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Optimization of Culture Conditions for L-Lysine Fermentation by *Corynebacterium glutamicum*

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Abstract: The objective of this study was to improve the L-lysine production by *Corynebacterium glutamicum*. For this purpose culture conditions for the lysine fermentation by the bacteria were optimized. In this regard the role of various physical and nutritional parameters was examined. The culture was incubated in a 500 ml Erlenmeyer flask in a rotatory shaking incubator at 200 rpm. The appropriate conditions were obtained only when 50 ml medium was charged for fermentation at 30 °C, pH 7.5 and at 10% inoculum size. The finally selected medium per 100 ml distilled water formulated was 10 g glucose, 2.5 g ammonium sulfate, 2.0 g calcium carbonate, 0.5 g bactocasamino acid, 20 μg thiamine hydrochlorid, 5 μg D-biotin, 0.1 g potassium dihydrogen phosphate, 0.05 g magnesium sulfate heptahydrate, 0.2 mg ferrous sulfate heptahydrate and 0.2 mg manganese chloride tetrahydrate. The optimized culture conditions resulted into elevated amount of L-lysine.

Key words: *Corynebacterium glutamicum*, L-lysine fermentation, culture conditions, optimization

Introduction

Major improvements in the productivity of fermentation processes are generally ascribed to be the development of superior strains via mutation (Zaki *et al.*, 1982). In previous investigation a new autotrophic mutant of *Corynebacterium glutamicum* had been developed (Shah *et al.*, 2002). However, other parameters such as nutritional and physical environments to which an organism is exposed are also known to significantly alter product yield (Wang *et al.*, 1991; Coello *et al.*, 1992). Each bacterium has definite range of culture conditions for high production of lysine. Therefore it is essential to investigate the influence of culture condition on yield. Liu (1986) studied optimization conditions of different parameters for lysine production with cane molasses by *Berevi* bacterium species and achieved high yield after optimization. Roy and Chattarjee (1989) also studied the influence of culture conditions on glutamic acid production by *Globi formis*. To maximize the product, the effect of different physical and chemical factors were examined one by one using the basal medium. Strain MRLH-GHA10 was used throughout the optimization process.

Materials and Methods

Media composition: This research work was performed at the Department of Biological Sciences, Quaid-e-Azam University, Islamabad and at the Department of Chemistry, Gomal University, Dera Ismail Khan, during 1997-2001.

Complete medium: Beef extract (1g), bactopeptone (1g), glucose (2g) NaCl (0.25g) and agar (2g) per 100 ml distilled water.

Basal medium: The basal medium used for optimization of the culture conditions had the following composition per 100 ml distilled water. Glucose (10 g), ammonium sulfate (2 g), calcium carbonate (1g), bactocasamino acid (0.2 g), potassium dihydrogen phosphate (0.2 g) magnesium sulfate heptahydrate (0.02 mg), manganese chloride tetrahydrate (0.3 mg), thiamine hydrochlorid (10 μm) and d-biotin (10 μg).

Seed medium: Seed medium had the composition of glucose (5 g), bactocasamino acid (0.2 g), ammonium sulfate (1 g), potassium dihydrogen phosphate (0.1 g), magnesium sulfate heptahydrate (0.04 mg), ferrous sulfate heptahydrate (0.2 mg), manganese chloride (0.2 mg), d-biotin (5 μg) and thiamin (10 μg) per 100 ml distilled water.

Culture method: Glucose solution suspended with $CaCO_3$ was sterilized separately and mixed with other nutrients just before inoculation. Seed medium (50 ml) was inoculated in 250 ml Erlenmeyer flask in duplicate by loop having cells of mutant GHA10 from 24 hrs. old complete agar plates and incubated in a rotatory shaking incubator at 30 °C for 20 hrs.

Basal medium (50 ml) was inoculated in 500 ml Erlenmeyer flask with 5 ml with seed medium in duplicate and incubated in a rotatory shaking incubators at 30 °C for six days. Samples were taken on 6th day and centrifuged. The supernatants were examined for lysine. For the analysis of lysine, residual sugar and dry cell weight (DCW), the same procedures, were followed as discussed by Shah *et al.* (2002).

