

Improvement of cell growth and L-lysine production by genetically modified *Corynebacterium glutamicum* during growth on molasses

Jianzhong Xu · Junlan Zhang · Yanfeng Guo ·
Yugui Zai · Weiguo Zhang

Received: 2 June 2013 / Accepted: 6 August 2013 / Published online: 13 September 2013
© Society for Industrial Microbiology and Biotechnology 2013

Abstract Fructose-1,6-bisphosphatase (FBPase) and fructokinase (ScrK) have important roles in regenerating glucose-6-phosphate in the pentose phosphate pathway (PPP), and thus increasing L-lysine production. This article focuses on the development of L-lysine high-producing strains by heterologous expression of FBPase gene *fbp* and ScrK gene *scrK* in *C. glutamicum* *lysC^{fbr}* with molasses as the sole carbon source. Heterologous expression of *fbp* and *scrK* lead to a decrease of residual sugar in fermentation broth, and heterologous expression of *scrK* prevents the fructose efflux. Heterologous expression of *fbp* and *scrK* not only increases significantly the activity of corresponding enzymes but also improves cell growth during growth on molasses. FBPase activities are increased tenfold by heterologous expression of *fbp*, whereas the FBPase activity is only increase fourfold during co-expression of *scrK* and *fbp*. Compared with glucose, the DCW of heterologous expression strains are higher on molasses except co-expression of *fbp* and *scrK* strain. In addition, heterologous expression of *fbp* and *scrK* can strongly increase the L-lysine production with molasses as the sole carbon source. The highest increase (88.4 %) was observed for *C. glutamicum* *lysC^{fbr}* pDXW-8-*fbp-scrK*, but the increase was also significant for *C. glutamicum* *lysC^{fbr}* pDXW-8-*fbp* (47.2 %) and *C. glutamicum* *lysC^{fbr}* pDXW-8-*scrK* (36.8 %). By-products, such as glycerol and

dihydroxyacetone, are decreased by heterologous expression of *fbp* and *scrK*, whereas trehalose is only slightly increased. The strategy for enhancing L-lysine production by regeneration of glucose-6-phosphate in PPP may provide a reference to enhance the production of other amino acids during growth on molasses or starch.

Keywords L-Lysine production · Heterologous expression · Fructose-1,6-bisphosphatase · Fructokinase · Molasses

Introduction

L-Lysine is one of the eight essential amino acids for humans and animals. With its advantages, L-lysine has been applied in more and more fields, for example, feed additives [1], pharmaceuticals, cosmetics, and polymer materials [2]. With the continual enlarging of application range of L-lysine, the demands of L-lysine are rapidly increasing every year. Therefore, the least expensive fermentation substrates and optimal production strains are needed for fermentation in order to reduce the costs of production. Currently, the industrial L-lysine producers are almost auxotroph, regulatory and combined *Corynebacterium glutamicum*, or the closely related *Brevibacterium flavum* mutants, which have been obtained by repeated physical and/or chemical mutagenesis [3]. *C. glutamicum* can use a relatively broad substrate as carbon and energy sources, which include glucose, sucrose, fructose, pentose, ethanol, and organic acids, etc., because it possesses several different uptake systems to metabolize different carbon sources [4–6]. Glucose uptake in *C. glutamicum* occurs via a PTS^{Glc} and subsequently phosphorylated to glucose-6-phosphate [6], and 50 % of glucose-6-phosphate pass the

J. Xu (✉) · Y. Guo · Y. Zai · W. Zhang
The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, 1800# Lihu Road, WuXi 214122, People's Republic of China
e-mail: xujz126@126.com

J. Zhang
OriGene Biotechnology Co., Ltd, 88# Meiliang Road, WuXi 214122, People's Republic of China

pentose phosphate pathway to generate anabolic reducing equivalents [7]. Fructose is simultaneously phosphorylated by a PTS^{Fru} and a PTS^{Man} in *C. glutamicum* to give fructose-1-phosphate and fructose-6-phosphate, respectively [8]. The PTS^{Fru} is the predominant fraction, more than four-fifths of fructose via this mechanism to synthesis fructose-1-phosphate, and then synthesis fructose-1,6-bisphosphate [8]. However, *C. glutamicum* lacks completely a viable fructose-1,6-bisphosphatase (FBPase) for conversion of the fructose-1,6-bisphosphate into fructose-6-phosphate; 80 % of total fructose consumption enters glycolysis at the level of fructose-1,6-phosphate [7]. On the other hand, fructose-1,6-bisphosphate inhibits the key enzymes of the pentose phosphate pathway glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase [5]. Therefore, expression of the FBPase gene *fbp*, which catalyzes the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate and inorganic phosphate, is proposed to increase the pentose phosphate pathway flux as well as lysine production on fructose and sucrose [4, 5, 9, 10]. In addition, FBPase is necessary for *C. glutamicum* to grow on the carbon sources acetate, citrate, glutamate, and lactate [4]. *C. glutamicum* was found to take up and phosphorylate sucrose by a phosphoenolpyruvate-dependent sucrose phosphotransferase system (PTS^{Suc}) to yield sucrose-6-phosphate and subsequently hydrolyzed by an invertase to produce glucose-6-phosphate and fructose [8]. However, *C. glutamicum* does not possess such a fructokinase (ScrK) activity [11]; further metabolism of the intracellular fructose requires efflux of the fructose and reassimilation via the fructose PTS (PTS^{Fru}). In order to phosphorylate directly the fructose without any fructose efflux, heterologous expression of the ScrK gene *scrK* from *Clostridium acetobutylicum* (*C. acetobutylicum*) in *C. glutamicum* is proposed. On the other hand, ScrK not only directs fructose flux to the pentose phosphate pathway for the supply of NADPH but also modifies significantly the rigidity of phosphoenolpyruvate (PEP) node. Therefore, ScrK expression may be a potential strategy for enhancing L-lysine production during growth on sucrose or molasses.

Molasses, a waste product of the sugar industry, is widely used for the chemical synthesis of many organic chemicals by microbial fermentation, such as ethanol, amino acid, and glycerin, etc. [12, 13]. Reuse of molasses not only provides a solution to the environmental issue arising from the disposal of molasses but also helps in stabilizing the agricultural sector in the sugar-producing countries [14]. Molasses is a mixture of sugar, nonsugars, and water, from which no sugar crystallizes under any conceivable physical and technically optimum conditions, with no regard to time [13]. The sugar in beet molasses mainly includes sucrose, glucose, fructose, and raffinose, but the main component is sucrose. On the other hand, molasses contains not only large

amounts amino acid (glutamic acid, aspartic acid, leucine, etc.) pantothenic acid, inositol, and biotin, but also other growth substances (nicotinic acid, folic acid, thiamine, etc.). In addition, molasses contain a large variety of salts, for example, potassium and manganese. All in all, most components in molasses are advantageous to the growth of microorganisms. Molasses is widely used for amino acid fermentation by *Corynebacterium* or *Brevibacterium*, but most published work focuses on single glucose, or fructose, or sucrose as the carbon source [4, 8, 9, 15]. Although the molasses is used as a carbon source during the evaluation of the molecular targets for L-lysine production, this is only a fraction of the carbon source used in the fermentation medium [16]. As we have seen, the molasses used as the sole carbon source to L-lysine production has not been reported.

