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Efficient production of lysine from genetically modified *Corynebacterium glutamicum* by feedback inhibition resistant strain

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Abstract

Lysine is an essential amino acid required for the synthesis of proteins. Lysine deficiency leads to numerous irreversible damages to the brain and other body organs. It plays a vital role in the treatment of osteoporosis and Herpes Simplex Virus (HSV). Lysine is commonly produced through fermentation. However, its efficient production is still a bottle neck. The wild strains of microorganisms are unable to produce sufficient amino acid. The current study was designed to enhance the lysine production through feedback inhibition resistant strains. N-methyl-N-nitro-N-nitrosoguanidine (NTG) was utilized to mutate the *Corynebacterium glutamicum* strain B391 to increase the production of Lysine. *C. glutamicum* was used as fermenting agent and cultivated in molasses-based media. S-β-aminoethyl-L-cysteine (AEC) lysine analogue resistant mutants were selected. It was observed that 30 g/L and 55 g/L of lysine were produced by the parent and mutant strains respectively. The increase in lysine production was observed with 10% of sugar concentration in 100 mL of molasses media (6.5 pH, and temperature 30˚C) with inoculum size of 8%. Furthermore, two different molasses media with different ingredients (termed M1 and M2) were used for the lysine production. The lysine production M-2 was observed more prominent.

Introduction

Amino acids are the building blocks of proteins and play key role in various metabolic pathways. There are twenty amino acids and are classified into essential and non-essential amino acids. Essential amino acids are ingested as food supplements, while non-essential amino acids are synthesized by the body. Essential amino acids plays vital role for the cure of diseases as glutamine protects the permeability of the intestine, L-citrulline lowers down the blood pressure and L-lysine enhances the bone strengthening [1]. Eggs, beans, cereals, meat and fish are the natural source of the essential amino acids including L-lysine [2].

Lysine plays an important role in carnitine synthesis and lowers down the cholesterol level. The carnitine has the ability to coverts the fatty tissues into the energy for use. Lysine is also helpful for the treatment of Herpes Simplex Virus (HSV) [3]. Children and animals require lysine for an adequate milk production and body growth. Lysine is also used in food flavor and in food preservation [4]. Various microorganisms have been used commercially for the production of amino acids including L-lysine. *Corynebacterium* sp., *Escherichia, Serratia* and *Bacillus*, are the most commonly used organisms for the synthesis of L-lysine through fermentation. Fermentation is cheaper and environment friendly approach [5]. Therefore, research has been focus for the efficient production of amino acids through fermentation methods by using genetic engineering tools [6]. The homoserine-auxotrophic mutant of *C. glutamicum* produced large amount of lysine in liquid broth medium. *C. glutamicum* served as a major host for the production of amino acids and used as a flavor enhancer L-glutamate. Various genetic engineering tools such as mutation, recombination and gene cloning are used for hyper production of L-lysine. Besides, chemical mutagens such as acethyl methane sulfonate (EMS), N-methyl-N-nitro-N-nitrosoguanidine (NG) and (Methyl nitro nitroso guanidine) MNNG are also applicable. To increase the production of the desired and targeted metabolites, feedback inhibition mechanism can be altered through possible mutations [7]. The lysine analogue S-(β- aminoethyl)-L-cysteine (AEC) can use as a substitute for lysine in feedback inhibition. Therefore, a mutant capable of exhibiting resistance against AEC may accumulate the large amount of lysine in the fermentation medium [8]. The current study improved the production of lysine by utilizing *C. glutamicum* through feedback inhibition mechanism.

Materials and Methods

Strain

*C. glutamicum B*391 strain was obtained from the Department of Zoology, University of the Punjab, Lahore, Pakistan. The culture was further streaked on the nutrient agar medium, and culture of the isolated strain was preserved in 30% glycerol and placed at -80°C for further processing.

Culture media preparation

The inoculum was prepared by utilizing two different protocols as through LB-Broth media and molasses media, used for fermentation. Two different types of molasses-based media (Sucrose 10g; KH_2PO_4, 0.05g; K_2HPO_4, 0.05g; $MgSO_4.7H_2O$, 0.025g; $CaCO_3$ 2.0g; $(NH_4)_2SO_4$, 2.0g; NH_4NO_3, 2.0g; pH 7.0) were tested for the production of the amino acid. The prepared media solutions were labeled as MM-1($(NH_4)_2SO_4$ (nitrogen source) and MM-2 ($(NH_4)NO_3$ (nitrogen source). The molasses was diluted as 1:1 and pH were adjusted at 7.0 with 1N HCl.

