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FERMENTATIVE PRODUCTION OF L-LYSINE: FUNGAL FERMENTATION AND MUTAGENESIS-II A REVIEW

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ABSTRACT:

Lysine has been recognized as one of the most deficient essential amino acids. It is an economically important food and feed supplement. It has also got various pharmaceutical applications in the formulation of diets with balanced amino acid concentration and in amino acid infusions. Chemical, enzymatic and fermentation processes have been used to synthesize lysine. The objective of this review is to outline the research work of various scientists which provide important and beneficial information regarding the production of lysine through fungal fermentation and mutagenesis. Moreover, methods to improve the total yield and quality of lysine are also presented.

INTRODUCTION

In our previous attempts of this series (Shah *et al.,* 2002) we have reviewed the fermentative production of L-Lysine by bacteria (Shah *et al.,* 2002). In this review the production of Lysine through fungal fermentation and mutagenesis would be reviewed. Lysine has been recognized as one of the most deficient essential amino acids in the food supply of both human beings and meat producing animals because it is not synthesized biologically in the body (Pelczar *et al.,* 1993). Research on the possible utilization of wild strains revealed that many microorganisms, such as bacteria, yeast, filamentous fungi and actinomycetes, accumulated amino acid in culture containing a supplementary source of nitrogen. Efforts have recently been made to elucidate the mechanisms of microbial production of amino acids. The most outstanding results concern metabolic regulation and amino acid transport. The biosynthetic pathways of most amino acids are now well documented (Anonymous, 1992a, 1992b and 1992c) and the focus of attention has therefore, moved to metabolic control and its break down, including the genus and species specificity of the phenomenon (Aida, 1972). Fifteen amino acids were found in cell hydrolyzate, of which arginine (1.14 g/L) and L-lysine 0.4 g/L were the most abundant (Nakayama, 1972).

Two distinct biosynthetic pathways are known for L-lysine production. In certain actinomycetes fungi and algae the carbon skeleton of L-lysine arises from acetate and α-ketoglutarate by biosynthetic sequences that include α-aminoadipic acid (Anonymous, 1993). The other pathway has been found in bacteria, higher plants, blue green algae and certain fungi (some phycomycetes) and protozoa (Ohsumi *et al.,* 1994). The L-lysine carbon chain is synthesized from pyruvate and aspartate and α-ε-diaminopimelic acid is a key intermediate.

Aminoadipic Acid Pathway:

Studies on the biosynthesis of L-lysine in *Neurospora* began with the isolation of several different L-lysine requiring mutants of this organism. One such mutant was able to use α-

aminoadipic acid in place of L-lysine (Mitchell and Houlahan, 1948). In experiments on mutants that could grow on either L-lysine or α-aminoadipic acid, it was found that virtually all the radioactivity of C^{14}-α-aminoadipic acid supplied in the medium was incorporated into L-lysine (Windor, 1951). The result indicated that α-aminoadipic acid is a precursor of L-lysine in *Neurospora.* Certain L-lysine requiring mutants of yeast, *Ophiostoma multiannulatum,* grew when supplemented with DL-α-aminoadipie acid, L-α-aminoadipic acid and α-ketoadipic acid. Acetate condenses with α-ketoglutarate to yield homocitric acid, which by reaction analogous to the citric acid cycle, would yield homocitrate, oxaloglutarate and α-ketoadipate (Strassman and Weinhouse, 1953). The formation of α-aminoadipic acid from the α-ketoadipic acid takes place by transmination. Although the participation of α-aminoadipic acid is as an intermediate in L-lysine biosynthesis (Strassman and Weinhouse, 1953).

Studies on the utilization of radioactive α-aminoadipic acid by yeast cells have indicated that this compound is efficiently utilized for the synthesis of L-lysine (Larson *et al.,* 1963). α-aminoadipic acid -δ-semialdehyde has often been suggested as an intermediate between α-aminoadipic acid and L-lysine, and there is evidence for the formation of the aldehyde in the conversion of L-lysine to aminoadipic acid in animal tissue. An enzyme fraction obtained from yeast was found to catalyze the conversion of α-aminoadipic acid to the corresponding δ-aldehyde in the presence of adenosine triphosphate, reduced triphosphopyridine nucleotide and magnesium ions (Sagisaka and Shimura, 1962). A reasonable pathway for the formation of L-lysine from α-aminoadipic acid-δ-semialdehyde would seem to be transmination, or reductive amination; however, this compound exists in solution predominantly in the cyclic configuration and so far no convincing evidence has been reported for its enzymatic transformation to L-lysine. It has been suggested that α-N-acyl derivatives of α-aminoadipic acid and α-aminoadipic acid-δ-semi aldehyde are involved in L-lysine biosynthesis (Sagisaka and Shimura, 1962).

