World Inventia Publishers

Journal of Pharma Research

http://www.jprinfo.com/

Vol. 8, Issue 6, 2019 ISSN: 2319-5622 USA CODEN: JPROK3

Research Article

PRODUCTION AND CHARACTERIZATION OF L-LYSINE BY SMALL SCALE LABORATORY CULTURE METHOD BY SELECTED MICRO ORGANISMS

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Received on: 21-04-2019; Revised and Accepted on: 18-06-2019

ABSTRACT

Amino acids are the basic bio elements of proteins, which are the most important macromolecules for the functions of humans and animals. These are molecule containing an amine group, a carboxylic acid group, and a side chain that is specific to each amino acid. The key elements of Amino acids are Carbon, Hydrogen, Oxygen and Nitrogen. Amino acids are the basic structural building blocks of proteins and other bio molecules. They are also utilized as an energy source. Out of the twenty amino acids, economically found in most of living organisms, L-lysine is one of the nine amino acids which are essential for human and animal nutrition. L-lysine is useful as medicament, chemical agent, food material (food industry) and feed additive (animal food). Its demand has been steadily increasing in recent years and several hundred thousand tons of L-lysine (about 800,000 tones/year) are annually produced worldwide almost by microbial fermentation It has also some pharmaceutical applications in the formulation of diets with balanced amino acid composition and in amino acid infusions. Chemical, enzymatic and fermentation processes have been used to synthesize lysine. Present work made an attempt to produce L-lysine by microorganisms like Bacillus subtilis, E.coli, and Saccharomayces cereviciae. Finally the yield obtained after fermentation is more by Bacillus when compared to E.coli and Sachharomyces cereviciae. The present paper discuss about characterization of L-Lysine by the application of different chemical, Chromatographic and Spectroscopic methods also. L-lysine produced by Bacillus given positive results to above mention methods.

KEYWORDS: *Production, Characterization and L-Lysine.*

INTRODUCTION

Lysine is one of the essential amino acids not synthesized biologically in the body. Children and growing animals have a high requirement of lysine, since it is needed for bone formation. Lysine is generally recognized as the most deficient amino acid in the food supply of both man and domestic meat producing animals. Since animal feed, such as grain and defatted oil seeds contain only small quantities of lysine, poultry, cattle and other live stocks are unable to synthesize this amino acid. So it must be added to this feed stuff to provide adequate diet. Out of the twenty naturally occurring amino acids, L-Lysine ($C_6H_{14}N_2O_2$; MW 146.19) is one of the 9 essential (histidine, isoleucine, leucine, lysine, methionine, phenyl-alanine, threonine, tryptophan and valine) and commercially important amino acids, economically found in naturally occurring proteins of all living organisms. Its major commercial form is L-Lysine-HCl (L-Lysine monohydrochloride) L-lysine is commonly produced in a stable and non-hygroscopic hydrochlorinated form ($H_2N(CH_2)_4CHNH_2CO_2H.HCl\ 2H_2O$) of a purity higher than 98.5% and moisture content less than 1%. It is mainly used as a feed additive in the animal feed industry, mixed with various common livestock such as cereals which do not contain sufficient levels of L-Lysine for the livestock's nutritional requirements, in especially for single-stomach (monogastric) animals like broilers, poultry and swine and as a supplement for humans, improving the feed quality by increasing the absorption of other amino acids. L-Lysine can be produced either by a chemical or a biochemical method, which is more economic, even though relatively low yields are obtained during the extraction of L-Lysine, requiring specific installations and the use of expensive products method for long lasting solid lysine compositions, suitable for animal feed supplements, which don't agglomerate in the presence of moisture and can for time not necessitating the use of expensive purified L-Lysine. As a fine chemical, it is utilized in human medicine, in cosmetics and in the pharmaceutical industry, particularly as ingredients of infusion solutions for pharmaceutical applications and as precursor for industrial chemicals. Furthermore, a production method for industrially producing an optically active lysine derivative useful as a pharmaceutical inter-mediate is described in L-Lysine can be produced either by a chemical or a biochemical method, which is more economic, even though relatively low yields are

