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Enhanced L-lysine production through chemical mutagenesis in *Corynebacterium glutamicum* MTCC 25069

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Abstract

Lysine is one of the most commercially available amino acid, which is chiefly used as a feed additive, human medicine and as a dietary supplement. In the present day, *Corynebacterium glutamicum* is the most popularly known microorganism for the industrial biosynthesis of lysine. The purpose of this study was to improve the yield of lysine production by chemical mutagenesis. In this study the culture conditions were optimized and the maximum growth was observed at 37p C, pH-7.4 for 72 hours.

The chemical mutagen, N Ethyl N Nitroso Urea (ENU) was used to develop the auxotrophic mutants by treating the wild type strain with different concentrations of ENU (i.e.,35mM, 50mM, 70mM, and 100mM) at different time periods (i.e.,0, 5, 10, 15, 20, and 25mins). The obtained mutants were then inoculated in seed culture medium enriched with methionine and threonine and all the mutants obtained were screened for lysine concentrations.

The most potent auxotrophic mutant developed in this study produced 30.2 g/l lysine when the culture was exposed to ENU of 70mM concentration for 10 minutes.

Keywords: L-lysine, *Corynebacterium glutamicum,* N Ethyl N NitrosoUrea (ENU), mutagenesis, auxotrophs, seed culture medium.

Introduction

L Lysine is one of the most important essential amino acids which could be used in many biophysical mechanisms in the 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 cosmetics[7].

Corynebacterium glutamicum is a non-lethal and non-emulsifying gram-positive bacterium. It exhibits a low protease activity in the culture and

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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 proteins[15] 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 MTCC 25069 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 mutation in Homoserine dehydrogenase gene to cause the Homoserine Auxotrophs of C. glutamicum[21].

Generally, the Aspartyl â semialdehyde is produced in two ways. In the Krebs cycle of Corynebacterium glutamicum, the Oxaloacetic acid (OAA)[19,20] undergoes transamination reaction with the presence of *glutamate: oxaloacetate: transaminase* enzymes produce aspartyl â semi aldehyde which further produces homoserine and L L diaminopimelate (2,3 meso- DAP) by two different pathway[1]. The Aspartyl â semialdehyde is also formed from *Aspartate dehydrogenase* from Aspartyl phosphate which was formed from Aspartate by *Aspartate kinase*[2,19]. The aspartyl â-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 aspartylâ-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 *MetA*[6] and produces O-Acetylhomoserine which reacts with *Met B* synthesize Cystathionine further reacts with *C*[7] 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 threonine[11] by Threonine synthase in Homoserine pathway. Aspartyl â semi aldehyde reacts with *2,3 Dihydrodipicolinate synthase* produces 2,3 Dihydropicolinate 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)[3,9] 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 â-semialdehyde to react with 2,3 Dihydrodipicolinate synthase the enzyme to produce more amounts of "-diaminopimelate (" DAP) through ddh pathway.2,3 Dihydrodipicolinate synthase [35] converts aspartic β-semialdehyde to

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2,3 Dihydropicolinate. In the presence of reductase 2,3 Dihydropicolinate 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 *acetyltransferase* and *Succinyltransferase* pathways by overexpression of *ddh* by the ddh recombinant strain of C. glutamicum MTCC 25069 strain with constitutive promoter.

Materials and Methods

Chemicals used in this study: Seed culture medium: (Peptone 5 g/l, Yeast extract 3.75 g/l, NaCl 5 g/l, KH_2PO_4 25 g/l, K_2HPO_4 25 g/l,) [D-Glucose 10 g/l, $(NH_4)_2SO_4$ 17.46 g/l, $ZnSO_4$ 0.05 g/l, $FeSO_4.7H_2O$ 0.02 g/l, $MnSO_4.5H_2O$ 0.02 g/l, $MgSO_4.7H_20$ 0.04 g/l, Methionine 2 g/l, Threonine 2 g/l].

Optimization of Corynebacterium glutamicum MTCC 25069 : The culture conditions of *Corynebacterium glutamicum* were optimized to improve the the yield of L-lysine.

Optimal pH: In order to find optimal pH, the temperature and time of incubation were kept constant. The 24hr broth culture which was incubated at different pH levels was centrifuged and the quantification of protein was done by Lowry's method.

