JOURNAL OF PURE AND APPLIED MICROBIOLOGY, APRIL 2014. Vol. **8**(2), p. 1645-1656

Production of Malate from Xylose with *Aspergillus parasiticus*

Yue Wu[1,2], Lijun Pan[1]*, Shuo Li[2], Shaotong Jiang[1] and Xingjiang Li[1]*

[1]School of Biotechnology and Food Engineering, Hefei University of Technology, Hefei, Anhui Province, 230009, P.R. China.
[2]Food Department, Liaoning Agricultural Vocation-Technical College, Yingkou, Liaoning Province, 115009, P.R. China.

(Received: 05 July 2013; accepted: 24 August 2013)

The production of malate by xylose fermentation with *Aspergillus parasiticus* CICC40365 and the involved metabolic pathway were investigated in this paper. A Box-Behnken experimental design was employed to study the fermentation medium components on the basis of monofactorial experiment. The results showed that the optimal medium composition for malate production were as follows: the xylose, $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$, $(NH_4)_2SO_4$, yeast extract powder, $MgSO_4$ and $CaCO_3$ were 100.0g/L, 0.15g/L, 0.08g/L, 2.0 g/L, 3.0g/L, 0.20g/L and 80g/L, respectively; the malate yield of 53.71g/L was obtained from the optimal condition and it was 40.5% higher than that of original condition. The optimum fermentation conditions through single factor experiments were found to be a temperature of 32!, shake flask liquid volume of 60mL/250mL, inoculum ratio 8%(v/v) and incubation time of 7 days leading to the malate yield of 55.64g/L. Moreover, the preliminary analysis result from the xylose metabolic pathway indicated that the xylulokinase was the key rate-limiting enzyme for the xylose metabolism of this strain. Thus by fermentation processing optimization, the yield of malate was raised effectively.

Key words: Malate, *Aspergillus parasiticus*, Box-Behnken experimental design, Xylose.

L-malic acid LMA , also called hydroxyl-butane diacid, was an important organic acid generated in the organism metabolic process. It is a kind of white crystalline or crystal powder, and has specific acidity. LMA is also a natural organic acid which widely exists in the vegetables and fruits[1-2]. In 1967, L-malic acid was confirmed as a safe and nontoxic and edible organic acid which was widely used in the food, medicine, cosmetic and other industries as excellent acid condiment and antistaling agent[3-5].

Currently, L-malic acid can be produced through four primary ways[6-7]: (1). Direct extraction from the fruit and vegetable juice, however, it was difficult to realize industrial production because of the limitation of raw materials and high costs (2). Chemical synthesis via hydration of maleic or fumaric acid, yielding the racemate (3). Conversion of fumaric acid by immobilization of cells and immobilization of enzymes. The process has been researched by many people and employed in the industrial production, but the L-malic acid produced by this way had a low yield and a high cost, the feedstock fumaric acid which was prepared by chemical synthesis had potential security liability[8-9]. (4). There are two types of fermentation, namely, the direct fermentation and two-step fermentation. The direct fermentation is to produce L-malic acid from sugar or non-sugar carbon source for fermentation by single strain; two-step fermentation is to convert the sugar into fumaric acid first, then convert the fumaric acid into L-malic acid by two microbes with different functions. The latter is not suitable for industrial production, because of its strict to the fermentation conditions,

* To whom all correspondence should be addressed.
E-mail: lixingjiang1978@hfut.edu.cn; panlijun1955@hotmail.com.

the long incubation period and the low yield[10]. Nevertheless, the direct fermentation can avoid the drawbacks above, and more importantly, the products have high security, and become a favorite.

The purpose of the present work was to obtain the optimal fermentation parameters through single factor experiments and response surface methodology, the another purpose was to preliminarily analyse the xylose metabolic pathway of *Aspergillus parasiticus* CICC40365, which can provide some reference frame for the bioconversion of xylose in the lignocellulosic hydrolysate.

MATERIALS AND METHODS

Microorganisms

Aspergillus parasiticus CICC40365, preserved in the laboratory of School of Biotechnology and Food Engineering, the Institute of Agriculture Products Processing Technology, Hefei University of Technology, was used in this study.

Culture medium

The culture was maintained on Petri dishes of PDA (potato dextrose agar). And the fermentation medium in this test contained the following components (g/L): xylose 100, $(NH_4)_2SO_4$ 2, yeast extract powder 3, KH_2PO_4 0.2, $MgSO_4$ 0.1, $MnSO_4 \cdot H_2O$ 0.1, $FeSO_4 \cdot 7H_2O$ 0.05, $CaCO_3$ 80.

Activation culture

After inoculation from the original slant, the dishes were incubated in the constant temperature incubator at 32°C for 3-5 days and subsequently stored at 4°C.

Culture conditions

The suspension of spores was obtained by washing the cultures with sterile distilled water, (the suspension concentration was 1×10^7spores/mL), the fermentations were carried out at 32° for 7 days in 250 mL shake flasks filled with 50 mL of fermentation medium held on a rotary platform shake (200r/min) with inoculation ratio of 10%.

Analytical techniques

L-malic acid was determined by high performance liquid chromatography (HPLC) of biomass-free filtered broth. The fermentation broth was mixed with 0.5 mol/L sulfuric acid solution in a ratio of 1:1(v/v), stayed for 30 min in the 60° water bath. Centrifuge for 10 min at 8000 r/min, supernatant fluid was used for HPLC analysis. The analysis conditions were as follows: Chromatograph type Waters e2695 chromatographic column Ultimate®LP-C18, 5μm, 4.6×250 mm. The eluent was phosphate buffer, pH value of 2.7, the eluent flow rate was 0.8 mL/min, the sample injection volume was 20μm.

Reducing sugars were estimated by the dinitrosalicylic acid (DNS) method[11]. The biomass dry weight was determined by filtering the fermentation broth to collect the mycelia pellets and washing them with 0.01 mol/L hydrochloric acid to remove the residual calcium carbonate, and the washed biomass was dried at 80°C for 24h before weight analysis[12] Activities of XR, XDH were determined as described in the reference literature[13]. The XK activity was measured as described in the literature[14]. All values were measured in triplicate.

