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RESEARCH ARTICLE

Selection and Characterization of a Lysine Yielding Mutant of *Corynebacterium glutamicum* - a Soil Isolate from Pakistan

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ABSTRACT

L-lysine is the second limiting amino acid for poultry and supplemented in broiler feed for optimal performance. Lysine can be produced by inducing mutation in glutamate producing bacteria. The study was conducted to enhance lysine production from a local strain of *Corynebacterium glutamicum*. The bacterium was mutated by exposure to UV. Mutants resistant to s-2-aminoethyle L-cystein (AEC) and showing auxotrophy for L-homoserine were screened for lysine production qualitatively and quantitatively. A mutant showing highest production of lysine (8.2 mg/mL) was selected for optimization of physical and nutritional parameters for maximum production of lysine in shake flask. An initial pH 7.6, 30˚C temperature, 300 rpm and 60 h incubation time were the optimized values of physical requirements. Cane molasses and corn starch hydrolysate were required at 15% (w/v) in the fermentation media which provided around 9% total sugars to produce maximum lysine (17 to 18 mg/mL). When amonium sulphate was used at 3.5% (w/v) level in molasses or corn starch hydrolysate based fermentation media, production of lysine slightly increased above 18 mg/mL. It is concluded that industrial by products like cane molasses, corn steep liquor, and corn starch hydrolysate can be used as carbon and organic nitrogen sources in fermentation medium for scale up process of lysine production and this lysine enriched broth may be used in broiler feed later. However, more potent lysine producing mutant and additional *in vivo* trials would be required to commercialize this product.

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INTRODUCTION

Corynebacterium glutamicum (*C. glutamicum*) is one of the best studied bacterium due to its industrial value. Corynebacteria are Gram positive, non sporulating actinomycetes of irregular rod shape with high GC ratio in DNA. *C. glutamicum* can over produce large amounts of glutamic acid from non proteinaceous sources. This discovery led to the development of fermentation industry in Japan in late 1960s. Monosodium glutamate (MSG) is produced from wild *C. glutamicum* in presence of penicillin and under biotin limiting conditions. Lysine is produced after inducing mutation in the metabolic pathway towards aspartate driven amino acids. MSG is used as flavoring agent in human food and lysine being an essential amino acid and deficient in all cereal grains, finds its utilization in pharmaceutical, food and feed industry. Over the last 50 years, world demand of lysine is met through mutants of this bacterium and closely related species of *Brevibacterium* (Park and Lee, 2008; Becker *et al*., 2011). Majority of this production is being used in poultry and pork feed. Due to increasing market demand and price competition, the industry has shown consistency in developing efficient strains either through classical mutagenesis or by applying modern biotechnology tools. The absence of co-enzymes on the lysine production pathway, presence of two different genes to tackle high & low ammonium concentration, a unique cell membrane and ability to co-utilize different carbon sources makes *C. glutamicum* very useful bacterium for the mankind (Blombach *et al*., 2009; Blombach and Seibold, 2010).

Anastassiadis (2007) estimated worldwide annual production of lysine to be around $8x10^5$ tones. At present Pakistan feed industry imports all the needed lysine. With production of 542.74 million broilers in Pakistan (Anonymous, 2010-11), and minimum requirement of

lysine (HCl salt) around 0.1% in broiler feed formulations, there is huge potential for utilization of locally made lysine in broiler feed. This study was conducted to develop lysine producing mutant of local strain of *C. glutamicum* and to determine the optimal culture conditions for the high yield production of lysine.

