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Original Research Article

Improvement in the Production of L-Lysine by Over-expression of Aspartokinase (ASK) in *C. glutamicum* ATCC 21799

Hilda Rastegari[1], Mohsen Chiani[2], Azim Akbarzadeh[2], Sara Cheraghi[3], Zahra Saffari[2], Mohammad Reza Mehrabi[2], Ali Farhangi[2] and Soheil Ghassemi[2*]

[1]Department of Microbiology, Azad University, Jahrom, [2]Department of Pilot Biotechnology, Pasteur Institute of Iran, [3]Department of Biochemistry, Payam_E_Noor University, Tehran, Iran

***For correspondence: E-mail:** ghassemi_so@yahoo.com; **Tel:** +98 21 6696 88 56; **Fax:** + 98 21 6646 51 32*

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Abstract

***Purpose*:** To clone *Corynebacterium glutamicum* ATCC21799 aspartokinase gene (EC 2.7.2.4) using shuttle expression vector pEKEx2 in order to increase lysine production.
***Methods*:** *C. glutamicum* DNA was extracted and used for amplification of aspartokinase gene (ask) by cloning into an *E. coli/C. glutamicum* shuttle expression vector, pEKEx2. Initially, the recombinant vector transformed into *E. coli DH5*α and then into *C. glutamicum.*
***Results*:** Electrophoresis of recombinant protein by SDS-PAGE showed that the molecular weight of the recombinant protein was 42 KD. The induction of recombinant vector by IPTG had an inhibitory effect on cell growth due to over-expression of the cloned gene. The results of lysine assay by Chinard method showed that lysine production increased about two-fold, compared with the parent strain, as a result of increased copy numbers of *lysC* gene in recombinant strain.
***Conclusion*:** A *two-fold increase in lysine production was observed by* cloning of the ASK gene in *C. glutamicum rather than in E. coli,* due to the presence of lysine exporter channel which facilitates lysine extraction.

Keywords: *LysC* gene, *Corynebacterium glutamicum,* L- lysine, Cloning, Aspartokinase, E. coli

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INTRODUCTION

Amino acids as structural elements of proteins have been produced using microorganisms for nearly 50 years [1,2]. L-lysine, as one of the essential amino acids for human and animal nutrition, is second to L.glutamic acid in terms of large-scale production [3]. It is mainly used as a feed additive, medicament, chemical agent and nutritional supplement, and the demand for it has grown in recent years [4].

In 1957, *C. glutamicum* was recommended as an amino acid producing microorganism by Kinoshita *et al* [3]. It belongs to the large group of Gram-positive bacteria with high G+C content (about 53 - 55 %) that are isolated from soil. It is not motile and produces yellowish colonies. Today, *C. glutamicum* and its close relative *Brevibacterium flavum* are central to the industrial production of amino acids [3,5].

L-lysine is a member of aspartate family amino acids which belong to the aspartate biosynthetic

pathway [6]. This pathway in *C. glutamicum* is controlled by feedback inhibition of a few key enzymes such as aspartokinase [7]. It is the first enzyme in the aspartate pathway and is responsible for phosphorylation of aspartate [3]. It has an interesting structure and consists of two α-subunits (421 amino acid) and two β-subunits (171 amino acid).. The regulatory features of the kinase reside in the β-subunit [8]. Due to this peculiar feature, this enzyme and over-expression of the related gene (*lysC*) have been the subject of extensive research in the past. Also, literature evidence suggests that it has a vital role in lysine biosynthesis [5]. In the present study, we report the effect of *lysC* gene over-expression on lysine production in *C. glutamicum*.

EXPERIMENTAL

Bacterial strains and culture conditions

The bacterial strains and plasmids used are listed in Table 1. *E.coli DH5α* prepared from gene bank Pasteur institute of Iran was grown in Luria Bertani medium (LB) [9] and used for routine transformation. LB contains 10 g/l bacto tryptone, 5 g/l yeast extract and 5 g/l NaCl. *C. glutamicum ATCC 21799* homoserine axotroph which is resistant to lysine analog, aminoethyl cysteine, was purchased from ATCC. It was grown in LB medium as a pre-culture. Competent cells for electroporation were prepared in BHI (Brain Heart Infusion) medium consisting of 37 g/l brain heart infusion and 91 g/l sorbitol; the *C. glutamicum* cells were grown on BHI plates after electroporation.

