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Nutritional and Mutational Aspects of Lysine Production by *Corynebacterium glutamicum* Auxotrophs

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Abstract.- Mutants of different auxotrophic natures were developed from one of the regulatory mutants of *Corynebacterium glutamicum.* All the mutants were examined for the production of lysine, yield based on total sugar, percentage conversion of consumed sugar, and yield per gram dry cell weight, in three different fermentation media in stirred tank fermenter. The mutated MRLH-GHAIO accumulated 38.4 g/L lysine with 43.8% conversion and 2.4 g lysine per gram dry cell weight in FM_1 medium while in FM_2 medium it gave 32.8 g/L lysine with 39.1% conversion and 2.1g lysine per gram dry cell weight. The other auxotrophic mutant MRLH-LI, T3, MT8, MTA2, HI6, HA5 and GHI3 produced 27.5-33 g/L lysine with 32.22-36.8% conversion in FM_1 medium and 25-30.8g lysine with 30.4-37% conversion in FM_2 medium the yield per gram dry cell weight was 1.5-1.98 g/g in FM_1 and FM_2 medium. The yield based on total sugar increased as the amount of lysine increased. Lower yield was recorded in FM_3 medium. All the auxotrophic mutants showed higher productivity and conversion efficiency than parent strain.

Key words: Nutritional factors, mutants, lysine fermentation, *Corynebacterium glutamicum.*

INTRODUCTION

Over 40, 000 tons of L-lysine is used each year chiefly as a nutritional supplement in animal feed (Tosaka *et al*., 1983). Demand for lysine as a dietary supplement in poultry and swine production increased at an annual growth rate of 14.6% during 1980. In 1989 the world wide demand for lysine was 115200 metric tons and continuous growth is expected (Grace *et al*., 1996).

Prior to 1965 most of the commercially produced lysine was isolated for protein hydrolyzate. In 1996 out of the world production of 80,000 metric tons of lysine approximately 90,000 tons was produced by fermentation (Hasio and Glatz, 1996). Global lysine production increase continuously, because of major advances in the efficiency of fermentation synthesis of lysine (Anonymous, 1985).

Yamaguchi *et al*. (1986) worked out the biosynthetic pathway of lysine in glutamate producing bacteria and pointed out the control mechanism for lysine overproduction. In *Corynebacterium glutamicum* oxalacetate transaminate to aspartate which then by asprtate kinase convert to branched point, intermediate, aspartic beta semialdehyde, which by one route forms homoserine and by another lysine. Homoserine then form threonine on one side and methionine on the other. The production and activities of enzymes in the biosynthetic pathway are controlled by the mechasnism of feed back inhibition, repression and metabolic interlock due to which the wild strain can not accumulate lysine beyond its requirement. Threonine and methionine by concerted feedback inhibition stop the activities of aspartate kinase and as a result lysine production will stop. Similarly other side reactions of alanine, leucine and glutamate are also involved in effecting the yield of lysine. To achieve maximum production it is necessary to overcome these regulation by developing different auxotrophs. The present study focused on the development of auxotroph and over production of lysine in stirred tank fermentation, after releasing the various metabolic control mechanism and utilizing three different media.

The parent strain is a thialysine resistant mutant of *Corynebacterium glutamicum* developed by Shah and Hameed (2002) which produced 22g/L lysine. The parent strain was further mutated to

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different auxotrophs and the potency for lysine was studied in different nutritional media.

MATERIALS AND METHODS

Media composition

Complete, Minimal and Screening, Media were the same as described by Shah and Hameed (2002).

Fermentation medium1 (FM1)

Fermentation medium (FM1) was constituted (PH 7.5) after optimization studies and had the composition (g) per 100 ml distilled water. Glucose, 10 ; ammonium sulphate, 2.5; potassium dihydrogen phosphate, 0.1 ; magnesium sulphate seven hydrate, 0.05; calcium carbonate, 2 and bactocasamino acid, 0.5 along with ferrous sulphate seven hydrate, 0.002; manganese chloride tetrahydrate, 0.002; d-biotin, 5 μg ,thiamine hydrochloride, 20 μg.