Given that the advantages of molasses, such as resource-rich and advantageous to cell growth, the present study was focus on the development of a high-yielding strain for the production of L-lysine with the beet molasses as sole carbon source. Firstly, the strain which heterologous expression the ScrK gene *scrK* from *C. acetobutylicum* in *C. glutamicum* lysC^{fbr} was constructed, and designated as *C. glutamicum* lysC^{fbr} pDXW-8-*scrK*; secondly, the strain which heterologous expression FBPase gene *fbp* from *Escherichia coli* in *C. glutamicum* lysC^{fbr} was constructed, and designated as *C. glutamicum* lysC^{fbr} pDXW-8-*fbp*; finally, the strain which heterologous expression *fbp* and *scrK* in *C. glutamicum* lysC^{fbr} was constructed, and designated as *C. glutamicum* lysC^{fbr} pDXW-8-*fbp-scrK*. The study shows the first time that heterologous expression of *scrK* and *fbp* are beneficial to L-lysine production and cell growth during growth on molasses as sole carbon source.

Materials and methods

Strains, growth media, and culturing conditions

The strains, plasmids, and oligonucleotides used in this study are listed in Table 1. DNA was extracted from *E. coli* and *C. acetobutylicum* using the TaKaRa MiniBEST Universal Genomic DNA Extraction Kit according to the protocol supplied by the manufacturer (Dalian, China). The *E. coli* DNA was used as a template to amplify the *fbp* operon with *fbp*-F and *fbp*-R, and the *C. acetobutylicum* DNA was used to amplify the *scrK* operon with *scrK*-F and *scrK*-R. The plasmid construction and transformation were performed according to the descriptions of Sambrook and Russel [17] and van der Rest et al. [18] (Fig. 1). Firstly, we constructed the strains which are heterologous expression the ScrK gene *scrK* from *C. acetobutylicum* or/and FBPase gene *fbp* from *E. coli* in *C. glutamicum* lysC^{fbr} . Plasmids were extracted

Table 1 Strains, plasmids, and oligonucleotides used in this study

	Relevant characteristic(s) or sequence	Reference or purpose
Strains and plasmids		
<i>E. coli</i> BL21 (DE3)	F ⁻ ompT gal dcm lon hsdS _B (r _B m _B) λ(DE3)	Strata gene
<i>E. coli</i> MG1655	Type strain	Our lab
<i>C. acetobutylicum</i> ATCC 842	Type strain	ATCC
<i>C. glutamicum</i> lysC ^{fbr}	Exchange T311I in the <i>lysC</i> gene	Our lab
<i>C. glutamicum</i> lysC ^{fbr} pDXW-8- <i>fbp</i>	<i>C. glutamicum</i> lysC ^{fbr} harboring pDXW-8- <i>fbp</i>	This work
<i>C. glutamicum</i> lysC ^{fbr} pDXW-8- <i>scrK</i>	<i>C. glutamicum</i> lysC ^{fbr} harboring pDXW-8- <i>scrK</i>	This work
<i>C. glutamicum</i> lysC ^{fbr} pDXW-8- <i>fbp-scrK</i>	<i>C. glutamicum</i> lysC ^{fbr} harboring pDXW-8- <i>fbp-scrK</i>	This work
pDXW-8	Amp ^r and Km ^r <i>E. coli</i> - <i>C. glutamicum</i> shuttle vector	[31]
pDXW-8- <i>fbp</i>	pDXW-8 carrying <i>fbp</i> gene	This work
pDXW-8- <i>scrK</i>	pDXW-8 carrying <i>scrK</i> gene	This work
pDXW-8- <i>fbp-scrK</i>	pDXW-8 carrying <i>fbp</i> and <i>scrK</i> gene	This work
Oligonucleotides		
<i>fbp</i> -F	CATGCCATGGGAAAGGAGATATACCATGAAAACG TTAGGTGAAATTT	Amplifying <i>fbp</i> operon
<i>fbp</i> -R	CCGCTCGAGTTACGCGTCCGGGAACTCACGG	
<i>scrK</i> -F	CGGAATTCGAAAGGAGATATACCATGAATAATGT TTTATGTATAG	Amplifying <i>scrK</i> operon
<i>scrK</i> -R	CTAGCTAGCTAATCACCTTCAACTTC	

Restriction sites are *underlined*

from *E. coli* or *C. glutamicum* using the Tianpure Mini Plasmid Kit according to the protocol supplied by the manufacturer (Tiangen, Beijing, China), and then confirmed by restriction endonuclease reaction (Fig. 2). Luria–Bertani (LB) was used as the standard medium for *E. coli* BL21. LBG (LB supplemented with 5 g/l glucose) was used for *C. glutamicum* [19]. *E. coli* and *C. glutamicum* were respectively grown at 37 °C and 30 °C. When appropriate, *E. coli* and *C. glutamicum* strains were cultured with kanamycin (50 µg/ml) and 120 rpm, and a reduced concentration of kanamycin (25 µg/ml) was used to obtain recombinant strains of *C. glutamicum* [5].

Fermentations of molasses

Molasses were supplied by the COFCO Biochemical (Anhui) Co., Ltd., (Anhui, China). They were diluted ten-fold in CgXII-media without carbon source and subsequently filtered to remove the suspended components by filter (ϕ 0.45 µm). Diluted molasses were used as fermentation media, and sterilized at 121 °C for 15 min before employed in the fermentations.

For lysine fermentation, a preculture was inoculated from a fresh LBG-plate and cultivated overnight. After washing cells with CgXII-media without a carbon source [5], the main culture with minimal medium was inoculated with OD₆₀₀ of 1 in 60 ml of fermentation media in 500-ml Erlenmeyer flasks. The cultures were grown at 30 °C and 120 rpm agitation. Heterologous gene expression of

recombinant strains was induced by adding isopropyl-β-D-thio-galactoside (IPTG, final concentration 1 mM) at OD₆₀₀ = 1.6. As a control, molasses was replaced by glucose where the concentration was equivalent to the total sugar concentration of molasses.

Enzyme activity assay

The crude enzyme was prepared according to the methods previously described [4, 19]. After centrifugation at 4 °C for 30 min at 10,000 × g, the cell-free supernatant was immediately used to determine the enzyme activities. Protein concentrations were determined using the Bradford Protein Quantification Kit according to the protocol supplied by the manufacturer (Tiangen, Beijing, China) with bovine serum albumin as 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 FBPase assay was based on the protocol of Becker et al. [4]. The fructokinase assay was based on the method of Porter et al. [20].

Analytical methods

Determination of sugar concentration

The sugar concentration was determined according to the descriptions of Periyasamy et al. [21]. The sugar concentrations were determined at initial fermentation and final fermentation.