Recombinant *C. glutamicum* was cultivated on a medium consisting of glucose 100 g/l, soy bean flour 30 g//l, ammonium sulfate 70 g/l, $MgSO_4$ 0.8 g/l, $MnSO_4$ 0.01 g/l, KH_2PO_4 1 g/l, K_2HPO_4 1 g/l, $FeSO_4$ 0.006 g/l, methionine 0.2 g/l, valine 0.2 g/l, arginine 0.2 g/l, leucine 0.1 g/l, thiamine 0.5m g/l, pantothenic acid 5m g/l, biotin 1 mg/l, as a medium to analysis the lysine production. *C. glutamicum* and *E. coli* strains were cultured at 30 and 37 °C, respectively. The organisms were cultured aerobically in 50 ml cultures in 250 ml Erlenmeyer flasks on a rotating shaker at 150 rpm. Ampicillin and kanamycin were used in culture mediums to select the bacterial strains containing cloning vectors, at concentrations of 100 and 50 mg, respectively.

DNA Preparation

C. glutamicum chromosomal DNA was isolated according to Eikmanns *et al*. (11). Plasmid DNA from *E. coli DH5*α was prepared by alkaline lysis procedure [10]. Restriction endonuclease and other enzymes were purchased from Fermentas (Germany). DNA separation and visualization were carried out with 0.6 % agarose gel electrophoresis.

Table 1: Bacterial strains and plasmids

Strain of plasmid	Genotype/description	Source
C. glutamicum ATCC 21799	Lysin-producing strain AEC^R	ATCC
E.coli DH5α	*supE44 hsdRl7 recAl endAl grA96 Thi-l relAl*	NRGB**
Ptz57R/T pEKEx2	*lacZ α* Ap^{r*} Km^{r*} *E. coli-C. glutamicum* shuttle vector For regulated gene expression (P*tac lacI*q pBL1 *oriVC.g*. pUC18 *oriVE.c*.)	Fermentase Eikmanns et al. (1991)

Km^r and Ap^r indicate resistance to kanamycin and ampicillin, respectively.
AEC^R indicates resistance to S-2-aminoethyl-L-cysteine, a lysine analog.
** *National Recombinant Gene Bank, Pasteur Institute of Iran.*

Polymerase chain reaction (PCR)

Primers were designed based on *C. glutamicom ATCC21729* aspartokinase gene (ASK) by Gene Runner (version 3.05). The forward and reverse primers were 5'GAATTCGAAAGGTGCACA AAG3' and 5'GAGCTCAACTGCGATGGTG3' which are complementary to the sequences coding for the amino and carboxyl terminal of the mature protein, respectively. Both primers have additional codons to facilitate directional cloning into the Sac1 and EcoR1 restriction endonuclase sites of pEKEx2 vector. The reaction was performed with a master cycler gradient (Biorad). Amplification reaction was carried out with 1 µl of *C. glutamicum* ATCC21799 DNA(20Ml) and 1 µl of each primers (10 µM), 2 µl of 10x PCR buffer with $MgSO_4$, 14.4 µl double distilled H_2O, 0.4 µl dntps (10 m M), 0.2 Ml of pfu DNA polymerase (1 unit) in a final concentration of 20 µl. The PCR programs were as follows: pre-denaturation 5 min at 95°C for 1 cycle; 35 cycles of 45 sec at 94°C, 45 sec at 42°C, 45 sec at 72°C; and 10 min at 72°C for 1 cycle as post-extension. PCR products confirmed with marker on agarose gel by electrophoresis.