Fermentation medium 2 (FM2)

FM 2 had the same composition as(FM1) except molasses (total sugar 10 g) was added 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 and Potassium dihydrogen phosphate 0.01 g.

Fermentation medium 3 (FM3)

FM 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 loops of cells (Respective mutant) from 24 h old agar plate and incubated on shaking incubator at 28°C for 72 h at 100 rpm. Sample of each mutant (auxotrophic, regulatory) were centrifuged. The supernatants were analysed for L-lysine both qualitatively and quantitatively.

Inoculum preparation

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

Stirred tank fermentation

Sterilized 3L fermentation medium was taken in 6 L stirred tank fermentor. The fermentation was started after inoculation with too much seed medium under 500 rpm agitation and 1.5 vvm aeration at 30°C for six days. One ml of the broth was pipetted out with sterilized pipette at intervals. The pH of the broth was controlled automatically at 7.5 with 20% ammonium hydroxide. Tween 20 was used as antifoaming agent

Development and isolation of auxotrophic mutants

Exponentially growing cells ($2x10^8$ cells/ml) of thialysine resistant mutant of *Corynebacterium glutamicum* were irradiated by 30 watt UV germicidal lamp at distance of 15cm for 30 second., under the same parameter as discussed for the development of thialysine resistant mutants (Shah and Hameed, 2002). The UV irradiated cells (1 mL) were taken in 10 ml test tube in duplicate. Then 50 units of penicillin G were added and incubated for 14 h ., on shaking incubator at 28°C (100 rpm). After 14 h., penicillinase (100 units) was added. After 5 minutes, in first experiment, 1 ml solution containing 0.04% of each homoserine, alanine and glutamate was added. In the 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. All the experimental flasks were then incubated under the same conditionsof pH and temperature. After 7 h, 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 h. Colonies that did not grow on minimal agar and grew on minimal agar plus 0.04% amino acids 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 h., samples were taken and centrifuged at 1000rpm for 20minute, the supernatant were examined for L-lysine qualitatively and quantitatively.

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 overnight at room temperature. The supernatant was removed and filtered.

Lysine estimation

For the qualitative analysis of lysine, paper chromatographic technique of Momose and Takagi (1978) was used. Lysine was assayed by acidic ninhydrine copper reagent method Aiko *et al*. (1978). Sugar and starch concentration were determined by Calorimetric method (Dubios *et al.*, 1956).

Dry cell weight (dcw)

After centrifugation, 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 to100°C to constant weight.

Extraction and purification of l-lysine from the fermentation broth

At the end of the cultivation cells and calcium salts were removed from the broth by centrifugation at 10000 rpm for 20 minutes. Cells-free broth were decolorized with charcoal and again centrifuged. Cell-free broth was acidified with concentrated sulfuric acid to pH 2, and 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 and L-lysine was then eluted with 2 N ammonium hydroxide solutions. The eluent was concentrated, adjusted to pH 5.6 and further concentrated. Crystals of crude L-lysine HCl were precipitated from the solution by cooling under reduced pressure. The precipitate were decolourized with charcoal and again recrystalized.

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

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%). All L-lysine producing auxotrophs were separately isolated on complete agar medium and screened for lysine yield.

In the second experiment 151 colonies (30.2%) were isolated as auxotrophs. Among these, threonine auxotrophs were the 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%). All the L-lysine producing mutants were isolated and activated.

Third experiment showed 6.2% (31) leucine auxotrophs, 1.8% auxotrophs of other amino acid

and 1.4% of other substances. The numbers of revertant and dead cells were 4.2 and 5.8%, respectively.

Profile of dry cell weight,and L-lysine production

All mutants were cultured in 3 liter stirred tank fermentation for 6 days, at constant pH 7.5, aeration 1.5 vvm at 30°C. After every 24 h, dry cell weight, and L-lysine production were determined.