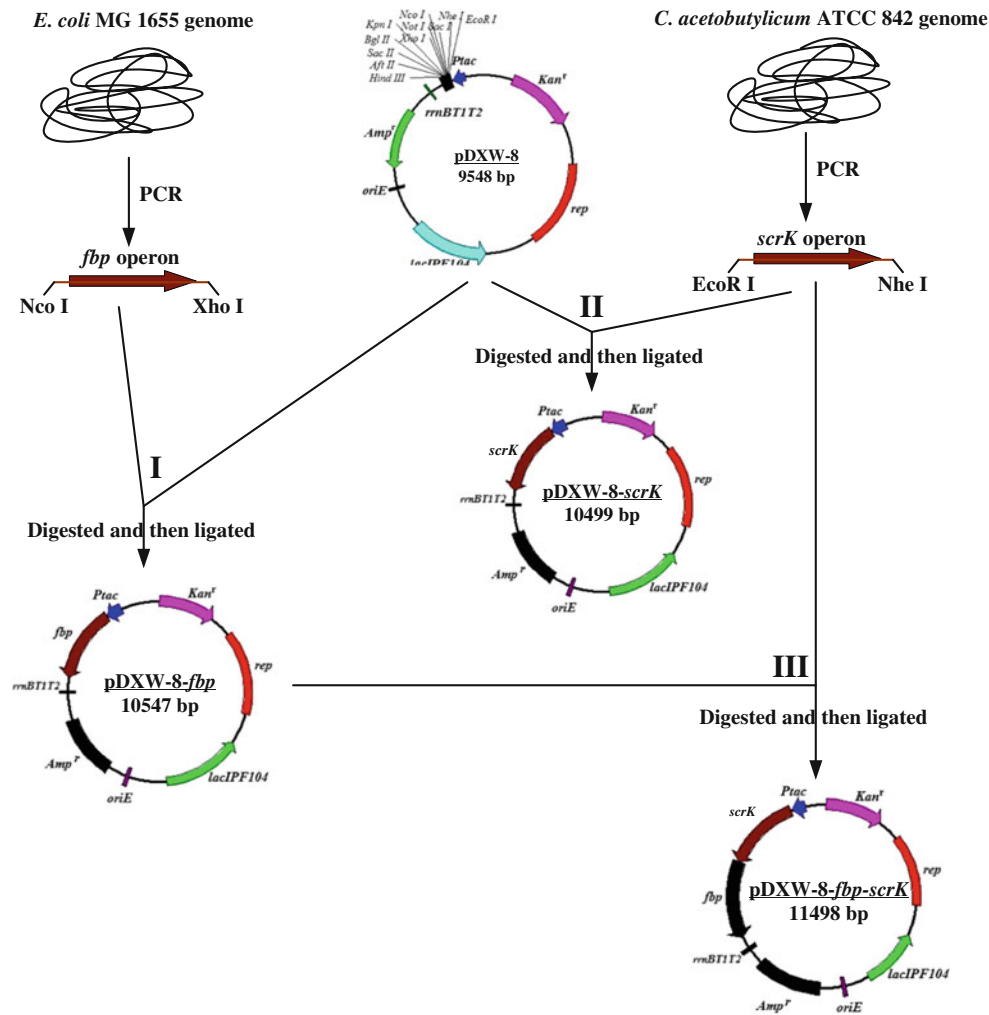


Fig. 1 Strategy used for construction of recombinant plasmids pDXW-8-*fbp*, pDXW-8-*scrK*, and pDXW-8-*fbp-scrK*

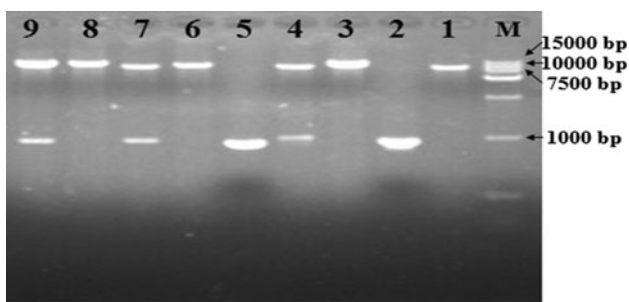


Fig. 2 Identification of recombinant plasmids pDXW-8-*fbp*, pDXW-8-*scrK*, and pDXW-8-*fbp-scrK* by enzyme digestion. *M* DNA marker DL15000 (TAKARA, Dalian, China), Lane 1 pDXW-8 *Hind*III digested, Lane 2 *fbp* PCR product, Lane 3 pDXW-8-*fbp* *Xho*I digested, Lane 4 pDXW-8-*fbp* *Xho*I and *Nco*I digested, Lane 5 *scrK* PCR product, Lane 6 pDXW-8-*scrK* *Eco*RI digested, Lane 7 pDXW-8-*scrK* *Eco*RI and *Nhe*I digested, Lane 8 pDXW-8-*fbp-scrK* *Eco*RI digested, Lane 9 pDXW-8-*fbp-scrK* *Eco*RI and *Nhe*I digested

Determination of glucose, fructose, sucrose, and trehalose concentration

Samples of 200 μ l were taken from Erlenmeyer flasks every 4 h. Then, 100 μ l of those samples was used to determine the glucose, fructose, sucrose, and trehalose concentration, and the other for measuring the cell growth and L-lysine production. The determinations of glucose, fructose, and trehalose in 1:10-diluted cultivation supernatant were carried out by high-performance liquid chromatography (HPLC, Agilent Technologies, Palo Alto, CA, USA) with a Sugar-Pak 1 6.5×300 -mm column at 70 $^{\circ}$ C using isocratic elution with ultrapure H_2O at a flow rate of 0.6 ml/min, and subsequent detection via determination of refraction indexes. The determination of sucrose was carried out by HPLC as described previously [4].

Determination of L-lysine, glycerol, and dihydroxyacetone concentration

The L-lysine concentration was determined by an SBA-40E immobilized enzyme biosensor (Shandong, China). The determinations of glycerol and dihydroxyacetone were in 1:10-diluted cultivation supernatant were carried out by HPLC with a Kromasil C-18 4.6×250 mm column (AkzoNobel, Amsterdam, The Netherlands) at 30 °C using isocratic elution with 20 mM KH_2PO_4 and 0.5 % acetonitrile at a flow rate of 0.8 ml/min, and subsequent detection via determination of UV absorption at 210 nm.

Determination of cell growth

One hundred microliters of cultures was used to measure the biomass concentration by a photometer at 600 nm after an appropriate dilution or by gravimetric analysis as described previously [19]. Under these experimental conditions, the correlation factor (g of biomass to OD_{600} nm) between dry cell weight and OD_{600} nm was determined as 0.341.

