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Improved Production of L-lysine by Over-expression of *Meso*-diaminopimelate Decarboxylase Enzyme of *Corynebacterium glutamicum* in *Escherichia coli*

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Abstract: The aim of this study is over-expression of *Meso*-diaminopimelate decarboxylase enzyme (EC 4.1.1.20) and enhancement of L-lysine production rate. The *C. glutamicum LysA* gene which encodes a *Meso*-diaminopimelate decarboxylase was cloned in *E. coli*. The cloned gene was sequenced; it encodes a 445 amino acids protein with molecular weight of 47 kD. Expression of the *LysA* gene in *E. coli* resulted in an increase in *Meso*-diaminopimelate decarboxylase activity, correlated with the presence in sodium dodecyl sulfate-polyacrylamid gels of a clear protein band that corresponds to this enzyme. 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. A two fold increase in lysine production rate was observed after introduction of the cloned gene into *E. coli*.

Key words: *LysA* gene, *C. glutamicum*, diaminopimelate decarboxylase, L-lysine

INTRODUCTION

L-Lysine has been produced with the aid of microorganisms for about 50 years (Hirose *et al*., 1985; Eggeling and Sahm, 1999). *Corynebacterium glutamicum* and *Bervibacterium lactofermentum* are *Coryneform bacteria* being used for the industrial production of L-lysine (Tryfona and Mak, 2005).

In 1957, Kinoshita discovered a potent amino acid-producing microorganism, *C. glutamicum* (initially named *Micrococcus glutamicum*), which provided a novel method for producing natural amino acids (Eikmanns *et al*., 1991). *C. glutamicum* is a Gram-positive, non-sporulating bacterium that is isolated from soil. It is not motile, with pleomorphic short rods producing yellowish colonies and having a DNA G+C content of 53-55% and requires biotin for growth.

L-lysine is mainly used as a feed additive in animal food industry, mixed with various common livestock's feed such as cereals which do not contain sufficient levels of L-lysine for the livestock's nutritional requirements (Anastassiadis, 2007). Furthermore it is used as a supplement in human diet, improving the food quality by increasing the absorption of other amino acids (Anastassiadis, 2007).

Lysine is an essential amino acid and belongs to aspartate biosynthetic pathway (Wittmann and Becker, 2007) which is responsible for biosynthesis of aspartate, asparagine, methionine, threonine, lysine and isoleucine (Grace *et al*., 1995). In *C. glutamicum* ATCC 21799 all pathways that biosynthesis other amino acids are blocked.

The bacteria synthesizes lysine through a pathway whose final step is the decarboxylation of *meso*-diaminopimelate (*meso*-DAP) (Wittmann and Becker, 2007). The so called DAP pathway has been well characterized in *Escherichia coli* (Cremer *et al*., 1991; Guillouet *et al*., 1999).

Meso-DAP decarboxylase catalyses the production of L-lysine from *meso*- DAP in *C. glutamicum*. *Meso*-DAP decarboxylase is a mainly important enzyme that over-expression of its gene (*lysA*) increases the L-lysine production (Hirono *et al*., 2000).

In this study, *lysA* gene was cloned to increase the production rate of L-lysine.

MATERIALS AND METHODS

This study was conducted at Pilot Biotechnology Department of Pasteur Institute of Iran in 2009.

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Table 1: Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source
C. glutamicum	Lysine-producing strain AEC^R	ATCC
E. coli DH5α	*supE44 hsdR17 recA1 endA1 grA96 thi-1 relA1*	NRGB**
E. coli BL21 (DE3)	*F– ompT hsdSB (rB–mB–) gal dcm (DE3)*	NRGB
Plasmid		
pTZ57R/T	*lacZα Ap*r*	Fermentase
pET28a	Km^r*	NRGB

*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

Strains, plasmids and media: The bacterial strains and plasmids used are listed in Table 1; *C. glutamicum* ATCC 21799 is a lysine producer which is resistant to S-2-amino ethyl-L-cysteine, a Lysine analog. The complex medium used for all strains was Luria-Bertani broth (Imaizumi *et al*., 2005). LB contains 10 g L^{-1} bacto tryptone, 5 g L^{-1} yeast extract and 5 g L^{-1} NaCl. *C. glutamicum* and *E. coli* strains were cultured at 30°C and 37°C, respectively. The organisms were cultured aerobically in 50 mL cultures in 250 mL Erlenmeyer flasks on a rotator shaker at 150 rpm. Ampicillin and kanamycin were used at the concentrations of 100 and 50 mg, respectively.