In FM1 and FM2 medium the dry cell weight increased to maximum at 3rd day and then decreased. In FM1 medium, maximum cell mass (18.8 g/L) was observed by MT8, T3 and L1, while other mutants gave 16.2 - 18 g/L dry cell weight in the fermentation broth. However, on first day of incubation 7 to 9.9 g/L dry cell weight was found. In case of FM2 medium, maximum dry cell weight (16.9 g/L) was found by GHA10 that and minimum by H16 and GH13, (13 g/L). In FM3 medium maximum dry cell weight was observed on 4th day of incubation and then decreased on 5th and 6th day. Mutant MRLH-L1 produced highest cell mass (15.7 g/L). The cell mass of other mutants were between 14-15 g/L (Figs. 1, 2, 3).

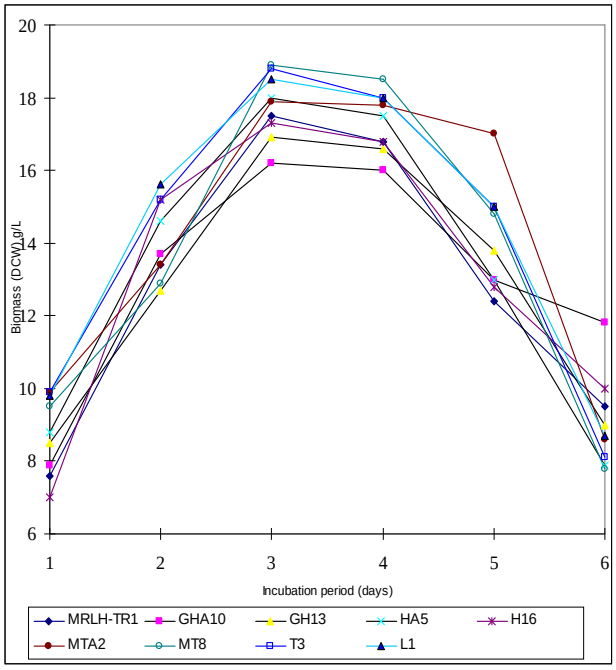

Fig. 1. Biomass (DCW) production by mutants in FM1 medium.

L-lysine production continuously increased upto 4th day of incubation and then slightly decreased. Maximum L-lysine was produced by GHA10 in FM1 medium, slightly less in FM2 medium and least in FM3 medium. On 4th day of incubation 22-38 g/L L-lysine was produced in FM1 medium. In FM2 medium 20.2 to 32.87 g/L yield was observed, while in FM3 medium 15-28 g/L L-lysine was accumulated in the fermentation broth (Fig. 4, 5, 6).

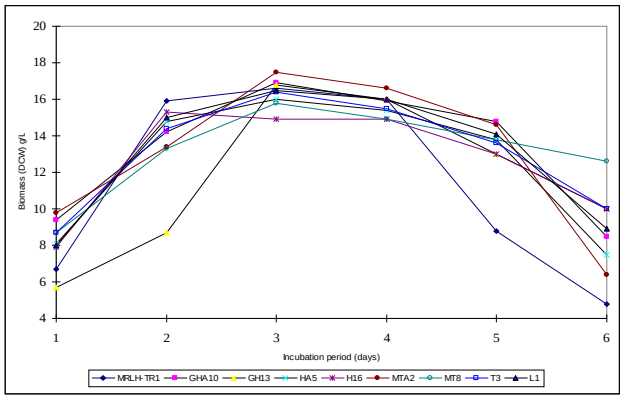

Fig. 2. Biomass (DCW) production by mutants in FM2 medium.