Results

The concentration of total sugar, glucose, fructose, and sucrose

There are differences in components and total sugar concentrations among companies within different counties. In order to have a rough idea of the consumption of sugar, the initial total sugar and residual sugar concentration were detected. The initial total sugar concentration of diluted molasses was 49.57 ± 3.73 g/l (Fig. 3). There were big differences in the residual sugar concentrations of fermentation broth between *C. glutamicum lysC^{fbr}* and other recombinant *C. glutamicum* strains (Fig. 3). The residual sugar concentrations of *C. glutamicum lysC^{fbr}*, *C. glutamicum lysC^{fbr} pDXW-8-fbp*, *C. glutamicum lysC^{fbr} pDXW-8-scrK*

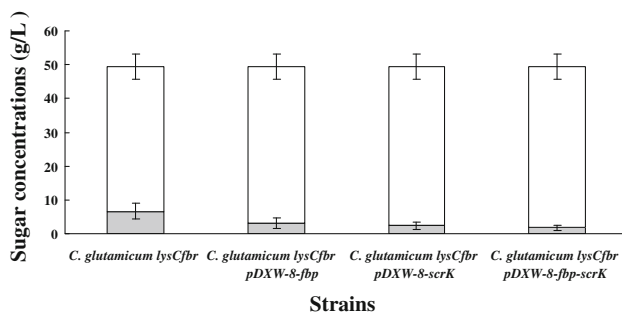


Fig. 3 Initial total sugar concentration and the residual sugar concentration after fermenting by various strains of *C. glutamicum*. The gray column indicates the residual sugar concentration and the white column indicates the consumption of sugar concentration

scrK, and *C. glutamicum lysC^{fbr} pDXW-8-fbp-scrK* were 6.62 ± 2.26 , 3.15 ± 1.53 , 2.38 ± 1.04 , and 1.71 ± 0.83 g/l, respectively. This phenomenon was also observed by detecting the concentration of glucose, sucrose, and fructose with HPLC in this study (Fig. 4). The concentration of glucose, sucrose, and fructose in diluted molasses was 0.94, 47.33, and 0.79 g/l, respectively. The concept that the sugar in beet molasses consists mainly of sucrose was known to everyone long ago [13]. It should be noted that glucose was firstly consumed, regardless the strains with tested (Fig. 4). The concentrations of sucrose and fructose were changeless during the lag phase of bacterial, regardless the strains with tested. However, the way of sucrose and fructose consumption was different between the tested strains with the passage of fermentation time. Most of the sucrose was consumed at 36 h for *C. glutamicum lysC^{fbr}*, whereas the concentration of fructose was increased to achieve a peak at 24 h (Fig. 4a). However, for *C. glutamicum lysC^{fbr} pDXW-8-fbp*, the concentration of sucrose had touched bottom at 44 h (0.55 g/l), whereas the fructose had peaked at 28 h (8.73 g/l). Interestingly, unlike *C. glutamicum lysC^{fbr}* and *C. glutamicum lysC^{fbr} pDXW-8-fbp*, the concentration of fructose had been at a low level (0.8 ± 0.05 g/l) for *C. glutamicum lysC^{fbr} pDXW-8-scrK* and *C. glutamicum lysC^{fbr} pDXW-8-fbp-scrK*, whereas the sucrose was completely consumed at 36 h (Fig. 4c, d).

In vitro activity of FBPase and fructokinase in the genetically defined *C. glutamicum* strains

FBPase exhibited a low basal in vitro activity in the *C. glutamicum lysC^{fbr}*, and the activity of FBPase was not affected by additional expression of *scrK* in *C. glutamicum lysC^{fbr}* (Table 2). Interestingly, the activity of FBPase from *C. glutamicum lysC^{fbr} pDXW-8-fbp* was 228 ± 5.46 mU/mg, which was significantly higher than that of *C. glutamicum lysC^{fbr} pDXW-8-fbp-scrK* (101 ± 4.97 mU/mg). For fructokinase, there was no detectable fructokinase in *C. glutamicum lysC^{fbr}* and *C. glutamicum lysC^{fbr} pDXW-8-fbp*. The result is consistent with the precious report [22] because of absence of fructokinase gene in *C. glutamicum*. For amplification of fructokinase, the recombinant strains *C. glutamicum lysC^{fbr} pDXW-8-scrK* and *C. glutamicum lysC^{fbr} pDXW-8-fbp-scrK* showed increased level of fructokinase activity (Table 2). The activity of fructokinase was not affected by additional expression of fructose-1,6-bisphosphatase gene (*fbp*). Overall, the enzyme data clearly showed that heterologous expression of *fbp* and *scrK* in *C. glutamicum lysC^{fbr}* were successful.

Growth performance of genetically defined *C. glutamicum* strains

To test the influences of the heterologous expressions of *fbp* and *scrK* for cell growth during growth on molasses,

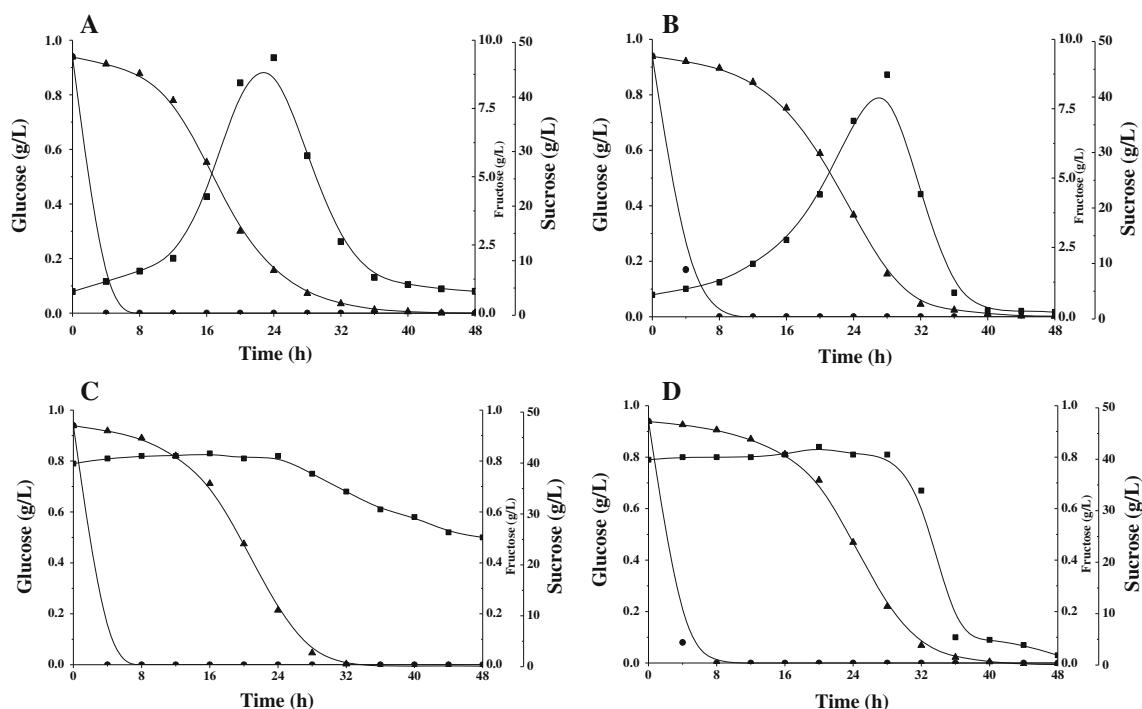


Fig. 4 Effect of heterogenous expression of fructose-1,6-bisphosphatase gene and fructokinase gene in *C. glutamicum* on glucose (filled circle), fructose (filled square), and sucrose (filled triangle)

catabolism in molasses minimal medium: **a** *C. glutamicum lysC^{fbr}*; **b** *C. glutamicum lysC^{fbr} pDXW-8-fbp*; **c** *C. glutamicum lysC^{fbr} pDXW-8-scrK*; **d** *C. glutamicum lysC^{fbr} pDXW-8-fbp-scrK*