Minimum Inhibitory Concentration (MIC)

MIC of s-(β-amino methyl)-L-cysteine (AEC) was determined against the wild type cells through the cup-plate bioassay technique [9]. The nutrient broth was used for seeding layer. The bioassay cups having the capacity of 0.8 mL were prepared with different concentrations (50-250 µg/mL) of AEC along with 0.1 M phosphate buffer (pH 7.0). The petri plates were incubated for 48 hours at 28˚C. The diameter of the inhibition zone was measured, and MIC was noted (**Suppl. Fig. 1**).

L-Lysine improvement

L-Lysine was improvement through mutagenesis through overnight growth having 30 mL (or 50 mL of MM-2) solution. The prepared culture was centrifuged for 7 min at 3,000 rpm and the process was repeated again having volume (7.5 mL) of citrate (0.1M) buffer (pH 5.5). The pellet was obtained and suspended [10]. NTG of 100 µg/mL concentration was prepared in one tube and incubated at 28°C and 150 rpm for 90 min in a vibratory incubator. In other tube, 0.2 mL of the simple citrate buffer was taken in order to keep balance considered as zero incubation. The prepared culture was taken out, centrifuged and washed with 4 mL phosphate buffer (pH 7.0). The

dilutions were prepared and inoculated on nutrient medium plates for further processing. The constituents of minimal medium [$CaCl_2$ 0.01g; $MnSO_4$ 0.01g; $ZnSO_4$ 0.001g; $CUSO_4$ 0.02g; $NiCl_2$ 0.02g; $(NH_4)_2SO_4$ 2.0g; urea 0.5g; KH_2PO_4 0.1g; K_2HPO_4 0.01g; $MgSO_4$ 0.25g; biotin 0.25g; glucose 2g/5ml] were adjusted with the pH of 7.2. AEC resistant mutants were obtained (**Table1**). One colony was picked with the help of sterile toothpick and stabbed on three separate plates carefully. The colonies were counted on the plate containing type 1 media and the colonies were seems healthy (**Fig. 1**) [11]. The lysine producing mutants were isolated as described [12]. The qualitative and quantitative estimation analyses of lysine production were performed. The substrate utilization assay was performed by commercially available glucose kit (**Suppl. Fig. 2**).

Table 1: The AEC resistant strains isolation and bacterial count

Type 1 media	Type 2 media	Type 3 media
AEC Con. Of 1 mg/ml	-------	-------
MM-2	MM-2	-------
Nutrient Agar	Nutrient Agar	Nutrient Agar

Estimation of reducing sugars

The reducing of sugar level was determined through DNS (ingredients: 3, 5-Dinitrosylsylic acid 10g; Rochelle salt (potassium sodium tartrate) 182g; phenol 3.0 mL; Sodium sulphite 0.5g and sodium hydroxide 10g).