MATERIALS AND METHODS

Mutation: A local soil isolate of *C. glutamicum* MRL-H strain (GenBank accession No. HM368259) was used for the study. Concepts of mutational treatment and selection of mutants were adapted from Shiio *et al*. (1990). Stock cultures were maintained on 1.8% agar slants of complete medium (CASO BOUILLON: 3 g/100mL). Broth culture was prepared by inoculating CASO broth with wild *C. glutamicum* cells from overnight plate culture on CASO agar. When culture OD_{600} reached at 7 to 8, cells were pelleted by centrifugation at 10^3 rpm for 5 minutes. Two washings were given with 0.1 *M* $MgSO_4.7H_2O$, and cells were re-suspended in 0.1 *M* $MgSO_4.7H_2O$ solution at a concentration of 10^8 cells per mL (OD_{600}= 1). This cell suspension (20 mL) was poured in an autoclaved petriplate and put under UV light (253.7 nm) for 180 s from a distance of 15 cm, using germicidal UV lamp G15T8 (15W, Sankyo Denki®, Japan). The petriplate was placed over a shaking plate form to ensure that all cells were exposed to UV. After exposure, 10 µL cell suspensions with 10^{-2} dilution were poured on CASO agar plates and incubated at 30˚C for 72 hours to check survival rate. Average number of colonies was calculated from five spots of 10 µL each and per cent survival rate was calculated.

Selection of regulatory (L-lysine analogue resistant) and L-homoserine auxotroph mutants: UV treated cells were enriched by culturing in minimal medium broth (MM1: $(NH4)_2SO_4$ 1 g, urea 0.3 g, K_2HPO_4 0.1 g, $MgSO_4.7H_2O$ 0.04 g, biotin 5 µg, glucose 2 g, $FeSO_4.7H_2O$ 0.2 mg, $MnSO_4.6H_2O$ 0.2 mg, NaCl 5 mg, distilled water to make 100 mL) supplemented with 10,000 units/mL penicillin G, 0.1 *M* magnesium sulfate and 20% (w/v) sucrose. Exponentially growing (non auxotroph) cells are killed under the action of penicillin whereas non growing mutants survive as quiescent cells. These may be auxotroph for many factors but selection of L-lysine analogue resistant {(s-2-aminoethyle, L-cystein), AEC^r} and L-homoserine auxotroph (hom^-) mutants was done by growing on MM1 agar medium supplemented with 10 m*M* AEC, 1 m*M* L-threonine and 0.1 m*M* L-homoserine, at 30˚C for 4 days. Colonies growing on this medium were further screened for L-homoserine auxotrophy by replica plate method (Kumar *et al*., 2002).

Qualitative selection of lysine producing colonies: One thousand and sixty one mutant colonies (AEC^r and hom^-) of *C. glutamicum* (MRL-H strain) were tested qualitatively for production of lysine using a tester strain of *E. coli* (JM109, Δ *lysA* deletion strain, and requiring lysine for growth). Lawn of *E. coli* was made with 1 mL suspension (OD_{600}=1) on MM1 agar medium supplemented with L-homoserine (0.01%) & pantothenic acid (0.001%) and was incubated at 37˚C overnight. No growth was seen next day. Then autoclaved paper discs were placed over the lawn and 2 µl of cell suspension (in MM1 broth) from single colonies of different mutants of *C. glutamicum* were poured over the paper discs and incubated at 30˚C for 7 days. Growth of *E. coli* around paper discs was indicative that some of the mutants were potent lysine producers (Kumar *et al*., 2002).

