American Journal of

Biochemistry and Molecular Biology

ISSN 2150-4210

Academic
Journals Inc.

www.academicjournals.com

OPEN ACCESS American Journal of Biochemistry and Molecular Biology

ISSN 2150-4210
DOI: 10.3923/ajbmb.2016.33.44

Research Article
Enhancing the Activity of Aspartate Kinase for an Overproduction of L-lysine by *Corynebacterium glutamicum*

[1]B. Viswanath, [2]S. Rajagopal, [2]P.A. Basha, [3]D.M. Rao, [4]P.S. Begum, [5]D. Rajakumari and [2]M.A. Razak

[1]Department of Bionanotechnology, Gachon University, San 65, Bokjeong Dong, Sujeong Gu, Seongnam Si, 461 701 Gyeonggi Do, Republic of Korea
[2]Department of Biotechnology, Rayalaseema University, 518002 Kurnool, Andhra Pradesh, India
[3]Department of Biotechnology, Sri Krishanadevaraya University, 515 003 Anantapur, Andhra Pradesh, India
[4]Department of Zoology, K.V.R. Govt College for Women, 518002 Kurnool, Andhra Pradesh, India
[5]Department of Zoology, Sri Venkateswara University, 517502 Tirupati, Andhra Pradesh, India

Abstract

Background: As in all the bacteria investigated, lysine biosynthesis in *Corynebacterium glutamicum* starts from aspartate and aspartate kinase is the principal enzyme involved in the lysine biosynthesis metabolic pathway. In *C. glutamicum*, this enzyme was regarded as potential target for improved production of lysine. Here the increased production of lysine by deregulating aspartate kinase gene in wild type *C. glutamicum* ATCC 13032 was described. **Materials and Methods:** Key regulatory enzyme aspartate kinase of *C. glutamicum* was manipulated by performing site-directed mutagenesis. The region coding for regulatory β-subunit in the aspartate kinase (lys C) gene was mutated by replacing the codon TCC-GTC to deregulate it from feedback inhibition, which resulted in improved lysine production. **Results:** About 4.2 g L^{-1} of more lysine yield was observed in the recombinant mutant compared to the wild type and the studies proved recombinants of *C. glutamicum* with feedback resistant aspartate kinase would be a potential option to increase the L-lysine production by biotechnological process. The mutant lysine producing strain reported in this investigation can be economical for industrial application. **Conclusion:** It was concluded that the metabolic engineering strategy reported here could serve as good concept for the development of microorganisms as capable cellular factories for the production of industrially important metabolites.

Key words: Aspartate kinase, *C. glutamicum*, feedback inhibition, L-lysine, metabolic engineering

Received: February 11, 2016 Accepted: March 07, 2016 Published: March 15, 2016

Citation: B. Viswanath, S. Rajagopal, P.A. Basha, D.M. Rao, P.S. Begum, D. Rajakumari and M.A. Razak, 2016. Enhancing the activity of aspartate kinase for an overproduction of L-lysine by *Corynebacterium glutamicum*. Am. J. Biochem. Mol. Biol., 6: 33-44.

Corresponding Author: M.A. Razak, Department of Biotechnology, Rayalaseema University, 518002 Kurnool, Andhra Pradesh, India

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Among the industrially important amino acids, L-lysine is in 1st position, which is used in pharmaceuticals, animal, human feeds and precursors for the production of peptides or agrochemicals[1]. As L-lysine has large applications, the demand for it is increasing constantly year by year. To minimize the gap between increasing demand and production of L-lysine, it has to be produced in large scale. The L-lysine plays a major role in building muscle protein, calcium absorption, recovering from surgery or sports injuries, body's production of hormones, enzymes, antibodies and in the treatment of Herpes simplex infection. As it is widely used in pharmaceuticals, cosmetics and polymer materials the market shows a growth potential[2] of 7-10% year^{-1}. From the total L-lysine manufactured commercially, about 80% is produced by biotechnological processes and 20% by chemical synthesis[3]. All traditional lysine amino acid producers have been created over many years by multiple rounds of random mutagenesis and selection[4]. Due to the unavoidable accumulation of undesired mutations during strain development, they typically showed growth deficiencies, low stress tolerance and by product formations that limit their production efficiency[5,6]. From the past 10 years the development of recombinant DNA techniques and detailed understanding of the biochemistry of metabolic reactions has increased the identification of genetic targets for improved lysine production including lysine biosynthesis[7-9]. The *C. glutamicum* is Gram positive, fast growing, non motile, non-sporulating micro-organism, which has been isolated from soil as natural producer of glutamate producer[10]. The *C. glutamicum* from long period has been used for the industrial production of various amino acids, primary metabolites and nucleotides. it is presently employed for the annual production of 2,160,000 t of glutamate and 1,480,000 t of lysine[11-14]. Many study efforts are being made to explore its potentiality as commodity chemical producer[15] and for its bioremediation application[16]. The complete genome sequence of two variants of *C. glutamicum* ATCC 13032 have been published[17,18], this genome sequence information has great impact for metabolic engineering of *C. glutamicum* for increased production of amino acids and vitamins[19]. Metabolic engineering has developed as a very powerful approach for redirection of metabolic pathways using genetic manipulation. In early 1980s it emerged as the new engineering science for directed modification of cellular properties and metabolism by modification of metabolic pathways using molecular biological techniques and recombinant DNA technology. Metabolic engineering along with omics technologies such as transcriptomics led to the production of a various amino acids, alcohols, organic acids, diamines and carotenoids[20]. Metabolically engineered products produced by *C. glutamicum* are succinate, proline, serine, α-ketoglutarate, lactate, pyruvate, pantothenate, isobutanol, 2-ketoisovalerate, 1,5-diaminopentane, various carotenoids, 1,4-diaminobutane, crude glycerol, hydrolysates, starch, amino sugars and cellubiose[21-26]. The tool box for metabolic engineering in *C. glutamicum* has been greatly expanded in the recent years. Aspartate Kinase (AK) (EC 2.7.2.4) is the enzyme that catalyzes the transfer of the C-phosphate group of ATP to aspartic acid. In most bacteria, the reaction is the first step of branched biosynthetic pathway for lysine, threonine, isoleucine and methionine and is known to be regulated by the end metabolites through feedback inhibition. For example, aspartate kinase from *Corynebacterium glutamicum* is concertedly inhibited by lysine and threonine, while aspartate kinase I and III from *Escherichia coli* is inhibited by threonine and lysine, respectively. Through metabolic engineering, strains were developed which have deregulated aspartate kinase. This was achieved by genetic engineering the 18 kDa β-subunit of the aspartate kinase enzyme which is very much essential for the high lysine and threonine producing strains. Overall, carbon flux in the lysine biosythesis pathway is regulated at two points: First, through feedback inhibition of aspartate kinase by the levels of both L-threonine and L-lysine and second through the control of the level of dihydrodipicolinate synthase. Therefore, increased production of L-lysine may be obtained in *Corynebacterium* species by deregulating and increasing the activity of these two enzymes. So aspartate kinase was chosen for the present study. In the present investigation metabolic engineering of the L-lysine biosynthesis pathway of *C. glutamicum* ATCC 13032 was done by deregulating the aspartate kinase enzyme. Amino acid substitution was done in the β-subunit of the aspartate kinase enzyme to make it deregulated, which resulted in increased production of L-lysine. Oligonucleotide directed site specific mutagenesis is the powerful tool adopted in the present study along with PCR method to generate point mutation in aspartate kinase gene to deregulate it.

