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BIOSYNTHESIS OF L-LYSINE BY *BACILLUS MEGATERIUM* THROUGH SUBMERGED FERMENTATION

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Abstract

L-lysine is an important and essential amino acid required in a great quantity for supplementation in a number of food and feed items. The present work aimed at the biosynthesis of Lysine by *B. megaterium* IIB187 through shake flask fermentation. Fifteen different fermentation media were studied in present work for enhancing the production of Lysine. From the fifteen fermentation media, FM-13 medium consisting of ammonium sulphate, glucose, potassium hydrogen phosphate, dipotassium phosphate, thiamine, casamino acid, manganese chloride and biotin was found to be the best medium for maximum lysine production i,e., 0.51g/L. By optimizing physical parameters including incubation temperature (30°C), incubation time (72 hrs) and initial pH of medium (7.0), *Bacillus megaterium* IIB187 produced 0.59 g/L Lysine in fermentation medium FM-13. Further screening of carbon and nitrogen sources and optimizations of nutritional parameters were also carried out. Glucose and ammonium sulphate were found as the best carbon and nitrogen sources, respectively. Maximum production of lysine i.e., 0.69g/L was found in the presence of 7% glucose, while 2.5% ammonium sulphate resulted in the highest amount of lysine production. Maximum of 1.2 g/L Lysine was obtained after fermentation under the optimized conditions.

Key words: Lysine, Culture medium, Optimization, Submerged fermentation, Amino acid.

Introduction

Lysine is an essential amino acid for animals and human which cannot be synthesized in their bodies but had been added in the food and feed for improving the food qualities (Stillings *et al*., 1971). Different methods of Lysine production can be used, such as enzymatic method, chemical synthesis, recombinant DNA technique, protoplasm fusion, protein hydrolyzate and fermentation (Nelofer *et al*., 2008; Anastassiadis, 2007). However, fermentation techniques seem to be practicable and most economical source of producing Lysine (Ekwealor & Orafu, 2003). Fermentation contributes 80% of the Lysine that is annually produced worldwide while the remaining 20% is contributed by other processes (Coello *et al*., 2002). As fermentation technique employs low pressure, low temperature, low cost carbon source and other requirements, therefore it is preferred over all other techniques (Nasab *et al*., 2007).

Many microorganisms have been found to synthesize L-lysine through fermentation. Some of the important bacteria include *Corynebacterium glutamicum* (Nelofer, 2008), *B. megaterium* (Ekwealor & Obeta, 2005), *B. linens, Streptomyces* (Shih & Shen, 2006), *B. flavum, B. lactofermentum, B. laterosporus* (Tosaka *et al*., 1979; Umerie *et al*., 2000) and *Bacillus subtilis* (Leuchtenberger, 1996). However, very limited literature is available on the synthesis of lysine from *Bacillus megaterium* through fermentation, so the present strain isolated indigenously may be proved very productive for the synthesis of said amino acid in the local industry.

Optimization of a fermentation process includes studying of different physical and chemical parameters to enhance the product yield however, the optimization of composition of the culture media is one of the most important and vital component of a bioprocess (Sassi *et al*., 1996; Adnan *et al.*, 2011). Lysine producing bacteria utilize various carbon and nitrogen sources besides many minor nutrients such as minerals and salts for higher yield of the end product (Eggeling, 2005). Each microorganism needs optimal ranges of cultural conditions and nutritional requirements for higher yield of the end product. That is why it is important to optimize the effects of different nutritional and cultural conditions including temperature, pH, agitation, incubation period and carbon & nitrogen sources for enhanced product yield. The objectives of present study were screening and optimization of different culture media for enhancing the biosynthesis of Lysine by *B. megaterium* IIB187 through fermentation in shake flasks.

Materials and Methods

Fermentation experiments: Bacterial strain of *Bacillus megaterium* IIB187 was used during present study which was taken from the culture bank of Institute of Industrial Biotechnology, GC University, Lahore. The strain was refreshed and maintained on nutrient agar plates. Fresh inoculum was prepared by taking loopful of bacterial strain in 250mL of conical flask which contained 25 mL of sterilized nutrient broth. The similar flasks containing 50 mL of culture medium were sterilized in an autoclave at 121ºC at 15 PSI for 20 min and cooled to room temperature. The flasks were the inoculated with 1mL of inoculum as prepared above and incubated in a shaking incubator at 30ºC for 24 hrs at a shaking speed of 160 rpm. At the end of fermentation, fermented broth was taken from the incubated flasks, centrifuged at 6000 rpm for 15 min and the supernatant was analyzed for measuring concentration of Lysine in the broth.

