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Full Length Research Paper

Studies on lysine production by *Bacillus megaterium*

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A Lysine-producing strain recovered from soil was found to produce large amount of the amino acid. The bacterium identified as *Bacillus megaterium* SP 14 accumulated a lysine yield of 3.56 mg/ml in a broth culture in 96 h. Fermentation experiments show that 8.0% (w/v) glucose and 4.0% (w/v) ammonium chloride used as sources of carbon and nitrogen, respectively, in a medium/fermenter volume ratio of 25.0%, influenced accumulation of the amino acid. Amino acids other than the aspartate family at 0.01% (w/v) stimulated growth and improved lysine yield. Addition of 0.01 unit/ml penicillin to the fermentation medium, immediately after inoculation, stimulated growth and appreciably enhanced lysine accumulation.

Key words: Lysine, amino acid, *Bacillus megaterium*, fermentation, penicillin.

INTRODUCTION

L-Lysine is nutritionally essential for humans and animals. It cannot be synthesized internally but may be added to food and feed materials to improve the protein quality (Stillings et al., 1971). However, fermentative methods seem to be most economical and practicable means of producing lysine, and many of such processes have been investigated (Schrumpf et al., 1992; Eggeling, 1994; Ekwealor and Orafu, 2003). The present report is on studies carried out to maximize yields of free lysine produced in a culture broth by a bacterial strain recovered from soil.

MATERIALS AND METHODS

Microorganism

Bacillus megaterium SP 14 was isolated from soil in Awka, Nigeria. It was maintained on nutrient agar (Oxoid) slants at 4°C. The medium for seed culture consists of peptone, 10.0 g; yeast extract, 10.0 g; NaCl, 5.0 g; distilled water, 1 L; pH adjusted to 7.0 with I N Na0H. The medium was sterilized at 121°C for 15 min. Two loopful of a 24 h slant culture was used to inoculate a 250 ml Erlenmeyer flask containing 50 ml of seed medium. The flask was incubated for 16-18 h on a rotary shaker at 120 rpm and 30°C.

Fermentation

The basal medium for fermentation experiments was composed of KH_2PO_4, 1.0 g; $MgSO_4.7H_20$, 0.4 g; $MnSO_4.H_20$, 2.0 mg; $FesO_4.7H_20$, 2.0 mg; $CaCO_3$, 50.0 g; glucose, 20.0 g; $(NH_4)_2SO_4$, 10.0 g; distilled water, 1 L; pH adjusted to 7.0 with 1 N NaOH and the medium sterilized at 115°C for 10 min. A 10% (v/v) seed culture was used to inoculate 100 ml Erlenmeyer flask containing 20 ml of fermentation medium. After 72 h incubation on a rotary shaker at 160 rpm and 30°C, growth and lysine accumulation were determined from the broth culture. Uninoculated flasks were kept as control. All values reported are an average of at least duplicates which agreed closely.

Analyses

Bacteria growth was determined turbidimetrically using Spectronic 21 spectrophotometer (Milton Roy Co.) at 660nm. Paper chromatography was employed for the detection of L-lysine in the culture broth and was run for 18-24 h on Whatman No. 1 filter paper by the ascending method. Solvent systems used include n-butanol:acetic acid:water (4:1:1, v/v) and phenol:water (5:1, v/v). The spots were visualized b spraying with a solution of 0.15%

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ninhydrin in butanol. Quantitative estimation of L-lysine in the supernatant fluid was determined by acidic ninhydrin method of Chinard (1952). Residual sugar was determined as glucose in the supernatant fluid by the colorimetric DNS method of Miller (1959).

RESULTS AND DISCUSSION

Isolation of lysine-producing bacteria

Soil sample (1.0 g) was serially diluted ten fold in sterile distilled water and 0.1 ml of soil suspensions plated on tryptone Soya agar (Oxoid) medium. The Petri dishes were incubated for 24 h at 30°C. Colonies on the plates were picked, purified and stored as agar slant cultures. The isolates were screened for lysine production on solid medium seeded with *Escherichia coli* 1099 or 5210 (lysine auxotrophs), following the method described by Halsall (1975). Of the tested broth cultures of lysine-producing bacteria, strain SP 14 produced a single minhydrin-positive spot which was detected on a paper chromatogram. Based on the characteristic features and with reference to Buchanan and Gibbon (1974), Bergey's manual of determinative bacteriology, it was concluded that strain SP 14 belongs to *B. megaterium*.

