}) of the control group and the experimental group were 6.17±0.29 and 8.06±0.39, respectively, the increasing extent was up to 30.63%. According to Eq. (6), the predict value of biomass under the optimal condition was 8.33 with 3.35% tolerance to the actual value, which proved that the regression equation approached the actual experiment well. And the acetate amount of the control group and the experimental group were 5.62±0.19 g·L^{-1} and 5.36±0.22 g·L^{-1}, respectively, the accumulation of acetate was decreased by 4.85%.

DISCUSSION

From fig. 1 and table 5, the dosage of pyruvate concentration was the prominent factor to the HLC production. It was advantageous for the synthesis of human-like collagen when the dosage of pyruvate was 8.23 mmol·L^{-1}. The accumulated pyruvate could be metabolized into acetyl coenzyme A (acetyl CoA) and then participate in tricarboxylic acid cycle (TCA), additional pyruvate could enhance the carbon flow belongs to those pathway. And also could enlarge the synthesis of amino acids which form HLC. Although, adding pyruvate could make a positive contribution to human-like collagen synthesis, the effect on biomass is still a question. The experiment below would try to give this question an answer.

In term of the optimization results of biomass CCD experiments, the concentration of pyruvate at 5th hour and at 11th hour was 8.60 mmol·L^{-1} and 8.31 mmol·L^{-1}, respectively. And the optimum dosage of pyruvate was very close to the dosage based on HLC CCD experiments. According to fig. 3 (b, c) and fig. 1, we found the effects of the dosage of pyruvate and the concentration of phosphate buffer on biomass were in accordance with on HLC production. From fig. 3, the change trend of biomass (OD_{600}) at the 4th hour disagreed to that at the 5th and the 11th hour, and it meant that both higher and lower concentration of buffer weren't favorable to cell growth at the 4th hour. In Paula Arense's research, when *Escherichia coli* was long-term exposed to the salt stress, in order to provide the precursors for biosynthesis, energy production by substrate-level phosphorylation (PTA – ACKA pathway) (fig. 2) and the anaplerotic function of the TCA cycle would be stimulated and enhanced (20). Although, the salt concentration in this study was not high as 0.8mol/L in Paula Arense's research, the *E. coli* was metabolized sugar into the intermediates and cell block-building, the accumulation of organic acids belongs to intermediates in broth could bring down the medium pH, bacteria would take more energy to against the changes of H^+ concentration for survival rather than for generation (21).

Based on the regression analysis of model Y_2 and Y_3 (table 7) and the response surface plot (fig. 3), the concentration of pyruvate was found to result in more noticeable effect on biomass than that of the phosphate buffer. This might be that, after pyruvate was added, both of the metabolic fluxes from pyruvate to the useful intermediates and by-products are increased, in order to gain more biomass, the balance between the positive effect and the negative effect should be considered comprehensively. And the change trend of biomass in fig. 3 (b, c) further proved that both too high and too low addition dosage of pyruvate were not benefit for cell growth and propagation. How this trend came from? In the light of central metabolic pathway (fig. 2), the major by-product, namely acetate, was noticed. A study of how the acetate changes after adding pyruvate was adopted.

According to fig. 4 (a, b) and fig. 3 (a), we found the changing trend of acetate amount was in line with the trend of biomass. First, fast growth rate means more glucose consumption, the metabolic flux of central metabolism pathway is increasing, meanwhile, fast growth rate also leads to an increasing in by-products metabolic flux (22). Second, fast growth rate demands additional energy, to bacteria, as the TCA cycle could not give enough energy, another way for product additional energy should be taken, the way of metabolic pyruvate to acetate by phosphotransacetylase/acetate kinase could generate ATP and NADH, which could give an extra

energy that demanded (23). Whereas, the acetate amount was at its minimum value at 11th hour, meanwhile the biomass was the maximum. As to this phenomenon, there was study has been shown that PEP is a co-substrate for glucose uptake into the cell by the PTS, an increase in pyruvate concentration relative to PEP can reduce the rate of glucose uptake, and then the decrease of glucose uptake rate could reduce the rate of acetate form because the form rate of acetate is directly related to the consumption rate of the usual substrate, glucose.

As to recombinant protein production, namely human-like collagen production, lower acetate concentration could lead to lower harm to protein synthesis. According to fig. 1 and fig. 4 (d), the trend of two pictures have been shown the HLC production was at its maximum value when the acetate was at its minimum value.

CONCLUSIONS

In this study, we obtained the optimal concentration of pyruvate and phosphate buffer for enhancing the production of HLC and biomass by RSM after screening. The final optimized concentration of pyruvate and buffer were 8.23mmol·$L^{-1}$and 0.17mol·L^{-1}, respectively. Under the optimal conditions, the HLC production, biomass and acetate were increased 11.72%, increased 28.72% and decreased 4.85% in comparison to the control group individually. The suitable addition of pyruvate could lead to a remarkable positive effect on the production of HLC and biomass.

ACKNOWLEDGEMENTS

This study was financially supported by the National Natural Science Foundation of China (21106111); the National High Technology Research and Development Program of China (863 Program, 2012AA021203, 2014AA022108); the Shaanxi Provincial Scientific Technology Research and Development Program (2012KJXX-28, 2013KJXX-28).