A possible solution to the problem of the conversion of α-aminoadipic acid-δ-semialdehyde to L-lysine has arisen from the discovery of saccharopine [ε-N-(L. glutaryl-2)-L-lysine] in yeast (Kuo *et al.,* 1964). They found that incubation of labeled α-aminoadipic acid with yeast cells led to formation of labeled saccharopine, the biosynthesis of saccharopine can be visualized in terms of Schiff base formation between α-aminoadipic acid-δ-semialdehyde and glutamate followed by reduction. It has been reported that the formation of saccharopine requires reduced diphosphopyridine nucleotide, and that saccharopine is converted to α-ketoglutarate and L-lysine by a diphosphopyridine nucleotide linked dehydrogenase.

A L-lysine requiring mutant of *Saccharomyces cerevisiae* has been reported to accumulate glutaric acid as well as α-aminoadipic acid (Mattoon and Haight, 1962). During these studies, Broquist *et al.* (1971) found that yeast accumulated large amounts of free L-lysine in the cell when incubated in the presence of α-aminoadipate, α-ketoadipate or α-hydroxy adipate. This occurred with various yeast such a *Saccharomyces cerevisiae* and *Torula utilis.* The L-lysine was readily extracted from the cells by hot water. Maximum accumulation of L-lysine reached 20% of cell weight corresponding to 60-83% conversion from substrate.

Diaminopimelic Acid Pathway:

The natural occurrence of ε-diaminopimelic acid was first reported by Work (1951), who

isolated this amino acid from hydrolyzates of *Corynebacteria diphtheria*. The studies of Work *et al.* (1951) and some other researchers (Work *et al.* 1955; Hoare and Work 1957; Meadow *et al.,* 1957) have shown that diaminopimelic acid is present in many bacteria and certain other microorganisms including several species of blue green algae. Diaminopimelic acid is a constituent of the cell walls of many of the organisms that contain this amino acid but it is not present in bacterial protein. Diaminopimelic acid was also found in a peptide in *Mycobacterium tuberculosis* (Meadow *et al.,* 1957).

Structural considerations suggested that diaminopimelic acid might be a precursor of L-lysine and this possibility was supported by the finding of a specific diaminopimelic acid decarboxylase in *E. coli* (Dewey and Work, 1952). A mutant of *E. coli* exhibited an absolute growth requirement for diaminopimelic acid and it was found that L-lysine exerted a sparing effect on the requirement for diaminopimelic acid. Certain L-lysine requiring mutants of *E. coli* were found to accumulate diaminopimelic acid and these did not exhibit diaminopimelic acid decarboxylase activity. These observations provided strong evidence for the participation of diaminopimelic acid in L-lysine biosynthesis.

Several approaches indicated that aspartate is a precursor of diaminopimelic acid (Arutyunyan, 1993). Thus, isotopic competition studies showed that aspartate competes with glucose in providing four of the carbon atoms of diaminopimelic acid and of L-lysine. Extracts of mutants that accumulated diaminopimelic acid catalyzed its synthesis, which is stimulated by addition of triphosphopyridine nucleotide, adenosine triphosphate, aspartate, succinates and pyruvate (Gilvarg, 1956; 1957; Rhuland and Bannister, 1956). A mutant of *E. coli* that exhibited an absolute requirement for diaminopimelic acid accumulated a compound which was identified as N-succinyl-L-α-, ε-diaminopimelic acid (Gilvarg, 1957; 1959).

After more studies on the *E. coli* mutant which accumulated N-succinyl-L-α, ε-diaminopimelic acid it was revealed that this organism could also accumulated N-succinyl-ε-keto-L-α-aminopimelic acid (Gilvarg, 1961). Transaminase activity capable of catalyzing reversible transamination between N-succinyl diaminopimelate and α-ketoglutarate was found in extracts of this mutant and also of the wild strain (Peterkofsky and Gilvarg, 1961).

Subsequent studies on the biosynthesis of N-succinyl-L-α-, ε-diaminopimelic acid by a mutant of *E. coli* established that the four-carbon moiety of aspartate is the precursor of the portion of the molecule that becomes succinylated (Edelman and Gilvarg, 1961). A very significant finding was the isolation of a mutant of *E. coli* that exhibited an absolute requirement for diaminopimelic acid, threonine, and methionine as well as relative requirement for L-lysine and isoleucine. This organism lacked aspartic acid β-semialdehyde dehydrogenase, the enzyme that catalyzes the formation of aspartic acid-β-semialdehyde from β-aspartyl phosphate. It is therefore, evident that aspartic acid β-semialdehyde is the branching point in the biosynthesis of several amino acid (Gilvarg, 1962). The branching reaction i.e., condensation of aspartic acid β-semialdehyde with pyruvate, is inhibited by L-lysine (Yugari and Gilvarg, 1962). This feed back effect thus effects the first reaction in the sequence that leads only to L-lysine. The available data indicates that diaminopimelic acid is an obligatory intermediate in the biosynthesis of L-lysine in *E. coli* and some other micro-organisms. An alternative path way of L-lysine formation not involving diaminopimelic acid was suggested by studies in which a mutant of *E. coli,* partially blocked in L-lysine biosynthesis, appeared to utilize diaminopimelic acid effectively for cell wall

diaminopimelic acid, but not for L-lysine synthesis (Meadow and Work, 1959). An interesting experiment carried out by Rhuland and Hamilton (1961) indicated that the diaminopimelic acid pathway is the only one leading to L-lysine biosynthesis in *E. coli.*

