REVIEW ARTICLE

L-Methionine production in 21st century: A paradigm shift from chemistry to microbiology

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ABSTRACT

Japan began large-scale fermentation production of amino acids more than 75 years ago. However, L-methionine is an exception that cannot be produced on a large scale by fermentation, and hence no successful production plant has been established. It is most likely due to a lack of knowledge about its bacterial biosynthesis, including feedback regulation. Due to its huge market demands, several trials have been made but have been unsuccessful. This review tries to give an overview of how it was discovered, how it is made on a large scale, how the global market works, the metabolic pathways for bacterial synthesis of stereospecific production of L-methionine, how it is exported outside of cells, recent progress, recovery from production media, problems, and possible futures in this century.

Keywords: L-methionine, Fermentation, Bacteria, Market, Extracellular, Metabolic.

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INTRODUCTION

L-methionine is the principal sulfur-containing essential amino acid, having a hydrophobic side chain with a thioester bond, which acts as a precursor for the biosynthesis of other sulfur-containing amino acids and their derivatives. It commonly appears as the initiating amino acid for protein synthesis that humans must obtain from food.[1,2] It plays a pivotal role in carbon metabolism, acting as a methyl group donor as S-adenosylmethionine. It also plays a significant role in forming hydrophobic protein-related structures. Deficiency of L-methionine leads to several pathophysiological states in human beings, like childhood rheumatic fever, excess hair loss, muscular paralysis, depression, Parkinson's disease, abnormal growth patterns, liver diseases, schizophrenia, etc.[3] Vegetables and pulses generally have lower L-methionine content. Hence, pure vegetarians commonly suffer from L-methionine deficiency symptoms. In addition, after the recommendation of the Complementary Medicine Evaluation Committee in April 2000 for L-methionine as generally safe for therapeutic and dietary supplements, especially among geriatric people without dietary restrictions, a rapid increase in the L-methionine market was reported.[4]

Methionine production was started in June 1948 as a DL mixture by Werner Schwarze of Chemiewerk Homberg AG, a subdivision of Degussa, via chemical synthesis. The DL-methionine synthesis uses several hazardous chemicals, like methyl mercaptan, a potent carcinogen. Furthermore, the process of separating L-methionine from this racemic mixture is a difficult task. Commonly, D-methionine is enzymatically converted to L-isomer, which is also very costly.[4] Despite significant operational costs, numerous health hazards, and the need for extensive technical skills, chemical synthesis methods are a common practice in methionine synthesis at industrial scales. Since the discovery of microbial fermentation of L-glutamic acid, due to strong feedback inhibition in the microbial biosynthetic routes and a lack of knowledge of the sulfur metabolism of microorganisms, the fermentation process for L-methionine from raw materials has been unsuccessful. However, with an understanding of metabolic engineering and several biotechnological tools, it is hoped that stereospecific L-methionine can be achieved from inexpensive indigenous raw materials through suitable microbial strain development.

This review attempts to summarize the current scenario and possible prospects of L-methionine production by fermentation.

Brief History of Methionine Discovery and its Industrial Production

The sulfur-containing amino acid methionine was discovered by J.H. Muller in 1921 with an incorrect formula. His colleague Odaka, in Japan, corrected the formula in 1925. Later on, the name methionine' was given by G. Barger and F.P. Coyne with its correct structure. After World War II, researchers re-investigated the initial findings related to the discovery of methionine. They were interested in synthetic methionine for the treatment of nutritional edema arising from chronic protein deficiency among the soldiers. The first synthetic DL-methionine (a racemic mixture) was reported by Werner Schwarze, Hans Weger, and Hermann Schulz of Degussa

