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MINI-REVIEW

Methionine production—a critical review

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Abstract This paper presents an updated critical review about several attempts to contribute methionine (Met) to the world market with an emphasis on fermentation processes, especially from natural biological sources. Analytical methods for the determination of methionine are reviewed as well as applications in feed, food, pharmacy, and medicine. Fermentation studies published within the last five decades are elucidated critically, mainly with respect to the sulfur balance, substrate yield, and the analytical validity. From all the published fermentation data, it can be concluded that up to now no more than 5 g/L methionine are achievable without using genetically modified organisms (GMOs). The highest L-methionine concentration from natural sources reached so far amounts to 35 g/L and is published as a patent using a GMO of *Escherichia coli*. The review closes with a comprehensive overview of the role and activities of global methionine manufacturers. Some current market data is also presented.

Keywords Methionine · Fermentation · Analytical methods · Sulfur balance · World market · Manufacturers

Introduction

Sulfur-containing amino acids had already been detected in 1847 at Liebig's laboratory by Fleitmann (1848). He discovered the heat instability of proteins in strong alkali solutions, liberating H_2S and NH_3. Later, Osborne (1902) determined in highly purified proteins two sulfur-containing amino acids and correctly attributed one of them to cysteine. The other was first isolated from casein and described later by Mueller (1923).Three years later, Barger and Coyne (1928) identified the chemical formula as -methylthiol-α-aminobutyric acid and suggested—agreeing with Dr. Mueller—the shorter name methionine (Fig. 1). In the following years, increasing work was done regarding the detection, analysis, as well as the role and the significance of methionine in biological systems. Already in the early 1950s, the importance of methionine in animal feed and food was discovered, and the first production plant (360 tons/year) was built by the Deutsche Gold- und Silber-Scheideanstalt (Degussa AG, since 1980 part of Evonik). Since that time, the numbers of publications increased continuously. The history of industrial amino acid production since the early 1970s was recently reviewed by Udaka (2008). Attempts to produce methionine by fermentation were reviewed by Roy et al. (1985), Mondal et al. (1996), Gomes and Kumar (2005), and Kumar and Gomes (2005).

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Basics

Methionine is—besides cysteine—one of the two sulfur-containing proteinogenic amino acids and is essential for life. In organisms, it can serve as precursor of cysteine. Due to the sulfur, responsible for disulfide bonds, which stabilizes tertiary structures of proteins, cysteine are mainly present in structural proteins such as collagen or keratin in skin, hair, feathers, and nails respectively. The highest methionine content of about 5 % can be found in albumins, especially egg albumin, which belongs to the water-soluble proteins (globulins). This is one reason for the high methionine demand of poultry.

Methionine exists in two isomers, L- and D-methionine, of which the L-form predominates in nature. Both forms can be metabolized in animals by a DL-racemase, which is important for the application of the chemically synthesized DL-methionine racemate as feed additive in industrial livestock farming (see below). Many studies since 1943 have shown that there is

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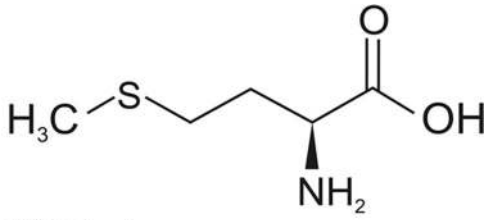
no significant difference in using L- or D-methionine in poultry diet (Goodson et al. 2012).

Most plants, fungi, and bacteria can synthesize methionine from carbohydrates, organic or inorganic nitrogen, and sulfur sources. However animals, including humans, depend on externally provided methionine sources. In organic farming, especially poultry and pig breeding, the supply with methionine has become a problem, since methionine is regarded as the first and third limiting amino acid in poultry and piglet feed, respectively (Jankowski et al. 2014), and the use of synthetic methionine in organic farming is banned in major countries (NPOP 2005; EC 2008; EC 2014a; NOP 2014).

Since about four decades, there has been increasing research activity on amino acid fermentation. Starting with glutamic acid as a commercial product in the 1970s, lysine, valine, and threonine followed. By now, numerous proteinogenic amino acids and some special pharmaceutical important amino acids are produced by fermentation (Verseck 2007). The role and the biotechnological production of essential amino acids were recently reviewed by Kyowa Hakko Kirin, Japan, one of the largest amino acid producers in the world (Mitsuhashi 2014).



L-Methionine

IUPAC:
L-2-amino-4-(methylthio)-butyric acid

Formula: $C_5H_{11}NO_2S$

CAS-Reg. No.: 63-68-3

Molar mass: 149.21 g/mol

Solubility in water (20 °C): 53 g/L

pK_a^1 (-COOH): 2.28 (25°C)

pK_a^2 (-NH_3): 9.21 (25°C)

Isoelectric point (*pI*): 5.74 (25°C)

Fig. 1 Formula and some important properties of methionine

Significance and utilization of methionine

Relevance in livestock

Most of the produced methionine is used for animal feed in livestock production. The chemically produced synthetic DL-methionine can be used for most applications. In 2013, the world market amounted to over 600,000 tons/year (see "Methionine market and industrial production" below). However, in organic farming, there is a ban or strong limitation in using synthetically produced methionine. The demand for "eco-methionine" based on natural resources without using genetically modified organisms (GMOs) will increase strongly in the future.

In the EU, the implementation rules allow a maximum percentage of 5 % non-organic proteins feed by the end of 2014 (EC 2008). An actual proposal to overhaul the CR 843/2007 will further strengthen the organic production and labeling, repealing the old CR 843/2007 (EC 2014a).

- Meanwhile, in the USA the use of synthetic DL-methionine in organic livestock production was banned by 2005, with two extensions until 2008 and 2010, respectively. Until the end of 2011, only 5 % non-organic ingredients including DL-methionine and the hydroxyl analogs were allowed (Fanatico 2010). In 2014, a further extension of only 3 lb methionine/ton of poultry feed (0.14 %) is allowed with further decreasing tendency (NOP 2014).
- China started implementation of their revised administrative measures for organic product certification from 20 November 2013 on 1 April 2014. The content of non-organic ingredients must be 5 % or lower.
- In 2005, India allowed a maximum of 15 % non-organic feed (dry matter) for ruminants and 20 % for non-ruminants, with a reduction of 5 % each by 2010. Exceptions are allowed under certain conditions. However, for example, synthetic appetizer, synthetic growth promoters, pure amino acids or abattoir waste, as well as GMOs are prohibited (NPOP 2005).

For this reason, the search for cost-saving feed-grade L-methionine meeting the rules of organic farming has recently intensified using all potential methionine-rich plants or animal material, residues, and waste, as well as the fermentation and enzymatic conversion of natural sources both without using GMOs.