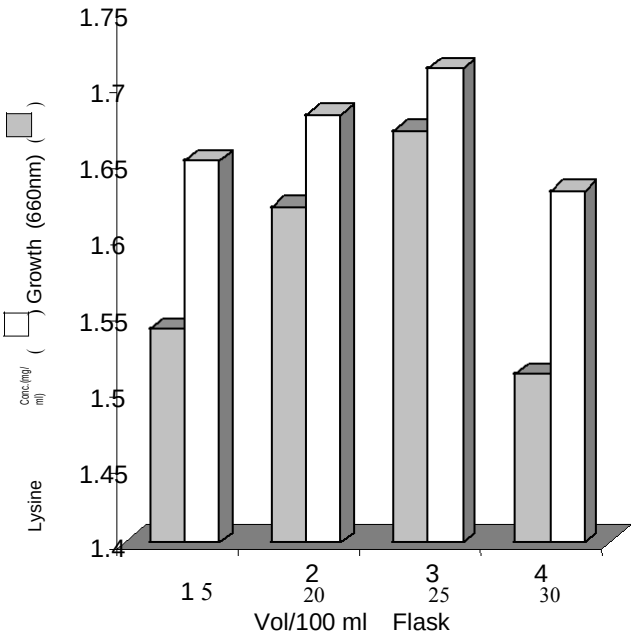

Figure 1. Influence of volume ratio of medium/fermenter on lysine production. Incubation time, 72 h; temperature 30°C; glucose, 20 g/l; $(NH_4)_2SO_4$, 10 g/l.

Paper chromatogram of L-lysine

After 24 h fermentation, the culture filtrate was submitted to a paper chromatography. The single ninhydrin-positive spot was assumed to be lysine judging from the comparative R_f values of the culture filtrate and the authentic lysine in the two solvent systems used (not shown).

Influence of medium/fermenter volume ratio

Since adequate aeration of fermetation medium is required for lysine production, the influence of oxygen supply on L-lysine formation was investigated. The shake culture experiments were performed by varying the volume of the medium in flasks shaken at 160 rpm. Judging from the results (Figure 1), lysine production increased with increasing medium volume ratio up to 25.0%. Further increasing the volume ratio to 30% caused a decrease in lysine synthesis. Thus, a volume ratio of 25% was chosen as the optimum for lysine production.

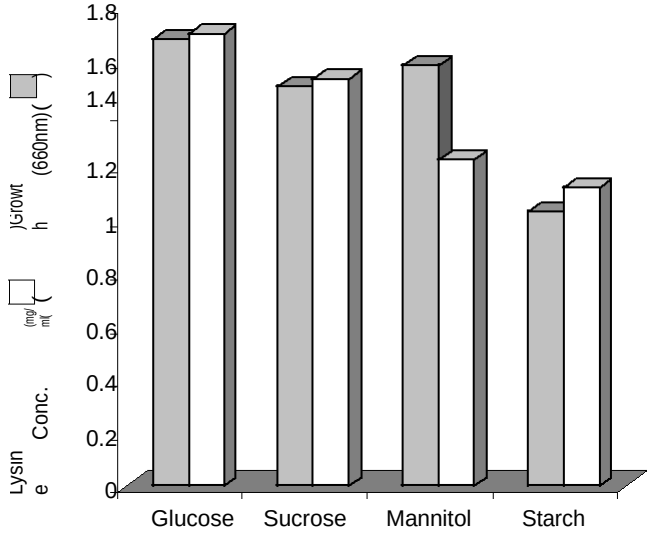

Figure 2. Influence of sources of carbon on lysine production. Incubation time, 72 h; temperature 30°C; glucose, 20 g/l; $(NH_4)_2SO_4$, 10 g/l; volume of medium, 25 ml/100 ml flask.

Influence of carbon source

The influence of sucrose, mannitol and starch on growth and lysine production was compared with that of glucose. Glucose in the basal medium was replaced by equivalent concentration of these carbon sources. Among the

substrates tested (Figure 2), glucose proved to be the best carbon source for lysine production. Results of the influence of varying levels of glucose studied (Figure 3) show that lysine production was a function of the initial sugar concentration in the fermentation medium. An increase in glucose concentration exceeding 8.0% resulted in poor lysine formation. The optimal glucose concentration of 8.0% was chosen for lysine production.

