Nutritional and Mutational Aspects of Indigenous Lysine Production by *Corynebacterium glutamicum:* Comprehensive Studies in Shake Flask Fermentation -1

[1]A. H. SHAH AND [2]A. HAMEED
[1]*Department of Biological Sciences*
Gomal University, D.I.Khan, Pakistan
[2]*Department of Biological Sciences*
Quaid-e-Azam University, Islamabad, Pakistan

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Summary: Mutants of different auxotrophic nature of *corynebacterium glutamicum* were developed. Each mutant was further investigated for the production of lysine in three different fermentation media on Shake flask. The potency of each mutant for the conversion of consumed sugar to lysine and yield based on per gram dry cell weight was also examined. Mutant MRLH.GHAIO produced 28.64 g/l lysine with 33.3% conversion and 3.36g lysine per gram dry cell weight in FMI medium and 26.35 g/L lysine with 30.42% conversion and 2.77g lysine per gram dry cell weight in FM_2 medium. The other auxotrophic mutant L_1, T_3, MT_8, MTA_2, H16, HA5 and GH_3 produced 18 8-27.53 g/L lysine with 24-31.7% conversion, 15.5-24.17 g/L lysine with 17.8 –28.3% conversion in FM_1 and FM_2 medium, respectively,

All mutants showed least yield and least conversion efficiency in fermentation medium3 and higher yield and conversion efficiency then the parent thialysine resistant mutant TR1.

Introduction

The significance of lysine for the human and domestic meat producing animal is already recognized. The recommended nutrient intake of lysine for normal adult person is 1.6 g/day. The deficiency of lysine in cereal protein has became quite an important problem in human nutrition. [1]

Lysine is the first limiting amino acid in virtually every cereal grain known to man [1]. Amino acids such as lysine and threonine offer great potential for improving the protein quality of cereal based human diets in third world countries where Kwashiorkor (protein deficiency disease) is so prevalent [2].

Researchers discovered that virtually all Chronic Fatigue Immune Dysfunction Syndrome (CFIDS) patients carry at least one of three different herpes viruses, Epstein-Barr virus, cytomegalovirus, and human herps-virus-6. These viruses are sometimes dormant but can flare up when other factors, such as stress, weakend the immune system. Herpes virus, however, require, the amino acid arginine for reproduction. Lysine by blocking the bio-availability of arginine, "starves", the virus into dormancy. Lysine also increases the body's level of interferon. Stauth pointed out that a chemical compound containing lysine raised interferon production in primates [3]. Thus, it is quite feasible that lysine could inhibit the symptoms of chronic fatigue syndrome. One of America's leading lysine researchers believes that lysine can inhibit Epstein-Barr virus and reports that lysine has effectively treated other herpes-related diseases, including Bell's Palsy (a facial paralysis) and Meniere's disease (an inner ear disorder). In addition, over the past few years there has been an increase in anecdotal evidence in the form of testimonial from supplement users that suggests that CFIDS does respond to various nutrients, including lysine. [3]. Nausea, dizziness and hypersensitivity to sound were observed in lysine deficiency in man [4].

The world wide demand of lysine as dietary supplement is increasing continuously. As a result of the major advances in the efficiency of fermentative synthesis of lysine, the present investigation have much significance.

The wild strain of *corynebacterium* could not accumulate because both lysine and threonine by concerted feedback inhibition, inhabit the synthesis of lysine [3]. The feedback inhibition was released by the development of different mutants that accumulated lysine.

Mutants of different species of bacteria have been investigated for the production of lysine. Among which *Brevibacterium lactofermentum Brevibacterium flavum and corynebacterium glutamicum* have significant metabolic pathway regarding the production of lysine. [6,7].

The present studies are focused on the development of different auxtrophic form thialysine resistant mutant of *corynebacterium glutamicum* with profile of yield of lysine with various changes that occurred on shake flask fermentation.

Results and Discussion

Population of Developed Auxotrophic Mutants

In the first experiment a total of 500 colonies were streaked and 140 different auxotrophs (28%) were isolated. Of the L-lysine producing auxotrophs, homoserine-alanine double auxotrophs were the most prevalent (14 %) and single auxotrophs of homoserine (4.2%) followed by glutamate homoserine double auxotrophs (3.6%), glutamate-homoserine-alanine triple auxotrophs (2.8%) and alanine auxotrophs (1.4%). Glutamate-alanine double auxotrophs and glutamate auxotrophs were not detected. Small number of auxotrophs of undesired amino acid and other substances were also detected. Some revertent and dead cells were also found. All L-lysine producing auxotrophs were separately isolated on complete agar medium and screened for highest yield.

In the second experiment 151 colonies (30.2%) were isolated as auxotrophs. Among these, threonine auxotrophs were most prevalent (8.4%) followed by methionine-threonine double auxotrophs (7.6%), methionine-threonine-alanine triple auxotrophs 5.8%, homoserine-alanine double auxotrophs (2.6%), homoserine single auxotrophs (1.8%), while auxotrophs of alanine, methionine and double auxotrophs of methionine-alanine were not detected. A small number of auxotrophs of undesired amino acids and other substances were also detected. Some revertent and dead were also found. All the L-lysine producing mutants were isolated and activated.

Third experiment showed 31(6.2%) leucine auxotrophs, 1.8% auxotrophs of other amino acid and 1.4% of other substances. The number of revertant and dead cells were 4.2% and 5.8%, respectively. Leucine auxotrophs were isolated and purified.

Profile of Dry Cell Weight, Change in pH and L-Lysine Production in Shake Flask Fermentation

After optimization, time course of different mutants were studied in shaking incubator (30°C, 200 rpm for 7 days). After every 24 hrs., dry cell weight, change in pH and the production of L-lysine were recorded.