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in 1946–47. The chemical synthesis of methionine was initiated with acrolein, mercaptan, and hydrogen cyanide. Notably, acrolein was synthesized from acetaldehyde and formaldehyde by Wagner and Schulz in 1936, which facilitated the commencement of research at this laboratory, which had relocated to Konstanz in 1946. Finally, in 1948, Werner Schwarze successfully produced 1 kg of DL-methionine at Chemiewerk Homburg AG, a pharmaceutical subdivision at Degussa. Within one year, a plant with a production capacity of 30 metric tons per month was established, which started production at a rate of 300 kg per month. The world's first pharmaceutical-grade DL-methionine (0.5 g/tablet) was launched by Chemiewerk Homburg AG with the name 'Thiomedon.'. However, the first report on animal feeding attempts using animal models was published in 1953, indicating that the use of DL-methionine improved laying capacity among the animals. Soon after the study, the Ministry of Agriculture in Bonn issued a license for the large-scale use of DL-methionine in feeding industries. The Wesseling factory in Germany started producing DL-methionine in 1967. In 1974, the methionine plant in Antwerp, Belgium, started producing DL-methionine with a capacity of 12,000 metric tons per year. Mobile, Alabama, USA, started production in 1977 with a capacity of 230,000 metric tons per year. In 2006, Antwerp set up a new facility with a capacity of 120,000 metric tons per year. Evonik started producing a complex in Singapore for animal feed in 2014. The second complex of Evonik was established in 2019 for Asian consumers, with a production capacity of 150,000 metric tons per year (Evonik Industries report). Conventional methods of methionine production are still not satisfactory due to huge operational costs and the use of several hazardous materials.[4]

Global Market and Production Companies for Methionine

With the increase in methionine demands in health sectors, including pharmaceutical industries and animal feed, its global market size is gradually increasing. The estimated global market size for L-methionine was USD 5.67 billion in 2021 and is expected to increase at a rate of 9.3% from 2022 to 2030. North America, with its huge demand for methionine in poultry and broiler farms, is receiving major attention in this context. North America (US and Canada), Latin America (Brazil, Argentina, Mexico, and other parts of Latin America), Europe (Germany, UK, Spain, France, Italy, Russia, and other parts of Europe), the Asian Pacific (China, India, Japan, Australia, South Korea, ASEAN, and other subcontinents of the Asian Pacific), the Middle East (GCC countries, Israel, and other countries of the Middle East), and Africa (South Africa, North Africa, and Central Africa) are the major consumers of L-methionine. Aquatic sectors are also showing interest in the consumption of L-methionine. Most significantly, the European Union imposed restrictions on the use of non-organic proteins within 5%, and no synthetic DL-methionine is permissible for use in organic farming. Synthetic methionine is also more expensive than methionine produced from natural sources. Recently, increased demand for liquid methionine added extra weight to the further hike in its global market demand. In 2018, Adisseo declared it would set up its third liquid methionine production plant in Nanjing, China, along with two other plants in Burgos, Spain, and Nanjing, China (adapted from a report published by Coherent Market Insights, December 2022).

The major companies for methionine production include Evonik (Germany), Adisseo (France), AJINOMOTO (Japan), Novus International (US), Phibro (US), Prinova Group (US), Sunrise Nutrachem (China), CJ CHEILJEDANG (South Korea), Chongqing Unisplenous Chemical (China), Sumitomo Chemical (Japan), etc.

Present Strategies for Methionine at an Industrial Scale

No report on the satisfactory amount of stereospecific L-methionine production by fermentation is available. Traditionally, DL-methionine is produced via chemical synthesis from methyl mercaptan, acrolein, and hydrogen cyanide via the following reactions,[8] summarized in Figure 1. The flow diagram mentions the detailed steps (Figures 1 and 2). The product is then acetylated to N-acetyl-DL-methionine, which is then converted to L-methionine by L-aminoacylase,[8] as shown in Figure 2.