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DOI: https://doi.org/10.5281/zenodo.3265338

obtained during the extraction of L-Lysine, requiring specific installations and the use of expensive products .The stereospecificity of amino acids and the steadily increasing L-Lysine demand necessitates indispensably their fermentative production (the Lisomer) over synthetic processes .Thus, L-Lysine-producing strains of the gram positive corynebacteria, especially Corynebacterium glutamicum, Brevibacterium flavum and Brevibacterium lactofermentum, have been used for the last fifty years for the industrial production of amino acids. Several hundred thousand tons of L-lysine (800,000tones/year) is presumably produced annually worldwide, almost exclusively using bacterial fermentations. Previous attempts were done by the production of L-Lysine using Corynebacterium, Brevibacterium and Yeast cell. Present work going to report production and characterization of L-Lysine by different microorganisms.

MATERIALS AND METHODS

Preparation of Fermentation media (Bacillus): Weighed 10gms of Lactose, 2gms of Ammonium sulphate and 5ml of Glycerol, 5gms of Meat extract, 2gms of Calcium carbonate and 1gm of Potassium dihydrogen Phosphate, 1 gm of Ferrous Sulphate in 250ml of Volumetric Flask. The Contents of the flask dissolved with distilled water, make up the volume to 250ml then add organism in the flask which placed on Rotary shaker for proper mixing of contents for about 24 hours. Maintain pH of the media about 7.5. If the pH of the media is acidic then maintain the basic conditions by adding NaOH. If the pH of the media is basic maintain the acidic conditions by adding HCl. After completion of proper mixing of the content of the flask It is subjected to rotary shaker incubator for 3-4 days.

Preparation of Fermentation media (E.Coli): Weighed 10gms of Dextrose, 2gms of Sodium Nitrate, 5ml of Glycerol and 2gms of Amylase, 2gms of Peptone Bacteriological powder. 1gm of Sodium Starch glycolate, 1gm of Potassium dihydrogen Phosphate. 1gm of Magnesium Sulphate transfer into 250ml of conical flask dissolved with distilled water. And make up the volume up to 250ml. After completion of proper mixing of the content of the flask It is subjected to rotary shaker incubator for 3-4 days.

Preparation of fermentation media (Saccharomyces): Weighed 10gms of Glucose, Ammonium sulphate 2gms and Potassium dihydrogen phosphate 1gm, Magnesium sulphate Heptahydrate 1gm and 5gms of Yeast Extract transfer into a 250ml of conical flask. Dissolved with distilled water and make up the volume up to 250 ml. The Content of the flask mixed thoroughly and add organism. The Flask was placed on rotary shaker for about 24 hours maintain the physicochemical conditions like pH and temperature. Maintain pH of the media about 5.3. If pH of the media is acidic then maintain the basic conditions by adding NaOH. If the pH of the media is acidic maintain the basic conditions by adding HCl. After completion of proper mixing of the content of the flask it is subjected to rotary shaker incubator for 3-4 days.

Equipments:

- Orbital shaker Incubator
- Rotary Shaker
- Centrifuge
- UV spectrophotometer
- Infrared spectroscopy.

RESULTS AND DISCUSSION

Yield obtained by Bacillus Subtilis, E. Coli, Saccharomyces (250 ml of production medium):

- Yield obtained by Bacillus subtilis was found to be 5%
- Yield obtained by yeast was found to be 1%
- Yield obtained by E.coli was found to be very less.

Qualitative and Quantitative Analysis of L-Lysine:

Qualitative and quantitative analysis of L-lysine is also an important point of consideration to obtain yield of the product. After centrifugation and filtration of fermentation broth, L-lysine is determined by various means of analytical methods.

Identification Test for L-Lysine:

Ninhydrin Test: Ninhydrin Test is also used in Amino Acid analysis of Protein. This test is due to a reaction between a Amino group of free Amino acid and Ninhydrin. Ninhydrin is a powerful oxidizing agent and its presents, Amino acid undergo oxidative de-amination liberating Ammonia, Carbon dioxide a corresponding aldehyde and reduced form of Ninhydrin. The Amine formed from a Amino group react with another molecule of Ninhydrin and is reduced product to give glue substance.

