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Over-production of α-ketoglutarate by the *Corynebacterium glutamate*

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ABSTRACT

Previous studies have shown that over-production of glutamate pathway is α-KG to glutamate by through the gdh of reduction.Therefore, deleting the gdh gene block the generation of glutamate. The strain with gdh distruped showed no L-gluDH activities, resulting in up to 14.7 times α-KG production than the original strain in flask culture. There results suggest that L-gluDH is the key enzyme in the generation of glutamate. Over-expression of pyruvate carboxylase can enhance CO_2 fixed and improve the metabolism stream of pyruvate to oxaloacetate. The result is that the strain C. glutamicum KGA-2pXMJ19pep α-KG production increased 15.7% more than the original strain. Our results can be applied in the industrial production of α-KG by using C.glutamate as producer.

Key words: α-KG, biosynthesis, gene disruption, overexpression, *Corynebacterium glutamicum*.

INTRODUCTION

α-KG (KGA) is an intermediate in the tricarboxylic acid cycle (TCA) and a major contributor to amino acid formation and nitrogen[1],[3]. So it has wide applications as a food additive and in the agrochemical and pharmaceutical industries [2], [3]. α-KG is also used in biochemical researches and medicine as a substrate for assaying activity of such enzymes as α-KG dehydrogenase, aspartate transaminase, and alanine transaminase for the diagnosis of broad spectrum of diseases (hepatitis, cute myocardial infarction, muscular dystrophy, dermatitis, and others) [9]. Besides, α-KG can protect from cyanide intoxication.

In general, there are three different α-KG production processes: chemical synthesis process, enzymatic process, and microbial fermentation process. Among those processes, the α-KG microbial production from sustainable, low-cost carbon source has the merits in terms of both high yield and high product purity [3]. However, chemical synthesis is still the main approach to produce α-KG in the industry. At present, low yield and application of toxic chemical and solvents increase the cost and limit its wider application [5],[6].

In microbes, α-KG is mainly synthesized by oxidative decarboxylation of isocitrate by isocitrate dehydrogenase in via TCA [7], and then is partly consumed to synthesized glutamate catalyzed by glutamate dehydrogenase (enconded by *gdh*). In recent years, several constructive and novel works concerning α-KG production by *Torulopsis glabrata* has indicated that α-KG could be over-synthesized by microorganisms [4],[8]. However, the period of α-KG

fermentation by *T. glabrata* seems a bit long (approximately 120h). Since α-KG is the precursor of glutamate, it makes sense that α-KG can be synthesized by glutamate producing *Corynebacterium glutamicum* with shorter fermentation period through *gdh* deletion.

Pyruvate carboxylase (encoded by *pc*) is a biotin-dependent and a key enzyme in the TCA reduction pathway, the pyruvate node comprises a series of metabolic pathways competing for carbon fluxes with L-glutamate synthesis, including L-alanine and L-lactate [10]. So the *pep* overexpression could enhance the carbon flux distribution flowing into the TCA cycle, and was expected to further improve α-KG production.

In this study, we attempted to construct α-KG producing strains from *C.glutamicum* KGA, which was used as a L-glutamate producer and had the advantages of high L-glutamate accumulation and short fermentation period [23]. Given these facts, enhancement of α-KG production was investigated by deletion of the gene *gdh* (encoding L-gluDH) and overexpression of the gene *pep*. Resulting strains exhibited a dramatic accumulation of α-KG in the culture medium.