MATERIALS AND METHODS

The wild-type *C. glutamicum* ATCC 13032 (American Type and Culture Collection, Manassas, USA) was used as the parent strain for metabolic engineering. The *C. glutamicum* ATCC 13032 was cultured on agar slopes containing peptone

(5 g), beef extract (3 g), NaCl (5 g), agar (15 g) and distilled water (1000 mL) was maintained at pH-7. *Escherichia coli* strain DH5α was used as a standard cloning host, while *E. coli* BL21strain was used as a host for recombinant expression of the aspartate kinase proteins. Vector pTZ57R/T (Fermentas, Germany) and vector pET-20b (+) (Fermentas, Germany) were used in this study. The purified PCR product was ligated into vectors according to the instructions of InsTAclone™ PCR cloning kit (Fermentas, Germany). The *E. coli* strains were generally grown at 37°C in liquid or solid luria-bertani (LB) medium supplemented with chloramphenicol (15 g mL^{-1}) or ampicillin (200 g mL^{-1}). Recombinant *E. coli* procedures were performed as protocol of Sambrook and Russell[27]. For production of AK proteins, overnight cultures of recombinant *E. coli* cells were grown at 37°C in LB medium until the optical density at 600 nm (OD600) was 0.6. Recombinant expression was induced by adding 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG) and then cells were grown at 25°C for 2 h before the growth temperature was changed to 16°C and cells were grown overnight. Cells were harvested by centrifugation (7,000 g, 15 min) and the pellets were stored at -20°C.

Overall experiment procedure: Cultured and propagated *C. glutamicum* ATCC 13032 strain on nutrient agar plates at 37°C. Fermentation studies were carried out for wild type *C. glutamicum* ATCC 13032. And later the following molecular biology study was carried out. Total genomic DNA was isolated by CTAB method. On the basis of sequence of lys C gene, gene specific primers are designed. Amplification of lys C gene by using PCR. After PCR amplification, the amplified PCR products were resolved by agarose gel electrophoresis. The PCR product's was cloned into TA cloning vector and expression vector pET-20b (+). The cloning and expression vectors with plasmids containing aspartate kinase gene was transformed into suitable competent cells (*E. coli* DH5α). To confirm the presence of aspartate kinase gene in expression vector restriction digestion was done. Selection and screening of active aspartate kinase expressing clones was done. The proteins are extracted from the above clones. The expression of the lys C was checked by SDS PAGE. The RNA was isolated from the active aspartate kinase expressing clones by using TRIzol® Max™ Bacterial RNA isolation kit and cDNA was synthesised from the isolated RNA and the synthesised cDNA was used for RT-PCR studies. The RT-PCR was performed to check the expression profile of aspartate kinase gene. Plasmid was isolated from the active aspartate kinase expressing clones. The isolated plasmid which posses the desired gene was used to perform site directed mutagenesis. Site directed mutagenesis was done in aspartate kinase gene by using Quick-change™ site directed mutagenesis kit. Mutagenic primers are used to create mutagenesis. The mutated (feedback resistant lys C) gene was transformed into XL1-blue supercompetent cells provided by Quick-change™ site directed mutagenesis kit. The XL1-blue supercompetent cells repair the nicks which are formed in the mutated plasmid during incorporation of the mutagenic primers. The mutated plasmid was isolated from the XL1-blue supercompetent cells and ligated into expression vector and transformed to *E. coli* DH5α. The mutated plasmid isolated from the *E. coli* DH5α cells was sent for sequencing to confirm the mutation. The mutated aspartate kinase gene containing plasmid isolated from the *E. coli* DH5α cells was transformed into *C. glutamicum* ATCC 13032 by electroporation method. The proteins are extracted from the above transformants which contain the feedback resistant lys C gene. The expression of the mutated lys C was checked by SDS-PAGE. The RNA was isolated from the active aspartate kinase expressing transformants and cDNA was syntheised from the RNA and cDNA was used for RT-PCR studies. The RT-PCR was performed to check the expression levels of mutated lys C gene. For confirming the transformation of mutated plasmid into the transformants the plasmid was isolated from them and sent for sequencing. Screening of transformants with FBR ASK gene was done by growing them on S-(2-aminoethyl)-L-cysteine. Fermentation studies are carried out on the recombinant strain, which posses feedback resistant ASK gene.

Chemicals: Glucose, yeast extract and tryptone were obtained from Difco Laboratories (Detroit, USA). All other chemicals were purchased from Sigma, Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland) and were of analytical grade.

Fermentation studies of *C. glutamicum* ATCC 13032 and recombinant mutant: The fermentation procedure described here was performed with both wild type and mutant *C. glutamicum.* Two loops of *C. glutamicum* cells were grown on nutrient agar strain maintenance plate for 24 h at 30°C were inoculated into 20 mL of Rich medium (Seed broth) in a 250 mL conical flask and cultivated for 24 h in orbital shaker at 30°C with 120 rpm. The seed broth was transferred to 180 mL of inoculum medium in an Erlenmeyer flask and was kept in orbital shaker at 30°C with 120 rpm for 40 h. This inoculum medium (Inoculum) was used to inoculate the fermentation medium in the new Brunswick bioreactor of 5 L and batch fermentation was carried out for 120 h. The aeration rate was set to 1.5 vvm by the integrated gas flow controller. A pH electrode (Mettler Toledo, Giessen, Germany) and automated

addition of 25% NH_4OH was used to maintain the pH at 7. The added volume was determined gravimetrically (Lab Balance Cupis, Sartorius, Gottingen, Germany). Dissolved oxygen was determined using a pO_2 electrode (Mettler Toledo, Giessen, Germany) and by variation of the stirrer speed. This was controlled by the process control software BaseLab (BASFSE, Ludwigshafen, Germany). Temperature was maintained at 30°C using jacket cooling. The CO_2 and O_2 in the exhaust gas were analyzed by a mass spectrometer. All processes data were monitored online and recorded by BaseLab. The composition of media's which were used in present fermentation process was as follow:

Rich medium: Glucose (10 g), yeast extract (10 g), peptone (10 g), NaCl (2.5 g) and distilled water (1.0 L). And the pH is maintained at 7.0.

