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An Overlooked Effect of Glycine Betaine on Fermentation: Prevents Caramelization and Increases the L-Lysine Production

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This article focuses on the effects of glycine betaine on preventing caramelization, and increasing DCW and L-lysine production. The additional glycine betaine not only decreased the browning intensity (decreased 4 times), and the concentrations of 5-hydroxymethylfurfural (decreased 7.8 times) and furfural (decreased 12 times), but also increased the availability of glucose (increased 17.5%) for L-lysine production. The DCW and L-lysine production were increased by adding no more than 20 mM glycine betaine, whereas the DCW and L-lysine production were decreased with the reduction of pH values, although pH had a better response to prevent caramelization than did glycine betaine. For L-lysine production, the highest increase (40%) was observed on the media with 20 mM glycine betaine. The crucial enzymes in glycolysis and L-lysine biosynthesis pathway were investigated. The results indicated that additional glycine betaine increases the activity of enzymes in glycolysis, in contrast to the effect of pH. All the results indicated that glycine betaine can be used to prevent caramelization and increase the L-lysine production. By applying this strategy, glucose would not be have to be separated from the culture media during autoclaving so that factories can save production costs and shorten the fermentation period.

Keywords: L-Lysine fermentation, glycine betaine, caramelization, glucose availability, caramelization products, browning intensity

Introduction

Industrial culture media for L-lysine fermentation contain high concentrations of carbohydrates (*e.g.*, glucose and molasses), which have adverse effects for fermentation, such as being non-enzymatic browning-prone during the autoclave sterilization and having high osmolarity. Non-enzymatic browning is the general cause of sugar products darkening [19], such as caramelization and Maillard. The former is the common name for a group of reactions that occur when carbohydrates are exposed to high temperatures with no amino groups, and the latter takes place in the presence of amino groups [18]. Caramelization is an important reaction in the food industry [19], but it is not so good for microbial fermentation. There are two main reasons for this phenomenon. One is that the sugar availability is decreased during the caramelization, because a lot of volatile compounds are produced during caramelization [4]. The other is that furfural and 5-hydroxymethylfurfural (5-HMF) are the major compounds in the caramelization product [8], which are highly toxic to microorganisms [24]. Modig *et al.* [15] have found that both aldehyde dehydrogenase (ALDH) and pyruvate dehydrogenase (PDH) were strongly inhibited by furfural and less strongly inhibited by 5-HMF. PDH, one of the most important enzymes in the tricarboxylic acid (TCA) cycle, catalyzes the

synthesis of acetyl-CoA. In addition, Banerjee *et al*. [1] have reported that furfural strongly inhibited the hexokinase (HK), which is an important enzyme in glycolysis. The TCA cycle and glycolysis are important for cell growth and metabolites biosynthesis. In order to relieve the restriction of caramelization products (CPs) to the cell growth and to increase the availability of sugar for L-lysine production, the caramelization should be as small as possible. Although caramelization was affected by pH, normally the optimal pH for *Brevibacterium* or *Corynebacterium* cell growth was 7.0–7.5 [21]. Therefore, another way should be found to minimize the caramelization during L-amino acid or other organic acids biosynthesis by *Brevibacterium* or *Corynebacterium*.

Glycine betaine is one of the major compatible solutes in plants. It is also commonly present in media for microbial growth. In *Corynebacterium glutamicum* and *Brevibacterium flavum*, glycine betaine is the preferred osmoregulator [22]. These organisms have been widely used to produce L-lysine in industry [25–27]. In order to resist the hyperosmotic stress, glycine betaine must be added into the culture broth during fermentation with *C. glutamicum* or *B. flavum* [16]. There are a lot of advantages of the additional glycine betaine: it (i) stimulates the growth rate of the bacterium in hyperosmotic stress condition [10]; (ii) stabilizes enzymes and resists the decrease of the cytoplasmic volume in cells exposed to hyperosmotic stress condition [22]; (iii) restores growth and invertase activity at high temperature *in vitro* [11]; and (iv) stimulates the respiration recovery rate [22]. In addition, *B. flavum* is unable to utilize glycine betaine as a sole carbon or nitrogen source, or degrade it [10]. Therefore, glycine betaine is the preferred osmoregulator for *B. flavum*. However, few people know that glycine betaine is able to prevent caramelization and increase the L-lysine production.

