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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Rational reformation of *Corynebacterium glutamicum* for producing L-lysine by one-step fermentation from raw corn starch

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Abstract

This article focuses on engineering *Corynebacterium glutamicum* to produce L-lysine efficiently from starch using combined method of "classical breeding" and "genome breeding." Firstly, a thermo-tolerable L-lysine-producing *C. glutamicum* strain $KT_{45\text{-}6}$ was obtained after multi-round of acclimatization at high temperature. Then, amylolytic enzymes were introduced into strain $KT_{45\text{-}6}$, and the resultant strains could use starch for cell growth and L-lysine production except the strain with expression of isoamylase. In addition, co-expression of amylolytic enzymes showed a good performance in starch degradation, cell growth and L-lysine production, especially co-expression of α-amylase (AA) and glucoamylase (GA). Moreover, L-lysine yield was increased by introducing AA-GA fusion protein (i.e., strain $KT_{45\text{-}6}$S-5), and finally reached to 23.9 ± 2.3 g/L in $CgXII^{IP}$M-medium. It is the first report of an engineered L-lysine-producing strain with maximum starch utilization that may be used as workhorse for producing amino acid using starch as the main feedstock.

Key points

- *Thermo-tolerable C. glutamicum was obtained by temperature-induced adaptive evolution.*
- *The fusion order between AA and GA affects the utilization efficiency of starch.*
- *C. glutamicum with starch utilization was constructed by optimizing amylases expression.*

Keywords *Corynebacterium glutamicum* · L-lysine production · Starch degradation · Amylolytic enzyme · Thermo-tolerance

Introduction

L-lysine, an essential amino acid for animal and human, has been widely applied in feed industry, food industry and pharmaceutical industry (Xu et al. 2019). According to statistics published by the China Biotech Fermentation Industry Association (http://www.cfia.org.cn/), the yield of L-lysine in China reached an all-time high of 2,533,000 tons in 2019. It is conceivable that the global demands for L-lysine will grow rapidly with the exploitation and extension of L-lysine's applications in the future. Although there are four methods to use for producing L-lysine (i.e., Proteolysis, Chemical synthesis, Microbial fermentation and Enzymatic synthesis), microbial fermentation is the common method in industry at present. *Corynebacterium glutamicum* and *Escherichia coli* as well as their derivatives are generally regarded as workhorses for L-lysine production by microbial fermentation (Sgobba et al. 2018; Xu et al. 2020). The main feedstock for L-lysine production is glucose (Tateno et al. 2007b), which accounts for about 40% to 50% of the whole production costs by microbial fermentation. Glucose is mainly produced from starch by inorganic acids or enzyme catalysis (Karim et al. 2019). However, the costs of energy and/or enzymes are high regardless of glucose produced from starch by acid-catalyzed hydrolysis or by enzyme-catalyzed hydrolysis (Tateno et al. 2007a). Therefore, how to increase the efficiency in the use of starch and/or to simplify the handling procedures of starch have

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become the key problems which need to be addressed in industrial L-lysine fermentations.

Starches are polysaccharides, which are widely existed in the natural plant. And they are formed from many α-glucose units that are interconnected by α-1,4- and/or α-1,6-glycosidic linkages, thus forming two polymers of glucose, i.e., amylose and amylopectin (Hu et al. 2020). Amylose is a linear polymer only with α-1,4-glycosidic linkages, while amylopectin is a multibranched polymer with α-1,4-glycosidic linkages and α-1,6-glycosidic linkages (Villas-Boas and Franco 2016). Starch coming from different sources possesses different contents of amylose and amylopectin, and the contents of amylopectin (i.e., 75 ~ 85%) is higher than that of amylose (i.e., 15 ~ 25%) in general (Zhao et al. 2020). Based on published research (Karim et al. 2019), more than 70% of starches are used to produce glucose. In the sugar industry, the production of glucose from starch normally involves two working procedures, i.e., liquefaction and saccharification (Guzmanmaldonado and Paredeslopez 1995; Karim et al. 2019). In liquefaction stage, starch is converted into a solution of soluble dextrins and maltose by a series of amylases (e.g., α-amylase, β-amylase, isoamylase and pullulanase), and then this liquefied solution is further hydrolyzed to glucose by glucoamylase in saccharification stage (Karim et al. 2019). It should be noted that these amylolytic enzymes in starch degradation have different function ways (Guzmanmaldonado and Paredeslopez 1995). α-Amylase (i.e., AA, EC 3.2.1.1, encoded by *amyA* gene) is an endo-type enzyme that randomly cleaves the α-1,4-glycosidic linkages in the interior of starch thus producing soluble maltodextrins as the main end product, while β-amylase (i.e., BA, EC 3.2.1.2, encoded by *amyB* gene) is an exo-type enzyme that cuts the α-1,4-glycosidic linkages from non-reducing end thus producing maltose as the end product (Das et al. 2018; Roy et al. 2013; Villas-Boas and Franco 2016). Isoamylase (i.e., IA, EC 3.2.1.9, encoded by *amyX* gene) and pullulanase (i.e., Pul, EC 3.2.1.41, encoded by *pulA* gene) specifically cleave the α-1,6-glycosidic linkages (Bi et al. 2020; Ghosh et al. 2020). Glucoamylase (i.e., GA, EC 3.2.1.3, encoded by *glaA* gene) not only cleaves the α-1,4-glycosidic linkages from non-reducing end but also slowly cuts the α-1,6-glycosidic linkages, thus producing glucose as the end product (Ghani et al. 2013; Karim et al. 2019; Lincoln et al. 2019). It should be noted that the above mentioned amylolytic enzymes show the optimally active at weak acidity (i.e., pH 4.5 ~ 7.0) and high temperature (i.e., > 50 °C) (Guzmanmaldonado and Paredeslopez 1995). Thus, the procedure of glucose production from starch is set at high temperature, whether in liquefaction stage (i.e., 90 ~ 110 °C) or in saccharification stage (i.e., 55 ~ 65 °C) (Parashar and Satyanarayana 2018; Zhao et al. 2007).

As the main workhorse for producing L-lysine in microbial fermentation, *C. glutamicum* grows on a variety of materials as carbon source (*e.g.*, mono-/di-saccharides, n-alkane, ethanol and organic acids) besides starch (Seibold et al. 2006; Xu et al. 2020), because it intrinsically lacks any amylolytic enzymes (Seibold et al. 2006). Many studies have attempted to modify *C. glutamicum* to directly utilize starch as a key resource for producing value-added chemicals, including L-lysine (Seibold et al. 2006; Sgobba et al. 2018; Tateno et al. 2007a, 2009; Tsuge et al. 2013). Tateno et al. (2007b) and Tsuge et al. (2013) have produced L-lysine and organic acids by cell surface-engineered *C. glutamicum* with α-amylase activity using starch as carbon source. In addition, heterologous over-expression and secretion of α-amylase in *C. glutamicum* could also be applied to produce L-lysine using starch as carbon source (Chen et al. 2020; Seibold et al. 2006; Tateno et al. 2007a, 2009). In recent years, researchers are trying to co-culture various strain combinations for value-added chemicals production from starch, for example, co-culturing *E. coli-C. glutamicum* for L-lysine production (Sgobba et al. 2018). Although these studies have obtained a series of meaningful results, it is hard to obtain the satisfactory L-lysine production from starch. Because the optimal temperature of amylolytic enzymes (i.e., > 50 °C) is higher than the growth temperature of *C. glutamicum* (i.e., 25 ~ 37 °C) (Abe et al. 1967).

At present study, *C. glutamicum* K-8 was improved by a series of strategies that allow it to grow at high temperature and to produce L-lysine from starch. Firstly, we isolated a thermo-tolerable *C. glutamicum* $KT_{45\text{-}6}$ derived from strain K-8 using temperature-induced mutagenesis-based adaptive laboratory evolution. And the effect of the adaptive strategy on fermentation performance of mutant strains was investigated at different culture temperature. Furthermore, different amylolytic enzymes were introduced into the thermotolerant mutant, and their effects on L-lysine production using corn starch as carbon source were studied. As a result, a high-temperature strain *C. glutamicum* $KT_{45\text{-}6}$S-5 (i.e., *C. glutamicum* $KT_{45\text{-}6}$ Δ*sigH::amyA-glaA*) derived from *C. glutamicum* K-8 was obtained, which produced 23.9 ± 2.3 g/L of L-lysine using corn starch as the main carbon source at 45 °C. As compared with existing strains reported by previous studies (Tateno et al. 2007a, 2007b, 2009), strain $KT_{45\text{-}6}$S-5 shows good high temperature tolerance and homologous expression of AA-GA fusion enzyme thus giving strain with maximum starch utilization for producing L-lysine.

Material and methods

Strains, growth medium, and conditions

Strains used in present study are listed in Table 1. *C. glutamicum* and *E. coli* were cultivated at Luria–Bertani (LB) medium and LBG (LB + Glucose) medium, respectively (Xu