Experimental design

Optimization of fermentation medium

On the basis of the single factor experiment, response surface methodology using Box-Behnken design was applied to study the response pattern and to identify the optimum combination of variables. The concentrations of $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$, yeast extract powder, $(NH_4)_2SO_4$ were chosen for the independent variables and were shown in Table 1. The yield of L-malic acid was used as the dependent output variable. RSM and analysis of variance (ANOVA) were performed using the design export 8.0 software. All experiments were conducted in triplicate.

Optimization of fermentation conditions

According to the optimized medium, the effects of inoculums ratio, shake flask liquid volume and fermentation temperature on the yield of L-malic acid were investigated through single factor experiment. The experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Single factor experiments

The effects of nitrogen sources and their concentration, the concentration of KH_2PO_4, $MgSO_4$, $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$ and $CaCO_3$ on the L-malic acid yield were investigated. The results were shown in Figs. 1-8. Data were expressed as means±SD.

Results from the Fig.1 showed that $(NH_4)_2SO_4$ was the best inorganic N for the L-malic acid production; yeast extract powder was the optimal organic N for the higher yield of the L-malic acid. It was probably because of the simple composition and stable properties of inorganic nitrogen source, it can be quickly utilized by the strain and be help to the growth and metabolism;

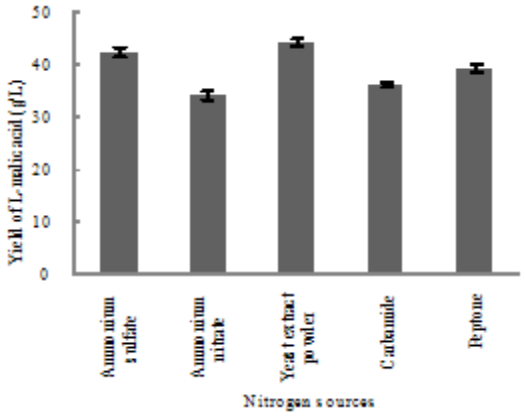

Fig. 1. Effect of different nitrogen sources on the yield of L-malic acid

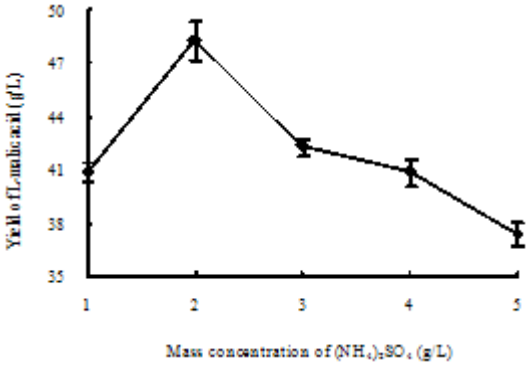

Fig. 2. Effect of concentration of $(NH_4)_2SO_4$ on the yield of L-malic acid

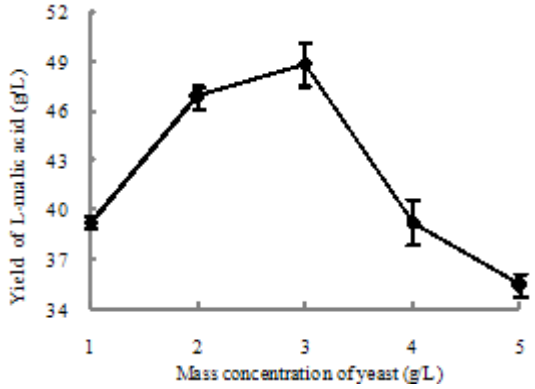

Fig. 3. Effect of mass concentration of yeast extract powder on the yield of L-malic acid

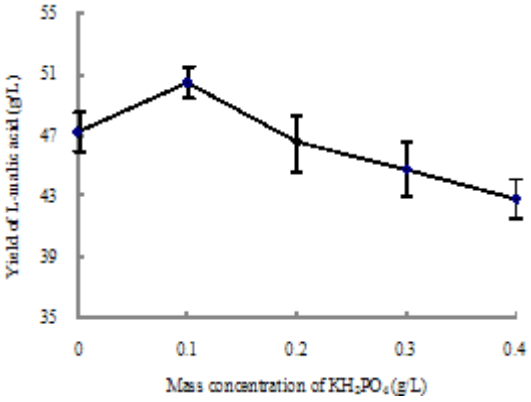

Fig. 4. Effect of mass concentration of KH_2PO_4 on the yield of L-malic acid

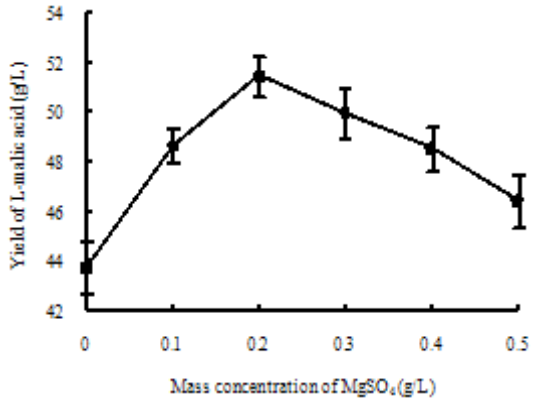

Fig. 5. Effect of mass concentration of $MgSO_4$ on the yield of L-malic acid

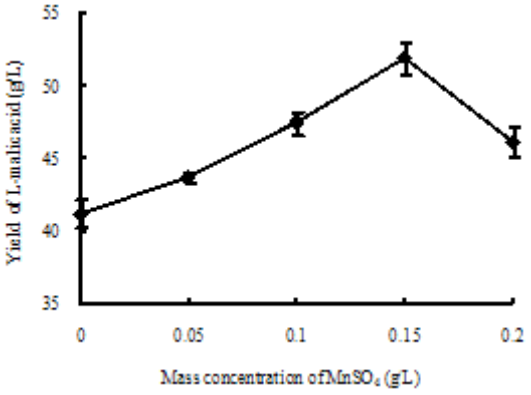

Fig. 6. Effect of mass concentration of $MnSO_4 \cdot H_2O$ on the yield of L-malic acid

the yeast extract powder contains abundant nutritional materials, such as protein, amino acid, a little carbohydrate and so on, it was conducive to the thallus growth, it promoted the accumulation of biomass and provided a good condition for fermentation. Therefore, $(NH_4)_2SO_4$ and yeast

