L LYSINE PRODUCTION BY CHEMICAL MUTAGENESIS OF HOMOSERINE DEHYDROGENASE OF *DDH* GENE IN *Corynebacterium glutamicum* ATCC13032

Bhushanam Vanasi¹, Laxminarayana Eppakayala² and Ramesh Malothu^{1,*}

¹Institute of Science and Technology, Jawaharlal Nehru Technological University Kakinada, Kakinada, Andhra Pradesh India

²Sreenidhi Institute of Science and Technology, Yamnampet, Ghatkesar, Hyderabad-501301 Telangana, India

*E-mail: ramesh_biotech@jntuk.edu.in

ABSTRACT

Auxotrophic mutant formed from *ddh* gene recombinant ATCC 13032 with blocked homoserine dehydrogenase showed increased yield of L Lysine of 24.89 g/l from normal *ddh* gene recombinant ATCC 13032 strain which had a yield of 20.66 g/l of L Lysine. The maximum yield of L Lysine for the auxotrophic mutant is attained at 7.5 PH, 30°C of temperature and an incubation time of 96 hrs. The Auxotrophic mutant of *ddh* recombinant *C. glutamicum* showed nearly 6.52 g/l more amount of l lysine than Auxotrophic mutant of wild type with 18.57 g/l of L Lysine. The Chemical mutagen ENU caused a mutation in the Homoserine serine dehydrogenase enzyme diverted the Aspartyl β semialdehyde to bind with 2,3Dihydrodipicolinate synthase to participate in the L Lysine synthesis through 2,3 meso- Diaminopimelate (Meso-Dap). Being a recombinant for diaminopimelate dehydrogenase (*ddh*) the auxotrophic mutant for the homoserine dehydrogenase follows the *ddh* pathway by overexpression of *ddh* by deviating the Acetyltransferase and Succinyl transferase is the reason for the high yield of L Lysine production.

Keywords: Lysine, *ddh*, 2, 3-meso DAP, ATCC 13032.

© RASĀYAN. All rights reserved

INTRODUCTION

L Lysine is one of the most important essential amino acids which could be used in many biophysical mechanisms in living organisms. *Corynebacterium glutamicum* is used to produce L Lysine commercially.¹ L-Lysine is an essential amino acid which is utilized in many biochemical reactions like phosphorylation and also used as an additive for fodder crops.² Annually around 80, 00, 00 tones were produced which made L Lysine second among global amino acid synthesis at industrial scale.^{3,4} Chemical synthesis, enzymatic method, fermentation, extraction from protein Hydrolysate, genetic engineering and protoplast fusions were several kinds of technologies employed in L Lysine synthesis from *Corynebacterium glutamicum*.^{3,26} L-lysine is one of the most deficient components found in the food of both human and animals. Animal feed generally contains a less quantity of L-lysine and is not synthesized by cattle, poultry or other livestock, so L-lysine will be added as a food supplement for animals to meet feed requirements.⁶ L-Lysine, one of the eight essential amino acids for animals and humans which is used as feed additives, dietary supplements and also as an ingredient of pharmaceuticals and cosmetic.^{5,7} *Corynebacterium glutamicum* is a non-lethal and non-emulsifying gram-positive bacterium. It exhibits a low protease activity in the culture and can secrete protease-sensitive proteins into the culture supernatant.¹⁴ *C. glutamicum* is a gram-negative bacteria with the absence of lipopolysaccharide removed in the production of therapeutic proteins¹⁵ increases the yield by reducing the purification steps. *C. glutamicum* is generally recognized as safe (GRAS) for the industrial biochemical production of L Lysine and L glutamate.¹⁶

Corynebacterium glutamicum is one of the major microorganisms used in amino acid synthesis. The *Corynebacterium glutamicum* is a rod-shaped bacteria, aerobic and gram-positive bacteria grows in the

soil, on the surfaces of vegetables and fruits,¹⁷ *C. glutamicum* has the capability to metabolize glucose, fructose, and sucrose.^{18, 29} *C. glutamicum* utilizes many different kinds of carbohydrates, organic acids, and alcohol as a carbon and energy source for rapid microbial growth and for many amino acids synthesis.^{24, 25} The glucose, or sucrose or any carbon source is utilized by the *Corynebacterium glutamicum* for L lysine synthesis by fermentation.²⁸ The time of incubation is reported for maximum L Lysine is between 48 hrs to 72 hrs.^{30,31} The ddh recombinant *Corynebacterium glutamicum* ATCC 13032 produces more amounts of L Lysine compared to Wild type. This is because of the expression of more amount of ddh which acts as an enzyme for the substrate 2,6 dicarboxylic acid with the participation of less number of enzymes. Chemical mutagenesis with ENU increased the yield of L Lysine in the mutant than the Wild type strain.²⁷ The ENU causes insertion or deletion mutation and shows its effect on protein synthesis. The ENU causes a mutation in Homoserine dehydrogenase gene to cause the Homoserine Auxotrophs of *C. glutamicum*.²¹