Table 1 Strains and plasmids used in present study

Contents	Relevant characteristic(s)	Reference
C. glutamicum strains		
ATCC13032	Wild-type strain	
JL-6	*C. glutamicum* AEC^r SD^r FP^s Met^l, a L-lysine-producing strain derived from *C. glutamicum* ATCC13032 after "classical breeding"	Xu et al. (2018)
K-8	A L-lysine producer derived from strain JL-6 after "genome breeding"	Xu et al. (2020)
$KT_{35\text{-}48}$	Thermotolerant L-lysine producer derived from strain K-8, growing well at 35 °C	This work
$KT_{38\text{-}15}$	Thermotolerant L-lysine producer derived from strain K-8, growing well at 38 °C	This work
$KT_{41\text{-}7}$	Thermotolerant L-lysine producer derived from strain K-8, growing well at 41 °C	This work
$KT_{43\text{-}31}$	Thermotolerant L-lysine producer derived from strain K-8, growing well at 43 °C	This work
$KT_{45\text{-}6}$	Thermotolerant L-lysine producer derived from strain K-8, growing well at 45 °C	This work
$KT_{45\text{-}6}$ Δ*sigH::amyA*	Strain $KT_{45\text{-}6}$ with heterologous expression of the gene *amyA*	This work
$KT_{45\text{-}6}$ Δ*sigH::amyB*	Strain $KT_{45\text{-}6}$ with heterologous expression of the gene *amyB*	This work
$KT_{45\text{-}6}$ Δ*sigH::amyX*	Strain $KT_{45\text{-}6}$ with heterologous expression of the gene *amyX*	This work
$KT_{45\text{-}6}$ Δ*sigH::pulA*	Strain $KT_{45\text{-}6}$ with heterologous expression of the gene *pulA*	This work
$KT_{45\text{-}6}$ Δ*sigH::glaA*	Strain $KT_{45\text{-}6}$ with heterologous expression of the gene *glaA*	This work
$KT_{45\text{-}6}$S-1	Strain $KT_{45\text{-}6}$ with co-expression of the genes *glaA* and *amyA*	This work
$KT_{45\text{-}6}$S-2	Strain $KT_{45\text{-}6}$ with co-expression of the genes *glaA* and *amyB*	This work
$KT_{45\text{-}6}$S-3	Strain $KT_{45\text{-}6}$ with co-expression of the genes *glaA* and *amyX*	This work
$KT_{45\text{-}6}$S-4	Strain $KT_{45\text{-}6}$ with co-expression of the genes *glaA* and *pulA*	This work
$KT_{45\text{-}6}$S-5	Strain $KT_{45\text{-}6}$ with expression of the fusion gene *amyA-glaA*	This work
$KT_{45\text{-}6}$S-6	Strain $KT_{45\text{-}6}$ with expression of the fusion gene *glaA-amyA*	This work
Plasmids		
pK18*mobsacB*	A vector for modification of *C. glutamicum* genome	Schafer et al. (1994)
pK18*mobsacB*/Δ*sigH*	pK18*mobsacB* with *sigH* deletion construct	Xu et al. (2020)
pK18*mobsacB*/Δ*ptsG*	pK18*mobsacB* with *ptsG* deletion construct	Xu et al. (2020)
pK18*mobsacB*/Δ*sigH::amyA*	pK18*mobsacB* with the cassette of *amyA* at *sigH* gene loci	This work
pK18*mobsacB*/Δ*sigH::amyB*	pK18*mobsacB* with the cassette of *amyB* at *sigH* gene loci	This work
pK18*mobsacB*/Δ*sigH::amyX*	pK18*mobsacB* with the cassette of *amyX* at *sigH* gene loci	This work
pK18*mobsacB*/Δ*sigH::pulA*	pK18*mobsacB* with the cassette of *pulA* at *sigH* gene loci	This work
pK18*mobsacB*/Δ*sigH::glaA*	pK18*mobsacB* with the cassette of *pulA* at *sigH* gene loci	This work
pK18*mobsacB*/Δ*ptsG::amyA*	pK18*mobsacB* with the cassette of *amyA* at *sigH* gene loci	This work
pK18*mobsacB*/Δ*ptsG::amyB*	pK18*mobsacB* with the cassette of *amyB* at *sigH* gene loci	This work
pK18*mobsacB*/Δ*ptsG::amyX*	pK18*mobsacB* with the cassette of *amyX* at *sigH* gene loci	This work
pK18*mobsacB*/Δ*ptsG::pulA*	pK18*mobsacB* with the cassette of *pulA* at *sigH* gene loci	This work
pK18*mobsacB*/Δ*sigH::glaA-amyA*	pK18*mobsacB* with the fusion cassette of *glaA-amyA* at *sigH* gene loci	This work
pK18*mobsacB*/Δ*sigH::amyA-glaA*	pK18*mobsacB* with the fusion cassette of *amyA-glaA* at *sigH* gene loci	This work

et al. 2014). Epo medium and LBHIS (LB + Brain Heart Infusion + Sorbitol) medium used in genetically modifying *C. glutamicum* were prepared based on the methods described by van der Rest et al. (1999). Unless stated otherwise, *C. glutamicum* was cultivated at 30 °C.

Batch shake-flask fermentation was referred to the methods reported by Xu et al. (2014). The modified CgXII-medium (i.e., $CgXII^{IP}$; CgXII + 0.25 g/L L-methionine + 0.6 mg/L biotin) without carbon source was applied as basal medium to produce L-lysine. The culture was operated in 50 mL $CgXII^{IP}$-medium with 40 g/L of glucose (i.e., $CgXII^{IP}$G-medium) or 10 g/L of glucose and 30 g/L of starch (i.e., $CgXII^{IP}$M-medium) or 40 g/L of starch (i.e., $CgXII^{IP}$S-medium), respectively. These medium were adjusted to pH 7.0 with 20% (w/v) NaOH.

Thermal tolerance assays

Tolerance for high temperature was examined by evaluating the growth of strain in LBG medium at different temperature. Cells in mid-log phase were scribed on LBG-plate or 1% (v/v) of cells in mid-log phase were inoculated

in LBG liquid medium, and then cultivated at 30 ~ 45 °C for 24 h.

Adaptive laboratory evolution for improving thermo-tolerance of *C. glutamicum*

LBG medium was applied to adaptive laboratory evolution. The procedure was according to the descriptions reported by Oide et al. (2015). In the first round of evolution, the initial temperature was set at 30 °C and eventually up to 35 °C as bacterial adaptation proceeded. On the 90th day, aliquots of the culture were spread on LBG-plate and incubated at 35 °C for 24 h. The colonies with big size were selected for fermentation test in shake flasks at 35 °C. Mutant with better fermentation performance was isolated and used as original strain for the next round of evolution. The second round (from 35 °C to 38 °C), the third round (from 38 °C to 41 °C), the fourth round (from 41 °C to 43 °C) and the fifth round (from 43 °C to 45 °C) of evolution were executed according to the abovementioned procedure.

Construction of recombinant *C. glutamicum* strains

The plasmids are listed in Table 1 and oligonucleotides are listed in Table S1. The modifications of *C. glutamicum* chromosome were referred to the published method (Xu et al. 2016). The cassettes of α-amylase (from *Streptococcus bovis*, Accession No. MZ821032), β-amylase (from *Bacillus aryabhattai*, Accession No. MZ821033), glucoamylase (from *Aspergillus niger*, Accession No. MZ821034), isoamylase (from *Bacillus lentus*, Accession No. MZ821035) and pullulanase (from *Bacillus thermoleovorans*, Accession No. MZ821036) with $P_{tac\text{-}M}$ promoter (Xu et al. 2011), signal sequence of gene *cspB* from *C. glutamicum* (Tateno et al. 2007a) and *rrnBT1T2* terminator were optimized for expression in *C. glutamicum* and were synthesized by GENEWIZ (Suzhou), Inc. (Suzhou, China). The procedure for construction of recombinant plasmids and strains was referred to the published methods (van der Rest et al. 1999; Xu et al. 2016), and the detail procedure was stated in Supplementary material and the building process was referred to Figure S1.

Enzyme activity assay

The qualitative analysis of enzyme activity was carried out by Iodine–Starch chromogenic assay (Seibold et al. 2006). Strains were cultivated on LBG-plate containing 5 g/L starch for 24 h, and then added the right amount of 0.1 mol/L of Lugol's solution to color. The colonies showing a colourless zone indicated the strain with amylolytic enzyme activity. The quantitative analysis of enzyme activity was performed using spectrophotometry. The activity of AA, BA, IA, GA and Pul in culture supernatants was determined using the method provided by Seibold et al. (2006), Duan et al. (2019), Chen et al. (1998), Karim et al. (2019), and Bi et al. (2020), respectively.

Analysis of the fermentation performance of different strains

Aliquots (200 µL) of the culture were taken from flasks every 2 or 4 h to analyze the biomass (i.e., dry cell weight; DCW) and L-lysine production. Total sugars were analyzed according to phenol–sulfuric acid method (Masuko et al., (2005). Biomass was assayed by spectrophotometer at 600 nm. Based on the previous results (Xu et al. 2014), 1 OD_{600} is the equal of 0.318 g DCW. Half of sample was diluted 100-fold, and then used to determine L-lysine concentration by SBA-40E immobilized enzyme biosensor (Shandong, China) (Xu et al., (2020).

Results

Temperature-induced mutagenesis-based adaptive evolution of *C. glutamicum* K-8

The optimal growth temperature of *C. glutamicum* and its relatives is between 25 °C and 37 °C (Abe et al. 1967). Thus, we first investigated thermotolerance of *C. glutamicum* K-8, its parental strain *C. glutamicum* JL-6 (Xu et al. 2018) and the wild-type strain *C. glutamicum* ATCC13032 on LBG-plate at various temperatures (Fig. 1a). All strains grew rapidly at 30 °C, and strain ATCC13032 even showed vigorous growth at 41 °C. However, strain K-8 and JL-6 exhibited no growth at 38 °C and above, implying that the intrinsic thermotolerance of strain JL-6 was lost after multiple rounds of random mutagenesis. The growth performance of these strains was also compared by cultivating them in shake flasks (Fig. 1b). Consistent with the results of LBG-plate assay, all strains grew well at 30 °C despite strain K-8 and JL-6 did worse than strain ATCC13032. While the growth of strain ATCC13032 was not significantly affect during temperature up to 41 °C, the growth of strain K-8 and JL-6 was significantly inhibited at 35 °C and above. All these results indicate that strain K-8 and JL-6 are not able to grow well at 35 °C and above.