Preparation of test solution:

i) Prepared 1% Amino acid solution in distilled water.
ii) Taken 1ml Test solution in dry test tube and 1ml distilled water in other test tube as a control.
iii) Pour few drops of 2% Ninhydrin in both the test tubes.
iv) Kept the test tubes in water bath for 5mins.
v) Development of blue or violet color is Obtained.

Observation: There is a positive result obtained for lysine produced by Bacillus, E.coli, Yeast.

Lysine- decarboxylase Test:

- The purpose is to see if the microbe can use the amino acid lysine as a source of carbon and energy for growth. Use of lysine is accomplished by the enzyme lysine decarboxylase
- A medium containing lysine and a pH indicator is used. When lysine is used, the pH of the medium rises and the indicator changes color.
- The medium used is lysine decarboxylase broth. The medium is a nutrient broth to which 0.5% lysine is added. An important component of the medium is a modest amount of glucose, necessary for the process to proceed. The pH indicator from cresol purple is purple at neutral or alkaline/basic pH but turns yellow at pH.< 5.2
- An inoculum from a pure culture is transferred aseptically to a sterile tube of lysine decarboxylase broth. The inoculated tube is incubated at 35-37 C for 24 hours and the preliminary results are determined. The microbe must first use the glucose present to cause the pH to drop. This is indicated by a change from purple to yellow. Once the medium has been acidified, the enzyme lysine decarboxylase is activated. The culture is incubated an additional 24 hours at 35-37 C to allow the microbe to now use the lysine. The final results are then obtained by observing the tube at 48 hours. Change back to purple from yellow indicates a positive test for lysine decarboxylase. Failure to turn yellow at 24 hours or to revert back to purple at 48 hours indicates a negative result.

Observation: The test result obtained for L-Lysine produced by Bacillus subtilis.

Seperation of Amino Acid by Thin Layer Chromotography Method:

Materials Required:

Reagents:

1. 2% solution of Individual Amino Acid
2. Solvent mixture of normal butanol, acetic acid and water in the ratio 4:1:5 by volume.
3. Ninhydrin reagent.

Requirements:

1. TLC plate 2. TLC chamber 3. Capillary tube 4. Reagent spray bottle 5. Beaker

Procedure:

1. Pour the solvent mixture in to the TLC chamber and close the chamber.
2. The chamber should not be disturbed for about 30 minutes so that the atmosphere in the jar becomes saturated with the solvent.
3. Cut the plate to the correct size and using a pencil (never ever use a pen) gently draw a straight line across the plate approximately 2 cm from the bottom
4. Using a capillary tube, a minute drop of amino acid is spotted on the line
5. Allow the spot to dry.
6. Spot the second amino acid on the plate [enough space should be provided between the spots].
7. Repeat the above step for spotting the unknown acid.
8. Place the plate in the TLC chamber as evenly as possible and lean it against the side (immerse the plate such that the line is above the solvent).
9. Allow capillary action to draw the solvent up the plate until it is approximately 1 cm from the end.

Remove the plate and immediately draw a pencil line across the solvent top Under a hood dry the plate with the aid of a blow dryer. Spray the dry plate with ninhydrin reagent. Dry the plates in hot air oven at 105°C for 5 min. [Ninhydrin will react with the faded spots of amino acids and make them visible as purple colored spots. After some time, mark the center of the spots, then measure the distance of the center of the spots from the origin and calculate the Rf values. Rf value can be calculated using the formula.

$$Rf = \frac{\text{Distance travelled by component}}{\text{Distance travelled by liquid}}$$

Rf value of Standard Lysine was found to be Rf = 0.7/4 = 0.14
Rf Value obtained for sample lysine is Rf = 0.6/5 = 0.12

Observation: Rf value Similar to standard L-Lysine

Wavelength determination by UV visible spectrophotometer:

A UV Spectrum of L-Lysine was recorded by scanning between 210-400nm. From the spectrum Lysine showed maximum absorbance at 270nm

Infra red spectroscopy of L-Lysine:

Infrared (IR) spectroscopy is one of the oldest and well established experimental techniques for the analysis of secondary structure of polypeptides and proteins. L-Lysine was structurally determined by Infra red spectroscopy.