Relevance in humans

Physiological significance

- The Met-derivative *s*-adenosyl methionine (SAM) serves as *methyl donor* and is involved in the synthesis of

metabolic intermediates such as lipoic acid or polyamine synthesis (e.g., spermine, spermidine).
- The Met-derivative *N*-formylmethionyl-tRNA (FMET) *initiates the protein biosynthesis.*
- Met is also involved in the *glutathione metabolism,* which is the *major antioxidant* in human cells as well as a cysteine and redox buffer (Nuttall et al. 1998; Jankowski et al. 2014).
- Drazic and Winter (2014) described the physical role of reversible methionine oxidation in vivo. Apart from antimicrobial effects, the methionine sulfoxide reductases (MSRS) play a key role in higher eukaryotes including human metabolism, e.g., regulation of protein function, and thus an important role in the *processes of aging* (Stadtman et al. 2005; Sohal and Orr 2012), *neurodegenerative diseases* (Gabbita et al. 1999), and *cancer* (De Luca et al. 2010), among others. They conclude that methionine oxidation as an inevitable consequence of aerobic life style regulates the activity of numerous proteins.
- Recent studies prove that methionine restriction can extend the lifespan of mammals (mice, rats), insects (*Drosophila melanogaster*) and yeast (*Saccharomyces cerevisiae*). However, whether this observation can be generalized is controversial (Perrone et al. 2013; Ables et al. 2014; Lee et al. 2014).

Some known methionine-related diseases

The influence of sulfur-containing amino acids on health has been reviewed by Townsend et al. (2004)

- Methionine deficit in food has been linked to diseases as toxemia, childhood rheumatic fever, muscle paralysis, hair loss, depression, schizophrenia, Parkinson's disease, liver deterioration, and impaired growth (Gomes and Kumar 2005)
- Some rare hereditary diseases in humans which are caused by defective methionine metabolism are cystathioninuria and homocystinuria=hypermethioninemia, which cause symptoms such as mental retardation, failure to thrive, thrombocytopenia, clubfoot, skeletal abnormalities, lens dislocation, and hearing defects. The Met level is strongly increased due to deregulated methionine metabolism (Dever and Elfarra 2010).

Applications of feed-grade L-methionine

- Feed-grade methionine is mainly used as *flavor* in food additives. In 2006, the consumption volume was 18.3 t in China alone and is strongly increasing. However, this accounts for only 15 % of the total food grade and pharmaceutical market.
- In pharmaceutical preparations, L-methionine is used in *hepatic therapeutics* and drugs for preventing hepatic impairments. A Met-deficient diet significantly upregulated proinflammatory and fibrotic genes, which was ameliorated by Met administration (Oz et al. 2008).
- Met is also used as a nutritive element in *infant milk* preparations, *parenteral nutrition*, *health foods*, and as a component of *sports supplements* (Ajinomoto 2014).

Sources of methionine

Chemical synthesis

DL-Methionine is mainly produced by chemical synthesis from methyl mercaptan, acrolein, and hydrogen cyanide (Lüssling et al. 1981; Pack 2004). The whole process has been running at Evonik Degussa, Germany, for 50 years and contributes with a capacity of 580,000 annual tons (Q4 2014) to 60 % of the DL-methionine worldwide capacity of about 1 million annual tons. However, against the background of decreasing fossil resources and the stronger environmental constraints (hazardous intermediates and waste), alternative, more sustainable, processes based on natural resources are gaining more and more interest.

Enzymatic conversion of DL-methionine to L-methionine

Since pharmaceutical and medical applications often need chiral pure L- or D-methionine, several enzymatic processes exist to convert the DL-racemate into the pure isomers.

The best-known and industrial-operated process is the enzymatic conversion of DL-methionine after acetylation to the *N*-acetyl DL-methionine. Only the L-isomer is subsequently enzymatically converted by L-amino acylase to get the L-methionine, which is separated, e.g., by alcoholic extraction or crystallization and purified by ion chromatography. The enzymatic step is conducted in an enzyme membrane reactor to retain the enzyme in a continuous process. Also, immobilization techniques of whole cells of enzyme producer (*Pseudomonas* sp., *Aspergillus oryzae*) in gelatine beads have been studied with a half-life of up to 70 days (Yuan et al. 2002).

The not-transformed D-*N*-acetyl methionine from the process undergoes racemization with acetic anhydride and recirculation (Woltinger et al. 2005). This process delivers several hundred tons per year of pharmaceutical-grade L-methionine, produced mainly by Rexim® in Nanning, China by Evonik, Germany.

A relatively new idea, which uses both isomers to obtain the pure L-form, has been proposed by Weckbecker and Hummel (2004) and Hummel et al. (2005). It comprises the microbial conversion of DL-methionine by a recombinant *Escherichia coli* host strain, which contains both enzymes D-amino acid oxidase (D-AAO) and leucine dehydrogenase (LeuDH). First, the D-methionine is deaminated to get a non-chiral keto-group. Then the amino group is restored by LeuDH to yield only L-methionine.

Fermentation from precursors

Another approach to achieve optical pure L-methionine is the enzymatic or fermentative conversion of chemically or biologically produced precursors. The enzymatic cleavage of 5′-monosubstitued hydantoin derivatives leads to optically pure L-amino acids. The history and biotechnological importance of the involved enzymes have been reviewed by Syldatk et al. (1999). In the late 1990s, Degussa tried to genetically optimize enzymes by directed evolution for a hydantoinase-based process using D-5-(2-methylthioethyl) hydantoin (D-MTEH) as precursor, which leads to the optically pure L-methionine (Wagner et al. 1996; May et al. 2000; May et al. 2002). This process is now used by Evonik Degussa's French subsidiary, Rexim, at their Wuming Plant, China, where up to 500 tons/year are being produced.

Other authors report the fermentation or enzymatic conversion of special precursors to produce L-methionine: CheilJedang (CJ), China describes a process starting from *o*-succinyl-L-homoserine (L-OSHS) (Kim et al. 2008). Another CJ Patent reports the enzymatic conversion of the precursor *o*-acetylhomoserine (OAHS) (Hong et al. 2012). An Arkema-CJ Patent from 2013 claims the enzymatic conversion of a precursor with gaseous methyl mercaptan (=methanethiol) (Fremy et al. 2013). However, because the precursors often are chemically synthesized or have to be produced in a first step by fermentation, there is no real advantage over the processes mentioned before. It could make sense for special applications in medicine or pharmacy or to establish a sustainable process without using petrochemical sources. Currently, a production plant in Kerteh, Malaysia is under construction, probably based on the described process by Arkema/CJ (see below).

Fermentation from natural sources

As mentioned above, the fermentation of L-methionine from natural resources could solve many problems. The main drawback is the very complex biosynthesis of methionine with manifold feedback inhibitions (Becker and Wittmann 2012). An additional issue is the sulfur source. Sulfur is usually provided as inorganic sulfate and has therefore been strongly reduced, before it can be transferred to methionine. Hence, the use of reduced sulfur sources in methionine fermentations could be beneficial (see below).

To the author's knowledge, there is no commercial fermentation plant for L-methionine from non-synthetic sources in the world, although many patents have been filed and some granted. Most feed methionine is supplied by chemical synthesis from petrochemical resources. One manufacturer is making great efforts in starting the production using a GMO of *E. coli*; however, some technical problems still have to be solved (MetEx 2014).

Alternatives to fermentative produced L-methionine

Naturally produced L-methionine can be found in fodder plants and animals. High levels of methionine are found in eggs (albumin, 5 %) and plant seeds. An overview of Met-rich materials used worldwide as animal feed was published in 2002 as a conference proceeding (FAO 2004).