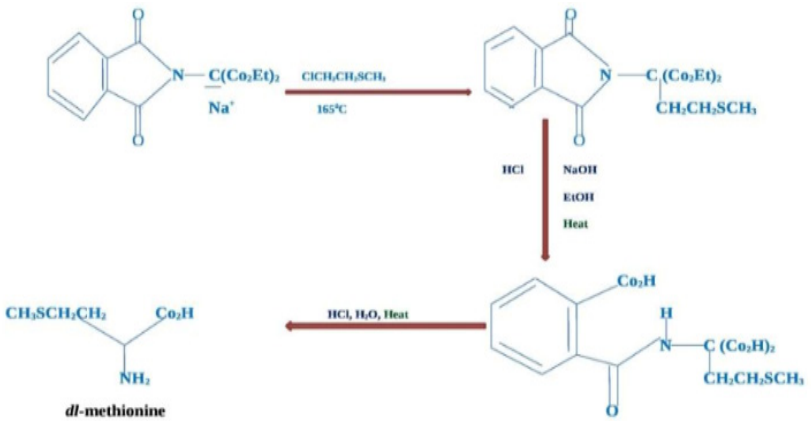

Figure 1: Chemical synthesis of *dl*-methionine

Metabolic Perspectives of Bacterial L-methionine Production

Biochemical pathways for L-methionine synthesis

Like other organisms, L-methionine appears to be comparatively less abundant than other amino acids in bacteria.[9] It is the key component of S-adenosyl methionine within the cellular microenvironment.[9] The estimated intracellular concentration of L-methionine in *Escherichia coli* is 5 mM during the exponential phase of growth in a glucose medium. Except for some endosymbiotic species, bacteria possess L-methionine synthesis systems. Methionine is derived from homoserine, a product of aspartate, via sequential reduction of the terminal carboxyl group. The formation of methionine is aimed at fulfilling the following mottos: (a) activation of homoserine by acetylation; (b) replacement of the hydroxyl side chain with a thiol group; (c) transfer of the methyl group to the thiol group for methionine production. Maximum studies on the microbial synthesis of L-glutamic acid have been carried out on *E. coli*. Information on L-methionine synthesis in other microorganisms is also available today. In microorganisms, enhancement of L-methionine biosynthesis is focused on removing negative regulation, regulation of metabolic flux analysis, and feedback inhibition of the enzymes involved in biosynthesis.[11-16] L-aspartate is converted to L-methionine by forming L-homocysteine through sequential reduction, sulfhydration reactions, and the methyl group transfer. There are two forms of methionine synthases (cobalamin dependent/MetH and cobalamin independent/MetE), both of which use 5-methyl-tetrahydrofolate. However, MetH showed almost forty times faster activity than MetE.[17] Besides these two traditional enzymes, two other non-traditional enzymes are also reported in this context: a short MetE-like enzyme identified in *Methanobacterium thermoautotrophicum* and another one is an unusual methionine synthase isolated from *Acinetobacter baylyi*.[18,19] Bacterial L-methionine synthesis is mostly studied in *E. coli* and *Corynebacterium glutamicum* (Figure 3).

In *E. coli*, L-methionine synthesis starts with the transfer of a succinyl group from succinyl CoA to the gamma hydroxyl group of L-homoserine by O-succinyltransferase (encoded by *metA*) to form activated homoserine called O-succinylhomoserine[1,20,21] In the next phase of reactions, trans-sulfurylation occurs through the transfer of the thiol group from cysteine to homoserine via the formation of O-succinyl homoserine and L-cystathione catalyzed by Cystathionine ¥ synthase (encoded by *metB*) and cystathionine β lyase (encoded by *metC*) to form L-homocysteine.[22] Finally, L-homocysteine is methylated to form L-methionine, which is catalyzed by two non-homologous enzymes: cobalamin-dependent methionine synthase or cobalamin-independent methionine synthase (encoded by either *metH* or *metE*, respectively).[1,23] On the other hand, *Corynebacterium glutamicum* utilizes two parallel pathways (transsulfuration and direct sulfhydration) for L-methionine synthesis,[24] as summarized in Figure 3.

Regulation of bacterial l-methionine synthesis at the molecular level

Bacterial L-methionine synthesis is regulated by repression and feedback inhibition.[25] Several attempts have been made to overcome such repression and feedback regulation by utilizing eco-friendly methods to produce the metabolically active L-isomer of methionine from non-toxic substances. However, no such fermentation plant has been established yet due to a lack of knowledge on the molecular understanding of the overall metabolic pathways and their