Optimal temperature: To find the optimal temperature, the pH and time of incubation were kept constant. The 24hr broth culture which was incubated at different pH levels was centrifuged and the quantification of protein was done by Lowry's method.

Optimal time: In order to find the optimal time, pH and temperature of the bacterial cultures were kept constant. The cultures were incubated at different time intervals. The samples were then collected and the total protein was estimated by lowry's method.

Mutation of Corynebacterium glutamicum MTCC 25069 : The bacterial strain *Corynebacterium glutamicum* MTCC 25069 was mutated by using the chemical mutagen N Ethyl N NitrosoUrea. The mutation of this bacteria was carried out at different concentrations of ENU (i.e., 35mM, 50mM, 70mM, and 100mM) and the auxotrophs were isolated as per given below. Seed culture medium was used for the growth of mutant bacterial cells.

Procedure: The wild type bacterial cells were grown on Luria Bertani (LB) broth and 10ml of LB broth was taken in eight falcon tubes and was inoculated with the growth. These tubes were incubated at 37p C for 24 hours and were used for mutagenesis purpose. After 24 hours the tubes were centrifuged at 10000 RPM for 5 minutes and the pellets were collected. The pellets were suspended in 3ml Sodium Citrate buffer (pH 4.1) and again centrifuged at 10000 RPM for 5 minutes. The pellets were again resuspended in Sodium Citrate buffer containing 1.2ml ENU solution. The cultures were incubated for 0, 5, 10, 15, 20 and 25 minutes. The growth culture without adding mutagen was also run along with the test samples

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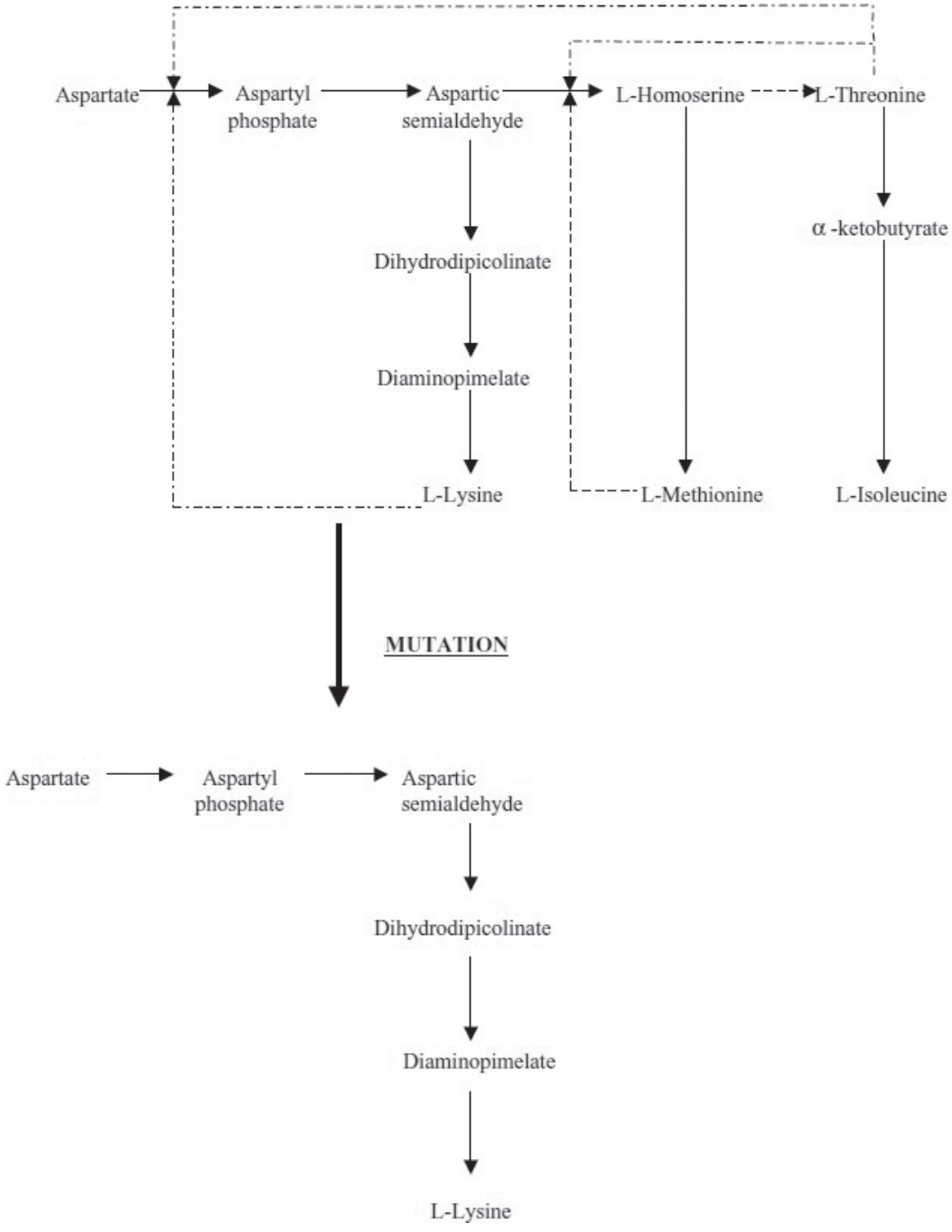

