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OPTIMIZATION OF FERMENTATION MEDIUM FOR L-LYSINE PRODUCTION BY *CORYNEBACTERIUM GLUTAMICUM*

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Abstract

Corynebacterium glutamicum is an industrially important microorganism used for amino acids production. The present work describes the production of L-lysine from *Corynebacterium glutamicum* IIB-C9 through submerged fermentation process. Fifteen fermentation media were screened in this study for L-lysine production. Out of these fifteen media, FM:13 was found a best one for maximum production of L-lysine i,e., 2.5g/L. The medium contained glucose, ammonium sulphate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, calcium carbonate, thiamine HCl, biotin, manganese chloride tetrahydrate and casamino acids. This medium was further supplemented with different nitrogen and carbon sources to increase the L-lysine production. Glucose, as best source of carbon and ammonium sulphate as a best nitrogen source were optimized. Finally 3.5 g/L of L-lysine was produced after 72 hrs incubation in FM:13 medium supplemented with 7% glucose and 2.5% ammonium sulphate

Keywords: Submerged Fermentation, Culture medium, Amino acids, Optimization, Production.

Introduction

In nine essential amino acids, Lysine is one of them which is used in nutrition of human and animal. It is the second highly produced amino acid at industrial scale with many hundred thousand tons of production (about 80,00,00 tones) per annum (Anastassiadis, 2007; Shah *et al*., 2002b). L-lysine generally identified as most deficient component in the food of both human and animals. As animal feed contains a small amount of L-lysine, similarly this amino acid is not synthesized by cattle, poultry and other livestock, so L-lysine will be added to diet of animal to meet feed requirements (Tosaka, 1983).

There are different ways of L-lysine production such as chemical synthesis, enzymatic method, extraction from protein hydrolyzate, fermentation technique, recombinant DNA technology and protoplast fusion (Anastassiadis, 2007; Nelofer *et al*., 2008). In these, fermentation method is practical and most economical method for production of Lysine. (Ekwealor & Obeta, 2005). *Corynebacterium glutamicum* is widely used for L-glutamate and lysine production at industrial scale (Ikeda, 2003). *C. glutamicum* has ability to use many kinds of carbohydrates, organic acids and alcohol as a carbon source and energy for rapid growth of microbes and also for production of many amino acids (Kircher & Pfeerle, 2001; Seibold *et al*., 2006). In the year 1996, out of world production of 80,000 metric tons of L-lysine approximately 90,000 tons was produced by fermentation (Hasio & Glatz, 1996).

Fermentative processes opened up a vast arena for the production of industrially important metabolites and L-lysine is one of them. L-lysine was discovered to be produced through fermentation by *Corynebacterium glutamicum* at Kyowa Hakko's plant in 1958 in Japan (Kelle *et al*., 2005). This method is preferred over all other methods because it employs low temperature, low pressure, low cost carbon sources and renders biological form of L-lysine as the final product (Nasab *et al*., 2007). Microorganisms that have been reported to produce L-lysine include *Corynebacterium glutamicum* (Nelofer *et al*., 2008), *Bacillus megaterium* (Ekwealor & Obeta, 2005), *Brevibacterium linens*, *Streptomyces Albulus IFO* (Shih *et al*., 2006), *Brevibacterium flavum*, *M. methylophilis* (Ishikawa *et al*., 2008), *B. lactofermentum* (Tosaka *et al*., 1979), *B. subtilis* and *Bacillus laterosporus* (Umerie *et al*., 2000). Among these, *C. glutamicum* has been most widely exploited industrially for L-lysine production (Pfefferle *et al*., 2003).

Productivity of fermentation processes are generally improved due to development of superior strains by mutation (Zaki *et al*., 1982). In investigations a new auxotrophic mutant of *Corynebacterium glutamicum* had been developed (Shah *et al*., 2002a). Moreover other nutritional and physical parameters also affect the growth and product yield of organism (Wang *et al*., 1991; Coello *et al*., 2002). Since each bacterium has definite range of culture conditions for better growth and for high production of L-lysine, therefore it is essential to investigate the effects of cultural conditions on bacterial growth and product yield.