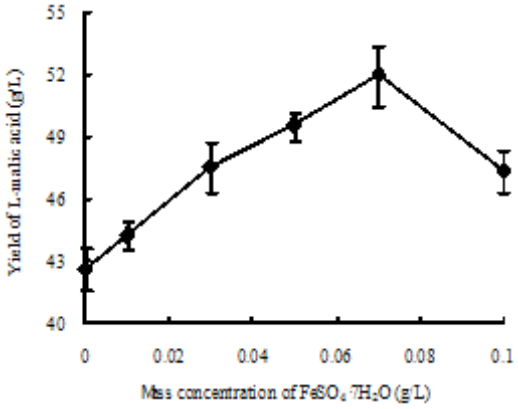

Fig. 7. Effect of mass concentration of $FeSO_4 \cdot 7H_2O$ on the yield of L-malic acid

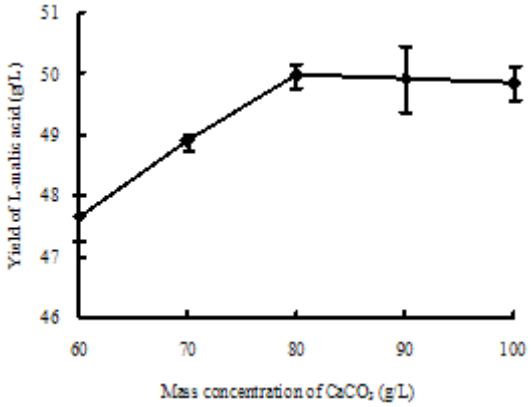

Fig. 8. Effect of mass concentration of $CaCO_3$ on the yield of L-malic acid

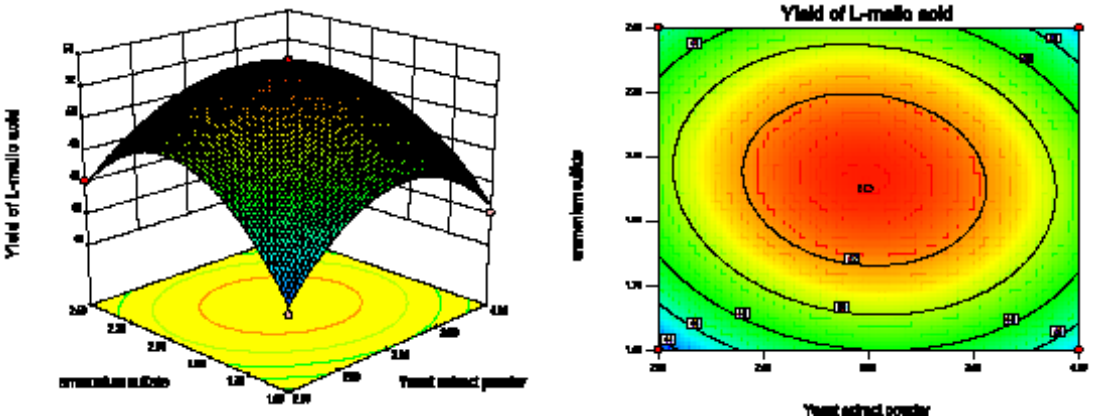

Fig.10. Response surface and contour plots for the interactive effects of $(NH_4)_2SO_4$ and yeast extract powder on yield of L-malic acid

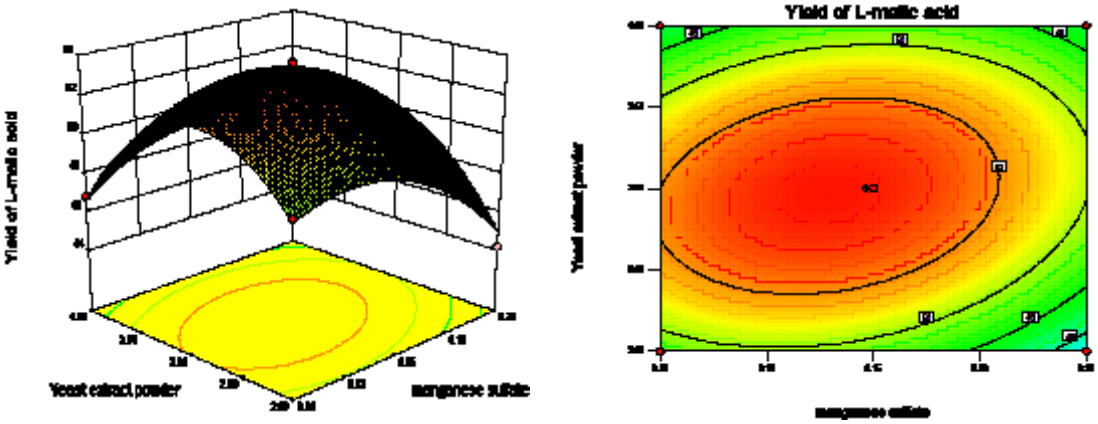

Fig.9. Response surface and contour plots for the interactive effects of yeast extract powder and $MnSO_4 \cdot H_2O$ on yield of L-malic acid

extract powder were chosen for the nitrogen sources.

From Fig.2, the yield of L-malic acid increased with the increasing of the concentration of $(NH_4)_2SO_4$, the yield was high when the concentration of $(NH_4)_2SO_4$ reached 2 g/L, but the yield of L-malic acid tended to decrease as the $(NH_4)_2SO_4$ was increased from 2~4 g/L. The result of variance analysis indicated that the effect of $(NH_4)_2SO_4$ concentration on the yield of L-malic acid was highly significant, so about 2g/L $(NH_4)_2SO_4$ was appropriate.