Fig. 1: Deregulation of lysine synthesis in auxotrophs (Nakayama, 1985).

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under similar conditions. After incubation, the samples were centrifuged at 10000 RPM for 5 minutes and the pellets were collected. The pellets were now washed with 3ml Sodium Citrate buffer and again centrifuged at 10000 RPM for 5 minutes to remove ENU and the resultant pellets were resuspended in 3ml of Sodium Citrate buffer. These pellets were inoculated in seed culture medium enriched with methionine and threonine and were incubated at 37p C for 3 days.

Isolation of auxotrophs: The growth obtained was inoculated in 1ml seed culture medium containing no methionine and threonine. To this, 100 units of penicillin G was added and incubated on shaking incubator for 20 hours at 37p C. After 20 hours, 50 units of penicillinase was added to each tube and left for 10 minutes.100ul of this growth was inoculated on seed culture medium plates without methionine and threonine and with seed culture medium with methionine and threonine. These plates were incubated at 37p C for 3 days. Colonies that grow on seed culture medium with methionine and threonine but not on seed culture medium without methionine and threonine were isolated. These isolated colonies were screened for lysine production. The samples were centrifuged at 15000 rpm for 10 minutes. The supernatant was collected and analyzed for lysine concentration.

Qualitative and quantitative analysis: For the qualitative analysis of lysine, paper chromatography was used whereas quantitative estimation was done by using the Ninhydrin method.

Results and Discussion :

Optimal pH: The pH of the media's were adjusted to 2, 5, 7.4, 9 and 11. From the values obtained from growth turbidity, (For total protein estimation) an efficient growth for *Corynebacterium glutamicum* MTCC 25069 was observed at an optimal pH of **7.4** (as shown in Fig. 3).

Optimal temperature: The cultures were incubated at different temperatures (i.e. 10p C, 25p C, 37p C, and 45p C). The optimal temperature required for the growth of *Corynebacterium glutamicum* MTCC 25069 is 37p C (as shown in Fig. 4).