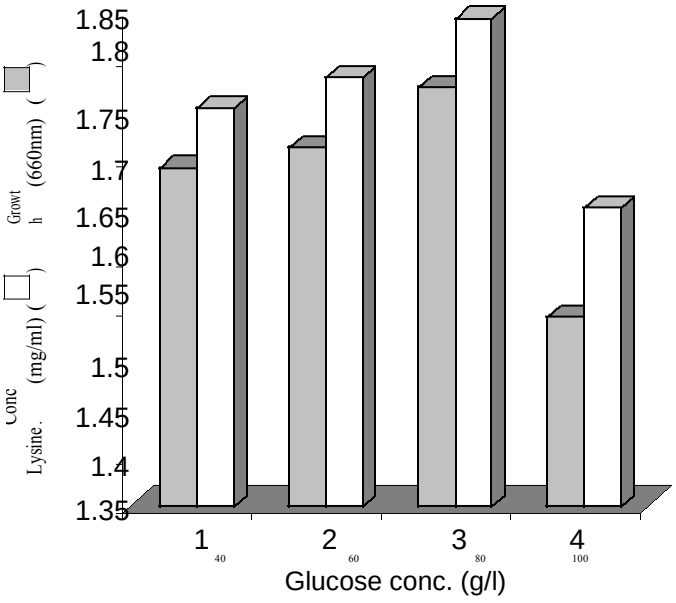

Figure 3. Influence of glucose concentration on lysine production. Incubation time, 72 h; temperature 30°C; glucose, 20 g/l; $(NH_4)_2SO_4$, 10 g/l; volume of medium, 25ml/100ml flask.

Influence of nitrogen source

In order to investigate the influence of ammonium chloride, diammonium hydrogen phosphate, ammonium dihydrogen phosphate, ammonium acetate and potassium nitrate on growth and lysine accumulation, ammonium sulphate of basal medium was replaced by equimolar concentration of the various nitrogen sources. The results presented in Figure 4, show that ammonium sulphate was the best medium for lysine production and was chosen as the nitrogen source for shake flask cultures. From the results of influence of varying levels of ammonium sulphate studied (Figure 5), lysine production was observed to be a function of nitrogen concentration up to 4.0%, beyond which accumulation of lysine decreased. This decrease, as suggested by Pham et al. (1992), may be attributed to osmotic pressure exerted by high nitrogen concentration, which may have adversely

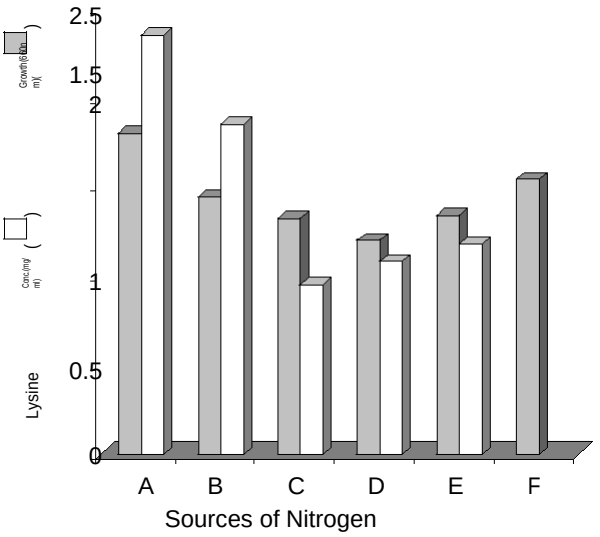

Figure 4. Influence of sources of nitrogen on lysine production. Incubation time, 72 h; temperature 30°C; glucose, 80 g/l; volume of medium, 25ml/100ml flask. A, $(NH_4)_2SO_4$; B, NH4Cl; C, $NH_4H_2PO_4$; D, $(NH_4)_2HPO_4$; E, CH_3COONH_4; F, KNO_3.

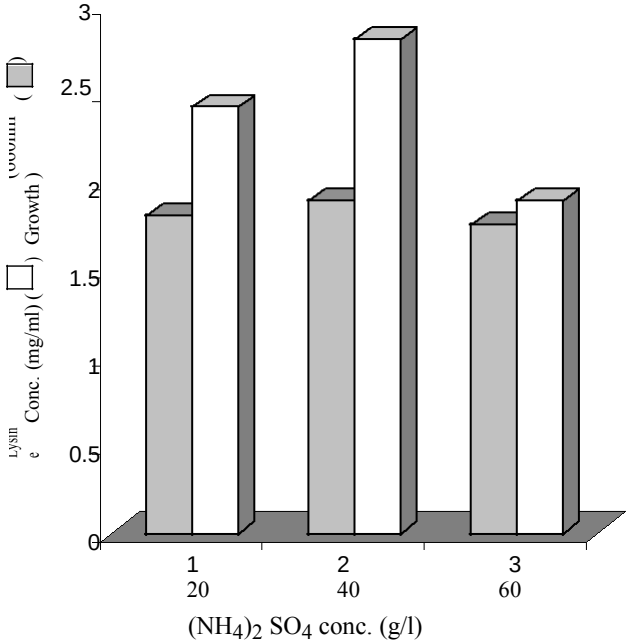

Figure 5. Influence of ammonium sulphate concentration on lysine production. Incubation time, 72 h; temperature 30°C; glucose, 80 g/l; volume of medium, 25ml/100ml flask.

