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MASS PRODUCTION AND RECOVERY OF L- LYSINE BY MICROBIAL FERMENTATION USING *BREVIBACTERIUM FLAVUM*

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ABSTRACT

A simple and cheap method of lysine production was developed which can be exploited on commercial scale to save foreign exchange. In present study lysine was produced at mass scale in 10 litre flask with 5 litre fermentation medium having pre-treated molasses (15 brix) as substrate by *Brevibacterium flavum*. Lysine content in fermented broth was 2.0167 percent. It was recovered from broth by passing through ion exchange resin column, using elution buffer (tris-HCl, pH 9). Crude brown crystals of lysine (40gm/500mL) were concentrated by evaporation (80˚C) of fermented broth and recovered with 95% ethanol at 4ºC. It was decolourized with 0.5% activated charcoal, pale yellow pure crystals of Lysine (25 gm) were obtained with 95% ethanol. High concentration of lysine in the form of crystals was produced with 62.5 percent purity and concentrated fermented broth having 8% lysine can be safely used in poultry rations as a source of Lysine. It was concluded that microbial production of lysine by fermentation is a cheap and valuable method than others being used in industry.

Key words: Lysine; *Brevibacterium flavum*; feed industry; ion exchange resin; fermentation

INTRODUCTION

L- Lysine, 2, 6 diaminohexanoic acid ($C_6H_{14}N_2O_2$) is a basic amino acid having two amino groups, one, on α- position and other at Ɛ- position (Tome and Bos 2007; Rao *et al*., 2011; Malothu *et al*., 2012). L-Lysine is generally deficientin the food supply of man and meat producing animals (Pellett and Ghosh 2004).It must be added infeed to provide a balanced diet (Nasab *et al*., 2007). Supplementation of wheat based foods with lysine improves protein quality, resulting in better growth rate and body tissue synthesis. L- Lysine promotes cell division and is necessary for carnitine production, which is a vital substance for long chain fatty acids oxidation along with generation of ATPs (Rambabu, 2007). It plays a critical role with arginine in collagen synthesis (Sokalingam *et al*. 2012). Highest proportion of these two amino acids is present in histone nucleoprotein (Peter, 2010). The use of L- Lysine is helpful to overcome angina pectoris. It is an essential ingredient to clean arteries, important for cancer prevention (Javaid *et al*., 2012). Lysine supports bone health by insuring adequate absorption of calcium and therefore prevents osteoporosis. It has an important role in production of antibodies for healthy immune system. It is the integral component of musculature (Haas, 2006).

Both chemical and biochemical methods are used for lysine production (Anastassiadis, 2007; Nelofer *et al*., 2008). From the commercially manufactured lysine, 80% is manufactured by biochemical method and only 20% by chemical means (Malothu *et al*., 2012). Among biochemical methods, fermentation is the most economical for the lysine production (Ekwealor and Obeta, 2005). The microbial process of fermentation is currently gaining importance because of Lysine production requires industrial and agricultural wastes or by- products as substrate. Microbial fermentation provides 100% L-amino acids whereas by chemical method 50% D and 50% L- amino acids are obtained (Khan *et al*. 2006).

Thus, L- Lysine is being produced on industrial scale using *Corynebacterium glutamicum* as fermenting agent. Othersub-species used include *Brevibacterium flavum, Brevibacterium lactofermentum, Corynebacterium lilium, Brevibacterium dicvaricatum* (Kircher and Pfeerle 2001; Sindelar and Wendisch, 2007). *Brevibacterium flavum* is considered the most suitable organism for the lysine production (Ali *et al*. 2009). In present study *Brevibacterium flavum* was used as fermenting agent. It is goal of animal industry to produce the protein of higher quality (meat, milk) from less expensive protein sources. As Pakistan is a developing country, a huge amount of foreign exchange is utilized in import of this essential amino acid for feed industry. The purpose of present study was to develop a cheap method for the Lysine production locally.

MATERIALS AND METHODS

Organism: *Brevibacterium flavum* (NRC- 207 F Rev. 2/78) was procured from the International University Tokoyo, Japan. The organism was revived and maintained on nutrient agar plates at pH 7.0± 0.2 (fig 1).

Pre Treatment of Molasses: First hand molasses (as a substrate) in equal volume was mixed with water and kept at 40ºC for 5 hours then centrifuged at 5000 rpm for 5 min. The pH was adjusted at 3.0 with 0.1 N H_2SO_4. Thereafter it was allowed to stand at room temperature for overnight and centrifuged at 3000 rpm for 15 minutes. Supernatant was taken to make its bricks (12- 15) with refracto meter (Inamdar, 1994). The purpose of pre-treatment was to increase the reducing sugar contents and eliminate the sludge (Shafaghat *et al*., 2010).

