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Emerging Trends on L-Lysine Fermentation

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INTRODUCTION

Amino acids are the basic bioelements of protein, which are the most important macromolecule for the functions of human and animals. Out of the 20 L-amino acids that the body needs for growth and tissue repair. L-lysine is one of the 9 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 year and several hundred thousand tones of L-lysine (about 800,000 tones/year) are annually produced worldwide almost microbial fermentation.

The stereo specificity amino acids (the L isomer) makes fermentation advantageous compared with synthetic process. Mutual auxotrophic or resistant to certain chemicals strains of so – called gram positive coryneform bacterial are generally used, including the genera ***Brevibacterium*** and ***Corynebacterium*** united to genus.

The significance of Research & Development increased Rapidly since the discovery of fermentation amino acids production in the fifties, leading to Innovation fermentation process which replaced the classical manufauring method of L-lysine like acid hydrolysis. L-lysine is separated and purified by suitable downstream process involving classical separation of Extraction methods (Ultrafilteration or Centrifugation, Separation or ion exchange, Extraction, Crystallization, Drying) and is sold as powder. Alternatively, spray dried pellet or liquid fermentation broth can be used as animal feed supplement. On behalf of today's strong competition in amino acid industry,

biotechnology companies are continuously aiming in innovative research development and use complex managements concepts and business strategies, towards gaining market leadership in the field of amino acid production.

Sources:

In addition to supplements, certain food contain large amounts of L-lysine, these includes dairy products, apricots, apples, pears, figs, avocades, salmon, sword fish, turnips, tomatoes and soyabeans, legumes (e.g. lima beans and lentils) also

provide essential lysine.

Dietry Sources

The human nutritional requirement is 1 – 1.5g daily. It is limiting amino acids (the essential amino acid and found in the smallest quantity in the particular food stuff) in all cereals grains, but in plentiful in all pulses (legumes). Food that contain significant amount of lysine include

1. Red Meat (14,200 – 15,000 PPM)
2. Eggs
3. Buffalo Gourd (10,130 – 33,000 PPM) in seed.
4. Water cress (1,340 – 26,800 PPM) in seed.
5. Soyabean (24,290 – 26,560 PPM) in seed.
6. Carob, Locust Bean, St. Johns, Bread (26,320 PPM) in seed.
7. Carob, common Bean as Black Bean, Green Bean,Haricot, Haricot Beans, Haricot Vert, Kitnery Bean, Garden Bean, Popping Bean, Snap Bean, Wax Bean (21,390 – 25,700 PPM) in sprout seeding.
8. Mesquite.
9. Spinach (1,740 – 20,664 PPM)
10. Asparagus Pea
11. Pea (3,170 – 14,995 PPM) in seed.
12. Amaranth, Quinoa
13. Buck wheat
14. Lentil (7,120 – 23,735 PPM) in sprout seedling.

Good source of lysine are food rich in protein including meat (specifically red meat, lamb, pork and poultry), cheese (particularly parmesan), certain fish (such as cod and sardines) and egg.

Properties

L-Lysine is necessary building block for all protein in the body. L-lysine plays a major role in calcium absorption; building muscle protein; recovery from surgery or sports injuries; and the body production of hormones and antibodies.

L-Lysine uasage

1. L-lysine is an exact configuration of the lysine molecule frequently uses in dietary supplements.
2. L-lysine is essential for collagens synthesis and it may bvital to bone health because it appears to enhance the body suck and up and preserve calcium.
3. L-lysine supplement can speed healing time and decrease the chance of reumat breakouts of the herpes problem creates due to harmful organism.

4. Lysine appears to enhance the body absorb and preserve calcium. 5. Reduces the feeling of helplessness, counteract

fatigue, and enhance in the absorption of calcium must to suitable growth.

6. L-lysine appears work with equivalent amounts of amino acids arginine. 7. L-lysine enhances get the better absorption of calcium from the digestive tract and beneficial effect loss of calcium in the urine. 8. Lysine strengthens circulation and enhance the defence mechanism of body.
9. L-lysine supplement is one of the best option existing for the favorable effects of herpes simplex virus problem created due to harmful organisms, especially oral forms.
10. L-lysine has also been used to pleasure sores caused by the herpes simplex and herpes zoster virus.