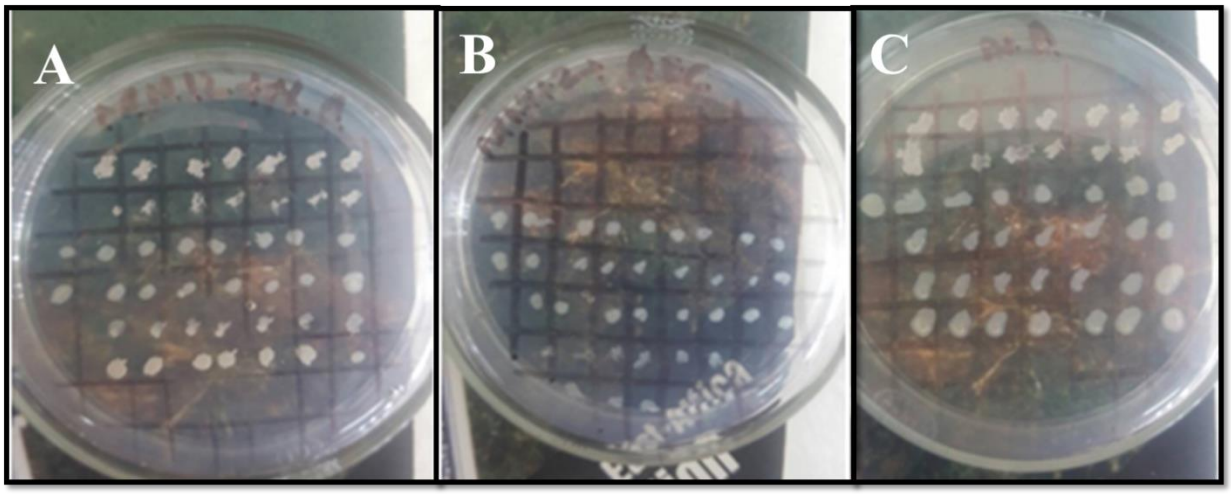

Fig. 1: Different growth media was utilized to analyze the maximum yield **A)** MM-2+ Nutrient Agar was utilized **B)** MM-2+AEC was used **C)** Nutrient Agar was applied

Comparison of two methods for assaying reducing sugars in the determination of carbohydrase activities

The selected ingredients were prepared with 600 mL distilled water in 2 L flask and stirred magnetically. The reducing sugars of the sample were calculated from standard curve by recording absorbance at 550 nm. The samples were collected with the difference of 12 hours. The maximum amount of L-lysine was obtained through 10% sugar concentration.

Chromatography

Quantitative analyses of amino acids were done by using paper chromatography.

Quantitative paper chromatography of amino acid mixtures by spot comparison methods

Paper chromatography approach was used to detect the amino acids [11]. The spots present closely were distinguished through electrophoresis technique. To determine the quantitative estimation of desired amino acids on the chromatography paper was cut and eluted in 3 mL of methanol solution (**Suppl. Fig. 3**). The solution in methanol was determined through spectrophotometer at 550 nm. The values of lysine were quantified through standard amino acids. The cell mass estimation was performed on spectrophotometer at 610 nm (**Suppl. Fig. 4**). The optimization for hyper production of *L*-Lysine, effect

of pH, effect of inoculum size, effect of temperature and growth kinetic parameters [13] were analyzed. The parameters of substrate utilization were μ - specific growth rate per hour (slope of time in hours on x-axis and lnX on y-axis), t_d was the doubling time (h) (0.693/μ), Q_s was the rate of substrate consumption (g/L.h), (slope of time in hours on x-axis and substrate (S) on y-axis), Q_x was rate of cell mass formation (g/L.h), (slope of time in hours on x-axis and cell mass (X) on y-axis), $Y_{x/s}$ was the cell yield coefficient **(**g cells/g Sub):

$$Y_{x/s} = \frac{X_m - X_o}{S_o - S_m}$$

q_s was the specific rate of substrate utilization (g sub/g cells/h) and $q_s = \mu / Y_{x/s}$. Similarly, the product formation parameters were also calculated as Q_p was the rate of L-Lysine formation (g/L.h), (slope of time in hours on x-axis and L-Lysine (P) on y-axis), $Y_{p/s}$ was the product yield of L-Lysine (g lys/g sub)

$$Y_{p/s} = \frac{P_m - P_o}{S_o - S_m}$$

$Y_{p/x}$ was the specific yield of L-Lysine (g lys/g cells)

$$Y_{p/x} = \frac{P_m - P_o}{X_o - X_m}$$

q_p was the specific rate of L-Lysine formation (g lys/g cells/h)

$$q_p = \mu . Y_{p/x}$$

For optimizing the parameters, the organisms were grown during the time course to reveal the specific growth rate (μh^{-1}), doubling time (t_d h), specific rate of substrate utilization (q_s), rate of substrate consumption (Q_s), rate of cell mass formation (Q_x), cell yield coefficient ($Y_{x/s}$), volumetric rate of *L*-Lysine formation (Q_{lys}), specific rate of L-Lysine formation q_{lys}, product yield of *L*-Lysine ($Y_{lys/s}$) and specific yield of *L*-Lysine ($Y_{lys/s}$) for biosynthesis of L-lysine.

Statistical analysis

The observed data was subjected to calculate the Mean ± standard error of mean (SEM). One-way Analysis of Variance (ANOVA) was applied to evaluate the means among the columns. The graphs were generated by utilizing the slide write software. Graph Pad in Stat 3 was used to determine the significant differences among the mean values. The p-values (≤0.05) were considered statistically significant.

Results

Microscopic and cultural characteristics

The characterization of the strain was performed through microscopy. The organism showed purple color after gram staining and having V shaped structure (**Fig. 2**).