Analysis of L-Lysine

Thin layer chromatography: Thin layer chromatographic method was used for qualitative detection of L-lysine in the broth. For this purpose, 10 µl sample from the supernatant (Fermented broth) was taken and spotted on aluminum TLC plate of 0.2 mm thickness. L-lysine standard solution (10 µl) was also spotted at the TLC plate. The plates were air dried and subjected to solvent system. TLC was continued untill the solvent traveled 3/4th part of TLC plate. The mobile phase used was mixture of n-butanol, acetic acid and water (2:4:8, v/v). After completion of chromatographic process, TLC plates were air dried and were sprayed with a solution of 0.25% ninhydrin solution. The plates were then heated at 110°C for 7 min in the oven and the purple color was recorded (Pyle *et al*., 1989).

Ninhydrin ferric reagent method: Quantitation of L-Lysine was done by using Ninhydrin ferric method of Hsieh *et al*., (1995). In this method, 550µl of reagent A (Methylecellosolve, ferric chloride and KCl) and 350µl of reagent B (Ninhydrin in KCl) was used. The mixture was heated at 100°C for about 15 min in water bath. After cooling at room temperature, 110µl of DMSO and 5ml of deionized water were added to it. Then absorbance of the reaction mixture was recorded at 480nm by using a Spectrophotometer.

Glucose estimation: DNS method of Miller (1959) was used for measuring residual sugar found in culture medium. For this purpose, fermented broth was taken from the flasks and after centrifugation was used for analysis of residual sugar.

Culture media: Culture medium is an important source of essential nutrients for microbial growth and for production of useful products by microorganisms. In present study, following 15 different culture media were screened for Lysine production (Table 1).

Table 1. Different culture media used for the production of L-Lysine by *B. megaterium*.