In this paper, to investigate the effects of the additional glycine betaine on preventing caramelization and increasing the L-lysine production, different concentrations of glycine betaine were added to the media before autoclaving and the residual glucose after autoclaving was detected. In addition, the effects of glycine betaine on caramelization, cell growth, and L-lysine production were compared with the effects of pH. This study shows for the first time that glycine betaine is able to prevent caramelization and increase the L-lysine production. Although the increase of the residual glucose and L-lysine production are not large, it is important to note that even the smallest effect has a great impact on the economy of the industrial manufacture for the mass-produced amino acid.

Materials and Methods

Strains, Growth Media, and Culturing Conditions

Brevibacterium flavum XQ-8 (*B. flavum* XQ-8) was screened by repeating random mutagenesis and selected from wild-type strain *B. flavum* ATCC 14067. *B. flavum* XQ-8 was resistant to s-2-aminoethyl-L-cysteine (AEC) and Sulfaguanidine (SG), and was a L-threonine auxotroph. This strain was used as the working strain.

For L-lysine fermentation, a preculture was inoculated from a fresh LBG-plate (LB supplemented with 5 g/l glucose) and cultivated overnight. After washing the cells with CgXII-media without carbon source (20 g/l $(NH_4)_2SO_4$, 5 g/l urea, 1 g/l KH_2PO_4, 1 g/l K_2HPO_4, 0.25 g/l $MgSO_4 \cdot H_2O$, 42 g/l 3-morpholinopronesulfonic acid, 10 mg/l $CaCl_2$, 10 mg/l $FeSO_4 \cdot 7H_2O$, 10 mg/l $MnSO_4 \cdot H_2O$, 10 mg/l $ZnSO_4 \cdot 7H_2O$, 0.2 mg/l $CuSO_4$, 0.02 mg/l $NiCl_2 \cdot 6H_2O$, 0.2 mg/l biotin, and 0.03 mg/l protocatechuic acid) [9], the main culture with minimal media was inoculated with OD_{600} of 1 in 60 ml CgXIIG-media (CgXII supplemented with 40 g/l glucose) in 500 ml Erlenmeyer flasks. To investigate the effects of glycine betaine, glycine betaine was added into the CgXIIG-media to different final concentrations (*i.e.*, 5, 10, 15, 20, 25, and 30 mM). These media were adjusted to pH 7.0. To investigate the effects of pH value, the CgXIIG-media were adjusted to different gradient pH values (*i.e.*, pH 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0) with HCl or NaOH. The LBG-media and CgXIIG-media were sterilized at 121°C, 0.1 MPa for 20 min. In addition, 0.4 g/l of sterile L-threonine was added into the culture media. The cultures were cultivated at 30°C and 120 rpm agitation. In order to eliminate the effect of osmotic pressure, 1.0 g/l of sterile L-proline as a compatible solute was added into the culture media after 12 h of incubation [28].

Detection of the Browning Intensity at OD_{420} and the Concentrations of 5-HMF and Furfural

The browning intensity of culture media after autoclaving was measured at room temperature at 420 nm (OD_{420}) [17]. 5-HMF and furfural were considered to be a precursor of polymers that lead to the development of brown color during caramelization [1]. Therefore, the concentrations of 5-HMF and furfural were detected after autoclaving. Samples, and 5-HMF and furfural standard solution (Sigma-Aldrich, USA) were filtered (0.45 μm) and analyzed by high pressure liquid chromatography (HPLC; Dionex, USA) with a Kromasil C-18 4.6 × 250 mm column (AkzoNobel, Amsterdam, The Netherlands) according to the previous methods reported by Quintas *et al*. [18] and Mao *et al*. [13], respectively.

Assay of Residual Glucose

The 100 μl samples were diluted 100 times and used later for assaying the residual glucose by an SBA-40E immobilized enzyme biosensor (Shandong, China).

Assay of Cell Growth and L-Lysine Production

First, 200 µl samples were taken from the Erlenmeyer flasks every 4 h. Then 100 µl of cultures was used to measure the biomass concentration by photometry (Unic, Shanghai, China) at 600 nm after an appropriate dilution or by gravimetric analysis as described previously [6]. Under these experimental conditions, the correlation factor (g of DCW to OD_{600}) between dry cell weight (DCW) and OD_{600} was determined as 0.327 g DCW l^{-1} OD^{-1}. The other 100 µl samples were diluted 100 times and used later for determining the L-lysine concentration by an SBA-40E immobilized enzyme biosensor (Shandong, China). The conversion rate of glucose was calculated according to the formula

the total of L-lysine production / the total of glucose.