Determination of MIC and mutagenesis of the bacterial strains

The experiments were performed for the determination of the minimum inhibitory concentration of L-lysine analogue S-β-aminoethyl-L-cysteine (AEC). The concentration of AEC was observed between the ranges from 0.5-1.5 mg/ml. The higher zone was appeared at higher concentration of AEC and the diameter of the zone gradually became smaller with the increase of concentration. The minimum concentration of AEC was selected having small zone and 1 mg/ml (**Fig. 3**) was observed. The survival rate of organism decreased as the concentration of the mutagen NTG increased from 0-50 μg/ml and the survival rate was observed 3-4% having treatment of 100 μg/ml for 2 h (**Fig. 2**).

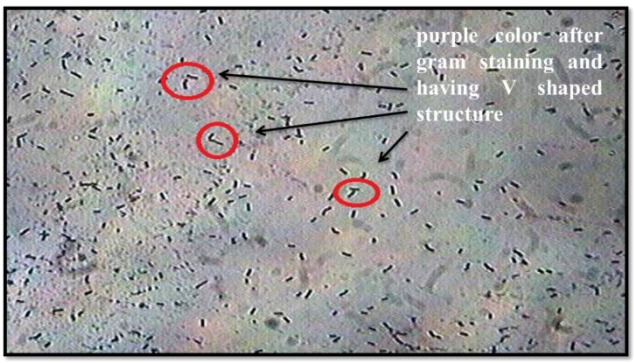

Fig. 2: Microscopic image of *Corynebacterium glutamicum*

Production of L-lysine

In present study, the mutants of *C. glutamicum* B391 were developed for the efficient production of L-lysine. The mutants were developed having resistance against AEC and the production of lysine was higher. The parent and the mutant strains were tested and

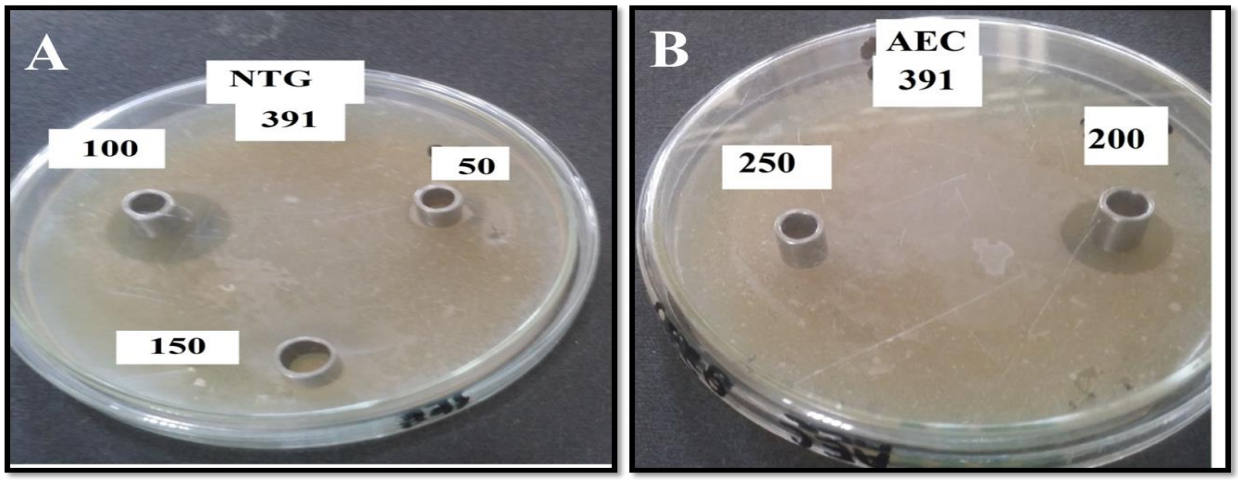

Fig. 3: The cup-plate bioassay technique for minimum inhibitory concentration (MIC) showed inhibition zone for 391 strain **A)** NTG mutagen growth were observed at 50, 100, 150 and 391 concentration **B)** AEC mutagen growth were observed at 200, 250 and 391 concentration

compared under different conditions for the production of L-lysine. It was observed that the parent strain produced ~30 g/L while the mutant strain showed ~50 g/L of L-lysine production. The increase in production was achieved after the optimization of different conditions including sugar concentration in molasses, pH, inoculum size and temperature. Interestingly, it was observed that the production of L-lysine from 15-17% of sugar concentration was increased, and the generated mutant may have the ability to increase the production.