Modifications

L-lysine can be modified through acetylation, methylation, sumoylation, needylation, biotinylation and carboxylation which tends to modify the function of the protein of which modified lysine residue (s) are a part.

Clinical Significance

It has been suggested that lysine may be beneficial for those with herpes simplex infections. However more research is needed to fully substantiate these claims. For more information, refer to Herpes simplex – lysine.

There are lysine conjugates that show promise in the treatment of cancer, by causing cancerous cells to destroy themselves when the drug is combined with the use of phototherapy, while leaving non- cancerous cells unharmed. While chemically insignificant to lysine itself, it is worth nothing that lysine is attached to dextroamphetamine to form the prodrug hisdexamfetamine (vyvanse). In the gastrointestinal tract, the lysine molecule is closed from the dexatroamphetamine, thereby making oral administration is necessary.

Disadvantage of L-lysine

L-lysine supplements have the potential to cause stomach pain and diarrhea in some users by the uses of lysine. Although the use of lysine, although very high dosage (more than 140 gram a day) and very large dosage of lysine (10 to 30 gram a day) increase toxicity of aminoglycoside antibodies, such as gentamicin, neomycin and streptomycine. It is dangerous to drink milk at the same time of taking lysine.

Chemical Composition of Formation L-lysine

1. 75g / l glucose.
2. 40g / l Ammonium Sulfate
3. 40g / l $CaCO3$
4. 100g / L Corn Step Liquor
5. 1g / KH_2PO4
6. 0.4g / L $MgSO_37H_2O$
7. 0.01g / L $FeSO_47H_2O$
8. 6mg / L $MnSO_4 4H_2O$
9. 300µg / L biotin
10. 500µg / L thiamine – HCl
11. 0.01g / L Pantothenic acid
12. pH 7.6 – 8.0

Alternatively L-lysine Production Chemical Composition

1. 30g / L glucose
2. 20.0 g/L Soyhydrolysate
3. 0.5g / L ammonium sulfate
4. 3.0g / L urea
5. 1.0 g / L KH2PO4
6. 0.5g / L MgSO4 7H2O
7. 0.002g / L MnSO4
8. 0.0001 g/L biotin
9. 0.0001 g/L thiamine – Het

The effect of various amino acids (1-3g /L) L-lysine production was investigated. The addition of certain amino acid such as arginine, asparagus acid, isolenic or valive enhanced L-lysine production in contrary to lercine furthocermane, the degree of inhibitory on bacterial growth caused by AEC (S-2 aminoethy cystemine) was reduced by the addition of 1 g/L of arginine to the culture medium.

REVIEW OF LITERRATURE

Tissue carnitive deficiency and Tryglyceride accumulation and concomitant impairment in fatty acid oxidetory Research Paper Reports (ARTICLES): Latifa Khan and Mahtab Banji. National Institute of Nutrition, ICMR, Hyderabad 500-007, India.

Since the essential amino acids lysine is a precursor of carnitive, the effect of cereal diet insupplement with limiting amino acids or carnitive, on carnitive and lipid levels in tissue and fatty acid oxidation in heart of rate were examined. Male weanling rates were fed either a dict providing approximately 5% protein from wheat severely impaired growth and dimished carnitive level in plasma and skeletal muscle, but not heart, or liver. Oxidation of [1 – 14C] palmitate by the 600 x g supernatant fraction of heart homogenerated was also markedly impaired in wheat died fed rates and could not be fully restored by the addition of carnitive to the incubation mixture. Triglyceride content of heart, skeletal muscle and liver and total lipids in liver were markedly elevated in these rates. Supplentation of wheat diet with 0.2% carnitive produced significant increase in plasma, muscle and liver carnitive level, restore fatty acid oxidation and reduced the triglyceride level of the tissues, without affecting growth.