Generally, The Aspartyl β semialdehyde is produced in two ways. In the Krebs cycle of *Corynebacterium glutamicum*, the Oxaloacetic acid (OAA)^{19, 20} undergoes a transamination reaction with the presence of *glutamate: oxaloacetate: transaminase* enzymes produce aspartyl β semi aldehyde which further produces homoserine and L L diaminopimelate (2,3 meso- DAP) by two different pathways¹. The Aspartyl β semialdehyde is also formed from *Aspartate dehydrogenase* from Aspartyl phosphate which was formed from Aspartate by *Aspartate kinase*.^{2,19} The aspartyl β -semi aldehyde acts as a common substrate to produce L Lysine through L L diaminopimelate(2,3-DAP) and Methionine or threonine through homoserine.^{1,28} The aspartyl β -semi aldehyde converts to Homoserine by reacting with *homoserine dehydrogenase*,⁴ which participates in the Homoserine pathway in the production of Threonine and Methionine.^{22,23} Homoserine reacts with *Met^f* and produces O- Acetylhomoserine which reacts with *Met B* synthesize Cystathionine further reacts with *C⁷* to produce Homocysteine finally reacts with *Met E* or *Met H* to produce methionine or Homoserine reacts with homoserine kinase produces L homoserine phosphate and converts to threonine¹¹ by Threonine synthase in Homoserine pathway. Aspartyl β semi aldehyde reacts with 2,3 *Dihydrodipicolinate synthase* produces 2,3 Dihydrodipicolinate which further reduces to 2,6 Dicarboxylic acids by 2,3 *Dihydrodipicolinate reductase*. *Corynebacterium glutamicum* chose three kinds of enzymes namely *Acetyltransferase* or *Succinyl Transferase* or *diaminopimelate dehydrogenase (ddh)* to produce L L diaminopimelate (2,3 meso DAP). The LL diaminopimelate (2,3 meso DAP) converts to L Lysine by *Lysine synthase*. By Recombination with ddh gene with a constitutive promoter enhances the productivity of L Lysine by diverting the acetyltransferase and succinic transferase pathway to ddh pathway. The Chemical Mutagen N-nitroso-N-ethyl urea(ENU)^{8,9} has the capability to cause deletion or insertion mutation in the *Homoserine dehydrogenase*¹⁸ enzyme and blocks the Homoserine Pathway which generally leads to the production of threonine and Methionine. This block in the homoserine pathway diverts the aspartic β -semialdehyde to react with 2,3 Dihydrodipicolinate synthase the enzyme to produce more amounts of Σ -diaminopimelate (Σ DAP) through ddh pathway. 2,3 Dihydrodipicolinate synthase³⁵ converts aspartic β -semialdehyde to 2,3 Dihydrodipicolinate. In the presence of reductase 2,3 Dihydrodipicolinate reduces to Piperidine 2,6, dicarboxylic acid. The formation of DAP will be done by binding of Piperidine 2,6, dicarboxylic acid with three different enzymes acetyltransferase or Succinyl transferase or diaminopimelate dehydrogenase enzymes leads to three different pathways for the L Lysine production through DAP. 2,6 dicarboxylic acid reacts with *acetyl transferase* produces the N – acetyl 2 –amino 6-keto L-pimelate which reacts with the enzyme *aminotransferase* produces N- Acetyl-L-L-diaminopimelate produces L-L-DAP by *deacetylase* in the acetyltransferase pathway which is a three-step pathway. 2,6 dicarboxylate reacts with Succinyl transferase to produce N Succinyl 2- amino 6 keto L pimelate which reacts with *dap C* Produces N Succinyl – L-L diaminopimelate which again reacts with *dap E* gives L L diaminopimelate is also a three-step pathway. The ddh recombinant strain produces more L L diaminopimelate (2,3 meso DAP) by overexpression of ddh enzyme which follows ddh pathway for the production of L L diaminopimelate (2,3 meso-DAP) by reacting with 2,6 Dicarboxylic acid as substrate by eliminating the more number of reactions that were in the remaining *acetyl transferase* and *Succinyl transferase* pathways by overexpression of *ddh* by the ddh recombinant strain of *C. glutamicum* ATCC 13032 strain with constitutive promoter. The general events that took place in the Lysine production are illustrated in Fig. - 1.

Fig.-1: The Homoserine Pathway for Threonine and Methionine Synthesis and Acetyltransferase, Succinyl Synthase and Diaminopimelate dehydrogenase Pathways in the Synthesis of L Lysine through L L diaminopimelate (2,3 meso- DAP).

EXPERIMENTAL

Bacterial Cell Cultures

The bacterial strain used in this research is a recombinant of *ddh* gene with a constitutive promoter of *C. glutamicum* (ATCC13032) developed by cloning³² in the lab of Ramesh Malothu, school of biotechnology, JNTUK which had an increased yield of 20.66 g/L of l-lysine.

Recombinant strain developed in our laboratory from the ATCC 13032 wild type *C. glutamicum*, by

recombining *ddh* gene with a constitutive promoter was utilized in the process of chemical mutagenesis with-nitroso-N-ethyl urea (ENU). The mutagenic power of N-ethyl-N-nitrosourea (ENU) stems from the generation of diazomethane.^{33,34}

Chemical Mutagenesis Method

Seed culture medium (D-Glucose 10 g/l, Peptone 5 g/l, Yeast extract 3.75 g/l, NaCl 5 g/l, (NH₄)₂SO₄ 17.5 g/l, K₂HPO₄ 25 g/l, KH₂PO₄ 25 g/l, Threonine 20 g/l, Methionine 20 g/l, ZnSO₄ 0.5 g/l, MgSO₄ 7H₂O 25 g/l, FeSO₄ 7H₂O 1 g/l and MnSO₄ 5H₂O 0.5 g/l), LB medium (Tryptone 10 g/l, NaCl 10 g/l and Yeast 5 g/l). The *ddh* recombinant ATCC 13032 strain was grown on LB medium and collected into test tubes with 3 ml each into 5 test tubes which were used for inducing the chemical mutation. Then the cell cultures were incubated for 24 hrs. at 37°C in an orbital shaker. Centrifuge the tubes at 10000xg for 5mins and collect the pellets. These collected pellets were suspended in 3 ml sodium citrate buffer and again centrifuge these pellets at 10000xg for 5 mins. The pellet is collected and resuspended into a 3 ml buffer containing sodium citrate buffer with a PH of 4.1 and 1.2 ml of N-nitroso-N-ethyl urea (ENU) with 100 nm concentration. The cultures were incubated separately for 0,5,10,15,20,25 and 30 min and centrifugation has done at 10000xg for 5 mins after the stipulated time. The pellets were collected and suspended in 3 ml sodium citrate buffer to wash the mutagen ENU and the resulted pellets were again suspended in the 3ml sodium citrate buffer to remove the traces of ENU. These pellets are incubated at 30°C for 3 days in the threonine and Methionine enriched seed culture media.

Isolation of Auxotrophs

The growth obtained after 3 days of incubation was inoculated in 1 ml seed culture media without Threonine or Methionine and penicillin G is added. Incubated for 20 hrs. in 30 °c in an orbital shaker. 50 units of penicillinase were added to each tube and left for 10 minutes. 100µl of this growth inoculated on to seed culture media with threonine and methionine and also onto the seed culture media without threonine or methionine and incubated for 3 days at 30 °C in the orbital shaker. After the time of incubation is completed the samples were screened for Lysine production. The samples were centrifuged at 15000xg for 10 min. The supernatant is collected for lysine analysis. Quantitative analysis of L Lysine was done by SDS PAGE.

Optimization of Fermentation Parameters

Different parameters like Concentration of ENU, Time of exposure of ENU, PH of the culture media, Temperature and time were tested to find the better growing conditions of the recombinant strain when treated with ENU for the high amount of production of L Lysine was tested.

In the chemical mutagenesis, we tested for different concentrations of 25, 35, 50, 75, 100 and 120 nm of the concentration of ENU and different times of exposure of ENU of 5,10,15,20,25 and 30 minutes to the Bacterial strain were tested at Constant Temperature, Time, and Ph. Which are optimized conditions of wild type *C. glutamicum* for L-Lysine production.¹³ The recombinant strain after treating with ENU, temperature and time of incubation was kept constant and tested for different pH values of 6, 6.5, 7.0, 7.5, 8 and 8.5. After checked for different PH the ENU treated *ddh* recombinant cultures were tested for different temperatures of 10, 25, 29, 30,31 and 35 by keeping the Volume, pH and time of incubation constant. The 100 nm ENU treated recombinant *C. glutamicum* was tested for different time periods of incubation 24, 48,72, and 96 hrs.by keeping the pH at 7.5 and temperature 30 °C constant.