Cloning of aspartate kinase (ASK) gene into pTZ57R/T cloning vector

Targeted gene amplification was performed by Pfu DNA polymerase with proof-reading properties to decrease errors. After extention,

PCR product was purified from gel by agarose gel extraction kit (Intron), to add a nucleotide at the 3' end , the purified product was mixed with 1 µl PCR buffer (10x), 1 µl MgCl2 . 1 µl dATP (200 µm) and 1 u taq DNA polymerase and incubated in 72 ^{o}C for 30 min. The fragment with overhang A and pTZ57R/T were mixed and ligated at 22 °C with T4 DNA ligase overnight. Efficient competent *E. coli DH5α* cells (20 µl), prepared by $CaCl_2$ procedure according to standard protocols, and were mixed with 2 µl of ligation mix for transformation. Screening was performed by culturing transformed *E. coli DH5α* on LB agar (containing 50 mg ampicillin). Recombinant plasmid DNA was extracted and sequencing was performed by dideoxy method [10].

Cloning in shuttle expression vector pEKEx2

Recombinant plasmid pTZ57R/T (containing *lysC* gene) was digested with *EcoRI* and *SacI* restriction enzymes. The resulting 1266 bp fragment was purified from an agarose gel by agarose gel extraction kit (Intron). The fragment was then sub-cloned in pEKEx2 plasmid. The ligation mixtures were transformed into efficient competent *E.coli DH5α* cells, which were prepared by heat shock method.

The presence of recombinant plasmid pEKEx2 was confirmed by PCR and the recombinant plasmid was extracted and digested with *EcoRI* and *SacI* restriction enzymes. The ligation mixtures, containing recombinant plasmid pEKEx2/lysC, were transformed into efficient competent *C. glutamicum* ATCC21799 cells [12] by electroporation. It was performed at 25 µF, 200 Ω, 2500 V.

Expression test and SDS PAGE analysis

Cultures of *C. glutamicum ATCC21799* containing plasmid pEKEx2 (with *lysC* gene) and *C. glutamicum* containing plasmid (without an insert) as a negative control were grown at 30 ^{o}C with aeration (200 rpm) for 24 h until they reached an optical density of 0.5 at 600 nm; then 15 µg/ml IPTG was added and protein expression allowed to occur by further incubation 52 h at 30 ^{o}C. Liquid cultures (5 ml each) were collected in different time points (24, 48 , 68, 72, 74, 76 h) after incubation. They were spinned down and pelleted by centrifugation at 10000 g for 20 min and the supernatant used for lysine assay. Five milliliters of the liquid culture was collected at the same time too, washed twice in 0.9 % NaCl, harvested by centrifugation, suspended in 100 mM Tris- Hcl (pH 8), 1 mM EDTA (pH 8) and sonicated with an ultrasonic apparatus with 100 % amplitude and 70 % cycle at 10 min (Hielscher). Cell debris was eliminated by centrifugation at 100000 g for 20 min. Thereafter, protein separation and visualization were carried out with 15 % SDS- PAGE.

Amino acid assay

L-lysine was analyzed by Chinard method [13]. In this method, ninhydrin was used to detect the production of lysine. Ninhydrin (2, 2-Dihydroxyindane-1, and 3- Dione) is a chemical used to detect ammonia or primary and secondary amines. On reacting with lysine at approximately pH 1, a red color develops.

Statistical analysis

The data (mean ± standard deviation) were analyzed by Student's t-test using IBM Statistics SPSS software, version 19. Differences between data were considered to be significant at $p < 0.05$.

RESULTS

lysC gene (1266 bp fragment)

lysC gene, isolated by the polymerase chain reaction (PCR) from *C. glutamicum* ATCC21799, is shown in Fig 1. In accordance with the known DNA sequence of *lysC* gene, the length of the amplified DNA fragment was calculated to be 1266 bp, including the extensions of the PCR primers.