Fig.3 showed that the yield of L-malic acid increased gradually with the increase of yeast extract powder concentration, L-malic acid yield reached a maximum (48.79 g/L) when the yeast extract powder concentration was 3 g/L; while the yield dropped down as the yeast extract powder concentration was higher than 3g/L. An explanation for the changes was that yeast powder, which has

Table 1. Independent variable values of the process and the corresponding levels

Independent variable	Symbol		Levels		
	Uncoded	Coded	-1	0	1
$MnSO_4$ (g/L)	x_1	X_1	0.10	0.15	0.20
$FeSO_4 \cdot 7H_2O$ (g/L)	x_2	X_2	0.05	0.08	0.10
Yeast extract powder (g/L)	x_3	X_3	2	3	4
$(NH_4)_2SO_4$ (g/L)	x_4	X_4	1.5	2.0	2.5

Table 2. Box-Behnken central composite design matrix and corresponding experimental results

Run No.	X_1	X_2	X_3	X_4	Yield of L-malic acid (g/L)
1	1	0	0	1	45.2±0.37
2	0	-1	0	-1	42.85±0.29
3	0	-1	0	1	42.47±0.45
4	0	0	0	0	52.75±0.35
5	0	1	0	-1	44.17±0.44
6	0	0	0	0	53.63±0.61
7	1	1	0	0	47.5±0.58
8	-1	0	0	1	47.85±0.49
9	1	0	1	0	46.17±0.29
10	0	0	-1	1	46.16±0.38
11	-1	-1	0	0	45.52±0.61
12	0	1	0	1	47.49±0.64
13	0	1	1	0	48.73±0.27
14	-1	1	0	0	50.19±0.42
15	0	0	1	-1	44.14±0.35
16	-1	0	1	0	46.82±0.27
17	0	-1	1	0	42.17±0.42
18	0	1	-1	0	46.16±0.71
19	1	-1	0	0	44.83±0.55
20	1	0	-1	0	44.15±0.36
21	-1	0	0	-1	46.52±0.31
22	0	0	0	0	53.66±0.66
23	-1	0	-1	0	49.31±0.47
24	0	-1	-1	0	45.5±0.38
25	0	0	1	1	44.45±0.68
26	0	0	-1	-1	42.65±0.43
27	0	0	0	0	53.04±0.52
28	1	0	0	-1	46.87±0.34
29	0	0	0	0	53.67±0.54

a complex component and is rich in proteins, peptides and free amino acids etcetera, can efficiently stimulate mycelium pellet growth. Too much yeast powder may result in the over exuberant thallus and consequently affect the accumulation of metabolites. The concentration of yeast extract powder was a significant factor for the L-malic acid production; 3g/L yeast extract powder was selected as the suitable condition for further optimization.

The concentration of KH_2PO_4 was varied from 0~0.4 g/L (Fig.4), the yield of L-malic acid increased first and then dropped down, the yield reached a maximum at 0.1 g/L. The reason for this phenomenon was that phosphate can have influences on the cell concentration, biomass growth rates and other aspects; however, the nitrogen source in the medium probably contained phosphorus, if excess KH_2PO_4 was added in the medium, it may cause growth too strong and decreased the production. So the optimal concentration of KH_2PO_4 was 0.1 g/L.

From Fig.5 we can see that when the concentration of $MgSO_4$ increased to 0.2 g/L, the yield of L-malic acid came to the maximum, 51.48 g/

Table 3. Analysis of variance for the fitted quadratic regression equation

Source of variance	Sum of squares	df	Mean square	F value	P-value (Prob>F)
Model	340.62	14	24.23	49.17	<0.0001
X_1	11.00	1	11.00	22.24	0.0003
X_2	36.40	1	36.40	73.57	<0.0001
X_3	0.18	1	0.18	0.35	0.5613
X_4	3.43	1	3.43	6.94	0.0196
X_1X_2	1.00	1	1.00	2.02	0.1770
X_1X_3	5.09	1	5.09	10.28	0.0063
X_1X_4	2.25	1	2.25	4.55	0.0512
X_2X_3	8.70	1	8.70	17.59	0.0009
X_2X_4	3.42	1	3.42	6.92	0.0198
X_3X_4	2.56	1	2.56	5.17	0.0392
X_1^2	34.41	1	34.41	69.55	<0.0001
X_2^2	102.34	1	102.34	206.84	<0.0001
X_3^2	110.02	1	110.02	222.35	<0.0001
X_4^2	150.51	1	150.51	304.21	<0.0001
Residual	6.93	14	0.49		
Lack of fit	6.19	10	0.62	3.38	0.1259
Pure of error	0.73	4	0.18		
Cor total	347.55	28			

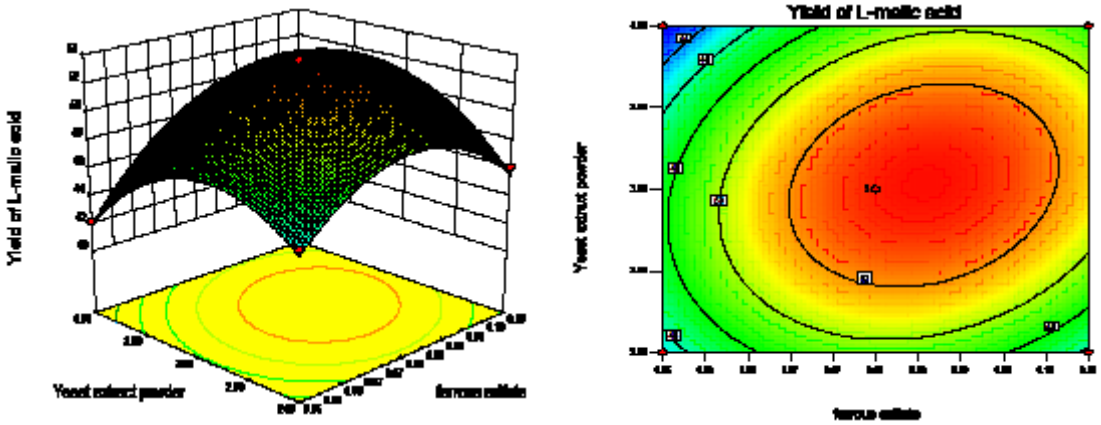

Fig.11. Response surface and contour plots for the interactive effects of yeast extract powder and $FeSO_4 \cdot 7H_2O$ on yield of L-malic acid

L, but it gradually decreased with the continuous increase of the $MgSO_4$ concentration. This is because Mg^{2+} acted as the coenzyme and activator of some enzymes, a small amount of Mg^{2+} could improve growth and metabolism of the strain, but high concentration can inhabit its growth and then affected the L-malic acid yield [15]. The effect of $MgSO_4$ was significant. Therefore, 0.2 g/L was optimal for the L-malic acid fermentation.