Results and Discussion

Physical parameters

Effect of aeration: Oxygen, an important nutrient, is usually supplied to flask through vigorous shaking on a rotatory or reciprocal shaker. Under conditions of insufficient oxygen large amount of lactic and succinic acids were accumulated, while excess of oxygen increased the amount of α-keto glutaric acid. It was found that both over abundance and meager aeration were undesirable. The former being inhibitory to cell growth and the latter to L-lysine production (Hallaert *et al.*, 1987; Inbar *et al.*, 1985; Liu, 1986). Different volumes of basal medium (25, 50, 75, 100 and 150 ml) were poured into 500 ml flask and inoculated with cell suspension from seed broth (10% inoculum). Growth and transformation were carried out at 28°C on rotatory shaking incubator. Residual sugar and L-lysine production were measured. Maximum L-lysine production (15.05 g/L) and sugar utilization (6.7%) were observed in the flask containing 50 ml broth volume. As the liquid volume increased, L-lysine production and sugar utilization decreased, as observed with 150 ml culture volume only 8.55 g/L L-lysine produced and 4.85% sugar utilized (Fig. 1). Inbar *et al.* (1985) reported the appearance of lactate and succinate at low aeration that was attributed to the increased glycolysis. Hadj-Sassi *et al.* (1996) found that oxygen limitation caused a decrease in substrate consumption rate and conversion efficiency of substrate into lysine.

Effect of agitation: Inoculum vessel containing liquid medium was agitated to provide homogeneity. The effect of agitation on L-lysine fermentation in rotatory shaking incubator and the stirred tank were examined . In shake flask, as the agitation increased form 50 to 200 rpm, L-lysine production increased rapidly and then leveled off. Maximum yield was observed at 200 rpm, although the sugar utilization increased from 50 to 300 rpm (Fig. 2). Wang *et al.* (1991) worked at 200 rpm for fermentation of L-