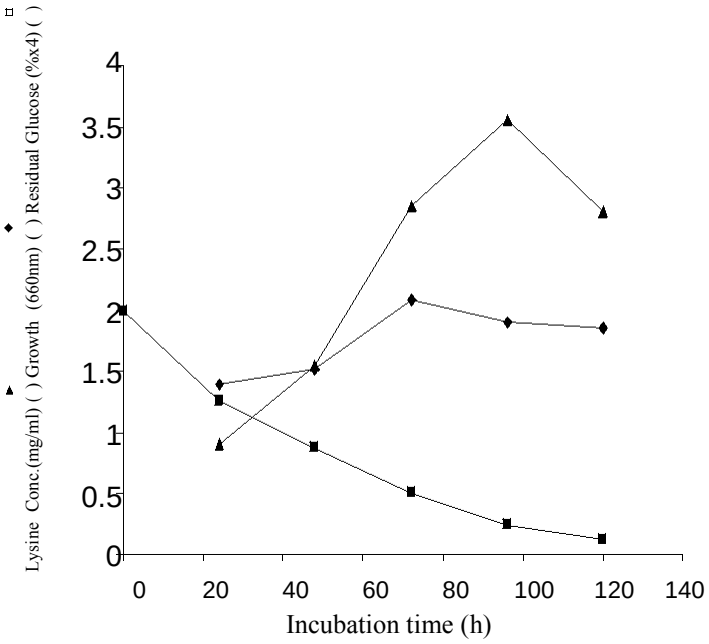

Figure 6. Culture under optimal condition. Temperature 30°C; glucose, 80 g/l; (NH4)2SO4, 40 g/l; volume of medium, 25ml/100ml flask.

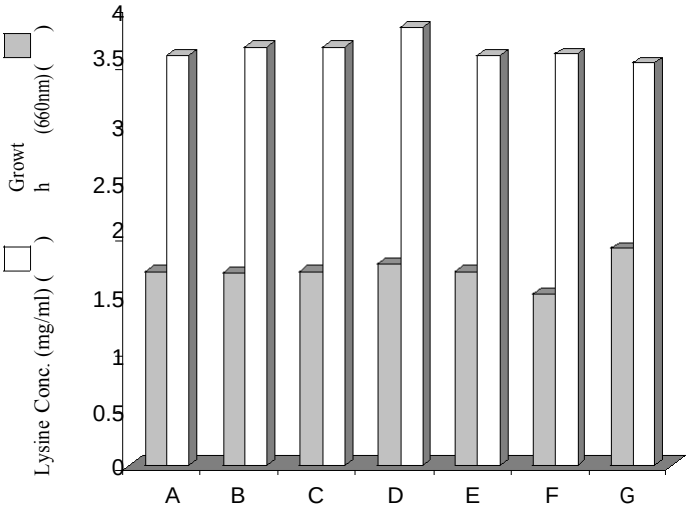

Figure 7. Influence of growth-producing substances on lysine production. A, yeast extract; B, peptone; C, yeast extract; D, soyabean; E, defatted soyabean; F, casein; and G, control (no growth promoting substance).

affected the organism's growth and lysine accumulation. Ammonium sulphate at a concentration of 4.0% was chosen for optimum lysine production.

Influence of fermentation time

Some of the most favourable culture conditions for L-lysine accumulation were investigated by shake flask experiments. From the result presented in Figure 6, a lysine yield of 3.56 mg/ml was obtained and the residual glucose was about 1.0% after 96 h fermentation. It was concluded from this experiment that 8.0% glucose and 4.0% ammonium sulphate added as carbon and nitrogen sources, respectively, to the basal medium in a medium/fermenter volume ratio of 25.0%, were the optimum cultural conditions for growth and lysine production by strain SP 14.

Influence of growth promoting substances

In this study, the influence of 0.1% (w/v) growth-promoting substances such as peptone, yeast extract, soya bean, defatted soya bean and casein on lysine production was examined. The fermentation process was carried out for 96 h at optimum cultural conditions. The results in Figure 7 indicate that growth promoting substances used retarded growth but stimulated lysine accumulation. These results contrasted with the views of Chao and Foster (1959) and Tauro et al. (1963), who reported a retardation in glutamic acid production by *Bacillus subtilis* and lysine accumulation in *Ustilago maydis*, respectively. The difference in lysine yields observed between defatted and non-defatted soyabeans, indicates that fatty acid substance(s) in the non-defatted soyabeans may have contributed to the increased lysine accumulation.