Dry cell weight smoothly increased till 3rd day in FM1 and FM2 medium, then was approximately constant till 6th day and then decreased. The parent strain (thialysine resistant mutant TR1) showed maximum cell mass on 3rd day (11.5 g/L) and least (9.4 g/L) by mutant T3 on the same day. On the first day of incubation dry cell weight was between 3.8 to 4.8 g/L, while on the last day of incubation was from 3.5 to 6.9 g/L, among all the mutant in FM1 medium. In FM2 medium, on first day the dry cell weight was from 3.1 to 6 g/L, and on 7th day 3.5 to 6.5 g/L was observed. In case of FM3 medium, low growth was seen. In most of the mutants dry cell weight increased up to 5th day and was constant on 6th day, then decreased on 7th day. Maximum cell mass 10 g/L was observed by MT8 on 4th day and by MTA2 on 5th day (Fig. 1, 2, 3.).

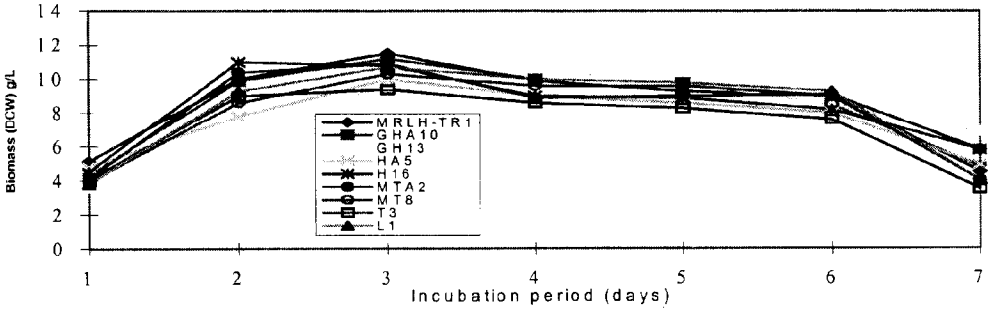

Fig. 1: Biomass (DCW) production in FM 1 medium by different mutants of *Corynebacterium glutamicum*.

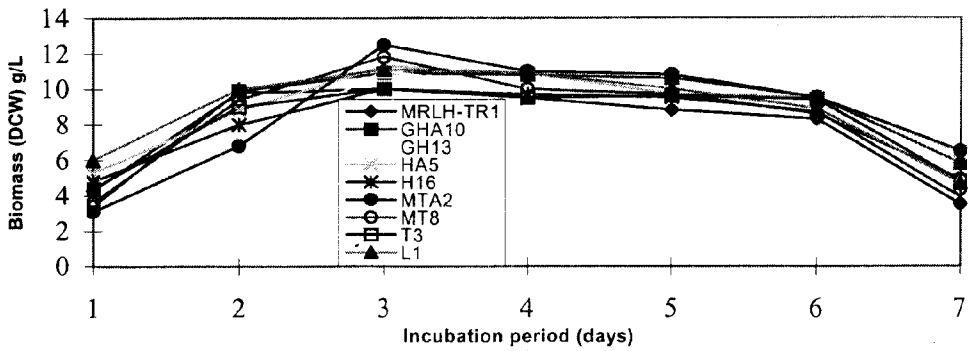

Fig. 2: Biomass (DCW) production in FM2 medium by different mutants of *Corynebacterium glutamicum*.

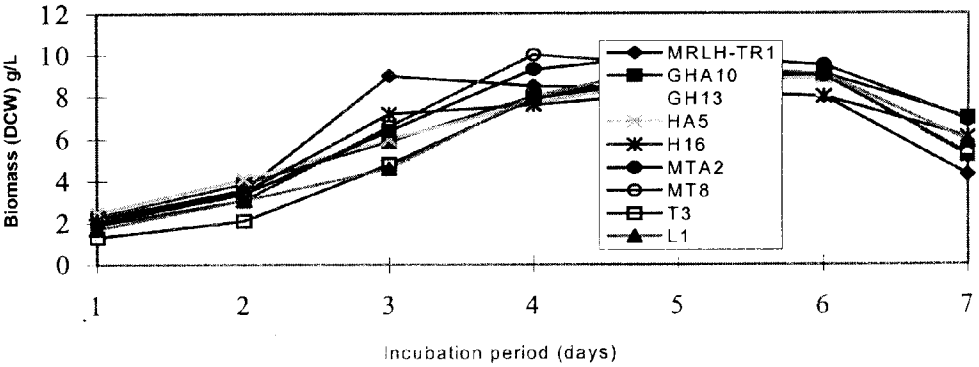

Fig. 3: Biomass (DCW) production in FM3 medium by different mutants of *Corynebacterium glutamicum*.

Change in pH was noted after every 24 hrs. pH decreased from starting pH 7.5 to 5.5 on 3rd day, then increased to 7.5. In most of the cases, pH again increased on 7th day in both FM1 and FM2 medium. In FM3 medium pH decrease to 6.0 on 2nd day, and 3rd day, further increased till 7th day. Final pH was more alkaline (8.3) in starch medium (FM3) on last days of incubation than in glucose medium (Fig. 4, 5, 6).

L-lysine accumulation increased smoothly upto 6th day and then decreased. Highest yield (28.64 g/L) was observed in FM1 medium by GHA10. While the other mutants [L_1, T_3, MT 8, MTA2 H16 HA5 and GH3] showed 14-27.53g/L L-lysine on 6th day. On first day 1-2 g/L L-lysine was observed. In FM2 medium yield was slightly less than in FM1 medium. GHA10 produced 26.35 g/L L-lysine. The other mutant showed 12.8 to 24.7 g/L L-lysine. In FM3 medium (Starch) yield was the least. On first day 0.56-2 g/L L-lysine was produced and on 6th day 10-21 g/L L-lysine was observed (Fig. 7, 8, 9).

Profile of Mutants for L-Lysine Production, Percentage Conversion of Consumed Sugar to L-Lysine, Yield Base on Total Sugar and Yield Base on Per Gram Dry Cell Weight in Different Fermentation Media

From the data of shake flask different mutants were compared for L-lysine production. Yield based on total sugar, percentage conversion of consumed sugar to L-lysine and yield per gram dry cell weight were determined in different media.

Glutamate-homoserine-alanine (MRLH-GHA-10) triple auxotrophic mutant produced maximum yield in shake flask (28.64 g/L L-lysine), compared to the parent strain MRLH-TR1, which produced 14 g/L L-lysine. Mutants H16 and MTA2 gave equal amount of yield (23 g/L) and mutants T3 and L1 also gave equal yield (18.3 to 18.8 g/L). The other mutants GH13, HA5 and MT8 produced 27.53, 25 and 20 g/L L-lysine, respectively in the fermentation broth after 6th day of incubation in FM1 medium.