The main objective of the work was to optimize the fermentation media for the production of L- lysine by *C. glutamicum* through submerged fermentation in the Erlenmeyer flasks to economize the production of said amino acid at industrial scale.

Materials and Methods

Microorganism: A bacterial strain *Corynebacterium glutamicum* was obtained from stock culture of Institute of Industrial Biotechnology, GC University Lahore, Pakistan. The strain was refreshed and maintained on nutrient agar slants.

Fermentation experiments: Vegetative inoculum was used during fermentation which was prepared by inoculating a loopful of bacteria from the slant into 250mL Erlenmeyer flasks containing nutrient broth. The Erlenmeyer flasks were incubated in a rotary shaker at 30ºC for 24hrs.

Submerged fermentation was performed in a 250ml of the Erlenmeyer flask with 25ml of fermentation medium. The flasks were inoculated with bacterial strain and placed in shaking incubator at 30ºC for specified time period. The broth taken from the fermentation medium was centrifuged and analyzed for the presence of L-lysine.

Culture Media

FM: 1. Glucose 10 g/L, peptone 10 g/L, meat extract 5 g/L, yeast extract 2 g/L, sodium chloride 2.5 g/L (Modified Baullin medium).

FM:2. Clarified cane molasses 20g/L, ammonium sulphate 40g/L, calcium carbonate 20 g/l, sodium chloride 2 g/L, magnesium sulphate 0.4 g/L, potassium dihydrogen phosphate 0.5g/L, di-potasium hydrogen phosphate 1.0g/L (Nelofer *et al*., 2007).

FM: 3. Glucose 50 g/L, urea 5.0 g/L, ammonium sulphate 5.0 g/L, magnesium sulphate heptahydrate 0.25 g/L, potassium dihydrogen phosphate 0.5 g/L, di potassium hydrogen phosphate 0.5 g/L, ferrous sulphate heptahydrate 0.01 g/L, manganese sulphate monohydrate 0.01 g/L, Calcium chloride 0.01 g/L, zinc sulphate heptahydrate 1 mg/L, copper sulphate 0.2 mg/L, nickle chloride 0.02 mg/L, Biotin 200 µg/L (Broer *et al*., 1993).

FM: 4. Glucose 70 g/L, peptone 10g/L, sodium chloride 3.0 g/L, potassium dihydrogen phosphate 0.5 g/L, yeast extract 5g/L, dipotassium hydrogen phosphate 1.5 g/L, manganese sulphate 0.5 g/L, ammonium sulphate 5 g/L, biotin 25 µg/L (Slightly modified Anastassidis, 2007).

FM: 5. Glucose 80 g/L, dipotassium hydrogen phosphate 1.2g/L, potassium dihydrogen phosphate 1.2g/L, manganese sulphate dihydrate 12 mg/L, ammonium sulphate 60 g/L, biotin 2 mg/L, magnesium sulphate heptahydrate 0.5 g/L, ferrous sulphate 2 mg/L, calcium pentothenate 2 mg/L, cornsteep liquor 40 g/L (Dry form) (Gulbler *et al*., 1994).

FM:6. Glucose 5 g/L, tryptone 5 g/L, yeast extract 3 g/L, sodium chloride 5 g/L (Modified LB medium).

FM: 7. Glucose30g/L, urea 3 g/L, ammonium sulphate 20g/L, magnesium sulphate 0.5 g/L, potassium dihydrogen phosphate 1 g/L, manganese sulphate monohydrate 4.2 mg/L, Biotin 100 µg/L, thiamine 100 µg/L, soya hydrolysate 20 g/L (Oh *et al*., 1993).

FM: 8. Sucrose 50 g/L, calcium carbonate 30g/L, ammonium sulphate 30 g/L, Urea 2 g/L, magnesium sulphate heptahydrate 0.5 g/L, potassium dihydrogen phosphate 0.05 g/L, di-potassium hydrogen phosphate 0.05 g/L, ferrous sulphate heptahydrate 0.01 g/L, manganese sulphate tetrahydrate 10 mg/L, sodium chloride 1.0 g/L, zinc sulphate 10 mg/L (Bathe *et al*., 2004).