Inoculum medium: $CaCl_2.2H_2O$ (1 g), $(NH_4).2SO_4$ (30 g), $MgSO_4.7H_2O$ (0.4 g), NaCl (0.05 g), $MnSO_4.H_2O$ (0.0076 g), $FeSO_4.7H_2O$ (0.001 g), KH_2PO_4 (0.5 g), K_2HPO_4 (0.5 g), Urea (2 g), yeast extract (1 g), peptone (1 g), D-glucose (10 g), thiamine (0.2 mg), D-biotin (0.5 mg) and distilled water (1.0 L). The pH is maintained at 7.0.

Fermentation medium: $CaCl_2.2H_2O$ (1 g), $(NH_4).2SO_4$ (30 g), $MgSO_4.7H_2O$ (0.4 g), NaCl (0.05 g), $MnSO_4.H_2O$ (0.0076 g), $FeSO_4.7H_2O$ (0.001 g), KH_2PO_4 (1 g), K_2HPO_4 (1 g), Urea (2 g), yeast extract (1.5 g), peptone (2 g), D-glucose (100 g), thiamine (0.2 mg), D-biotin (0.5 mg), L-serine (0.1 mg) and distilled water (1.0 L). The pH is maintained at 7.0.

Fermentation conditions: The optimized operational conditions for maximal yield and productivity of L-lysine by free cells of *C. glutamicum* were followed according to Razak and Viswanath[28,29].

Analytical estimation of L-lysine and substrates: Cell concentration was determined photometrically at 660 nm. Supernatant from batch fermentation was used for quantification of substrates and products were obtained by separation of the biomass by centrifugation (8500 g, 5 min, 4°C). The L-lysine was estimated by the method of Chinard[30]. Glucose concentration was determined by anthrone method[31,32]. Residual sugar was determined as glucose in the supernatant fluid by the colorimetric DNS method of Miller[33]. Biomass in the broth was estimated in 1 mL of the sample by centrifugation and dried in an oven at 105°C until constant cell weight obtained. Bradford[34] method was used for determining protein concentration by using bovine serum albumin as a standard.

Cloning and expression of aspartate kinase gene: Genomic DNA from the *C. glutamicum* ATCC 13032 and mutant was isolated by CTAB method. The quantity and quality of the isolated DNA was checked in UV-Vis spectrophotomer (Eppendorf Biophotometer). Based on the gene sequence of aspartate kinase, gene specific primers were designed manually to amplify aspartate kinase gene. The primers were synthesized from Eurofins (MWG), Bangalore, India. The sequences of the primers used are forward primer-GAATTCCCGTTCCGCCAGCTCGTGAAATG and reverse primer GCGGCCTTGCACCAACAACTGCGATGTGG. The aspartate kinase gene was amplified using forward and reverse primers, which are specific for aspartate kinase gene in a gradient thermal cycler (Labnet USA). The PCR amplified product was purified by using GenElute™ PCR clean-up kit (Sigma). The purified PCR product was ligated into vector pTZ57R/T (Fermentas, Germany) for cloning and in vector pET-20b (+) (Fermentas, Germany) for expression and later transformed into *E. coli* DH5α and BL21 cells according to the instructions of InsTAclone™ PCR cloning kit (Fermentas, Germany). Standard methods such as PCR, restriction digestion and ligation were carried out according to manufacturer's manual. The *E. coli* competent cells were transformed by heat shock method[35]. All cloned DNA fragments were proven by sequencing. Expression analysis of aspartate kinase gene was done by real time PCR. The proteins were isolated from the clones and analyzed by SDS-PAGE. The RNA was isolated from the clones and cDNA synthesised from the isolated RNA was used as the template for the real time PCR reactions. Triplicate real time PCR reactions were performed using 2X-SYBR green supermix (Ambion Biosystems, USA), 1.5 pmole of forward and reverse primers and required amount of cDNA template. The sequences of the primers used for RT-PCR were as forward primer GACTGCTGGTGAGCGTATTTC and reverse primer ACGACCTGGAG GACATAAC.

Quantitative real time polymerase chain reaction (qRT-PCR): The cDNA synthesised from the RNA isolated from the wild type *C. glutamiucm* ATCC 13032 and mutant created by site directed mutagenesis was used as template for RT-PCR experiments. The primers were designed using Intigrated DNA Technologies (IDT) and possessed a GC content of 40-60%, Tm>50°C, primer length 20-24 nucleotides and expected amplicon sizes were 100-200 bp. Before going to real time

PCR, the reactions were normalized with gradient PCR for normalizing template and primer concentrations. The relative fold change of each of the selected gene was detected from the CT values. The comparative CT method eliminated the need for standard curves, but could only be used if PCR efficiencies are relatively equal between target and reference. Therefore, validation experiments were performed on 5-6 log dilutions of each of the target and reference to determine if their amplification efficiencies were equal. The real time polymerase chain reaction was performed in an applied-biosystems step-one real time PCR system (USA). The RT-PCR set up was followed by 40 cycles of 1 min at 95°C, 1 min at 57°C and 1 min at 72°C. Dissociation curves were obtained using a thermal melting profile performed after the qRT-PCR cycles: 95°C for 15 sec followed by a constant increase of the temperature between 60 and 95°C. Negative control reactions using untranscribed RNA were run with the main reactions to confirm absence of genomic DNA.

Site directed mutagenesis in aspartate kinase gene: Site directed mutagenesis in as partate kinase gene was done by using Quick-change™ site directed mutagenesis kit (Statagene). The protocol for site directed mutagenesis were followed as per the instruction mentioned in Quick-change™ site directed mutagenesis kit. The following forward mutagenic primer 5′ GAA ACC GTC GAT GTC ACC ACG 3′ and reverse mutagenic primer 5′ GAA ACC GTC GAT GTC ACC ACG 3′ used for the present experiment and are obtained from Eurofins (MWG), Bangalore, India. The mutagenic primers were designed for point mutation, where the amino acid serine (TCC) is replaced by valine (GTC).The position of the mutation is 235 and 236 nucleotides in the nucleotide sequence of the aspartate kinase gene. The position of the mutation is 79 amino acid in the amino acid sequence of the aspartate kinase gene. The mutation was made in β-subunit of the aspartate kinase gene so that the aspartate kinase gene is deregulated or made feedback resistant to L-lysine. The expression profile of the mutated gene i.e., feedback resistant aspartate kinase gene was checked by SDS-PAGE and RT-PCR experiments as described earlier. The mutated plasmid was isolated from the *E. coli* BL21 cells with feedback resistant ASK gene and transformed into *Corynebacterium glutamicum* ATCC 13032 by electroporation method.