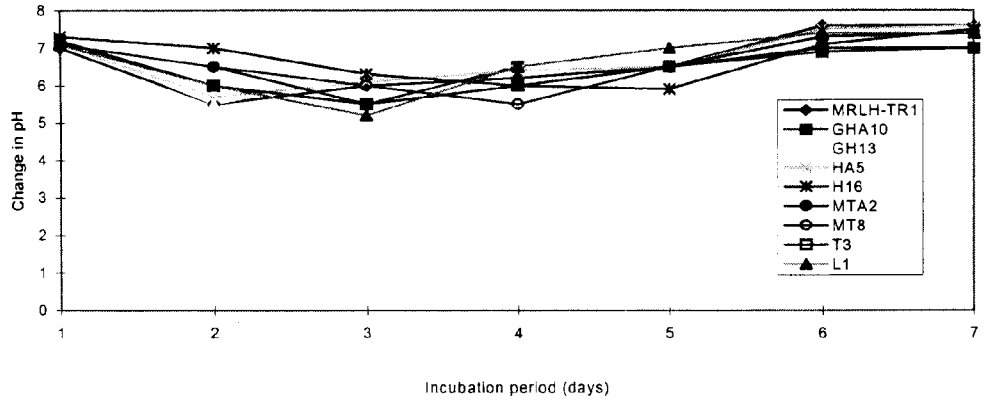

Fig. 4: Change in pH in FM1 medium by different mutants of *Corynebacterium glutamicum*.

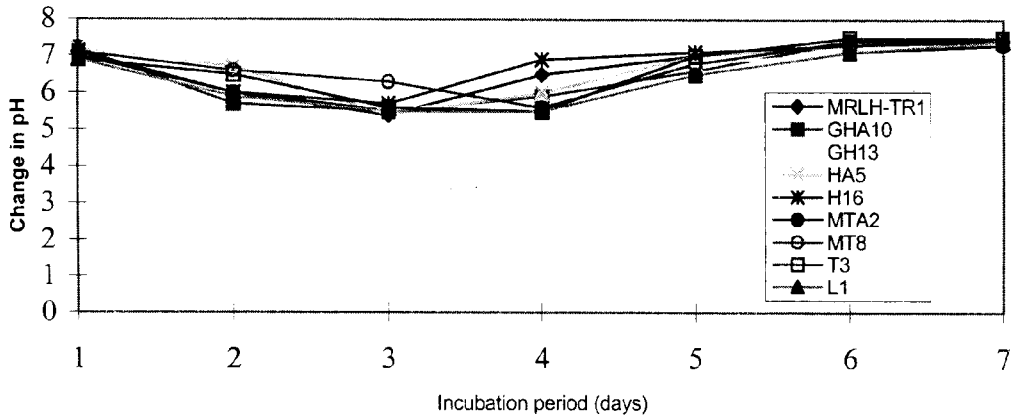

Fig. 5: Changes in pH in FM2 medium by different mutants of *Corynebacterium glutamicum*.

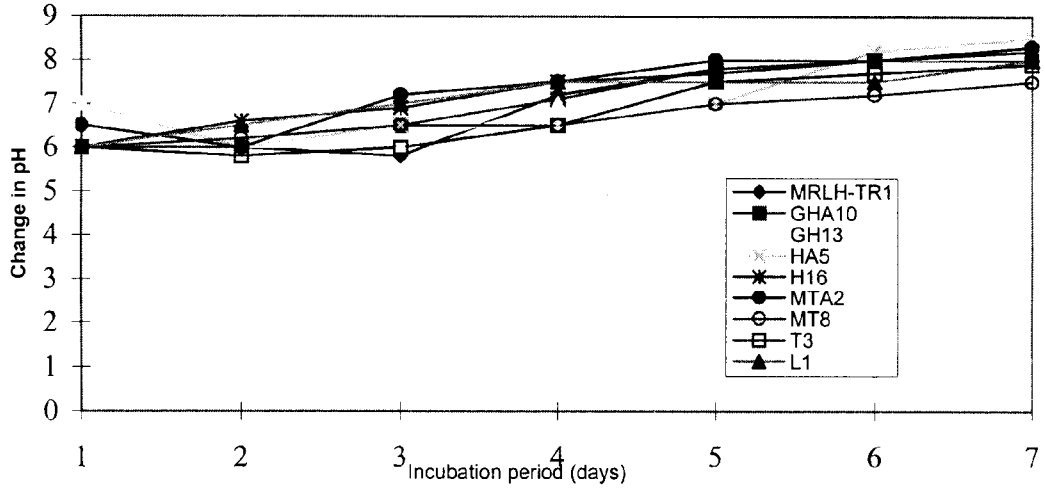

Fig. 6: Change in pH in FM3 medium by different mutants of *Corynebacterium glutamicum*.

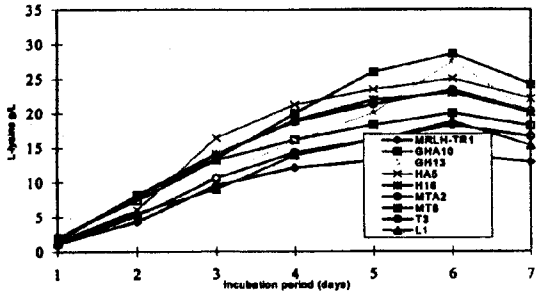

Fig. 7: L-lysine production FM1 medium by different mutants of *Corynebacterium glutamicum*.

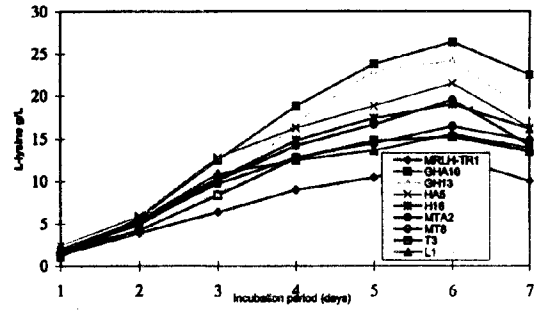

Fig. 8: L-lysine production in FM2 medium by different mutants of *Corynebacterium glutamicum*.