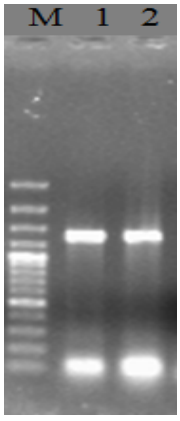

Figure 1: Agarose gel electrophoresis of PCR product obtained from amplification of *lysC* gene. Genomic DNA from *C. glutamicum* ATCC 21799 was used as template in PCR. ***Lane M*** contains a ladder as a size marker (Fermentase), ***Lane 1:*** amplified *LysC* gene with *pfu* DNA polymerase, ***Lane 2:*** amplified *LysC* gene with *Taq* DNA polymerase.

Cloning of lysC gene

Restriction analysis of the recombinant plasmids revealed that they contained the same 1266 bp

EcoRI - *SacI* DNA fragment. The recombinant pEKEx2 (contains *lysC* gene) vector was introduced into *C. glutamicum* and transformants were selected on BHI medium.

Expression of the lysC gene in C. glutamicum

The results of SDS–PAGE analysis of the proteins are shown in Fig 2. Cultures of recombinant *C. glutamicum* (*lysC* gene cloned in pEKEx2) and *C. glutamicum* containing plasmid (without an insert) as a negative control showed that the rate of growth decreased due to over-expression of cloned gene (Fig 3).
Analysis of a plot of RF versus log MW showed that the molecular weight of recombinant protein was 42 KD .

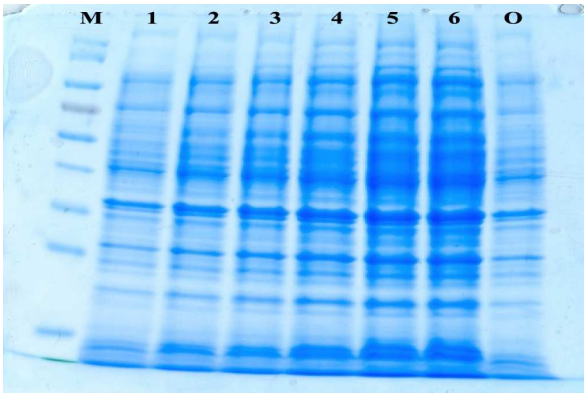

Figure 2: Electrophoretic profile of recombinant protein by SDS-PAGE. M = Molecular weight marker, O = negative control, Lanes 1 to 6 denote cell lysates from *C. glutamicum* clone expression *LysC* protein at 68,70,72,74,76 and 78 h, respectively, after induction of Isopropyl β-D-1-thiogalactopyranoside IPTG.

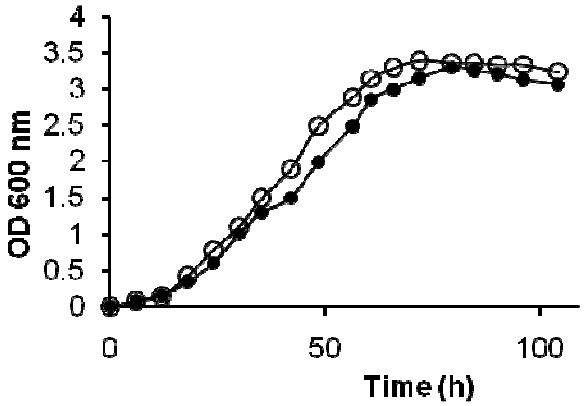

Figure 3: Comparison of growth curve of recombinant *C. glutamicum* containing pEkEx2 vector with insertion of *lysC* gene (○) with that of the parent strain without inset (●) in LB medium.

L-lysine assay

The results of lysine assay shown that the rate of lysine production in recombinant, compared to the wild strain (Fig. 5), increased about two fold even during the growing and stationary phases.

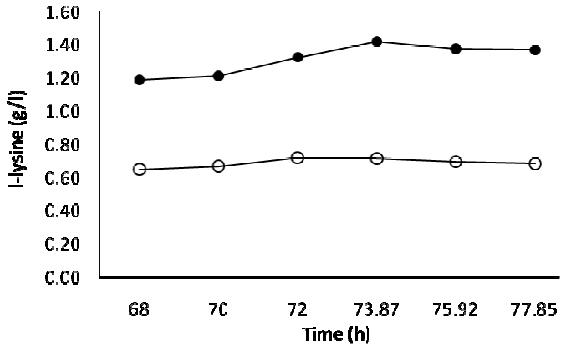

Figure 5: Comparison of the production of l-lysine in recombinant *C. glutamicum* (●) and parent strain (○) after 68, 70, 72, 74, 76 and 78 h culture.