Affinity purification of aspartate kinase proteins: Frozen pellets of recombinant *E. coli* BL21 cells were thawed on ice and resuspended in native binding buffer (50 mM $Na_2H_2PO_4$, 0.5 M NaCl, 10 mM imidazole, 1 mM dithiothreitol, pH 8.0) and cells were disrupted by sonication as Brautaset *et al.*[36] method. The Ni-nitrilotriacetic acid agarose kit (Qiagen) was used for proteins purification and the protocol was followed according to manufacturer's instructions. Native elution buffer (250 mM imidazole) was used for elution of AK proteins and finally dialyzed against dialysis buffer (pH 8.0, 50 mM NaCl, 50 mM tris-HCl, 1 mM dithiothreitol) for overnight at 4°C. The purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the purity of each AK protein was estimated by visual inspection of the resulting images. Protein concentrations were determined spectrophotometrically by using the Bradford assay at 595 nm (Bio-Rad) and bovine serum albumin as a reference. The purified enzymes were immediately subjected to biochemical analysis and were frozen in liquid N_2 and stored at -80°C.

Screening of recombinants with feedback resistant aspartate kinase gene: All the recombinants which are resistant to the S-(2-aminoethyl)-L-cysteine (lysine analogue) grow on the agar plates which contain S-(2-aminoethyl)-L-cysteine indicating that they contain the feedback resistant aspartate kinase gene. The *C. glutamicum* without feedback resistant aspartate kinase gene show no growth on the agar plates with S-(2-aminoethyl)-L-cysteine. About 50 mg of S-(2-aminoethyl)-L-cysteine was added to the 100 mL of LB agar medium composition and poured into petriplates. Recombinants with feedback resistant aspartate kinase gene only grow on S-(2-aminoethyl)-L-cysteine containing plates.

RESULTS

The batch fermentation was carried out in the stirred tank bioreactor, wild type *C. glutamicum* 13032 produced 40 g L^{-1} of lysine, biomass of 14.70 g L^{-1}, residual sugar of 9.14 g L^{-1} and glucose utilized is 90.86 g L^{-1}. The *C. glutamicum* ATCC 13032 mutant produced 44.2 g L^{-1} of lysine, biomass of 15.95 g L^{-1}, residual sugar of 11.93 g L^{-1} and glucose utilized is 91.11 g L^{-1}. As the aspartate kinase in mutant was deregulated from feedback inhibition, increased production of lysine (4 g L^{-1}) was observed. The fermentation results of wild type and mutant *C. glutamicum* (Fig. 1).

Amplification of aspartate kinase gene: The genomic DNA isolated from *C. glutamicum* ATCC 13032 and genomic DNA of mutant created by site directed mutagenesis were used as the template to amplify aspartate kinase gene and feedback resistant aspartate kinase gene. By using the forward and reverse gene specific primers, aspartate kinase gene was

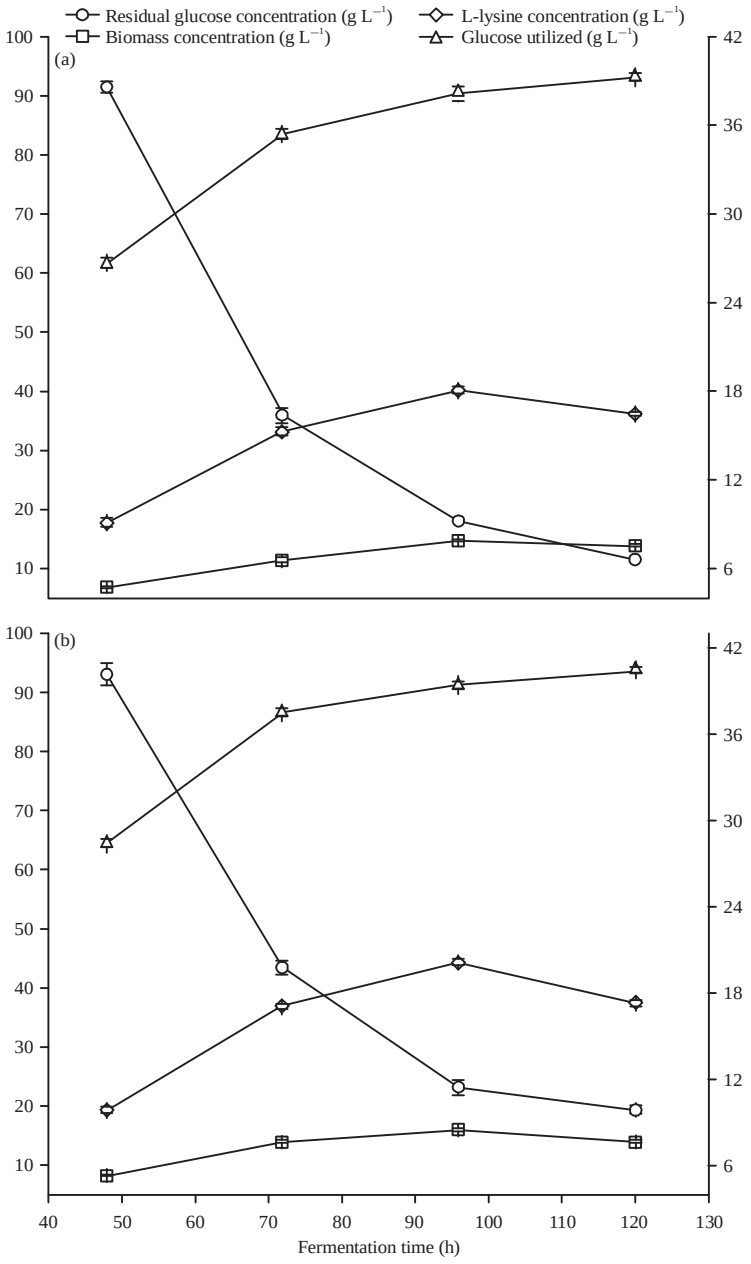

Fig. 1(a-b): Different upstream parameter for biotechnological production of L-lysine was studied by conducting batch fermentation in mew Brunswick 5 L bioreactor for 120 h by using, (a) Wild type and (b) Mutant *Corynebacterium glutamicum* ATCC 13032, which is having feedback sensitive aspartate kinase. The sample was collected from the bioreactor under sterile conditions and different parameters were investigated using appropriate protocols

amplified and sequence was submitted to NCBI (GenBank Accession No. KJ868806). The amplified PCR products were analysed in analytical agarose gel electrophoresis and the results were illustrated in Fig. 2. In the present study, plasmid DNAs extracted from positive clones after cloning the aspartate kinase gene into the TA and pET vectors were subjected to EcoR1 and BamH1 digestion and the products were analyzed in an analytical agarose gel alongside a DNA molecular weight marker (Fig. 3 and 4).