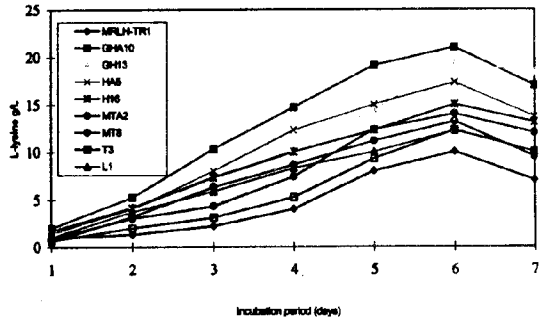

Fig. 9: L-lysine production in FM3 medium by different mutants of *Corynebacterium glutamicum*.

In FM2 medium the yield was lower than FM1 medium, where GHA10 accumulated 26.35 g/L L-lysine, as compared to the parent strain MRLH-TR1, which produced 12.8 g/L L-lysine. H16 and MTA2 produced (19.02-19.5g/L) showing decreased of L-lysine, followed by T3 and L1, which produced 15.5 - 15.2 g/L yield. While, GH13, HA5, and MT8 produced, 24.17, 21.5 and 16.4 g/L L-lysine, respectively. In FM3 medium the most potent triple auxotrophic (GHA10) mutant gave 21 g/L L-lysine, while the parent strain produced 10 g/L. Mutants MRLH-L1, T3, MT8, MTA2, H16 produced 12.24 - 15 g/L L-lysine. The double auxotrophic mutants HA5 and GH13 yielded 21.5 and 24.17 g/L L-lysine, respectively (Fig. 10).

Among the media maximum conversion occurred in FM1 medium, less in FM2 medium and least in FM3 medium. The conversion in most potent mutant GHA10 was 33.3%, 30.42% and 19% in FM1, FM2 and FM3 medium, respectively.

Approximately equal percentage of conversion (22.3 - 24.5%) was found by L1, T3 and MT8 in FM1 medium on shake flask. Same proportion of conversion (28.6 %) was found in MTA2 and H16. Mutants such as HA5 and GH13 also gave equal conversion 31.1 - 31.7%. While in the parent strain 18% conversion was found.

In FM2 medium T3 and L1 showed approximately same conversion (17.3 - 17.8%). MT8, MTA2 and H16 gave 22.3 to 22.8% conversion. In FM3 medium the parent strain RT1 gave 9% conversion, while in the other auxotrophic mutants, except GHA10, from 10.4 to 16.6% (Fig. 11).

The percentage yield based on total sugar was found nearly in same proportion among all mutants, as in L-lysine production, in FM1, FM2, FM3 medium. The most potent mutant GHA10 gave 29.2%, 27.1% and 14.2% in FM1, FM2 and FM3 medium, respectively, as compared to the parent strain RT1 which gave 14.34% in FM1 medium, 13.12% in FM2 and 6.71 in FM3 medium (Fig. 12).

The yield per gram of dry cell weight was determined in shake flask, as well as in stirred tank in FM1, FM2 and FM3 medium. In FM1 medium GHA10, which is the most potent mutant produced 3.36 g/g and by the parent strain TR1, 1.55 g/g yield was found. Homoserine alanine double auxotroph (HA5) and glutamate homoserine double auxotroph (GH13) synthesized equal amount (3.1 g/g D.C.W.). In H16 2.8 g L-lysine was found by per gram dry cell weight, while in the other auxotroph 2 - 2.57 g L-lysine per gram dry cell weight were found. In case of FM2 medium, L1, T3 and MT8 gave 1.6 to 1.9 g L-lysine per gram dry cell weight. While in other

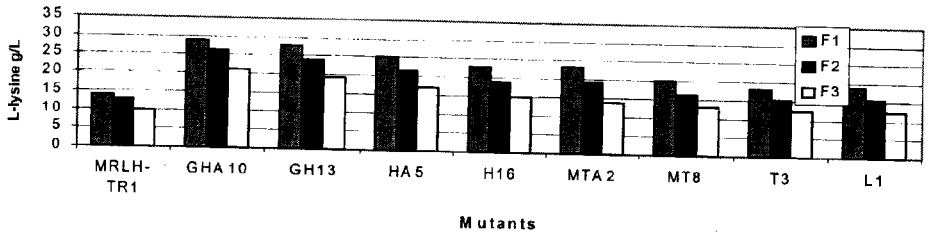

Fig. 10: Maximum L-lysine produced in FM1, FM2 and FM3 medium by mutants of *Corynebacterium glutamicum*.

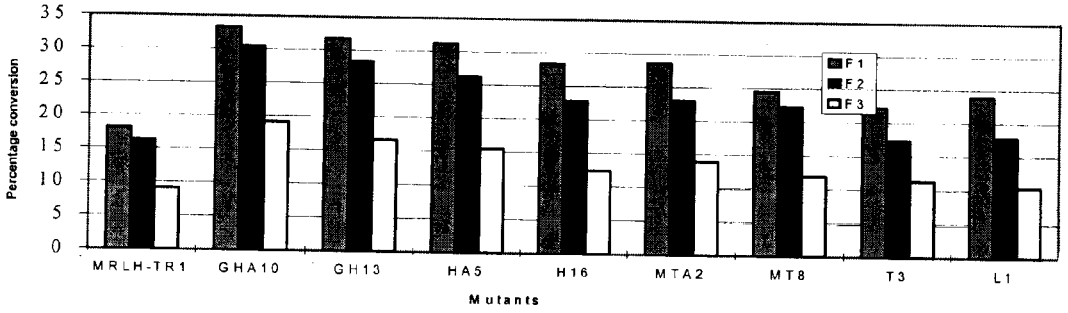

Fig. 11: Percentage conversion of consumed sugar to lysine in FM1, FM2 and FM3 medium by mutants of *Corynebacterium glutamicum*.