Enzyme Activity Assay

The crude enzyme was prepared according to the previous methods reported by Hou *et al*. [6]. After centrifugation at 4°C for 30 min at 10,000 ×*g*, the cell-free supernatants were immediately used to determine the enzyme activities. Protein concentrations were determined using the Bradford Protein Quantification Kit (Tiangen, Beijing, China) with bovine serum albumin as the standard. The analyses of enzyme activities and protein concentrations were done in triplicate. Specific activity was given as the number of mU/mg of protein. The activities of PDH [2], HK [23], AK [7], and DDH [3] were determined according to the previous reports.

Results

The Browning Intensity of Culture Media and the Concentration of 5-HMF in Culture Media

The color changes were different among the culture media with different concentrations of glycine betaine or different gradient pH values after autoclave sterilization (data not shown). These results were consistent with the detected results *via* spectrophotometry at 420 nm (Fig. 1A). The browning intensity of culture media without glycine betaine and pH = 7.0 was OD_{420} = 0.185 ± 0.0106 − almost 4 times as many as the media containing 30 mM glycine betaine (OD_{420} = 0.047 ± 0.0024) and 6.6 times as many as pH = 2.0 (OD_{420} = 0.028 ± 0.0032). It is worth pointing out that the effect of glycine betaine on the browning intensity was worse than that of pH (Fig. 1A). It is generally known that 5-HMF and furfural are the precursors of colored

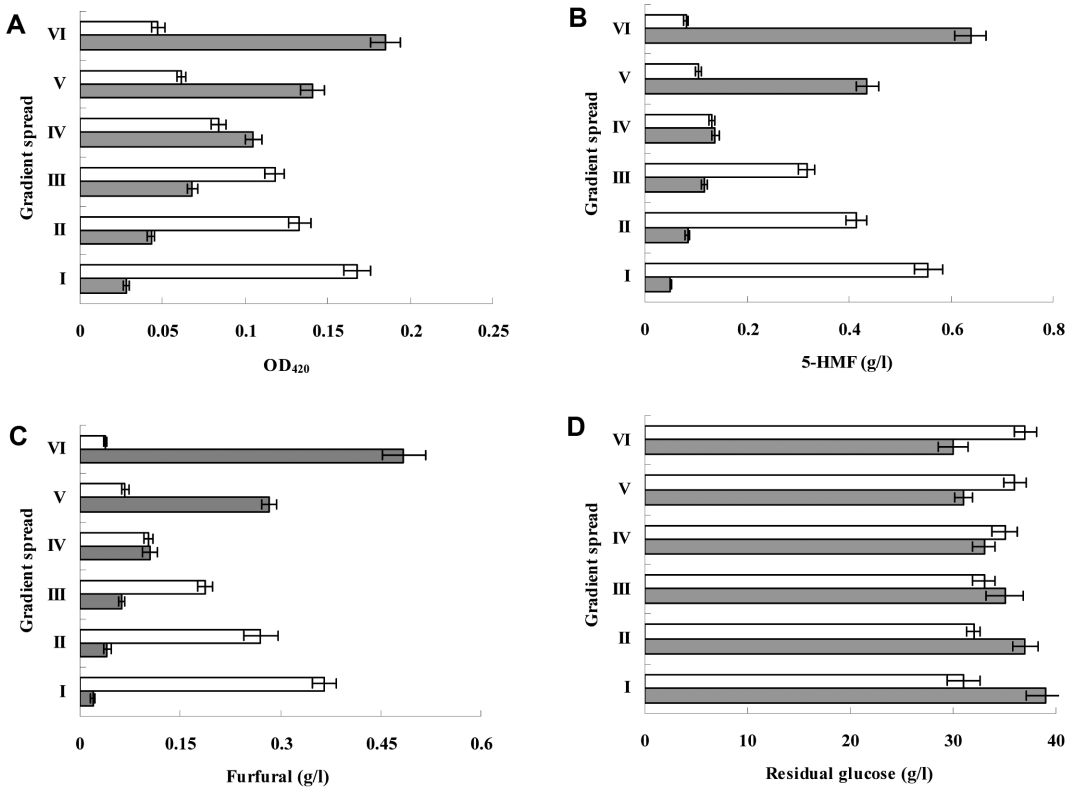

Fig. 1. Effects of glycine betaine (*white column*) and pH (*gray column*) on the browning intensity OD_{420} (**A**), the concentration of 5-HMF (**B**), the concentration of furfural (**C**), and the concentration of residual glucose (**D**) after autoclaving.