SDS-PAGE analysis of expression of ASK gene and feedback ASK gene: The lys C/asd gene cluster of *Corynebacterium glutamicum* ATCC 13032 was cloned and sequenced. The lys C locus coding for aspartokinase consists of two in-frame

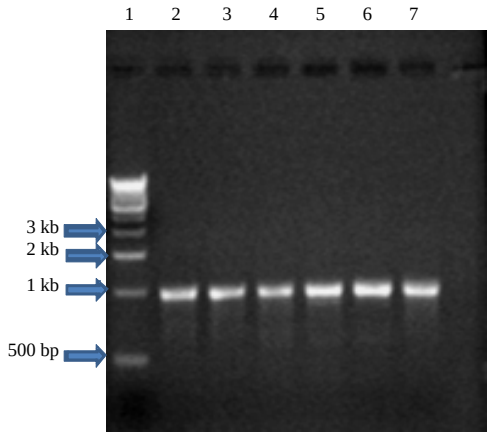

Fig. 2: Gel image of amplification of asparate kinase gene. Genomic DNA from wild type *Corynebacterium glutamicum* ATCC 13032 was isolated and using aspartate kinase gene specific reverse and forward primers aspartate kinase gene of 1 kb was amplified. And later the amplified PCR product was purified and sent for sequencing, Lane 1: 1 kb ladder, Lane2-7: PCR product of 13032

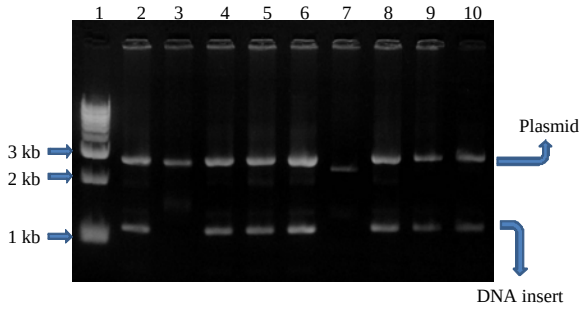

Fig. 3: Purified PCR product was ligated into vector pTZ57R/T (Fermentas, Germany) for cloning and to confirm the presence of wild type aspartat kinase gene in positive clones. The plasmid from them is isolated and subjected to restriction digestion, a band at 2.8 kb is of plasmid DNA and band above 1 kb of aspartat kinase gene from wild type *Corynebacterium glutamicum* 13032, Lane 1: 1 kb ladder, Lane 2-10: Recombinant of 13032

overlapping genes, lys C alpha encoding a protein of 421 amino acids (46,300) and lys C beta encoding a protein of 172 amino acids (18,600). The *C. glutamicum* aspartokinase was purified and found to contain two proteins of 47,000 and 18,000 KD. The expression of wild type aspartate kinase gene and feedback resistant aspartate kinase gene was analyzed by SDS-PAGE (Fig. 5).

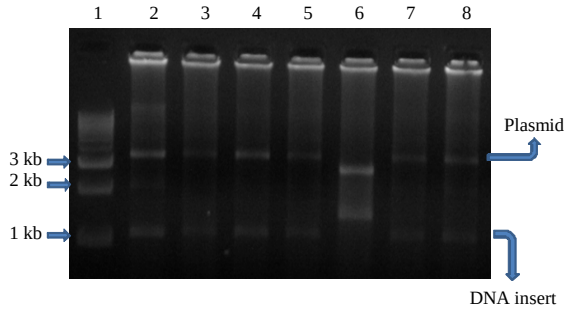

Fig. 4: Purified PCR product cloned into vector pET-20b (+) (Fermentas, Germany) for expression and later transformed into *E. coli.* To confirm the presence of wild type aspartat kinase gene in positive clones. The plasmid from them is isolated and subjected to restriction digestion, a band at 3.2 kb is of plasmid DNA and band at 1.2 kb of aspartat kinase gene from wild type *Corynebacterium glutamicum* 13032, Lane 1: 1 kb ladder, Lane 2-8: Recombinant plasmid of *Corynebacterium glutamicum*

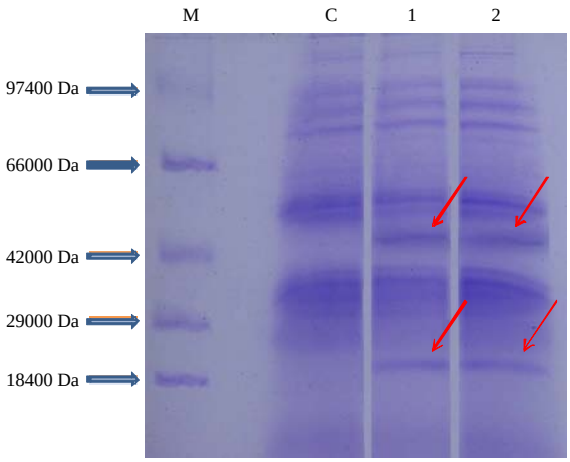

Fig. 5: SDS-PAGE analysis of expression of ASK gene. The Ni-nitrilotriacetic acid agarose kit (Qiagen) was used for proteins purification. Native elution buffer (250 mM imidazole) was used for elution of AK proteins and finally dialyzed against dialysis buffer (pH 8.0, 50 mM NaCl, 50 mM tris-HCl, 1 mM dithiothreitol). The *Corynebacterium glutamicum* aspartokinase was purified and found to contain two proteins of 47,000 and 18,000 KD. Two bands of predicted size can be seen in the image. The expression of feedback resistant aspartate kinase gene was analyzed by SDS-PAGE, M: Molecular weight ladder, C: Control, 1-2: ATCC 13032 ASK gene

RT-PCR studies: The RNA isolated from the wild type *C. glutamiucm* ATCC 13032 and mutant created by site directed mutagenesis which was used as template for the synthesis of cDNA copies were analysed in analytical agarose gel electrophoresis. The aspartate kinase gene cloned in the expression vector pET20b (+) was expressed. The Ct values obtained were used to calculate the initial quantity of each specific cDNA by extrapolating from the standard curve assessed the primer set. The amount of cDNA copies is correlated to the amount of mRNA in each sample. The number of cDNA copies in each sample for each gene was calculated from the respective standard curve made up with 10-fold serial dilutions of cloned plasmid DNA with the specific primer sets.

The aspartate kinase gene that displayed an expression levels with a mean value of 5 log initial cDNA copies before mutation had showed 6 log initial cDNA copies after site directed mutagenesis. The RT-PCR experiment was conducted to investigate the expression levels of the normal and feedback resistant aspartate kinase gene. The real time PCR analysis clearly indicated the expression of the FBR aspartate kinase gene (Fig. 6). The 3-D Structure of FBR aspartate kinase gene with S79V mutation is illustrated in Fig. 7.