Table 2 In vitro specific activity of FBPase and ScrK in recombinant *C. glutamicum* strains during growth on molasses

Specific activity (mU/mg)	<i>C. glutamicum</i> strains			
	<i>lysC^{fbr}</i>	<i>lysC^{fbr} pDXW-8-fbp</i>	<i>lysC^{fbr} pDXW-8-scrK</i>	<i>lysC^{fbr} pDXW-8-fbp-scrK</i>
FBPase	23 ± 0.33	228 ± 5.46	23 ± 0.72	101 ± 4.97
ScrK	ND	ND	53 ± 0.25	51 ± 1.6

Each value represents the mean with standard error of three replicative experiments, corresponding deviations are given
ND not detectable

we analyzed the DCW of these recombinant strains as compared to growth on glucose. There were differences in the DCW and maximal specific growth rate among the recombinant strains during growth on molasses. The DCW of recombinant strains were higher than the starting strain *C. glutamicum lysC^{fbr}* during growth on molasses. The DCW of *C. glutamicum lysC^{fbr}*, *C. glutamicum lysC^{fbr} pDXW-8-fbp*, *C. glutamicum lysC^{fbr} pDXW-8-scrK*, and *C. glutamicum lysC^{fbr} pDXW-8-fbp-scrK* were 19.22, 22.13, 22.71, and 20.25 g/l, respectively, during growth on molasses. However, compared with the molasses, the DCW of these strains were different during growth on glucose, 22.3, 21.07, 22.1, and 21.07 g/l, respectively. The maximal specific growth rate was decreased by heterologous expression of *fbp* or *scrK* as compared to *C. glutamicum lysC^{fbr}*. The maximal specific growth rate of *C. glutamicum lysC^{fbr}*, *C. glutamicum lysC^{fbr} pDXW-8-fbp*, *C. glutamicum*

lysC^{fbr} pDXW-8-scrK and *C. glutamicum lysC^{fbr} pDXW-8-fbp-scrK* was 0.295, 0.243, 0.253, and 0.232/h, respectively, during growth on molasses. Interestingly, the maximal specific growth rate of *C. glutamicum lysC^{fbr} pDXW-8-scrK* was lower than *C. glutamicum lysC^{fbr}*, whereas the DCW of it was highest among the four tested strains (Figs. 5, 6). It should be noted that heterologous expression of *scrK* has no effect on cell growth during growth on glucose (Fig. 6). It is generally known that no fructose was synthesized during growth on glucose, so expression of *scrK* is ineffective.

L-Lysine production of genetically defined *C. glutamicum* strains

As compared to growth on glucose, *C. glutamicum lysC^{fbr}* showed a lower L-lysine production during growth on

molasses, 34.5 and 25 mM, respectively. This is due to the main carbon source of molasses, which contains mainly sucrose as carbon source. The additional heterologous expression of *fbp* and/or *scrK* in *C. glutamicum lysC^{fbr}* led to a significant increase in the L-lysine production on molasses (Fig. 5). The highest increase (88.4 %) was observed for *C. glutamicum lysC^{fbr} pDXW-*fbp-scrK**, but the increase was also significant for *C. glutamicum lysC^{fbr} pDXW-8-*fbp** (47.2 %) and *C. glutamicum lysC^{fbr} pDXW-8-*scrK** (36.8 %). Compared with heterologous expression of *fbp*, heterologous expression of *scrK* led to little increase

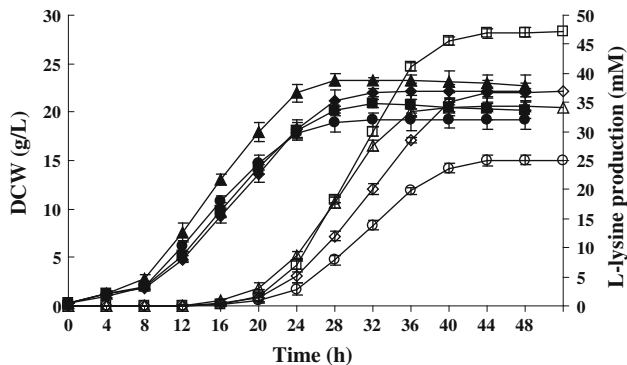


Fig. 5 Effect of heterogenous expression of fructose-1,6-bisphosphatase gene and fructokinase gene in *C. glutamicum* on DCW and L-lysine production. Symbols are as follows: filled circle DCW of *C. glutamicum lysC^{fbr}*, filled diamond DCW of *C. glutamicum lysC^{fbr} pDXW-8-*fbp**, filled triangle DCW of *C. glutamicum lysC^{fbr} pDXW-8-*scrK**, filled square DCW of *C. glutamicum lysC^{fbr} pDXW-8-*fbp-scrK**, open circle L-lysine production of *C. glutamicum lysC^{fbr}*, open diamond L-lysine production of *C. glutamicum lysC^{fbr} pDXW-8-*fbp**, open triangle L-lysine production of *C. glutamicum lysC^{fbr} pDXW-8-*scrK**, open square L-lysine production of *C. glutamicum lysC^{fbr} pDXW-8-*fbp-scrK**. Each value represents mean with standard error of three replicative experiments. The standard errors are shown as bars

L-lysine production on molasses, but the L-lysine production was similar to growth on glucose, 34.5 and 34.2 mM, respectively (Fig. 5). Although the L-lysine production of *C. glutamicum lysC^{fbr} pDXW-8-*scrK** was not higher, the time of reaching the peak was shortest among these strains (reached peak at 36 h). In addition, *C. glutamicum lysC^{fbr} pDXW-8-*fbp-scrK** showed the biggest the maximal specific L-lysine production rate among these strains (data not shown). It is worth pointing out that heterologous expression of *fbp* in *C. glutamicum lysC^{fbr}* leads to a significant increase (32.2 %), whereas heterologous expression of *scrK* in *C. glutamicum lysC^{fbr}* was invalid for increasing L-lysine production during growth on glucose (Fig. 6). This is, however, not a consequence of an unexpressed *scrK*. In fact, the expression of *scrK* was successfully expressed in *C. glutamicum* (Table 2).