To give strain K-8 with thermo-tolerance, adaptive laboratory evolution by gradually increasing temperature was carried out in strain K-8. In the first round of evolution, about 80 colonies were isolated after 60 days of unintermittent evolution. Whether grown in LBG-medium or in $CgXII^{IP}G$-medium, these evolved strains showed increased growth at 35 °C compared to the strain K-8 (Fig. 2a and Table 2). Among these evolved strains,

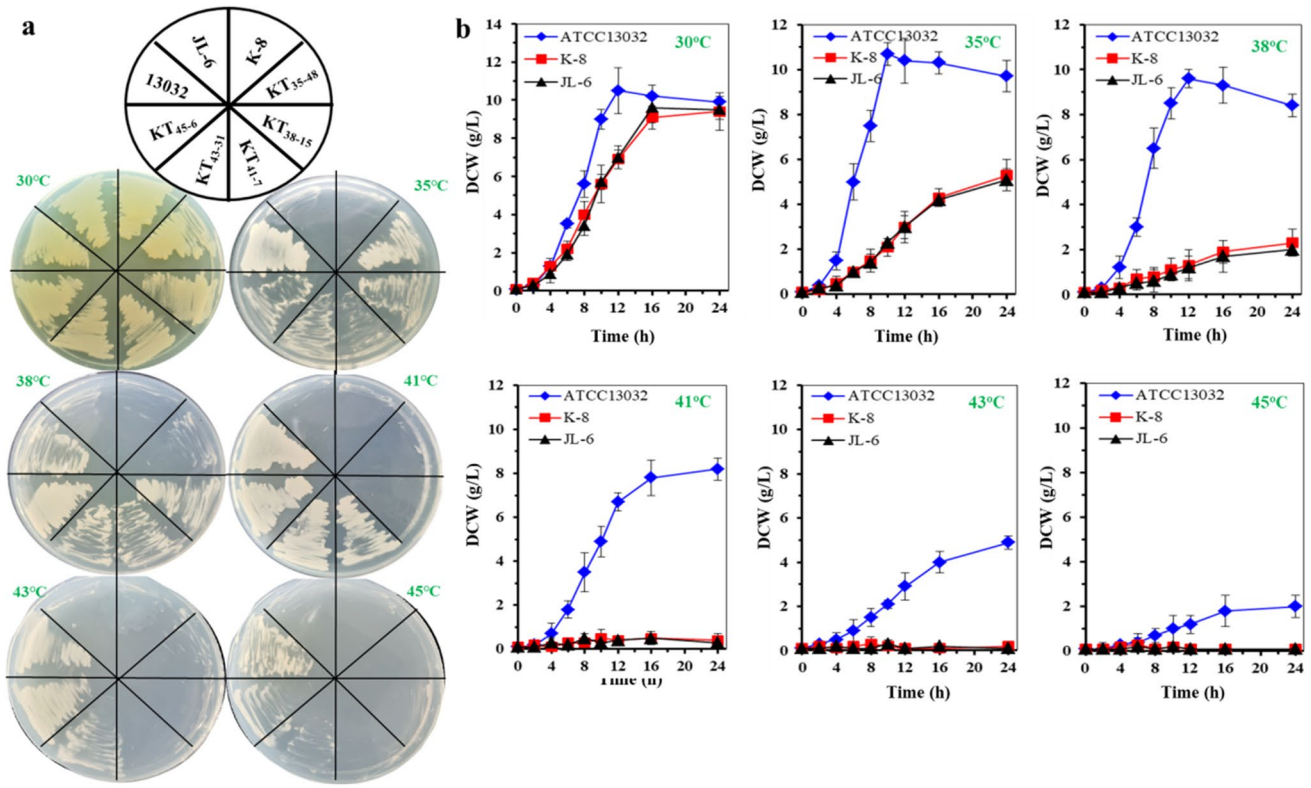

Fig. 1 The growth performance of different strains at elevated temperature. **a** The strains were cultivated on LBG-plate at various temperatures. **b** The strains were cultivated in shake flasks at various temperatures. The data are mean values obtained from three independent experiments with error bar

however, more than half of mutants showed an obvious decrease of L-lysine production. The negative mutants accounted for about 64% of all evolved strains (Fig. 2b). Among these evolved strains, strain *C. glutamicum* $KT_{35\text{-}48}$ showed a slight increase of L-lysine production (from 26.7 ± 3.5 g/L to 27.4 ± 2.9 g/L), thus it was used as the original strain for the next round of evolution. In a follow-up experiment, 57 colonies, 29 colonies, 32 colonies, and 18 colonies were isolated in the second round, the third round, the fourth round, and the fifth round of evolution, respectively (Fig. 2c–f). It should be noted that the final DCW and the maximal growth rates (μ_{max}) of the evolved strains at the elevated temperatures were lower than that of strain K-8 at 30 °C (Fig. 2 and Table 2). Especially, DCW of 18 candidate strains from the fifth round of evolution at 45 °C was limited to about 67% of strain K-8 at 30 °C (Fig. 2f). Unexpectedly, the L-lysine production of all 18 candidate strains at 45 °C was decreased as compared with strain K-8 at 30 °C (Fig. 2b). Strain *C. glutamicum* $KT_{45\text{-}6}$ grew well at 45 °C and exhibited a little decrease of L-lysine production in comparison with strain K-8 at 30 °C after 48 h of batch fermentation (23.8 ± 3.2 g/L vs. 26.7 ± 3.5 g/L; Fig. 2b). In addition, there were no notable differences in cell shape or size between the strain $KT_{45\text{-}6}$ and the parental strain K-8 (Fig. 3). Therefore, the thermo-tolerable strain $KT_{45\text{-}6}$ was used as the initial strain for producing L-lysine from starch.

Expression of heterologous amylolytic enzymes in the thermotolerant *C. glutamicum* $KT_{45\text{-}6}$

To produce L-lysine from starch, we modified *C. glutamicum* $KT_{45\text{-}6}$ to obtain a recombinant *C. glutamicum* strain with amylolytic enzymes activity. For the stable expression of enzymes in cells, a gene was constructed for integration into the *C. glutamicum* $KT_{45\text{-}6}$ chromosome using the recombinant mobilizable plasmid derived from pK18*mobsacB* (Xu et al. 2016). The amylolytic enzyme-coding gene was expressed under the constitutive promoter *tacM* (Xu et al. 2011). The recombinant mobilizable plasmids with different amylolytic enzyme-coding gene cassettes were introduced into *C. glutamicum* $KT_{45\text{-}6}$ to achieve two round of homologous recombination (see "Materials and methods"), and the resultant transformants were named strain $KT_{45\text{-}6}$ Δ*sigH::amyA*, $KT_{45\text{-}6}$ Δ*sigH::amyB*, $KT_{45\text{-}6}$ Δ*sigH::amyX*, $KT_{45\text{-}6}$ Δ*sigH::pulA* and $KT_{45\text{-}6}$ Δ*sigH::glaA*, respectively. To investigate the expression of heterologous amylolytic

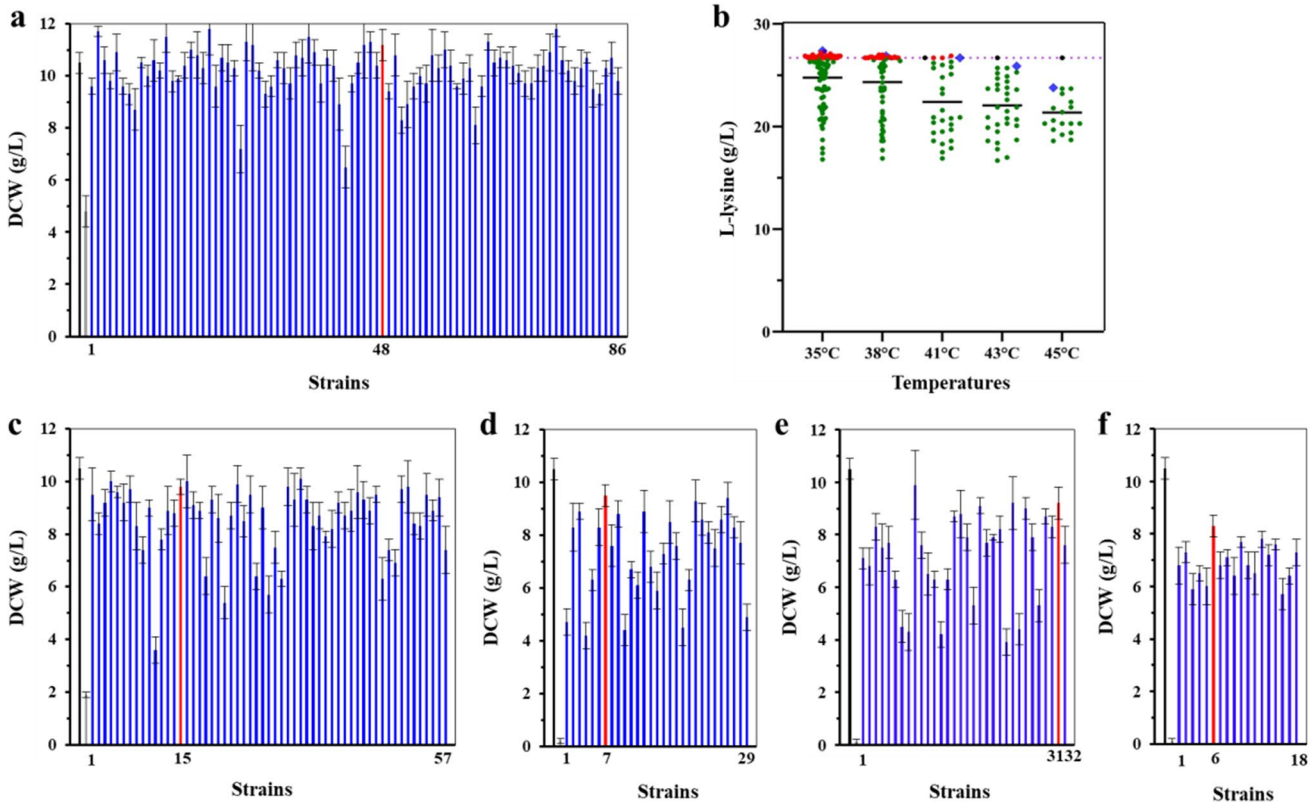

Fig. 2 Temperature-induced adaptive evolution to endow *C. glutamicum* K-8 with the thermo-tolerance. **a** and **c**–**f** The dry cell weight (DCW) of evolved strains at 35 °C (**a**), at 38 °C (**c**), at 41 °C (**d**), at 43 °C (**e**), and at 45 °C (**f**). The red column represents the candidate strain in this study. **b** The L-lysine production of evolved strains and original strain K-8 at elevated temperature. The black dot represents the L-lysine production of original strain K-8 at 30 °C. The red dot represents the L-lysine production of evolved strains at elevated temperatures. The blue diamond represents the candidate strain in this study. The purple dot line represents the level of L-lysine production in strain K-8 at 30 °C. The data are mean values obtained from three independent experiments with error bar

Table 2 Summary of cell growth and L-lysine production by different *C. glutamicum* strains at various temperatures to discuss the influences of temperature on L-lysine production