S. No.	Medium	Composition
1.	FM-1	Molasses 25g/L, CaCO3- 15 g/L, $NH_4(SO_4)_2$- 25g/L, MgSO4- 0.4 g/L, NaCl- 2 g/L, KH_2PO_4 - 0.7g/L, K_2HPO_4 - 0.7g/L (Nelofer, 2007).
2.	FM-2	D-Glucose 75 g/L, $(NH_4)_2SO_4$- 5 g/L, peptone 10g/L, KH_2PO_4 - 0.6 g/L, NaCl- 3.0 g/L, Yeast Extract 5g/L, manganese sulphate 0.5 g/L, K_2HPO_4 - 1.2 g/L, biotin 20 µg/L (Anastasidis, 2007).
3.	FM-3	D-glucose 85 g/L, $(NH_4)_2SO_4$- 60 g/L, K_2HPO_4 - 1.0 g/L, potassium di-hydrogen phosphate 1.0 g/L, ferrous sulphate 2 mg/L, manganese sulphate dihydrate 12 mg/L, MgSO4. $7H_2O$- 0.6 g/L, biotin 3 mg/L, corn steep 35 g/L (Gulbler *et al*., 1994).
4.	FM-4	D-Glucose 65 g/L, Yeast Extract 3 g/L, NaCl- 6 g/L, tryptone 6 g/L (Modified LB medium).
5.	FM-5	D-Glucose 95 g/L, $(NH_4)_2SO_4$- 24 g/L, thiamine HCl- 150 ug/L, $MgSO_4$. $7H_2O$- 0.6 g/L, KH_2PO_4 0.6 g/L, biotin 250 ug/L (Coello *et al*., 2002).
6.	FM-6	$(NH_4)_2SO_4$- 25 g/L, $CaCO_3$ - 20 g/L, Corn steep 90 g/L, KH_2PO_4 - 1.0 g/L, MgSO4. $7H_2O$- 0.4 g/L, $MnCl_2$ - 3 mg/L, biotin 200 ug/L, thiamine HCl- 350 ug/L, NaCl- 2.5 g/L (Rehman *et al*., 2012).
7.	FM-7	D-Glucose 15 g/L, Yeast Extract 2.5 g/L, meat extract 5 g/L, peptone 7.5 g/L, NaCl- 2.2 g/L (Baullin medium).
8.	FM-8	D-Glucose 55 g/L, urea 2.5.0 g/L, $(NH_4)_2SO_4$- 4.0 g/L, MgSO. $7H_2O$- 0.25 g/L, K_2HPO_4 - 0.5 g/L, KH_2PO_4 - 0.5 g/L, $FeSO_4 \cdot 7H_2O$ - 0.02 g/L, $CaCO_3$ - 0.03 g/L, $CuSO_4$- 0.3 g/L, $ZnSO_4$. $7H_2O$- 2 mg/L, $MnSO_4$. H_2O- 0.04 g/L, Biotin 150 µg/L, $NiCl_2$ - 0.03 mg/L (Broer *et al*., 1993).
9.	FM-9	D-Glucose 35g/L, urea 3 g/L, $(NH_4)_2SO_4$- 20g/L, soya hydrolysate 20 g/L, $MgSO_4$. $7H_2O$- 0.6 g/L, KH_2PO_4 - 1 g/L, Biotin 100 µg/L, $MnSO_4.H_2O$- 4.2 mg/L, thiamine- 100 µg/L, (Oh *et al*., 1993).
10.	FM-10	$CaCO_3$ – 35 g/L, Sucrose 45 g/L, $(NH_4)_2SO_4$- 27 g/L, Urea 2 g/L, KH_2PO_4 - 0.05 g/L, $FeSO_4 \cdot 7H_2O$ - 0.01 g/L, K_2HPO_4 - 0.05 g/L, $MnSO_4.3H_2O$- 10 mg/L, $ZnSO_4$- 10 mg/L, NaCl- 1 g/L (Bathe *et al*., 2004).
11.	FM-11	D- Glucose 40 g/L, peptone 2.5 g/L, malt extract 2.5 g/L, yeast extract 2.5 g/L, $MgSO_4$. $7H_2O$- 0.2 g/L, K_2HPO_4 – 1.5 g/L (Rattray & Fox, 1997).
12.	FM-12	Urea 6 g/L, D-Glucose 55 g/L, $MgSO_4$. $7H_2O$- 0.5 g/L, KH_2PO_4 - 1.0 g/L, thiamine HCl 75 µg/L, $FeSO_4.7H_2O$ 0.01 g/L and biotin 1 µg/L, (Calik *et al*., 2001).
13.	FM-13	$(NH_4)_2SO_4$- 10 g/L, D- Glucose 52 g/L, K_2HPO_4 - 0.1 g/L, MgSO. $7H_2O$- 5 mg/L, KH_2PO_4 - 0.1 g/L, $CaCO_3$ -15 g/L, $FeSO_4 \cdot 7H_2O$ - 1 mg/L, casamino acid 2.0 g/L, biotin 50 ug/L, thiamine HCl 120 ug/L, $MnCl_2$. 4 H_2O- 1 mg/L (modified, Shah *et al*., 2012).
14.	FM-14	Peptone 5.5 g/L, D-Glucose 45 g/L, yeast extract 4.5 g/L, $(NH_4)_2SO_4$- 22 g/L, $CaCl_2$ - 0.7 g/L, KH_2PO_4 - 0.5g/L, K_2HPO_4 - 0.6 g/L (Locally designed).
15.	FM-15	$(NH_4)_2SO_4$- 25 g/L, D- Glucose 110 g/L, KH_2PO_4 - 2.0 g/L, $CaCO_3$ -20 g/L, $MgSO_4$. $7H_2O$- 0.5 g/L, casamino acid 5 g/L, $MnCl_2$. 4 H_2O- 0.02 g/L, $FeSO_4 \cdot 7H_2O$ - 0.6 g/L, biotin 60 ug/L, thiamine HCl- 115 ug/L (Shah *et al*., 2012).