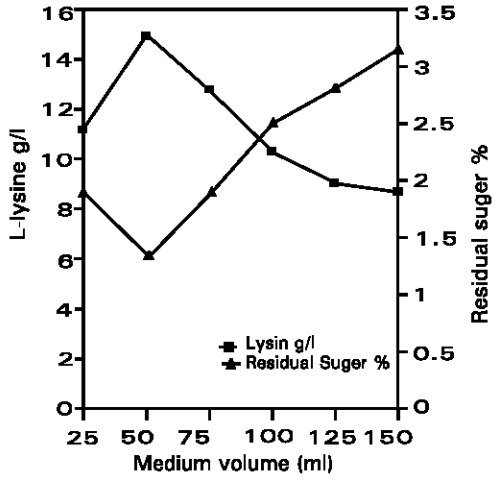

Fig.1: Effect of aeration on L-lysine production

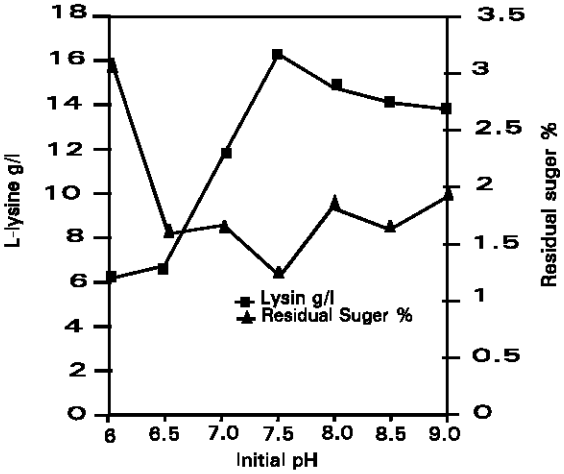

Fig.4: Effect of initial pH on L-lysine production

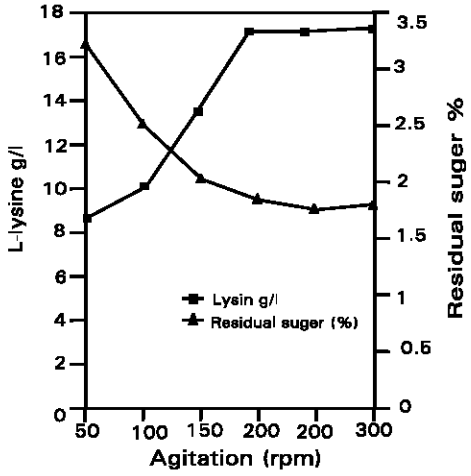

Fig.2: Effect of agitation on L-lysine production

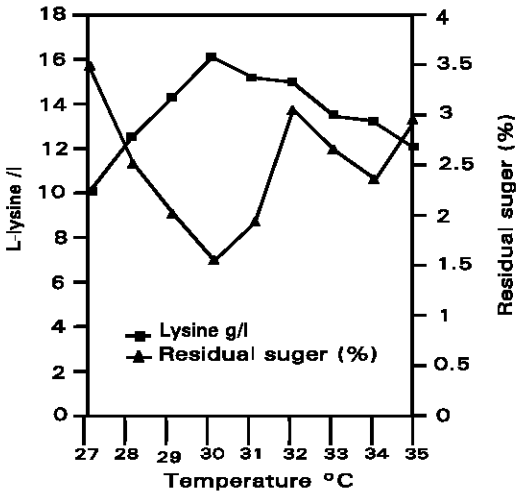

Fig.5: Effect of temperature on L-lysine production

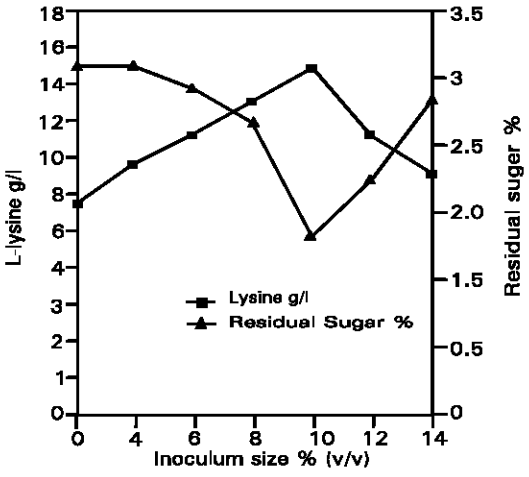

Fig.3: Effect of inoculum size on L-lysine production

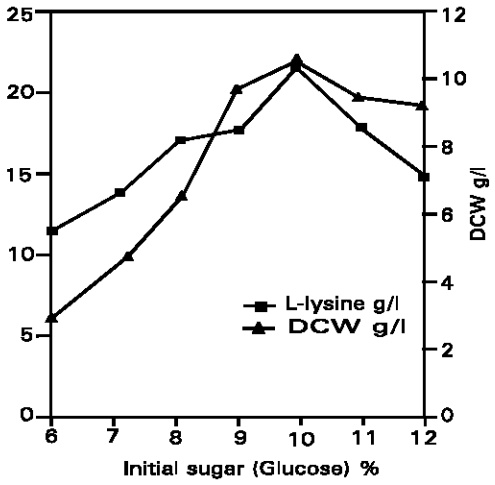

Fig.6: Effect of initial sager (Glucose) on L-lysine production

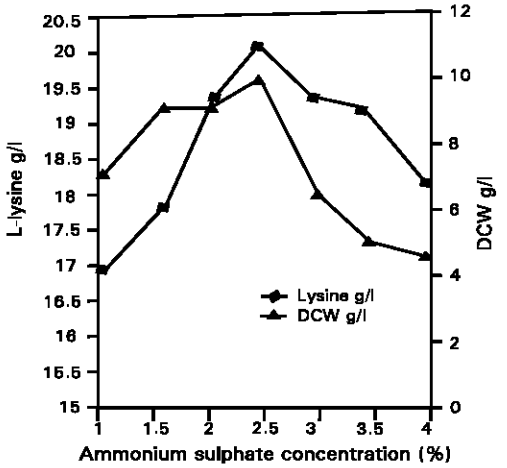