C. glutamicum strains	Temperature	LBG-medium		$CgXII^{IP}G$-medium		
		DCW (g/L)	μ_{max} (h^{-1})	DCW (g/L)	μ_{max} (h^{-1})	L-lysine (g/L)
K-8	30 °C	3.1 ± 0.2	0.251	10.5 ± 0.4	0.233	26.7 ± 3.5
KT_{35-48}	30 °C	3.1 ± 0.5	0.252	9.8 ± 0.7	0.228	26.9 ± 1.4
	35 °C	3.3 ± 0.3	0.256	11.2 ± 0.6	0.235	27.4 ± 2.9
KT_{38-15}	30 °C	3.0 ± 0.1	0.243	9.9 ± 0.4	0.229	26.7 ± 3.4
	38 °C	2.9 ± 0.4	0.228	9.8 ± 0.3	0.224	27.1 ± 4.3
KT_{41-7}	30 °C	3.1 ± 0.5	0.240	10.0 ± 1.3	0.227	26.8 ± 4.2
	41 °C	2.9 ± 0.2	0.223	9.5 ± 0.4	0.218	26.8 ± 1.6
KT_{43-31}	30 °C	2.9 ± 0.2	0.236	9.8 ± 0.8	0.226	26.8 ± 3.3
	43 °C	2.7 ± 0.3	0.219	9.2 ± 1.1	0.193	25.3 ± 2.7
KT_{45-6}	30 °C	2.9 ± 0.5	0.235	9.5 ± 0.7	0.223	26.2 ± 2.5
	45 °C	2.6 ± 0.1	0.211	8.3 ± 0.4	0.184	23.8 ± 3.2

All data are mean values obtained from three independent experiments with error value (± SD)

enzyme-coding gene, the abovementioned transformants and strain KT_{45-6} were grown on starch-containing LBG-plate at 45 °C for 24 h and then dipped in Lugol's solution. All of transformants could degrade starch, but not for strain KT_{45-6} (Fig. 4a). There results indicated that all of transformants express the amylolytic enzyme-coding gene and secrete amylolytic enzyme into the medium. The expression of heterologous amylolytic enzyme-coding gene in these

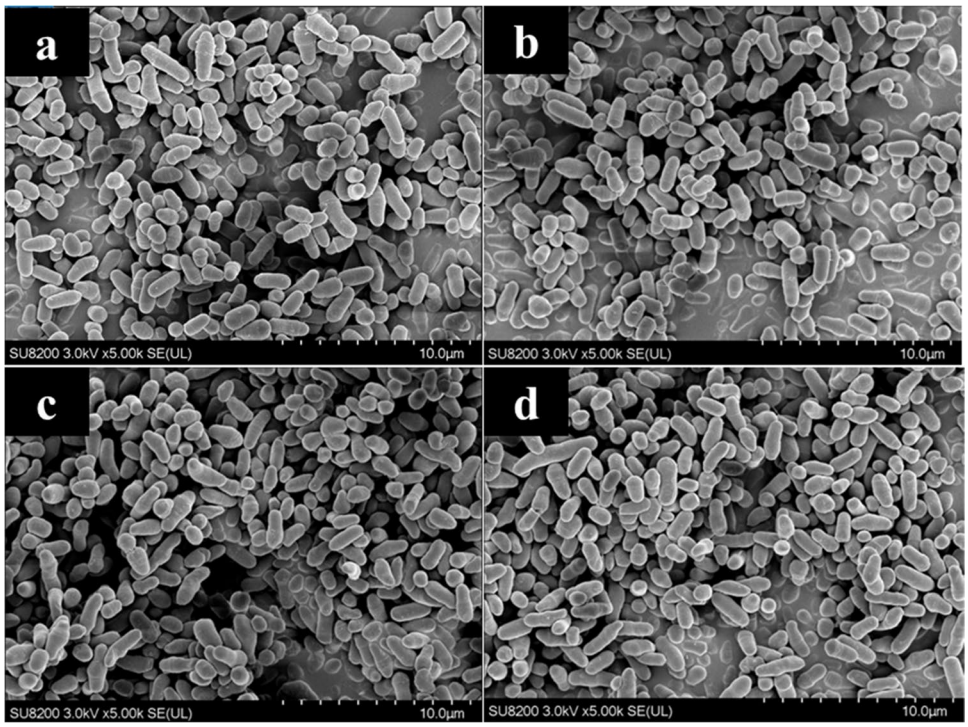

Fig. 3 The cell shape and size of strains analyzed by scanning electron microscope (SEM). **a** *C. glutamicum* ATCC13032. **b** *C. glutamicum* JL-6. **c** *C. glutamicum* K-8. **d** *C. glutamicum* $KT_{45\text{-}6}$

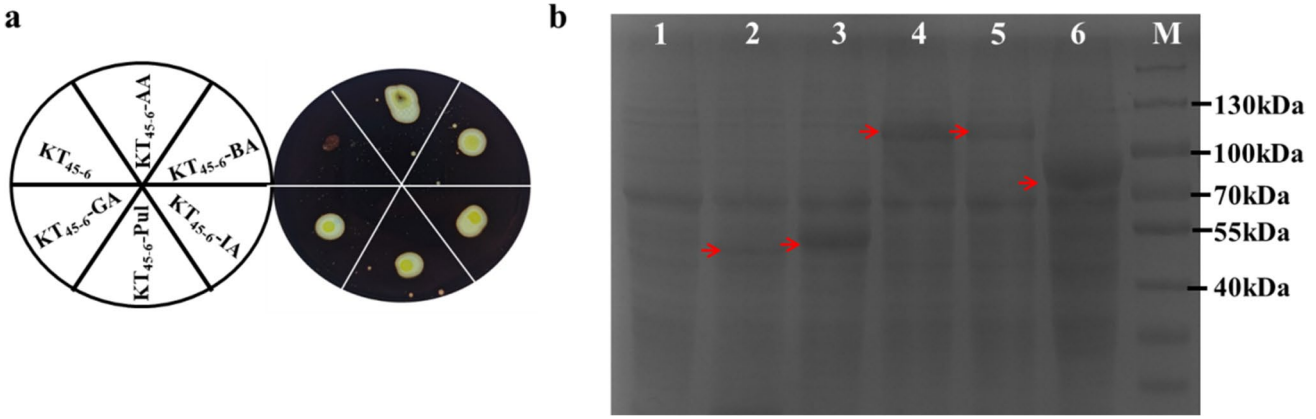

Fig. 4 Analyzing the enzyme activity in strain *C. glutamicum* $KT_{45\text{-}6}$ with single expression of amylolytic enzyme. **a** The starch degradation of different *C. glutamicum* strains on LBG-plate containing 0.5% (w/v) starch at 45 °C. **b** SDS-PAGE analyses of amylolytic enzymes in culture supernatant from different *C. glutamicum* strains. Lane M: protein molecular weight marker; Lane 1: strain $KT_{45\text{-}6}$, Lane 2: strain $KT_{45\text{-}6}$ Δ*sigH::amyA*, Lane 3: strain $KT_{45\text{-}6}$ Δ*sigH::amyB*, Lane 4: strain $KT_{45\text{-}6}$ Δ*sigH::amyX*, Lane 5: strain $KT_{45\text{-}6}$ Δ*sigH::pulA*, and Lane 6: strain $KT_{45\text{-}6}$ Δ*sigH::glaA*. The red arrows represent the target protein

transformants was also tested by SDS-PAGE analysis and enzyme analysis. The results of SDS-PAGE analysis showed a prominent protein band at molecular mass (Fig. 4b), and the mass of these proteins was consistent with the estimated size of the amylolytic enzymes. In addition, all of transformants exhibited an activity of amylolytic enzymes in the culture supernatants besides strain $KT_{45\text{-}6}$ (Table 3). Furthermore, we also found the activity of amylolytic enzymes in the cell pellet of all transformants (Table 3).

To investigate the effect of heterologous amylolytic enzymes on growth in the thermotolerant L-lysine-producing strain $KT_{45\text{-}6}$ using starch as substrate, the above-mentioned transformants and strain $KT_{45\text{-}6}$ were cultivated in $CgXII^{IP}$G-medium, $CgXII^{IP}$M-medium and $CgXII^{IP}$S-medium, respectively. In $CgXII^{IP}$G-medium and $CgXII^{IP}$M-medium, all of strains showed vigorous growth (Fig. 5a, b). However, strain $KT_{45\text{-}6}$ did not grow in $CgXII^{IP}$S-medium, while transformants exhibited the

Table 3 Distribution of five amylolytic enzyme activities in different *C. glutamicum* strains to analyze expression level of the amylolytic enzyme-coding gene in *C. glutamicum*

C. glutamicum strains	Ingredients	Enzyme activity (U/L)[a]				
		AA	BA	IA	Pul	GA
$KT_{45\text{-}6}$	Culture supernatant	-	-	-	-	-
	Cell pellet	-	-	-	-	-
$KT_{45\text{-}6}$ Δ*sigH::amyA*	Culture supernatant	82.9 ± 5.2	-	-	-	-
	Cell pellet	57.4 ± 6.1	-	-	-	-
$KT_{45\text{-}6}$ Δ*sigH::amyB*	Culture supernatant	-	86.2 ± 8.5	-	-	-
	Cell pellet	-	56.5 ± 4.7	-	-	-
$KT_{45\text{-}6}$ Δ*sigH::amyX*	Culture supernatant	-	-	1086.3 ± 62.8	-	-
	Cell pellet	-	-	645.7 ± 73.6	-	-
$KT_{45\text{-}6}$ Δ*sigH::pulA*	Culture supernatant	-	-	-	63.2 ± 5.7	-
	Cell pellet	-	-	-	38.9 ± 4.6	-
$KT_{45\text{-}6}$ Δ*sigH::glaA*	Culture supernatant	-	-	-	-	49.3 ± 2.4
	Cell pellet	-	-	-	-	34.6 ± 3.9
$KT_{45\text{-}6}$S-1[b]	Culture supernatant	88.4 ± 7.6	-	-	-	37.5 ± 4.3
	Cell pellet	60.7 ± 4.7	-	-	-	22.8 ± 1.5
$KT_{45\text{-}6}$S-2[b]	Culture supernatant	-	92.5 ± 7.8	-	-	32.9 ± 2.7
	Cell pellet	-	61.2 ± 7.4	-	-	20.4 ± 3.2
$KT_{45\text{-}6}$S-3[c]	Culture supernatant	-	-	876.7 ± 76.4	-	51.6 ± 4.5
	Cell pellet	-	-	514.2 ± 47.3	-	36.5 ± 3.3
$KT_{45\text{-}6}$S-4[c]	Culture supernatant	-	-	-	60.3 ± 6.4	58.5 ± 6.8
	Cell pellet	-	-	-	35.8 ± 2.8	41.2 ± 3.7
$KT_{45\text{-}6}$S-5[c]	Culture supernatant	97.3 ± 8.4	-	-	-	51.4 ± 6.2
	Cell pellet	62.8 ± 7.3	-	-	-	35.7 ± 2.3
$KT_{45\text{-}6}$S-6[c]	Culture supernatant	69.8 ± 5.7	-	-	-	38.7 ± 3.4
	Cell pellet	53.4 ± 6.4	-	-	-	24.1 ± 3.5