Supplementation of wheat died with lysine and threonine at 0.2% level (keeping the food intake the same as the unsupplemented group). It improves the growth of muscle carnitive levels and palmitate oxidation, and dimished the triglycerids level of tissues. Food restriction percentage raise palmitate oxidating nutrient, and the supply has to ensured either through adequate intake of precursor amino acids lysine and methionine or through performed carnitive

L-lysine dehydrogenase deficiency in a patient with congential lysine intolerance – W.BURGI, R. RICHTERICH & J.P.COLOMBO

Medizinisch – Chemisches Institute for Universilaat, Bern and Zentrallaboraterinn des Kantonsspibls Arm

University – Kinderklink, Bern
Recently, a new metabolic defect, which was belive to arise from congential lysine intolerance was described in a 3 month old baby girl. An enzymatic impairement in the pathway for the degradation of lysine seemed the most probable explanation of the main finding (i.e. high plasma lysine levels). Because the enzymes responsible for the metabolism
Cell line (strain) development
FLOW DIAGRAM PROCESS L – lysine fermentation
Classic
Mutagenesis
Recombinant DNA
technology

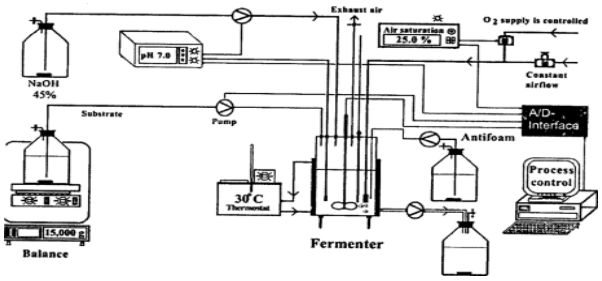
PROCESS DETAILS
L-Lysine Production
L-Lysine is mainly produced by fermentation using strains of corynebacteria, especially Cornebacterium glutamicum, which comprises a multi – step process including fermentation, cell separation by centrifugation or ultrafiltration, product separation and purification, exaporation and drying, because of L-lysine's great importance, efforts are constantly being made in

order to improve the fermentation process, comprising strain and process development as well as media optimization and downstream processing. Batch, fed batch, repeated batch and repeated fed batch (feeding during cultivation) are used for the production of L-lysine and other L-amino acids, operating in mixing tank or air lift fermenters. A continuous process may serve as an alternative application for the industrial production L=lysine. However only a few successful attempts can be found in the international bibliography regarding continuous fermentation of L-Lysine, because of technical difficulties and microbiological instability problems. The development of continuous fermentation processes for the production of citric acid and gluconic acid as well as L-lysine have shown numerous of the last 100 years. Thus, a continuous production of L-lysine also including the repeated fed batch mode seems to be very promising for future applications.
The development of new multi-step biotechnological process requires three steps. Those are –
i. Identification and characterization of a suitable biological system (microorganism, biocatalyst)
ii. Increase of bioreactor productivity by systematic media optimization and adaptation of fermentation technology.
iii. Downstream process (cell separation by centrifugation or ultrafiltration, separation, evaporation and drying)

of lysine in human beings had not previously been investigate, efforts were made to find an enzyme for the existence of a either to unknown enzyme, l-lysine, nicotinamide adenine dinucleotide (NAD), oxidoreductase (deaminating) (trival name;L-lysine dehydrogenase) in human liverhomogenates.

Fermentation Purification Technology Dev

Medium VO, T, pH, etc
Metabolic engineering
Cell Separation Ion Exchange Chromatography Drying