The yield of L-malic acid showed a rising

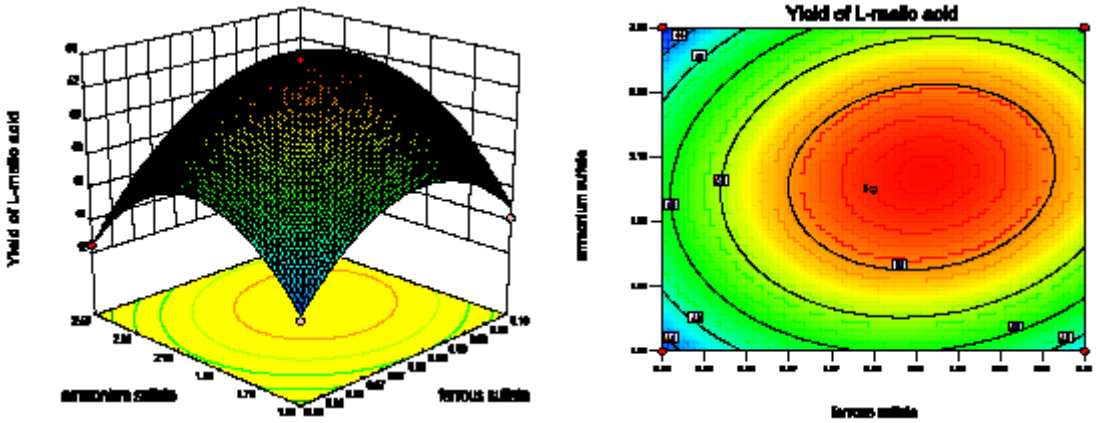

Fig.12. Response surface and contour plots for the interactive effects of $(NH_4)_2SO_4$ and $FeSO_4 \cdot 7H_2O$ on yield of L-malic acid

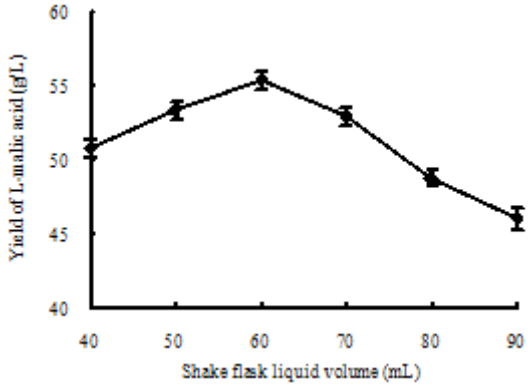

Fig.13. Effect of shake flask liquid volume on yield of L-malic acid

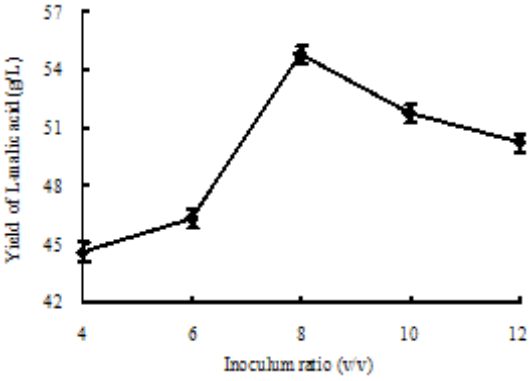

Fig.14. Effect of the inoculum ratio on yield of L-malic acid

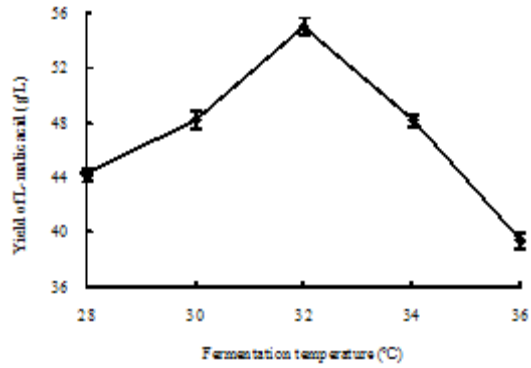

Fig.15. Effect of the fermentation temperature on yield of L-malic acid

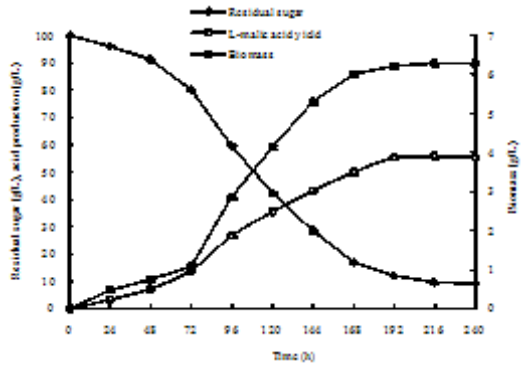

Fig.16. Fermentation curve of *Aspergillus parasiticus* CICC40365 under optimized condition

trend when the concentration of $MnSO_4 \cdot H_2O$ was less than 0.15 g/L, but it reduced slightly at the $MnSO_4 \cdot H_2O$ concentration above 0.15 g/L(Fig.6). Manganese ions participate in synthesis of a variety of enzymes, and it is likely to be the activator of xylulokinase, thereby Mn^{2+} raised the metabolic capability of this strain for xylose-utilizing. The variance analysis showed that $MnSO_4 \cdot H_2O$ was highly significant, so the suitable concentration of $MnSO_4 \cdot H_2O$ was about 0.15 g/L.

The concentration of $FeSO_4 \cdot 7H_2O$ varied from 0~0.1 g/L, the yield of L-malic acid reached the maximum (51.96 g/L) when the $FeSO_4 \cdot 7H_2O$ concentration was 0.07 g/L and then decreased