Results and Discussion

Figure 1 shows that *B. megaterium* IIB187 produced maximum Lysine (0.51 g/L) in FM13 medium. Moreover this strain produced 0.41 g/L Lysine in FM-8, 0.38 g/L in FM-12, 0.37 g/L in FM-14, 0.34 g/L in FM-4, FM-6 and in FM-15 medium while in FM-5 medium, *B. megaterium* IIB187 produced 0.33 g/L of Lysine. In rest of culture media, this strain produced less than 3.0 g/L of Lysine and a minimum of 0.13 g/L Lysine was produced in FM-2 medium (Fig. 1). The culture medium FM-13 showed higher quantity of Lysine production as compared to all other fermentation media so it was selected for further studies and optimization of other parameters.

Culture medium FM-13 have all the necessary constituents which are helpful to the bacterial culture for production of L-lysine. *B. megaterium* IIB187 utilize these nutrients in metabolic pathway of Lysine biosynthesis. Results of present study are in similarity to the results reported by Oh *et al*., (1993) who also got maximum production of Lysine by using same constituents in the culture medium. Shah *et al*., (2012) also reported maximum Lysine production in the fermentation medium, which had the same constituents as in FM-13 medium. So the present study is also an agreement to the previous studies reported earlier.

Fermentation conditions: "Medium: FM13; Incubation temperature: 35°C; pH: 7.0; Agitation rate: 200 rpm"

Lysine production rate was studies ranging from 24 hrs of incubation to 144 hrs with the interval of 24hrs. Figure 3 shows L-lysine yield, residual sugar and dry cell mass produced during fermentation at respective incubation periods. From the results it was found that *B. megaterium* IIB187 produced 0.23 g/L L-lysine after 24 hrs of fermentation which increased gradually to 0.38 g/L in 48 hrs and reached maximum (0.50 g/L) in 72 hrs, then started decreasing as the incubation period was increased and reached minimum (0.46 g/L) after 144 hrs of incubation period. Dry cell mass was also calculated with respect to incubation period. It was found that the cell mass of *B. megaterium* IIB187 kept on increasing as the incubation period passed and it was maximum at the end of fermentation (after 144 hrs). Estimation of residual sugar showed a gradual depletion in sugar with the increase in time of incubation hence resulted in enhanced Lysine and biomass production (Fig. 2).

Microorganisms have specific viable time period during which these yield maximum concentration of metabolites. Then after the specific fermentation time period, microorganisms start to die and there is decline in production of metabolites (Cocaign *et al*., 1993). Most of Lysine production by *Bacillus* species were completed in about 72 hrs to 100 hrs and has been reported by Anastassiadis (2007). Adnan *et al*., (2011) determined the time profiles of bacterial isolates under both optimized and un-optimized conditions and concluded that maximum Lysine yield was observed after 48 h to 72 hrs of incubation period. Maximum Lysine production after 72 hrs was also reported by (Ishii *et al*., 1997). Sano *et al*., (1990) reported 72 hrs of incubation period, a best one for Lysine production.

Fermentation conditions: "Medium: FM-13; Incubation temperature: 30°C; pH: 7.0; Agitation rate: 200 rpm"

Effect of various initial pH values (from 6.0 to 8.5) of fermentation medium on the production of Lysine was studied. Results showed that maximum Lysine production was observed at an initial pH of 7.0 while minimum amount of lysine (3.0 g/L) was observed at pH 6.0. Increasing pH value from 7.0 resulted in decreased production of lysine (Fig. 3). Dry cell mass was maximum (2.8 g/L) at 7.0 pH while it was minimum (1.9 g/L) at 6.0 pH. Residual sugar in the broth was found minimum at pH 7.0 where L-lysine yield and dry cell mass were highest.

The pH of medium is considered very important factor for the biosynthesis of microbial secondary metabolites. The pH affects the permeability characteristics of cell membrane which cause either ions uptake or loss in nutrient media during microbial growth. Therefore, pH is considered a strong and very important influencing factor of culture medium for microbial growth and product yield. Basic components, such as potassium and sodium hydroxide, calcium carbonate, ammonia, urea and inorganic acids are used as controlling the pH of medium (Nakamura *et al*., 2000). Present results are in line with Broer (1993) who also suggested that the suitable pH for L-lysine production from bacterial isolates was 7.0–7.5. Sattar *et al*., (2008) reported enhanced L-lysine yield at pH 7.0, whereas Rehman (2012) reported higher L-lysine yield at pH 7.5.