MICROORGAMISMS AND STRAIN DEVELOPMENT
The fermentative production of amino acids started in the fifties (1956-57) with the discovery of glutamic acid producing bacteria by Japanese scientists. Since then, most of development work was devoted to the improvement of mutant strains involving classical and modern microbial genetics, while efficiency of commercial L-lysine production has steadily increased by the isolation of higly producing mutant strains. Microorganisms employed in microbial amino acid production are divided into 4 classes, including the wildtype, auxotrophic mutant, regulatory mutant and auxotrophic regulatory mutant strains. Mutant auxotrophic or resistant to certain chemicals strains of so called L-glutamic acid producing *Corynebacterium* or *Brevibacterium* sp are employed for L-lysine production. Process improvement for producing

larger amounts of l-lysine using microorganisms remains a continual attempt, whereas the continuous development of classical and modern genetics resulted in the development of superior stains imparted with properties adavantageous for the commercial productioof L-lysine. Selected mutant starins with improved productivity characteristics are used today in industry for the production of L-lysine, which are obtained by various methods including classical mutagenesis, plasma fusion, genetic engineering and any other techniques commonly used for mutation of microorganism. L-lysine yields (g product / g added carbon source x 100) between 40 and 50% are disclosed.
Microorganisms employed in microbial processes for the production of amino acids are divided into 4 major classes; wild-type, auxotropic mutant(auxotrophy), regulatory mutant and auxotrophic regulatory mutant strain (sensitivity and

analog resistance) cited in Mutants of Coryne bacterium and related organisms enable the inexpensive production of amino acids by direct fermentation of cheap carbon sources (e.g. molasses, acetic acid and ethanol).

Classical Mutagenesis: Classical mutagenesis of microorganisms comprises exposing to ultraviolet light irradiation (UV), X-ray irradiation, radiation irradiation(e.g. exposing to UV radiation at 30^0C for about 180 sec) and chemical mutagen treatment at room temperature, 30 or 32^0C for 10 – 30 min followed by mutant selection conducting replication on selective minimal agar plate media or LB agar plates. Alternatively, genes relevant to a resistance (resistant mutants) or genes controlling the synthesis of selected

amino acids (regulatory negatiove mutants) can be isolated from L-glutamic acid producing coryneform bacteria, then be subjected to a mutation treatment in vitro in order to ibtain a resistant, regulatory or recombinant mutant gene type which will substitute the corresponding wild type gene on the bacterial chromosome by established homologous recombination techniques. Auxotropic or to certain chemical and antimetabolies resistant 1 cysteine; acyl – lysine, methylated acyl-lysine, oxo-lysine, lysine hydroxamate and regulatory negative mutant strain of so-called coryneform bacteria with increased productivity are used as biocatalysts for the excretion of amino acids, including the genera.

Mutagenesis by protoplast Fusion

A process for the production of L-lysine is described in comprises culturing a protoplast fusion mutant strain derived by protoplast fusion between strains of *Corynebacterium glutamicum* and *Brevibacterium lactofermentum.*

Mutagenesis by Recombinant DNA Technology

More recent developments of recombinant DNA technology and molecular biology have also been used in order to improve L-lysine producing strains e.g. of *Corynebacterium glutamicum* or *Brevibacterium*, by amplifying individual biosynthesis genes, integrating genes into chromosomal DNA or inserting genes coding for enzymes being higly rate determining for the

biosynthesis of L-amino acid into one or at least two plasmid vectors, which have compatible replicating origins different fromeach other. For example the subunits carrying the biotin-carboxyl carrier protein domain and the biotincarboxylase domain in the nucleotide sequence (accBC gene) encoding the enzyme acetyl – CoA carboxylase, an lysC allele cosding for a feedback resistant aspartate kinase, a recombinant DNA fragment conferring resistant to aminoethylcysteine, the dapA gene coding for Dihydrodipicolinate systhase, the asd gene coding for aspartate semiadehyde dehydrogenase and nucleotide sequence coding for the accDA gene are amplified and in particularly overexpressed. Intracellular activity of one or more important enzymes can increase by increasing the copy number of the corresponding L lysine biosynthetic pathway gene(s) (gene amplication) in a host cell chromosome and the expression (over expression) by using a stronger promoter or a gene encoding for a corresponding enzyme with higer activity and optionally combining these measures, or mutating the promoter and regulatory region or the ribosome binding site located upstream from the structural gene and increasing the promoter strength.