Optimization of culture conditions and nutritional requirements: A total of 558 lysine producing mutants (around which sufficient growth of *E. coli* was seen) were selected for quantitative production of lysine in screening medium (per 100 mL: glucose 5 g, $(NH_4)_2SO_4$ 1 g, $CaCO_3$ 1 g, KH_2PO_4 0.3 g, K_2HPO_4 0.7 g, $FeSO_4.7H_2O$ 0.2 g, $MgSO_4.7H_2O$ 0.01 mg, $MnCl_2.4H_2O$ 0.2 mg, biotin 10 µg, thiamine hydrochloride 20 µg, L-homoserine 0.04 g, pH 7.2). Whereas basal medium (per 100 mL: glucose 8 g, $(NH_4)_2SO_4$ 2 g, bactocasamino acids 0.2g, KH_2PO_4 0.2 g, $FeSO_4.7H_2O$ 0.3, $MgSO_4.7H_2O$ 0.02 mg, $MnCl_2.4H_2O$ 0.3 mg, biotin 10 µg, thiamine hydrochloride 20 µg, pH 7.6) was used for optimization of culture conditions for lysine production. Three fermentation media (FM1, FM2 and FM3) were used for comparative requirement of total sugars (carbon) from different sources and requirement of inorganic nitrogen. FM1 comprised of glucose 10 g, $(NH_4)_2SO_4$ 2.5 g, $CaCO_3$ 0.2 g, bactocasamino acids 0.5 g, KH_2PO_4 0.1 g, $MgSO_4.7H_2O$ 0.05 g, $MnCl_2$ $4H_2O$ 0.2 mg, biotin 5 µg, thiamine hydrochloride 20 µg, and distilled water to make 100 mL. FM2 comprised of sugar cane molasses 20 g, $(NH_4)_2SO_4$ 2.5 g, $CaCO_3$ 0.2 g, bactocasamino acids 0.2 g, KH_2PO_4 0.1 g, $MgSO_4.7H_2O$ 0.1 g, $MnCl_2$ $4H_2O$ 0.2 mg, biotin10 µg, thiamine hydrochloride 20 µg and distilled water to make 100 mL. FM3 comprised of corn steep liquor 10 g, corn starch hydrolysate 10 g, $(NH_4)_2SO_4$ 2.5 g, urea 0.5 g, $CaCO_3$ 2 g, KH_2PO_4 0.1 g, $MgSO_4.7H_2O$ 0.05 g, $FeSO_4.7H_2O$ 0.001 g, $MnCl_2.4H_2O$ 0.4 mg, biotin 20 µg, thiamine hydrochloride 40 µg, sodium chloride 0.25 g and distilled water to make 100 mL. Initial pH of all three media was adjusted at 7.6. Composition of screening, basal and three fermentation media were followed after certain modifications as described by Shah and Hameed (2004).

In glucose based media, glucose was separately sterilized as 40 % stock solution. Cane molasses and corn steep liquor were centrifuged at 10000 rpm for three hours and supernatant were inactivated as separate entity by keeping in dry air oven at 80˚C overnight. Corn starch hydrolysate was kept at 80˚C in dry air oven for six hours. Calcium carbonate was also dry sterilized by keeping in dry air oven at 180˚C overnight. Biotin, thiamine and amino acids were syringe filtered (0.22 µm). Ten per cent working volume in flasks, 10% (v/v) over night broth culture of seed medium as inoculum and 150 rpm were taken as fixed parameters for optimization of physical parameters in shake flask cultures. All values of results are average of three replica of each flask/experiment.

Quantitative analysis of L-lysine: Lysine was quantified in culture supernatant by ninhydrin-ferric reagent assay as described by Hsieh *et al.* (1995). Concentrations of unknown samples (culture supernatant) were calculated using linear regression analysis equation derived from standard curve, $y = 0.291x - 0.033$ where y = absorbance at 470 nano meter (nm) and x = lysine (g/100 mL).

Determination of total sugars: Total sugars in cane molasses and corn starch hydrolysate were determined by colorimetric method (Anonymous, 2001). The amount of total sugars in samples were determined with reference to linear regression equation obtained from standard curve, y = 0.138x +0.412 where y = absorbance at 490 nm and x = glucose concentration (µg/mL).

RESULTS

After exposure to UV, 99.9 % kill rate was achieved and mutants (AEC^r and hom^-) were selected for maximum lysine production as described earlier. Mutant No.116 produced maximum lysine (8.2 mg/mL) in screening medium at 30˚C after 48 h of incubation in shake flask cultures (150 rpm). None of the other mutants could accumulate lysine more than 5 mg/mL. So mutant No.116 was further selected for optimization of culture conditions.