FM: 9. Glucose 30 g/L, malt extract 3 g/L, peptone 3.0 g/L, yeast extract 2.5 g/L, di-potassium hydrogen phosphate 2.5 g/L, magnesium sulphate heptahydrate 0.2 g/L (Slightly modified, Rattray and Fox, 1997).

FM: 10. Glucose 60 g/L, urea 6 g/L, magnesium sulphate heptahydrate 0.4 g/L, potassium di-hydrogen phosphate 1.0 g/L, FeSO4.7H2O 0.01 g/L, biotin 1 µg/L, thiamine HCl 80 µg/L (Calik *et al*., 2001).

FM: 11. Glucose 30 g/L, peptone 5 g/l, yeast extract 2.5g/L, ammonium sulphate 30 g/L, biotin 2 mg/L, calcium chloride 0.5 g/L, potassium dihydrogen phosphate 0.5g/L, dipotassium hydrogen phosphate 0.5 g/L (Locally designed).

FM: 12. Glucose 100 g/L, ammonium sulphate 25 g/L, potassium dihydrogen phosphate 1.0 g/L, magnesium sulphate seven hydrate 0.5 g/L, calcium carbonate 20 g/L, casamino acid 5 g/L, ferrous sulphate seven hydrate 0.5 g/L, manganese chloride tetrahydrate 0.02 g/L, biotin 50 ug/L, thiamine hydrochloride 120 ug/L, pH 7.2 (Shah *et al*., 2012).

FM: 13. Glucose 50 g/L, ammonium sulphate 20g/L, dipotassium hydrogen phosphate 0.1 g/L, potassium dihydrogen phosphate0.1 g/L, magnesium sulphate seven hydrate 5 mg/L, calcium carbonate 15 g/L, casamino acid 2.0 g/L, ferrous sulphate seven hydrate 1 mg/L, manganese chloride tetrahydrate 1 mg/L, biotin 50 ug/L, thiamine hydrochloride 120 ug/L (modified, Shah *et al*., 2012).

FM: 14. Glucose 100 g/L, ammonium sulphate 30 g/L, thiamine hydrochloride 300 ug/L, magnesium sulphate seven hydrate 0.4 g/L, potassium dihydrogen phosphate 0.5 g/L, biotin 400 ug/L, PH 7.2 (Coello *et al*., 2002).

FM: 15. Corn steep liquor 100 g/L, ammonium sulphate 25 g/L, calcium carbonate 20 g/L, potassium dihydrogen phosphate 1.0 g/L, magnesium sulphate seven hydrate 0.5 g/L, ferrous sulphate seven hydrate 0.01 g/L, manganese chloride tetrahydrate 4 mg/L, biotin 200 ug/L, thiamine hydrochloride 400 ug/L, sodium chloride 2.5 g/L, pH 7.2 (modified Rehman *et al*., 2012).

Analysis of L-lysine: The quantitative analysis of L-lysine was carried out by Ninhydrin ferric reagent method (Hsieh *et al*., 1995). In this method 20µl of sample was mixed in 660µl of reagent A (Methylecellosolve 373ml, 50% ferric chloride solution 30ml and 0.1M KCl solution 600 ml) and 370µl of reagent B (1% Ninhydrin in 0.1M KCl solution). This solution was then subjected to heating up to 100˚C for 20 min in a water bath. Then its temperature was lowered to room temperature and 100µl of DMSO was poured to this mixture to dissolve the coloured product. Then 3ml of deionized or distilled water was added and absorbance was taken at 470nm with a photospectrometer. The results were obtained after a comparison to a standard L-lysine solution.

Glucose estimation: Residual glucose was determined by using DNS method (Miller, 1959). Fermentation broth was centrifuged and then used for the estimation of residual glucose.