Fig.7: Effect of ammonium sulphate concentration on L-lysine production

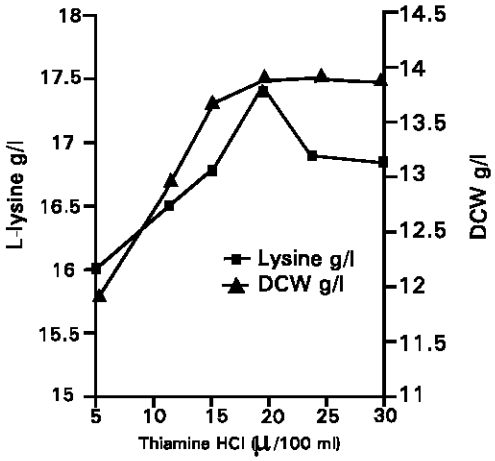

Fig.10: Effect of thiamine HCl on L-lysine production

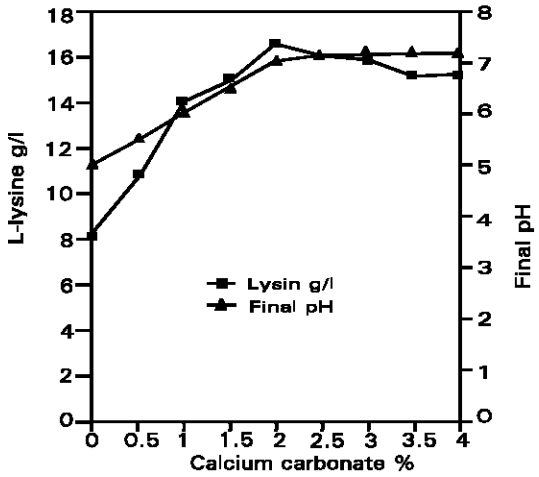

Fig.8 : Effect of calcium carbonate on L-lysine production

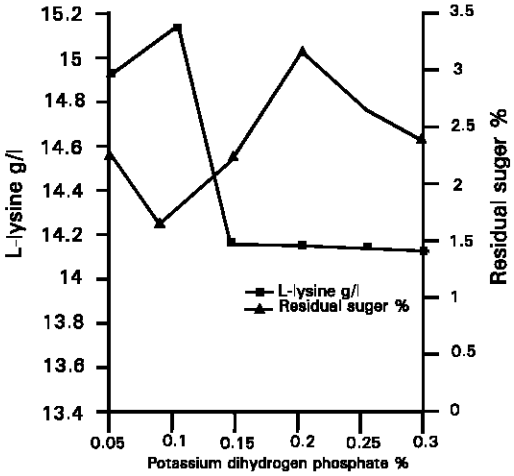

Fig.11: Effect of potassium dihydrogen phosphate on L-lysine production

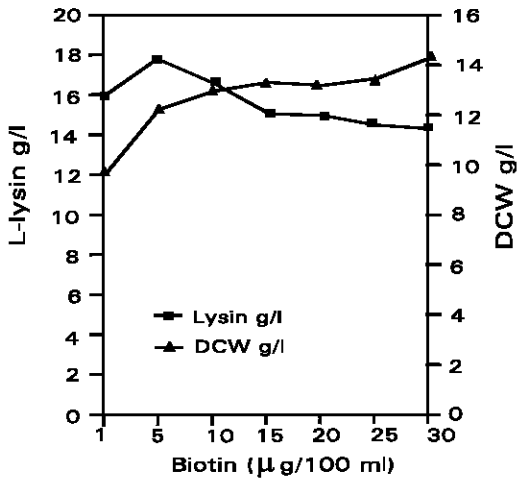

Fig. 9 : Effect of biotin on L-lysine production

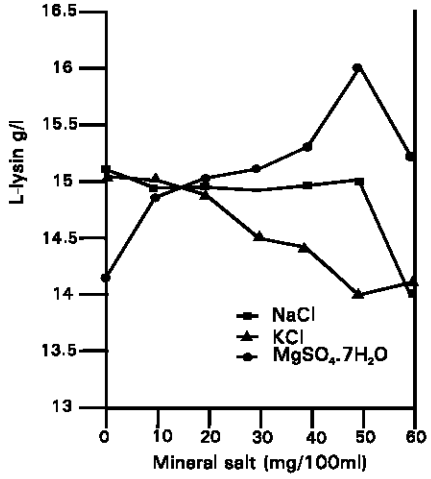

Fig.12: Effect of mineral salt (NaCl, KCl, $MgSO_4$, $7H_2O$) on L-lysine production