DNA isolation and manipulation: Total DNA from *C. glutamicum* was isolated by 2.5 mg mL^{-1} lysozyme and 1% Sodium Dodecyl Sulfate (SDS). Plasmid DNA from *E. coli* DH5α was prepared by alkaline lysis procedure (Maniatis *et al*., 1982). Restriction endo-nuclease and other enzymes were obtained from Fermentase. DNA separation and visualization were carried out with 0.6% agarose gels by standard methods.

PCR: In order to amplify *LysA* gene-specific fragment from genomic *C. glutamicum,* two polynucleotides were designed by Gene Runner software. The sequence of the coding strand primer is 5′GCTAGCGAAGATGTA ACAATGGC3′. It has an extension of six nucleotides at the 5′ end which completes a *NheI* restriction site (underlined). The sequence of the complementary strand is 5′AAGCTTAAGAAACCCAGAAACCC3′. It also has an extension of six nucleotides at the 5′ end which completes a *HindIII* site (underlined).

PCR was performed with a 20 μL mixture containing 2 μL PCR buffer (10 x), 0.2 mM (each) dNTP, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 1 μg of template DNA and 1 U of the *pfu* DNA polymerase. The temperature profile of the 30 cycles of PCR was 45 sec at 95°C, 45 sec at 68°C and 170 sec at 72°C. PCR products (5 μL of a 20 μL reaction mixture) were analyzed on 1.0% agarose gels. PCR-amplified DNA was separated from excess nucleotides and primers by gel extract kit of Intron.

DNA techniques: *pfu* DNA polymerase has not extension activity in PCR so it cannot be cloned in pTZ57R/T plasmid. Gel extracted product was mixed with 10 μL mixture of 200 μM dATP, 1 U *Taq* DNA polymerase, 1.5 mM $MgCl_2$ and 1 μL PCR buffer (10x). The temperature of cycle was 30 min at 72°C. Then the fragment with overhang A and pTZ57R/T were mixed and ligated at 22°C with T_4 DNA ligase overnight. The ligation mixtures were transformed into *E. coli* DH5α in order to amplify the gene. Recombinant plasmid DNA was isolated after transformation of *E. coli* DH5α with alkaline lysis procedure (Maniatis *et al*., 1982). Sequencing was performed by the dideoxy method (Maniatis *et al*., 1982).

Cloning in expression vector: Recombinant plasmid containing *lysA* gene was digested with *HindIII* and *NheI*. The resulting 1432 bp fragment was purified from an agarose gel by agarose gel extraction kit (Intron). The fragment was then sub-cloned in pET28a plasmid. The ligation mixtures were transformed into *E. coli* BL21 (DE3). *E. coli* BL21 (DE3) was competented by $CaCl_2$ method (Maniatis *et al*., 1982).

Gene expression in *E. coli*: Cultures of recombinant *E. coli* BL21 (DE3) (*lysA* gene cloned in pET28a) and *E. coli* BL21 (DE3) containing plasmid pET28a (without an insert) as a negative control were grown at 37°C with aeration (150 rpm) for 3 to 4 h, until they reached an optical density of 0.5 at 600 nm and then 0.1 mM of IPTG was added and protein expression was allowed to occur by 4h incubation at 37°C. Cultures were 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, 70% cycle at 5 min (Hielscher). Cell debris was eliminated by centrifugation at 100000 g for 20 min. Then protein separation and visualization were carried out with 12% SDS-PAGE.

Amino acid assay: L-Lysine was analyzed by Chinard (1952) method. In this method, ninhydrin would be 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. When reacting with lysine at pH about 1, a red color would be evolved.