Effect of sugar concentrations on L-Lysine production

Different concentrations of sugar were tested for the parent and the mutant strains of *C. glutamicum.* Organism was cultivated on 7.5%, 10%, 12.5% and 14% of sugar concentrations maintained through molasses-based media. The production with utilization of the substrate was increased (**Table 2**). With the increase of glucose concentration, the production rate of L-lysine was decreased. At 10% of glucose concentration, the parent strain produced 23 g/L, while the mutant strain produced 44g/L after 36–48 h of incubation. The sugar in the medium for mutant strain and parent strain were 10 g/L and 9 g/L respectively.

Effect of pH and temperature on *L*-Lysine production

The pH optimum was estimated for the maximum L-lysine production and the cell mass formation in the fermentation media was incubated at 28˚C to 30˚C. The effect of initial pH of fermentation media on L-Lysine yield was observed between the ranges of 6.5-7.5. Various experiments were performed with the pH of 7 and similar results were observed. It was observed that the maximum production of L-lysine was obtained at pH 6.5 and the increase and decrease of pH reduces the production of L-lysine (**Table 3**).

Effect of temperature on L-lysine production

The temperature is an important factor that effects the production. The optimum temperature for maximum production of lysine was determined and observed the ranges of 26˚C - 32˚C. The maximum production of the temperature was observed ranges from 30˚C - 32˚C for the parent and mutant strains. It was observed that the maximum yield was obtained between 30˚C - 32˚C. It was also observed that the increase in temperature (>32˚C) cause reduction in the yield of L-lysine. At 30˚C, the parent strain showed maximum yield of 30 g/L while the mutant strain yielded for 55g/L (**Table 4**). With the increase in temperature, the production of lysine from both parent and mutant strains were reduced for 4.3 g/L and 8 g/L respectively.

Effect of inoculum size on L-lysine production

During the kinetics study, the effect of inoculum size was also observed for *C. glutamicum* for parent and mutant strains. The inoculum size for kinetics study ranges from 4-10 % used for the fermentation media in shaking incubator (**Table 5**). It ranges from 6 to 8 % to increase the L-lysine production for parent and mutant strains. However, 8% can be used as optimum inoculum size for L-lysine production on industrial scale.

Table 2: The effect of sugar concentration on substrate utilization parameters through kinetic study

Glucose Conc. (%)	μ (h^{-1})		T_d (h)		q_s g sub/g cells/h		Qs g/l.h		Qx g/l.h		$Y_{x/s}$ g cells/g sub		Q_{lys} g/l.h		q lys g lys/g cells/h		$Y_{lys/s}$ g lys/g sub		Y lys/x g lys/g cells	
	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M
7.5	--	--	--	2.23	--	--	--	--	--	--	0.23	0.31	--	--	--	--	0.30	0.35	--	--
10	--	--	2.73	--	--	--	--	--	0.55	0.94	--	--	0.82	2.06	0.291	0.592	--	--	--	0.91
12.5	--	--	2.73	--	--	--	2.39	--	--	--	--	--	--	--	--	--	--	--	--	--
14	0.24	0.34	--	--	1.71	1.54	--	7.1	--	--	--	--	--	--	--	--	--	--	0.91	--

Table 3: The effect of pH concentrations on substrate utilization parameters through kinetic study

pH	μ (h^{-1})		T_d (h)		q_s g sub/g cells/h		Qs g/l.h		Qx g/l.h		$Y_{x/s}$ g cells/g sub		Q_{lys} g/l.h		q lys g lys/g cells/h		$Y_{lys/s}$ g lys/g sub		Y lys/x g lys/g cells	
	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M
6.5	--	--	--	--	--	--	0.89	2.12	--	--	--	--	--	--	0.255	--	--	--	--	--
7.0	0.24	0.33	3.15	2.23	--	0.941	--	--	0.86	1.51	0.54	0.34	0.81	1.79	--	--	0.31	0.54	1.17	1.52
7.25	--	--	--	--	0.923	--	--	--	--	--	--	--	--	--	--	0.392	--	--	--	--
14	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--

Table 4: The effect of temperature ranges on substrate utilization parameters through kinetic study