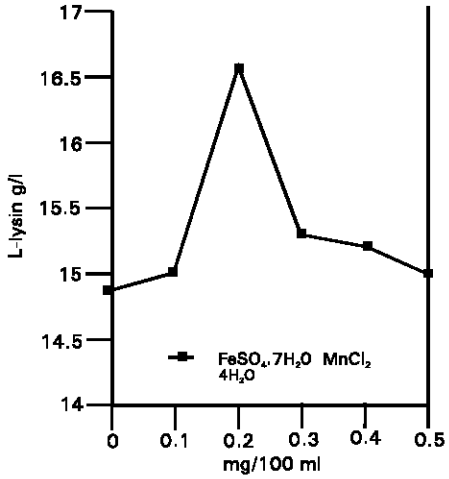

Fig.13 : Effect of ferrous sulphate and manganese chloride in glucose on L-lysine production

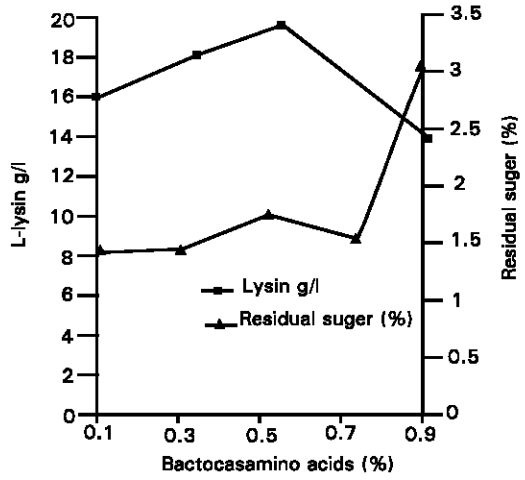

Fig.14 : Effect of bactocasaino acids on L-lysine production

lysine on rotatory shaker.

Effect of inoculum size: It is generally necessary to optimize inoculum density. Too low a density may give insufficient biomass and too high density may produce too much biomass and deplete the substrate of nutrients necessary for L-lysine fermentation. Inoculum size from 2-14% was studied on shake flask. The best fermentation of L-lysine was obtained at 10% inoculum size. The residual sugar percentage decreased to 1.5% and maximum L-lysine produced was 15.3 g/L. Above 10% inoculum sugar utilization decreased and L-lysine produced ceased (Fig. 3) because high density may produce too much biomass and deplete the substrate of nutrients necessary for product formation. Nakayama *et al.* (1961) utilized 10% inoculum size for various auxotrophic mutants. It may not be fair to say that 10% inoculum size is required in all cases of fermentation, since it depends upon the cell mass and the composition of the seed medium to be transferred. Hallaert *et al.* (1987) reported that inoculum size has a marked effect. Small inoculum size caused an increase in growth period, because too low a density may give insufficient biomass.

Effect of initial pH: Growth is usually very sensitive to variation in pH. Furthermore, the process of growth changes the pH of the medium therefore, the effect of different pH on L-lysine production was examined in shake flask. Basal media with different pH (6-9) were adjusted with 2M HCl and 1 M NaOH. After inoculation with cell suspension, the fermentation was carried out as usual. At pH 7.5 maximum L-lysine (16.5 g/L) was produced and maximum sugar was utilized. Above and below 7.5 the sugar utilization and L-lysine production slightly decreased (Fig. 4). Broer *et al.* (1993) found that the optimum pH for maximum velocity of transport by *Corynebacterium glutamicum* was 7.4-7.8. Kelle *et al.* (1996) studied the L-lysine transport by and *Corynebacterium glutamicum* found that pH value governed the transport activity and maximum L-lysine export rate was revealed at pH 7. Liu (1986) found that L-lysine synthesis decreased at pH lower than 6.5 but found no difference between 6.5 and 8.0. An initial pH of 7.5 was recommended for L-lysine production by *Corynebacterium glutamicum* (Broer and Kramer, 1991).

Effect of temperature: The growth rate of microorganism is a function of temperature through the reaction sequence, which make up the whole of metabolism. It is important to realize that shifts in temperature can alter the utilization rate of one component as compared to another, thus unbalancing the medium with respect to growth. The early depletion of a critical nutrient can shift the culture from balanced to unbalanced growth and changes its performance. Clearly, for reproducible growth rates temperature must be rigorously controlled. The effect of different temperature (27°C - 35°C) on L-lysine fermentation were carried out in shake flask. At 30°C maximum yield of L-lysine was observed. Above and below 30°C low sugar consumption and L-lysine accumulation was recorded (Fig. 5). Hilliger *et al.* (1984) observed the influence of temperature was one of the major fermentation parameters on growth and L-lysine formation of *Corynebacterium glutamicum* and suggested that 29°C is optimum temperature for both biomass and L-lysine yield. Over 29°C biomass and L-lysine excretion decreased, whereby temperature exceeding 32°C markedly reduced the specific L-lysine formation rate and the substrate conversion yield coefficient.