Plant protein is supplied, e.g., as soy or sesame cake, chick pea (Acharjee and Sarmah 2013), wheat, maize, or potato protein. One of the Met-rich seeds is the Brazil nut with up to 12 % methionine (Tao et al. 1987; Tu et al. 1998; Danneel 2005).

Animal protein has been researched recently. Potential sources are fast-growing animals such as insects and their larvae (Veldkamp et al. 2012; FAO/WUR 2013; van Huis 2013; Van Huis et al. 2013) or worms (Fanatico 2010).

The application of *reprocessed animal residues* (meat meal, fish meal, bone meal, feather meal) is—for health reasons (BSE, bird flu)—seen critically in many countries. Fishmeal, for example, has been banned in the EU since 2000 for ruminant nutrition but is still allowed for pigs, poultry, and fish. Fishmeal is still used in over 50 countries including the USA (Fanatico 2010; FAO 2014). One of the world's leading manufacturers of fishmeal, FF Skagen, Denmark, is certified in accordance with the Soil Association Organic Standards, Naturland, and the MSC, the Marine Stewardship Council Chain of Custody Standards (www.ffskagen.dk).

Single cell protein (*SCP*) was studied extensively in the 1970s. The most investigated cells were yeasts, algae, and methylotrophic bacteria. The protein content in those cells is usually about 50 % of the dry cell and can reach 85 % under optimized conditions (Goldberg 1985; Anupama and Ravindra 2000). Unfortunately some contaminants can produce mycotoxins, and yeasts are often deficient in methionine. After temporary enthusiasm, especially in the USSR in the 1980s (CIA 1999), many plants were closed for environmental and economic reasons (Tsepilova 2002). Today, only few plants in the world are running including the world leader, UniBio A/S from Denmark (www.unibio.dk), which turns natural gas into SCP using a patented U-loop technology. However, the sold product UniProtein® (Unibio 2014), with only 2 % of

methionine (19.8 g/kg dry matter), is not suited for the special demands of chicken and pig breeding.

A substantial drawback of feeding protein rich plants or other complex amino acid sources is the potential imbalance of the major essential amino acids. If only one amino acid is limiting in the feed, the other amino acids are not assimilated and cause nitrogen waste. This fact led to the concept of feeding according to animal demand. Therefore, it is important to provide the most relevant amino acids as isolated substances or in a suitable concentration mix. In the case of poultry breeding, methionine has to be isolated either by fermentation, or by enzymatic treatment of Met-rich feedstock (feathers, hairs, nails, nuts, pea), or by the hydrolyses of proteins, followed by separation and purification (Verseck 2007; Srivastava et al. 2011; Stahel et al. 2014; Zhang et al. 2014).

Another approach is to transform genes of methionine-rich material (proteins) to fodder plants (e.g., potato, canola) to influence their amino acid content and balance. (Altenbach et al. 1992; Tu et al. 1998; Lee et al. 2003)

There is no ultimate solution to filling the protein gap, especially for methionine, in organic farming. There will probably be packages of measures based on local and operational conditions (Früh 2014; Willer and Lernoud 2014).

Biotechnical approaches to gain methionine

Biochemical fundamentals

There are numerous bacteria and yeasts which are able to overproduce amino acids under adequate conditions. However, because of the very complex regulation of the L-methionine syntheses, only a few strains are able to produce relevant amounts of methionine. Therefore they normally have to undergo several rounds of mutation and selection, or genetic manipulations as well as process optimization.

The major bacterial amino acid producer is *Corynebacterium glutamicum*, a gram-positive, facultative anaerobic, non-pathogenic soil bacterium (generally recognized as safe (GRAS)) that is used for the large-scale industrial production of the flavor enhancer L-glutamate (2.93 million tons in 2012), and the food additive L-lysine (1.95 million tons in 2012). Recent reviews relating to amino acid production or advances and developments of synthetic biology and metabolic engineering in *C. glutamicum* provide comprehensive overviews (Ikeda and Takeno 2013; Woo and Park 2014).

A detailed insight in biochemical methionine synthesis would exceed the scope of this review. Interested readers are referred to the very comprehensive reviews of Lee and Hwang (2003), Kumar and Gomes (2005), Figge (2007), and Becker and Wittmann (2012).

A simplified scheme of the biosynthesis of L-methionine in *C. glutamicum* is shown in Fig. 2. The direct synthesis of methionine starting from aspartate needs 1 ATP and 2 NADPH. For the incorporation of oxidized inorganic sulfate, in addition, 2 ATP, 1 GTP, and 4 NADPH are needed. This shows the strong influence of the sulfur source. If reduced sulfur (gaseous methanethiol or liquid dimethyl disulfide) is used, the energy balance could be improved by direct assimilation of these sulfur sources to methionine (Fig. 2, inset). There is evidence that this pathway (shortcut) may drastically improve the yield of methionine (Lievense 1993; Kiene et al. 1999; Krömer et al. 2006; Bolten et al. 2010). The described pathway is part of a branched amino acids metabolism leading to lysine (branch off from aspartate semi-aldehyde) and threonine and isoleucine (branch of from L-homoserine). Due to this fact, auxotrophs of lysine, threonine or isoleucine are favored for Met over-production, because some control mechanisms may be lost (Fig. 2).

The degradation of methionine to methanethiol, dimethyl disulfide or related compounds has long been known and extensively investigated. These compounds are, for example, responsible for the typical flavor of cooked cabbage, asparagus urine (Pelchat et al. 2011), and garlic or cheese (Martinez-Cuesta et al. 2013). It is therefore also used in the food industry as a flavor enhancer, especially in formulations of onions, garlic, and cheese. So, it should be no problem to also use it in methionine fermentation for organic application. The availability should also be no problem, because it is a commercial product. For example, Arkema's Paladin® contains dimethyl disulfide (DMDS) for agricultural soil fumigation to replace the phased out climate-damaging methyl bromide.

A potential natural N-source for methionine fermentation is glucosamine, which can be derived from the degradation of chitin, the most abundant biopolymer on earth (Himmel et al. 2007).

There are several publications and patents trying to increase methionine yield by optimizing the energy and redox balance, using reduced sulfur sources or a balanced supply of special precursors, as well as the transport of substrate and product into and out of the cell, respectively (Trötschel et al. 2005; Figge 2007; Figge et al. 2009; Dischert and Figge 2013a; Ikeda and Takeno 2013).

Strain screening and improvement

A general overview of methods and problems in strain improvement of processes yielding microbial products is given by Adrio and Demain (2006). They discussed and evaluated several methods of mutagenesis and screening/selection as well as recombinant DNA technologies.