DISCUSSION

The purpose of the present study was to clone *Corynebacterium glutamicum* ATCC21799 aspartokinase gene (EC 2.7.2.4) using shuttle expression vector pEKEx2, for the first time, in order to increase lysine production. Good amino acid producers have been isolated in the past by classical and random mutagenesis [5]. In recent years, physiology, biochemistry and molecular biology of amino acid biosynthesis in *C. glutamicum* have been studied intensively. This organism is known as a potent amino acid producing bacterium [3] and the regulation of amino acid biosynthesis pathways in this organism is much simpler than in others [6]. A series of experiments identified aspartokinse as a potential lysine-limiting step together with lysine exporter and diyhydrodipicolinate synthase. Indeed, the flux control coefficient for ASK is higher than those for others [5, 14]. The gene for the feedback resistant aspartokinase alone is enough to achieve lysine secretion in the wild type [15].

C. glutamicum ATCC21799 was used because it has feedback-resistant aspartate kinase and all pathways that biosynthesize other amino acids are blocked. Thus, it would be effective in the production larger amounts of lysine. Purification of recombinant lysine is a major problem in other microorganisms, such as *E.coli* strains, because of lysine accumulates in cells; in contrast, *C. glutamicum* has a lysine exporter channel and lysine can be extracted from the medium [16], and in continuous production processes, the bacterium cells can remain alive for a longer time, and thus gives better results.

It has been reported that over-expression of feedback resistant ASK produces more lysine

than the parent strain [5, 15]. In our study, after the introduction of the cloned gene into *C. glutamicum*, lysine assay with Chinard method showed that the rate of lysine production was higher in the recombinant strain, and also about two-fold increase in the specific activity of *lysC* gene was observed. This finding is in agreement with the other studies [5, 15].

The induction of cloned gene by IPTG has been shown to have an inhibitory effect on cell growth due to over-expression of the cloned gene product [17,18]. As a result, higher copies and higher activity of ASK yielded higher lysine productivity, albeit at the expense of reduced cell growth, as reported in a previous study [5]. The induction of recombinant strain by higher IPTG concentration (250 µl) has been shown not to result in larger amounts of clone gene product. It thus seems that over-expression of enzymes in the product pathway gives rise to an inverse relation between flux increase towards product and growth limitation.

In order to explain this type of physiological behavior, some metabolic models have been proposed [5]. As a result, attenuation in growth is observed in ASK over-expressing recombinant strains. It is proposed that it is possible to counterbalance the negative effect in growth from one gene (ASK) over-expression by increasing anaplerotic activity through coordinated over-expression of more than one gene in the pathway, for example, ASK and pyruvate carboxylase (pyc) [5]. Increase in the number of copied genes in lysine biosynthesis pathway and increase in their expression using optimized medium and conditions and stronger promoters [4] would increase lysine production rate. These stronger promoters can also increase production rate in the fermentation process. Hirono *et al*. expressed the most important enzymes which play an important role in lysine biosynthesis pathway; they are diaminopimelate decarboxylase, dihydrodipicolinate reductase, diaminopimelate dehyrogenase and dihydrodipicolinate synthase [19].

CONCLUSION

We found in this study genetic engineering of *lysC* resulted in overexpression of aspartokinase and a two-fold increased lysine production as a consequence of redistribution of the fluxes at aspartokinase bottleneck in the lysine biosynthesis pathway. In the near future, it is expected that the genetic changes in lysine production pathway will provide a significant improvement in the regulatory process involved in amino acid overexpression by *C. glutamicum* especially in continuous fermentation processes because of the secretory features of some cell membrane proteins. Lysine has been widely supplied on an industrial scale by fermentation process using mutants of *C. glutamicum*.

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