Cultural conditions: "Medium: FM13; Incubation temperature: 30°C; pH: 7.0; Agitation rate: 200 rpm"

For the optimization of the incubation temperature, the bacterial culture was grown at various temperatures from 25 to 40°C with an increase of 5°C. The strain produced 0.41 g/L of Lysine at 25°C and a maximum of 0.59 g/L at 30°C. Maximum dry cell mass (4.1 g/L) was produced at 30°C where L-lysine production was also in maximum amount and minimum dry cell mass (2.5 g/L) by this strain was recorded at 25°C. Residual sugar was also estimated and minimum residual sugar 2.4 g/L was recorded at 30°C (Fig. 4).

Incubation temperature is very important and critical factor for fermentative production of microbial products. Growth rate of microorganisms represents function of temperature through the reaction sequence which makes up the whole metabolism. Maximum product yield in the present case was observed at 30°C. Above and below 30°C, less L-lysine yield, low dry cell mass and low sugar consumption were recorded. Hilliger *et al*., (1984) reported that 30°C was considered a good incubation temperature for fermentative production of Lysine. Ohnishi *et al*., (2003) described that at an industrial scale most of L-lysine synthesis was carried out at 30°C.

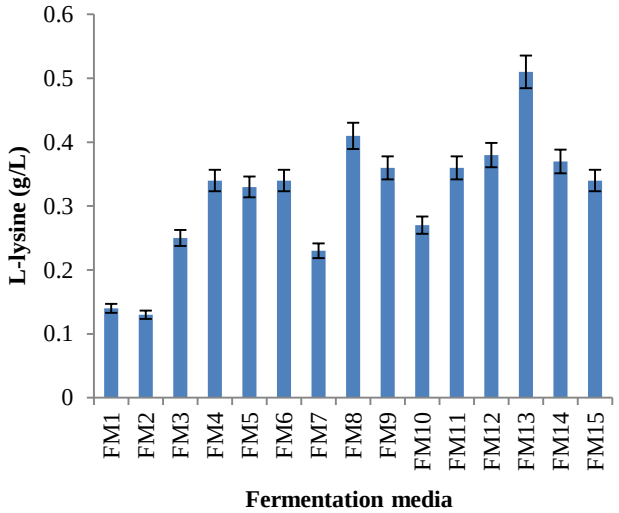

Fig. 1. Lysine production by *B. megaterium* IIB187 in different fermentation media
"All values are means of three replicates. Y-error bar represent standard error of mean"

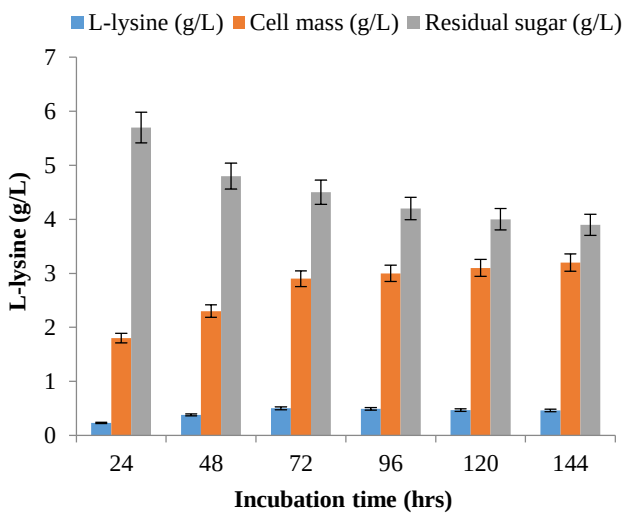

Fig. 2. Optimization of incubation time for L-Lysine production by *B. megaterium*
"All values are means of three replicates. Y-error bar represent standard error of mean"