By checking for all the parameters we chose the best-adapted values of chemically mutated recombinant strain which had given a high amount of yield of L Lysine to culture the recombinant strain and compared with Wild Type *C. Glutamicum* for productivity of L Lysine.¹²

Culturing the Chemical Mutagen ENU (100 nm) Treated *ddh* Recombinant Strain of *C. glutamicum* ATCC 13032 Under Optimized Conditions

After optimizing the different conditions of Temperature, PH and time of incubation 20 min chemical mutagen ENU (100 nm) exposed *ddh* gene recombinant strain ATCC 13032 was cultured in under these conditions. The ENU treated *ddh* recombinant was cultured at 30°C of temperature, 7.5 pH and with an incubation time of 96 hrs. was tested for the productivity of L Lysine from mutant *ddh* recombinant strain

compared with normal wild type ATCC 13032 *C. glutamicum* mutant.²⁴

Molecular Docking Analysis of Aspartyl β semi aldehyde with Homoserine Dehydrogenase and 2,3 Dihydrodipicolinate Synthase

Molecular docking was performed for Aspartyl β semi aldehyde with Homoserine dehydrogenase and 2,3 Dihydrodipicolinate synthase to find the bonding interactions between the Protein and ligand. The amino acid sequence of Homoserine dehydrogenase enzyme of *C. glutamicum* ATCC 13032 with accession number NP_600409.1 and 2,3Dihydrodipicolinate synthase enzyme of *C. glutamicum* ATCC 13032 with accession number NP_601846.1 was collected and from NCBI and checked verified in the UniProt and the sequences from UniProt is used to build a protein model by Homology modeling in the SWISS-MODEL Server belongs to Swiss Institute of Bioinformatics (SIB). Protein model quality built by Swiss model server analyzed through the PDBsum database. After checking the Ramachandran plot and RMSD values we choose the protein models of Homoserine dehydrogenase and 2,3 Dihydrodipicolinate synthase to dock with ligand Aspartyl β semialdehyde in the PyREX software. Finally, the image analysis and amino acid interactions in the Protein-ligand are generated in the Discovery studio.

RESULTS AND DISCUSSION

Chemical Mutagenesis

Effect of Concentration of ENU on the Recombinant Strain of *C. glutamicum*

The amount of ENU used to treat plays a pivotal role in causing Mutagenesis in the bacterial species. The recombinant *C. glutamicum* showed high productivity of L Lysine of 23.28 g/l of yield by keeping the temperature, time of incubation, and PH constant at 100 nm concentration of ENU. Optimized conditions to grow the wild type *C. glutamicum* ATCC 13032 strain for maximum yield of L Lysine of 96 hrs. time of incubation, 30 C of temperature and PH 7.5. Kept constant by checking the yield for 25,35,50,75, 100 and 120 nm of concentration produced 20.66 g/l, 20.11g/l, 21.05 g/l, 22.10 g/l, 23.28 g/l and 18.17 g/l of L Lysine respectively. We got high productivity of L Lysine at 100 nm concentration of ENU 23.28 g/l for Auxotrophic ddh recombinant mutant and a high yield of 16.23 g/l for Auxotrophic mutant of wild type *C. glutamicum* when compared to 35 nm, 50 nm,75nm concentrations of ENU.

Table-1: Table for L Lysine Production at Different ENU Concentrations

S.No.	Time of Incubation (Hours)	Temperature (0 °C)	The Concentration of ENU Treated.	pH	L-Lysine Concentration Auxotrophic Wild Type	L-Lysine Concentration Auxotrophic Mutant
1	96	30	25	7.5	13.26	20.11
2	96	30	35	7.5	13.50	20.66
3	96	30	50	7.5	14.40	21.05
4	96	30	75	7.5	15.80	22.10
5	96	30.0	100	7.5	16.23	23.28
6	96	30.0	120	7.5	14.99	18.17

The amounts of L Lysine produced shown increased trend from 25 nm concentration up to a maximum amount of L Lysine achieved at 100 nm concentration of ENU 23.28 g/l which is increased from 20.66g/l represented in table 1 and plotted in the Fig 1 by taking L Lysine concentration on Y-axis and Concentration of ENU on X-axis. The pattern of increase of L Lysine production shown illustrated in Fig.-1.

The Effect of Time of Exposure of ENU on Recombinant *C. glutamicum* ATCC 13032

The ENU treated recombinant *C. glutamicum* showed maximum yield of L Lysine of 24.02 g/l for 20 mins of exposure of chemical mutagen at 100 nm of concentration were compared to different times of exposure chemical mutagen at 100 nm concentration for 5, 10, 15,25 and 30 mins. The effect of ENU on the L Lysine is recorded maximum at 20 min of exposure before washing with citrate buffer in the process of creating the Auxotrophic mutant⁴ from the recombinant ATCC 13032. The increased productivity of L

Lysine Yield by taking the time of exposure of ENU is represented in the Fig 2 by plotting the Fig between Time of exposure of ENU on X-axis and Concentration of L Lysine on Y axis from the values of table 2 got for different times of exposure of ENU by keeping Time of incubation 96 hrs., Temperature 30 C and 100 nm Concentration of ENU and PH of 7.5 Constant. The trend of the Fig increased from 5 mins of exposure till 20 mins of exposure recorded 20.22 g/l, 22.67 g/l, 23.17 g/l and 24.02 g/l for 5 mins, 10mins, 15mins and 20 mins of exposure to ENU respectively. The maximum yield of L Lysine achieved by the Auxotrophic mutant of ddh recombinant is 24.02 g/l and for Auxotrophic mutant of the wild type strain is 16.48 g/l at 20 mins of exposure of ENU. After 25 mins of exposure decreased the amount of L Lysine yield to 22.23 g/l and at 30 min of exposure, it was 21.56 g/l.

Fig.-2: A Plot between Concentration of ENU exposed Vs Concentration of L Lysine produced. The L-Lysine concentration showed an increased pattern with an increase in the concentration from 25 nm till 100 nm with a maximum yield of 23.28 g/l and decreased at 125 nm concentration at constant values, time of Incubation 96 hrs., Temperature 30°C and PH 7.5.

The amounts of L Lysine produced shown increased trend from 25 nm concentration up to a maximum amount of L Lysine achieved at 100 nm concentration of ENU 23.28 g/l which is increased from 20.66g/l represented in Table-1 and plotted in the Fig.-1 by taking L Lysine concentration on Y-axis and Concentration of ENU on X-axis. The pattern of increase of L Lysine production shown illustrated in Fig.-2.