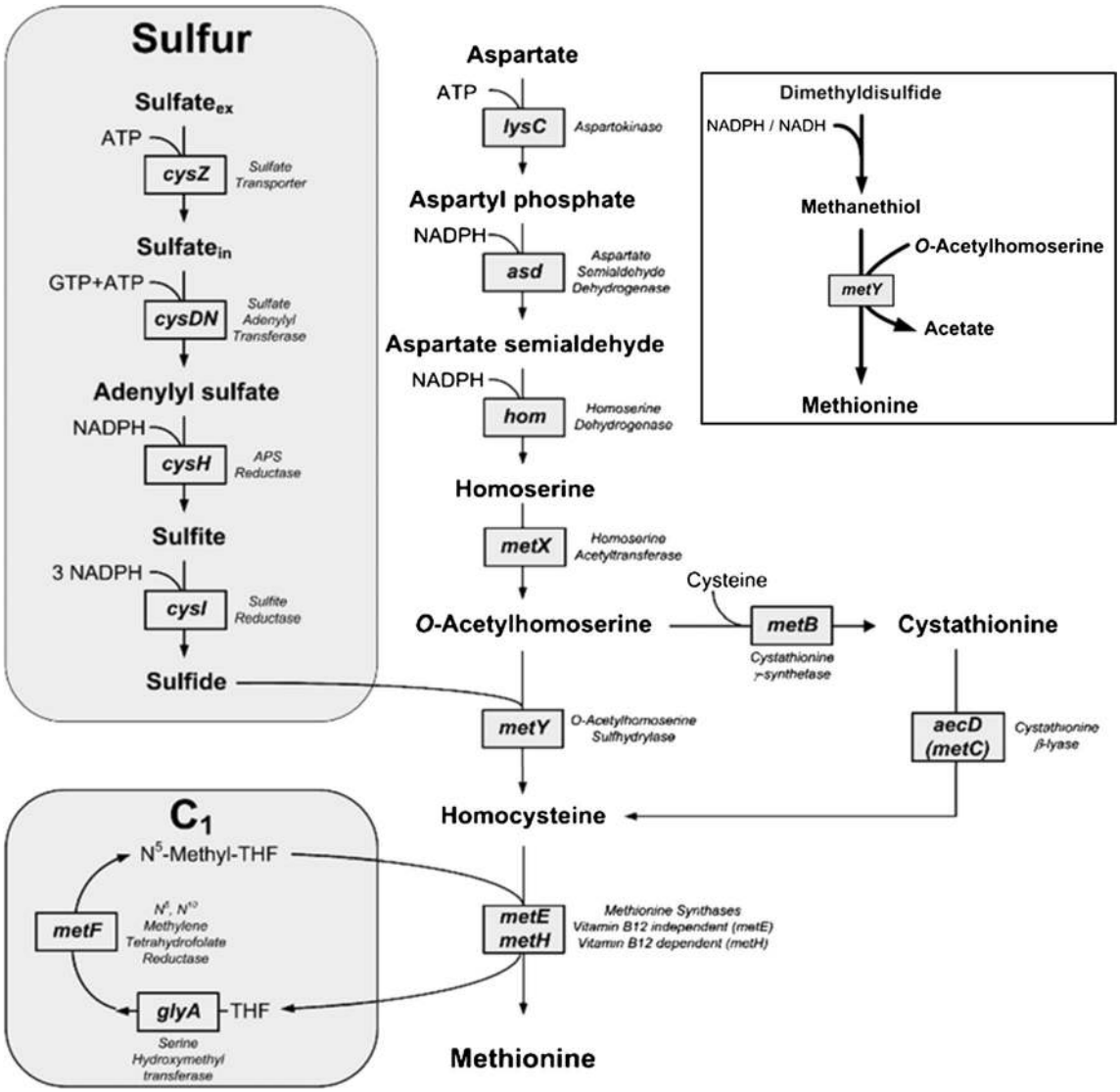


Fig. 2 Simplified methionine pathway in *Corynebacterium glutamicum*; adapted from Bolten et al. (2010). The *inset* shows the proposed pathway (shortcut) from *O*-acetyl-homoserine to L-methionine when using strongly reduced sulfur sources

Natural and induced mutants

Several studies have been done using classical screening methods for natural bacteria or yeast to produce methionine in excess, which is internally stored or excreted into the medium. Some of the succeeding studies are summarized in Table 2 and discussed afterwards with regard to the sulfur balance, the analytical issues, and the published results. The success of those studies was disillusioning and additional efforts are being made to speed up the screening. After the finding that methionine analogs could act as feedback regulators without influencing other essential reactions within the cell, Met analogs, such as α-methyl-DL-methionine (AMM), DL-ethionine (ET), DL-norleucine (NL), are widely used as indicators to detect Met-overproducers (Rowbury and Woods 1961; Lawrence et al. 1968). Organisms which grow in the presence of Met analogs are obviously resistant due to defects in the feedback regulation and should therefore produce methionine in excess. First attempts to elucidate the inhibition mechanism of DL-ethionine in *C. glutamicum* are published by Mampel et al. (2005). They found a single gene encoding for a carboxylate-amine ligase (NCgl2640), which is responsible for resistance to DL-ethionine. The knockout of NCgl2640 conferred ethionine resistance.

Other useful natural mutants suitable for methionine over-production should be lysine or/and threonine–auxotrophs, which should show (i) less inhibition in the highly branched methionine pathway and (ii) achieve better yields due to unbranched carbon flux towards methionine, too (Gomes and Kumar 2005). Because

those mutants rarely occur in nature, the screening procedures were expended by rounds of induced mutation, either by chemical agents (e.g., NTG) or by ultraviolet (UV) radiation.

Protoplast fusion

Protoplast fusion has proven to be successful in transferring useful industrial properties in yeast, e.g., osmotolerance (Legmann and Margalith 1986) or substrate utilization (Farahnak et al. 1986; Pina et al. 1986). Studies to enhance the internal methionine pool in food or fodder yeasts focused mainly on the genera *Saccharomyces* and *Candida* (Brigidi et al. 1988). The yielded pool concentrations of methionine reached about 5 mg/g dry cells, which means a 20-fold improvement compared with the wild-type strain. The released methionine was not the focus of investigation. Brigidi et al. (1988) reported also a stable DL-ethionine resistant auxotrophic *S. cerevisiae* to overproduce methionine using NTG mutation and protoplast fusion with *Saccharomyces uvarum*. The hybrids produced a maximum of 4 mg/g dry cells and 20 mg/L methionine, respectively.

Genome engineering

The control of genes within the branched and highly regulated methionine pathway is an ambitious task. Starting with genetic engineering of plants to increase the methionine content of seeds (Altenbach et al. 1989), in the middle of the 1980s, bacteria or yeasts were also included. In the early 1990s, when the knowledge of gene-manipulation technology in *C. glutamicum* had proceeded, the work concentrated—besides *E. coli*—on this organism. In 2003 when the whole genome of *C. glutamicum* had been sequenced (Nakagawa et al. 2000; Kalinowski et al. 2003), the systematic and specific genome manipulation was implemented, later supported by systems biology approaches.

There are some excellent overviews about metabolic engineering of methionine synthesis with the main focus on *E. coli* (Figge 2007) and *C. glutamicum* with respect to synthetic biology (Woo and Park 2014). The first author also holds patents assigned to the French company Metabolic Explorer regarding the bio-fermentation of L-methionine by a genetically engineered *E. coli* (Dischert and Figge 2013a; Dischert and Figge 2013b; Dischert et al. 2013). An associated industrial process is on the way to commercialization (see below).