REFERENCES

Bailey JE (1991). Toward a science of metabolic engineering. *Science*, **252**(5013): 1668-1675.

Cherrington AC, Hinton M and Chopra I (1990). Effect of short-chain organic acids on macromolecular synthesis in *Escherichia coli*. *J Appl Microbiol.,* **68**(1): 69-74.

Fan DD, Duan MR, Mi Y, Song JR, Xi JF, Wang DW and Wang GZ (2002). High-density fermentation of recombinant *E. coli* for production of human-like collagen. *J Ind Eng Chem* (China), **53**(7): 752-754.

Guo JQ, Luo YE, Fan DD, Gao PF, Ma XX and Zhu CH (2010). Analysis of metabolic products by response surface methodology for production of human-like Collagen II. *Chin J Chem Eng.*, **18**(5): 830-836.

Hu K, Cui FZ, Lü Q, Ma J, Feng QL, Xu L and Fan DD (2007). Preparation of fibroin/recombinant human-like collagen scaffold to promote fibroblasts compatibility. *J Biomed Mater Res.*, **84**(2): 483-490.

Ishizaki A, Tripetchkul S, Tonokawa M, Shi ZP and Shimizu K (1994). PH-mediated control methods for continuous ethanol fermentation using *Zymomonas mobilis*. *J Ferm Bioengin.*, **77**(5): 541-547.

Jacky LS, Lorraine PY, Hans VW and Lonnie OI (1995). Protein burden in *Zymomonas mobilis*: Negative flux and growth control due to overproduction of glycolytic enzymes. *Microbiology*, **141**(9): 2329-2337.

Korz DJ, Rinas U, Hellmuth K, Sander EA and Deckwer WD (1995). Simple fed-batch technique for high cell density cultivation of *Escherichia coli*. *J Biotechnol.*, **39**(1): 59-65.

Koh BT, Nakashimada U and Pfeiffer M (1992). Comparison of acetate inhibition on growth of host and recombinant *Escherichia coli* K12 strains. *Biotechnol Lett*., **14**(12): 1115-1118.

Luo YE, Fan DD, Ma XX, Wang DW, Mi Y, Hua XF and Li WH (2005). Process control for production of human-like collagen in fed-batch culture of *Escherichia coli* BL21. *Chin J Chem Eng.*, **13**(2): 276-279.

Luo YE, Fan DD, Shang LA, Shi Hui J, Ma XX, Mi Y and Zhao CF (2008). Analysis of metabolic flux in *Escherichia coli* expressing human-like collagen in fed-batch culture. *Biotechnol Lett.*, **30**(4): 637-643.

Ma M and Fan DD (2007). Hemostatic effect and histocompatibility of chitosan-human-like collagen on rabbit liver wound surface. *J the Fourth Military Medical University* (China). **12**: 1122-1124.

March JC, Eiteman MA and Altman E (2002). Expression of an anaplerotic enzyme, pyruvate carboxylase, improves recombinant protein production in *Escherichia coli*. *Appl Environ Microbiol.*, **68**(11): 5620-5624.

Paula A, Vicente B, José LI and Manuel C (2010). Metabolic adaptation of *Escherichia coli* to long-term exposure to salt stress. *Process Biochemistry*, **45**: 1459-1467.

Stephanopoulos G (1999). Metabolic fluxes and metabolic engineering. *Metab Eng.*, **1**(1): 1-11.

Stephanopoulos G and Kelleher J (2001). How to make a superior cell. *Science*, **292**(5524): 2024-2025.

Wang Y, Cui FZ, Jiao YP, Hu K and Fan DD (2008). Modification of bone graft by blending with lecithin to improve hydrophilicity and biocompatibility. *Biomed Mater.*, **3**(1): 1-6.

Wong MS, Wu S, Causey TB, Bennett GN and San KY (2008). Reduction of acetate accumulation in *Escherichia coli* cultures for increased recombinant protein production. *Metab Eng.*, **10**(2): 97-108.

Yang YT, Bennett GN and San KY (2001). The effects of feed and intracellular pyruvate levels on the redistribution of metabolic fluxes in *Escherichia coli*.

Metab Eng., **3**(2): 115-123.

Yin MW, Nan YM and Wang XM (1994). Improvement of the spectrophotometric method for the determination of hydroxyproline. *J Henan Medical University* (China), **29**: 74-77.

Zhai Y and Cui FZ (2006). Recombinant human-like collagen directed growth of hydroxyapatite nanocrystals. *J Cryst Growth*, **291**(1): 202-206.

Zhu CH, Fan DD, Duan ZG, Xue WJ, Shang LA, Chen FL and Luo YE (2009). Initial investigation of novel human-like collagen/chitosan scaffold for vascular tissue engineering. *J Biomed Mater Res.*, **89**(3): 829-840.

Zhu CH, Fan DD, Ma XX, Xue WJ, Yu YY, Luo YE, Liu BW and Chen L (2009). Effects of chitosan on properties of novel human-like collagen/chitosan hybrid vascular scaffold. *J Bioact Compat Pol.*, **24**(6): 560-576.