The Effect of Time of Exposure of ENU on Recombinant *C. glutamicum* ATCC 13032

The ENU treated recombinant *C. glutamicum* showed maximum yield of L Lysine of 24.02 g/l for 20 mins of exposure of chemical mutagen at 100 nm of concentration were compared to different times of exposure chemical mutagen at 100 nm concentration for 5, 10, 15, 25 and 30 mins. The effect of ENU on the L Lysine is recorded maximum at 20 min of exposure before washing with citrate buffer in the process of creating the Auxotrophic mutant⁴ from the recombinant ATCC 13032. The increased productivity of L Lysine Yield by taking the time of exposure of ENU is represented in the Fig.-2 by plotting the Figure between Time of exposure of ENU on X-axis and Concentration of L Lysine on Y axis from the values of Table-2 got for different times of exposure of ENU by keeping Time of incubation 96 hrs., Temperature 30 C and 100 nm Concentration of ENU and PH of 7.5 Constant. The trend of the Fig increased from 5 mins of exposure till 20 mins of the exposure recorded 20.22 g/l, 22.67 g/l, 23.17 g/l and 24.02 g/l for 5 mins, 10mins, 15mins and 20 mins of exposure to ENU respectively. The maximum yield of L Lysine achieved by the Auxotrophic mutant of ddh recombinant is 24.02 g/l and for Auxotrophic mutant of the wild type strain is 16.48 g/l at 20 mins of exposure of ENU. After 25 mins of exposure decreased the amount of L Lysine yield to 22.23 g/l and at 30 min of exposure, it was 21.56 g/l.

Table-2: Table for L Lysine Production at Different Times of Exposure of ENU to ddh Recombinant Strain.

S.No.	Time of Incubation (hours)	Temperature ($^{\circ}\text{C}$)	Time of exposure of ENU In Mins.	Concentration of ENU in Nanomolar	pH	L-Lysine concentration Auxotrophic Wild Type	L-Lysine concentration Auxotrophic Mutant
1	96	30	5	100	7.5	13.05	20.22
2	96	30	10	100	7.5	14.78	22.67
3	96	30	15	100	7.5	15.23	23.17
4	96	30	20	100	7.5	16.48	24.02
5	96	30	25	100	7.5	15.54	22.23
6	96	30	30	100	7.5	14.23	21.56

Fig.-3: A Plot between Time of Exposure of 100 nM ENU and L. Lysine Yield. The L Lysine concentration showed an increased pattern with an increase in the Time of exposure of ENU from 5 mins to 20 mins. with a maximum yield of 24.02 g/l and 16.48g/l for Recombinant and wild type mutants respectively and decreased at 25 mins. of ENU exposure at a constant time of Incubation 96 hrs., Temperature 30°C and PH 7.5.

Isolation of Auxotrophic Mutant

After three days of incubation, the strains that show growth in the presence of Methionine or Threonine or the presence of both the amino acids were considered as auxotrophic mutants and were isolated and cultured. Due to a lack of homoserine dehydrogenase, the mutant strains utilize the Threonine and methionine supplemented in the media. The auxotrophic mutants require more quantity of threonine and methionine in their media signifies the lack of Homoserine pathway that could occur in the normal strains of both Wild types as well as ddh recombinant varieties of *C. glutamicum* ATCC 13032. These auxotrophic mutants were used further to know the optimized conditions of PH, Temperature and time of incubation.¹⁰

The Chemical mutagen had shown its maximum activity at 100 nm by blocking the Homoserine dehydrogenase a key enzyme in the production of Threonine and methionine. The ENU caused mutation in the Homoserine dehydrogenase gene which generally combines with Aspartyl B Semi aldehyde to produce the homoserine. This blockage in the Homoserine pathway favors the DAP pathway by combining with 2,3, Dihydrodipicolinate synthase produced 2,3, Dihydrodipicolinate which reduces to 2,6, a dicarboxylic acid.

Optimization of PH, Temperature and Time of Incubation for Chemical mutagen ENU treated ddh recombinant strain of *C. glutamicum* ATCC 13032

Effect of pH on the Recombinant strain of *C. glutamicum*

The ENU concentration used to mutate the recombinant strain is 100 Nm which was an optimized condition for high productivity of L Lysine was used which was treated on Recombinant strain for 20

mins. The recombinant strain has shown maximum lysine productivity of 22.16 g/l at 7.5 pHs when compared to ENU mutated wild type strain ATCC 13032 at the same 7.5 pHs. The Productivity of l lysine is increased considerably in the recombinant strain for all the Ph values when compared with wild type strain. But both the strains had shown maximum productivity at 7.5 pH.

The trend of production of L Lysine increased from pH 6.0 to 7.5 and shown decreased productivity for pH 8 and PH 8.5. The L Lysine yield recorded maximum at pH 7.5 with an L Lysine concentration of 24.32 g/l. 16.50 g/l, 18.17 g/l, 22.16 g/l, 24.32 g/l, 19.17 g/l and 17.29 g/l of L Lysine yield was reported for 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 pH values respectively represented in the Table-3. The L Lysine yield recorded maximum at 7.5 pH with 24.32 g/l for Auxotrophic recombinant mutant and 17.02 g/l for an auxotrophic mutant of wild type *C. glutamicum*. The Fig was plotted by taking PH values on the X-axis and Yield on the Y-axis and illustrated the trend of increase in the productivity of L Lysine in Fig-3.

Table-3: Table for L Lysine Production at Different Times of Exposure of ENU to ddh Recombinant Strain.

S.No.	Time of Incubation (hours)	Temperature (°C)	pH	L-Lysine concentration Auxotrophic Wild Type	L-Lysine concentration Auxotrophic Mutant
1	96	30	6.0	14.78	16.50
2	96	30	6.5	15.02	18.17
3	96	30	7.0	16.35	22.16
4	96	30	7.5	17.02	24.32
5	96	30	8.0	16.21	19.17
6	96	30	8.5	13.23	17.29

The pH showed its impact on media utilization and glucose consumption by maximum uptake of glucose at 7.5 pH had supported the increased participation of the *C. glutamicum* in the Dap pathway for the L-Lysine synthesis. The pH had shown its impact on the fluidity of bacterial cell wall and Plasma membrane of the *C. glutamicum* at 7.5 which helped in glucose consumption.

Fig.-4: Plot between a pH of the Media and L Lysine Yield. The L Lysine concentration showed increased pattern with increase with the increase in value of pH, from 6.0 to 7.5 reached the maximum yield of 24.32 g/l L-Lysine and decreased for further increase in the PH by keeping time of Incubation for 96 hrs., Temperature 30°C constant after treating with ENU (100 nm) with 20mins of exposure before washing.