I: 5 mM glycine betaine or pH = 2.0; II: 10 mM glycine betaine or pH = 3.0; III: 15 mM glycine betaine or pH = 4.0; IV: 20 mM glycine betaine or pH = 5.0; V: 25 mM glycine betaine or pH = 6.0; VI: 30 mM glycine betaine or pH = 7.0. The standard errors are shown as bars.

compounds in the caramelization [18]. Therefore, we detected the concentrations of 5-HMF and furfural in culture media with different concentrations of glycine betaine or different gradient pH values using HPLC (Fig. 1B). The 5-HMF concentration of media without glycine betaine and pH = 7.0 was 0.638 ± 0.0511 g/l – almost 7.8 times as many as the media containing 30 mM of glycine betaine (0.081 ± 0.0058 g/l) and 12.8 times as many as pH = 2.0 (0.050 ± 0.0051 g/l). In addition, the 5-HMF concentration of media was decreased with the increase of glycine betaine concentration or the decrease of pH value (Fig. 1B). Similar to the effect on 5-HMF, the furfural concentration of media was decreased with the increase of glycine betaine concentration or the decrease of pH value (Fig. 1C). The furfural concentration of media without glycine betaine and pH = 7.0 was 0.445 ± 0.0319 g/l – almost 12 times as many as the media containing 30 mM of glycine betaine (0.037 ± 0.0021 g/l) and 23.4 times as many as pH = 2.0 (0.019 ± 0.0026 g/l).

Concentration of Residual Glucose After Autoclaving

A lot of volatile compounds were synthesized during caramelization, such as hydrocarbons, aliphatic aldehydes, ketones *etc.* [4], which led to the decrease of the glucose availability. To investigate the effect of glycine betaine or pH on preventing glucose degradation, the residual glucose of culture media with different concentrations of glycine betaine or different gradient pH values after autoclaving was detected by an SBA-40E immobilized enzyme biosensor (see Material and Methods). The glycine betaine or pH strongly affected the concentration of residual glucose after autoclaving (Fig. 1D). The residual glucose concentration of culture media without glycine betaine and pH = 7.0 was 30 ± 1.73 g/l, whereas it was increased with the increase of glycine betaine concentration or the decrease of the pH value (Fig. 1D). As compared with the media without glycine betaine and pH = 7.0, the residual glucose concentration was increased to 37 ± 1.45 g/l by adding 30 mM glycine betaine into the media or increased to 39 ± 2.14 g/l at pH = 2.0.

Growth Performance of *B. flavum* XQ-8 Under Different Culture Conditions

To investigate the influences of the glycine betaine and pH on cell growth during L-lysine fermentation, we analyzed the DCW and growth curve of *B. flavum* XQ-8 during growth on culture media with different concentrations of glycine betaine or different gradient pH values. There was a small difference in DCW and maximal specific growth rate (u) among the culture media containing different concentrations of glycine betaine, whereas there was a big difference in DCW and maximal specific growth rate among the different gradient pH values (Figs. 2A, 2B, and 2C). The DCW of *B. flavum* XQ-8 remained approximately 12.50 g/l on culture media with different concentrations of glycine betaine (Fig. 2A and Table 1). However, *B. flavum* XQ-8 could not grow on media with pH = 2.0, and the growth of *B. flavum* XQ-8 was strongly retarded during pH ≤ 5.0 (Fig. 2B). It should be noted that the growth of *B. flavum* XQ-8 was also retarded on culture media with 25 or 30 mM glycine betaine (Fig. 2C).

Table 1. The DCW, L-lysine yield, biomass-specific L-lysine yield ($Y_{P/X}$), and substrate-specific L-lysine yield ($Y_{P/S}$) of *B. flavum* QX-8 in shake flask during growth on CgXIIG medium containing different concentrations of glycine betaine or at different pHs.