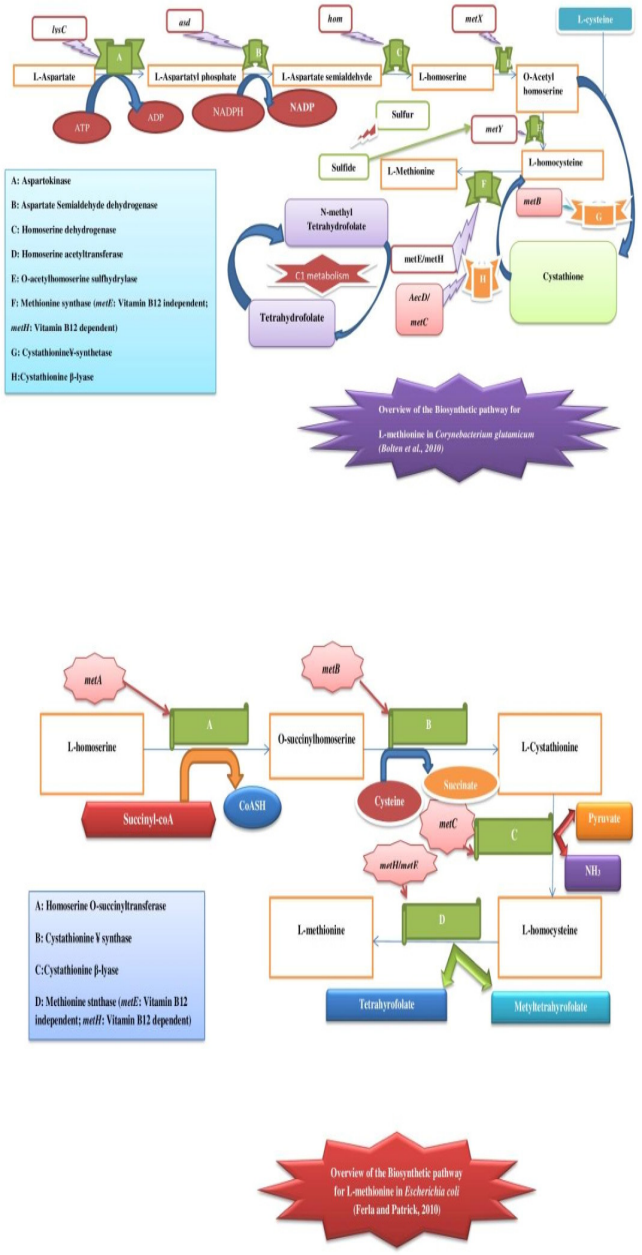

Figure 3: Bacterial Biosynthesis of L-methionine using *Corynebacterium glutamicum* and *Escherichia coli* as two model organisms

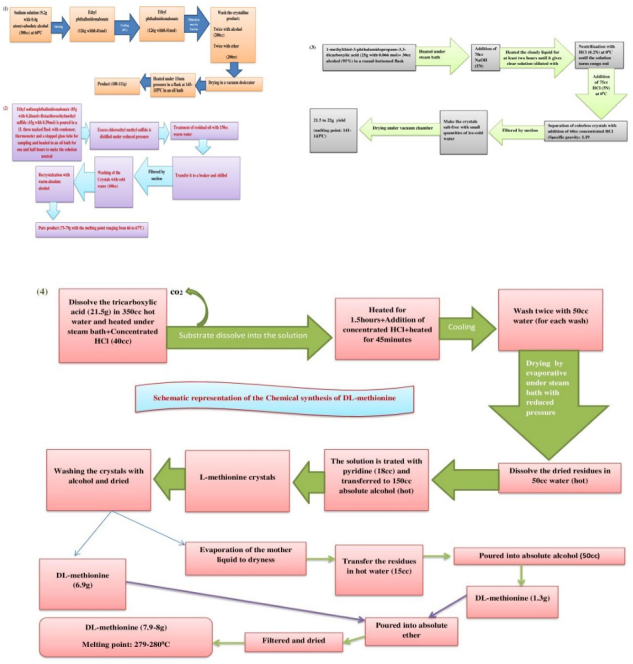

Figure 2: Chemical synthesis of *dl*-methionine and enzymatic conversion of D-methionine to L-methionine