RESULTS

PCR amplification and cloning of the *C. glutamicum* ATCC 21799 *LysA* gene: Two degenerated olignucleotides were designed in order to be amplified by PCR. As shown in Fig. 1, amplification with genomic DNA from *C. glutamicum* ATCC 21799 as a template produced specific DNA fragment with identical size.

In accordance with the known DNA sequence of *LysA* gene, the length of the amplified DNA fragment was calculated to be 1432 bp, including the extensions of the PCR primers. The PCR product obtained from *C. glutamicum* genomic DNA was cloned in pTZ57R/T and the DNA sequence was determined.

Cloning of the *LysA* fragment: A gene of *C. glutamicum* was expressed in *E. coli*. *LysA* catalyzes the reaction of L-lysine formations from *meso*-DAP. Cloning of the *LysA* gene was done as follows: recombinant pTZ57R/T (containing *LysA*) was cleaved with *HindIII* and *NheI* and fragment of approximately 1432 bp were ligated with *HindIII* and *NheI* digested vector pET28a. After transformation with the ligation products, the transformants were selected on LB medium containing kanamycin. Restriction analysis of the plasmids isolated from six clones revealed that they all contained the same 1432 bp *HindIII-NheI* DNA fragment.

Expression of the *LysA* in *E. coli* BL21 (DE3): SDS-polyacrylamid gel electrophoresis analysis of the proteins has been shown (Fig. 2). Cultures of recombinant *E. coli* BL21 (DE3) (*lysA* gene cloned in pET28a) and *E. coli* BL21 (DE3) containing plasmid pET28a (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 Rf versus log MW plot showed that the molecular weight of recombinant protein was 50.29 kD (Fig. 4).

L-Lysine assay: *E. coli* BL21 (DE3) containing recombinant plasmid (pET28a containing *LysA* gene) was cultured in LB medium for the measurement of lysine production. The results showed significantly that the rate of lysine production in recombinant strain compared with the wild strain (Fig. 5) increased even during the growing and stationary phases.

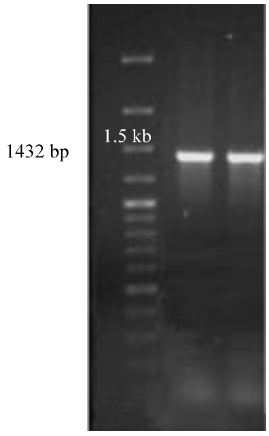

Fig. 1: Agarose gel electrophoresis of PCR product obtained from amplification of *LysA* gene. Genomic DNA from *C. glutamicum* ATCC 21799 was used as template. Lane M contains a ladder as a size marker (Fermentase), Lane 1: amplified *LysA* gene with *pfu* DNA polymerase, Lane 2: amplified *LysA* gene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

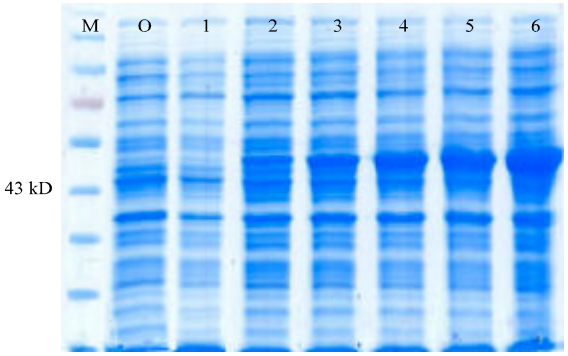

Fig. 2: Electrophoretic profile of recombinant protein by SDS-PAGE. M: Molecular weight marker, O: negative control, lane 1 to lane 6, cell lysates from *E. coli* clone expression *LysA* protein at 0, 60, 120, 180, 240 and 300 min after induction of IPTG

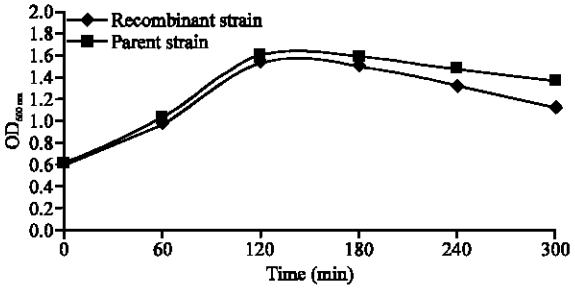

Fig. 3: Growth curve of recombinant *E. coli* BL21 (DE3) (*lysA* gene cloned in pET28a), compare with negative control