Fermentation medium

In addition to physical parameters like pH, agitation and aeration rate, air saturation, temperature, dissolved CO2 and foaming, medium composition is a very important factor strongly influencing fermentation processes, pften being object of extensive process development and optimization studies. The culture medium must satisfy in a suitable manner the requirements of

microbial growth and production. Defined media acquiring pure growth requiring nutrients and essential additives or alternatively undefined media containing natural organic substances such as soybeamhydrolyzate, corn step liquour, yeast extract or peptone are used for L-lysine fermentation. Common fermentation media for L-lysine production contain various carbon and nitrogen sources, inorganic ions and trace elements, amino acids, vitamins (biotine, thiamine – HCl, Nicothin amide) and numerous complex organic compounds. An over – expression of genes is also achieved by optimizing the composition of the media and the culture technique in addition to physiological and genetic parameters. E coli culture medium for L-lysine production contains one or more types of inorganic salts, including potassium monohydrogen phosphate (K2HPO4), magnesium sulfate (MgSo4), sodium chloride (NaCl), magnesium chloride (MgCl2), ferrous chloride (FeCl2), ferrous sulfate (FeSO4) and manganese sulfate (MnSO4), one or more organic compounds such as yeast extract, peptone, meat extract, corn steep liquor and a soyabean or wheat effluent. Furthermore they require suitable amounts of a saccharide strating material, vitamins and the like as required. Ethylene glycol resistant mutants has been reported to be insensitive to concentrated aqueous solutions of sodium chloride, potassium chloride, ammonium chloride, ammonium sulfate, potassium sulfate, sodium sulfate, glucose, fructose, sucrose, and maltose which would inhibit the growth of the parent strains. Additionally, calcium carbonate is added to shake flask cultures acting as buffer.

Carbon Source

Mutants of *Corynebacterium* and related microorganisms enable the inexpensive production of amino acids from cheap renewable carbon sources by direct fermentation. Various carbohydrates are utilized individually or as a mixture for the production of L-lysine such as glucose, fructose, sucrose, molasses (Sucrose, glucose, fructose etc), maltose, blackstrap molasses, starch hydrolyzate) lactose, maltose, starch and starch hydrolysates, cellulose, cellulose hydrolysate, organic acids such as acetic acid, propionic acid, benzoic acid, formic acid, malic acid, citric acid, benzoic acid, formic acid, malic

acid and fumaric acid, alcohols such as ethanol, propanol, inositol and glycerol and certainly hydrocarbons, oil and fats such as soyabean oil, sunflower oil, groundnut oil and coconut oil as well as fatty acids such as e.g.palmitic acid, stearic acid and linoleic acid, those substnces may be used individually or as mixtures.

Nitrogen Source

Various sources of nitrogen are utilized individually or as mixtures for the commercial and pilot scale production of L-lysine including inorganic compounds such as gaseous and aqueous ammonia, ammonium sulfate, ammonium nitrate, ammonium phosphate, ammonium chloride, ammonium acetate and ammonium carbonate. Alternatively, natural nitrogen containing organic materials like soyabean-hydrolzate, soyaprotein HCl – hydrolyzate, soyabean meal, soyabean cake hydrolysare, corn steep liquour, casein

hydrolysate, yeast extract, meat extract, malt extract, urea, peptose, and amino acids may also be utilized.

Inorganic Salts, Trace Elements and Growth Factors

Further components are necessarily added to fermentation media at the initiation and / or intermittently during the course of L-lysine fermentation, such as inorganic salts of various metals, like magnesium (e.g. magnesium sulfate), manganese and zinc or traces of other metals. Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium salts are commonly used as source of phosphorous for the production of L-lysine. Essential growth factors such as amino acid and vitamins(e.g. vitamin B1) and suitable precursors are also added to the culture in addition to the substances mentioned above, one – off batch or intermittently during the cultivation.

Influence of Oxygen

No significant information has been found in patent literature regarding the effect of air saturation and a little is known regarding the real effect of oxygen on L-lysine fermentation. L-lysine fermentation is an aerobic process

demanding large amounts of oxygen and strongly influenced by the air saturation in bioreactor. Lactic acid is formed as a byproduct under anaerobic conditions, which is re-consumed after the establishment of aerobic conditions. Aerobic conditions are maintained by aseptically adding to the culture oxygen containing gaseous mixtures. Cultivation of L-lysine producing microorganisms is carried out with shaking of shake flasks or by the aeration of stirring bioreactors. Enormous effects of air saturation on continuous L-lysine production by B. *lactofermentum* have been found in chemo stat process development experiments.