EXPERIMENTAL SECTION

Materials

Strains and plasmids used in this work are listed in Table 1.From *Corynebacterium glutamicum* KGA breeding high yield in the α-KG strain, as a production of α-KG of the original strain. The strain was routinely grown at 32 °C in CgIII medium containing 1% peptone, 0.25%NaCl, 1% yeast extract and 2% glucose. For *Corynebacterium glutamicum* KGA competent cell production, *Corynebacterium glutamicum* GDK-9 Single colony transfer to 5mL of BHIS medium (BactoTM brain heart infusion 37g/L; D-sorbitol 91g/L) wave pipe for the overnight training, again with 2 ml bacteria liquid transfer to 50 ml BHIS shake flask medium. The cells were grown continuously until they reached an absorbance of 1.75 at 578 nm. Then the cells were harvested and with TG buffer (1m mol/L Tris-HCl; 10% glycerol; pH7.5) washing and 10% glycerol were in turn washing cells 3 times. Add 1 ml glycerin heavy suspension, each EP tube is installed 100 ml bacteria liquid, deposited in the -80°C refrigerators spare. For the production of α-KG in flask culture, the seed culture was carried out at 32°C for 6-7 h with shaking (200 r/min) in seed medium (pH7.0, adjusted with NaOH) containing 25g/L glucose; 33mL/L Corn steep liquor; 22mL/L Soybean protein hydrolysate; 2.2g/L $K_2HPO_4 \cdot 3H_2O$; 0.9g/L $MgSO_4 \cdot 7H_2O$; 3g/L urea. One milliliter of seed culture was transferred to 10 ml of fermentation medium (pH7.0, adjusted with NAOH) containing 80g/L glucose; 1.1g/L molasses; 33mL/L Corn steep liquor; 2.83g/L $Na_2HPO_4 \cdot 12H_2O$; 1.33g/L KCl;0.233mg/L VB_1; 2.33mg/L $MnSO_4$; 2.33mg/L $FeSO_4$; 1.83g/L $MgSO_4 \cdot 7H_2O$. The cells were grown for 34 h at 34°C, and the temperature sequence control (lift 0.5°C per 4 hours). *Escherichia coli* were grown in LB medium (5g/L Yeast Extract; 10g/L Trypton; 10g/L NaCl) at 37°C.

Table 1 strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Reference or source
Corynebacterium glutamicum		
KGA	The α-KG-producing strain	This work
KGA-2	*C.glutamicum* GDK-9 with *gdh* deletion	This work
KGA-2pXMJ19pep	*C.glutamicum* KGA-2 harboring pXMJ19pep	This work
Escherichia coli		
DH5αMCR	F^-φ80d*lac*Z△(lacZYA-argF)U169 *recA1 endA1 hsdR17*(rk^- mk^+)*supE44* λ^-thi-1gyA96 *relA1*	[12]
Plasmids		
pK18mobsacB	Integration vector, Km^r $oriV_{Ec}$	[13,14]
pK18mobsacB△*gdh*	Plasmid carrying the△*gdh*	This work
pXMJ19	Expression vector, p*tac*, *lac*I^q, Cm^r	[15]
pXMJ19*pep*	Plasmid carrying the *pep* gene encoding Pyruvic carboxylase	This work

Km^r: resistance to kanamycin; Cm^r: resistance to chloramphenicol.

Plasmid construction and recombinant DNA techniques

All restriction and modification enzymes used for the manipulation of DNA were purchased from Takara Biotechnology (Dalian) Co., Ltd. Plasmid isolation and DNA purification kits were purchased from Biomed. All primers used in this study were synthesized by Beijing Liuhe and Huada Genomics Technology Co., Ltd. DNA sequencing analysis was done by Beijing Liuhe and Huada Genomics Technology Co., Ltd. PCR amplification was carried out with a mastercycler@ personal (Eppendorf) by using Taq DNA polymerase (by Fermentas, Tianjin, China).Plasmid pK18mobsacB and pXMJ19 are an *E.coli-Corynebacterium* shuttle vector by insertion of multiple cloning sits. Other molecular biological methods used in this lab are described elsewhere.

Table 2 Primer sequences used in this lab

Primer	Sequence(5'-3')
gdh 01S	TAT CTG AGC TCT GGT CAT ATC TGT GCG ACA C
gdh 01A	TGC ACA GGT CTT GAA GAT GTT CAT TTC CTC GTT CCC ATC TC*G*
gdh 02S	GAA CAT CTT CAA GAC CTG TGC AGA GAC CGC AGC AGA GTA TG
gdh 02A	TAT CTA AGC TTA GGG AAG CCT TGA GGT TGG
pep 01S	GCA TAC AAG CTT GCG GTG TAC CCA TCA TTG TC
pep 02A	GTA TAC GAG CTC CCA AAT CCA ACT CAC CCA TCT C
Km 01S	CAG AGT CCC GCT CAG AAG
Km 02A	ACT GGA TGG CTT TCT TGC
Cm 01S	ATA GAA GGC GGC GGT GGA AT
Cm 02A	GTC GGA TCA GCT TGAGTA GG

Construction of strains

To obtain the *gdh* mutant from *C.glutamicum* KGA, a 2.744 kb complete gene fragment of *gdh* mutant was amplified by PCR from the total chromosomal DNA by using the primers *gdh01S* and primers *gdh02A*.The PCR product was cloned into *Hind* III and *Eco* RI site of pK18mobsacB vector to obtain kanamycin resistance *gdh* gene fragment. Which was transformed into *C. glutamicum* KGA to integrate into the chromosomal *gdh* locus by a single crossover, generating the strain named *C. glutamicum* KGA-2.