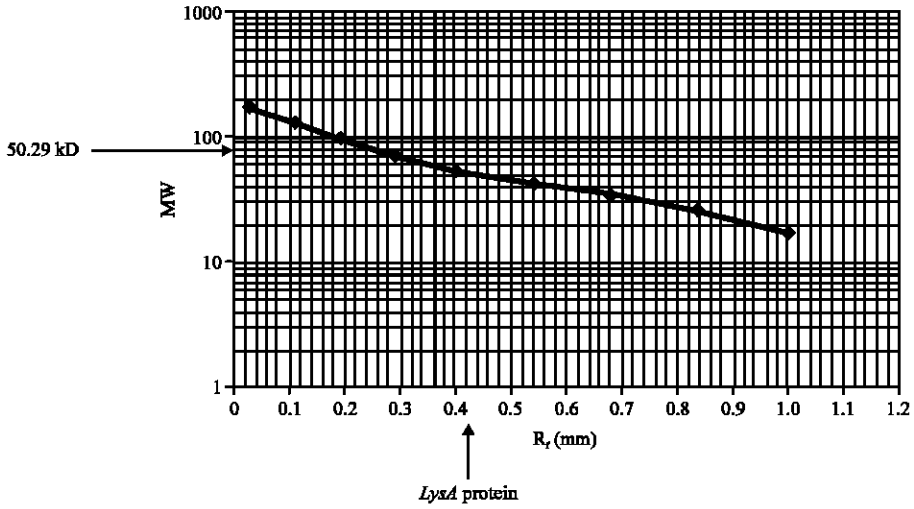

Fig. 4: Molecular weight of recombinant protein in SDS-PAGE

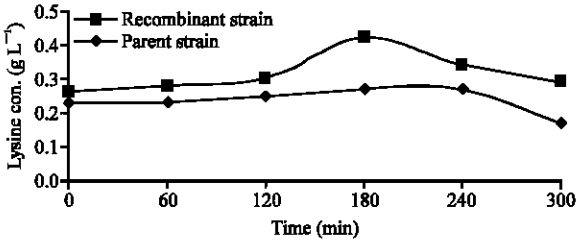

Fig. 5: The effect of cloned gene on L-lysine production in *E. coli*

DISCUSSION

We report here the cloning of the *LysA* gene of *C. glutamicum* in *E. coli*. The plasmid harboring the cloned gene directed the synthesis of *LysA* in *E. coli*.

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 (Bentley and Kompala, 1991; Tsuchiya and Morinaga, 1988). A two-fold increase in the specific activity of *LysA* was observed after the introduction of the cloned gene into *E. coli*. The induction of recombinant strain by high IPTG concentration (250 μL) has been shown to have not more clone gene product.

TAA is stop codon of 3'end of LysA gene. Analysis with plot of R_f versus log MW showed that the molecular weight of recombinant protein is 50.29 kD which indicates that His-taq sequence has been attached to C-terminal of recombinant protein.

Lysine assay with Chinard method showed that the rate of lysine production was increased in recombinant strain cultured in LB medium.

In this study for the first time, the expression rate of *Meso*-diaminopimelate decarboxylase enzyme in expression vector was investigated and was increased significantly.

After cloning, with respect to the increase in expression of *lysA* gene in *E. coli*, it could be expected that the mentioned increase in *C. glutamicum* would also lead to the increase in lysine production rate. In some studies, this increase has been reported about fifteen fold of increase in enzymatic activity (Patriee *et al*., 1988).

Increase in the number of copied genes in lysine biosynthesis pathway and increase in their expression by using stronger promoters, would increase the lysine production rate. These stronger promoters could also increase the production rate in fermentation process (Anastassiadis, 2007).

Hirano *et al*. (2000) expressed the most important enzymes which play an important role in lysine biosynthesis pathway as: diaminopimelate decarboxylase, dihydrodipicolinate reductase, diaminopimelate dehydrogenase and dihydrodipicolinate synthase. Our studies also showed the importance of these enzymes.