By-product production of genetically defined *C. glutamicum* strains

Glycerol and dihydroxyacetone were the leading by-products for molasses-grown cells of *C. glutamicum lysC^{fbr}*, which is also accompanied by little trehalose (Table 3). The comparative analysis for the other recombinant strains revealed that heterologous expression of *fbp* or/and *scrK* led to strong decrease in glycerol and dihydroxyacetone yields (Table 3). Interestingly, single expression of *scrK* in *C. glutamicum lysC^{fbr}* led to a larger decrease in glycerol and dihydroxyacetone production as compared to single expression of *fbp* (Table 3). In addition, the concentrations of glycerol and dihydroxyacetone were lowest during co-expression of *fbp* and *scrK*, 0.5 ± 0.07 and 1.7 ± 0.09 mM, respectively. However, the trehalose formation was increased by heterologous expression of *fbp* or/

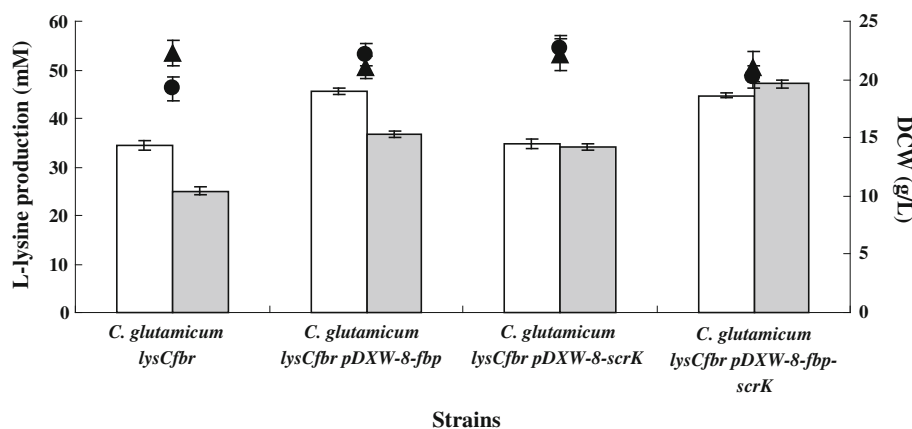


Fig. 6 Comparison of DCW and L-lysine production of recombinant *C. glutamicum* strains during growth on glucose with growth on molasses minimal medium. The white column indicates the L-lysine production of recombinant strains during growth on glucose and the gray column indicates the L-lysine production of recombinant strains

during growth on molasses. Symbols are as follows: filled triangle the DCW of recombinant strains during growth on glucose, filled circle the DCW of recombinant strains during growth on molasses. Each value represents mean with standard error of three replicative experiments. The standard errors are shown as bars

Table 3 Yield of glycerol, dihydroxyacetone, and trehalose in the stages of L-lysine production by recombinant *C. glutamicum* strains during growth on molasses

<i>C. glutamicum</i> strains	Glycerol (mM)	Dihydroxyacetone (mM)	Trehalose (mM)
<i>lysC^{fbr}</i>	3.3 ± 0.27	11.2 ± 0.56	0.7 ± 0.15
<i>lysC^{fbr} pDXW-8-fbp</i>	1.4 ± 0.14	4.5 ± 0.18	0.9 ± 0.10
<i>lysC^{fbr} pDXW-8-scrK</i>	0.6 ± 0.12	2.1 ± 0.20	1.3 ± 0.06
<i>lysC^{fbr} pDXW-8-fbp-scrK</i>	0.5 ± 0.07	1.7 ± 0.09	1.4 ± 0.31

Each value represents the mean with standard error of three replicative experiments; corresponding deviations are given

and *scrK* as compared to the *C. glutamicum lysC^{fbr}* (Table 3). Among them, heterologous expression of *scrK* played a major role in the increase of trehalose. These results showed that heterologous expression of *fbp* and *scrK* was best for minimizing glycerol and dihydroxyacetone, especially co-expression of *fbp* and *scrK*, during growth on molasses.

Discussion

Molasses is widely used for product many organic chemicals by microbial fermentation, such as ethanol, amino acid, and glycerin, etc. [12–14], and is beneficial to cell growth [13]. In the present study, we focus on the development of a high-yielding strain for the production of L-lysine with beet molasses as the sole carbon source. Although molasses is widely used for L-lysine fermentation by *Corynebacterium* or *Brevibacterium* [16], the molasses is firstly used as the sole carbon source to L-lysine production. In order to increase the L-lysine production and cell growth during growth on molasses, the heterologous *fbp* from *E. coli* and *scrK* from *C. acetobutylicum* are firstly expressed in *C. glutamicum lysC^{fbr}*.

In order to enhance the L-lysine production, it is necessary to increase the consumption of sugar. There are big differences of sugar consumption between *C. glutamicum lysC^{fbr}* and recombinant *C. glutamicum lysC^{fbr}* strains (Fig. 3). The variation of sugar consumption may be related to components of molasses and gene expression. Molasses contain multiple sugars, for example, glucose, sucrose, fructose, and raffinose [13]. In addition, the differences of various sugar uptake capacity were found in *C. glutamicum* [5]. Glucose was preferred by *C. glutamicum*, and next being sucrose and fructose [5, 23], and this is exactly why glucose is firstly consumed, regardless of the strain tested (Fig. 4). Interestingly, the way of sucrose and fructose consumption was different between the tested

strains with the passage of fermentation time (Fig. 4). This may be related to the metabolic methods of fructose and sucrose in *C. glutamicum* and recombined *C. glutamicum* strains. Sucrose is ingested by *C. glutamicum* via PTS^{Suc} to yield intracellular sucrose-6-phosphate and subsequently hydrolyzed to produce glucose-6-phosphate and liberated fructose [8], but in the absence of fructokinase, further metabolism of the liberated fructose requires efflux of the fructose [23]. In this way, the concentration of sucrose is decreased, whereas the concentration of fructose is increased during the initial stage of fermentation. Finally, the fructose in culture was reassimilated by PTS^{Fru} so that the concentration of fructose is decreased with the fermentation progress. Heterologous expression of *scrK* in *C. glutamicum lysC^{fbr}* and *C. glutamicum lysC^{fbr} pDXW-8-fbp* not only increases the activity of fructokinase but also prevents the efflux of the fructose (Table 2; Fig. 4c, d). However, the FB Pase activity is affected by additional expression of *scrK* in *C. glutamicum lysC^{fbr} pDXW-8-fbp* (Table 2). Expression of *scrK* modifies the transportation of sucrose. The intercellular free fructose derived from sucrose was directly phosphorylated to fructose-6-phosphate and thus without fructose efflux [22]. In this way, the concentration of fructose-6-phosphate is increased, whereas the concentration of fructose-1,6-bisphosphate is decreased during expression of *scrK*. Hereby, the activity of FB Pase from *C. glutamicum lysC^{fbr} pDXW-8-fbp-scrK* is decreased.