Table No. 1: Fermentation media for Bacillus subtilis

Materials	Quantity
Lactose	10 gm
Ammonium sulphate	2 gm
Meat extract	5 gm
Glycerol	5 ml
Calcium carbonate	2 gm
Potassium di hydrogen phosphate	1 gm
Ferrous sulphate	1 gm

Table No. 2: Fermentation media for E.coli

Materials	Quantity
Dextrose	10 gm
Sodium nitrate	2 gm
Glycerol	5 ml
Amylase	2 gm
Peptone bacteriological powder	2 gm
Sodium starch glycolate	1 gm
Potassium di hydrogen phosphate	1 gm
Magnesium sulphate	1 gm

Table No. 3: Fermentation media for Saccharomyces cerveciae

Materials	Quantity
Glucose	10 gm
Ammonium sulphate	2 gm
Potassium di hydrogen phosphate	1 gm
Magnesium sulphate hepta hydrate	1 gm
Yeast extract	5 gm

Fig. 1: Production media in orbital shaker Incubator

Fig. 2: Results for Ninhydrin test

Fig. 3: Results for Lysin –decarboxylase test

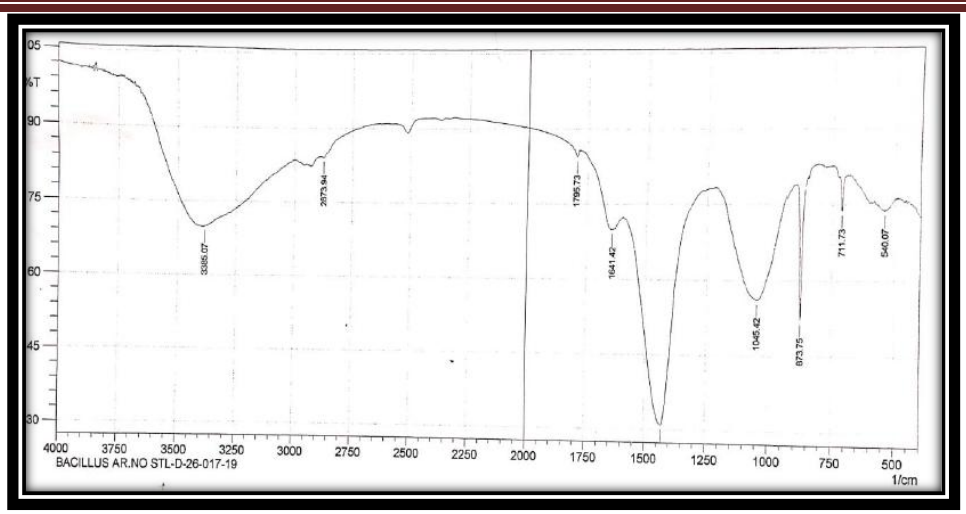

Fig. 4: IR spectra of lysine produced by Bacillus subtilis

CONCLUSION

L-lysine production by means of fermentation methods applying a variety of strains of microorganisms obtained either by solid state fermentation and submerged fermentation with different production media. Present work was attempted by production of Lysine by Small scale laboratory culture method using microorganisms like Bacillus, E.coli and Yeast.

Progressive results and maximum yield of Lysine obtained with Bacillus subtilis and it was confirmed by both chemical and analytical tests. Lysine produced by three cultures showed positive results for ninhydrin and Lysine produced by Bacillus given confirmed result with Lysine decarboxylase test.

It was further characterized by thin layer Chromatography, Spectroscopy and IR infrared spectra. A little work has been devoted to the fermentation process development and optimization, still leaving large opportunities for further improvements in terms of improving productivity concentration and yield of L-Lysine

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How to cite this article:

V. Asha Ranjani et al. PRODUCTION AND CHARACTERIZATION OF L-LYSINE BY SMALL SCALE LABORATORY CULTURE METHOD BY SELECTED MICRO ORGANISMS. J Pharm Res 2019;8(6):424-428. **DOI: https://doi.org/10.5281/zenodo.3265338**

Conflict of interest: The authors have declared that no conflict of interest exists.

Source of support: Nils