Down streaming: Fermented broth taken from medium was subjected to centrifugation at 6000rpm for 20 min in a glass centrifuge tube. Then pellet was dried at 80˚C in a hot air oven and the dried pellet was weighed.

Results

Fifteen different fermentation media (FM:1 to FM:15) were screened on the basis of L-lysine production in 250 ml shaking flasks at 30˚C for 72hrs and 200 rpm. Out of these media, fermentation medium FM:13 showed maximum yield i,e., 2.5g/L of L-lysine in 250mL of Erlenmeyer shaking flasks (Fig. 1). Effect of different carbon sources (glucose, sucrose, fructose, molasses, lactose and starch) on the production of L-lysine by C. *glutamicum* was studied. These carbon sources were used in fermentation medium at the concentration of 5% each. Maximum L-lysine (2.6g/L) was produced when the medium FM: 13 was supplemented with glucose as a carbon source. In other carbon sources L-lysine was produced (sucrose 1.9g/L , fructose 1.6g/L , molasses 1.4g/L, lactose 1.7g/L, starch 1.5g/L) in less quantity as compared to glucose as a carbon source (Fig. 2). To optimize glucose in fermentation medium, its different concentrations (4% to 9%) were used in FM:13. Maximum L-lysine (3.3g/L) was produced in FM:13, when it was supplemented with 7% glucose (Fig. 3).

Six nitrogen sources (potassium nitrate, ammonium sulphate, peptone, yeast extract, urea and corn steep liquor) were used in optimized fermentation medium for studying their effects on production of L-lysine by *C. glutamicum*. These all nitrogen sources were used in concentration of 2% each in FM:13. Maximum lysine production (2.6g/L) was observed in the presence of ammonium sulphate in the fermentation medium. But other nitrogen sources showed less L-lysine yield (peptone 2.2g/L, urea 1.4 g/L, yeast extract 2.0 g/L, potassium nitrate 1.2 g/L and corn steep liquor 2.1g/L) as compared to ammonium sulphate (Fig. 4). Different concentrations of ammonium sulphate were optimized for L-lysine production in FM:13. Maximum 3.5g/L L-lysine was produced by FM:13 medium in the presence of 2.5% ammonium sulphate (Fig. 5). Cell biomass and residual sugar were also calculated in the fermentation medium. It was concluded that cell biomass increases with the increase in product yield and residual sugar gradual decreases with increase of product yield (Figs. 2 and 4).

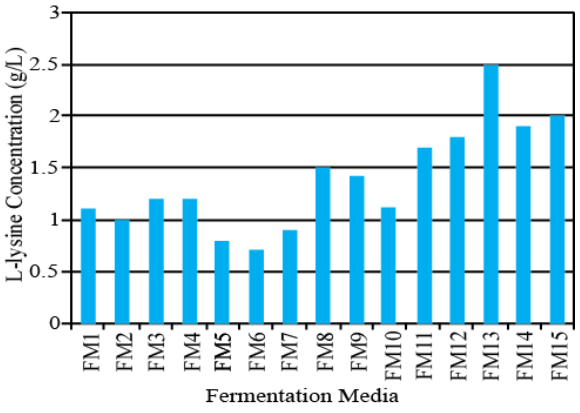

Fig. 1. Screening of different culture media for the production of L-lysine by *C. glutamicum.*

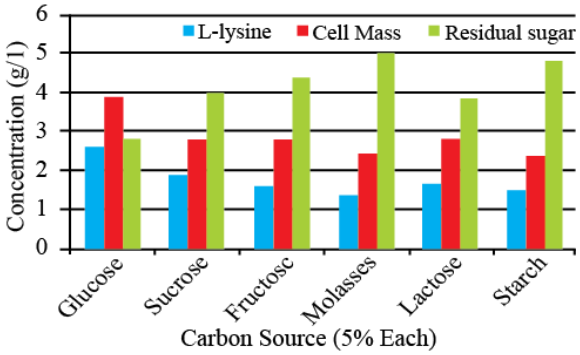

Fig. 2. Effect of different carbon sources on the production of L-lysine by *C. glutamicum.*