Fermentation: Inoculum medium was prepared as described by Nasab *et al*. (2007). The optical density of the culture was adjusted to 0.6 at 600 nm by diluting with sterilized distilled water (Athar *et al*., 2009).The basal medium for fermentation was prepared by adding $MgSO_4.7H_2O$ (4gm), $KH_2PO_4.H_2O$ (2gm), NaCl(1gm), $(NH_4)_2SO_4$(2.5gm), $CaCO_3$(40gm), urea (5gm), corn steep liquor (6mL),vitamin B complex (2-3mL) perlitre. The basal medium having molasses (12- 15 brix) was inoculated with fresh inoculum (4% v/v) of *Brevibacteriumflavum*. The fermentation was carried out by using above pre-optimized conditions on orbital shaker at 180 rpm at 30ºC for 72 hours (Nasab *et al.*, 2007).

Mass scale production of Lysine: Pilot scale production of lysine was carried out through fermentation by applying the optimal values; (15 brix) molasses, (200gm) $CaCO_3$, (20gm) $MgSO_4$. $7H_2O$, (5.0gm)NaCl, (10gm) KH_2PO_4 and (12.5gm)$(NH_4)_2SO_4$ in 10 litre volume flask having 5 litre fermentation medium with *Brevibacterium flavum* at 30ºC for 72 hrs.The growth medium was aerated with air pump and stirred with magnetic stirrer.

Recovery of Lysine: The biomass liquor was autoclaved for 20 minutes at 115ºC, cells lysed with acetone to release intracellular lysine. Cellular debris was separated by centrifugation at 10,000 rpm for 10 minutes and concentrated at 80ºC in water bath up to 500 mL of the total volume 2.5 L (Tanaka *et al*., 1978). A glass column (28 cm × 4 cm) was packed with ion exchange resin (50 g) by following the method of Rodney (1993).The concentrated centrifuged fermented broth (5mL) having pH 2, was applied on of cation exchange resin (Dowex-60) for the separation of Lysine. Sample was fractionally eluted in labelled test tubes up to 2mL each, with sodium citrate buffer pH 3, 6 and 9 (trisHCl). Elute fraction was collected until there was no violet spot appeared on chromatographic paper with 0.2% ninhydrin in acetone.

Crystallization: The fractions containing lysine obtained by eluting with tris HCl buffer (pH 9) were concentrated to $1/4^{th}$ of volume on water bath at 80ºC. Then crystallization of L-lysine was carried out firstly by acidification of fraction with 8N HCl and adjusted its pH 4. Three volumes of 95% ethanol were added to concentrate fractions to get the crude brown crystals of lysine at 4ºC, crystals were re-dissolved in distilled water and decolourized with 0.5% activated charcoal. Concentrated and yellowish colour pure crystals of Lysine were obtained after addition of 95% ethanol (Su *et al*., 1990). TLC of lysine crystals (100mg/100mL H_2O) along with standard solution was performed as described by Stock and Rice (1967).

RESULTS AND DISCUSSION

Due to the increasing demand of Lysine for poultry feed sector in Pakistan, a prompt action is awaiting for the establishment of Lysine producing industry locally. Therefore a complete model was designed in this study which can be practically exploited. To get our target, Mass production of Lysine was examined in 10 litre flask having 5 litre of fermentation media, molasses was used as a carbon source, sample was collected after every 8 hours to determine the increase in lysine production, no lysine was observed in zero hour and 8 hours sample (fig 2) as bacterial cells were in their lag phase and adapt themselves to growth conditions (Druzina *et al*., 2011).The lysine production was started at just start of log phase after 16 hours (0.1233% lysine),where micro-organism exponentially grows and produce amino acids, enzymes and other metabolites. A rapid raise of lysine was observed after 24- 32 hours (0.5067- 1.076% lysine) with the promotion of exponential phase. The raise of Lysine titre continued and after 70 hours, the maximum Lysine 2.0167% was observed. With further no increase of lysine titre, probably depletion of essential nutrient and formation of inhibitory and regulatory products as described by Javaid *et al*. (2012). The Lysine content (2.0167 %) was estimated from fermented broth by following Chaves *et al*., (1988). This method was developed to determine Lysine content in fermentation media having metabolites as vitamins and other amino acids that did not interfere in reaction for lysine estimation. While (17- 18 mg/mL) Lysine was estimated by Rehman *et al*. (2012) following spectrophotometric method of Hsieh *et al*. (1995), using UV- mutated *Corynebacterium glutamicum* as fermenting agent. Rao *et al*. (2011) followed Chinard (1952) for quantitative assay of Lysine (45.34 g/L) and Nasab *et al*. (2007) estimated Lysine (48g/L) after 96 hours of incubation by using thin layer chromatography (TLC) method.