Exponentially growing cells cultured in LBG-medium in shake flasks were used for analysis

[a]AA, BA, IA, Pul, and GA represent α-amylase, β-amylase, isoamylase, pullulanase and glucoamylase, respectively; "-" represents not detected

[b]The activity of AA, BA, and Pul is the difference between the reducing power measured by the dinitrosalicylic acid method and the GA activity

[c]The activity of IA is the difference between the increased absorbance per min at 610 nm and the GA activity

All data are mean values obtained from three independent experiments with error value (± SD)

poor growth vigor in $CgXII^{IP}$S-medium as compared with in $CgXII^{IP}$G-medium and $CgXII^{IP}$M-medium (Fig. 5c). It should be noted that transformants showed the highest final DCW (> 8.0 g/L) and μ_{max} (~ 0.22 h^{-1}) in $CgXII^{IP}$M-medium except strain $KT_{45\text{-}6}$ Δ*sigH::amyX* (Fig. 5a–c). As expected, starch in $CgXII^{IP}$M-medium was completely degraded at 12 h, whereas the starch in $CgXII^{IP}$S-medium was completely degraded at 36 h because of the poor growth vigor of transformants in $CgXII^{IP}$S-medium (Data not shown). These results implied the ability of all of transformants to use starch as substrate for growth. Among these transformants, the strain with heterologous expression of β-amylase (i.e., strain $KT_{45\text{-}6}$ Δ*sigH::amyB*) showed the highest final DCW and the μ_{max} in $CgXII^{IP}$M-medium and $CgXII^{IP}$S-medium, closely followed by strain $KT_{45\text{-}6}$ Δ*sigH::amyA* and $KT_{45\text{-}6}$ Δ*sigH::glaA*, while strain $KT_{45\text{-}6}$ Δ*sigH::amyX* had the worst cell growth (Fig. 5b, c).

To investigate whether these transformants could use starch as feedstock for L-lysine production, different strains were cultivated in $CgXII^{IP}$G-medium, $CgXII^{IP}$M-medium and $CgXII^{IP}$S-medium at 45 °C to analyze the concentration of L-lysine. In $CgXII^{IP}$G-medium, all of strains begun to produce L-lysine after 10 h and accumulated about 23 g/L of L-lysine (Fig. 6a). In contrast, only strains with amylolytic enzymes activity were able to produce L-lysine in $CgXII^{IP}$M-medium and $CgXII^{IP}$S-medium, except strain $KT_{45\text{-}6}$ Δ*sigH::amyX* (Fig. 6b, c). This result indicated that strain $KT_{45\text{-}6}$ Δ*sigH::amyA*, $KT_{45\text{-}6}$ Δ*sigH::amyB*, $KT_{45\text{-}6}$ Δ*sigH::glaA* and $KT_{45\text{-}6}$ Δ*sigH::pluA* can use starch as feedstock for L-lysine production. Strain $KT_{45\text{-}6}$ Δ*sigH::amyA* exhibited the best L-lysine production, followed by strain

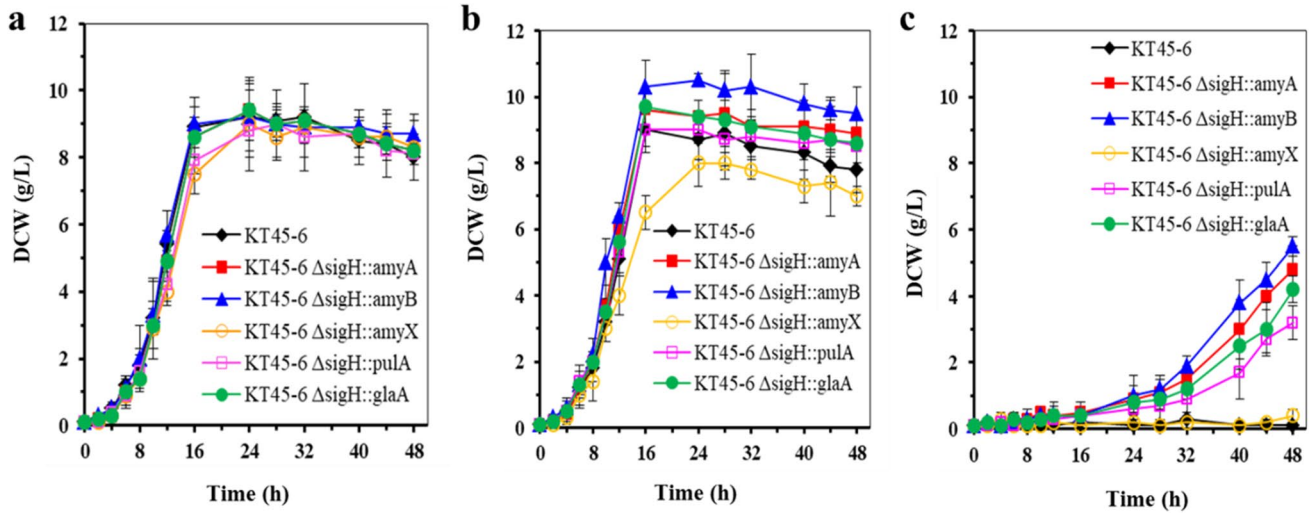

Fig. 5 The growth performance of different *C. glutamicum* strains using different sugar as carbon source. **a** Strains in CgXII^{IP}G-medium containing 4% (w/v) glucose. **b** Strains in CgXII^{IP}M-medium containing 1% (w/v) glucose plus 3% (w/v) starch. **c** Strains in CgXII^{IP}S-medium containing 4% (w/v) starch. The data are mean values obtained from three independent experiments

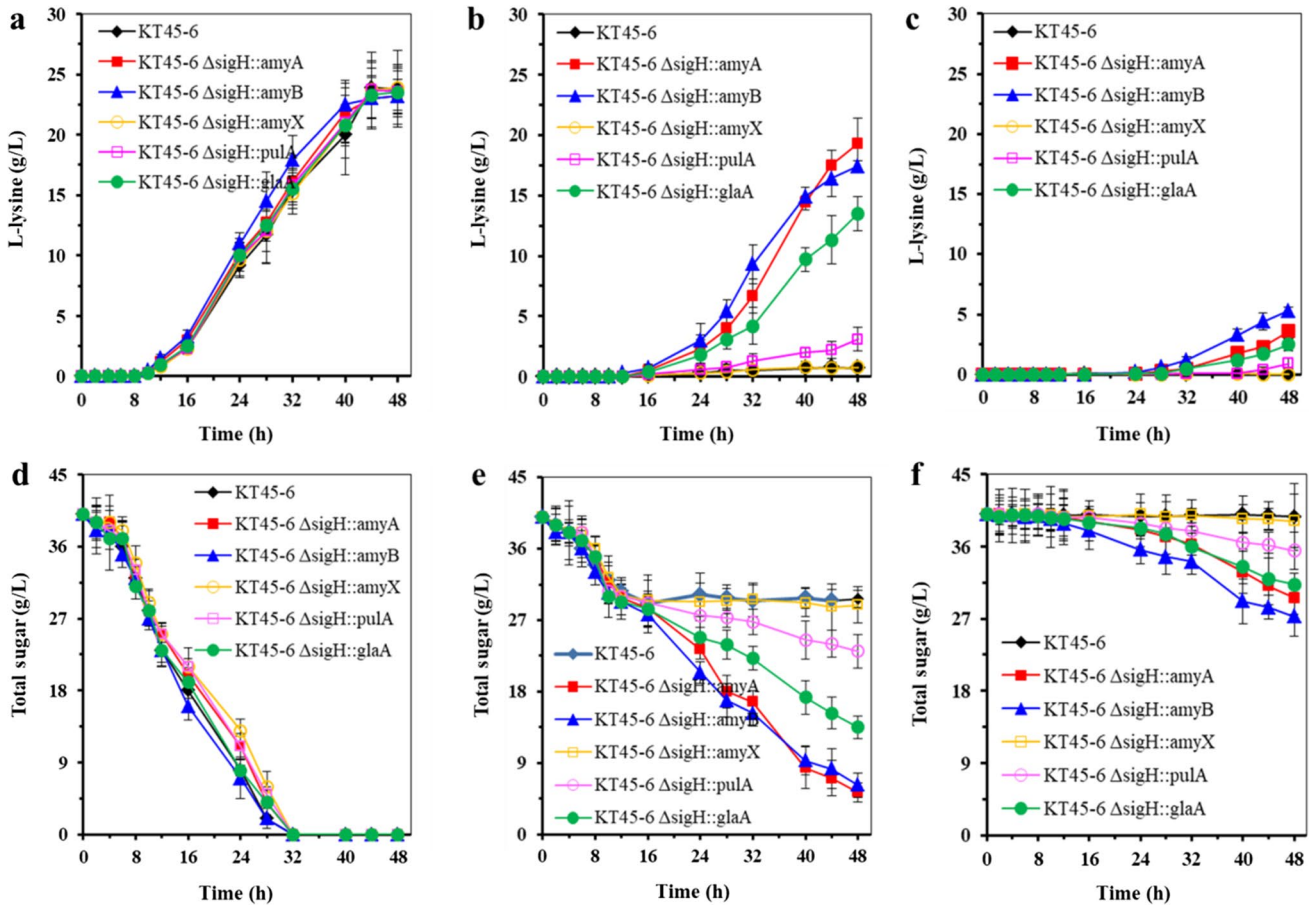

Fig. 6 The L-lysine production and total sugar consumption of different *C. glutamicum* strains using different sugar as carbon source. **a–c** L-lysine production in CgXII^{IP}G-medium, CgXII^{IP}M-medium and CgXII^{IP}S-medium, respectively. **d–f** Total sugar in CgXII^{IP}G-medium, CgXII^{IP}M-medium and CgXII^{IP}S-medium, respectively. The data are mean values obtained from three independent experiments with error bar