To obtain the *pep* mutant from *C.glutamicum* KGA-2, a 3.98kb complete gene fragment of *pep* mutant was amplified by PCR from the total chromosomal DNA by using the primers *pep01S* and primers *pep02A*. The PCR product was cloned into *Hind* III and *Sac* I site of pXMJ19 vector to obtain chloramphenicol resistance pep gene fragment. Which was transformed into *C. glutamicum* KGA-2, generating the strain named *C. glutamicum* KGA-2pXMJ19pep [16],[17],[18].

Determination method

Using Agilent 1200 high performance liquid chromatograph (made in gemany), detector VWD (variable wavelength UV detector),as the standard-type injector autosampler, injection volume 20uL;Column Eclipse XDB-C18,3.5μm, 150mm ×4.6 mm;0.05mol/L $(NH_4)_2HPO_4$ as the mobile phase, H_3PO_4 adjusted to pH=2;column temperature of 30°C;flow rate of 1mL/min. Detection wavelength of 215nm.

Fermentation

Fermentations were carried out in 5L bioreactors (Shanghai Baoxing Bio-engineering Equipment Co., Shanghai, China). The *C.glutamicum* seed culture was grown in shake-flasks (100 ml seed medium in a 1L shake flask, 200 rpm, 32°C) to an $OD_{600\times20}$ about 0.7-1.0 within 7h and was transferred to the 5L production medium for α-KG production with 10% (v/v) inoculum size. Glucose concentration was kept close to 2.0% (20.0g/L).The pH was controlled by the addition of 20% NaOH and maintained at approximately 7.2. The ammonium concentration was kept not be higher than 0.1mmol/L. The concentration of dissolved oxygen was 20.0–50.0% during the whole process.

Enzyme assays

The strains grown in LB medium were collected when they reached 2.5 at A_{600}, and washed twice or triple with phosphate-buffered saline (0.14mol/L NaCl, 2.7mmol/L KCl, 10mmol/L Na_2HPO_4, and 1.8mmol/L KH_2PO_4, pH7.5). Finally resuspended in PBS followed by disruption with Noise isolating tamber (NingBo, scientz, biotechnology Co., LTD. China).The supernatant was collected by centrifugation and used as crude enzyme solution. L-gluDH activity was measured by a spectrophotometer at 25°C in 3ml reaction system containing of 2.5ml 0.1mol/L Tris-HCl (pH7.5), 0.1ml 1mol/L NH_4Cl, 0.15ml 0.2mol/L α-KG, 0.2ml 0.25mmol/L NADPH and 0.05ml of crude enzyme solution. The L-gluDH activity was determined by measuring the decrease in absorbance at 340nm. One unit of enzyme was defined as 1umol of NADPH or NADP consumed per minute.

Cells grown in LB medium were collected at 4 °C when they reached 2.5 at $A_{600,}$ and washed twice with phosphate-buffered saline (0.14mol/L NaCl, 2.7mmol/L KCl, 10mmol/L Na_2HPO_4, and 1.8mmol/L KH_2PO_4, pH7.5). And finally resuspended in PBS followed by disruption with Noise isolating tamber (Ning Bo, scientz, biotechnology Co., LTD. China).The supernatant was collected by centrifugation and used as crude enzyme solution. Pyruvate carboxylase activity was measured by a spectrophotometer at 25°C in 1ml reaction system containing of 100mmol/L Tris-HCl (pH7.8), 10mmol/L pyruvate, 10mmol/L HCO_3^-, 2.5mmol/L MgATP, 2.5mmol/L $MgCl_2$, 0.25mmol/L acetyl-CoA, and 0.1ml of crude enzyme solution. The PC activity was determined by measuring in absorbance at 415nm one unit of enzyme was defined as generated 1umol of oxaloacetic acid per minute [19],[20],[21].