DISCUSSION

The biotechnological production of L-lysine by *Corynebacterium glutamicum* needs a continuous development of the lysine production process with a unique focus on optimization of the production strains. This includes the recognition and performance of genetic modifications that appear beneficial for production. The preliminary steps of strain optimization were based on accessible regulatory and metabolic information for *C. glutamicum*. In the present study, the biosynthesis of L-lysine was released from feedback inhibition by lysine and threonine through the introduction of the amino acid substitution in the β-subunit of lys C gene encoding aspartate kinase, which is the key regulatory enzyme of the lysine pathway. This modification results in a feedback resistant aspartokinase variant and deregulation of the lysine pathway. Moreover, in this study, it was succeeded in establishing an engineered *C. glutamicum* with novel mutation in aspartate kinase gene that can produce more L-lysine, which have wider range of applications, especially food and medical related uses. Furthermore, the suitable tunable gene expression system for deregulated aspartate kinase gene in *C. glutamicum* was established that

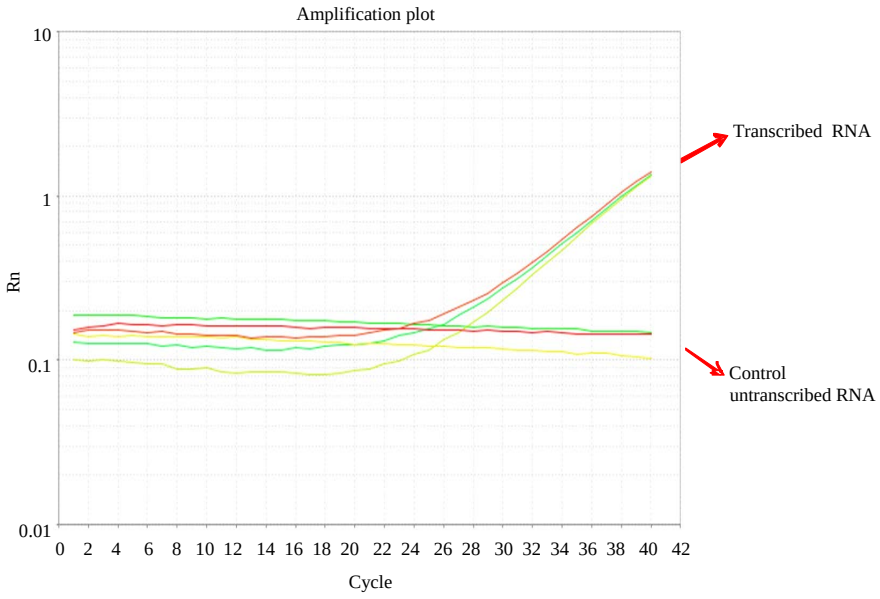

Fig. 6: RT-PCR amplification plot of feedback resistant ASK gene of wild type *C. glutamicum* 13032 mutant. The cDNA synthesised from the RNA isolated from the wild type *Corynebacterium glutamicum* ATCC 13032 was used as template for RT-PCR experiments. The primers were designed using Intigrated DNA Technologies (IDT) and possessed a GC content of 40-60%, Tm>50°C, primer length 20-24 nucleotides and expected amplicon sizes were 100-200 bp. The Ct values obtained were used to calculate the initial quantity of each specific cDNA by extrapolating from the standard curve

Fig. 7: It is the 3-D structure of feedback resistance aspartate kinase gene which is having 2 α-subunits and 2-β subunits. Deregulation of aspartate kinase gene can be obtained by mutating in β-subunits region. In the above image, aspartate kinase gene is mutated at 79 position of amino acid, there serine is replaced by valine

could help to increase the production L-lysine production under inducing conditions. In this study, key methodology was established the substitution of codon TCC with GTC in aspartate kinase gene through site directed mutagenesis to deregulate the aspartate kinase from feedback regulation. The obtained mutant secreted an increased amount of lysine than the wild type. The expression profile of feedback resistant aspartate kinase gene was studied by using RT-PCR technology and SDS-PAGE. The mutant showed an overall growth behaviour (specific growth rate, glucose uptake rate, biomass yield) which was very parallel to that of the parent strain, but differed in slightly increased lysine formation. Major parameters like specific growth rate or yield for biomass, lysine and by-products remained constant throughout the cultivation. This clearly shows that both strains were in metabolic steady-state. Analysis of the recombinant mutant strain under high-cell-density conditions confirmed that they it displayed increased L-lysine production compared to the wild type. Biotechnological production of L-lysine by *C. glutamicum* 13032 was estimated at 40 g L^{-1} whereas, *C. glutamicum* 13032 mutant produced 44.2 g L^{-1} of lysine respectively. The mutant of *C. glutamicum* 13032 with feedback resistant aspartate kinase gene showed 4 g L^{-1} more yield of L-lysine compared to the *C. glutamicum* 13032. Cremer *et al.*[37] illustrated a rational approach to the analysis of L-lysine production with *C. glutamicum* by overexpression of the several biosynthetic enzymes. Each of the six genes that are involved in the pathway of L-aspartate to L-lysine was overexpressed independently in the wild type and a mutant with a feedback resistant aspartate kinase. Higher specific activities of each of the enzymes were observed in the recombinant *C. glutamicum* strains[37]. Analysis of L-lysine formation revealed that overexpression of the gene for the feedback-resistant aspartate kinase alone is enough to achieve L-lysine secretion in the wild type. Follettie *et al.*[38] reported the *C. flavum* N13 ask and asd genes encoding for aspartate semialdehyde dehydrogenase and feedback-insensitive aspartokinase have been investigated throughly. The existence of the intact ask-asd operon on the plasmid results in a 6-fold increase in aspartokinase activity and in a 23-fold increase in aspartate semialdehyde dehydrogenase activity. Similar results were attained by Kalinowski *et al.*[39,40] who showed that the d eregulation of the aspartokinase in *C. glutamicum* DG 52-5 was due to a mutation of serine in position 301 to a tyrosine. Deregulated aspartokinases of *C. glutamicum* characterized, so far contain mutations in the carboxy terminus of the enzyme, a regulatory role of the P subunit has been proposed[38]. In this present investigation, 10-fold increases in the aspartokinase activity was attained and higher L-lysine production in *C. glutamicum* than *C. flavum* N13. Yoshida *et al.*[41] reported that the crystal structure of the regulatory subunit of an α2 β2-type AK from *C. glutamicum* (CgAKβ) complexed with its effectors Thr and in combination with site directed mutagenesis, revealed several characteristics of the feedback inhibition mechanism in *C. glutamicum* AK. A pseudo wild type strain KK-25 with a resistant aspartate kinase attained via conjugal transfer showed a very low lysine yield. The *C. glutamicum* production strains DG 52-5 and MH20-22B obtained through classical mutagenesis and screening had comparable low lysine secretion rates and low lysine yields. The newly engineered strain with feedback resistant aspartate kinase obtained in our study had more lysine yield compared to DG 52-5 and MH20-22B[42]. The screening, analysis and availability of positive mutations will significantly contribute to development of lysine overproducing strains. Application of site-directed mutagenesis to *C. glutamicum* 13032 strain revealed the key role of deregulated aspartate kinase.