Lysine has been widely supplied on an industrial scale by the fermentation process using mutants of *C. glutamicum*. We have an interest in its effect on lysine productivity and the introduction of the gene into some lysine-producing mutants. The results will give significant information on strain improvement.

REFERENCES

Anastassiadis, S., 2007. L-Lysine fermentation. Biotechnology, 1: 11-24.

Bentley, W. and S. Kompala, 1991. Dynamics of induced CAT expression in *E. coli*. Biotechnol Bioeng., 38: 749-760.

Chinard, F.P., 1952. Photometric estimation of Proline and Ornithine. Bio. Chem., 199: 91-95.

Cremer, J., L. Eggeling and H. Sahm, 1991. Control of the lysine biosynthesis sequence in Corynebacterium glutamicum as analyzed by overexpression of the individual corresponding genes. Applied Environ. Microbiol., 57: 1746-1752.

Eggeling, L. and H. Sahm, 1999. L-glutamate and L-lysine: Traditional products with impetuous developments. Applied Microbial. Biotechnol., 52: 146-153.

Eikmanns, B.J., M. Kricher and D.J. Reinscheid, 1991. Discrimination of corynebacterium glutamicum, brevibacterium flavum and brevibacterium lactofermentum by restriction pattern analysis of DNA adjacent to the hom gene. FEMS Microbiol. Lett., 82: 203-208.

Grace, E.N.C., S.M.J. Mike, T.N. Thuchien, E.G. Marcel, T.F. Maximillian, J.S. Anthony and S. Gregory, 1995. Effect of Inducible thrB Expression on Amino Acid Production in Corynebacterium lactofermentum ATCC 21799. Applied Environ. Microbiol., 61: 74-78.

Guillouet, S., A.A. Rodal, G.H. An, P.A. Lessard and A.J. Sinskey, 1999. Expression of the escherichia coli catabolic threonine dehydratase in corynebacterium glutamicum and its effect on isoleucine production. Applied Environ. Microbiol., 65: 3100-3107.

Hirono, S., M. Sugimoto, E. Nakan, M. Izui, A. Hayakawa, Y. Yoshihara and T. Nakamatsu, 2000. Method of producing L-lysine. United States Patent, 6090597. http://www.freepatentsonline.com/6090597.html.

Hirose, Y.H., H. Encei and H. Shibai, 1985. L-Glutamic Acid Fermentation. In: Comprehensive Biotechnology, Moo-Young, M. (Ed.). Vol. 2, Pergamon Press, New York, pp: 593-600.

Imaizumi, A., R. Takikawa, C. Koseki, Y. Usuda and H. Yasueda *et al*., 2005. Improved production of l-lysine by disruption of stationary phase-specific rmf gene in *Escherichia coli*. J. Biotechnol., 117: 111-118.

Maniatis, T., E.F. Fritsch and J. Sambrook, 1982. Molecular Cloning: A Laboratory Manual. Vol. 2, Cold Spring Harbor University Press, Cold Spring Harbor, New York, pp: 545.

Patriee, Y., A.M. Sicard and A.J. Sinskey, 1988. Nucleotide sequence of the lysA gene of corynebacterium glutamicum and possible mechanisms for modulation of its expression. Mol. Gen. Genet., 212: 112-119.

Tryfona, T. and T.B. Mark, 2005. Fermentative production of lysine by *Corynebacterium glutamicum*: Transmembrane transport and metabolic flux analysis. Process Biochemis., 40: 499-508.

Tsuchiya, M. and Y. Morinaga, 1988. Genetic control systems of *Escherichia coli* can confer inducible expression of cloned genes in coryneform bacteria. Biotechnology, 6: 428-430.

Wittmann, C. and J. Becker, 2007. The L-Lysine Story: From Metabolic Pathways to Industrial Production. In: Amino Acid Biosynthesis-Pathways, Regulation and Metabolic Engineering, Wendisch, V.F. (Ed.). Springer, Berlin, Heidelberg pp: 39-70.