Effect of Temperature on the Recombinant Strain of *C. glutamicum*

Here the strain treated with 100 Nm concentration of ENU which was exposed to ENU for 20 mins was tested. The incubation was kept for different temperatures and pH of 7.5 and time of incubation 96 hrs.

was kept constant. The recombinant strain showed an increased productivity of L Lysine with the maximum amount of L Lysine was 24.20 g/l at 30 °C of temperature. All the recombinant strains showed an increased pattern in the L Lysine production till 30 °C and productivity decreased for 31 °C and 32 °C of temperature. L Lysine yield of 17.11 g/l, 19.52 g/l, 22.78 g/l, 24.20 g/l, 22.16 g/l and 18.17 g/l for temperatures 27.0 °C, 28.0 °C, 29.0 °C, 30.0 °C, 31.0 °C and 32.0 °C respectively was reported is represented in the Table-4. The Auxotrophic mutant of ddh recombinant strain had shown the maximum L Lysine productivity of 24.20 g/l and wild type auxotrophic mutant shown 18.13 maximum L Lysine yield at 30 °C. The trend for the increase in the productivity of L Lysine is illustrated in Fig.-4 by taking the pH on the X-axis and L Lysine Yield on Y axis.

Table-4: Table for L Lysine Production at Different Temperatures of ENU treated ddh Recombinant *C. glutamicum*.

S.No.	Time of Incubation (hours)	Temperature (°C)	pH	L-Lysine concentration Auxotrophic Wild Type	L-Lysine concentration Auxotrophic Mutant
1	96	27	7.5	15.23	17.11
2	96	28	7.5	16.07	19.52
3	96	29	7.5	17.45	22.78
4	96	30	7.5	18.13	24.2
5	96	31	7.5	17.66	22.16
6	96	32	7.5	16.52	18.17

The L Lysine concentration showed an increased pattern with an increase in temperature, from 27 °C to 30 °C reached the maximum yield of 24.20 g/l and 18.13 g/l L- Lysine respectively for Auxotrophic mutants of ddh recombinant and wild type and decreased for a further increase in the temp. by keeping the time of Incubation for 96 hrs., pH 7.5 constant after treating with ENU (100 nm) with 20 mins of exposure (Fig.-4).

The temperature had shown its significant effect on the rate of metabolism favoring the enzymes and substrates that participates in the synthesis of Amino acids. The temperature of 30 °C had favored the enzyme-substrate complex formations and showed its impact on the increased rate of reactions by reducing the activation energies of catalytic enzymes that were involved in the L Lysine pathway by keeping the Time, Volume and pH are constant.

Fig.-5: A Plot between Changes in the Temperature vs L-Lysine Yield

Effect of Time of incubation on ENU treated recombinant strain of *C. glutamicum*

The recombinant strain has shown maximum productivity of 24.16 g/l after 72 hrs. and also shown increased productivity when compared with ENU mutated wild type strain. The time of incubation is 96

hrs. for wild type strain which is also the same for both Recombinant and ENU treated Recombinant strains of *C. glutamicum* ATCC13032. The Time of incubation signifies the growth kinetics of *C. glutamicum*. Further incubating the strain showed the decrease in the quantity of L lysine production due to the strain entering into death phase between 96 to 120 hrs. of incubation.

The L Lysine productivity increased while the time of incubation is being increased. The L Lysine productivity for 12 hrs., 24 hrs., 48 hrs., 72 hrs., 96 hrs., and 120 hrs. was 10.23 g/l, 13.21 g/l, 17.16 g/l, 18.68, g/l, 24.16 g/l and 18.17 g/l respectively represented in the Table-5 and plotted in Fig.-5 by taking Time of incubation on the X-axis and L Lysine yield on the Y-axis. The trend of the time of incubation increased from 12 hrs. to 96 hrs. reached the maximum L Lysine concentration of 24.16 g/l for Auxotrophic recombinant mutant and 17.98 g/l for Auxotrophic mutant of L Lysine further decreased to 18.17 g/l and 15.23 for Recombinant and Wild type mutants respectively at 120 hrs. of incubation.

Table-5: Table for L Lysine Production at Different Times of Incubation of ENU Treated *ddh* Recombinant *C. glutamicum*

S.No.	Time of Incubation (hours)	Temperature (°C)	pH	L-Lysine Concentration Auxotrophic Wild Type	L-Lysine Concentration Auxotrophic Mutant
1	12	30	7.5	14.56	15.78
2	24	30	7.5	15.11	16.43
3	36	30	7.5	16.32	17.16
4	48	30	7.5	16.43	18.68
5	72	30	7.5	17.98	24.16
6	96	30	7.5	15.23	18.17

The L Lysine concentration showed an increased pattern with an increase with the increase in time of incubation, from 12 hrs. to 96 hrs. reached the maximum yield of 24.16 L and 17.98 g/l Lysine for Auxotrophic recombinant mutant and auxotrophic wild type mutant respectively and decreased for a further increase in the time by keeping temperature 30°C., PH 7.5 constant after treating with ENU(100nm) with 20 mins of exposure before washing.

The *Corynebacterium glutamicum* had entered the stationary phase after the 96 hrs. of incubation is the reason for and production of L Lysine increased substantially after the logarithmic phase and reached the maximum amount of L Lysine production between 72 hrs. to 96 hrs. of incubation.

Fig.-6: A Plot between Time of Incubation for *ddh* gene Recombinant *C. glutamicum* and L Lysine yield.

Comparative Analysis of Maximum Yields of L Lysine for Auxotrophic *ddh* Recombinant Mutant and Auxotrophic Wild Type Mutant at Optimized Conditions

By optimizing the growth of ENU treated *ddh* Recombinant ATCC 13032 strain of *C. glutamicum* we got

23.28g/l of L Lysine and 16.2 g/l of L lysine for wild type auxotrophic mutant for 100 nm concentration of ENU. By treating the ENU (100 nm) concentration for 20 minutes we got the better yield of 24.02 g/l for ddh recombinant strain and 16.48 g/l for the Wild type. After isolating the Auxotrophic mutant for Threonine and Methionine after exposing to ENU (100 nm) for 20 mins of time of exposure of ENU before washing with Citrate buffer was checked for different pH, different Temperatures and for different times of incubation for ddh gene recombinant ATCC auxotrophic mutant of *C. glutamicum* and wild type auxotrophic mutant. we got a better yield of L Lysine of 24.32 g/L for the ddh Recombinant auxotrophic mutant and 17.02 g/l for wild type auxotrophic mutant at 7.5 pH. We got a better yield of 24.20 g/l of Lysine for Auxotrophic mutant of ddh recombinant *C. glutamicum* and 18.13 g/l for wild type auxotrophic mutant at 30°C of temperature and high productivity of 24.16 g/l and 17.98 g/l of L Lysine respectively after 96 hrs. of time of Incubation (Fig.-6). Hence the best optimizing conditions for ENU treated ddh Recombinant mutant strain of *Corynebacterium glutamicum* shown for high yield of L Lysine at the 100 nm concentration of ENU with an exposure time of 20 mins created an Auxotroph which shows a better yield of L Lysine at 7.5 pH, 30°C of temperature and 96 hrs. of Incubation. The Optimized Parameters and amount of L Lysine Produced for Recombinant mutant and quantity of L Lysine produced by wild type auxotrophic mutant at the same conditions of recombinant strain was tabulated in Table-6.