After completion of culturing, Lysine can be recovered by conventional means ion exchange resin,

extraction with solvent, precipitation, adsorption and chromatography. Cation exchangers provide an efficient and appropriate method for purification of Lysine from fermented broth. The results of cation exchange resin are in line with Nakayama *et al*. (1973) and Jaffari *et al*. (1989), they also packed the column with resin and loaded sample of pH (3- 0.5) with same protocols, but reported 2N NH_4OH (pH 12) to elute Lysine.The sodium citrate buffers of increasing pH, from pH 3, pH 6 and trisHCl of pH 9 were used in present study. Concentrated fermented broth of pH 2 was loaded on highly acidic ion exchange, to bind all Lysine present in broth. The use of increasing pH buffers facilitated the elution of acidic, neutral amino acids and to separate Lysine from other metabolites respectively. Lysine was eluted with tris HCl (pH 9) as its isoelectric point is between 9- 10 pH, that ensured its complete recovery (Zurawick, 1999). Same method of ion exchange resin was described by Csapo *et al*. (2008) with increasing pH of elution buffers because at pH 2 all the amino acids binds with resins with no chromatographic divisions, waiting for change in pH. The comparison of ion exchange resin and HPLC was described regarding isoelectric points of amino acids. Isoelectric point of an amino acid is the pH at which amino acid dispose no electric charge. Crude crystals of lysine HCl were obtained which was of brown colour, the results of crystallization was in line with Su *et al*. (1990) which also decolourized it with 0.5% activated charcoal and pale yellow crystals of Lysine (fig.3), were obtained with 95% ethanol. Lysine thus crystallized in this study was 62.5% pure while Su *et al*. (1990), reported more purity (99.2%) in his study.