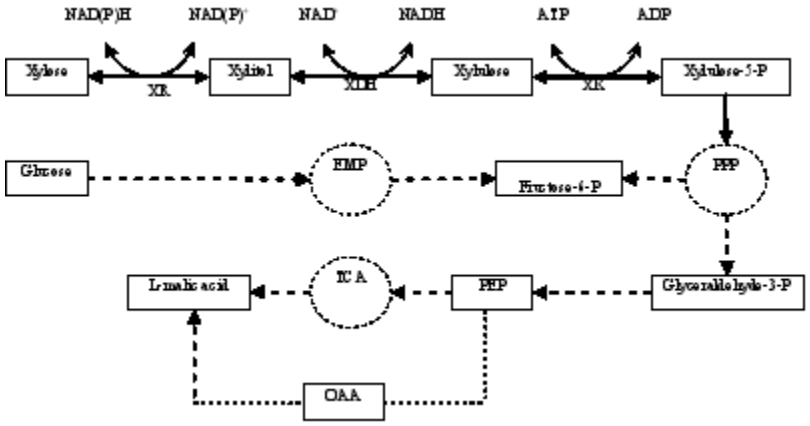

Fig.17. Main metabolic pathway of xylose

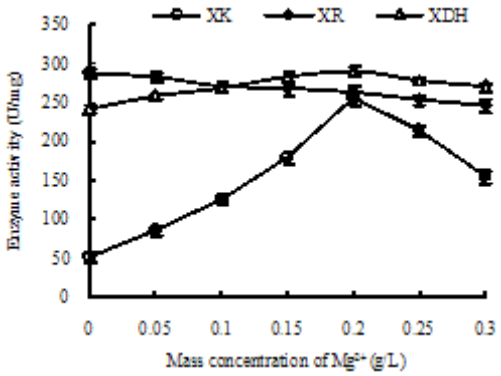

Fig.18. Effect of mass concentration of Mg^{2+} on the activity of XR0XDH and XK

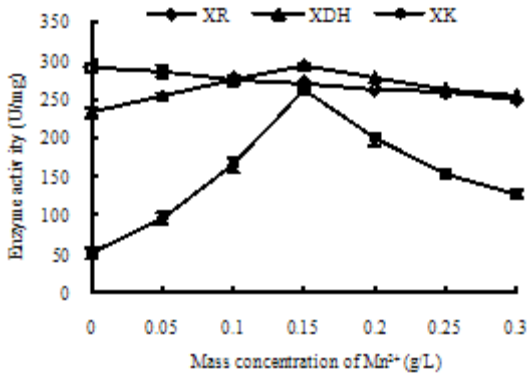

Fig.19 . Effect of mass concentration of Mn^{2+} on the activity of XR0XDH and XK

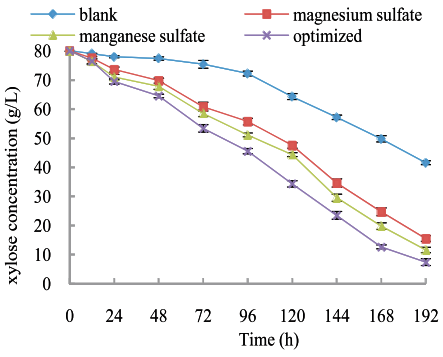

Fig. 20. Consumption of strain to the xylose under different conditions

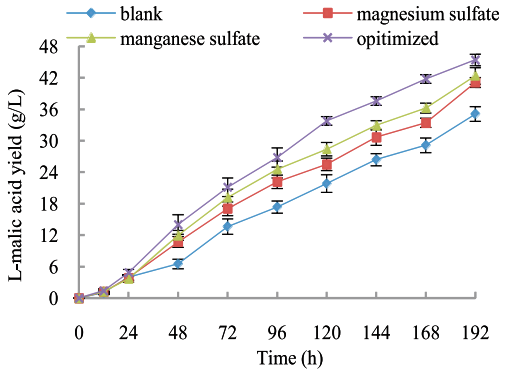

Fig. 21. Time courses of L-malic acid yield under different conditions

slightly (Fig.7). $FeSO_4 \cdot 7H_2O$ was identified a significant factor for L-malic acid production through the variance analysis. Hence, suitable concentration of $FeSO_4 \cdot 7H_2O$ was 0.07 g/L.

Fig.8 showed that as the concentration of $CaCO_3$ constantly increased, the yield of L-malic acid enhanced correspondingly. $CaCO_3$, used as neutralizer, was advantaged to produce L-malic acid. When the $CaCO_3$ concentration was 80 g/L, the yield of L-malic acid was at a maximum, 49.98 g/L, however, the yield almost remained the same above 80g/L. This is probably because excessive $CaCO_3$ may lead to insufficiency of the dissolved oxygen. Above all, 80 g/L $CaCO_3$ was appropriate.

Results of response surface optimization

Experimental results for the Box-Behnken central composite design

Factors and levels in the response surface analysis for optimizing medium composition were shown in Table 1.

Fitting the model and significance test

The Design - Expert software is used to analyze the experimental data for fitting a multiple quadratic regression model, which is an empirical relationship between the L-malic acid yield and the test variables in coded units, as given in the following equation:

$$Y=53.35-0.96X_1+1.74X_2-0.12X_3+0.54X_4-0.50X_1X_2+1.13X_1X_3-0.75X_1X_4+1.47X_2X_3+0.93X_2X_4-0.80X_3X_4-2.30X_1^2-3.97X_2^2-4.12X_3^2-4.82X_4^2$$

The result of variance Analysis for regression equation and partial regression coefficient was showed in table 3

The regression model $P<0.0001$ and the lack of fit $P>0.05$ indicated that the model was significant; the Lack of Fit was not significant relative to the pure of error. The coefficient of determination, R^2 is 0.9801, implied that the sample variation of 98.01% for L-malic production is attributed to the independent variables, namely, the concentration of $MnSO_4$, $FeSO_4 \cdot 7H_2O$, yeast extract powder, $(NH_4)_2SO_4$. $X_1 0 X_2 0 X_4 0 X_1 X_3 0 X_2 X_3 0 X_2 X_4 0 X_3 X_4 0 X_1^2 0 X_2^2 0 X_3^2 0 X_4^2$ in the regression equation were highly significant to the yield of L-malic acid, and the X_1X_4 was significant.

The 3D surface plots were drawn to illustrate the main and interactive effects of the independent variables on the dependent one. These graphs were drawn by imposing a constant value (i.e. the central points of the interval taken into consideration to one independent variable).

The effects of the concentration of $MnSO_4$, $FeSO_4 \cdot 7H_2O$, yeast extract powder, $(NH_4)_2SO_4$ and the interaction on the L-malic acid yield were shown in Figs. 9~12 with two variables kept at optimum level and varying another two within the experimental range. In general, exploration of the response surfaces indicated a complex interaction between the variables[16-18].

Fig.9 depicted the influence of yeast extract powder concentration and $MnSO_4 \cdot H_2O$ concentration; the increase of east extract powder concentration and $MnSO_4 \cdot H_2O$ concentration both resulted in a higher L-malic acid yield, the oval contour implied the two factors had strong interaction. Fig.10 showed the effect of yeast extract powder concentration and $(NH_4)_2SO_4$ concentration on L-malic acid yield. L-malic acid yield elevated first and then reduced with the concentration of yeast extract powder and $(NH_4)_2SO_4$ increase, the response surface of the slope was steep, which demonstrated that the interaction of these two variables were significant. Likewise, characteristics of Fig.11 and Fig.12 were similar to that of Fig.10.