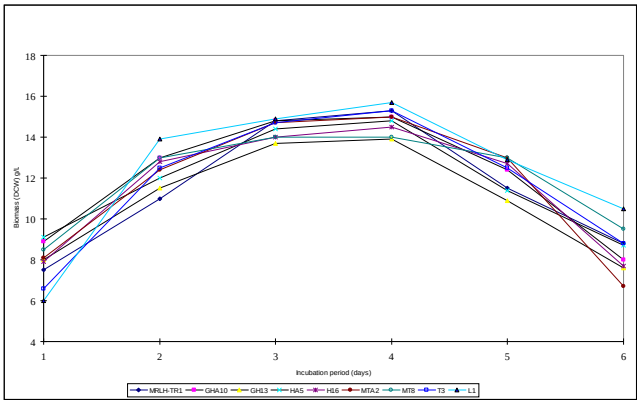

Fig. 3. Biomass (DCW) production by mutants in FM3 medium.

L-lysine production by mutants

Different mutants were compared for L-lysine production, yield based on total sugar, conversion of consumed sugar to L-lysine (%) and yield per gram dry cell weight in different media.

In FM1 medium the most potent mutant

GHA10 produced 38.4 g/L L-lysine, as compared to the parent strain TR1, which produced 22 g/L L-lysine. The other auxotrophic mutants accumulated 26.2 - 33 g/L L-lysine. In FM2 medium GHA10 gave maximum yield (32.8 g/L) and TR1 gave 20.2 g/L L-lysine. While the rest of the auxotrophic mutants produced from 23.6 to 30.8 g/L L-lysine. MTA2 and H16 produced approximately equal yield (30.1 - 30.6 g/L L-lysine). In FM3 medium MRLH-L1 and T3 produced approximately equal amounts of L-lysine (16.1-16.6 g/L). MT8 and MTA2 also gave similar yield of lysine (18 - 18.6 g/L). The other auxotrophic mutants H16, HA5, GH13 accumulated 20.2 to 25 g/L L-lysine.The most potent mutant GHA10 gave maximum 28.6 g/L and TR1 accumulated 15 g/L L-lysine (Fig. 7).

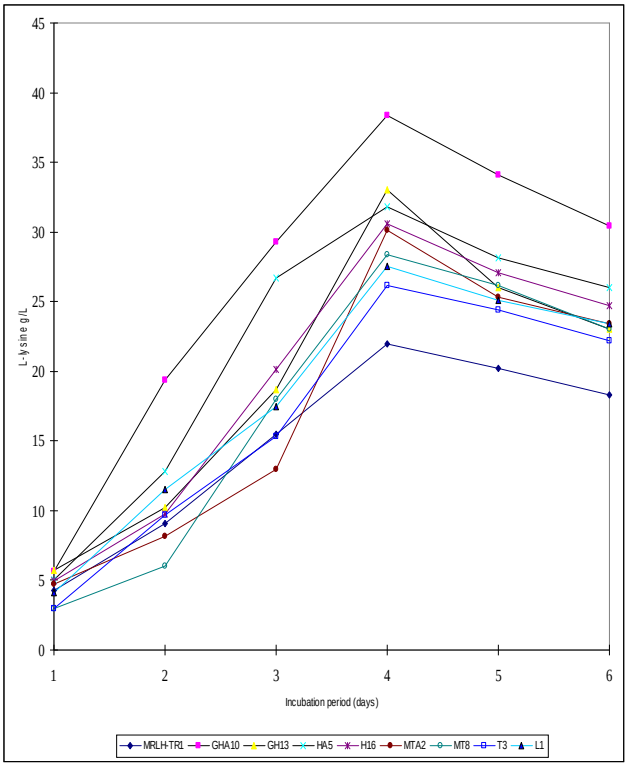

Fig. 4. Lysine production by muants in FM1 medium.

Among the media maximum conversion occurred in FM1 medium followed by FM2 and FM3 media respectively. The conversion of consumed sugar to lysine by the most potent mutant GHA10 was3.8%, 39.2% and 24.7% in FM1, FM2 and FM3 media respectively.