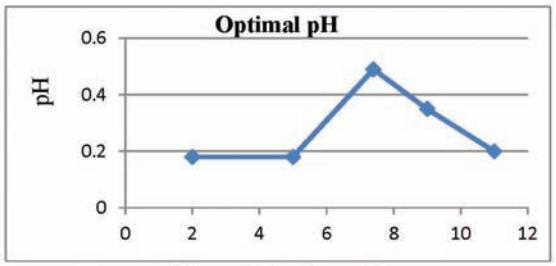

Fig. 2: Optimal pH

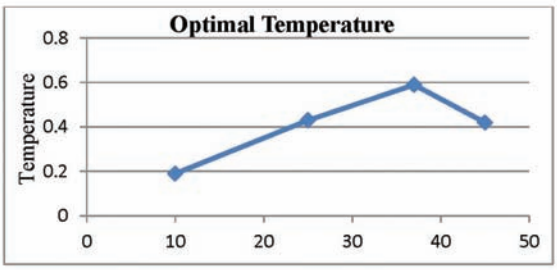

Fig. 3: Optimal temperature

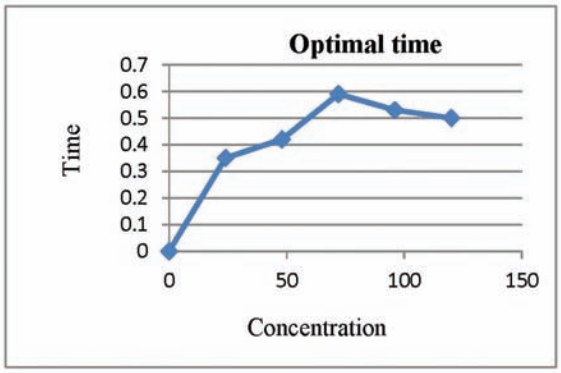

Fig. 4: Optimal time

Optimal time: Fermentation was carried out for 120 hours and for every 24 hours the samples were collected to measure growth turbidity.The growth turbidity has increased after 48 hours and was maximum at 72 hours. Later the concentration of protein gradually decreased (as shown in Fig. 5).

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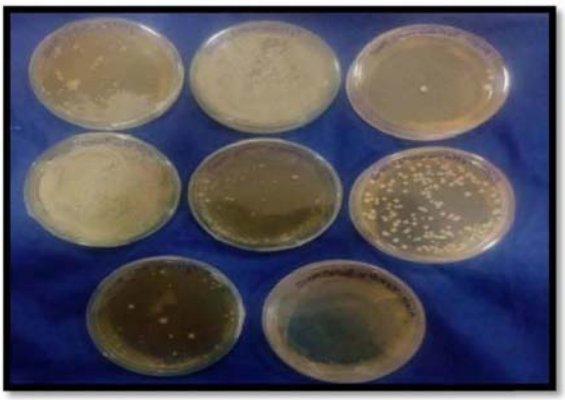

Fig. 5: Culture plates treated with 35mM concentration of ENU.

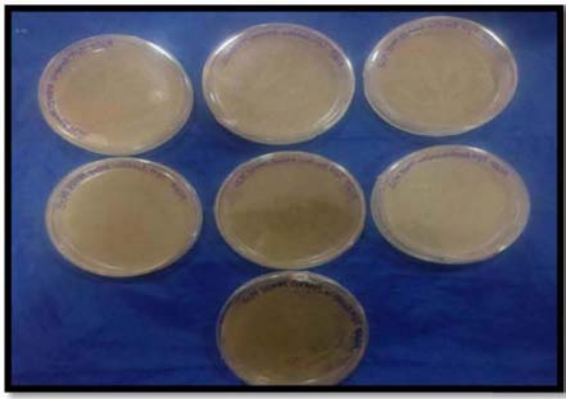

Fig. 6: Culture plates treated with 50mM concentration of ENU.

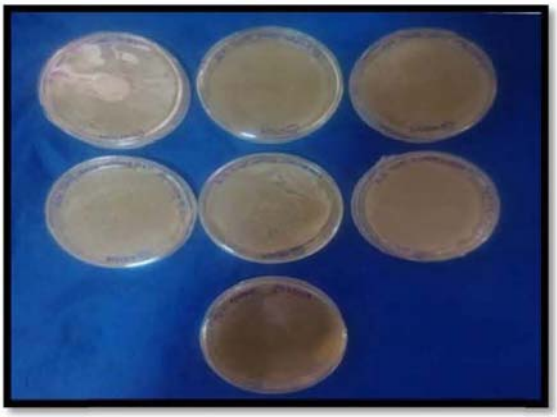

Fig. 7: Culture plates treated with 70mM concentration of ENU.

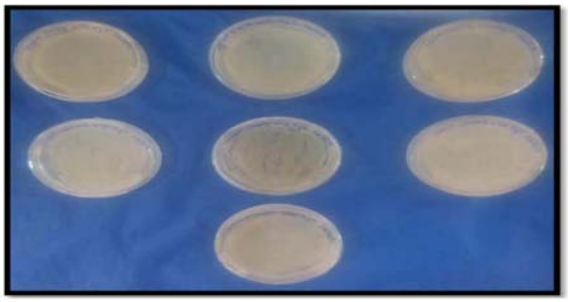

Fig. 8: Culture plates treated with 100mM concentration of ENU.