Optimization results and model Verification

The regression model was analyzed by response surface, when the predictive value of L-malic acid yield reached a maximum ,the levels of factors were as follows: $MnSO_4 \cdot H_2O$ 0.14 g/L $FeSO_4 \cdot 7H_2O$ 0.08 g/L yeast extract powder 2.98 g/L $(NH_4)_2SO_4$ 2.05 g/L; predictive value 53.712 g/L. when the significane level±=0.05, the 95% prediction interval of L-malic acid was [53.0594 g/L 54.364g/L].

To verify the accuracy of the regression model prediction, the optimal conditions were rounding to $MnSO_4 \cdot H_2O$ 0.14 g/L $FeSO_4 \cdot 7H_2O$ 0.08 g/L yeast extract powder 3.0 g/L $(NH_4)_2SO_4$ 2.0 g/L. vetification tests were conducted for five repeats, the average of L-malic acid was 53.58±0.37g/L, and this value fell in the 95% predictive interval of the response value. It implied that this regression model can be applied to predict the xylose fermentation for L-malic acid by *Aspergillus parasiticus* CICC40365. The optimal conditions by rounding were in accordance with the central points in Table 2.

Optimization results of fermentation conditions

Effects of inoculum ratio, shake flask

liquid volume and fermentation temperature on the yield of L-malic acid were investigated through single factor experiments. The results were shown in Figs. 13, 14 and 15. Data were expressed as means±SD.

Fig.13 showed that L-malic acid yield was on the rise when the shake flask liquid volume increased from 40-60 mL, because too low liquid volume led to high dissolved oxygen and reducing the water activity; when the shake flask liquid volume was higher than 60 mL, the yield gradually decreased. An explanation for this was that dissolved oxygen going down caused the inadequate fermentation[19]. Therefore, 60mL/250mL was selected as the optimal shake flask liquid volume.

The L-malic acid yield increased when the inoculum ratio was in the range of 4%-8%; while the inoculum ratio surpassed 8%, the yield slightly decreased (Fig. 14). It is probably because excessive inoculum ratio may lead to mycelium cluster and have an effect on the dissolved oxygen; insufficient inoculums ratio resulted in inadequate fermentation and affected the acid production. Thus, 8% inoculums ratio was suitable.

With the increase of fermentation temperature, L - malic acid production gradually increased. When the temperature was 32°C, the L-malic acid yield came to the maximum; When the temperature is higher than 32°C, L - malic acid production is on the decline. Above all, 32°C was an optimum temperature for fermentation.

Fermentation curve of L-malic acid by *Aspergillus parasiticus* CICC40365

The fermentation curve of Aspergillus parasiticus CICC40365 under optimized condition was given in Fig.16.

Fig.16 depicted that both L-malic yield and biomass increased slowly in 0~72h, and the consumption of xylose was also slow in the period; L-malic yield and biomass increased rapidly in 72h~192h, at the same time, the consumption of xylose was fast; at 192h~240h L-malic acid yield was almost stable, the final yield was 55.54 g/L, the biomass also didn't increase any more, it stayed at 6.24 g/L, the concentration of xylose dropped to below 10g/L.

Xylose metabolic mechanism analysis of *Aspergillus parasiticus* CICC40365

In the nature, the xylose metabolic route of the yeast and filamentous fungi was shown in Fig. 17. The xylose was converted into xylitol by catalysis of the xylose reductase (XR, EC 1.1.1.21), then the xylitol was transformed to xylulose by xylitol dehydrogenase (XDH, EC 1.1.1.9), the xylulose-5-phosphate was produced through the action of xylulokinase (XK, EC 2.7.1.17) phosphorylation. The X5P entered into the pentose phosphate pathway (PPP)[20-22]. The intermediate glyceraldehydes-3-phosphoric acid, the intermediate product in the PPP, was generated into pyruvic acid through glycolytic pathway; pyruvic acid was converted into L-malic acid through TCA cycle or by the action of Pyruvate carboxylase and malic dehydrogenase.

Fig.17 indicated that XR, XDH and XK were the key enzymes in the metabolic pathway. The coenzyme and activator of these enzymes were mainly connected with Mg^{2+}, Mn^{2+} or other metal ions, And all of the kinases needed the activation of Mg^{2+}or Mn^{2+} in the process of transferring the phosphoric acid on the ATP to the receptors. Therefore, basing on steady fermentation medium components and fermentation conditions, the effects of Mg^{2+}, Mn^{2+} on the enzyme activities of XR, XDH and XK were investigated, the results were given in Fig.18 and Fig. 19.

Figs. 18 and 19 showed that when the medium didn't contain Mg^{2+}, Mn^{2+}, the activity of XK was significantly less than that of XR and XDH; this indicated that XK was the key rate-limiting enzyme in the xylose metabolic process. The low activity of XK resulted in the accumulation of xylitol and xylulose in the strain, and also inhibited the activity of XR and XDH; as a result, the metabolic capability of this train for xylose was greatly reduced. Mg^{2+} and Mn^{2+} had an obvious effect on the activity of XK when they were in a certain concentration range, and the activation increased with the concentration of Mg^{2+}, Mn^{2+}. The activity of XK was at a maximum while the concentration of Mg^{2+} and Mn^{2+} were 0.2 g/L, 0.15 g/L, respectively, meanwhile, the yield of L-malic acid was high under the concentration. In addition, the XDH can be activated by Mg^{2+}, Mn^{2+}, but the concentration of Mg^{2+}, Mn^{2+} had little impact on the activation of XDH; on the contrary, the activity of XR was restrained by Mg^{2+}, Mn^{2+} to a certain extent.

As the activity of XK was enhanced by activation of Mg^{2+} and Mn^{2+}, both the xylose utilization of this strain and L-malic acid were improved (Figs. 20 and 21). This further illustrated that XK was a key rate-limiting enzyme in the xylose metabolic pathway. The activation level of Mn^{2+} to the XK was a little higher than that of Mg^{2+}, moreover, the combination of them had an obvious positive interaction on the activity of XK.