	Gradients	DCW (g/l)	L-lysine (g/l)	$Y_{P/X}$ (g [g DCW]$^{-1}$)	$Y_{P/S}$ (g [g glucose]$^{-1}$)
Glycine betaine	5 mM	12.63	16	1.267	0.400
	10 mM	12.91	19	1.472	0.475
	15 mM	12.55	19	1.514	0.475
	20 mM	12.65	21	1.661	0.525
	25 mM	12.35	20	1.619	0.500
	30 mM	11.45	17	1.485	0.425
pH	2	0.12	0	0	0
	3	3.38	3.0	0.789	0.075
	4	5.67	5.4	0.952	0.133
	5	9.37	9.8	1.046	0.210
	6	11.78	13.5	1.146	0.338
	7	12.15	15	1.235	0.375

All data are mean values of three determinations of three independent experiments with errors ≤7%.

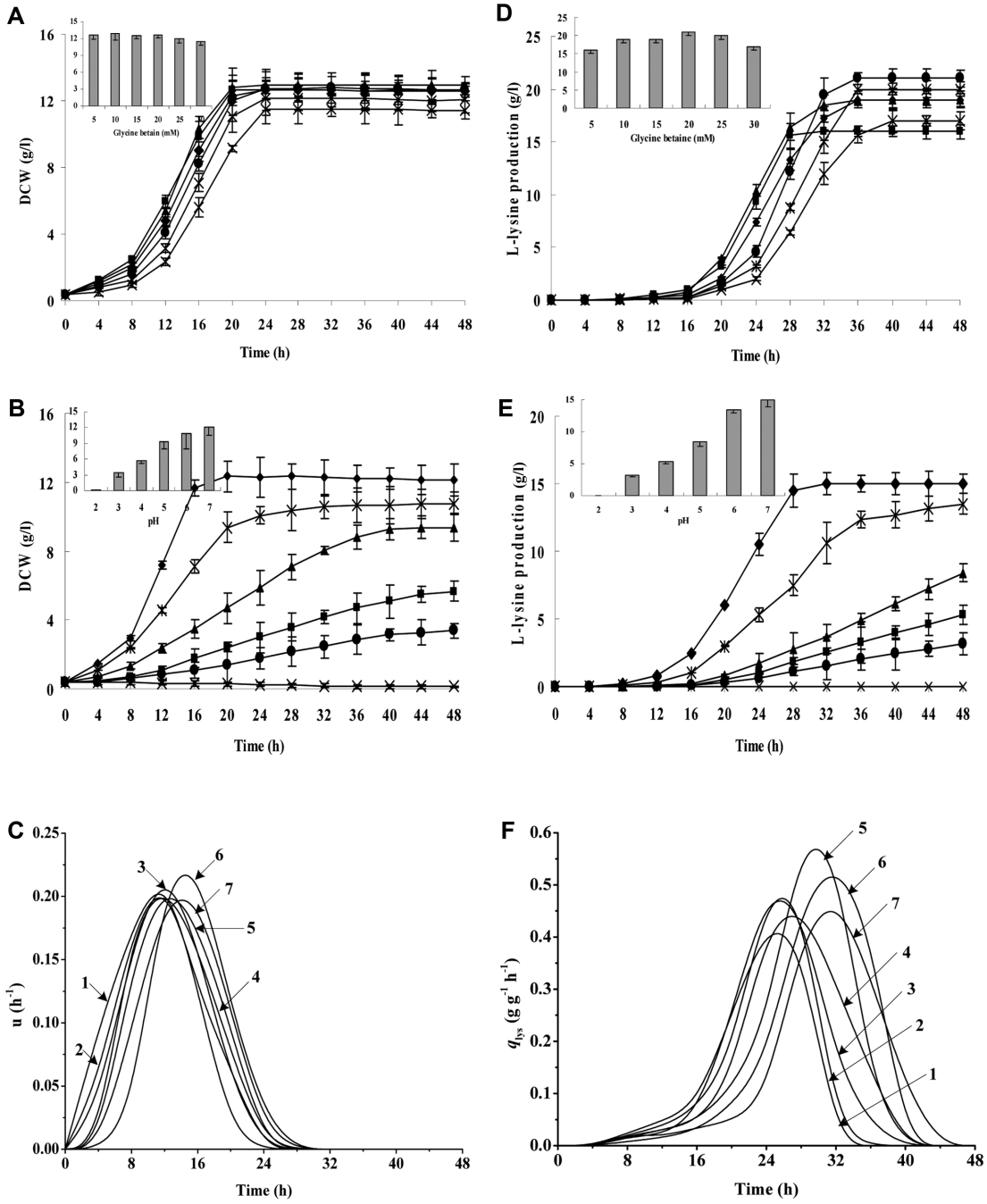

Fig. 2. The growth performance and L-lysine production of *B. flavum* XQ-8 in shake flask during growth on CgXIIG medium with different concentrations of glycine betaine or at different pHs.