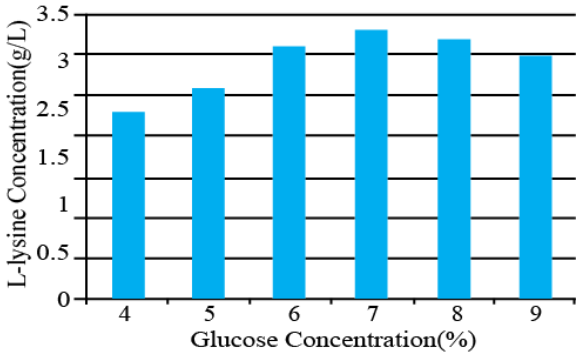

Fig 3: Effect of different concentrations of Glucose for L-lysine production

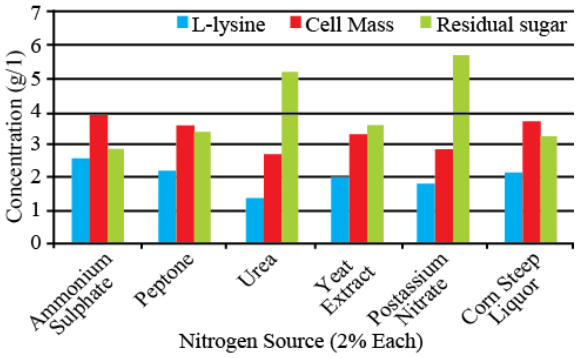

Fig. 4. Effect of different nitrogen sources on the production of L-lysine by *C. glutamicum.*

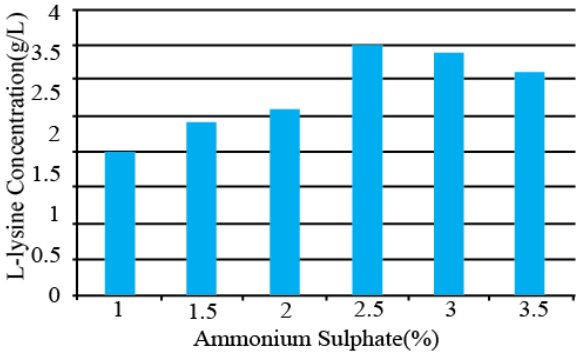

Fig. 5. Effect of different concentrations of Ammonium sulphate for L-lysine production

Discussion

The medium FM:13 contained all the essential growth promoting factors and nutrients that *C. glutamicum* can easily take up and directly use them in its metabolic pathway for enhanced production of L-lysine. These results are in accordance to the work of Oh *et al*., (1993), who used some of constituents of FM:13 in fermentation medium for increasing L-lysine yield. Moreover Shah et al., (2002) also improved L-lysine yield by using modified FM: 13 medium. Glucose is more efficient carbon source as compared to other sources (Cerning *et al*., 1994) so maximum L-lysine production was observed in the presence of glucose as carbon source. Similar results were reported by Nasri *et al*., (1989) who showed increased product yield and cell biomass in fermentation medium containing glucose carbon source. Kiefer *et al*., (2004) also demonstrated that glucose is the best carbon source among other carbon sources. That is why maximum product yield and cell biomass has been obtained by using glucose as carbon source.

Since one L-lysine molecule contains two amide groups so for its production best nitrogen source is required in fermentation medium. Nitrogen is essential component in the fermentation medium for L-lysine production (Ferreira & Duarte (1991), therefore the selection of best and suitable nitrogen source was very important in the present studies. The results reported in present study are similar to the results reported by Hsiao & Glatz (1996), they improved L-lysine production by using ammonium sulphate as nitrogen source in the fermentation medium.

Conclusions

It is concluded from the present studies that the production of L-lysine from *Corynebacterium glutamicum* can be substantially enhanced by optimizing the culture medium. Different ingredients have an important role in the metabolic pathway of the organism for L-lysine production. Carbon and nitrogen sources have also been found to have influential role in the amino acid production.

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