Nutritional parameters

Effect of carbon source: Glutamic acid producing bacteria can utilize various carbon sources, such as glucose, fructose, sucrose, maltose, ribose or xylose. Glucose concentration was investigated n a study (Hirose *et al.*, 1985) and it was found that higher concentration of glucose inhibited bacterial growth along with low yield. For this purpose the effects of different concentrations of glucose on L-lysine production were examined. On shake flask glucose from 6 to 12% was added in the culture medium in different flask. It was observed that 10 % glucose concentration gave the best L-lysine production. Above 10% glucose there was a decrease in DCW because increased concentration of the glucose inhibited the growth of *Corynebacterium glutamicum*. L-lysine produced at 10% glucose was 21 g/L (Fig. 6). Ferreria and Durate (1991) also used 10 % glucose for maximum yield of L-lysine by *Corynebacterium glutamicum* fluoropyruvate sensitive mutant and reported similar results. Hadj-Sassi *et al.* (1988) reported that initial concentration of glucose influenced the production of L-lysine by *Corynebacterium* sp. in batch culture and found that specific production rate was obtained at 65 g/L of glucose.

Effect of nitrogen source: The ample supply of a suitable nitrogen source is essential for L-lysine fermentation, since this molecule contains 19.16% nitrogen. Ammonium salts, such as ammonium chloride, or ammonium sulfate are assimilable. The ammonium ion is detrimental to both cell growth and product formation and its concentration in the medium must be maintained at a low level (Hirose *et al.*, 1985). In this study the effect of different concentrations of ammonium sulfate were studied. Maximum L-lysine (20 g/L) was accumulated at 2.5% ammonium sulfate concentration, while above and below 9.5% the cell mass, as well as the yield decreased. The DCW increased up to 2.5% ammonium

sulfate and then decreased, because more than 2.5% inhibited the growth (Fig. 7). Ammonium sulfate was used as the nitrogen source for L-lysine production by number of workers (Zaki *et al.*, 1982; Ferreira and Durate, 1991; Hsiao and Glatz, 1996). Wang *et al.* (1991) suggested more than 2% ammonium sulfate for high yield of L-lysine and high concentration inhibited cell growth.

Effect of calcium carbonate: In fermentation process, the pH of the broth decreased due to accumulation of pyruvic acid, lactic acid, gluconic acid etc. As a result, the bacterial growth ceased with concomitant decrease in the yield. Calcium carbonate neutralized the pH of the broth, acting as internal neutralizing agent, it would be useful for shortening the fermentation time. The effect of 1 to 4% calcium carbonate was evaluated. At 2% maximum yield of L-lysine (16.52 g/L) was obtained. At the same time fermentation without calcium carbonate was also studied. At this condition the pH decreased dramatically and the L-lysine yield was about one half (Fig. 8). Thus, as mentioned before, pH is one of the most important factors effecting microbial propagation. As nutrients are consumed and converted into product during the fermentation process, the pH changes markedly in the absence of suitable control mechanism. In order to maintain optimal pH, reagents like calcium carbonate must be added to the culture medium at the beginning of the fermentation (Kinoshita, 1972). Thus, calcium carbonate was used as internal neutralizing agent. Wang *et al.* (1991) reported that though the pH of the fermenter automatically controlled by ammonia water, but still small amount of $CaCO_3$ must be added. It eliminates the lag phase of cell growth, thereby shortening fermentation time.