(**A**) The DCW on media with 5 mM (■), 10 mM (▲), 15 mM (◆), 20 mM (●), 25 mM (*), and 30 mM (×) glycine betaine; (**B**) the DCW on media at pH = 2.0 (×), pH = 3.0 (●), pH = 4.0 (■), pH = 5.0 (▲), pH = 6.0 (*), and pH = 7.0 (◆); (**C**) the specific growth rate (u) of *B. flavum* XQ-8 on media at pH 7.0 with 0 mM (line 1), 5 mM (line 2), 10 mM (line 3), 15 mM (line 4), 20 mM (line 5), 25 mM (line 6), and 30 mM (line 7) glycine betaine. (**D**) The L-lysine production on media with 5 mM (■), 10 mM (▲), 15 mM (◆), 20 mM (●), 25 mM (*), and 30 mM (×) glycine betaine; (**E**) the L-lysine production on media at pH = 2.0 (×), pH = 3.0 (●), pH = 4.0 (■), pH = 5.0 (▲), pH = 6.0 (*), and pH = 7.0 (◆). (**F**) The specific L-lysine production rate (q_{lys}) of *B. flavum* XQ-8 on media at pH 7.0 with 0 mM (line 1), 5 mM (line 2), 10 mM (line 3), 15 mM (line 4), 20 mM (line 5), 25 mM (line 6), and 30 mM (line 7) glycine betaine. The histogram in Figs. 2A, 2B, 2D, and 2E represents the total DCW and L-lysine production at the end of fermentation. The standard errors are shown as bars.

L-Lysine Production of *B. flavum* XQ-8 Under Different Culture Conditions

As compared with growth on culture media without glycine betaine, *B. flavum* QX-8 showed a higher L-lysine production during growth on media with 5, 10, 15, 20, 25, and 30 mM glycine betaine, at 15 ± 0.76, 16 ± 0.67, 19 ± 1.03, 19 ± 0.68, 21 ± 0.76, 20 ± 0.73, and 17 ± 0.87 g/l, respectively (Fig. 2D, Table 1). However, the L-lysine production was decreased with the decrease of pH values (Fig. 2E). The highest increase (40%) was observed for media with 20 mM glycine betaine, but the increase was also significant for media with 25 mM (33.3%) and 10 or 15 mM (26.7%) glycine betaine. Although the L-lysine production of *B. flavum* QX-8 was higher, the time of reaching the maximum on media with 15, 20, and 25 mM glycine betaine was longer (peak reached at 32 h) than on media without glycine betaine or pH = 7.0 (peak reached at 28 h, Fig. 2F). As shown in Fig. 2E, the L-lysine production was rising constantly during growth on media with pH ≤ 5.0 except on media with pH = 2.0. In addition, the maximal specific L-lysine production rate (q_{lys}) of *B. flavum* QX-8 on media with different concentrations of glycine betaine was higher than on media without glycine betaine (Fig. 2F). Moreover, the biomass-specific L-lysine yield ($Y_{P/X}$) and substrate-specific L-lysine yield ($Y_{P/S}$) of *B. flavum* QX-8 on medium with different concentration of glycine betaine were better than on media with different gradient pH values (Table 1). It is worth pointing out that the highest $Y_{P/X}$ ($Y_{P/X}$ = 1.661 g [g DCW]$^{-1}$) and $Y_{P/S}$ ($Y_{P/S}$ = 0.525 g [g glucose]$^{-1}$) were observed during growth on media with 20 mM glycine betaine.

Activity of Crucial Enzymes in Glycolysis and L-Lysine Biosynthesis Pathway in *B. flavum* QX-8 During Cultivation on Different Conditions

Previous research has reported that 5-HMF and furfural strongly inhibit the activities of HK and PDH of glycolysis [1, 15]. To investigate the effects of glycine betaine and pH on the crucial enzymes in glycolysis and L-lysine biosynthesis pathway, we detected the specific activities of HK and PDH (belonging to glycolysis), and AK and DDH (belonging to the L-lysine biosynthesis pathway). As shown in Table 2, the activity of HK and PDH were increased by adding glycine betaine, whereas the activity of AK and DDH changed very little under different concentrations of glycine betaine. Interestingly, the activities of HK, PDH, AK, and DDH were strongly decreased with the decrease of pH value. It is important to note that the activities of HK and PDH were slightly decreased when adding more than 20 mM glycine betaine.