$KT_{45\text{-}6}$ Δ*sigH::amyB*, whereas strain $KT_{45\text{-}6}$ Δ*sigH::pluA* was the worst (Fig. 6b, c). Strain $KT_{45\text{-}6}$ Δ*sigH::amyA* begun to produce L-lysine after 16 h and accumulated about 19.3 ± 2.1 g/L of L-lysine in $CgXII^{IP}$M-medium, which were worse than those of strain $KT_{45\text{-}6}$ in $CgXII^{IP}$G-medium (Fig. 6a, b). However, the consumption rate of sugar of all strains in $CgXII^{IP}$M-medium and $CgXII^{IP}$S-medium was lower than that in $CgXII^{IP}$G-medium, particularly in $CgXII^{IP}$S-medium (Fig. 6d–f). As expected, strain $KT_{45\text{-}6}$ Δ*sigH::amyA* showed the highest consumption rate of sugar, and 34.7 g of total sugar was consumed after 48 h during cultivated in $CgXII^{IP}$M-medium (Fig. 6e).

Co-expression of different amylolytic enzymes enhanced starch degradation and L-lysine production

Based on the procedure of glucose production from starch in industry, AA, BA, IA and Pul are usually used in liquefaction stage to produce soluble dextrins, while GA is often used for producing glucose from dextrins in saccharification stage (Karim et al. 2019). To simulate the production requests of industrial glucose production in microorganism, we co-expressed the enzymes used in liquefaction stage and in saccharification stage (i.e., co-expression of AA and GA, BA and GA, IA and GA or Pul and GA), and the resultant transformants were named strain $KT_{45\text{-}6}$ Δ*sigH::amyA* Δ*ptsG::glaA* (i.e., strain $KT_{45\text{-}6}$S-1), $KT_{45\text{-}6}$ Δ*sigH::amyB* Δ*ptsG::glaA* (i.e., strain $KT_{45\text{-}6}$S-2), $KT_{45\text{-}6}$ Δ*sigH::amyX* Δ*ptsG::glaA* (i.e., strain $KT_{45\text{-}6}$S-3) and $KT_{45\text{-}6}$ Δ*sigH::pulA* Δ*ptsG::glaA* (i.e., strain $KT_{45\text{-}6}$S-4), respectively. All of these strains were able to degrade starch (Fig. 7a) and exhibited an activity of amylolytic enzymes in the culture supernatants (Table 3). In addition, a prominent protein band was observed at the appropriate locations from SDS-PAGE through analyzing the culture supernatants of these strains (Fig. 7b). These results suggested that the amylolytic enzymes-coding genes in strain were expressed and secreted into the medium. As expected, all of strains could grow in $CgXII^{IP}$M-medium and $CgXII^{IP}$S-medium, but they are the worse in $CgXII^{IP}$S-medium (Fig. 8a, b). However, it is worth noting that the growth of strains with co-expression of amylolytic enzymes is higher than that of strains with single-expression of amylolytic enzymes in $CgXII^{IP}$S-medium (Fig. 5c and Fig. 8b). In addition, the highest final DCW (10.2 ± 1.0 g/L) and μ_{max} (0.23 h^{-1}) in $CgXII^{IP}$M-medium were observed in strain $KT_{45\text{-}6}$S-1, which were higher than that of strain $KT_{45\text{-}6}$ Δ*sigH::amyA* (8.9 ± 0.7 g/L and 0.22 h^{-1}, respectively) and $KT_{45\text{-}6}$ Δ*sigH::glaA* (8.5 ± 0.3 g/L and 0.20 h^{-1}, respectively; Fig. 5b and Fig. 8a).

To investigate the effect of co-expression of amylolytic enzymes on L-lysine production using starch as substrate, the abovementioned strains were cultivated in $CgXII^{IP}$G-medium, $CgXII^{IP}$M-medium and $CgXII^{IP}$S-medium, respectively. As a control, strains with single-expression of amylolytic enzymes did the same. Interestingly, strains with co-expression of amylolytic enzymes produced more L-lysine than strains with single-expression of amylolytic enzymes in $CgXII^{IP}$M-medium and $CgXII^{IP}$S-medium (Fig. 6b, c and Fig. 8c, d). The L-lysine production is the best in strain $KT_{45\text{-}6}$S-1, followed by strain $KT_{45\text{-}6}$S-4 (Fig. 8c, d). Strain $KT_{45\text{-}6}$S-1 accumulated 21.7 ± 1.8 g/L of L-lysine in $CgXII^{IP}$M-medium, which was only about 8.8% lower than that of strain $KT_{45\text{-}6}$ in $CgXII^{IP}$G-medium (Fig. 6a and Fig. 8c). Although strain $KT_{45\text{-}6}$S-3 exhibited the worst fermentation performance, the L-lysine yield of this strain was higher than that of strain $KT_{45\text{-}6}$ Δ*sigH::amyX* and $KT_{45\text{-}6}$ Δ*sigH::glaA* (Fig. 6c, d

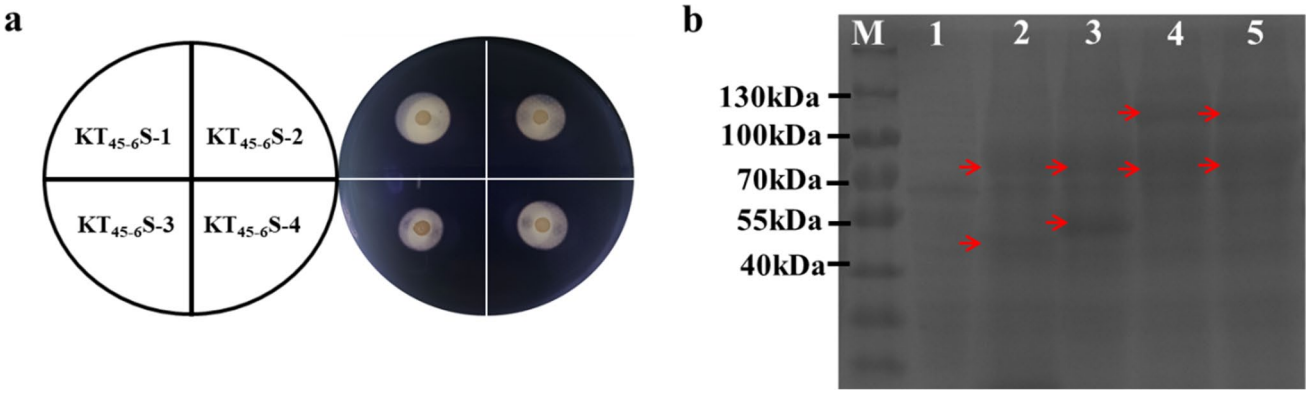

Fig. 7 Analyzing the enzyme activity in strain *C. glutamicum* $KT_{45\text{-}6}$ with co-expression of amylolytic enzyme. **a** The starch degradation of strains on LBG-plate containing 0.5% (w/v) starch at 45 °C. **b** SDS-PAGE analyses of amylolytic enzymes in culture supernatant from different *C. glutamicum* strains. Lane M: protein molecular weight marker; Lane 1: strain $KT_{45\text{-}6}$, Lane 2: strain $KT_{45\text{-}6}$S-1, Lane 3: strain $KT_{45\text{-}6}$S-2, Lane 4: strain $KT_{45\text{-}6}$S-3, Lane 5: strain $KT_{45\text{-}6}$S-4. The red arrows represent the target protein