Effect of initial pH: Different pH values of basal medium were adjusted before sterilization with 2 *N* HCl and 1 *N* sodium hydroxide. At pH 7.6, maximum lysine (12.5 mg/mL) was produced after 60 h of incubation (Fig. 1).

Effect of incubation time: Lysine concentration gradually increased in fermentation medium after 36 h of incubation and reached at its peak (12.5 mg/mL) at 60th h and then leveled off (Fig. 2).

Effect of agitation: Lysine production increased (at optimized temperature and pH) as rpm were increased from 150 to 300 in shaking incubator. Maximum production (15.1 mg/mL) was observed at 300 rpm (Fig. 3).

Effect of temperature: Maximum yield of lysine (12.5 mg/mL) was observed at 30˚C after 60 h of incubation (Fig. 4).

Effect of carbon sources: Effect of various glucose concentrations (8, 9, 10 and 11% w/v) on production of lysine were studied in fermentation medium (FM1) at 30˚C. Maximum (17.5 mg/mL) lysine yield was observed at 9% concentration (Fig. 5). Total sugar contents in sugar cane molasses were 64.3% (w/w). It was used as carbon source in FM2 medium at different concentrations i.e. 10, 15 and 20% w/v (6.43, 9.65 and 12.86% based on total sugars). Maximum (17.3 mg/mL) lysine yield was observed at 15% (w/v) concentration in FM2 Medium followed by 13.6 mg/mL at 10% and 11.2 mg/mL at 20% (w/v) molasses in FM2 medium.

Total sugar contents in corn starch hydrolysate were 57.25 % (w/w). It was used as carbon source in FM3 medium at different concentrations i.e. 10, 15 and 20% w/v (5.73, 8.59 and 11.46% based on total sugars). Maximum (17 mg/mL) lysine yield was observed at 15% (w/v) corn starch hydrolysate concentration followed by 14 mg L-lysine/mL at 10% and 11.9 mg/mL at 20% level.

Effect of nitrogen source: Lysine yield increased as concentration of ammonium sulphate was increased from 2.5 to 4%, and then declined (Fig. 6).

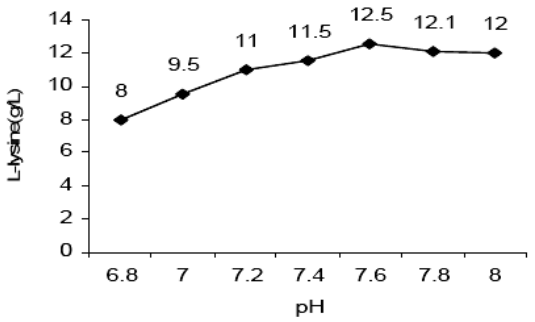

Fig. 1: Effect of pH on production of Lysine by mutant No.116 (Shake Flask, 300 rpm, 30˚C, basal medium)

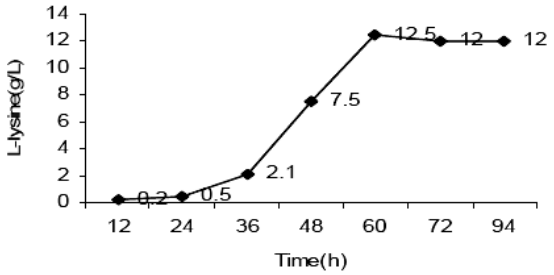

Fig. 2: Time of incubation for the production of Lysine by Mutant No.116 (Shake Flask, 300 rpm, 30˚C, basal medium)

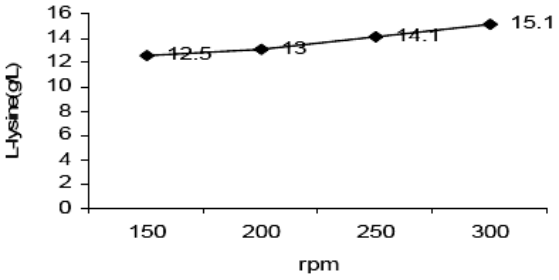

Fig. 3: Effect of agitation on production of Lysine by Mutant No.116 (Shake Flask, 300 rpm, 30˚C, basal medium)