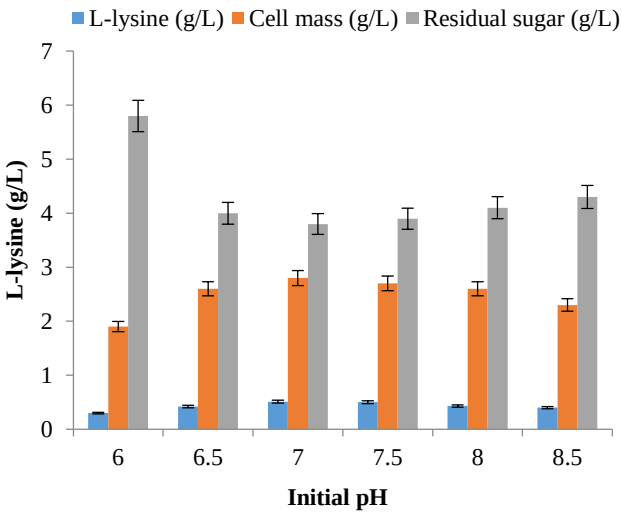

Fig. 3. Effect of initial pH on the production of L-lysine by *B. megaterium* IIB187
"All values are means of three replicates. Y-error bar represent standard error of mean"

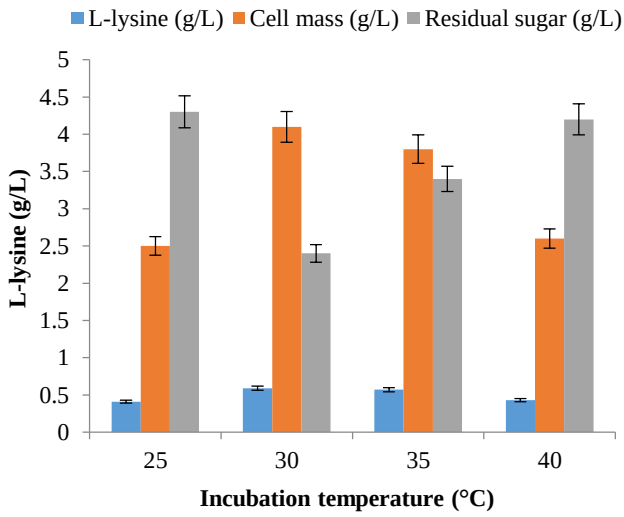

Fig. 4. Optimization of incubation temperature for production of L-lysine by *B. megaterium* IIB187
"All values are means of three replicates. Y-error bar represent standard error of mean"

Conditions of fermentation:- "Medium: FM13; Agitation: 200 rpm; Incubation period: 72 hrs; Initial pH: 7.0"

Six carbon sources including sucrose, glucose, fructose, molasses, starch and lactose were screened for L-lysine production. The maximum quantity of Lysine (0.60 g/L) was produced when glucose as carbon source was used in culture medium. All the other carbon sources resulted in lesser amount of lysine in the broth such as 0.45 g/L by sucrose, 0.36 g/L by fructose, 0.35 g/L by molasses, 0.47 g/L by lactose and 0.38 g/L of L-lysine was produced by starch. Dry cell mass was also checked and the bacterial culture showed dry cell mass as 4.2 g/L in sucrose, 2.2 g/L in fructose, 2.1 g/L in molasses, 2.7 g/L in lactose and 2.3 g/L of lysine in the presence of starch. Utilization of sugar was also studied and minimum residual sugar (2.5 g/L) was found in culture medium which contained glucose as a carbon source (Fig. 5).

Carbon source plays structural and functional role for microbial cells and it is necessary component of microbial growth. It causes major effects on microbial production of metabolites and other byproducts. This result correlates with the result obtained by Nasri *et al.*, (1989) who also reported highest yield of L-lysine in a medium which contained glucose as carbon source. Kiefer *et al.*, (2002) described the glucose a best carbon source. Cerning *et al.*, (1994) compared different carbon sources and described glucose is highly efficient among all other sources. As the maximum lysine biosynthesis was found in the fermentation medium containing glucose as a carbon source. Therefore glucose was selected as a best carbon source and further optimization was done by using glucose in the fermentation medium.