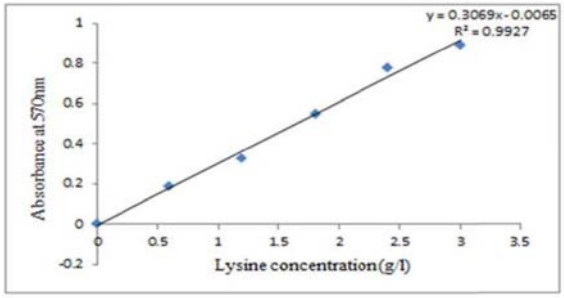

Fig. 9: Standard graph for Ninhydrin method.

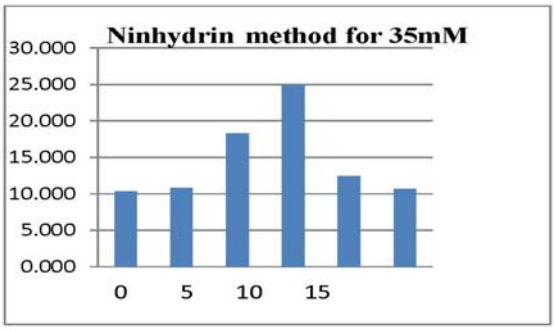

Fig. 10: Ninhydrin method for 35mM.

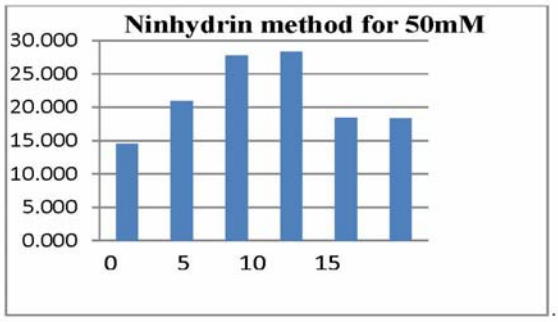

Fig. 11: Ninhydrin method for 50mM.

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Table 1: Concentration of Lysine in mutants developed from Corynebacterium glutamicum MTCC 25069

Mutants	Lysine
Wild type	concentration (g/l)
MTCC 25069	14.959
A0	10.318
A5	10.835
A10	18.275
A15	24.873
A20	12.453
A25	10.706
B0	14.507
B5	20.927
B10	27.784
B15	28.302
B20	18.437
B25	18.356
C0	26.701
C5	30.275
C10	24.356
C15	19.051
C20	18.614
C25	17.838
D0	11.790
D5	10.027
D10	10.431
D15	6.970
D20	13.180
D25	5.790

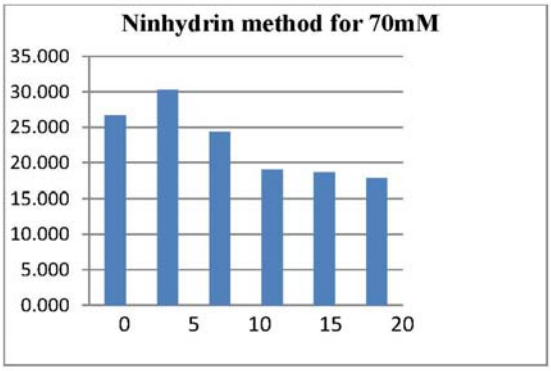

Fig. 12: Ninhydrin method for 70mM.

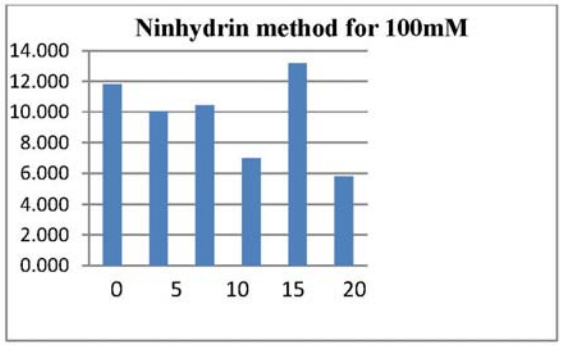

Fig. 13: Lysine estimation by Ninhydrin method for 100mM.

Well no.1 and 2: reference
Well no 3 to 6: A15, B15, C5, D20.