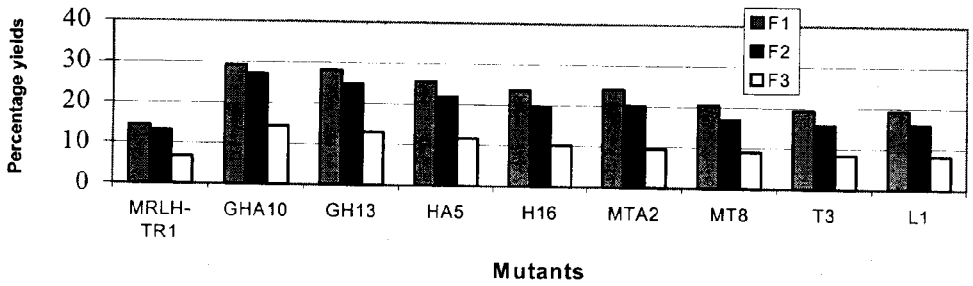

Fig. 12: Yield percentage based on total sugar in FM1, FM2 and FM3 medium by mutants of *Corynebacterium glutamicum*.

mutant from 2 - 2.77 g L-lysine per gram dry cell weight was produced. In FM3 medium, GHA10 and GH13 gave 2.3 g L-lysine per gram DCW, while other auxotrophs gave 1.35 to 1.9 g L-lysine per gram DCW. The parent strain TR1 gave 1.54 and 1.25 g/g in FM2 and FM3 medium, respectively (Fig. 13).

Extraction and Purification of L-Lysine

Ion exchange resins have been widely applied for the extraction and purification of amino acids. About 1.5 liter broth was centrifuged then one liter of the supernatant containing 38 g/L L-lysine was passed into the column of cation exchange resins, Amberlit IR120, plus in ammonium form. L-lysine was eluted by 2N ammonium hydroxide solution. The eluent was concentrated to 200 ml, adjusted to pH 5.6 and further concentrated to 100 ml. Crystal of crude L-lysine were precipitated out from the solution after freeze drying. Decolourized with charcoal and again recrystalized, as a result 37.16 g L-lysine HCl (29.73 g/L L-lysine) was isolated with 78.2% recovery.

L-lysine, one of the essential amino acids used in food and feed stuffs, and as an active ingredient in

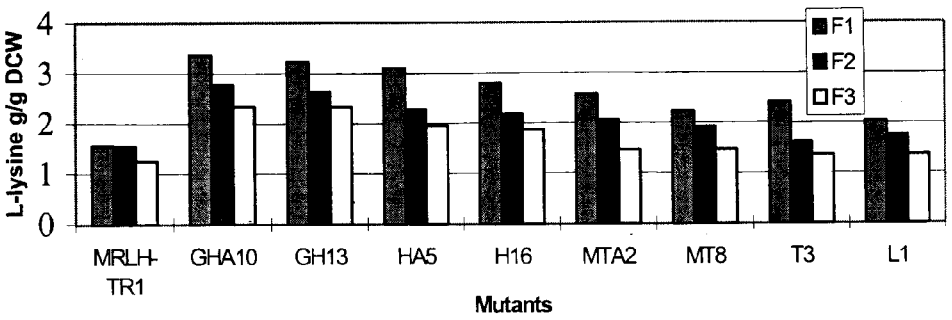

Fig. 13: Yield based on per gram dry cell weight in FM1, FM2 and FM3 medium by mutants of *Corynebacterium glutamicum*.

pharmaceuticals, has been produced by microorganisms as a primary metabolite.

This fermentation process has been studied by number of researchers. [6,7,11]. The biosynthetic pathway for L-lysine production is known [5,13].

The production and activities of the enzymes in the biosynthetic pathway are controlled by feed back inhibition, repression and metabolic interlock mechanism to avoid over production In *Corynebacterium glutamicum,* threonine and L-lysine stop the activity of aspartokinase by concerted feed back inhibition, due to which the synthesis of aspartic semialdehyde will stop as that of the L-lysine and threonine. Homoserine dehydrogenase is inhibited by threonine and is repressed by methionine. For the microbial production of L-lysine, changes in the biosynthetic pathway are necessary, which includes blockage of side reactions for conversion to another amino acid and to stop catabolism of said amino acid.

The efflux of metabolite from cell pool to minimize its effect as effector of feed back inhibition and repression is also important. Mutagenesis is a means by which change can be intorduced in the metabolic process. Production could also be increased by reducing inhibitory factors for growth and optimizing the culture conditions. The most direct and general method for over production is the genetic removal of feed back control. The blockage of homoserine synthesis at homoserine dehydrogenase results in the release of the concerted feed back inhibition by threonine and L-lysine on asparto-kinase. The aspartic semialdehyde produced proceeds to L-lysine through the L-lysine biosynthetic path-way. Another effective technique for the over production of L-lysine is the development of regulatory mutants, which are insensitive to feed back inhibition or repression. Thus, if aspartokinase is genetically altered to become resistant to the concerted feed back inhibition by threonine and L-lysine, then higher yield of L-lysine would be produced, independent of concentration of threonine. There have been many cases of over production of L-lysine by such mutants, which are generally obtained as mutant resistant to the analogue of L-lysine [14].

Single, double and triple auxotrophs were generated in three different experiments from MRLH-TR1, when homoserine, alanine and glutamate were added in the medium. Out of 140 auxotrophs from 500 colonies screened were found. Maximum number was of homoserine-alanine double auxotrophic mutants, while glutamate and glutamate-alanine deficient mutants were not detected. In second experiment methionine, threonine and alanine were supplied in selective medium. Auxotrophs of threonine, methionine-threonine double auxotrophic mutant and triple auxotrophic mutant of methionine-threonine-alanine were dominate in the population, while no auxotrophs of methionine plus alanine, methionine, alanine were found. Third mutagenesis experiment resulted in the production of leucine auxotrophs (6.2%), when enriched with leucine after UV radiation and penicillin addition. Liu and Shyang [15] reported generation of maximum auxotrophs of methionine when selected in methionine plus threonine medium and reported no glutamate and leucine negative mutants.