Temp. (C^o)	μ(h^{-1})		T_d(h)		q_s g sub/g cells/h		Qs g/l.h		Qx g/l.h		$Y_{x/s}$ g cells/g sub		Q_{lys} g/l.h		q lys g lys/g cells/h		$Y_{lys/s}$ g lys/g sub		Y lys/x g lys/g cells	
	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M
26	--	--	3.64	2.16	--	--	--	--	--	--	0.27	--	--	--	--	--	--	--	--	--
28	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1.63
30	0.23	0.34	--	--	0.95	1.32	1.27	2.10	0.50	--	--	0.33	0.94	2.21	0.287	0.726	0.31	0.55	1.25	--
32	--	--	--	--	--	--	--	--	--	0.76	--	--	--	--	--	--	--	--	--	--

Table 5: The effect of inoculum sizes on substrate utilization parameters through kinetic study

Inoculum (%)	μ (h^{-1})		T_d (h)		q_s g sub/g cells/h		Qs g/l.h		Qx g/l.h		$Y_{x/s}$ g cells/g sub		Q_{lys} g/l.h		q lys g lys/g cells/h		$Y_{lys/s}$ g lys/g sub		Y lys/x g lys/g cells	
	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M
4	--	--	3.31	2.23	--	--	--	--	--	0.76	--	--	--	--	--	--	--	--	0.48	1.61
6	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
8	0.23	0.33	--	--	0.750	1.21	--	2.91	--	--	0.31	0.36	0.87	1.68	0.12	0.49	0.31	0.54	--	--
10	--	--	--	--	--	--	--	--	0.74	--	--	--	--	--	--	--	--	--	--	--

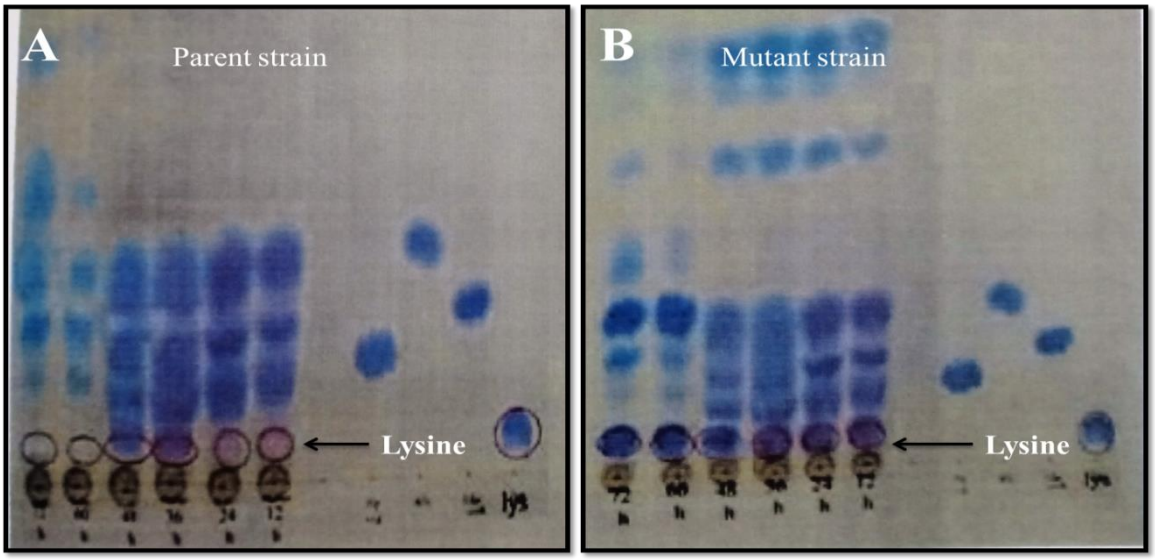

Fig. 4: Paper chromatogram exhibited the amino acids produced by **A**) parent strain with lysine production **B**) mutant strain (spots in red circle showed L-lysine)

Paper chromatography of amino acids by wild and mutant strain

During fermentation, different amino acids were observed in the fermentation broth including glutamic acid, leucine, isoleucine, alanine, lysine and aspartic acid. After using optimum conditions during fermentation, the results of paper chromatography showed that the mutant strain produced lysine in higher quantity as compared to the parent strain and showed confirmation of L-lysine by paper chromatography (**Fig. 4**).