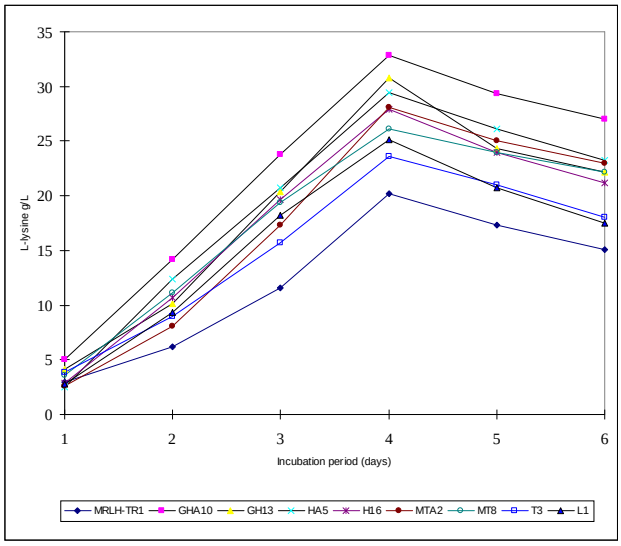

Fig. 5. L-lysine production by mutants in FM2 medium.

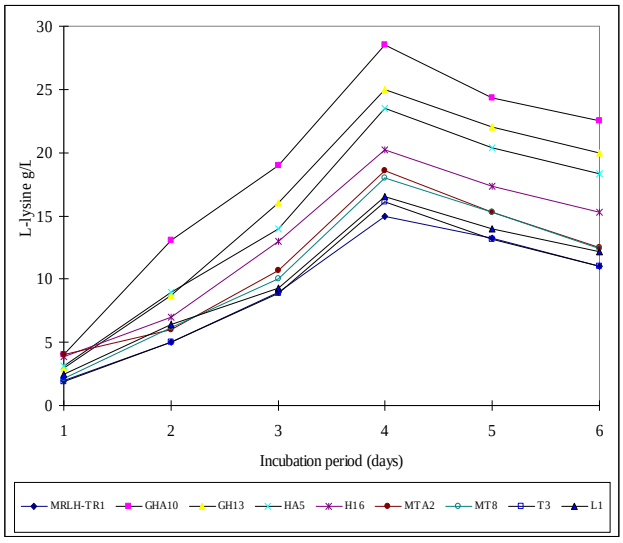

Fig. 6. L-lysine production by mutants in FM3 medium.

The most potent GHA10 gave 43.8% conversion in FM1 medium, among the rest of the mutants the parent strain TR1 gave 26.1% conversion. In FM2 medium in most of auxotrophs approximately same % conversion was observed as

in FM1 medium, except the parent strain, which gave 23.8% and GHA10, which gave 39.2%,. In FM3 medium lower conversion was found among all mutants than FM1 and FM2 medium. The parent strain gave 12.98%, while rest of the mutants showed 15.5 to 24.7% conversion and sugars to lysine (Fig. 8).

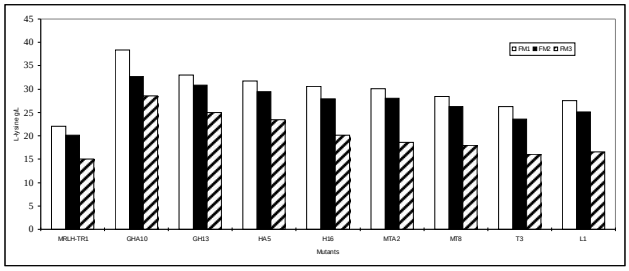

Fig. 7. Maximum L-lysine produced by mutants in FM1, FM2 and FM3 medium.