Table-6: Table for L Lysine production for ddh recombinant mutant and wild type mutant at the optimized parameters of ddh recombinant mutant.

S. No.	Optimized Parameters	L Lysine in Wt.mutant (g/l)	L Lysine in AR Mutant(g/l)
1.	Conc. Of ENU (100 nm)	16.12	23.28
2.	Time of exposure (20 min.)	16.48	24.02
3.	PH (7.5)	17.02	24.32
4.	Temperature (30°C)	18.13	24.20
5.	Time of incubation 96 hrs.	17.98	24.16

Fig.-7: Maximum Yield of L Lysine for Auxotrophic Wild Type Mutant (WT) and auxotrophic ddh recombinant mutant (AR Mutant) based on Optimized parameters of Recombinant strain.

Molecular docking analysis of Aspartyl β Semi Aldehyde Ligand With Homoserine Dehydrogenase Protein

Homology model is developed in the SWISS-MODEL server for the amino acid sequence collected from UniProt.³⁷ The Protein model built in the Swiss model³⁶ collected in the .pdb format. The protein model is further analyzed in PDBsum³⁷ database to check the protein quality for docking. The Ramachandran plot had shown nearly 91.5% of residues in the favorable regions and RMS distance from planarity is around 2 signified good protein quality. The ligand of aspartyl semi aldehyde collected from PubChem is used to dock in the PyREX³⁵ software with the Modeled Homoserine dehydrogenase protein. The ligand Aspartyl β semi aldehyde had shown its interaction with glycine GLY (151) with a hydrogen bond and also a carbon-hydrogen bond with glycine GLY (288). Proline PRO (B: 287) Alanine ALA (B:289), tyrosine TYR (B: 178,155), Glycine GLY (B:268, 151), Asparagine ASN (B:270) and Leucine LEU (B: 154) are the interacting amino acids with the ligand in Homoserine dehydrogenase. Homoserine

dehydrogenase enzyme is with one conventional hydrogen bond. The Homoserine dehydrogenase showed good interaction with the ligand Aspartyl β semi aldehyde with a binding energy of -5.2 Kcal (Fig.-7). Homoserine dehydrogenase had nine interacting amino acids is the reason for having high binding energy even though having only a hydrogen bond and a Carbon-hydrogen bond³⁶.

Molecular Docking Analysis of Aspartyl β Semi Aldehyde Ligand With 2,3Dihydrodipicolinate Synthase

Homology model is developed in the SWISS-MODEL server for the amino acid sequence 2,3 Dihydrodipicolinate synthase collected from UniProt. The Protein model built in the Swiss model collected in the .pdb format. The protein model is further analyzed in the PDBsum database to check the protein quality for docking. The Ramachandran plot had shown nearly 90.05% of residues in the favorable regions and RMS distance from planarity is between 1.5 to a signifying good quality of modeled protein. The ligand of aspartyl semi aldehyde collected from PubChem is used to dock in the PyREX software with the Modeled 2,3 Dihydrodipicolinate synthase protein. The ligand Aspartyl β semi aldehyde had shown its interaction with Leucine (B:206), Serine (B:261), Aspartate ASP (B:205) and Isoleucine (B:257) with a conventional hydrogen bond. 2,3, Dihydrodipicolinate synthase is with 4 conventional hydrogen bonds. Leucine LEU (B:206), Aspartate ASP (B:205), Serine SER (B:261) and Isoleucine ILE (B:257) were the interacting amino acids of 2,3, Dihydrodipicolinate synthase. The 2,3Dihydrodipicolinate synthase showed good interaction with the ligand Aspartyl semi aldehyde with binding energy of -4.4 Kcal (Fig.-8). Even though it had fewer amino acid interactions but due to the presence of 4 hydrogen bonds it can able to show considerable binding with the Ligand³⁷.

RMS distances from planarity

Fig.-8: (a) Ligand Aspartyl β semialdehyde, (b) Protein Homoserine dehydrogenase (c and d) Protein-ligand interaction (Docking in Pyrex), (e) lid plot analysis (PDBsum) of Ligand Aspartyl β semi aldehyde with Protein Homoserine dehydrogenase: GLY (151), H-bond and C-H bond with GLY (288). PRO (B: 287), ALA (B:289), TYR (B: 178,155), GLY (B:268, 151), ASN (B:270) and LEU (B: 154) are the interacting amino acids in protein Homoserine dehydrogenase with the ligand Aspartyl β semi aldehyde, (f) Ramachandran plot for HSD with 91.5% favored regions,(g) RMSD value of HSD between 1.5 to 2.0 for HSD signifying the good quality of Protein to be docked with the ligand.

Table-7: Ramachandran Plot Statistics Representing the Quality of Proteins Used for Modeling

S.No.	Plot Statistics	Homoserine Dehydrogenase		2,3 Dihydrodipicolinate Synthase	
1	Residues in most favored regions [A,B,L]	1124	91.5%	447	90.5%
2	Residues in additional allowed regions [a,b,l,p]	92	7.5%	42	8.5%
3	Residues in generously allowed regions [$\sim$ a, $\sim$ b, $\sim$ l, $\sim$ p]	8	0.7%	2	0.4%
4	Residues in disallowed regions	4	0.3%	3	0.6%
5	Number of non-glycine and non Proline residues	1228	100.0%	494	100%
6	A number of end-residues (excl. Gly and Pro)	8		4	
7	Number of glycine residues (shown as triangles)	92		24	
8	Number of Proline residues	48		18	
9	Total number of residues	1376		540	

The aspartyl β semi aldehyde had shown higher binding energy of -5.2 k. cal with homoserine dehydrogenase with 9 interacting amino acids and two hydrogen bonds than 2,3 Dihydrodipicolinate synthase enzyme with - 4.5 k.cal of binding energy (Fig.-9), four interacting amino acids with four Hydrogen bonds. As the chemical mutagen, ENU caused a mutation in the homoserine dehydrogenase caused protein not to express or might have produced a protein which could not bind properly with Aspartyl β semi aldehyde diverted the Aspartyl semialdehyde to bind with 2,3 Dihydrodipicolinate synthase enzyme entered the dap pathway. As the binding energy is more for homoserine dehydrogenase enzyme it showed higher reactivity with the Aspartyl β semi aldehyde but lack of it caused the Aspartyl semialdehyde to bind with 2,3 Dihydrodipicolinate synthase which directed to L-Lysine synthesis through producing L L diaminopimelate. Being a ddh recombinant strain the *Corynebacterium glutamicum*

The L Lysine yield in the newly developed auxotrophic mutant of ddh recombinant strain of *Corynebacterium glutamicum* ATCC 13032 strain in the presence of Homoserine based amino acids

Auxotrophic ddh recombinant strain in the presence of methionine and threonine had produced g/l of L Lysine, in the presence of Threonine only had produced 22.34 g/l and 23.54 g/l in methionine only and 22.92 g/l of L Lysine in the absence of both methionine and Threonine from the auxotrophic recombinant mutant.