Fig. 14: SDS-PAGE gel showing lysine bands

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Isolation of auxotrophs: The Auxotrophs were isolated after spreading chemically (ENU) mutated samples onto the seed culture medium containing methionine and threonine and on to the seed culture medium without methionine and threonine. Colonies that grow only on methionine and threonine containing plates but not on plates without methionine and threonine were considered as auxotrophs. These auxotrophs were isolated after several repeats and a reduced growth was observed on the plates which were analyzed for lysine production. (From Fig 6 to Fig 9)

Quantitative analysis:

Ninhydrin method: This method was used to quantify lysine concentration in both mutated samples and in the wild type strain of *Corynebacterium glutamicum* MTCC 25069.

Here y = absorbance at 440nm and x = Concentration of lysine (g/l) (Fig. 11).

The concentration of lysine was calculated and presented in Table 15:

Out of 24 mutants, only 13 (represented in bold in the table) showed an increase in the lysine

yield, compared to the wild type strain which is grown in the seed culture medium.

In both the sets A and B, the mutants obtained by treating the cultures with ENU for 15 minutes showed higher lysine concentration compared to the remaining samples.

In set A, the concentration of lysine has increased only in A10 and A15 mutants. Whereas in set B all the mutants showed an increase in lysine yield except B0.

All the mutants of set C showed an increase in the lysine concentration with the highest maximum yield of 30.275g/l by C5 mutant. All the mutants of set D showed a negative effect on the mutants as there was no increase in the lysine yield.

Graphs were plotted for each set of ENU concentrations from the values obtained from the Ninhydrin test (i.e., for 35mM,50mM,70mM, and 100mM).

From the (Fig. 16), it can be interpreted that, at 35mM concentration of ENU, the mutant sample treated for 15 minutes showed the highest lysine concentration compared to the remaining mutant samples.

From (Fig. 13), it can be inferred that the mutant sample obtained by treating the wild type strain with 50mM ENU for 15 minutes produced the highest lysine concentration.

From the (Fig. 14), it can be interpreted that, at 70mM concentration of ENU, the mutant sample at 5 minutes showed the highest maximum lysine concentration.

The mutant samples obtained by treating the wild type strain with 100mM concentration of ENU at different time periods showed different concentrations of lysine, with the highest yield at 20minutes (Fig. 15).

SDS-PAGE: This method was performed to confirm whether lysine is present in the mutated sample and wild type strain.

At different concentrations of ENU, the mutant samples producing the highest lysine concentrations from each set were selected (i.e., A15, B15, C5, and D20).

In the above gel picture, the first two wells are loaded with L-lysine (reference) and the remaining wells are loaded with samples. After staining and destaining of the gel, bands were visible and they stand along the same line with lysine and by comparing with the reference (L-lysine) it can be inferred that lysine was present in the mutant samples (Fig. 16).

Conclusion

According to lysine production statistics in 2015, approximately 2.4 metric million tons of L-lysine is required every year, but there is an enormous shortage in the production of this amino acid as only 2 million tons of L-lysine is produced annually. Due to the increasing demand in the world market for this amino acid, there is a growing interest in the development and optimization in the fermentative process of L-lysine production.

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Of all the bacteria present, *Corynebacterium glutamicum* is an industrially important microorganism for the production of various amino acids, hence it has been selected for this study. Mutagenesis is carried out by using N Ethyl N NitrosoUrea (ENU) which is one of the potent alkylating chemical mutagens. (ENU) adds alkyl groups (an Ethyl group in ENU) to many positions on all four bases, mutagenesis is best correlated with an addition to the oxygen at the 6th position of guanine to create an O-6 alkylguanine. This addition results in GC '! AT transitions and would lead to direct mispairing with thymine.

In this study, the bacterial culture conditions were optimized and the maximum growth was observed at 37p C, pH 7.4 for 72 hours. The auxotrophic mutants were obtained by treating the wild type bacterial strain with different concentrations of ENU (i.e. 35mM,50mM,70mM, and 100mM) for different time periods (i.e 0, 5, 10, 15, 20 and 25 minutes). 13 out of 24 mutants showed a significant difference in the yield and productivity compared to the wild type strain.

The auxotrophic mutant treated with 70mM ENU for 5 minutes showed the highest lysine concentration of 30.2g/l.

From this, we can conclude that the mutagenic effect of ENU was significant when cells of *Corynebacterium glutamicum* MTCC 25069 were treated with 70mM concentration of ENU for 5 minutes.

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