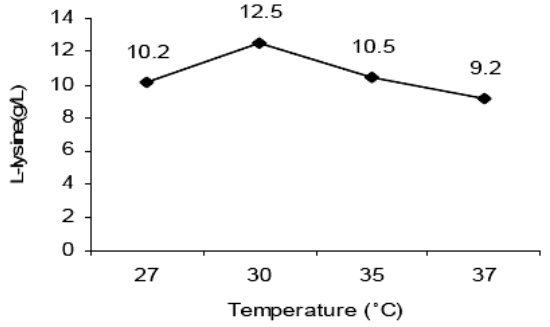

Fig. 4: Effect of incubation temperature on production of Lysine by mutant No.116 (Shake Flask, 300 rpm, 30˚C, basal medium)

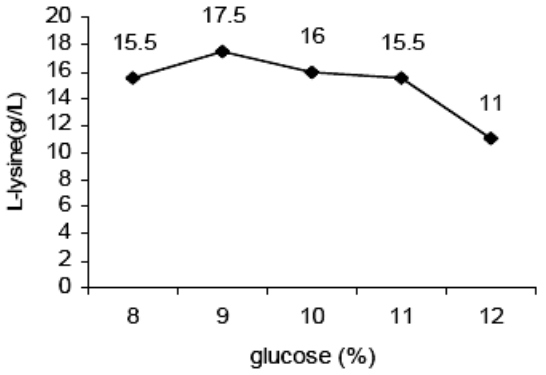

Fig. 5: Effect of glucose concentration on production of Lysine by mutant No.116 (Shake Flask, 300 rpm, 30˚C, Fermentation Medium 1)

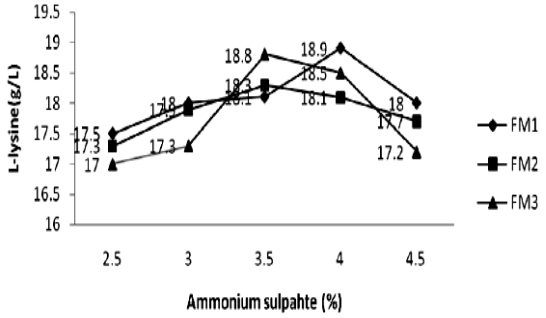

Fig. 6: Effect of amounium sulphate in different media on production of Lysine (shake flask, 300 rpm, 30˚C). FM1: Glucose based fermentation medium; FM2: Cane molasses based fermentation medium; FM3: Corn steep Liquor based fermentation Medium

DISCUSSION

Strains of *C. glutamicum* are the best lysine producer after mutation. For the purpose, physical as well as chemical methods can be applied (Patnaik, 2008). UV rays of 254 nm wavelength are the most potent one. The most important products of UV radiation are dimmers (thymine-thymine, thymine-cytosine) formed between adjacent pyrimidines or between pyrimidines of complimentary strands, which results in cross links (Goosen and Moolenaar, 2008). Important biosynthetic pathways in *C. glutamicum* for production of amino acids and vitamins are derived from L-aspartate or pyruvate. Lysine biosynthesis is well controlled in *C. glutamicum* through enzymes for feedback inhibition, repression and interlocking mechanisms. Intracellular lysine and L-threonine cease the activity of aspartokinase by concerted feedback inhibition. Thus conversion of aspartate to aspartylphosphate is being stopped. Another important enzyme on the way is homoserine dehydrogenase which is inhibited by L-threonine and repressed by L-methionine (Blombach *et al*., 2009).