RMS distances from planarity

Fig.-9: (a) Ligand Aspartyl β semialdehyde, (b) Protien2,3 Dihydrodipicolinate synthase, (c and d) Protein-ligand interaction, (e) lid plot analysis of Ligand Aspartyl β semi aldehyde with ProtienProtien 2,3 Dihydrodipicolinate synthase : LEU (B:206), SER (B:261), ASP (B:205) and ILU (B:257) are the interacting amino acids of protien2,3 Dihydrodipicolinate synthase with 4 conventional H-bonds formed with the ligand Aspartyl β semi aldehyde, (f). Ramachandran plot for DHPS with 90.05% favored regions, (g) RMSD value of HSD between 1.5 to 2.0 for DHPS signifying the good quality of Protein to be docked with the ligand.

Fig.-10: L Lysine Yield of Auxotrophic mutant of ddh Recombinant strain of *C. glutamicum*.

In the presence of both Methionine and Threonine, the L Lysine Yield is 24.89 g/l, in the presence of threonine only the L Lysine yield is 23.54 g/l, methionine only is 22.34 g/l and in the absence of Methionine and threonine is 21.92 g/l for the Auxotrophic mutant of ddh recombinant ATCC 13032 strain of *C. glutamicum* (Fig.-10).

The ddh Recombinant mutant produced more amounts of L Lysine under because of the effect chemical mutagenesis of ENU by blocking the *Homoserine dehydrogenase* of homoserine pathway diverting the aspartate β -semialdehyde to bind with 2,3 Dihydrodipicolinate synthase. The auxotrophic recombinant mutant had shown a yield 24.89 g/l L Lysine in the presence of threonine and Methionine. 23.54, g/l in the presence of threonine only and 22.34 g/l in the methionine only and 22.92 g/l without threonine and methionine in the media signifies the mutation had occurred in the Homoserine dehydrogenase which participates in the Homoserine pathway of the auxotrophic mutant of ddh recombinant *Corynebacterium glutamicum* ATCC 13032. Binding energy for the protein homoserine dehydrogenase of -5.2 K. Cal with one conventional hydrogen bond binds more strongly with -0.7 k.cal greater than 2,3 Dihydrodipicolinate synthase with -4.5 K.Cal with 4 conventional hydrogen bonds with aspartate β -semialdehyde. Due to the lack of Homoserine dehydrogenase in the auxotrophic mutant of ddh recombinant, the energy utilized in the synthesis of Threonine and Methionine by Homoserine pathway will be utilized in the L Lysine synthesis by binding with 2,3 Dihydrodipicolinate synthase to produce L Lysine through L L diaminopimelate (2,3 meso-DAP). Even the number of hydrogen bonds and interacting amino acids of the proteins play an important role in the diverting the Homoserine pathway to DAP pathway can be analyzed in the molecular docking analysis of Protein and ligand.

Being a recombinant for ddh gene, the recombinant mutant expressed more amounts of DDH enzyme which further diverts the strain from entering 3 enzymes involved pathways of acetyltransferase and Succinyl transferase to a single enzyme involved ddh pathway in the production of L L diaminopimelate (2,3 meso-DAP) for the production of High yield of L Lysine.

The wild type mutant and ddh recombinant mutant had Chemical mutagen ENU induced blockage in the homoserine pathway and overexpression of ddh gene directed DAP synthesis in ddh recombinant strain with the involvement of fewer enzymes compared with Acetyltransferase and Succinyl transferase pathways lead to increased production of L Lysine than auxotrophic mutant Wild Type of *C. glutamicum*. Nearly 4.23 g/l amount of L Lysine was increased in this newly developed Strain when compared with normal ddh recombinant strain which had a yield of 20.66 g/l L Lysine. The L Lysine Yield of Auxotrophic mutant of ddh recombinant is 6.32 g/l L Lysine Yield more than auxotrophic mutant wild type with a yield of 18.57 g/l L Lysine. In the case of Auxotrophic mutant of ddh recombinant strain shown the maximum yield in the presence of both threonine and methionine of 24.89 g/l L Lysine, 23.54 g/l L Lysine in the presence of threonine only, 22.34 g/l L Lysine in the presence of only methionine, and finally 22.92 g/l of L Lysine in the absence of both methionine and threonine. From this study, the

amount of lysine production enhanced is discussed on the basis of molecular docking which further supported our results. Hence we developed an auxotrophic mutant from the ddh gene recombinant of *Corynebacterium glutamicum* ATCC 13032 which will be helpful in industrial L Lysine Production through this new Auxotrophic mutant (Fig.-11).

Fig.-11: Yield of L Lysine of Auxotrophic mutants of ddh recombinant and Wild Type *C. glutamicum* at Optimized conditions of ddh Recombinant mutant. R- L Lysine in an auxotrophic mutant of ddh Recombinant *C. glutamicum* ATCC 13032 is 24.89(g/l). L- Lysine in an auxotrophic mutant of wild type *C. glutamicum* ATCC 13032 18.57 (g/l).

CONCLUSION

By culturing the chemically mutated ddh recombinant ATCC 13032 strain the L Lysine yield was increased to 24.89 g/l at optimized parameters of PH 7.5, temperature 30 C and 96 hrs. of time of incubation compared to an auxotrophic mutant of wild type strain with a yield of 18.57 g/l of L Lysine.

ACKNOWLEDGEMENT

I sincerely thank Science and Engineering Research Board (SB/FTP/ETA-168/2012, Dt: .20-5-2013,DST,Govt, New Delhi-110070) for founding this entire project and also to JNTUK Kakinada for enabling me to continue my research project.