tight regulation in bacterial models. In a cell-free extract of the methylotroph strain, OM33 indicated that homoserine-o-transferase acetylated L-homoserine. In this model, the enzyme responded to feedback inhibition by S-adenosyl-L-methionine. Another ethionine-resistant mutant, OE120, derived from OM33, was able to maximally overcome such feedback inhibition by S-adenosyl-L-methionine maximally, indicating an important role of homoserine-o-transferase in L-methionine synthesis.[26] *Met*J mutants of *E. coli* showed *an* enhanced level of non-repressible enzymes for L-methionine synthesis and S-adenosyl-L-methionine, which was initially suspected to act as a regulatory locus or code for enzymes involved in L-methionine biosynthesis.[27] Repression of the microbial biosynthesis in *E. coli* is MetJ protein and S-adenosyl-L-methionine. Apart from the repressor MetJ and co-repressor S-adenosyl-L-methionine, another protein called MetR has been identified and characterized as a trans-activator for *metE* and *metH* gene expression. Transcription of metRisR is in turn controlled by the L-methionine precursor L-homocysteine. Cobamide-containing MetH holoenzyme represses the expression of the metE gene.[28] It was also confirmed earlier, used in the plasmid pRSE562 with the *E. coli met*E and *met*R genes. In this paper, it was clearly shown that the product of the *met*R gene acted as a trans-activator for *met*E gene expression, and the metR gene had an autogenous pattern of regulation and showed repression with the MetJ protein.[29] In an in vitro study of the *E. coli* genes *met*B, metL, and *metJ*, it was found that the *met*B and *met*L genes were inhibited by the MetJ protein, whereas the *metJ* gene is showing partial auto-regulation by its gene product.[30] In *Streptococcus* mutans, homocysteine plays an important role in the regulation of *Met*R activity.[31] In *Staphylococcus aureus*, *Cod*Y activity, T-box, and mRNA decay control the synthesis as well as the stability of *met*ICFE-*mdh* mRNA.[32] Site-directed mutagenesis of *met*A (encoding L-homoserine O-succinyltransferase) and overexpression of *metAfbr*, metC, and *yje*H played a pivotal role in improved L-methionine production up to 1.93 L in submerged fermentation. Further improvement up to 2.51 g/L in the shake flask mode of fermentation was achieved with the deletion of *pykA* and *pykF* genes in a strain derived from *E. coli*. L-methionine production was further enhanced by 52.9% by improved expression of cys*E*fbr, serAfbr, and *cysDN*.[33]

Extracellular excretion of L-methionine

Amino acid fermentation gained attention in this century as a part of the green revolution in the context of amino acid overproduction. To maximize production, not only the synthetic pathways but also the extracellular export mechanisms have gained central focus in scientific communities. L-methionine export from *Corynebacterium glutamicum* was characterized by the DNA microarray technique for the identification of genes for membrane-associated exporter proteins, which are certainly overexpressed at high levels of intracellular L-methionine. In a series of experiments involving deletion, complementation, and overexpression of *Brn*FE, it was proven that this two-component amino acid exporter efficiently exports both methionine and isoleucine at the same rate. Furthermore, its gene cluster expression is dependent on the Lrp-type transcription factor, which is highly induced by an elevated level of intracellular L-methionine concentration.[34-36] Recent studies have identified YjeH as a potent L-methionine exporter.[37,38]

Recent Trends in Enhancement Strategies for L-Methionine Fermentation

Amino acid fermentation on an industrial scale has lasted more than 75 years since its beginning in the 1950s. Though the initial phases started with the aim of overproducing L-glutamic acid, later on, other amino acids (like lysine, threonine, etc.) gained importance in the scientific fraternity. With the advancement of biotechnology and genetic engineering, especially after the recommendation of the Complementary Medicines Evaluation Committee (CMEC) in April 2000 for L-methionine as a safe dietary supplement as well as a therapeutic agent without having substrate specificity, L-methionine fermentation gained central focus. However, due to a lack of knowledge of metabolic pathways for microbial synthesis and strong feedback regulation, no successful plant has been established until now. However, recent trends in research in biotechnological tools and techniques, as well as progress in new insights into biochemical pathways for microbial synthesis of L-methionine, including its extracellular export, throw light on the possible emergence of a new dimension in its large-scale

(industrial-scale) production using modern fermentation technologies. Disruption of *met*J with overexpression of genes encoding MetA and L-methionine exporter yejH led to a tenfold improvement in L-methionine production in *E. coli*. Deletion of *met*A and *met*B from an auxotrophic *E. coli* (MG1655) and supplementation with *met*X and *met*Y from *Cyclobacterium marinum,* along with deletion of *met*J with overexpression of yjeH, enhanced L-methionine production up to 160 folds.[11] Very recently, dynamic deregulation of metabolism appeared as an effective tool for overproduction of L-methionine in a non-auxotrophic *E. coli* strain. This study repaired L-lysine production by *in situ* complementation of the *lys*A gene, followed by *in situ* promoter replacement with $Pfli_A$ (dynamically regulated) to construct the desired non-auxotrophic strain. In addition, pathways for central metabolism and L-cysteine catabolism were also modified to achieve the high L-methionine-yielding strain. The strain produced up to 17.74 g/L L-methionine in a 5L bioreactor without further addition of L-amino acids.[39] An L-homoserine auxotroph, *Alcaligenes faecalis* ATCXT3624, was developed from a wild strain by induced mutation, which could produce L-methionine up to 20.1 mg/ml in submerged fermentation.[40] In another study, the addition of 30 g/L $CaCO_3$ to the fermentation medium would increase the production of L-methionine up to 1.48 g/L in *E. coli* W3110BL at the cost of 0.09 mol/mol glucose, which was 57.45% higher than the control.[41]