Determination of methionine

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) methods for the determination of amino acids have been common since the early 1960s. The basics have been investigated by Spackman et al. (1958). There are several approaches depending on the available equipment, the origin of sample, as well as the desired sensitivity and selectivity:

- Reversed-phase (RP) chromatography of underivatized amino acids and direct detection using UV light, fluorescence, electrochemical detection, evaporating light scattering detection (ELSD), or mass spectrometry (MS) (Agrafiotou et al. 2009).
- Separation of underivatized amino acids and fluorescence detection after post-column reaction with ninhydrin (amino acid analyzer, AAA) EU-Standard method 1998 (EC 1998)
- Ion-exchange separation of underivatized amino acids and post-column reaction with ninhydrin or *o*-phthalaldehyde (OPA; AAA)
- Hydrophilic interaction liquid chromatography (HILIC) without derivatization coupled with MS (Person et al. 2005). This method was developed for sensitive determination of taurine and methionine in high-carbon energy drinks with detection limits of 20 and 50 μg/L, respectively.
- RP separation after pre-column derivatization with ninhydrin or OPA, and detection using two ultraviolet/visible (UV/Vis) detectors at different wavelengths and fluorescence, respectively (Krömer et al. 2005). This recently developed method also allows the determination of all methionine intermediates in *C. glutamicum* with high precision.
- Ultra-performance liquid chromatography (UPLC) separation combined with MS is a recently developed method for fast quantitation of methionine pathway metabolites in liver tissue (van Liempd et al. 2013).
- A variety of other HPLC methods using pre- or post-column derivatization with numerous reagents for special purpose (Coppex and Walz 2000).

For detailed information, the reader is referred to the reviews of Sarwar and Botting (1993) or Peace and Gilani (2005).

Gas chromatography

Since amino acids are not volatile, gas chromatographic methods are only applicable if the amino acids are converted to volatile analytes (e.g., ester or ether). The analysis of amino acids by means of gas chromatography (GC) is not very common. However, new developments in automatic sample pretreatment in combination with a capillary GC and flame ionization detection (FID) allow very selective, fast, and reliable determination of amino acids (Husek and Sweeley 1991; Husek 2000; Husek and Simek 2001). A kit based on Husek's

studies has been commercially available since 2005 as EZ:faast™, which enables the quantitative determination of up to 32 free or protein-bound amino acids, also from complex matrices, in less than 15 min (Phenomenex 2005). Hartwich (2008) implemented this method in a high-performance screening combined with a turbidimetric microbial assay (TMA; see below).

Thin-layer chromatography

Thin layer chromatography (TLC) equals paper chromatography (PC) but with much higher resolution and precision due to the technical advancements of the stationary phases (silica gel, aluminum oxide, etc.). Sample application, development, and documentation/calculation can be conducted with automated systems (high-performance TLC (HPTLC)) (Mohammad and Zehra 2007; Shewiyo et al. 2012).

A comprehensive overview about HPLC, GC, and TLC techniques for the determination of amino acids was recently presented by Dolowy and Pyka (2014).

Capillary electrophoreses

Capillary electrophoresis (CE) is the transformation of gel electrophoreses onto an inert or coated capillary. The analytes are dissolved in an electrolyte buffer and separated according to their mobility in an electrical field. Detection can be achieved similar to HPLC techniques (UV/Vis, fluorescence, electrochemical, MS). The selectivity can be modified within a wide range by changing the mobile buffer system, the pH value of the buffer or by adding modifiers to the buffer, as well as by introducing special capillary coatings. An example for the rapid separation of essential amino acids including methionine is given by Cavazza et al. (2000). Optimization of the separation of methionine and betaine in pharmaceutical formulations, e.g., has recently been published by Vitali et al. (2014)

Microbial tests

The TMA is based on the growth of a Met-auxotrophic bacterium or yeast, which is, under defined conditions, directly related to the methionine concentration and be measured as turbidity or via optical density (OD) in a spectrophotometer (Hartwich 2008). More selective and sensitive is a method, developed for bioavailable methionine in animal feed (Froelich et al. 2002). More sophisticated methods rely on auxotroph-based biosensors (see below). An approach for the determination of methionine in animal feed without hydrolyzation is reported by Froelich and Ricke (2005). The TMA method is also applicable for the rapid screening of the methionine content in plants (Wright and Orman 1995).

Biological sensors

Sensors are particularly suitable for rather fast qualitative analysis, if pretreatment of the sample is not possible or time consuming. The application of amino acid sensors in the food and drink industry has been reviewed by Mello and Kubota (2002). However, special methionine sensors are not mentioned.

Some new methods based on biological systems (whole cells, enzymes) have been developed for the determination of methionine, mainly for application in medical samples, such as blood plasma, tissue or even in living systems, e.g., in systems biology. A single cell biosensor based on *C. glutamicum* was developed recently for the detection of intracellular methionine and branched amino acids, which could improve strain development (Mustafi et al. 2012). The sensor-plasmid was transformed in a *C. glutamicum* wild-type strain, which induced a methionine-dependent fluorescence (FRET). The dynamic range of this system is greater than 78, at a linear range 0.2–23.5 mM methionine within the cell. *E. coli*-based biosensors for detection of methionine were recently reviewed by Froelich and Ricke (2005) and Chalova et al. (2010). Such sensors are mainly used in therapeutic medicine and during screening of fodder plants. Quite recently, a GMO-based nanosensor was developed for the analysis of metabolic fluxes in system biology as well as to establish high throughput screening systems for bacteria and yeast cells (Mohsin and Ahmad 2014).

Chemical analytical methods

Chemical reactions of methionine, useful for spectral-analytical purposes (spectroscopic methods (SM)), have been reviewed by Greenstein and Wintz (1961). There are an immense number of studies concerning colorimetric methods to estimate amino acids, because before 1960 few other feasible methods existed. The methods mostly used are combinations of PC and colorimetric detection and also single colorimetric methods without preceding separation. Almost all of these methods based upon reactions with either nitroprusside or ninhydrin reagent. Both reactions generate chromophores, which can be measured in a UV/Vis spectrophotometer. Since 1942, most of the studies have tried to improve the reliability of the methods, either by stabilizing the reagents used or by adding special modifiers to mask interferences. Originally developed for protein hydrolyzates, the application to more complex matrices such as bacterial culture broths exposed additional shortcomings of these methods. The most used methods for quantitative determination of methionine in fermentation or culture broths are summarized in Table 1.

Table 1 Analytical methods used for determination of L-methionine in fermentation broth

Method	Description	References
AAA	Amino acid analyzer (HPLC with pre- or post-column derivatization)	Spackman et al. (1958) and EC (1998)
HPLC	High-performance liquid chromatography with direct detection methods (ELSD, UV/VIS, refractive index (RI), MS)	Schuster (1980), Cobb et al. (2001), and Agrafiotou et al. (2009)
GC	Gas chromatography after derivatization and detection with FID or MS	Husek and Simek (2001) and Nozal et al. (2004)
PC	Paper chromatography	Fink et al. (1963)
CPC	Circular paper chromatography	Giri and Rao (1952)
SM1	Spectrometric with nitroprusside (specific)	Greenstein and Wintz (1961)
SM2	Spectrometric with acidic ninhydrin (nonspecific)	Moore and Stein (1948)
SM3	Spectrometric with acidic ninhydrin (nonspecific), modified	Chinard (1952)
SM4	Spectrometric with acidic ninhydrin (nonspecific), modified	Work (1957) based on Chinard (1952)
SM5	Spectrometric with acidic ninhydrin (nonspecific), modified	Kawerau and Wieland (1951)
TMA	Turbidimetric microbial assay (indirectly, using Met auxotrophs)	Wright and Orman (1995)