CONCLUSIONS

The medium for L-malic fermentation by *Aspergillus parasiticus* CICC40365 was optimized through response surface analysis, the optimal medium composition was as follows: the xylose, $(NH_4)_2SO_4$, yeast extract powder, $MgSO_4$, $MnSO_4 \cdot H_2O$ $FeSO_4 \cdot 7H_2O$ and $CaCO_3$ were 100.0 g/L, 2.0 g/L, 3.0 g/L, 0.20 g/L, 0.15g/L, 0.08 g/L and 80 g/L, respectively. The yield of malic acid from the optimal condition was 53.58±0.37 g/L; this value was in the 95% prediction interval of the regression model, which implied regression equation has good prediction on the L-malic acid yield. The optimum fermentation condition was obtained by single factor experiments, it was inoculums ratio 8%, shake flask liquid volume 60mL/250mL, rotating speed 170r/min, fermentation temperature 32°C for 7 days; The L-malic acid yield was 55.64 g/L under the condition. From the preliminary study on the xylose metabolic pathway of *Aspergillus parasiticus* CICC40365 we found that XK was the key rate-limiting enzyme, and it can be significantly activate by Mg^{2+} and Mn^{2+}. The activation of XK can enhance the xylose metabolism of the strain, and improve the L-malic acid yield. The research results indicated that *Aspergillus parasiticus* CICC40365 could make good use of xylose for fermentation to produce L-malic acid, its production and xylose utilization efficiency were improved. Results of the study can provide some references for bioconversion of the xylose in the lignocellulose hydrolyte.

ACKNOWLEDGEMENTS

Financial supports from National natural Science Foundation of China (Project No: 31071636/31101352) and Science and Technology Plan Projects of Anhui Province (No: 1206c0805017) are gratefully acknowledged.

REFERENCES

1. Wu JL, Wu QP, Huang JM, et al. Effects of L-malate on physical stamina and activities of enzymes related to the malate-aspartate shuttle in liver of mice. *Physiol Res*, 2007; **56**(2): 213-220.
2. Peleg Y, Rokeml J.S, Goldberg I. A simple Plate-assay for the screening of L-malic acid producing microorganisms. *FEMs Microbiol Lett*, 1990; **67**: 233-236.
3. Goldbergl I, Rokeml J.S, PINES O. Organic acids: old metabolites, new themes. *J Chem Technol and Biotechnol*, 2006; **81**(10): 1601-1611.
4. Taing O, Taing K. Production of malic and succinic acids by sugar-tolerate yeast Zygosaccharomyces rouxii. *Eur Food Res Technol*, 2007; **224**: 343–347.
5. Pines O, Even-Ram S, Elnathan N, et al. The cytosolic pathway of L-malic acid synthesis in Saccharomyces cerevisiae: the role of fumarase. *Appl Microbiol Biotechol*, 1996; **46**: 393-399.
6. Battat E, Peleg Y. Optimization of L-malic acid production by *Aspergillus flavus* in a stirred fermentor. *Biotechnol Bioeng*, 1991; **37**(11):1108-1116.
7. Peleg Y, Barak A, Scrutton M.C, et al. Malic acid accumulation by Aspergillus flavus. III. 13C-NMR and isoenzyme analysis. *Appl Microbiol Biotechnol*. 1989; **32**: 334–339.
8. Takao S, Yokota A, Tanida M. L-malic acid fermentation by a mixed culture of *Rhizopus arrhizus* and *Paecilomyces varioti*. *J Ferment Technol*, 1983; **61**: 643-645.
9. Tian SD, Wu YN, Xie SY, Yang J. Research on one step fermentation of L-malic acid. *Food Science Technology* , 2008; **6**: 106-108
10. Yoav P, Barry S, Israel G. Malic acid accumulation by *Aspergillus flavus*. *Appl Microbiol Biotechnol*, 1988; **28**: 69-75.
11. Miller, G.L. Use of DNS reagent for determination of reducing sugars. *Anal Chem*, 1959; **31**: 426-428.
12. Liao w, Liu Y, Frear C, et al. A new approach of pellet formation of a filamentous fungus-*Rhizopus orgzae*. *Bioresource Technol*, 2007; **98**: 3415-3423.
13. Jin YS, Jeffries TW, Changing flux of xylose metabolites by altering expression of xylose reductase and xylitol dehydrogenase in recombinant *Saccharomyces cerevisiae*. *Appl Biochem Biotechnol*, 2003; **106**(1/3): 277-285.
14. Luccio E.D, Petschacher B, Voegtli J, et al.

Structural and Kinetic Studies of induced Fit in xylulosekinase from *Escherichia coli*. *J Mol Biol*, 2007; **265**(3): 783-798.
15. Liu JJ, Zhao XY, Tian YJ, Li PW, Zhang JX, Liu LP, Han YL. Studies on fermentation conditions of L-malic acid produced by *Aspergillus flavus* HA5800 from sugar. *Food and Fermentation Industries*, 2005; **31**(1):5-9.
16. JL Casas Lopez, JA Sanchez Perez, JM Fernandez Sevilla, et al. fermentation optimization for the production of lovastatin by *Aspergillus terreus*: use of response surface methodology. *J Chem Technol and Biotechnol*, 2004; **79**: 1119-1126.
17. Liu JJ, Zhao XY, Tian YJ, Li PW, Zhang JX, Liu LP, Han YL. Studies on fermentation conditions of L-malic acid produced by *Aspergillus flavus* HA5800 from sugar. *Food and Fermentation Industries*, 2005; **31**(1):5-9.
18. Li W, Du W, Liu D. Optimization of whole cell-catalyzed methanolysis of soybean oil for biodiesel production using response surface methodology. *J Mol Catal B Enzym*, 2007; **45**(3/4): 122-127.
19. Ambati P, Ayyanna C. Optimizing medium constituents and fermentation conditions for citric acid production from Palmyra jaggery using response surface method. *World J Microbiol Biotechnol*, 2001; **17**(4): 331-335.
20. Han WQ, Wang F, Tan TW, Deng L. The effect of inoculums concentration and liquid volume on fumaric acid production from raw tapioca flour by *Rhizopus arrhizus*. *Journal of Beijing University of Chemical Technology (Natural Science Edition)*, 2011; **38**(5):95-99.
21. Zhang Y, Ma RQ, Hong HZ, Zhang W, Chen M, Lu W. Metabolic engineering for microbial production of ethanol from xylose: a review. *Chinese Journal of Biotechnology*, 2010; **26**(10):1436-1443.
22. Karhumaa K, Fromanger R, Marie F, *et al*. High activity of xylose reductase and xylitol dehydrogenase improves xylose fermentation by recombinant *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol*, 2007; **73**: 1039-1046.