Effect of thiamine HCl and biotin: Glutamic acid producing bacteria require biotin for growth and its concentration must be strictly controlled for the maximum yield of the L-lysine. Young and Chipley (1984) investigated the role of biotin in L-lysine production in *Brevibacterium lactofermentum* and found that biotin treated cell took up more glucose, than did the control one. Biotin, apparently, caused some compositional changes in the cell wall membrane complex, allowing an increase in uptake of glucose. The result of uptake studies and fatty acid analysis suggested that biotin effected the cell surface, probably the bacterial membrane. It is well known that bacterial membrane plays an important role as a charged barrier. This mechanism might also regulate the amount of L-lysine released by the cells. Tosaka *et al.* (1979 a, b), suggested that the effect might be due to the activation of pyruvate carboxylase by biotin. In these investigations the effect of 1-30 μg per 100 ml biotin on L-lysine production examined. The best production obtained at 5 μg per 100 ml. The cell mass increased up to 15 μg per 100 ml of biotin and then became constant (Fig. 9). Thiamine is also the desired nutrient for the said bacterium, as it increased bacterial growth and hence of L-lysine production. Cell mass increased up 20 μg per 100 ml of thiamine HCl and then leveled off. Maximum yield (17.5 g/L L-lysine) was observed at 20 μg per 100 ml thiamine HCl (Fig. 10).

Effect of potassium dihydrogen phosphate and other mineral salts: Hydrogen, oxygen, sulfur and phosphorus are the essential elements for all organisms. Hydrogen and oxygen form part of many organic compounds. Sulfur is needed for the biosynthesis of amino acids such as cystine, methionine etc. Phosphorus is essential for the synthesis of nucleic acids and adenosine triphosphate (ATP), a compound that is extremely important for energy storage and transfer. Many other essential elements are required, though in smaller amounts than the element already stated. They may facilitate the transport of materials across cell membrane. For example, Fe^{+2} is required for such enzymes as cytochromes, catalase and succinic acid dehydrogenase. Moreover, essential elements are often required as cofactors for enzymes. Other mineral elements are also needed, but usually in extremely small amount, example of the trace element include manganese (Mn^{+2}), magnesium (Mg^{+2}) etc. They are required to activate enzymes (Pelczar *et al.*, 1993). Fe and Mn are the most important of the trace elements as they play a role in the excretion of primary metabolites (Dunn, 1985). Keeping in view the importance of these elements, the effect of different concentrations of potassium dihydrogen phosphate (KH_2PO_4) on the yield of L-lysine was examined. Potassium dihydrogen phosphate at 0.1 g per 100 ml produced maximum L-lysine with high sugar utilization. Above 0.1 g the yield was decreased (Fig. 11).

Different amounts of sodium chloride, potassium chloride, magnesium sulfate, manganese chloride and ferrous sulfate were added to the basal medium separately and cultured as usual. Sodium chloride and potassium chloride has no profound effect on fermentation, but a small decrease in yield was observed if potassium chloride (KCl) was added in more than 10 mg / 100 ml. The effect of magnesium sulfate (0-60 mg per 100 ml) on L-lysine production was investigated. The maximum yield was obtained at 50 mg per 100 ml. A slight decrease in yield was observed above 50 mg per 100 ml of magnesium sulfate (Fig. 12). Sen and Chattarjee (1985) reported that salt of micro nutrient stimulated the growth and enhanced the L-lysine yield. The effect of ferrous sulfate and manganese chloride (0-0.5 mg per 100 ml) on L-lysine production was examined (Fig. 13). The best yield (16.5 g/L L-lysine) was found at 0.2 mg per 100 ml each of $FeSO_4$ and $MnCl_2$.

Effect of bactocasamino acids: Bactocasamino acids contain all amino acids required both for auxotrophs and prototrophs for L-lysine production. Sen and Chatterjee (1985) found that bactocasamino acids significantly improve the yield of L-lysine in synthetic medium. So instead of using different amino acids for different auxotrophs, bactocasamino acids was recommended for all mutants for maximum production of L-lysine. Bactocasamino acids (0.1-0.9 g per 100 ml) was examined for L-lysine production. At 0.5 g per 100 ml of bactocasamino acids, the maximum yield was obtained. As the concentration of bactocasamino acids increased, L-lysine accumulation increased up to 0.5 g per 100 ml and then decreased. However, the residual sugar was constant up to 0.7 g per 100 ml and then slight increase was observed (Fig. 14). Thus, 0.5 g per 100 ml bactocasamino acids was used throughout. It was concluded that using the basal medium, different nutritional and physical factors might significantly alter the overall production of L-lysine by the fermentation process.

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