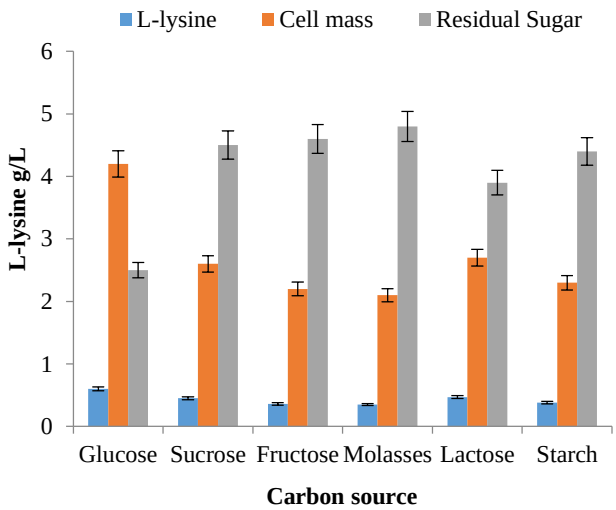

Fig. 5. Screening of carbon sources during production of L-lysine by *B. megaterium* IIB187
"All values are means of three replicates. Y-error bar represent standard error of mean"

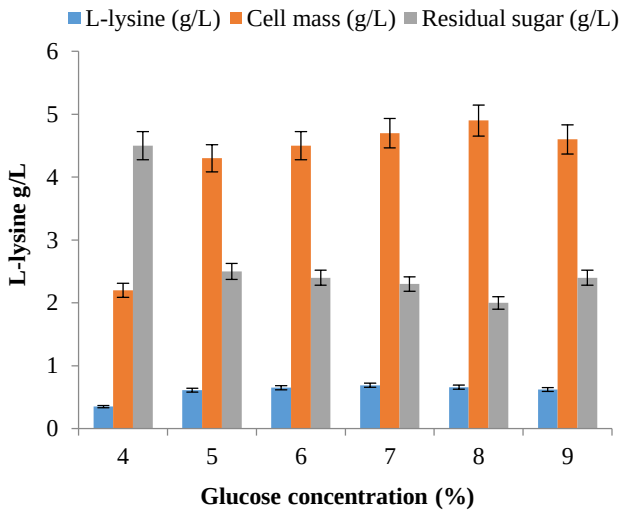

Fig. 6. Optimization of glucose concentration in the fermentation medium for biosynthesis of L-lysine by *B. megaterium* IIB187
"All values are means of three replicates. Y-error bar represent standard error of mean"

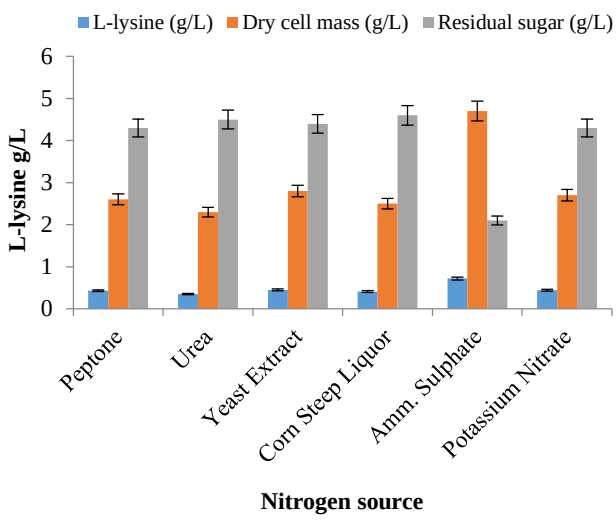

Fig. 7. Screening of nitrogen sources for production of L-lysine by *B. megaterium* IIB187
"All values are means of three replicates. Y-error bar represent standard error of mean"

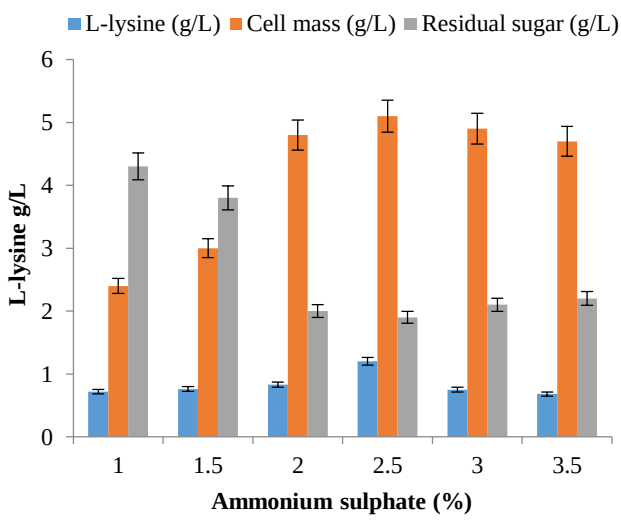

Fig. 8. Optimization of amount of ammonium sulphate for production of L-lysine by *B. megaterium* IIB187
"All values are means of three replicates. Y-error bar represent standard error of mean"