Real-time RT-PCR

The RT-PCR technology uses UltraSYBR two-step quantitative PCR kit (Beijing Kangwei Century Biotech Co., Ltd.).The Reverse transcription reaction system containing of 4ul dNTP Mix, 2ul Primer Mix, 1ul RNA template, 4ul 5×RT Buffer , 2ul DTT , 1ul SuperRT and 7ul RNase-Free water. The Reverse transcription reaction condition is incubate at 42°C for 50min and 70°C for 15 min. After completion of the reaction, put it a brief centrifugation and placed on ice to cool. The RT-qPCR reaction system containing of 15ul 2×UltraSYBR Mixture (with ROX), 0.5ul 10mmol/L forward primer, 0.5ul 10mmol/L reverse primer, 4ul cDNA template and 10ul RNase-Free water. The cycling conditions comprised 10min polymerase activation at 95°C and 40 cycles at 95°C for 15 sec and 60°C for 60s[24].

Analytics

During the fermentation process, 1ml samples were taken from the cultures, centrifuged at 4°C and 13000 g for 5 min. Glucose was determined with a biosensor (Institute of Biology, Shang dong Academy of Science, Shanghai, China). Amino acids were analyzed by using high-performance liquid chromatography.

RESULTS AND DISCUSSION

The deletion of *gdh* gene and over-pression of *pep* gene

Detection of *gdh* gene was confirmed by PCR amplification, sequencing (data not shown) and enzyme activities. As shown in Table 3, L-gluDH activity was not detectable in *C.glutamicum* KGA-2, indicating that *gdh* gene has been successfully deleted.

Table 3 Activities of glutamate dehydrogenase (L-gluDH), pyruvate carboxylase in the engineered *Corynebacterium glutamicum* strains

Strain	Activity(U/min/mg)	
	L-gluDH	PC
KGA	9.4±0.8	-
KGA-2	ND	4.3±0.5
KGA-2pXMJ19pep	-	7.2±0.7

Notes: Values are the means ± standard deviations of three independent determinations. ND, enzyme activities were not detectable by using the assay method described in the text.

To confirm the effect *gdh* gene deletion on α-KG production, adjusted pH with ammonia or NAOH was performed when production of α-KG. In the case of adjusted pH with ammonia, the removal of L-gluDH resulted in a higher accumulation of α-KG after 34h, while in the *C.glutamicun* KGA strain， the accumulation of α-KG is less than 1g/L (Fig 2), and the accumulate amount of glutamate is opposite. However, only a very small amount of glutamate accumulation is mainly due to the lack of the enzyme, while glutamate synthase and glutamate dehydrogenase of *Corynebacterium glutamicum* is not essential for glutamate synthesis [11],[22]. These results indicate that α-KG to L-glutamate synthesis and metabolism hindered is caused by glutamate dehydrogenase deletion.