Discussion

Bacteria adapt the environment for survival and utilize different kind of substances and being used in various industries [14]. It is a rare mechanism that an organism produces an amino acid for a feedback inhibition mechanism to prevent the organism for synthesis of the amino acids more than the body requirement. The aim of present study was to enhance the synthesis of lysine through the isolated strain of *C. glutamicum* 391. The followed [11] protocol have the potential to achieve the goals of current project. The significant pathways in *C. glutamicum* for the production of the amino acids derived from the L-Aspartate. The lysine biosynthesis in *C. glutamicum* is controlled through the feedback inhibition and repression mechanism [15]. To achieve the hyper-lysine production, the organism should improve the feedback inhibition resistance. AEC (lysine analogue) for false feedback inhibition will be lacking feedback mechanism. The MIC of the organism for the isolation of AEC resistant mutant was 0.01 g/ml [16]. Petri plates and the cells were treated with the UV light through the distance of 15 cm leads to pour on CASO agar plate for the recording of survival rate [17]. NTG was used as mutagen to mutate the organism. Although, the function of NTG was not clear however the induction of mutation at replication site leads to the clustering of mutations. The dose of mutagenesis was maintained at 100 µg/ml for 2 h [16]. The mutant strains were used for hyper productions of required lysine [18]. The production depends on various parameters including sugar concentration, pH, temperature and inoculum size. The growth for 10 % sugar concentration yielded maximum for parent and mutant strains (**Table 1**), and the observed results were reconciled with reported evidences [19].

Sugar is an important element for microorganism to utilize as carbon source. Various concentrations of glucose (7.5-14%) were maintained through molasses base media. At 10% sugar concentration, maximum lysine production was observed (**Table 1**). Another important factor in the production of lysine was the maintenance of pH. Different ranges of pH (6.5- 7.5) were being studied it was observed that 6.5 to 7 pH showed the maximum optimum conditions for growth and production (**Table 2**). Hence, pH was also considered as an important factor in lysine production. C. *glutamicum* released α-amylase from *Streptococcus bovis* 148 (AmyA) to produce amino acids directly from the starch. Lysine fermentation was carried out by *C. glutamicum* recombinant in the

growth medium along with starch (50 g/L). The fermentation was carried out at different temperatures and pH and the maximum amount of lysine was obtained at 30˚ C and at pH 7. The amount of starch was also reduced at 18 g/L, by utilizing starch at 8 g/L of lysine was obtained after 24 h. The inoculum size is also a significant element to produce higher yield. The inoculum size helps to produce high amount of biomass while low density may be insufficient to produce the biomass. The inoculum size is also an important factor in kinetics studies. To get better yield, the inoculum size should be optimized.

In present study, the higher production was observed with 6 % of inoculum size. Various amount of inoculum size ranged from 4-10% were utilized and maximum yield was observed. The optimum inoculum size was considered as 8% to produce high yield (**Table 4**).

Temperature is also an important parameter for maximum production and directly effects the yield. The higher temperature may damage the production while low temperature may not initiate the production according to the capacity. Various temperature ranges from 26˚C to 32˚C were utilized for maximum production. It was observed that the optimum temperature was ranging from 28˚C to 30˚C (**Table 3**) and results were reconciled with previous studies [20]. The optimum pH of 7 for production was also reported [21]. The mutant developed has the ability to produce maximum level of lysine by using a cheaper substrate source molasses for cost effective production of L-lysine. Interestingly, the mutation of *C. glutamicum* strain 391 has not been reported for lysine production.

Conclusion

The production of both parent and mutant strains were studied through kinetics parameters. The observed results showed that the higher lysine production was observed with 10 % sugar, at 7 pH, with inoculum size of 6% and temperature at 30°C. The mutant strain produced 55 g/L lysine after feed back inhibition through NTG. Two molasses media were also tested M1 and M2 for the production of lysine and M2 showed better results as compared to M1.

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Conflict of interest

The authors declare no conflict of interest.

References

[1] Elango R, Laviano A. Protein and amino acids: key players in modulating health and disease. Current Opinion in Clinical Nutrition & Metabolic Care. 2017;20:69-70.

[2] Mohanty B, Mahanty A, Ganguly S, Sankar T, Chakraborty K, Rangasamy A, et al. Amino acid compositions of 27 food fishes and their importance in clinical nutrition. Journal of amino acids. 2014;2014.

[3] Breau RH, Kokolo MB, Punjani N, Cagiannos I, Beck A, Niznick N, et al. The effects of lysine analogs during pelvic surgery: a systematic review and meta-analysis. Transfusion medicine reviews. 2014;28:145-55.