***Conditions of fermentation:-** "Medium: FM13; Agitation: 200 rpm; Incubation period: 72 hrs; Initial pH: 7.0"

For enhanced production of L-lysine, 4 to 9% of glucose was used in the medium to optimize its concentration. Maximum (0.69 g/L) L-lysine was found at a concentration of 7% glucose and minimum (0.35 g/L) Lysine in the presence of 4% glucose in fermentation medium. Maximum amount of dry cell mass (4.9 g/L) was found at 8% of glucose and minimum dry cell mass (2.2 g/L) was observed in the presence of 4% glucose in fermentation medium (Fig. 6). Ferreria & Durate (1991) reported that 8 to 10% glucose concentration was proved best for L-lysine production. Residual sugar concentrations during Lysine biosynthesis was also reported by Hirose & Shibai (1985) and they were concluded that over concentration of sugar in the culture medium inhibited the microbial growth rate along with decreased production of L-lysine and dry cell mass. While low concentration of glucose also caused low product yield and dry cell mass. Sassi *et al.*, (1990) also concluded that initial concentrations of sugar influenced biosynthesis of Lysine during fermentation and it was found that higher yield was observed at 70 g/L of D-glucose.

Conditions for fermentation:- "Medium: FM13; Agitation: 200 rpm; Incubation period: 72 hrs; Initial pH: 7.0"

Six different nitrogen sources including ammonium sulphate, corn steep liquor, yeast extract, potassium nitrate, peptone and urea were screened in the culture medium for optimum production of lysine. All these nitrogen sources were added in FM-13 medium and it was observed that maximum Lysine yield (0.72g/L) was found in medium

containing ammonium sulphate. While other nitrogen sources produced lower Lysine i.e., urea 0.35 g/L, peptone 0.43g/L, potassium nitrate 0.44 g/L, corn steep liquor 0.41g/L and Yeast Extract 0.45 g/L (Fig. 8). Ammonium sulphate concentration (1 to 3.5%) was also optimized in FM-13 for lysine production by *B. megaterium*. Maximum Lysine was synthesized in medium containing 2.5% of ammonium sulphate. Dry cell mass and remaining glucose were also examined and it was found that cell mass increased with increase in the product concentration and decrease in residual sugar (Figs. 7 and 8).

***Conditions of fermentation:-** "Medium: FM13; Agitation: 200 rpm; Incubation period: 72 hrs; Initial pH: 7.0"

The ample supply of a suitable nitrogen source is essential for L-lysine fermentation, since this molecule contains 19.16% nitrogen. Ammonium ion is harmful at higher concentrations for microbial growth and for product formation so it must be kept at lower levels (Hirose *et al*., 1985). Less amount of ammonium sulphate caused low product yield and dry cell mass but higher concentration of ammonium sulphate also rendered the less product and cell mass biosynthesis. So it was necessary to optimize the different concentrations of ammonium sulphate in the fermentation medium to find its optimum concentration for enhancing L-lysine production by *B. megaterium* IIB 187. Our results are in accordance to the results of Shah *et al*., (2002) in which they reported maximum L-lysine in culture medium which contained 2.5% amm. sulphate as nitrogen source. Dry cell masse and sugar utilization were also high at 2.5% ammonium sulphate concentration in the fermentation medium. Zaki *et al*., (1982) also reported maximum L-lysine production by using ammonium sulphate at concentration of 2.5%. Ferreira & Durate (1991), Hsiao & Glatz (1996) and Wang *et al*., (1991) also suggested more than 2% ammonium sulphate in culture medium for maximum production of Lysine.

***Conditions for fermentation:-** "Medium: FM13; Agitation: 200 rpm; Incubation period: 72 hrs; Initial pH: 7.0"

Conclusions

It is concluded from the present study that *B. megaterium* IIB187 is an excellent source of L-lysine production through submerged fermentation. The production of lysine can be enhanced many folds by optimizing different nutritional and cultural parameters of fermentation. The present strain may be used by local industry for the indigenous production of L-Lysine.

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