Table 2. Specific activity of enzymes of *B. flavum* QX-8 in shake flask during growth on CgXIIG medium containing different concentrations of glycine betaine or at different pHs.

	Gradients	Specific activity (mU [mg protein]$^{-1}$)			
		PDH	HK	AK	DDH
Glycine betaine	5 mM	49	322	128	195
	10 mM	53	335	127	197
	15 mM	58	344	126	193
	20 mM	65	353	129	202
	25 mM	51	326	128	199
	30 mM	45	309	125	195
pH	2	-	-	-	-
	3	16	107	28	61
	4	21	165	36	75
	5	34	206	53	98
	6	41	273	86	132
	7	43	305	126	193

PDH: Pyruvate dehydrogenase; HK: Hexokinase; AK: Aspartokinase; DDH: Diaminopimelate dehydrogenase. Symbol "-": No detection; All data are mean values of three determinations of three independent experiments with errors ≤10%.

Discussion

Industrial culture media for L-lysine fermentation contain high concentrations of carbohydrates, such as glucose and molasses, which will inevitably lead to a high-intensity caramelization during autoclaving. In the present study, we focussed on the overlooked effect of glycine betaine on L-lysine fermentation. Although glycine betaine is wildely used as osmoregulators in L-lysine fermentation by *Corynebacterium* or *Brevibacterium* [16], the viewpoint that the glycine betaine is able to prevent caramelization and increase the L-lysine production is presented for the first time.

There were big differences of color change in CgXIIG medium containing different concentrations of glycine betaine or at different pHs (Fig. 1A). The variation of color change is related to the intensity of caramelization, which leads to the color changes [18]. The typical brown color developed during caramelization is attributed to the production of polymeric products [19], and it is well known that 5-HMF and furfural are the precursors of polymeric products [18]. Thus, the intensity of browning and the concentration of 5-HMF and furfural can be used

as measures to determine the intensity of caramelization of culture media. The intensity of browning (OD_{420}) and the concentrations of 5-HMF and furfural were decreased by adding glycine betaine or decreasing pH values. With the addition of more glycine betaine or the lower pH, the intensity of browning and the 5-HMF and furfural concentrations decreased even more (Figs. 1A, 1B, and 1C). The above experimental results show that glycine betaine is able to prevent caramelization. Interestingly, in the case of the effect on browning intensity and the concentration of 5-HMF concentration of furfural, pH appeared to work better than glycine betaine (Figs. 1A, 1B, and 1C).

In order to enhance the L-lysine production, it is necessary to increase the availability of sugar and to maintain a certain mount of biomass concentration. As shown in Fig. 1D, the glycine betaine and pH affected strongly the concentration of glucose after autoclaving. The residual glucose concentration of culture media was increased by adding different concentrations of glycine betaine or decreasing the pH values (Fig. 1D). On the one hand, some non-volatile polymeric products were produced [19], which were not utilized by the microorganism. On the other hand, a lot of volatile compounds were synthesized during caramelization, such as hydrocarbons, aliphatic aldehydes, ketones, *etc.* [4], which will dissipate into the ambient air. The above-mentioned results directly affect the availability of glucose for L-lysine production. In addition to the availability of glucose, biomass is an important factor for L-lysine production. There were small differences in the total DCW and specific growth rate among the culture media containing no more than 20 mM glycine betaine (Figs. 2A, 2C). This does not mean that additional glycine betaine does not affect the cell growth. Lee *et al.* [12] have reported that fermentation in media containing 5-HMF or furfural showed decreased cell growth and longer times to reach the maximum production. As showed in Figs. 1B, and 1C, the concentrations of 5-HMF and furfural are decreased by adding glycine betaine. In addition, the availability of glucose is increased by adding glycine betaine (Fig. 1D). Based on the above results, the DCW should be gradually increased with increasing the addition of glycine betaine. However, the DCW is regulated by at least three factors: (i) the availability of glucose [25]; (ii) the concentration of inhibitory compounds, such as 5-HMF and furfural [12]; and (iii) the osmotic pressure of the culture media [16]. The osmotic pressure will lead to cell lysis or dehydration, thereby preventing cell growth [16]. Additional glycine betaine will change the concentration of substrate and then increase the osmotic pressure, because glycine betaine is not metabolized by *C. glutamicum* [5]. In addition, Meury [14] has found that the cell growth was decreased early in the addition of glycine betaine, indicating that exogenous glycine betaine may harm cell growth. Although additional glycine betaine leads to an increase in the availability of glucose and to a decrease in the concentrations of 5-HMF and furfural, it is also harmful for cell growth and increases the osmotic pressure. Therefore, the difference of DCW among the different concentrations of glycine betaine is small. Interestingly, there was a big difference in DCW and maximal specific growth rate among the different gradient pH values. The optimal pH for *Brevibacterium* or *Corynebacterium* cell growth was 7.0–7.5; this may be a reason why the DCW and maximal specific growth rate had a big difference among the different gradient pH values [21]. It is strange that the DCW of *B. flavum* XQ-8 on media with 25 and 30 mM glycine betaine was lower than on media without glycine betaine or at pH = 7.0 (Fig. 2A). Although the reason for the DCW decrease and growth retardation is obscure, the initial growth retardation phenomenon is similar to previous reports [14].