Sources of analytical errors

Spectroscopic methods

All spectroscopic and colorimetric methods (SMx) in Table 1 suffer from interferences with matrix effects (e.g., salts, proteins, and related analytes) as well as from measuring conditions (pH, T, reagents). Therefore the purity of the sample can have strong influence on the analytical results. SM should therefore only be used in combination with separation or purification techniques, such as PC, TLC, or HPLC. Most methods used in Table 1 were not evaluated or proven for methionine in fermentation broth by the authors. In addition, due to strong dilution of the sample, the measured values have to be multiplied with the dilution factor afterwards, leading to a strong increase of systematical errors. Chinard (1952) pointed out the importance of removing interfering substances, which, for example, was not executed by Shakoori et al. (2012), who only discriminated the amino acids by wave length. Giri et al. (1952) reported that methods combined with PC cannot be used for methionine, since overlapping with valine always takes place. The authors recommended the application of the platinic iodide test (Winegard et al. 1948) for determination of methionine. Obviously, this note was not considered by Banik and Majumdar (1975). So, the risk of incorrect measurement is high and the results are questionable.

TMA methods

The major sources of errors in quantitative analysis by TMA are internal-stored methionine, the methionine released by lysed cells in old cultures or peptides/proteins after enzymatic hydrolyzation, which gives false-positive results. This has to be taken into account if used in screening tests. It is essential to optimize the experimental conditions including the pre-culture of the auxotroph to minimize such side effects. When this is not possible, the test requires additional certification by an independent method.

Other

The other discussed methods also have all their intrinsic error sources; however, they are generally known and can be neglected, when the methods are used according to good laboratory practice (GLP).

Methionine fermentation

Sulfur and substrate balance

Methionine contains 21.5 % sulfur (MW_{sulfur}/MW_{Met}). For each gram of methionine, the production strain needs 0.22 g of sulfur (e.g., 1.7 g/L $MgSO_4 \cdot 7H_2O$ or 0.9 g/L $(NH_4)_2SO_4$), exclusive of the sulfur needed for biomass production. Based on these calculations, a lot of the published data summarized in Tables 2 and 3 is highly questionable and needs to be reviewed.

Literature overview

In some publications yields of more than 30 % ($g_{Met}/g_{glucose}$) are reported. The maximum theoretical values for *E. coli* and *C. glutamicum* were calculated based on flux analysis and extensively discussed by Krömer et al. (2006). They published values for *C. glutamicum* between 49.3 %, using inorganic sulfate as sulfur source and 92.9 % using methanethiol. However in vivo, maximum achieved yields do not exceed 20 % (Figge et al. 2009).

Table 2 S-balances of published experimental data relating to biological L-methionine production using wild-type strains without mutation

References	Strain	S-content in medium (g/L)	Max. theoretical Met (g/L)	Measured Met (g/L)	Analytical method (refer to Table 1)
Roy et al. (1984)	*Bacillus megaterium* B71 wild-type strain	n.a.	n.a.	0.072	PC, MT
Mondal et al. (1990)	*Nocardia polychromogenes* *Brevibacterium ammoniagenes*	0.02	0.1	1.7 2.4	TMA
Mondal (1993)	*N. polychromogenes* *B. ammoniagenes*	0.02	0.1	5.0[a] 6.5[a]	TMA, SM3
Anike and Okafor (2008)	*Lactobacilli* isolated from *Cassava* pulp	4.84	>20	1.35–3.48[b]	SM2, modified (Rosen 1957)
Nwachukwu and Ekwealor (2009)	*Streptomyces* sp.	0.04	0.2	3.7[a]	PC, SM1
Ali et al. (2011)	*Bacillus* sp.	0.04	0.2	10[a]	SM2
Dike and Ekwealor (2012)	*Bacillus cereus* isolated from soil	2.4	11	1.1–1.9	SM1
Ozulu et al. (2012)	Bacteria isolated from soil	2.4	11	0.5–1.4	TMA, SM1
Shakoori et al. (2012)	*Bacillus anthracis* *Bacillus cereus* *Escherichia coli* *Bacillus sp.*	< 0.1 <0.1 <0.5 <0.5	< 0.5 <0.5 <2.5 <2.5	12.52[a] 11.2[a] 13[a] 8.12[a]	SM2
Venkata Narayana et al. (2013)	*Corynebacterium glutamicum* MTCC2745	4.8	22	5.6	PC, SM1
Anakwenze et al. (2014)	*Bacillus thuringiensis* EC1	2.4	11	3.2	SM1

[a] Measured methionine concentration not achievable due to insufficient sulfur in the medium

[b] Glucose balance highly questionable, since 3.5 g/L met from 10 g/L glucose is not reliable under the given conditions (see text)

Mondal reported methionine concentrations in the range of 4 to 25 g/L in several papers between 1990 and 1996 (Mondal et al. 1990; Mondal 1993; Mondal and Chatterjee 1994; Mondal et al. 1994a; Mondal et al. 1994b; Mondal et al. 1996). All data based on fermentations in Alfoldi-medium (Alfoldi 1958), which contained only 20 mg/L sulfur, i.e., sufficient for only 0.1 g/L methionine. Table 2 shows results with wild-type strains, whereas Table 3 shows data of mutants. Some of the fermentations took place in the presence of DL-ethionine. It may be that the ethionine sulfur was assimilated or that ethionine interfered with the quantitative determination of methionine (Joson and Klug 1956)

Anike and Okafor (2008) reported up to 3.5 g/L methionine produced by *Lactobacillus plantarum*, which was isolated from cassava pulp. The sulfur balance is correct, however the modified ninhydrin method according to Rosen (1957) cannot distinguish between methionine and other amino acids, and so probably the sum of all is determined. Further evidence is given by the methionine yield of nearly 0.35 g/g. This value is indeed theoretically possible (Krömer et al. 2006), but never reached so far with inorganic sulfate. The best yields of 0.24 were reached by Dischert and Figge (2013a) with an *E. coli* GMO, extensively optimized with regard to yield. So, published results with higher Met yields than 20 % reached with a wild-type strain are rather questionable.

Nwachukwu and Ekwealor (2009) reported the production of 3 g/L methionine by a wild-type soil bacterium without providing any sulfur to the medium. In a subsequent paper regarding a new screening method by using a Met-auxotrophic indicator organism, they very well addressed the problem of sulfur. However, in that paper, all methionine concentrations are significantly lower (Ozulu et al. 2012). Recently, a new publication of the same group reported on a wild-type strain of *Bacillus thuringiensis*, isolated from fermented oil beans to over-producing methionine. In this work, further optimization of the process (e.g., N- and C-source, pO_2, vitamins, trace metals) could increase the methionine concentration from initially 1.9 to 3.2 g/L (Anakwenze et al. 2014). In this case, all experimental conditions were feasible except the questionable analytical method SM1 of Greenstein and Wintz (1961).

In the work of Ali et al. (2011), several fermentations were conducted using different media yielding methionine concentrations of 6–10 g/L. The highest methionine concentration of 10 g/L was reported in a medium with only 40 mg/L sulfur (FM6), which is of course not achievable.