CONCLUSION

A lot of studies have been conducted on how each gene involved in amino acid biosynthesis affects L-lysine production by amplifying or deletion of each of the genes and develop a recombinant *Corynebacterium.* Elimination of non-essential genes causes the reduced use of lysine which favours the unnecessary lysine consumption and also favours

the lysine production under same condition. Metabolic flux analysis provided valuable information in understanding the cellular response resulting from genetic engineering to visualize metabolic imbalance to guide further strain engineering. Especially for the optimization of lysine production in *C. glutamicum*, the studies related to central metabolism and lysine biosynthetic pathway has turned out to be crucial. Metabolic engineering benefits from target identification through biochemical insight, flux analysis or genomics, transcriptomics, proteomics, metabolomics and other systems biology approaches. In the past, the main biotechnological fermentation products were amino acids and reduced products (e.g., ethanol or lactate) that are formed naturally by the respective microorganisms under anaerobic conditions, which means when metabolic end product yields are high and loss of substrate to cell mass and CO_2 are low.

Recent years have seen an increasing interest in engineering *C. glutamicum* for production of organic acids. Here, it was reported a new engineered strain capable of lysine production, which has 10-fold high lysine production capacity than wild type strain. The present study provided further insight into AK regulation and function and also provided valuable information for engineering L-lysine producing *C. glutamicum* strains. The results provided in this report show the importance of the deregulation of aspartate kinase and site directed mutagenesis for over production of L-lysine in *C. glutamicum.* The new engineered strain in this study promises to create new opportunities in bioprocess performance and over produce L-lysine for therapeutic and biomedical applications. The recombinant strain described here may provide a basis for future metabolic engineering to obtain further increases in L-lysine production by *C. glutamicum*. The newly engineered *C. glutamicum* potentially serves as a food-grade and biomedically applicable platform for the production of L-lysine. Further studies need to be performed on this newly engineered *C. glutamicum* to understand substrate binding and catalysis of its feedback resistant aspartate kinase gene compared to wild type feedback sensitive aspartate kinase. Although, the present study is a serious effort towards development of newly engineered *C. glutamicum* strain with L-lysine overproducing capacity, the final goal is to fit in further beneficial mutations in the metabolic pathways of this newly engineered *C. glutamicum* strain. So that L-lysine could be produced by more cost effective process by using low grade, low cost sugars, such as molasses, as a carbon source. These study approaches will open the opportunity for developing new engineered strains, which are more efficient and lysine overproducing. Furthermore, it was believe that an increasing number of targets for new *C. glutamicum* strain development will be recognized as the result of transcriptome, proteome, genome, metabolome, flux and other systems biology analysis.

ACKNOWLEDGMENTS

Abdul Razak is thankful to Science and Engineering Research Board (SERB), Govt. of India for awarding Start up Research Grant (Young Scientist) (YSS/2015/000272) for the project entitled "Construction of novel synthetic heterologous pathway for the production and polymerization of green biodegradable polyester precursors by *Corynebacterium glutamicum*".

SIGNIFICANT STATEMENTS

The literature studies revealed that overproduction of L-lysine by *Corynebacterium glutamicum* can be obtained by mutating the β-subunit of the aspartate kinase gene, it can be made insensitive to the feedback inhibition of threonine or lysine formed as the end product. In this article, it was included by making the point mutation which replaces the codon TCC-GTC in aspartate kinase gene at β-subunit region, which helps to overproduction of the lysine. It was constructed the recombinant strain which expressed the feedback resistant aspartate kinase genes which is responsible for over production of lysine. Deregulated or feedback resistant aspartate kinase gene obtained by site-directed mutagenesis led to higher lysine yield than wild type.

REFERENCES

1. Leuchtenberger, W., K. Huthmacher and K. Drauz, 2005. Biotechnological production of amino acids and derivatives: Current status and prospects. Applied Microbiol. Biotechnol., 69: 1-8.
2. Koffas, M. and G. Stephanopoulos, 2005. Strain improvement by metabolic engineering: lysine production as a case study for systems biology. Curr. Opin. Biotechnol., 16: 361-366.
3. Coello, N., L. Brito and M. Nonus, 2000. Biosynthesis of L-lysine by *Corynebacterium glutamicum* grown on fish silage. Bioresour. Technol., 73: 221-225.
4. Kelle, R., T. Hermann and B. Bathe, 2005. L-Lysine Production. In: Handbook of Corynebacterium Glutamicum, Eggeling, L. and M. Bott (Eds.). CRC Press, Boca Raton, FL., USA., ISBN: 9781420039696, pp: 465-488.
5. Ohnishi, J., S. Mitsuhashi, M. Hayashi, S. Ando, H. Yokoi, K. Ochiai and M. Ikeda, 2002. A novel methodology employing *Corynebacterium glutamicum* genome information to generate a new L-lysine-producing mutant. Applied Microbiol. Biotechnol., 58: 217-223.