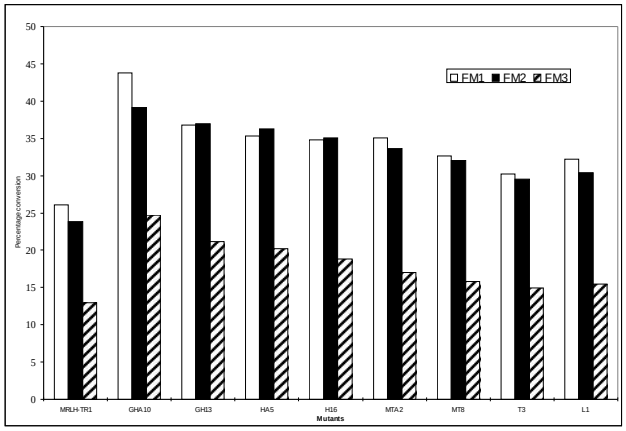

Fig. 8. Percentage conversion of consumed sugars to lysine by mutants in FM1, FM2 and FM3 medium.

Conversion of sugar to lysine

The mutants which gave high yield of L-lysine was also gave high percentage yield based on total sugar. The most potent mutant GHA10 gave 39.3% in FM1 medium. 33.7% in FM2 medium and 19.3% in FM3 medium. While the parent strain RT1 gave 22%, 20.7% and 10.13% in FM1, FM2 and FM3 medium, respectively (Fig. 9).

The yield in gram per gram dry cell weight g/gdcw by triple auxotrophic mutant GHA10 was 2.4 g/g, 2.1 g/g and 1.9 g/g in FM1, FM2 and FM2 medium, respectively. While in other auxotrophs 1.45 to 1.98 g/g L-lysine per gram dry cell weight in FM1 and FM2 medium and from 1 to 1.8 g/g in FM3 medium. The parent strain TR1 gave 0.98 - 1.3 g L-lysine per gram dry cell weight in FM1, FM2 and FM3 medium (Fig. 10).

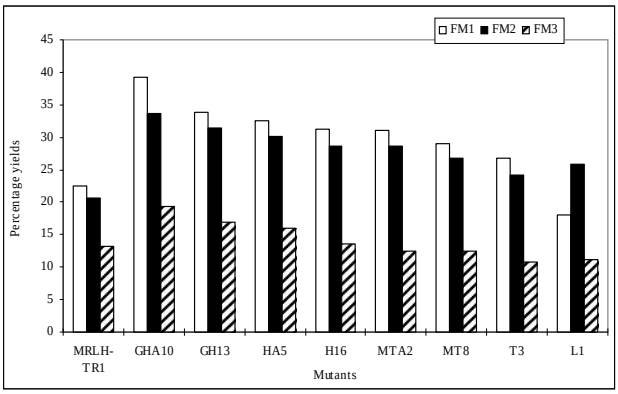

Fig. 9. Yield percentage based on total sugar by mutants in FM1, FM2 and FM3 medium.

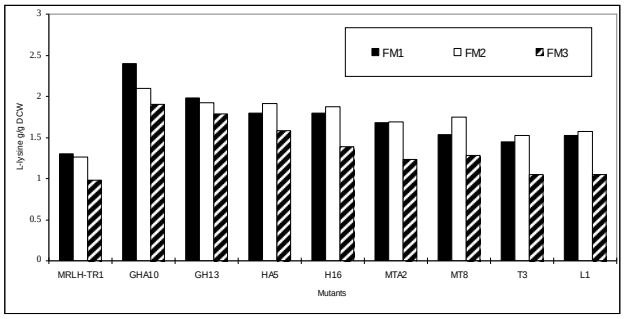

Fig. 10. Yield based on per gram dry cell weight by mutants in FM1, FM2 and FM3 medium.

Out of 8 different auxotrophs studied for L-lysine production, the triple auxotrophic mutant of glutamate-homoserine-alanine (MRLH-GHA10) was found to be the most potent mutant for L-lysine production.MRLH-GHA10 showed 38.4 g/L L-lysine on 4th day in FM1 medium in stirred tank fermentation at 30°C (pH 7.5, aeration 1.5 vvm, agitation 500 rpm).

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 at 1000 rpm for 20minute and one liter of the supernatant containing 38 g/L L-lysine was passed through 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. Crystals of crude L-lysine were precipitated out from the solution after freeze drying.The crystals were decolourized with charcoal and recrystalized. As a result 37.16 g L-lysine HCl (29.73 g/L L-lysine) was isolated with 78.2% recovery. The melting point found was 263, which was the same as reported by Windholz (1976). Zaki *et al*. (1982) reported 81% of L-lysine recovery from culture broth by separation on cation exchange resins.