Constraints and Possible Overcome Strategies

With the increasing market demand for L-methionine in the health sector, it is desired to establish plants for the overproduction of stereospecific L-isomer of methionine. Several trials have been conducted over the last many decades, but no such plant has been established for L-methionine production by fermentation without using hazardous materials. In the meantime, the United States has banned the usage of synthetic DL-methionine in farming sectors; only methionine produced from natural sources is permissible. Complex manufacturing processes, extensive technological skills, and substantial operational costs also severely constrain the global production of L-methionine. Classical selection methods for L-methionine overproducers and applications of random mutagenesis were unsuccessful in terms of microbial L-methionine fermentation.[42] This is possibly due to a lack of knowledge of sulfur assimilation and feedback inhibition and repression in the microbial biosynthesis of L-methionine pathways, and its extracellular export routes and mechanisms. In the last decade, L-methionine production by fermentation was reported to be only up to 5 g/L, which is not satisfactory as far as its market demand is concerned.[43] Plackett-Burman and Box-Behnken designs were used to statistically optimize L-methionine production by a genetically engineered *E. coli* MET-3 strain, which could produce 12.8 g/L L-methionine under optimized conditions. Mohany *et al*. (2021) wrote an extensive review of recent trends in research, especially focusing on the identification, characterization, and patterns of expression of different microbial L-methionine transporters, and reported four families of L-methionine exporters and importers are essential for the further understanding of its overproduction.[36] Extensive scientific research has been carried out to solve this problem. Genetic engineering could solve the problem. However, very recently, Hazra et *al*. (2023) found a way to develop an L-methionine overproducer using *Alcaligenes faecalis* ATCXT3624, which could produce up to 20.1 mg/ml of L-methionine in submerged fermentation using synthetic medium before optimizing the medium ingredients.[40]

Recovery of Methionine from Fermentation Medium

A few years ago, Xiong *et al*. (2019) developed a novel method for the separation of L-methionine from the fermentation broth based on the adsorptive properties of ten macroporous resins, among which D72 showed the highest adsorptive capacity (52.37 mg/g) with a desorption rate of 99.12%. The adsorption showed physisorption with pseudo-second-order kinetics, which fit well with the Sips model. For maximum efficacy, the pH of the medium was adjusted to 2.0. The loading volume was 55 mL, with a loading flow rate of 2 BV/h. $NH_3.H_2O$ was used as an eluent with a flow rate of 2 BV/h. The column height and diameter are 14:1. Finally, the recovery and purity for L-methionine were 82.37 and 85.69%, respectively.[44]

Current Market Status & Possible Future Scenario

The Complementary Medicines Committee (CMEC) recommended L-methionine as a safe ingredient for therapeutic agents and a dietary supplement that does not exert any substrate-specific restrictions. Based on this recommendation, which came into force in April 2000, the global demand for this stereospecific amino acid has drastically increased, especially in dietary supplements for geriatric patients, aiming to minimize overall healthcare costs. Very recently, FMI analysts surveyed the global market for L-methionine, concluding that the total market size was US$3.36 billion in 2022 and is expected to increase at a CAGR of 5.9% from 2022 to 2032. In the next few years, sports fields are also expected to be the most promising area for expanding the L-methionine global market. The pharmaceutical industry is also expected to be a future consumer of L-methionine. The animal feed industry is emerging as one of the most prominent consumers of L-methionine, significantly improving meat quality. With advancements in biotechnology and genetic engineering, the L-methionine industry is expected to emerge as one of the significant fermentation industries, serving the fields of food supplements for sports science, geriatric healthcare, perfusion, and therapeutics, as well as the animal feed industry. It will also dominate a significant fraction of the global market economy in this millennium.

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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.