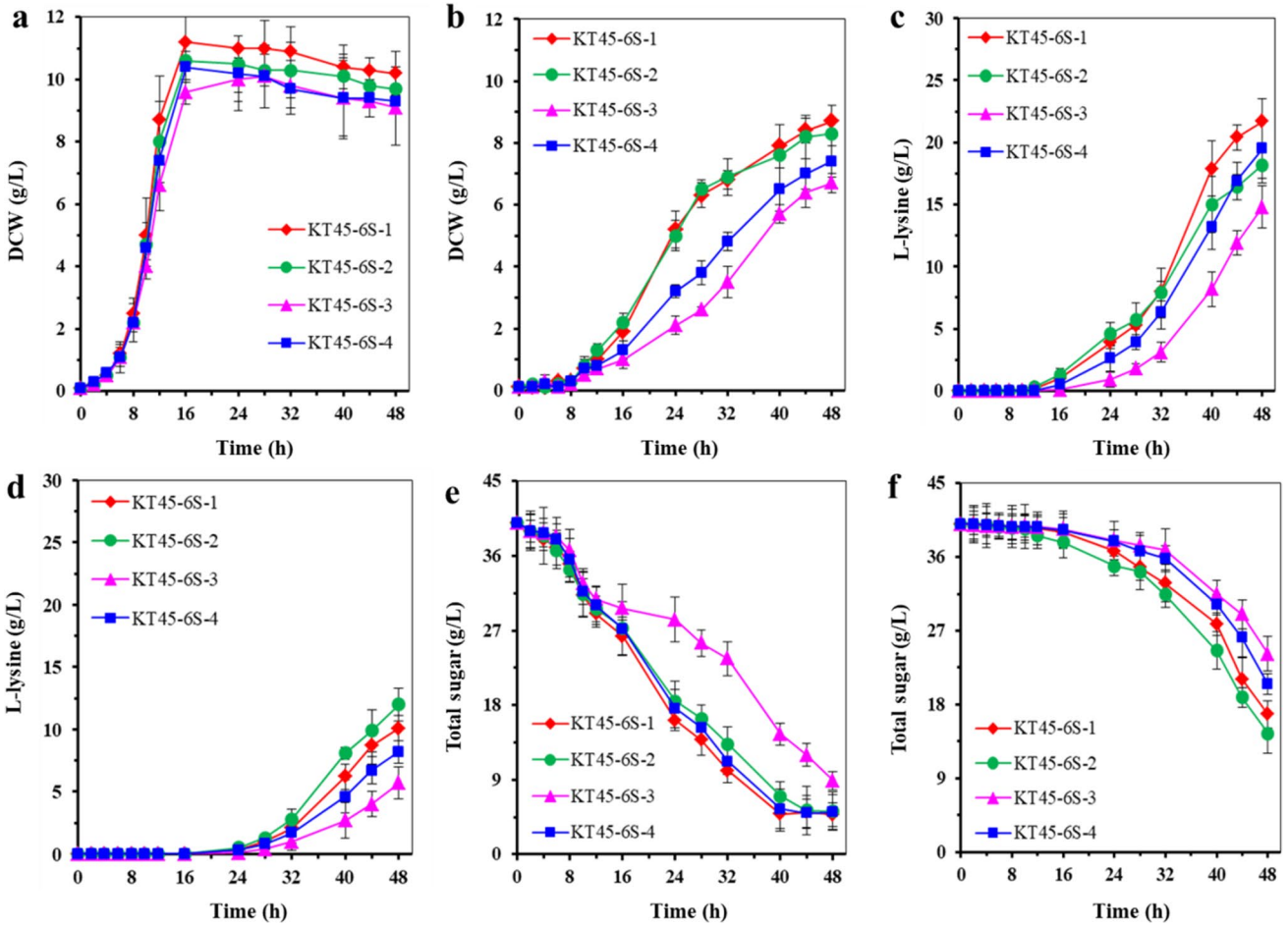

Fig. 8 The fermentation performance of different *C. glutamicum* strains using different sugar as carbon source. **a**, **b** The cell growth in CgXII^{IP}M-medium (**a**) and CgXII^{IP}S-medium (**b**). **c**, **d** The L-lysine production in CgXII^{IP}M-medium (**c**) and CgXII^{IP}S-medium (**d**). **e**, **f** Total sugar in CgXII^{IP}M-medium (**e**) and CgXII^{IP}S-medium (**f**). The data are mean values obtained from three independent experiments with error bar

and Fig. 8c, d). Not surprisingly, more total sugar was consumed in strains with co-expression of amylolytic enzymes (Fig. 8e, f). This result indicated that co-expression of amylolytic enzymes is beneficial to give strain with strong hydrolysis ability of starch. However, it is important to point out that the fermentation performance of all the abovementioned strains in CgXII^{IP}M-medium is better than that in CgXII^{IP}S-medium (Fig. 8c, d).

The effect of AA-GA and GA-AA fusion enzymes on starch degradation and L-lysine production

Based on the above results, co-expression of AA and GA in strain $KT_{45\text{-}6}$ has a significant impact on starch degradation and L-lysine production. Considering that recombinant multifunctional fusion enzyme has several advantages in catalyzing sequential reactions (Wang et al. 2007), genes encoding AA and GA were fused with a linker and expressed in the thermotolerant L-lysine-producing strain $KT_{45\text{-}6}$. In the AA-GA fusion enzyme, the C terminus of AA was fused with the N terminus of GA in frame. By contrast, the GA-AA fusion enzyme was inverted (Fig. 9a). And the corresponding strains were named *C. glutamicum* $KT_{45\text{-}6}$S-5 and *C. glutamicum* $KT_{45\text{-}6}$S-6, respectively. Recombinant enzymes produced in strain $KT_{45\text{-}6}$S-5 and $KT_{45\text{-}6}$S-6 was used to analyze by SDS-PAGE (Fig. 9b), which matched the theoretical molecular mass from the deduced amino acid sequence. Strain $KT_{45\text{-}6}$S-5 had similar or even better biodegradability of starch than strain $KT_{45\text{-}6}$S-1, whereas $KT_{45\text{-}6}$S-6 did worse (Fig. 9c). In addition, enzyme activity assay once again showed that strain $KT_{45\text{-}6}$S-5 exhibited the highest activity of amylolytic enzymes (97.3 ± 8.4 U/L of AA and 51.4 ± 6.2 U/L of GA) while $KT_{45\text{-}6}$S-6 had only 108.5 U/L of amylolytic enzyme activity (i.e., 69.8 ± 5.7 U/L of AA and 38.7 ± 3.4

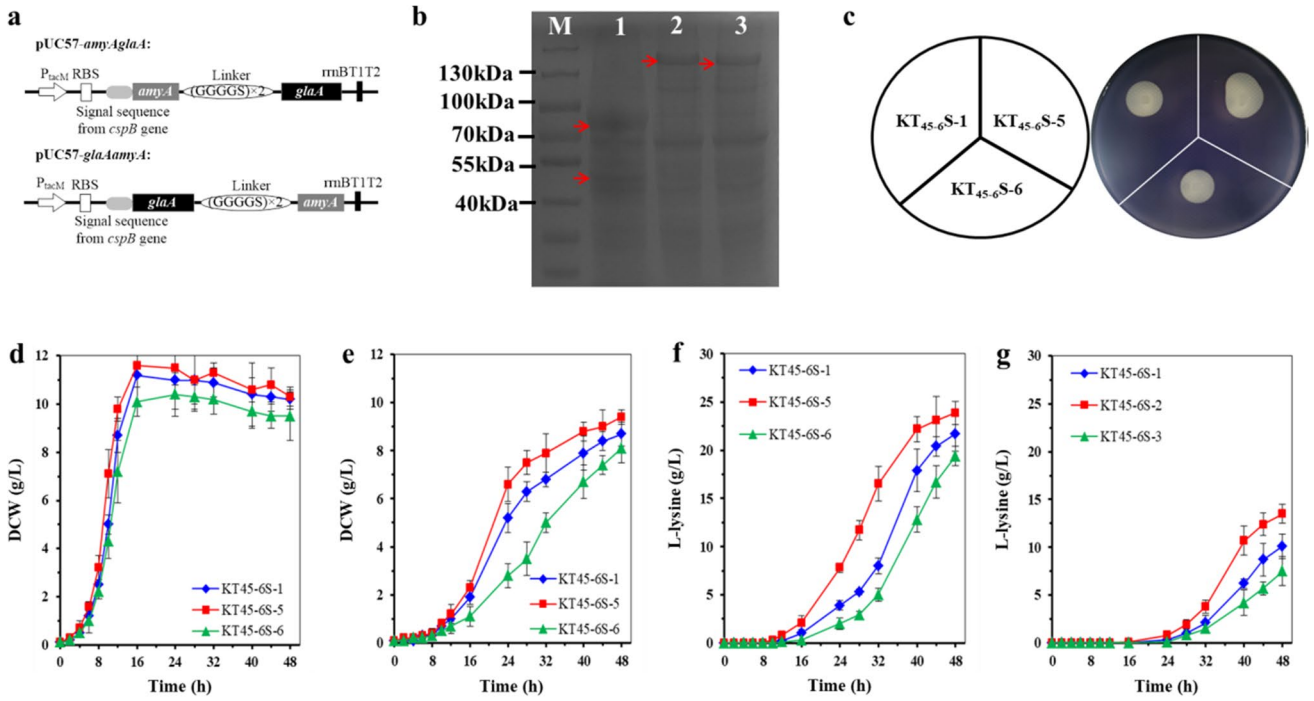

Fig. 9 Starch degradation and fermentation performance of different *C. glutamicum* strains with AA-GA or GA-AA fusion enzyme. **a** Construction process of the recombinant plasmids for expression fusion protein. **b** SDS-PAGE analyses of amylolytic enzymes in culture supernatant from different *C. glutamicum* strains. Lane M: protein molecular weight marker; Lane 1: strain $KT_{45\text{-}6}S$-1, Lane 2: strain $KT_{45\text{-}6}S$-5, Lane 3: strain $KT_{45\text{-}6}S$-6. **c** The starch degradation of strains on LBG-plate containing 0.5% (w/v) starch at 45 °C. **d**, **e** The cell growth in $CgXII^{IP}$M-medium (**d**) and $CgXII^{IP}$S-medium (**e**). **f**, **g** The L-lysine production in $CgXII^{IP}$M-medium (**f**) and $CgXII^{IP}$S-medium (**g**). The data are mean values obtained from three independent experiments with error bar

U/L of GA) in culture supernatants (Table 3). As anticipated, strain $KT_{45\text{-}6}S$-5 and $KT_{45\text{-}6}S$-6 could use starch as carbon source for cell growth and L-lysine production (Fig. 9c, d). However, strain $KT_{45\text{-}6}S$-5 showed the best performance of growth and L-lysine production in $CgXII^{IP}$M-medium and $CgXII^{IP}$S-medium. In $CgXII^{IP}$M-medium, strain $KT_{45\text{-}6}S$-5 begun to produce L-lysine after 10 h and accumulated 23.9 ± 2.3 g/L of L-lysine (Fig. 9f). Encouragingly, expression of AA-GA fusion enzyme could significantly promote performance of growth and L-lysine production in $CgXII^{IP}$S-medium (Fig. 9e, g). The highest final DCW (9.4 ± 0.3 g/L) and μ_{max} (0.15 h^{-1}) in $CgXII^{IP}$S-medium were observed in strain $KT_{45\text{-}6}S$-5, which were higher than that of strain $KT_{45\text{-}6}S$-1 (8.7 ± 0.5 g/L and 0.12 h^{-1}, respectively; Fig. 8b and Fig. 9e). In addition, L-lysine was firstly accumulated at 16 h and finally to about 13.5 ± 1.0 g/L in strain $KT_{45\text{-}6}S$-5 during cultivated in $CgXII^{IP}$S-medium (Fig. 9g). It is worth noting that strain $KT_{45\text{-}6}S$-5 showed the better fermentation performance during cultivated in $CgXII^{IP}$S-medium and $CgXII^{IP}$M-medium at 45℃ that of at 30℃ (Fig. S2a-f). In addition, it showed the similar fermentation performance with the parental strain K-8 during cultivated in $CgXI^{IIP}$G-medium at 30℃ (Fig. S2g-i). Strangely, expression of GA-AA fusion enzyme seems to be slanted against starch degradation. Strain $KT_{45\text{-}6}S$-6 showed the worst performance of growth and L-lysine production during used starch as carbon source (Fig. 9e, g).