Influence of Temperature

A wide range of optimum and operational temperatures have been disclosed in the international patent bibliography for L-lysine fermentation, in order to protect the whole usable range.

Influence of pH

The pH is a very important factor strongly influencing microbial fermentations. Basic compounds such as sodium hydroxide, potassium

hydroxide, ammonium hydroxide, calcium carbonate, urea, ammonia and gaseous ammonia or inorganinc acid compounds such as phosphoric or sulfuric acid and organic acids are utilized for controlling pH in L-lysine cultures at a pH ranging from 5 to 9 discloses L-lysine production by mutant strains at a pH spectrum between 5 and 8.5 between 6.0 to 9.0, in the range from 6 to 8 and a pH in the range of 5.8 to 8.5, preferably 6.5 to 7.5

Antifoaming

Foaming occurring during fermentation is controlled by the addition of antifoams such as fattyt acid polyglycol esters or silicone and polyproplyne.

Plasmid Protection

Suitable selectively acting substances e.g. antibiotics are added into the medium inorder to maintain the stability of plasmids.

L-Lysine Fermentation Result

A little of the work described in the patent literature has been devoted to the fermentation process development and optimization, compared with the genetic improvement of L-lysine production strains. According to international patent bibliography, bacterial strain and process development has resulted in the continuous increase of L-Lysine titers and yield the last 50 years of L-lysine fermentation. Fermentation usually operates until concentration of L-lysine or of desire product reaches a maximum. This target is normally achieved within 10 hours to 160 hours for L-lysine production.

Downstream Processes of L-lysine

Product separation and purification is very important factor enormously affecting fermentation process effectivenss and production costs, steadily requesting improvements in the recovery process of amino acids, especially L-lysine. L-lysine is recovred from fermentation both in various ways. For many years L- lysine HCl solid has been produced following various steps such as fermentation, separation, purification, crystallization and drying. L lysine od resultant culture both can be recovred by known conventional of L lysine from culture both. After cell separation by cell filteration or

centrifugation, L-lysine amy be recovred from fermentation both by an ion exchange step and therafter concentrated by evaporation and spray dying.

Cell Separation

The fraction of L-lysine fermentation broth is obtained by any suitable separating methods such as ultrafiltration or centrifugation. In order to overcome difficulties occurring in due of high viscosity of culture broth, filterability can be improved by adding a mineral acid to adjust the pH to 1 – 3 or by adding an alkali to adjust the pH to at least 9 and then

heating the culture broth to =80^0C (100^0C) for at least 5 minutes.

Solid Feed Additive of L-lysine

It is described that a process for the production of an amino acid feed supplement which still contain a most of fermentation broth solids describes a fermentation process for the preparation of stable long lasting L-lysine sulphate for animal nutrition, adding sulfuric acid or ammonium sulphate. Concentrated l-lysine fermentation solutions have the same nutritive properties as purified lysine and biomass, other medium components or their derived products do not have any detrimental effect on animal health.

CONCLUSION

L-lysine production by means of fermentation methods applying a variety of corynebacterium and other microorganisms obtained either by classical mutagenesis or recombinant technology still continuous to remain a first runner in biotechnological production of bulk chemicals. It is assumed that no other microbial process has been undertaken to such extensive efforts of gaining better mutant strains. A little work has been devoted to the fermentation process development and optimization, still leaving large opportunities for further improvements in term of improving productivity for further improvements in terms of improving productivity concentration and yield of L-lysine. The results (about 100g / 1 L Lysine) obtained in continuous cultures (chemostat) at low residence times using a regular L lysine producing ATCC strain confirmed those thoughts. In comparison, the legendary fermentation production of citric acid is reflecting the development of fermentation technology and process development occurring in the last 100 years of modern biotechnology.

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