REFERENCES

1. B. Mallaiah, T. Giri, E. Laxminarayana, M. Thirumala Chary and K. Shashikala, *Organic and Medicinal Chemistry Letters*, **4**, 16 (2014)
2. Min Ju Lee and Pil Kim, *Frontiers in Microbiology*(2018), [DOI:10.3389/fmicb.2018.02523](https://doi.org/10.3389/fmicb.2018.02523)
3. V. F. Wendisch and Bott M, *CRC Press. Taylor & Francis*, pp. 377–396(2005), [DOI:10.1201/9781420039696](https://doi.org/10.1201/9781420039696)
4. S. Anastasiadis. *Recent Patents Biotechnology*, **1**, 11(2007) , [DOI:10.2174/187220807779813947](https://doi.org/10.2174/187220807779813947)
5. A. H. Shah, A. Hameed, and G.M. Khan. *International Journal of Medical Sciences*, **2**, 52 (2002), [DOI:10.3923/jms.2002.152.157](https://doi.org/10.3923/jms.2002.152.157)
6. O. Tosaka, *Trends in Biotechnology*, **1**, 70(1983) , [DOI:10.1016/0167-7799\(83\)90055-0](https://doi.org/10.1016/0167-7799(83)90055-0)
7. M. Ikeda, *Advances in Biochemical Engineering/Biotechnology*, **79**, 1(2003, [DOI:10.1016/0167-7799\(83\)90055-0](https://doi.org/10.1016/0167-7799(83)90055-0)
8. J. Z. Xu, M. Han, J. L. Zhang, Y. F. Guo and W. G. Zhang. *Amino Acids*, **46**, 2165(2014), [DOI:10.1007/s00726-014-1768-1](https://doi.org/10.1007/s00726-014-1768-1)
9. M. Hilliger, F. Haenel and J. Menz, *Journal of Applied Microbiology* **24**, 437(1984), [DOI:10.1002/jobm.19840240704](https://doi.org/10.1002/jobm.19840240704)
10. Leuchtenberger W, Huthmacher K and Drauz K. *Applied Microbiology and Biotechnology*, **69**, 1 (2005), [DOI:10.1007/s00253-005-0155-y](https://doi.org/10.1007/s00253-005-0155-y)
11. M. Patek , *Springer, Heidelberg*, 778(2007).
12. R. S. Hanson, R. L. Dillingham, P. Olson, G. H. Lee, D. Cue and F. J. Schendel, *Kluwer Academic Publishers, Dordrecht*, 227(1996) , [DOI:10.1007/978-94-009-0213-8_31](https://doi.org/10.1007/978-94-009-0213-8_31)

13. T. Tryfona and T. Mak, *Biochemistry*, **40**, 499(2005) , [DOI:10.1093/nar/gku989](#)
14. S. Hirono, M. Sugimoto, E. Nakan, M. Izui and Y. Hayakawa, United States Patent 6090597(2000).
15. L. Liu, H. Yang, H. D. Shin, J. Li, G. Du and J. Chen, *Applied Microbiology and Biotechnology* , **97**, 9597 (2013) , [DOI:10.1007/s00253-013-5250-x](#).
16. J. Y. Lee, Y. A. Na, E. Kim, H. S. Lee and P. Kim, *Journal of Microbiology and Biotechnology*. **26**, 1341(2016), [DOI:10.4014/jmb.2016.2607.1341](#)
17. Van Ooyen J, Noack S, Bott M, Reth and L. Eggeling, *Biotechnology & Bioengineering*, **109**, 2070(2012) , [DOI:10.1002/bit.24486](#)
18. H. Ozaki, I. Shiio, *Agricultural and Biological Chemistry*, **47**, 1569(1983), [DOI:10.1080/00021369.1983.10865820](#)
19. G. E. Colón, M. S. Jetten, T. T. Nguyen, M. E. Gubler, M. T. Follettie, A. J. Sinskey and G. Stephanopoulos, *Applied and Environmental Microbiology*, **61**, 74 (1995).
20. D. J. Reinscheid, W. Kronmeyer, L. Eggeling, B. Eikmanns and H. Sahm, *Applied and Environmental Microbiology*, **60**, 126(1994)
21. M. Kircher, W. Pfeerle, *Chemosphere*, **43**, 27 (2001).
22. G. M. Seibold, S. Auchter, J. Berens, Kalinowski and Eikmanns, *Journal of Biotechnology*, **124**, 381 (2006) , [DOI:10.1016/j.jbiotec.2005.12.027](#)
23. R. Q. Nelofer, M. Syed and X. Nadeem, *Turkish Journal of Biochemistry*, **33**, 50 (2002).
24. A. H. Shah, G. M. Hameed and Khan, *Pakistan Journal of Biological Sciences*, **5**, 80(2002), [DOI:10.3923/pjbs.2002.80.83](#)
25. A. H. Shah, S.U. Hameed, A. H. Rehman, *Pakistan Journal of Zoology*, **44(1)**, 141(2012).
26. C. B. Pham, H. Kataoka, and M. Matsumura, *International Center for Biotechnology.*, **16**, 506509 (1993).
27. M. V. Matos and N. Coello, *Acta Científica Venezolana*, **50**, 233 (1999).
28. D. Hanahan, *DNA Cloning*, **1**. IRL Press, Oxford (1985).
29. J. Ohnishi, A. S. Mitsuhashi, M. Hayashi, S. Ando, K. Yokoi, K. Ochiai and M. Ikeda, *Applied Microbiology and Biotechnology*, **58**, 217 (2002, [DOI:10.1007/s00253-001-0883-6](#)
30. C. Wittmann and E. Heinzle, *Applied and Environmental Microbiology* **68**, 5843 (2002).
31. H. Nishida, M. Nishiyama, K. Nobuyuki, T. Kosuge, T. Hoshino and H. Yamane, *Genome Research* **9**, 175 (1999) , [DOI:10.1101/gr.9.12.1175](#)
32. A. Waterhouse, M. Bertoni, S. Bienert, G. Studer, G. Tauriello and Gumieny, *Nucleic Acids Research*, **46**, W296 (2018) , [DOI:10.1093/nar/gky427](#)
33. R. A. Laskowski, J. Jabłońska, L. Pravda, R. S. S. Vařeková, J. M. Thornton, *Protein Science*, **27**, 129 (2018) , [DOI:10.1002/pro.3289](#)
34. R. A. Laskowski, M. W. MacArthur, D. S. S. Moss and Thornton, *Journal of Applied Crystallography*, **26**, 283 (1993) , [DOI:10.1107/S0021889892009944](#)
35. M. Kalia, V. K. Yadav, P. K. Singh, S. Dohare, D. Sharma, S. S. Narvi and V. Agarwal, *Biotech*, **9(2)**, 40 (2019) , [DOI:10.1007/s13205-019-1567-1](#)
36. Uni Prot and Consortium, *Nucleic Acids Research*, **43**, D204 (2015) , [DOI:10.1093/nar/gku989](#)
37. T. P. Dao, D. C. Nguyen, T. H. Tran, P. V. Thinh and V. Q. Hieu, *Rasayan Journal of Chemistry* **12(2)**, 666 (2019) , [DOI:10.31788/RJC.2019.1225107](#)
38. A. A. Parikesit and U. S. F. Tambunan, *Rasayan Journal of Chemistry*, **11(3)**, 1133(2018), [DOI:10.31788/RJC.2018.1133038](#)

[RJC-5416/2019]