Aspartate and pyruvate or closely related compounds are also precursors of dipicolinic acid (Powell and Strange, 1957). A plausible pathway would be the condensation of aspartic acid β-semialdehyde and pyruvate to yield 2, 3-dihydrodipicolinic acid, followed by oxidation of the latter compound to dipicolinic acid. An alternative pathway is suggested by the observation of non-enzymatic conversion of 2, 6-diketopimelic acid (which might be formed from DAP or perhaps by another route) in the presence of ammonia to dipicolinic acid (Powell and Strange, 1959).

The accumulation of DAP in a culture broth by L-lysine auxotrophic mutant of *E. coli* was found first by Devis (1952) which was later studied as a process for L-lysine production. The amount of DAP produced by *E. coli* ATTCC 12, 408 reached 9 g/L. The whole broth including the cells was then incubated with *Aerobactor aerogines* (ATCC 12, 409) after addition of small amount of toluene and 100% of DAP was decarboxylated to yield L-lysine. DAP could be used in fermentation for the production of L-lysine; (Nakayama, 1972) producing 100% yield of L-lysine after 20 hrs. at 35^{o}C in a fermentor by *Aerobactor aerogenes* ATTCC 12.

Diaminopimelate decarboxylase (meso-2, 6-diamino pimelate carboxylase) an enzyme of L-lysine synthesis, has been extracted from the higher plant *Lemna perpusilla* grown on a new chemically defined medium (Shimura and Vogal, 1966). This finding supported earlier conclusions that the diaminopimelate-L-lysine path is characteristic of an evolutionary continuum ranging from bacteria to higher plant. The lemna enzyme exhibits considerable activity without added cofactor, whereas the *E. coli,* even in crude extracts, does not (While and Kelly, 1965).

Mutagenesis:

Microbial metabolites of possible industrial interest are normally produced initially in very low levels. However, when the product turns out to be of commercial value, programs of media development and strain improvement are initiated (Tempest *et al.,* 1983; Richard and Bhattacharjee, 1992). The over production of desired metabolite can be achieved by the genetic removal of feed back control, which is possible through mutation (Annonymous, 1985). The important thing is the rate at which mutation can be induced and the nature of mutation itself: Is it "tight or leaky"? Is it conditional i.e., the activity, osmosis or temperature is remedial? And is it revertible? In most cases tight mutations are required because activity is totally abolished. Non-revertibility is also desirable because it decreases the rate of "strain degeneration" (Jacobson, 1981).

The most potent (efficient) chemical mutagens are alkylating agents, e.g. ethyl methane sulfonate (EMS) and the nitroso compound such as N-methyl-N-nitro-N-nitrosoguanidine (NG). The alkylating agent EMS or NG can be used in strains, which are resistant to UV light. Damage to DNA from physical agents such as heat, ultraviolet light and ionization radiation such as X-ray and γ-irradiation can also result in gene mutation (Jacobson, 1981).

Ultraviolet light has been recommended as a mutagen of first choice. It can induce both base pair substitution and frame shifts mutations. Both lethality and mutation have been shown to decrease when culture are incubated in the dark under non-growth conditions (Bridge, 1976). The intrastrand cyclobutane pyrimidine dimer is the predominant DNA lesion produced by UV light (254 nm). Many other lesions have been reported such as hydration across 5-6 double bonds of pyrimidines and dimers with the amino acid cystein (Stelow and Carrier, 1966).

In cellular systems the pyrimidine dimer is the major cause of lethal and mutagenic damage. Furthermore, the reversal, or alteration and the then removal of pyrimidine dimer allows error free repair of UV damaged DNA (Sutherland, 1981). Secondly, pyrimidine dimers can be excised from DNA by excision repair mechanisms. The third mechanism called post-replication repair occurs after DNA synthesis. The terms "recombinational repair" and "daughter strand gap repair" have also been used to describe this specific system. The processes of photoreactivation, excision repair and post replication recombination repair are error free and almost certainly act to reduce mutant yield although there is a clear evidence to suggest that a low level of mistake does occur with, e.g., excision repair (Bridge and Mottershead, 1971). Depending on the mutation system under study, transition, frameshifts, deletions and duplication have been reported. Mutation can arise from pyrimidine dimer or other photo lesions and the type of mutation that result depends on the site of the primary lesion of the gene.

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