Discussion

Starches are natural polysaccharides, and they are closely related to sugar industry (Karim et al. 2019). Although *C. glutamicum* cannot utilize starch directly (Tateno et al. 2007b), many studies have attempted to modify *C. glutamicum* to directly utilize starch as a key resource for producing value-added chemicals (Chen et al. 2020; Sgobba et al. 2018; Tsuge et al. 2013). However, the present achievements are also hard to obtain the satisfactory L-lysine production from starch in industry. In this study, we devote to improve *C. glutamicum* strain for efficiently producing L-lysine using raw corn starch as the key carbon source. In order to improve cell growth and L-lysine production on starch, an L-lysine-producing strain *C. glutamicum* K-8

was consecutively modified to give strain K-8 with high-efficiency degradability of starch at high temperature, for example temperature-induced mutagenesis-based adaptive laboratory evolution, introduction of different amylolytic enzymes as well as optimization of combinations and expressing systems of amylolytic enzymes. As a result, a high-temperature strain *C. glutamicum* $KT_{45\text{-}6}$S-5 was obtained, which produced 23.9 ± 2.3 g/L of L-lysine using starch as the main carbon source at 45 °C. It is the first report that the classically derived mutant strain was modified to improve temperature tolerance and to co-expression of amylolytic enzymes and thus to increase starch utilization for producing L-lysine.

The optimal growth temperatures of *C. glutamicum* and its relatives are between 25 °C and 37 °C (Abe et al. (1967). However, the L-lysine-producing strain used in this work (i.e., *C. glutamicum* K-8) exhibited the bad growth at 35 °C and above (Fig. 1). The parental strain of K-8 is *C. glutamicum* JL-6, which was derived from *C. glutamicum* ATCC 13,032 by "classical breeding" (Xu et al. 2018). Ohnishi et al. (2003) also found that strains modified by "classical breeding" showed a worse growth at elevated temperatures (e.g., 35 °C) than strains modified by "genome breeding." One possible reason is that the intrinsic thermo-tolerance was lost in classically derived strains after random mutagenesis. However, many studies pointed out that high-temperature fermentation could accelerate fermentation progress, increase the yield of target products and reduce the dosage of cooling water, thus cutting the cost of production (Eiichiro et al. 1996; Hou et al. 2012; Ohnishi et al. 2003). In addition, improving the thermotolerance of strain K-8 should help increase the yield and productivity of L-lysine during used starch as carbon source because amylolytic enzymes are optimal activity at high temperature (i.e., > 50 °C)(Leveque et al. 2000). Stress-induced mutagenesis-based adaptive evolution is a valid method in improving microbial stresses-tolerance and generating robust microbial strains (Zhu et al. 2014). Strain *C. glutamicum* $KT_{45\text{-}6}$ was obtained after 5 round of temperature-induced mutagenesis-based adaptive evolution, which grows well at 45 °C and accumulates 23.8 ± 3.2 g/L of L-lysine after 48 h of batch fermentation at 45 °C (Fig. 2b). It may be due to the DNA-repair mechanisms in cell and disruption of metabolic pathways (Leszczewicz and Walczak 2019; Oide et al. 2015; Wang et al. 2019a), the positive mutants are no more than 30% of all mutants (Fig. 2).

Previous study demonstrated that wild-type *C. glutamicum* cannot utilize starch directly (Tateno et al. 2007b), whereas the recombinant *C. glutamicum* strains with α-amylase activity exhibited the ability to degrade starch (Seibold et al. 2006; Tateno et al. 2007a, 2007b). Except for α-amylase, many amylolytic enzymes are also widely used in starch degradation, such as β-amylase, glucoamylase, isoamylase, and pullulanase (Guzmanmaldonado and Paredeslopez 1995; Karim et al. 2019). Our results indicated that all recombinant strains with different amylolytic enzymes can degrade starch at different levels (Fig. 4b), but not all recombinant strains can used starch as the only carbon source for cell growth and L-lysine production (Fig. 5a–c and Fig. 6). Based on the data published by Guzmanmaldonado and Paredeslopez (1995), these amylolytic enzymes in starch degradation have a different function way. IA is a glycogen-/starch-debranching enzyme that hydrolyzes the α-1,6-glucosidic linkage specific in α-glucans with more than 3 glucose residues to only produces amylose (Park et al. 2007). This might be why strain $KT_{45\text{-}6}$ Δ*sigH::amyX* cannot grow and produce L-lysine in $CgXII^{IP}$S-medium (Fig. 5c and Fig. 6c). In contrast, Pul, as another debranching enzyme, can hydrolyze the α-1,6-glucosidic linkage in α-glucans with lower than 3 glucose residues to produce maltose and maltotriose (Wang et al. 2019b), which in turn could help strain $KT_{45\text{-}6}$ Δ*sigH::pulA* grow in $CgXII^{IP}$S-medium (Fig. 5c). Since maltose is the main degradation products of starch after BA hydrolysis (Duan et al. 2019), strain $KT_{45\text{-}6}$ Δ*sigH::amyB* with BA activity showed the best performance of growth in $CgXII^{IP}$M-medium and $CgXII^{IP}$S-medium (Fig. 5b, c). However, the L-lysine production of strain $KT_{45\text{-}6}$ Δ*sigH::amyB* is lower than that of strain $KT_{45\text{-}6}$ Δ*sigH::amyA* in $CgXII^{IP}$M-medium (Fig. 6b). That may be because more carbon source in strain $KT_{45\text{-}6}$ Δ*sigH::amyB* are used to cell growth rather than L-lysine production (Moon et al. 2005; Seibold et al. 2006). It should be noted that all recombinant strains showed the worse performance of growth and L-lysine production in $CgXII^{IP}$S-medium than in $CgXII^{IP}$M-medium (Fig. 5b, c and Fig. 6b, c). Similar results were achieved in another research reported by Seibold et al. (2006). A possible explanation could be that some of starch hydrolysis products are not usable for growth and L-lysine production (Seibold et al. 2006). In addition, the results of phenol–sulfuric acid colorimetric assay indicated that the consuming rate of total sugar by strains in $CgXII^{IP}$S-medium is lower than that in $CgXII^{IP}$M-medium (Fig. 6b, c). This might be another possible reason.

In sugar industry, two kinds of amylolytic enzymes are used to degrade starch for producing glucose (Karim et al. 2019). Some studies also pointed out that co-expression of amylolytic enzymes in yeast or in *Aspergillus niger* is beneficial to starch degradation and cell growth (Inokuma et al. 2015; Juge et al. 2006; Murai et al. 1999). In this study, GA was co-expressed with AA, BA, IA and Pul in a thermotolerant L-lysine-producer *C. glutamicum* $KT_{45\text{-}6}$. As shown in Fig. 4a and Fig. 7a, all of recombinant strains with co-expression or single-expression of amylolytic enzymes are able to degrade starch, but the recombinant strains with co-expression of amylolytic enzymes showed the better degradability than strains with

single-expression of amylolytic enzymes. More strikingly, the growth and L-lysine production of strains with co-expression of amylolytic enzymes in CgXII^{IP}M-medium and CgXII^{IP}S-medium is higher than that of strains with single-expression of amylolytic enzymes, especially in CgXII^{IP}S-medium (Fig. 5b, c, Fig. 6b, c, and Fig. 8a–d). These similar results were also obtained in previous studies (Inokuma et al. 2015; Murai et al. 1999). As is known to all, GA from *Aspergillus niger* possesses the non-catalytic substrate binding domains (SBD), which accelerates the rate of hydrolysis of substrate (Juge et al. 2006). In addition, soluble dextrin is believed to be the best substrate for GA (Karim et al. 2019; Shigechi et al. 2004). Murai et al. (1999) also pointed out that the recombinant *S. cerevisiae* with expression of GA only forms a small colourless zone around the colony. Therefore, strain $KT_{45\text{-}6}$S-1 (i.e., co-expression of AA and GA in strain $KT_{45\text{-}6}$) showed the biggest colourless zone as well as the best cell growth and L-lysine production using starch as carbon source (Fig. 7 and Fig. 8). Seo et al. (2000) pointed out that recombinant multifunctional fusion enzyme is beneficial to improve catalytic activities of freestanding enzymes because of a proximity effect. And our results also indicated that expression of AA-GA fusion enzyme in strain $KT_{45\text{-}6}$ (i.e., strain $KT_{45\text{-}6}$S-5) significantly increases catalytic activities and promotes the performance of growth and L-lysine production using starch as carbon source (Table 3 and Fig. 9c–g). This is because the efficiency of hydrolyzing starch to fermentable sugars was greatly improved (Juge et al. 2006). However, expression of GA-AA fusion enzyme seems to be slanted against starch degradation (Fig. 9e, g). Similar results were also obtained in previous studies, in which bifunctional fusion enzyme TSBA (i.e., Trehalose synthase-β-amylase fusion protein) showed higher catalytic activities than that of BA/TS, but BATS showed similar activities with BA/TS because of the different *K*m value and k_{cat} value for starch (Wang et al. 2007). Therefore, we speculate that AA-GA fusion enzyme showed the best catalytic efficiencies (i.e., k_{cat}/K_m), whereas GA-AA fusion enzyme showed the worst catalytic efficiencies for starch.

In conclusion, a thermotolerant L-lysine producer *C. glutamicum* $KT_{45\text{-}6}$ has been achieved by temperature-induced mutagenesis-based adaptive laboratory evolution. Additionally, this thermotolerant L-lysine producer $KT_{45\text{-}6}$ can be used to produce L-lysine from corn starch with high efficiency by introduction of AA and GA. Furthermore, a recombinant bifunctional fusion enzyme AA-GA can increase the efficiency of hydrolyzing starch to fermentable sugars, thus improving the performance of growth and L-lysine production of the recombinant strain. These results provide a convenient and efficient way to produce L-lysine direct from raw starch for industrial applications.

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Author contribution LML and JZX conceived and designed the experiments. WGZ contributed new reagents or analytical tools. CLL and HZR performed the experiments and analyzed the data. CLL, HZR and JZX wrote the paper. All authors read and approved the final manuscript.

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Data availability Data and materials will be made available on reasonable request.

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