Shakoori et al. (2012) screened several soil organisms with regard to methionine over-production. They also used different media and found five strains that produced between 8 and 12 g/L, whereas the sulfur only allows methionin concentrations of a maximum of 2.5 g/L.

Venkata Narayana et al. (2013) used a *C. glutamicum* wild-type strain for methionine fermentation. They could increase the methionine concentration to 5.6 g/L by means of comprehensive process optimization. However, the less-reliable methionine analysis method makes the results questionable, although the sulfur and glucose concentrations are sufficient.

Table 3 S-balances of published experimental data relating to biological L-methionine production using wild-type strains after mutation

References	Strain	Sulfur in medium (g/L)	Max. theor. Met [g/L]	measured Met (g/L)	Analytical method (refer to Table 1)
Dulaney et al. (1964)	*Ustilago maydis* UV- and NM mutation	0.13	0.6	6.5[a]	PC and TMA after (Difco 1953)
Nakayama et al. 1973	*C. glutamicum* ATCC® 21608™ (mutated ATCC 13032)	4.85	22	3.4	n.a.
Komatsu et al. (1974)	*Candida petrophilum* ET-resistant mutant	0.24	1.1	Pool-Met; 3.9 mg/g DCM; <0.046 g/L[b]	TMA, AAA
Banik and Majumdar (1974, 1975)	*C. glutamicum* (formerly *Micrococcus glutamicus*) EMS, gamma- and X-ray mutation	0.04	0.9	2[a] 4.5[a]	CPC, PC
Yamada et al. (1982)	Methylotrophic bacterium OE120 ET-resistant mutant	1.2	5.6	0.42	TMA, PC
Tani et al. (1988)	*Candida boidinii* no. 2201 UV mutation, ET-resistant	0.6	2.8	Pool-Met; 16 mg/g DCM; <0.05 g/L[b]	TMA
Roy et al. (1989)	*B. megaterium* B71 multianalog-resistant mutant	>5	>23.5	4.5	PC, TMA
Pham et al. (1992)	*C. glutamicum* ATCC® 21608™ patent deposit	2.6	12	3.6	SM1
Mondal and Chatterjee (1994)	*Brevibacterium heali* ET-resistant NTG mutants	0.02	0.1	13[a]	TMA, SM1
Mondal et al. (1994a)	*Brevibacterium heali* ET-resistant NTG mutants	0.02	0.1	25.5[a]	TMA, SM1
Mondal et al. (1994b)	*Brevibacterium heali* ET-resistant NTG mutant, double auxotrophic	0.02	0.1	5.5[a]	TMA, SM1
Kitamoto and Nakahara (1994)	*Kluyveromyces fragilis* M-81 from whey-permeate ET-resistant UV mutant	0.02 1 % peptone; 0.5 % yeast extract	n.d.	0.15 pool-Met; 14.2 mg/g DCM; 0.120 g/L	TMA, AAA
Mondal et al. (1996)	*Brevibacterium heali* mutant	0.02	0.1	5.5[a]	MT, SM3
Chattopadhyay et al. (1995)	*E. coli* K12, NTG mutants	0.24	1.13	2[a]	PC, SM5
Sharma and Gomes (2001)	*Corynebacterium lilium*=*C. glutamicum* conti-culture	0.04	0.18	2[a]	SM1
Kumar et al. (2003)	*Corynebacterium lilium*=*C. glutamicum* NTG, UV mutation	0.8	3.7	2.3	SM3
Reershemius (2008) and Willke et al. (2010)	*C. glutamicum* KY10574[c]	2.4	11	1.45	GC, MS

[a] Measured methionine concentration not achievable due to insufficient sulfur in the medium

[b] Calculation based on biomass data provided by the authors

[c] Strain provided by Kyowa Hakko Kirin

Dulaney et al. (1964) reported on a lysine auxotrophic *U. maydis*, which should produce 6.5 g/L methionine from only 0.13 g/L sulfur, a highly questionable result. Methionine was determined qualitatively by ninhydrin reaction after paper-chromatographic separation and quantitatively after Difco manual (Difco 1935), which is based on TMA. They not only mentioned the difficulties of analysis and the unusual results but also cited the results in a following paper. The producer strain has been lost, so no further experiments could be conducted.

Banik and Majumdar (1974, 1975) also found a methionine over-producing strain which should yield 3 g/L methionine (after optimization up to 4.5 g/L) from only 0.04 g/L sulfur, also a highly questionable result. However, the elemental analysis of the product after separation on acid Dowex 50 should fit with methionine, e.g., 21.5 % sulfur content. Quantification was conducted by PC and successive ninhydrin reaction. The source of the additional sulfur is not clear. No further experiments or discussion were provided.

Chattopadhyay et al. (1995) used NTG- mutants of *E. coli* K-12, which are resistant to a threonine and a methionine analog. They reported threonine and methionine concentrations of 2 g/L each but without providing sufficient sulfur in the medium. The analytical method of PC using ninhydrin reaction is not selective and can provide false-positive results, maybe through sulfur-containing methionine analogs, which were components of the used AM-medium.

Sharma and Gomes (2001) conducted continuous experiments for methionine production under different oxygen conditions using *Corynebacterium lilium* NL-87, now also regarded as *C. glutamicum* NL-87. They reported methionine concentrations of up to 2 g/L, whereas the medium contained only 40 mg/L sulfur. The used nitroprusside method (Greenstein and Wintz 1961) provided obviously much too high results.

In Table 4, important work using GMOs are shown, most of them pending or issued patents.

All presented studies on methionine over-production using GMOs considered the sulfur and substrate balances as well as adequate fermentation conditions. The analytical data are reliable and comprehensible. So, the reported data seems to be correct. Thus methionine concentrations up to 35 g/L are achievable with great efforts; however, there are also current industrial patents which documented only 0.55 g/L. All concentrations above 5 g/L are published by the same scientific group of Metabolic Explorer, France with one exception: Möckel et al. (2002) reported 16 g/L methionine produced by a genetically engineered *C. glutamicum* strain from only 50 g/L glucose, which is a very good yield of 0.32 g/g, never reached so far. This patent to Degussa AG is not mentioned further, although the results are comparatively promising. The strain is deposited at DSMZ, Braunschweig, Germany as DSM 13556.

Recovery of methionine from fermented broth

Process development, up- and downstream processing, as well as process scale up is not part of this review. For details, please refer to Hermann (2003) and Eggeling and Sahm (2009, 2011). Here, only the basic process steps are listed, regarding the separation and purification of amino acids which can be applied in combination or alone (Boy et al. 2005).