As compared with growth on culture media without glycine betaine, *B. flavum* QX-8 showed a higher L-lysine production during growth on media with different concentrations of glycine betaine (Fig. 2D and Table 1). This is because the additional glycine betaine increases the availability of glucose but decreases the concentrations of 5-HMF and furfural (Figs. 1B, 1C, and 1D). However, interestingly, the increase of L-lysine production is not consistent with the increase in concentration of glycine betaine. We can find that the DCW of *B. flavum* XQ-8 was decreased by adding 25 or 30 mM glycine betaine, which may be the reason for the decrease of L-lysine production during cultivation on culture media with 25 or 30 mM glycine betaine. Moreover, the glucose was not completely consumed during growth on CgXIIG medium containing 25 or 30 mM glycine betaine (data not shown). It should be noted that the time of reaching the maximal L-lysine production on media with more than 5 mM glycine betaine was longer (reached peak at ≥32 h) than on media without glycine betaine or with 5 mM glycine betaine (peak reached at 28 h, Fig. 2F) for reasons of DCW. However, interestingly, the L-lysine production was decreased with the decrease of pH value, although pH usually does better work than glycine betaine on preventing caramelization (Fig. 2E). The decrease of DCW may be at least partly to blame. Because of the increase of the glucose availability, the biomass specific L-lysine yield ($Y_{P/X}$) and the substrate-specific

L-lysine yield ($Y_{P/S}$) were increased by adding glycine betaine (Table 1).The highest $Y_{P/X}$ and $Y_{P/S}$ were observed when adding 20 mM glycine betaine into culture media. This indicates that 20 mM glycine betaine is helpful to increase the L-lysine production during cultivation on media with 40 g/l glucose. Interestingly, although the availability of glucose for L-lysine production was increased, the $Y_{P/X}$ and $Y_{P/S}$ decreased with the decrease of pH value.

As described previously, the activities of HK and PDH were increased by adding no more than 20 mM glycine betaine because of the decrease of the 5-HMF and furfural concentrations [1, 15]. It is strange that the activities of HK and PDH were slightly decreased when adding more than 20 mM glycine betaine, but the reasons are not clear. We speculate this may be the effect of excessive glycine betaine. Interestingly, the activities of AK and DDH changed very little under different concentrations of glycine betaine, indicating that 5-HMF or furfural does not affect the activities of AK and DDH. It is strange that the activities of HK, PDH, AK, and DDH were decreased with the decrease of pH value (Table 2). The reason could be that the transcription and translation of the enzymes' genes are interrupted at a low pH value [20].

In conclusion, we have studied the effect of glycine betaine on the L-lysine production, and demonstrated that glycine betaine is able to prevent caramelization and increase the L-lysine production. These results provide a new strategy that adding glycine betaine into culture media can avoid caramelization during autoclaving. By applying the principle of this strategy, glucose need no longer be separated from the culture media during autoclaving and the wastage of glucose *via* caramelization is decreased, so that factories can save production costs and shorten the fermentation period.

Acknowledgments

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