6. Park, J.H., S.Y. Lee, T.Y. Kim and H.U. Kim, 2008. Application of systems biology for bioprocess development. Trends Biotechnol., 26: 404-412.
7. Becker, J., C. Klopprogge, A. Herold, O. Zelder, C.J. Bolten and C. Wittmann, 2007. Metabolic flux engineering of L-lysine production in *Corynebacterium glutamicum*-over expression and modification of G6P dehydrogenase. J. Biotechnol., 132: 99-109.
8. Marx, A., S. Hans, B. Mockel, B. Bathe and A.A. de Graaf, 2003. Metabolic phenotype of phosphoglucose isomerase mutants of *Corynebacterium glutamicum*. J. Biotechnol., 104: 185-197.
9. Ohnishi, J., R. Katahira, S. Mitsuhashi, S. Kakita and M. Ikeda, 2005. A novel gnd mutation leading to increased L-lysine production in *Corynebacterium glutamicum*. FEMS Microbiol. Lett., 242: 265-274.
10. Kinoshita, S., S. Udaka and M. Shimono, 1957. Studies on the amino acid fermentation: Part I. production of L-glutamic acid by various microorganisms. J. General Applied Microbiol., 3: 193-205.
11. Ajinomoto, 2010. Food Products Business. Ajinomoto Co. Inc., Tokyo.
12. Ajinomoto, 2011. Feed-use Amino Acids Business. Ajinomoto Co. Inc., Tokyo.
13. Demain, A.L., 2000. Microbial biotechnology. Trends Biotechnol., 18: 26-31.
14. Herman, T., 2003. Industrial production of amino acids by Coryneform bacteria. J. Biotechnol., 104: 155-172.
15. Inui, M., S. Murakami, S. Okino, H. Kawaguchi, A.A. Vertes and H. Yukawa, 2004. Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions. J. Mol. Microbiol. Biotechnol., 7: 182-196.
16. Shen, X.H., C.Y. Jiang, Y. Huang, Z.P. Liu and S.J. Liu, 2005. Functional identification of novel genes involved in the glutathione-independent gentisate pathway in *Corynebacterium glutamicum*. Applied Environ. Microbiol., 71: 3442-3452.
17. Ikeda, M. and S. Nakagawa, 2003. The *Corynebacterium glutamicum* genome: Features and impacts on biotechnological processes. Applied Microbiol. Biotechnol., 62: 99-109.
18. Kalinowski, J., B. Bathe, D. Bartels, N. Bischoff and M. Bott *et al*., 2003. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. J. Biotechnol., 104: 5-25.
19. Wendisch, V.F., M. Bott and B.J. Eikmanns, 2006. Metabolic engineering of *Escherichia coli* and *Corynebacterium glutamicum* for biotechnological production of organic acids and amino acids. Curr. Opinion Microbiol., 9: 268-274.
20. Zahoor, A., S.N. Lindner and V.F. Wendisch, 2012. Metabolic engineering of *Corynebacterium glutamicum* aimed at alternative carbon sources and new products. Comput. Structural Biotechnol. J., 3: 1-11.
21. Song, Y., K.I. Matsumoto, T. Tanaka, A. Kondo and S. Taguchi, 2013. Single-step production of polyhydroxybutyrate from starch by using α-amylase cell-surface displaying system of *Corynebacterium glutamicum*. J. Biosci. Bioeng., 115: 12-14.
22. Gopinath, V., A. Murali, K.S. Dhar and K.M. Nampoothiri, 2012. *Corynebacterium glutamicum* as a potent biocatalyst for the bioconversion of pentose sugars to value-added products. Applied Microbiol. Biotechnol., 93: 95-106.
23. Meiswinkel, T.M., V. Gopinath, S.N. Lindner, K.M. Nampoothiri and V.F. Wendisch, 2013. Accelerated pentose utilization by *Corynebacterium glutamicum* for accelerated production of lysine, glutamate, ornithine and putrescine. Microbial Biotechnol., 6: 131-140.
24. Adachi, N., C. Takahashi, N. Ono-Murota, R. Yamaguchi, T. Tanaka and A. Kondo, 2013. Direct L-lysine production from cellobiose by *Corynebacterium glutamicum* displaying beta-glucosidase on its cell surface. Applied Microbiol. Biotechnol., 97: 7165-7172.
25. Uhde, A., J.W. Youn, T. Maeda, L. Clermont and C. Matano *et al*., 2013. Glucosamine as carbon source for amino acid-producing *Corynebacterium glutamicum*. Applied Microbiol. Biotechnol., 97: 1679-1687.
26. Razak, M.A. and B. Viswanath, 2015. Comparative studies for the biotechnological production of l-Lysine by immobilized cells of wild-type *Corynebacterium glutamicum* ATCC 13032 and mutant MH 20-22 B. 3 Biotech, 5: 765-774.
27. Sambrook, J. and D.W. Russell, 2001. Molecular Cloning: A Laboratory Manual. Vol. 1, CSHL Press, USA., ISBN: 9780879695774, Pages: 2344.
28. Razak, M.A. and B. Viswanath, 2015. Optimization of fermentation upstream parameters and immobilization of *Corynebacterium glutamicum* MH 20-22 B cells to enhance the production of l-lysine. 3 Biotech, 5: 531-540.
29. Razak, M.A. and B. Viswanath, 2015. Role of *Corynebacterium glutamicum* in White Biotechnology with Special Reference to L-Lysine Production. In: New Horizons in Biotechnology, Viswanath, B. and G. Indravathi (Eds.). Paramount Publishing House, India, pp: 60-67.
30. Chinard, F.P., 1952. Photometric estimation of Proline and Ornithine. Bio. Chem., 199: 91-95.
31. Morris, D.L., 1948. Micobiol Physiology. John Wiley and Sons, New York, Pages: 161.
32. Neish, A.C., 1952. Analytical Methods for Bacterial Fermentation. National Research Council, Manual, Canada, Pages: 263.
33. Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.

34. Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.
35. Sambrook, J., E.F. Fritsch and T.A. Maniatis, 1989. Molecular Cloning: A Laboratory Manual. 2nd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY., USA., ISBN-13: 9780879695774, Pages: 397.
36. Brautaset, T., M.D. Williams, R.D. Dillingham, C. Kaufmann, A. Bennaars, E. Crabbe and M.C. Flickinger, 2003. Role of the *Bacillus methanolicus* citrate synthase II gene, *citY*, in regulating the secretion of glutamate in L-lysine-secreting mutants. Applied Environ. Microbiol., 69: 3986-3995.
37. 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.
38. Follettie, M.T., O.P. Peoples, C. Agoropoulou and A.J. Sinskey, 1993. Gene structure and expression of the *Corynebacterium flavum* N13 ask-asd operon. J. Bacteriol., 175: 4096-4103.
39. Kalinowski, J., B. Bachmann, L. Eggeling, H. Sahm and A. Puhler, 1991. Genetic and biochemical analysis of the aspartokinase from *Corynebacterium glutamicum*. Mol. Microbiol., 5: 1197-1204.
40. Kalinowski, J., B. Bachmann, G. Thierbach and A. Puhler, 1990. Aspartokinase genes lysCα and lysCβ overlap and are adjacent to the aspartate β-semialdehyde dehydrogenase gene asd in *Corynebacterium glutamicum*. Mol. General Genet., 224: 317-324.
41. Yoshida, A., T. Tomita, T. Kuzuyama and M. Nishiyama, 2007. Purification, crystallization and preliminary X-ray analysis of the regulatory subunit of aspartate kinase from *Thermus thermophilus*. Acta Crystallographica Sect. F: Struct. Biol. Crystallization Commun., 63: 96-98.
42. Broer, S. and R. Kramer, 1991. Lysine excretion by *Corynebacterium glutamicum*. I. Identification of a specific carrier system. Eur. J. Biochem., 202: 131-135.