The DCW of recombinant *C. glutamicum lysC^{fbr}* strains is increased by heterologous expression of *fbp* or *scrK* (Fig. 5), however, the specific growth rate is decreased by expression of *fbp* or *scrK*. This same phenomenon was found in previous works [4, 22]. This is because heterologous expression of *fbp* and/or *scrK* leads to a redirection of carbon from glycolysis toward the PPP and an increased NADPH supply, thus meeting the requirement of NADPH for biomass synthesis [4]. On the other hand, heterologous expression of *fbp* and *scrK* lead to a decrease in the flux of TCA (see Figure 1 of Georgi et al., [5]). The *scrK*-expression strains grow faster than the *fbp*-expression strains (Fig. 4). This could be because of the difference of characteristics between ScrK and FB Pase. The intercellular fructose is catalyzed to synthesize the fructose-6-phosphate by ScrK, thus preventing the effusion of fructose [24]. The synthetic fructose-6-phosphate has two metabolic pathways: (1) entering glycolysis via phosphofructokinase and (2) entering glycconeogenesis via glucose-6-phosphate isomerase, thus utilizing by strains. FB Pase catalyzes the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate and inorganic phosphate [25]. The intracellular fructose must be transferred to extracellular, subsequently ingested by PTS^{Fru} to synthesize fructose-1,6-bisphosphate. Therefore, heterologous expression of *fbp* should not

directly utilize intracellular fructose by strains. Interestingly, Moon et al. [22] reported that growth of the *C. glutamicum* ATCC13032 harboring the fructokinase gene was noticeably retarded during the initial stage of growth, whereas this phenomenon does not appear in our experiment. This may be due to the difference of starting strains or even media. As compared to growth on glucose, the DCW of *C. glutamicum lysC^{fbt} pDXW-8-fbp* and *C. glutamicum lysC^{fbt} pDXW-8-scrK* were higher during growth on molasses (Fig. 6). Apart from the fact that the growth substances are abundant in molasses [13], heterologous expression of *fbp* and *scrK* plays a major role in the increase of biomass. Interestingly, co-expression of *fbp* and *scrK* leads to a small decrease in biomass (Figs. 5, 6). This may be related to the fact that heterologous co-expression of *fbp* and *scrK* influence the intracellular distribution of the carbon source, with a shift from biomass towards amino acid production [4, 22].

Apart from the benefit for cell growth, Becker et al. [4] and Georgi et al. [5] have reported that overexpression of *fbp* in *C. glutamicum lysC^{fbt}* led to a strong increase in L-lysine production during growth on glucose, fructose, and sucrose. In addition, Becker et al. [4] thinks that overexpression of FBPase was beneficial to L-lysine production by *C. glutamicum* with starch or molasses as the carbon source, and Georgi et al. [5] have also pointed out that FBPase was a limiting factor for L-lysine production by *C. glutamicum* with sucrose as the carbon source. However, the FBPase from *C. glutamicum* is negatively regulated at the metabolic level by phosphoenolpyruvate or AMP [26] and inhibited by fructose 2,6-bisphosphate [27]. Fortunately, the FBPase from *E. coli* is not inactivated by catabolite and is insensitive to fructose-2,6-bisphosphate ([28]; Jianzhong Xu et al. unpublished results). Therefore, expression of *E. coli fbp* is beneficial in increasing L-lysine production and decreasing by-products (Figs. 5, 6; Table 3). It is worth pointing out that the improvements of L-lysine production by *C. glutamicum lysC^{fbt}* with heterologous *fbp* expression are superior to the previous results, which are obtained via homologous *fbp* expression in *C. glutamicum lysC^{T311I}* during growth on fructose and sucrose [4]. It may be that *E. coli* FBPase has removed the inactivation or inhibitions of fructose-2,6-diphosphate, AMP and catabolite, thus catalyzing effectively the formation of fructose-6-phosphate from fructose-2,6-diphosphate. Expression of the *scrK* in *C. glutamicum* can directly phosphorylate intracellular fructose into fructose-6-phosphate instead of fructose-1-phosphate, thus changing the route of fructose entry into metabolism [22]. In addition, fructokinase uses ATP as a phosphoryl donor rather than phosphoenolpyruvate. Therefore, fructokinase expression would seem to be a potential strategy for enhancing amino acid production. As compared with heterologous

expression of *fbp*, however, heterologous expression of *scrK* leads to a smaller increase in L-lysine production (Fig. 5). We find the DCW was increased more by heterologous expression of *scrK* as compared with expression of *fbp*. So, we deduce that heterologous expression of *scrK* led to more C-source enter to synthesize biomass than to produce the L-lysine.

The differences in carbon-metabolite accumulation are indicative of a different pattern of carbon-flux distribution through the central metabolic pathways [7]. Heterologous expression of *fbp* and *scrK* changes the carbon-metabolite accumulation as compared to starting strains *C. glutamicum lysC^{fbt}* [3, 4, 22]. Therefore, the L-lysine production is strongly increased, whereas the by-products are greatly decreased by expression of *fbp* and *scrK* ([4] and Table 3). However, *fbp* and/or *scrK* expression lead to a slight increase in trehalose. Glucose-6-phosphate is used as a precursor to synthesis trehalose [15], which is increased by expression of *fbp* and *scrK*. Therefore, reducing the concentration of glucose-6-phosphate is beneficial to reducing the concentration of trehalose. Enhancing the PPP flux may be a promising method for reducing the concentration of glucose-6-phosphate. In this regard, expression of the glucose-6-phosphate dehydrogenase gene *zwf* or introduction of the modified variants of 6-phosphogluconate dehydrogenase or glucose-6-phosphate dehydrogenase has been suggested [15, 29]. In addition, enhancing the PPP flux is also of critical importance for L-lysine production [30].

In conclusion, we have studied the development of a high-yielding strain for the production of L-lysine, and demonstrated that heterologous expression of *fbp* and *scrK* are beneficial to increasing the biomass and L-lysine production with beet molasses as the sole carbon source. On the basis of these and previous results [4, 15, 22], we regard that strategies which lead to an increased PPP flux could increase L-lysine production or other NADPH-dependent compounds. In addition, expression of *C. acetobutylicum scrK* can effectively stop intracellular fructose effusion and enhance the L-lysine production during growth on molasses.

Acknowledgments This work was financially supported by the Program of Chinese 863 National High-Tech Research and Development Plan Project (No. 2008AA02Z212).

References

1. Jakobsen ØM, Brautaset T, Degnes KF, Heggset TMB, Balzer S, Flickinger MC et al (2009) Overexpression of wild-type aspartokinase increases L-lysine production in the thermotolerant methylotrophic bacterium *Bacillus methanolicus*. Appl Environ Microbiol 75:652–661. doi:10.1128/AEM.01176-08