To get potent lysine producing mutants, 90-99.9% inactivation is an utmost necessity (Kumar *et al*., 2002). For commercial production of L-lysine, the most potent mutants are among regulatory or homoserine auxotrophs (hom^-) or methionine-threonine double auxotrophs. In our experiments, presence of AEC^r mutants was indicative that resistance to feed back inhibition (mutation in *lysC* gene) has been achieved. Similarly presence of hom^- mutants was indicative of mutation in *hom* gene resulting insufficient production of homoserine dehydrogenase.

Since aeration has pronounced effect on lysine production**,** 10% working volume (25 mL in 250 mL flask) and 10% seed inoculums were taken as basic parameters for optimization of pH, temperature, time of incubation and agitation conditions for maximum production of L-lysine (Zimmermann *et al*., 2006). Effect of initial pH of production medium on export system of lysine is an important factor. Export of lysine is governed by *lysE* and regulated by *lysG* genes (Park and Lee, 2010). The optimum pH for lysine production had been reported to be above 7 and up to 8 for *C. glutamicum* (Takeshita *et al*., 2010). A sharp decrease in lysine production was noticed below pH value of 7.2, whereas decline was not as sharp with increase in pH from 7.6 up to 8 (Fig. 1). At pH value of 7.6, best production of lysine (12.5 mg/mL) in basal medium was in agreement with the results of Broer and Kramer (1991) and Hua *et al*. (2000). However, Broer *et al*. (1993) produced different mutants of *C. glutamicum* capable of excreting extra cellular lysine at different pH levels (7-7.8). Membrane potential was found to be an important factor influenced by pH of fermentation medium towards lysine excretion by the cells.

Temperature optimization revealed that the mutant under study was producing the best (12.5 mg /mL) at 30˚C. Most of industrial lysine production is being carried out below 35˚C from different strains of *C. glutamicum* (Ohnishi *et al*., 2003).

Lysine production increased from 12.5 mg/mL to 15.1 mg/mL in shake flask cultures when rpm were increased from 150 to 300. Under aerobic conditions, sufficient oxaloacetate is available for aspartate conversion and then flows towards lysine formation. However under anaerobic conditions, carbon metabolism shifts towards formation of ethanol, lactic acid and succinate (Wendisch *et al.*, 2006).

Maximum production of lysine reached at its peak after 60 h of incubation and then slightly decreased in shake flask cultures (Fig. 2). Most of the industrial production of lysine from *Corynebacterium/ Brevibacterium* species are being completed around 72 h of incubation but range of completion time within 30 to 100 h has been reported (Anastassiadis, 2007).

Laboratory grade glucose (D), sugar cane molasses and corn starch hydrolysate were used as carbon source. Maximum lysine production was achieved around 9% available sugars regardless of the source provided. Optimum requirement of carbon source (either glucose or sucrose) by most of the industrial mutants is around 10 % (Yakota and Shiio, 1988). However, Sassi *et al.* (1996) reported 18% glucose requirement for the yield of 60 g lysine per liter of fermentation broth. Cane molasses and starch hydrolysate are used in industrial amino acid production as cheep carbon source and *C. glutamicum* is capable of co-utilizing these carbon back bones for making amino acid molecules (Teramoto *et al*., 2011). Production results of our experiments by using glucose, molasses or corn starch hydrolysate as carbon source for the production of lysine are consistent with genomic structure and function of *C. glutamicum* (Blombach and Seibold, 2010).

Optimum levels of lysine were obtained when ammonium sulphate was used around 3.5 and 4% level in all three fermentation media (Fig. 6). Since lysine molecule contains two amide radicals, supply of nitrogen molecule is needed for its biosynthesis. Ammonium sulphate concentration from 2.5 to 4.5% level was tested in all three fermentation media. Optimum level in FM 3 was at 3.5% and for FM 1 and FM 2 it was around 4%. For growth of *C. glutamicum* inorganic ammonium is preferred source of nitrogen but it may be utilized from organic sources, too (Rehm and Burkovski, 2011). A bit lower requirement of ammonium sulphate in FM 3 may be due to fulfillment of the need of amino group form amino acids present in corn steep liquor.

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