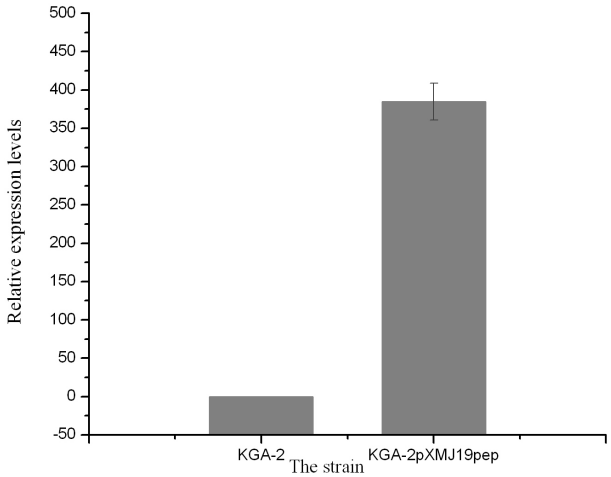


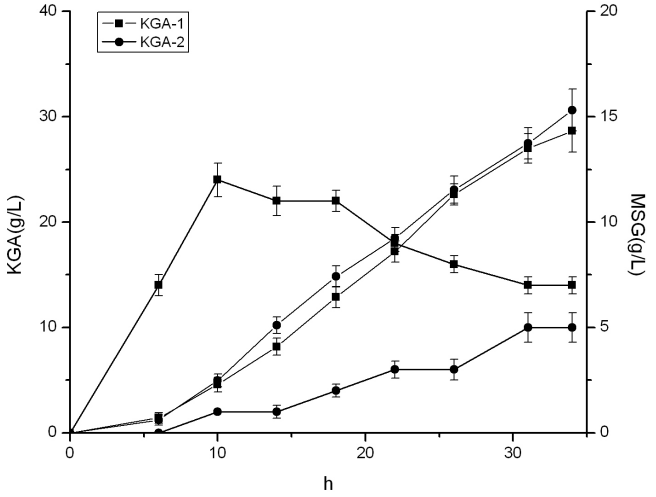
Fig.1. Relative expression levels of *pep* gene in the strain of KGA-2 and KGA-2pXMJ19pep. The latter is approximately 400 times than the former

The *pep* gene of overexpression was confirmed by PCR amplification, sequencing and PC activities. As shown in

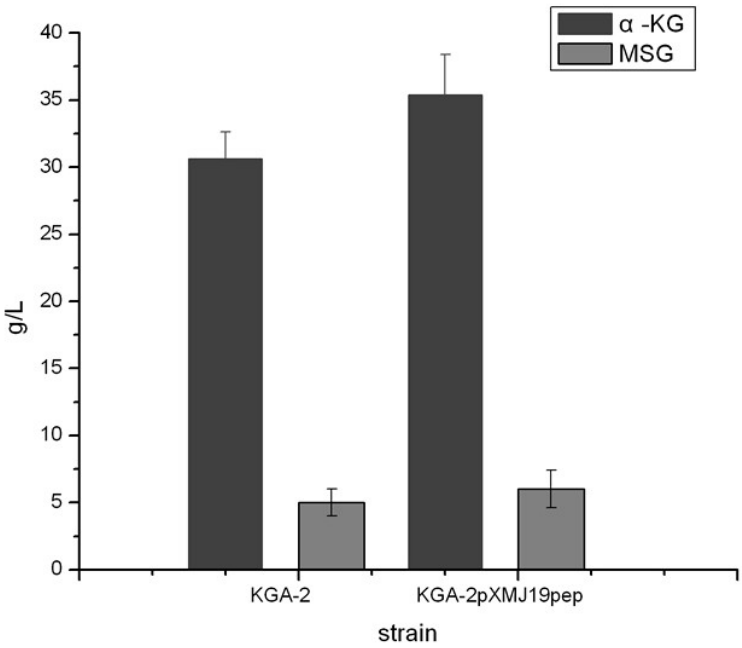
Table 3, the PC activity of the *C.glutamicum* KGA-2pXMJ19pep is 1.6 times than the *C.glutamicum* KGA-2, indicating that the pXMJ19pep has been successfully into the *C.glutamicum* KGA-2.

Enhanced production of α-KG by their coordination

The production of α-KG was conducted with the constructed *C.glutamicum*KGA-2 in a flask containing 20mL of fermentation medium, which was described in material and methods. The production of α-KG in *C.glutamicum* KGA-2 reached up to 14.7g/L. However, the *C.glutamicum* KGA just only reached to 0.93g/L after 34h in the fermentation, the accumulation of α-KG of the former is 15.8 times than that of the latter. Since an increase in α-KG synthesis amount leading to the reduction in the amount of glutamate synthase. So, these results demonstrate that the disruption of *gdh* gene prevented the conversion of α-KG to L-glutamate. The production of α-KG in strain KGA-2 reached up to 30.6g/L in a preliminary experiment using a 7.5L fermenter. However, the accumulation of α-KG in the strain KGA is less than 1g/L in the same fermenter (Fig 2).



(a) Time-dependent production of α-KG, the pH adjusts with NaOH



(b) Comparison of the concentration of α-KG with monosodium L-glutamate (MSG) after 34 h cultivation, the pH adjusts with ammonia

Fig.2. Production of α-KG from the metabolically engineered *Corynebacterium glutamicum*. (a) Time-dependent production of α-KG. (b) Comparison of the concentration of α-KG with monosodium L-glutamate (MSG) after 34 h cultivation

Values are means ±standard deviations of three independent experiments.

The effects of over-expression of *pep* gene involved in the metabolic pathways of pyruate to oxaloacetate in *C.glutamicum*. The pXMJ19pep was studied by measuring the activities of the enzymes encoded by the genes. As

shown in Table 3. *PEP* enzyme activity per unit volume, the strain KGA-2pXMJ19pep is 1.6 times than the strain KGA-2.