2. Kaffas M, Stephanopoulos G (2005) Strain improvement by metabolic engineering: lysine production as a case study for systems biology. *Curr Opin Biotechnol* 16:361–366. doi:10.1016/j.copbio.2005.04.010
3. Wittmann C, Becker J (2007) The L-lysine story: from metabolic pathways to industrial production. *Microbiol Monographs* 5:39–70. doi:10.1007/7171_2006_089
4. Becker J, Klopprogge C, Zelder O, Heinzle E, Wittmann C (2005) Amplified expression of fructose 1,6-bisphosphatase in *Corynebacterium glutamicum* increases in vivo flux through the pentose phosphate pathway and lysine production on different carbon sources. *Appl Environ Microbiol* 71:8587–8596. doi:10.1128/AEM.71.12.8587-8596.2005
5. Georgi T, Rittmann D, Wendisch VF (2005) Lysine and glutamate production by *Corynebacterium glutamicum* on glucose, fructose and sucrose: roles of malic enzyme and fructose-1,6-bisphosphatase. *Metab Eng* 7:291–301. doi:10.1016/j.ymben.2005.05.001
6. Lindner SN, Seibold GM, Henrich A, Krämer R, Wendisch VF (2011) Phosphotransferase system (PTS) independent glucose utilization in *Corynebacterium glutamicum* by inositol permeases and glucokinases. *Appl Environ Microbiol* 77:3571–3581. doi:10.1128/AEM.02713-10
7. Dominguez H, Rollin C, Guyonvarch A, Guerquin-Kern JL, Coccagn-Bousquet M, Lindley ND (1998) Carbon-flux distribution in the central metabolic pathways of *Corynebacterium glutamicum* during growth on fructose. *Eur J Biochem* 254:96–102. doi:10.1046/j.1432-1327.1998.2540096.x
8. Wittmann C, Kiefer P, Zelder O (2004) Metabolic fluxes in *Corynebacterium glutamicum* during lysine production with sucrose as carbon source. *Appl Environ Microbiol* 70:7277–7287. doi:10.1128/AEM.70.12.7277-7287.2004
9. Kiefer P, Heinzle E, Zelder O, Wittmann C (2004) Comparative metabolic flux analysis of lysine-producing *Corynebacterium glutamicum* cultured on glucose or fructose. *Appl Environ Microbiol* 70:229–239. doi:10.1128/AEM.70.1.229-239.2004
10. Pons A, Dussap CG, Pequinet C, Gros JB (1996) Metabolic flux distribution in *Corynebacterium melassecola* ATCC 17965 for various carbon sources. *Biotechnol Bioeng* 51:77–189. doi:10.1002/(SICI)1097-0290(19960720)51:2<177:AID-BIT7>3.0.CO;2-G
11. Peng X, Okai N, Vertès AA, Inatomi K, Inui M, Yukawa H (2011) Characterization of the mannitol catabolic operon of *Corynebacterium glutamicum*. *Appl Microbiol Biotechnol* 91:1375–1387. doi:10.1007/s00253-011-3352-x
12. Kopsahelis N, Nisiotou A, Kourkoutas Y, Panas P, Nycha GJE, Kanellaki M (2009) Molecular characterization and molasses fermentation performance of a wild yeast strain operating in an extremely wide temperature range. *Bioresour Technol* 100:4854–4862. doi:10.1016/j.biortech.2009.05.011
13. Olbrich H (2006) The molasses. *Biotechnologie-Kempe GmbH, Berlin*
14. Kopsahelis N, Agouridis N, Bekatorou A, Kanellaki M (2007) Comparative study of spent grains and delignified spent grains as yeast supports for alcohol production from molasses. *Bioresour Technol* 98:1440–1447. doi:10.1016/j.biortech.2006.03.030
15. Becker J, Klopprogge C, Herold A, Zelder O, Bolten CJ, Wittmann C (2007) Metabolic flux engineering of L-lysine production in *Corynebacterium glutamicum*—over expression and modification of G6P dehydrogenase. *J Biotechnol* 138:99–109. doi:10.1016/j.jbiotec.2007.05.026
16. Becker J, Zelder O, Häfner S, Schröder H, Wittmann C (2011) From zero to hero—design-based systems metabolic engineering of *Corynebacterium glutamicum* for L-lysine production. *Metab Eng* 13:159–168. doi:10.1016/j.ymben.2011.01.003
17. Sambrook J, Russel DV (2001) *Molecular cloning: a laboratory manual*, 3rd edn. Cold Spring Harbor Laboratory Press, New York
18. van der Rest ME, Lange C, Molenaar D (1999) A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. *Appl Microbiol Biotechnol* 52:541–545
19. Hou XH, Ge XY, Wu D, Qian H, Zhang WG (2012) Improvement of L-valine production at high temperature in *Brevibacterium flavum* by overexpressing *ilvEBN^c* genes. *J Ind Microbiol Biotechnol* 39:63–72. doi:10.1007/s10295-011-1000-1
20. Porter EV, Chassy BM, Holmlund CE (1980) Partial purification and properties of a mannofructokinase from *Streptococcus mutans* SL-1. *Infect Immun* 30:43–50
21. Periyasamy S, Venkatachalam S, Ramasamy S, Srinivasan V (2009) Production of bio-ethanol from sugar molasses using *Saccharomyces cerevisiae*. *Modern Appl Sci* 3:32–37
22. Moon MW, Kim HJ, Oh TK, Shin CS, Lee JS, Kim SJ, Lee JK (2005) Analyses of enzyme II gene mutants for sugar transport and heterologous expression of fructokinase gene in *Corynebacterium glutamicum* ATCC 13032. *FEMS Microbiol Lett* 244:259–266. doi:10.1016/j.femsle.2005.01.053
23. Dominguez H, Lingdley ND (1996) Complete sucrose metabolism requires fructose phosphotransferase activity in *Corynebacterium glutamicum* to ensure phosphorylation of liberated fructose. *Appl Environ Microbiol* 62:3878–3880
24. Aulkemeyer P, Ebner R, Heilenmann G, Jahreis K, Schmid K, Wrieden S, Lengeler JW (1991) Molecular analysis of two fructokinases involved in sucrose metabolism of enteric bacteria. *Mol Microbiol* 5:2913–2922. doi:10.1111/j.1365-2958.1991.tb01851.x
25. Myung S, Wang Y, Zhang YHP (2010) Fructose-1,6-bisphosphatase from a hyper-thermophilic bacterium *Thermotoga maritima*: characterization, metabolite stability, and its implications. *Process Biochem* 45:1882–1887. doi:10.1016/j.procbio.2010.03.017
26. Rittmann D, Schaffer S, Wendisch VF, Sahn H (2003) Fructose-1,6-bisphosphatase from *Corynebacterium glutamicum*: expression and deletion of the *fbp* gene and biochemical characterization of the enzyme. *Arch Microbiol* 180:285–292. doi:10.1007/s00203-003-0588-6
27. Schaftingen EV, Hers HG (1981) Inhibition of fructose-1,6-bisphosphatase by fructose 2,6-bisphosphate. *Proc Natl Acad Sci USA* 78(5):2861–2863
28. Navas MA, Gancedo JM (1996) The regulatory characteristics of yeast fructose-1,6-bisphosphatase confer only a small selective advantage. *J Bacteriol* 178:1809–1812
29. Ohnishi J, Katahira R, Mitsuhashi S, Kakita S, Ikeda M (2005) A novel gnd mutation leading to increased L-lysine production in *Corynebacterium glutamicum*. *FEMS Microbiol Lett* 242:265–274. doi:10.1016/j.femsle.2004.11.014
30. Marx A, Eikmanns BJ, Sahn H, de Graaf AA, Eggeling L (1999) Response of the central metabolism in *Corynebacterium glutamicum* to the use of an NADH-dependent glutamate dehydrogenase. *Metab Eng* 1:35–48. doi:10.1006/mben.1998.0106
31. Xu DQ, Tan YZ, Huan XJ, Hu XQ, Wang XY (2010) Construction of a novel shuttle vector for use in *Brevibacterium flavum*, an industrial amino acid producer. *J Microbiol Methods* 80:86–92. doi:10.1016/j.mimet.2009.11.003