All isolated auxotrophic mutants were examined for L-lysine production. The most potent glutamate homoserine-alanine triple auxotrophs (MRLH-GHA10) produced 28.64 g/L L-lysine in the fermentation liquor, which was more than the parent strain

(MRLH-TR1). The mutant of glutamate-homoserine double auxotroph (MRLH-GH13) produced 27.53 g/L L-lysine, which is less than that produced by MRLH-GHA10. The reason is that GH13 is glutamate and homoserine negative, but GHA10 in addition to glutamate, homoserine, is also alanine negative.

Kinoshita *et al.* [16] reported that glutamic acid producing bacteria possess glutamic acid dehydrogenase, which catalyses the dehydrogenation not only of glutamic acid, but also of α.ε-diamino-pimelic acid, a precursor of L-lysine. They also mentioned that a mutant of *Corynebacterium glutamicum,* which are glutamate negative posses glutamic acid dehydrogenase, but lack of isocitrate dehydrogenases, catalyses the production of α-Ketoglutarate in TCA Cycle.

Ishino *et al.* [17] reported that *Corynebacterium glutamicum* and other glutamate producing bacteria possess diaminopimelate dehydrogenase (DDH), which form meso-diaminopimelate (meso-DAP) directly to form L-lysine. They further revealed that glutamate dehydrogenase (GDH) had no meso-α, ε-diaminopimelate dehydrogenase (DDH) activity. The work of Ishino *et al.* [17] and Yamaguchi *et al.* [5] lead to the conclusion that both DAP and DAP dehydrogenase bypaths exist in *Corynebacterium glutamicum.*

Homoserine-alanine double auxotrophic mutant (MRLH-HA5) and homoserine single auxotrophic mutant (MRLH-H16) produced 25.0 g/L and 23.0 g/L L-lysine, respectively. MRLH-HA5 is homoserine negative as well as alanine negative, while MRLH16 is only homoserine negative, due to which HA5 showed higher productivity. However, less potent than GH13. It is due to the blockage of different side reactions. Chancharoensin and Amarate [18] isolated homoserine negative mutant of *Corynebacterium glutamicum*. Schendal *et al.* [19] isolated (Hom^-, AEC^r) mutant of methylotrophic *Bacillus species*. Shiio *et al.* [20] isolated (Hom^-) mutant of *Brevibacterium flavum* for L-lysine production.

The maximum L-lysine producing triple auxotrophic mutant of methionine-threonine-alanine produced 23.4 g/L L-lysine, which was approximately the same as by H16 (Hom^-) mutant because in MTA2 ($Meth^-$, Thr, Ala). The pathway is blocked after homoserine, so aspartic semialdehyde is also converted to homoserine, while in H16 the pathway is blocked at the step of production of homoserine. Double auxotrophic mutant MRLH-MT8 produced 20 g/L L-lysine, less than MTA2, because in MTA2, in addition to methionine and threonine, the side reaction of alanine is also blocked. Bhattacharyya and Samanta [21] developed methionine-threonine double auxotrophic and AEC resistant mutant of *Arthrobacter globiformis*. Liu and Shyang [15] isolated auxotroph of threonine, methionine and threonine for L-lysine production.

The threonine negative mutants, when screened for L-lysine production, maximum concentration of 18.25 g/L was produced by MRLH-T3. The increase production of L-lysine was due to removal of concerted feed back by threonine. Nakayama *et al.* [22] patented the strain of *Brevibacterium flavum* having a requirement of threonine and resistance to feed back inhibition of L-lysine and threonine.

Apart from feed back inhibition and side reaction, metabolic interlock regulates the synthesis of amino acid. By the release of this regulation the productivity of amino acid increases [23]. In L-lysine synthesis, L-leucine acts as an metabolic interlock, which repressed the activity of dihydrodipicolinate synthetase. L-lysine synthesis of AEC mutant of *Brevibacterium flavum* is inhibited by over addition of L-leucine and caused accumulation of L-alanine and valine. L-lysine activated leucine synthesis. Leucine negative mutant of AEC strain increased the production of L-lysine from 16 to 41% [24].

In one experiment of mutagenesis, leucine auxotrophs were selected and the maximum yield in screening medium was 18.8 g/L L-lysine (MRLH-L1) which showed same potency as threonine auxotroph. Cremer *et al.* [25] studied the enzymes regulation of L-lysine biosynthetic pathway in *Corynebacterium glutamicum* and found that aspartate semialdehyde dehydrogenase (ASD), dihydrodipicolinate synthetase (DHDS), dihydropicolinate reductase and diaminopimelate dehydrogenase were not an influence in this specific activity, nor inhibited any of the aspartate family of amino acids. HDH was repressed by methionine to 15% its original activity and inhibited by threonine. Inclusion of leucine in the growth medium resulted in a two fold increase of HDH specific activity. The flow of ASP to L-lysine on homoserine was influenced by

the activity of ADH or DHDS. The two fold increase in HDH activity resulted in decrease in L-lysine formation. [5]

The maximum yield of L-lysine was estimated in at optimum conditions [37] by all the mutants derived. Maximum yield was achieved after 6 days incubation at 30°C, When different media were compared for L-lysine production by these mutants, 14 to 28.6 g/L L-lysine accumulated in FM1 medium (glucose medium), while 12.8 to 26 g/L observed in FM2 medium (Molasses). The yield was least in starch (FM3) medium, with a range of 10-21 g/L.

Ajinomoto Co. obtained high L-lysine production by a patent [26] at low cost after 7 days of incubation from pulmycin resistant mutant of *Corynebacterium glutamicum* (AEC^r, Hom^-), *Corynebacterium acetacidophyllum* (AEC^r plus Hom^-) and *Brevibacterium lactofermentum* (AEC^r + Hom^-). Maximum L-lysine production (36-45 g/L) was also observed in 96-120 h with strains of *Brevibacterium flavum*, using saccharose and non-standard nitrogen source [27].

Nasri *et al.* [28] reported maximum L-lysine after 5 days by *Corynebacterium sp.* Plachy and Ulbert [29] showed the maximum production of L-lysine (45 g/L) after 4th day in 20 L fermentor by mutant strain of *Corynebacterium glutamicum*. Glucose was the best carbon source for the production of L-lysine. Yakoto and Shiio [30] described 40 g/L of L-lysine production in 10% glucose medium by threonine negative mutant of AEC resistance *Brevibacterium flavum*. Another *Corynebacterium glutamicum* strain utilized glucose (20 g/L) as carbon source and showed specific L-lysine production as 1.3 g/L/h in Shaking culture [31].