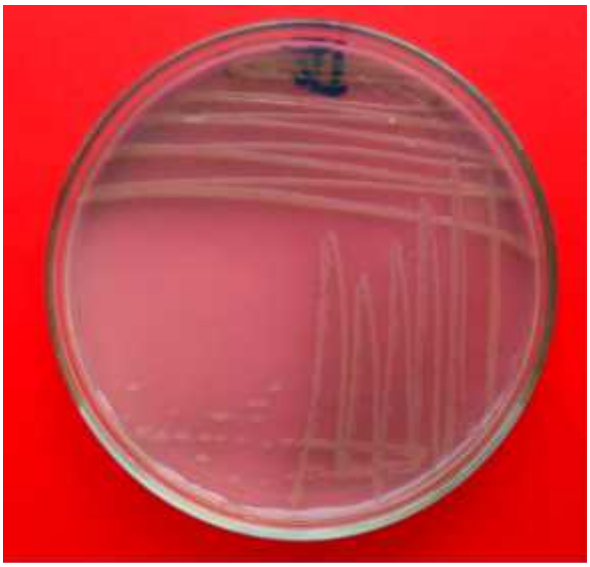

Figure 1: *Brevibacterium flavum* on nutrient agar plates at 7± 0.2 pH

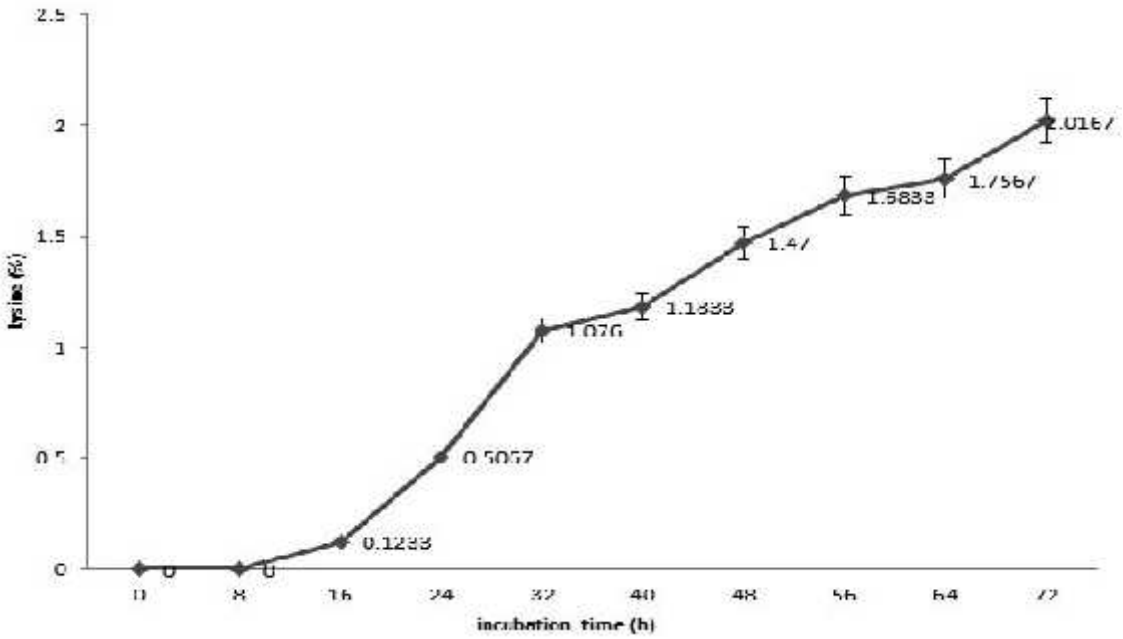

Figure 2: Increasing rate of Lysine production after every 8 hours interval of total 72 hours of incubation with *Brevibacterium flavum*, at 180 rpm and 30ºC

Error bars represent the increasing percentage of Lysine with increasing time intervals and has maximum percentage after 72 hours of incubation. (h)- represents hour; (%)- represents percentage

Identification of amino acids is extremely important for amino acid fermentation as well as for evaluation of protein structure. TLC is an important authentic technique used for the identification of amino acids. Different spraying reagents were used to visualize spots and ninhydrin is one of them due to its high sensitivity (Laskar *et al*., 2001). Deep violet colour spots with same R_f value as that of the standard and sample were observed after spraying with 0.2% ninhydrin in acetone. That clearly indicated that the lysine crystals obtained in this study were of high purity as compared by the standard Lysine. Furthermore, there were no other spots appeared except Lysine spots (fig. 4). This was also indicated that Lysine produced in the study is almost pure Lysine. Nasab *et al*. (2007) also shown the presence of Lysine in fermented broth by paper chromatography using solvent; n-butanol, acetic acid and distilled water (2:2:1). Ekwealor and Obeta (2005) employed paper chromatography for the detection of Lysine in fermented broth. Solvent systems used include n- butanol: acetic acid: water (4:1:1, v/v) and phenol: water (5:1, v/v). Lysine and threonine was selectively separated and quantitatively determined using TLC by Nabi and Khan (2003) from a mixture of amino acids present in a commercially available drug.

Based on the observations, it is concluded that very high concentration of Lysine in the form of Lysine crystals can be prepared, with 62.5% purity. However the concentrated form of broth containing 8% Lysine can be safely used in poultry rations as a source of Lysine. It is a simple and cheap method for Lysine production which can be exploited on commercial scale to save annual foreign exchange.

Figure 3: Pale yellow Crystals of L-Lysine obtained after recrystallization of crude brown Lysine crystals

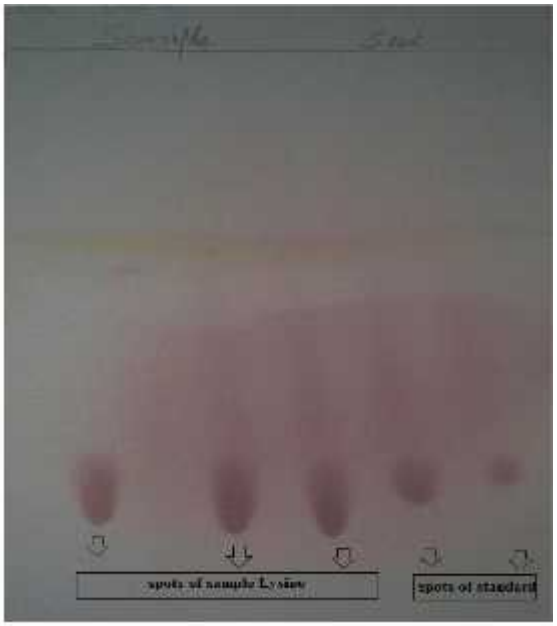

Figure 4: Thin layer chromatography of crystallized Lysine spots and standard Lysine spots.

Mobile Phase: chloroform, methanol and 17% ammonia (2: 2: 1), on the right side first three spots are of sample Lysine and next two spots are of standard lysine

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