Through mutagenesis a change can be introduced at genetic level that is then translated into metabolic pathways. 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 step results in the release of the concerted feed back inhibition by threonine and L-lysine on aspartokinase. The aspartic semialdehyde produced proceeds to L-lysine through the L-lysine biosynthetic pathway. Another effective technique for the overproduction 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 concentration of L-lysine would be produced, independent of concentration of threonine.

Kinoshita *et al*. (1960) reported that glutamic acid producing bacteria possess glutamic acid dehydrogenase, which catalyses the dehydrogenation not only of glutamic acid, but also of α.ε-diaminopimelic acid, a precursor of L-lysine. They also mentioned that a mutant of *Corynebacterium glutamicum*, which is glutamate negative posses glutamic acid dehydrogenase, but lack of isocitrate dehydrogenases, catalyses the production of α-Ketoglutarate in TCA Cycle.

Ishino *et al*. (1984) 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*. (1984) and Yamaguchi *et al*. (1986) lead to the conclusion that both DAP and DAP dehydrogenase by paths exist in *Corynebacterium glutamicum*.

Effect of carbon sources

The carbon source used was glucose, molasses and starch hydrolysate. The effect of initial sugar concentration on L-lysine accumulation showed highest production at 10% initial sugar. With higher sugar concentration the cell mass and L-lysine yield decreased. Bacteria are very sensitive to osmotic pressure of the medium and for this reason the fermentation product accumulated in medium was low above 10% sugar.

Initial concentration of glucose influenced the production of L-lysine by *Corynebacterium sp*. in batch culture has also previously been found to production rates at 65 g/L of glucose (Hadj-Sassi *et al*. 1988). Ferreira and Duarate (1991) also used 10% glucose for maximum yield of L-lysine from *Corynebacterium glutamicum* fluoropyruvate sensitive mutant and reported similar results.

Molasses concentration used was calibrated to 10% sugar after clarification. This concentration yielded 26.35 g/L L-lysine in shake flask (Anonymous, 1992). Nakayama *et al*. (1973) also used molasses at 10% sugar concentration for *Corynebacterium glutamicum*. The *Corynebacterium glutamicum* could not hydrolyze starch therefore, starch hydrolyzate was used. The concentration of starch hydrolyzate was optimized by using 9-17% in medium as carbon source for L-lysine biosynthesis. Optimum concentration found was 15% and above this the yield and the cell mass were approximately constant. Smekal *et al*. (1983) reported that *Corynebacterium glutamicum* (AEC^r.

Hom$^-$) accumulate L-lysine in the fermentation with standard carbon source, such as molasses, as well as non standard, such as hydrolyzate of cereal starch. Nasri *et al*. (1989) reported maximum L-lysine after 5 days by *Corynebacterium sp*. Plachy and Ulbert (1985) 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 (1988), 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 (Ferreira and Duarate, 1991).

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*. (1988) observed decreased of L-lysine production and cell mass after a substitution of sugar by molasses in a mutant of *Brevibacterium flavum*. Nakayama *et al*. (1973) 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 found 15 to 28 g/L of fermentation broth. Similar results were observed by Pelechova *et al*. (1983), where only 10-12 g/L L-lysine was produced when paper hydrolysate was used as carbon source. Kubota *et al*. (1970) used 16g/dl sweet-potato starch hydrolyzate and obtained 5.20 g/dl L-lysine HCl by threonine-valine auxotroph of *Brevibacterium lactofermentum*.

CONCLUSIONS

In the present study 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 overproduction of lysine. 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 overproduction 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 natures 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). Hence, 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.

Glutamate negative mutants of glutamate producing strains accumulate more L-lysine, if small amounts optimal requirement (nutritive requirement) of glutamate is supplied from external source.

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