In the molasses medium lower concentration of L-lysine was observed than in glucose medium, due to inhibitory effect of molasses on growth and production. Plachy *et al.* [32] observed decrease of L-lysine production and cell mass after a substitution of sugar by molasses in mutant of *Brevibacterium flavum*.

Nakayama *et al.* [22] patented their work for the production of L-lysine by *Corynebacterium glutamicum* (Hom^-, Leu^-, AEC^r) using molasses (as 10% glucose) in mineral salt medium, recording a yield of 39.5 g/L.

Starch hydrolysate have relatively low content of monosaccharide, therefore the production of L-lysine was only 15 to 28 g/L of fermentation broth. Similar results were observed by Pelechova *et al.* [, where only 10-12 g/L L-lysine was produced when paper hydrolysate was as carbon source. Kubota *et al.* [32] used 16g/dl sweet-potato starch hydrolyzate and obtained 5.20 g/dl L-lysine HCl by threonine-valine auxotroph of *Brevibacterium lactofermentum*.

Hadj-Sassi *et al.* [33] showed 2.9 g of L-lysine per gram of cell mass by *Corynebacterium glutamicum*, using 17.5% glucose with conversion efficiency of 44%. Rate of biosynthesis was shown to be controlled by the rate of carbon source utilization in one chromogenic mutant of *Brevibacterium sp.* M27, 43 to 49 g/L L-lysine was produced with sugar conversion 45 to 49% [26]

The profile of L-lysine production showed that the parent strain (Thialysine resistant) produced low amount of L-lysine, GHA10 produced maximum, while GH13, HA5, H16, MTA2 and MT8 did not showed much variation in yield. In T3 and L1 almost equal yield was observed slightly less than the other group described.

Hagino *et al.* [34] reported 30 g/L L-lysine in molasses medium by thialysine resistant, homoserine negative mutants of *Bacillus lichinoformis*. In our isolates homoserine negative, thialysine resistant mutant H16 gave 28.6 g/L L-lysine in fermentor.

Methionine plus threonine double auxotrophic mutant, MT8, yielded 28 g/L in optimum conditions. Similar auxotrophs of *Arthrobacter globiformis* gave yield of 32 g/L [21].

Yakota and Shiio [29] reported 40 g/L L-lysine by AEC resistance and threonine negative mutants of *Brevibacterium flavum* in glucose medium. Similar mutants of *Corynebacterium glutamicum* yielded 26.8 g/L in our studies.

The maximum lysine was estimated at optimum condition by all mutants derived. Maximum yield was achieved after 6 days incubation the culture condition became more favourable on 6^{th} days of incubation. The dry cell mass increases upto 6^{th} day then decrease. pH decreased upto 3^{rd} day and then increase upto 75 till 7^{th} day. However residual sugar continously decreases. The most potent mutant was GHA10 while the most fevonable medium was FM_1

Maximum conversion of consumed sugar to lysine by the most potent mutant was in FM_1 slightly less then FM_2, the yield per gram of dry cell weight was also maximum by mutant GHA10 in FM_1 medium the percentage yield based on total sugar was found nearly in same proportion by all mutant, as in L.lysine production in FM_1 FM_2, and FM_3 medium.

Hadj-sassi *et al.* [33], grew the mutant strain of *Corynebacterium glutamicum* in medium containing 17.5% glucose, 5.5% ammonium sulphate and 2% yeast extract. Under laboratory conditions, it produced high amounts of L-lysine in this optimized medium. L-lysine production, residual sugar, and dry cell mass were measured as a function of fermentation time. It was observed that 1 g of cell mass produced 2.96 g of L-lysine. The conversion efficiency was found to be 44%.

Coello *et al.* [35], worked on the physiological aspect of L-lysine production. They observed that in case *Corynebacterium glutamicum,* phosphate limited cultures at low growth rates were favourable to L-lysine production. L-lysine was produced when a culture at low dilution rate (0.03/4) was established. A dilution rate of about 0.04/4 should be maintained in order to assure good productivity. L-lysine yield was 0.53 g/g under carbon limiting conditions the maintenance energy and growth yield of 0.03 g / g / h and 0.41 g/g, respectively were obtained. Under these limiting conditions the L-lysine production was not favoured even at lower dilution rates.

In the present studies different auxotrophic mutants were derived from the regulatory mutants (Thialysine resistant mutant). All showed higher L-lysine production than the parent strain (MRLH-TR1). This indicates that in the parent strain the aspartokinase was not completely insensitive to concerted feed back inhibition by L-lysine and L-threonine. Furthermore, it could produce homoserine, threonine, methionine instead of only L-lysine. Therefore, it is necessary to block the pathway between aspartic semialdehyde and homoserine to achieve over production. If the mutants are only auxotrophic of homoserine and not regulatory, then the amino acids in the medium, such as molasses, play the same role of concerted feed back inhibition. Thus, for over production it is essential that the strain must be auxotrophic, as well as a regulatory mutant.

The production of lysine not only depends upon the multiple nature of auxotrophic mutants but also depends upon the blocking of side reactions. Due to this reason, the triple auxotrophic mutant of glutamate-homoserine-alanine (MRLH-GHA10) produced a higher yield, than the triple auxotrophic mutant of methionine-threonine-alanine (MRLH-MTA2). It may be concluded that the side reactions of glutamate production *consumed* more sugar, and when blocked, produced more L-lysine than the side reactions of alanine.

Experimental

The parent strain is a thialysine resistant mutant of *corynebacterium glutamicum* developed by Shah and Hameed [8] For the activation of Lysine producing strains, all mutant were streaked on complete agar medium and incubated for 24 hours and restreaked on complete agar medium for next 24 hours.

Media Composition

Complete, Minimal, Screening, Media were the same as discussed by Shah and Hameed. [8]

Selection Medium

For the selection (screening) of different auxotrophs of amino acids, minimal medium was supplement with 0.04 g amino acid of desired auxotroph.