[4] Ezemba C, Ozokpo C, Anakwenze V, Anaukwu G, Ogbukagu C, Ekwealor C, et al. Lysine Production of *Microbacterium lacticum* by Submerged Fermentation Using Various Hydrocarbon, Sugar and Nitrogen Sources. Advances in Microbiology. 2016;6:797.

[5] Khan S, Rasool G, Nadeem S. Bioconversion of cane molasses into amino acids. Pak J Agric Sci. 2006;43:157-60.

[6] Ezemba C, Ekwealor C, Ekwealor I, Ozokpo C, Chukwujekwu C, Anakwenze V, et al. Microbacterium lacticum; a lysine producing bacterium isolated from oil-contaminated soil in South-East Nigeria. British Microbiology Research Journal. 2015;9.

[7] Garrad R, Bhattacharjee J. Lysine biosynthesis in selected pathogenic fungi: characterization of lysine auxotrophs and the cloned LYS1 gene of Candida albicans. Journal of bacteriology. 1992; 174:7379-84.

[8] Han J-K, Oh J-W, Lee H-H, Chung S, Hyun H-H, Lee J-H. Molecular cloning and expression of s-(2-aminoethyl)-L-cysteine resistant aspartokinase gene of *Corynebacterium glutamicum*. Biotechnology letters. 1991;13:721-6.

[9] Ouedrhiri W, Balouiri M, Bouhdid S, Moja S, Chahdi FO, Taleb M, et al. Mixture design of Origanum compactum, Origanum majorana and Thymus serpyllum essential oils: optimization of their antibacterial effect. Industrial Crops and Products. 2016; 89:1-9.

[10] Wang H, Tsai C, Kong D, Chan K, Abild-Pedersen F, Nørskov JK, et al. Transition-metal doped edge sites in vertically aligned MoS 2 catalysts for enhanced hydrogen evolution. Nano Research. 2015; 8:566-75.

[11] Nadeem S, Ikram A, Rana S, Yaqoob N, Qureshi M, Shakoori A. Enhanced L-lysine production by an Escherichia coli mutant WARN 30522 after MNNG treatment. Strain. 2001; 48:96.

[12] Costa-Ferreira M, Duarte JC. Amino acid accumulation by an analogue sensitive mutant of *Corynebacterium glutamicum*. Biotechnology letters. 1992; 14:1025-8.

[13] Albertini M, Fernandez-Yague M, Lázaro P, Herrero-Climent M, Rios-Santos J-V, Bullon P, et al. Advances in surfaces and osseointegration in implantology. Biomimetic surfaces. Medicina Oral, Patologia Oral Ycirugia Bucal. 2015;20:e316.

[14] DeLong EF, Pace NR. Environmental diversity of bacteria and archaea. Systematic biology. 2001; 50:470-8.

[15] Torruella G, Suga H, Riutort M, Peretó J, Ruiz-Trillo I. The evolutionary history of lysine biosynthesis pathways within eukaryotes. Journal of molecular evolution. 2009; 69:240-8.

[16] Kumar D, Garg S, Bisaria V, Sreekrishnan T, Gomes J. Production of methionine by a multi-analogue resistant mutant of *Corynebacterium lilium*. Process Biochemistry. 2003; 38:1165-71.

[17] Hameed A, Ahmed S. Selection and Characterization of a Lysine Yielding Mutant of *Corynebacterium glutamicum*- A Soil Isolate from Pakistan. Pakistan Veterinary Journal. 2012;32.

[18] Gupta A, Das S, Banik A. Induced mutation and selection of a high yielding strain of Micrococcus glutamicus for L-lysine production. International Journal of Pharma and Bio Science. 2012;3.

[19] Costa-Ferreira M, Duarte J. Glucose utilization by lysine-producing fluoroacetate-sensitive mutants of *Corynebacterium glutamicum*. Applied biochemistry and biotechnology. 1991; 27:251-7.

[20] Hilliger M, Haenel F, Menz J. Influence of temperature on growth and L-lysine formation in *Corynebacterium glutamicum*. Z ALLG MIKROBIOL. 1984; 24:437-41.

[21] Takeshita R, Ito H, Wachi M. A role of the cspA gene encoding a mycolyl transferase in the growth under alkaline conditions of *Corynebacterium glutamicum*. Bioscience, biotechnology, and biochemistry. 2010; 74:1617-23.