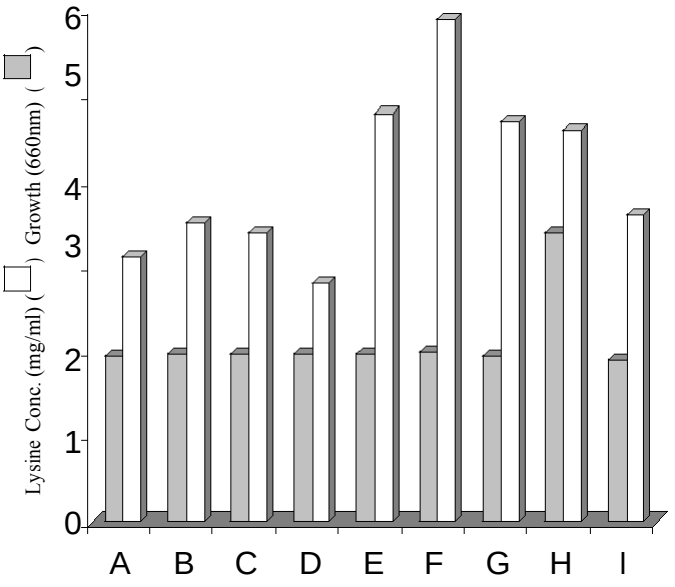

Figure 8. Influence of amino acids on lysine production. A, aspartic acid; B, methionine; C, threonine; D, isoleucine; E, glycine; F, ornithine; G, proline; H, dihydroxyphenylalanine; and I, control (No amino acid).

Table 1. Influence of penicillin and addition time on growth and lysine production by *B. megaterium* SP 14.

Parameter	Conc. (Unit/ml)	O	16 h	18 h	20 h	22 h	24 h
Growth (OD_{660nm})	0	1.91	-	-	-	-	-
	0.01	1.96	1.66	1.71	1.71	1.73	1.72
	0.03	1.91	1.79	1.82	1.83	1.85	1.86
	0.07	1.90	1.90	1.90	1.93	1.87	1.87
	0.67	1.83	1.94	1.96	1.98	1.89	1.88
	1.33	1.78	2.02	2.04	2.04	2.00	1.91
Lysine (mg/ml)	0	3.54	-	-	-	-	-
	0.01	6.68	1.03	1.14	1.17	1.28	1.21
	0.03	4.64	2.00	2.03	2.04	2.08	2.08
	0.07	4.04	3.02	3.08	3.34	2.14	2.13
	0.67	2.16	3.54	3.76	4.06	2.46	2.34
	1.33	1.50	4.84	4.88	4.88	4.40	3.22

Influence of amino acids

The influence of 0.01% (w/v) aspartic acid, methionine, threonine, isoleucine, glycine, ornithine, proline and dihydroxphenylalanine on lysine accumulation was studied. The fermentation process was at optimum cultural conditions. From the results presented in Figure 8, all the amino acids except members of the aspartate family, which included aspartic acid, methionine, threonine and isoleucine, stimulated growth and enhanced lysine accumulation. The inhibitory effect of the aspartate family, as suggested by Mindlin and Zaitseva (1966), may be due to repression or inhibition of specific enzymes which direct the biosynthetic pathway for producing only lysine.

Influence of penicillin and addition time

There have been reports (Shiio et al., 1963; Otsuka et al., 1964; Shiio and Uchio, 1969) on the effect of penicillin and its addition time on amino acid production, and these were investigated in the present study. Varying levels of penicillin were added to the culture medium at 0, 16, 18, 20h, 22 and 24 h. Optimum cultural conditions were maintained. The results of Table 1 show that growth and lysine accumulation were stimulated by penicillin. A change in permeability of the cell wall of the *Bacillus* strain, as suggested by Otsuka et al. (1964) and Demain and Birnbaum (1968), may have been responsible for the improved lysine yields.

The enhanced lysine accumulation at various addition time of penicillin obtained in this study, does not support the view of Nara et al. (1964). They reported that the addition of penicillin at an earlier or a later period than the critical time caused no effect on glutamate accumulation by *Micrococcus glutamicus*. From the results (Table 1), maximum lysine accumulation by strain SP 14 was obtained with 0.01 unit/ml penicillin, added at the start of the fermentation process (0 h). In contrast to this finding Shiio and Uchio (1969) noted that 20 unit/ml penicillin added 20 h after inoculation provided the maximum L-glutamic acid accumulation in *Corynebacterium hydrocarboclastus*.

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