Fermentation Medium (FM1)

Fermentation medium (FM1) was constituted after optimization studies had the following composition per 100 ml distilled water. Glucose 10 g, Ammonium sulphate 2.5 g, Potassium dihydrogen phosphate 0.1 g, Magnesium sulphate seven hydrate 0.05 g, Calcium carbonate 2 g, Bactocasamino acid 0.5 g, Ferrous sulphate seven hydrate 0.2 mg, Manganese chloride tetrahydrate 0.2 mg, d. Biotin 5 µg Thiamine hydrochloride 20 µg, pH 7.5

Fermentation Medium 2 (FM2)

Fermentation medium 2 had the same composition per 100 ml distilled water as fermentation medium (FM1) except molasses was used as total sugar (10 g) and the concentration of bactocasamino acid, ferrous sulphate, manganese chloride and magnesium sulphate was modified as : Bactocasamino acids 0.2 g, Ferrous sulphate seven hydrate 0.1 mg, Manganese chloride tetrahydrate 0.1 mg, Magnesium sulphate seven hydrate 0.5 mg, Potassium dihydrogen phosphate 0.01 g,

Fermentation Medium 3 (FM3)

Fermentation medium 3 had the same composition per 100 ml distilled water as FM1 except 15 g per 100 ml starch hydrolyzate was used as carbon source.

Culture Methods

Screening of mutants for L-lysine production

Screening media (20 ml) in 100 ml flask were inoculated by loop of cells (Respective required mutant) from 24 hrs. old complete agar plate and incubated on reciprocal shaking incubator at 28°C from 72 hrs. at 100 rpm. Sample of each mutants (auxotrophic, regulatory) were centrifuged. The supernatant were examined for L-lysine both qualitatively and quantitatively.

Batch Fermentation

Seed medium (50 ml) were inoculated in 250 ml Erlenmeyer flask in duplicates by loop of cells of most potent mutant (auxotrophic, regulatory) from 24 hrs. old complete agar plates and incubated on rotatory shaking incubator at 30°C for 20 hrs.

Fermentation medium (50 ml) was inoculated in 500 ml Erlenmeyer flask by 5 ml seed medium in duplicate and incubated on rotatory shaking incubator at 30°C for seven days. Sample were taken every day and centrifuged. The supernatant was examined for L-lysine [8].

Mutational Treatment

Development and Isolation of Auxotrophic Mutants

Exponentially growing cells (2 x 10^8 cells/ml) of thialysine resistant mutant were irradiated by 30watt UV germicidal for 30 Sec., under the same parameter as discussed for the development of thialysine resistant mutants [8]. One ml of the UV irradiated cells were taken in 10 ml test tube in duplicate. Then 50 units of penicilline G were added and incubated for 14 hrs., on reciprocal shaking incubator at 28°C with agitation 100 rpm. After 14 hrs., 100 units of penicillinase was added. After 5 minutes, in first experiment, 1 ml solution containing 0.04% each homoserine, alanine and glutamate was added. In second experiment, 1 ml solution containing 0.04% each methionine, threonine and alanine was added and in the third experiment, 1 ml solution containing 0.4% leucine was added for enrichment. Then incubated under the same parameters. After 7 hrs., 0.2 ml of the culture in each test tube was separately smeared on complete agar and incubated. Each colony was then streaked on minimal agar and bactocasamino acids. The colonies that had grown on bactocasamino acids were then restreaked on glutamate agar, glutamate-alanine agar, alanine agar, homoserine agar, homoserine-glutamate agar and homoserine-alanine agar from first experiment and on methionine, threonine, alanine, homoserine-alanine, methionine- alanine, threonine-alanine, methionine-threonine and methionine-threonine-alanine agar from the 2nd experiment of bactocasamino acid agar and on leucine agar from the 3rd experiment. All the plates were incubated for 48 hrs. Colonies that did not grow on minimal agar and grew on minimal agar plus 0.04% amino acid of their respective auxotrophs were isolated and activated.

Each auxotrophic, double auxotrophic and triple auxotrophic mutants were separately cultured in 20 ml auxotrophic screening media in 100 ml flask and incubated at 28°C agitation 100 rpm. After 72 hrs., samples were taken and centrifuged the supernatant were examined for L-lysine qualitatively and quantitatively [37].

Clarification of Molasses

Sugarcane molasses was provided by Murree Brewery Co. (Pvt.) Ltd., Rawalpindi, Pakistan. The clarification of molasses was done by diluting the molasses with 1.5 times of distilled water and adjusted to pH 9.5 and was placed in shaking water bath at 50°C for 10 minutes and then allowed to settle the solid material overnight at room temperature. The supernatant was removed and filtered [24].

Analytical Methods

For the qualitative analysis for lysine paper chromatographic technique of Momose and Takagi, [9] was used. lysine was assayed by acidic ninhydrine copper reagent method [10,11) Sugar and starch were determined by Calorimetric method [12].

Determination of Dry Cell Weight (Dcw)

After centrifugation of a few ml of one normal HCl was poured into the precipitate of bacterial cells and calcium carbonate to dissolve calcium carbonate. The remaining bacterial cells were washed with water and dried at 100°C until constant.

Extraction and Purification of L-Lysine from the Fermentation Broth

At the end of the cultivation cell and calcium salt were removed from the broth by centrifugation at 10000 rpm for 20 minutes. Cells-free broth were decolourised with charcoal and again centrifuged. Cell-free broth was acidified with concentrated sulfuric acid to pH 2, then pumped to a 2.5 x 50 cm (diameter x length) column packed with 200 ml of wet strong cation exchange resine in the ammonia from, which had been converted from 50 g of dry resine in the hydrogen form (Amberlit IR 120 plus, mesh size 40 micron. The column was washed with deionized water, L-lysine was then eluted with 2 N ammonium hydroxide solution. The eluent was concentrated, adjusted to pH 5.6 and further concentrated crystal of crude L-lysine. HCl were precipitated from the solution by cooling under reduced pressure. Decolourized with charcoal and again recrystalized.

For regeneration the column was rinsed with 600 ml of deionized water then regenerated with 600 ml of 1N NH_4OH and rinsed with 600 ml of deionized water. The flow rate for all steps were 1 ml /cm^2/min.

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