In the condition of the pH adjusts with NaOH, the production of α-KG was carried out with the strain KGA-2pXMJ19pep in the flask containing 20mL of fermentation medium, reached up to 16.4g/L, and the latter is 11.2% higher than the former. The production of α-KG in strain KGA-2pXMJ19pep reached up to 35.4g/L in a preliminary experiment using a 7.5L fermenter (Fig 2).

The α-KG original strain is the glutamate overproduction strain GDK-9, the α-KG located in glutamate classical pathway, and it is the glutamate precursor substance. In addition, in the strain *C.glutamicun* KGA, the L-gluDH is the key enzyme in the glutamamte synthetase and accumulation of α-KG. In the strain *C.glutamicun* KGA-2, the *gdh* gene knockout led to a massive accumulation of α-KG, the main reason is to prevent the α-KG to the metabolism of glutamate. But, this also led to increase the dissolved oxygen level in fermentation, there is possible *gdh* knockout reducing $NADP^+$ (NAD^+), the $NADP^+$ (NAD^+) with the coupling of the respiratory chain, and further led to weakened cell respiration. Meanwhile, with a small amount of lactic acid production and sugar consumption rate slows. In addition, the strain *C.glutamicun* KGA has the same effect with the strain *C.glutamicun* KGA-2 adjust pH with NaOH. This also proved L-gluDH is the key enzyme in accumulation of α-KG.

However, in the fermentation broth, as well as generates a small amount of glutamic acid, this is probably because the disruption of *gdh* can be compensated by the GS and GOGAT, the GS-GOGAT system in *C.glutamicum* is regulated in response to the presence and absence of L-gluDH. The results suggest that the L-gluDH is not essential for glutamate formation by *Corynebacterium glutamicum* [11].

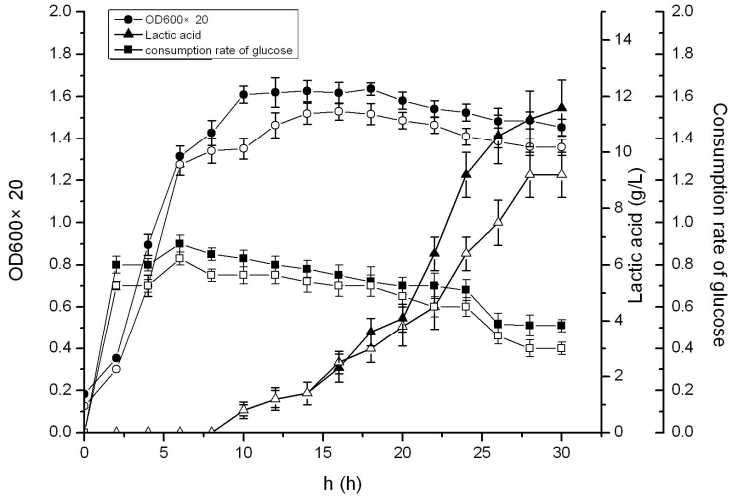


Fig.3. The level of dissolved oxygen, sugar consumption rate and vice acids (lactic acid) production in the α-KG fermentation process

After over-expression of pyruvate carboxylase in the strain KGA-2pXMJ19pep, biomass yield has slight increase. As shown in Fig 3. The possible reason is pyruvate carboxylase catalyzed oxaloacetate flow TCA pathway, on the other hand, oxaloacetate and L-malic acid into the mitochondria through its own transport mechanism involved in the TCA cycle, provide ATP and some intermediate metabolites needed for growth for the growth of strain, thus promote cell growth. As shown in Fig 3. This could also be the strain pXMJ19*pep* per unit volume of the PC activity of 1.67 times the strain KGA, while the accumulation of α-KG was only about 12%.The results suggest that increasing the carbon metabolic flux flows to the TCA cycle ,to improve the production of α-KG is also a viable means.

CONCLUSION

In conclusion, the enhanced biosynthesis of α-KG by using *C.gluyamicum* can be achieved by strengthen the metabolic pathways and modification of synthetic pathways. The over-expression of pyruvate carboxylase and the disruption of *gdh* resulted in the accumulation of α-KG. All of the above research can be applied for the industrial-scale production of α-KG from *C.glutamicum*.

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