- Separation of biomass and insoluble components at increased temperature to dissolve all the methionine.
- Ultrafiltration to remove proteins and other macromolecules
- Activated charcoal treatment to remove smaller impurities (salts, sugar, pigments)
- Concentration of the product by (vacuum) evaporation
- If further purification is necessary, adsorption of the methionine solution at low pH value onto a strongly acidic cation exchanger (e.g., Dowex 50, Amberlite IR 120, Lewatit MDS 1368)

Table 4 Published experimental data relating to biological L-methionine production *using GMO*

References	Strain	S-content in medium (g/L)	Max. theor. Met (g/L)	Measured Met (g/L)	Analytical method (refer to Table 1)
Nakamori et al. (1999)	*E. coli* JM109 GMO mutant TN1	1.24	5.8	0.91	TMA, AAA
Möckel et al. (2002)	*C. glutamicum* DSM 5715	6	28	1.4	AAA
	GMO thereof, patent deposited as DSM 13556			16	
Figge et al. (2007)	*E. coli,* GMO	>10	>50	25	GC-MS
Maier et al. (2004)	DSM 15421, GMO patent deposit	1.2	5.7	4.8	HPLC
Figge et al. (2009)	*C. glutamicum,* GMO	>10	>50	35[a]	HPLC
Park et al. (2007)	*C. glutamicum,* GMO	4.8	22	2.9	HPLC
Schneider et al. (2012)	*E. coli*, GMO	4.5	21	0.55	AAA
Dischert et al. (2013)	*E. coli*, GMO	>20	>100	30[a]	HPLC

[a] Calculation based on biomass data provided by the authors

- Elution and separation of methionine from the ion-exchange column with water
- (Cooling) crystallization
- Filtration and drying
- Recirculation of the mother liquor and washing fluids to the biomass fraction to save waste water

The biomass can be spray-dried and sold as methionine-rich feed additive. For feed purposes only, it can be economical to use the raw fermentation broth after spray drying. In this case, additional important amino acids and other nutrients are enriched as well. An example is the product Biolys® (Höfler et al. 2012).

The cation-exchange steps can be repeated several times until the desired purity is achieved. Some manufacturers offer methionine solutions. In this case, the crystallization and drying steps are not necessary.

A company which uses an ion-exclusion process on a large scale (500 m^3 resin) to isolate amino acids from molasses or other protein rich feedstocks is the Amino GmbH, Frellstedt, Germany (www.amino.de). The product portfolio is mainly focused on pharmaceutical-grade products, used in pharmaceutical and dietary products and clinical nutrition (Smolnik and Thommel 1995). In 1992, Gist Brocades, now DSM, has filed a method for preparation or extracting amino acids from manure (Sliejkhuis and Sander 1992). A patent for a method to recover methionine by crystallization from fermentation broth has been filed by BASF (Boy et al. 2005). The major amino acid producer Ajinomoto (see below) has patented a recovery process using ion exchange.

Methionine market and industrial production

The global DL-methionine market in 2013 was US$ 2.85 billion for 850,000 t (Feed Info, methionine average price 2013). The global market is to reach US$ 3 billion by 2015. At the end of June 2014, 1 metric ton of feed-grade DL-methionine (99 %) was sold at a price of $ 4.70–4.83/kg. In 2013, the wholesale price for feed-grade DL-methionine was about $ 4.20/kg. A global growth rate of 5.0–5.5 % can be expected during 2014 (FeedInfo 2014). The bulk of methionine is used in animal feed. In 2013, more than 600,000 t of DL-methionine were produced only for feed.

The market of food-grade L-methionine used for human nutrition additives and for medical applications amounts to only some 10,000 tons/year. However, due to the higher price of $ 30–250/kg (Ajinomoto 2014, $ 234/kg), the monetary value can reach the same order.

In 2002, the European Commission fined Degussa AG and Nippon Soda Company Ltd., respectively € 118 ($ 117) million and € 9 ($ 8.9) million for participating in a price-fixing cartel in methionine together with Aventis SA. Aventis SA (formerly Rhône-Poulenc) was granted full immunity from fines because it revealed the cartel's existence to the Commission and provided decisive evidence on its operation (Pieters 2002).

Some major global amino acid manufacturers

The current global production capacities of methionine are summarized in Table 5. Relevant details to the history, cooperation, and actual activities of most important amino acid producers subsequently follow in alphabetic order.

- **Adisseo** (France; www.adisseo.com/home.html; see also Aventis and ChemChina).
- **Ajinomoto** (Japan; www.ajiaminoscience.com)—Ajinomoto is the global leader in the manufacture and supply of L-amino acids, especially of pharmaceutical grade. So far, L-methionine is produced by optical resolution of the DL-form, which is manufactured by chemical synthesis starting from acrolein. The L-methionine capacity is rather low and only offered for R&D purposes. A fermentative process using a recombinant *E. coli* is filed for patent (Usuda and Kuruhashi 2009). However, the achieved concentration in the given example of about 0.25 g/L is much too low for an industrially feasible process.
- **Archer Daniels Midland (ADM) Alliance Nutrition** (USA; www.admani.com)—ADM Alliance Nutrition, a subsidiary of ADM, is a leading producer of livestock feed additives. They offer a rumen bypass methionine, which is protected against degradation in the rumen. Under the brand Stimerall™ P, a concentrated source of 80 % methionine in meal form is provided mainly for ruminants.
- **Arkema** (France) and CJ CheilJedang (South Korea; www.arkema.com; www.cj.co.kr/cj-en)—Arkema and CJ CheilJedang, a Korean food, feed, and biosciences company have built the world's first methyl mercaptan-integrated plant platform to produce bio-methionine for animal feed in Malaysia. The $ 450 million in costs would be split equally between the companies. The 80,000 tons/year facility should actually start at the end of 2013. Currently, start of operation is planned for Q4 2014. Arkema is bringing its knowledge of methyl mercaptan, a sulfur-based intermediate for the manufacture of methionine to the project (Arkema 2011). CJ contributes a bio-fermentation process to produce L-methionine from plant-based raw materials. Animals, CJ claims, can digest L-methionine more readily than DL-methionine, which currently dominates the feed market. The process is probably based

Table 5 Global production capacity of methionine in 2014

Manufacturer	Products	Production Site	Capacity [MT/y]	Output [MT/y]	Launch
Arkema/CJ-CheilJedang	L-Methionine from fermentation (GMO) using methyl mercaptan as S-source, Co-products: succinic and lactic acid	Kerteh, MYS	(80,000)		Q4 2014
ChemChina-BlueStar/Adisseo Nutrition Group Ltd., CHN Formerly: Aventis Animal Nutrition	DL-Met (powder) Smartamine®, Metasmart® (rumen-protected methionine MHA converted from 99 % DL-methionine (yield 0.8))	Nanjing, CHN	(70,000)	n.a.	2014
		Commentry, FRA	n.a.	n.a.	
		Les Roches, FRA	77,000	n.a.	2003
		Roussillon, FRA	n.a.	n.a.	
		Burgos, ESP	105,000	n.a.	2005
		Institute, USA	24,000	n.a.	1994
Evonik Degussa (SEA) Pte. Ltd, Evonik Industries DEU	99 % feed-grade DL-Met	Jurong Island, SGP	(150,000)	Slowly increasing	Q4 2014
		Wesseling, DEU Antwerpen, BEL	Total 430,000	n.a.	1971
		Mobile, USA			1974
					Exp. 2006
					1977
Evonik Rexim® Pharmaceutical Co., Ltd	Feed-grade L-methionine	Nanning, CHN	3000	n.a.	
Metabolic Explorer	L-Methionine by fermentation (GMO)	Nusajaya, MYS	n.a.	n.a.	2015
Novus international by Nippon Soda (Nisso), JPN	99 % feed-grade DL-Met MHA converted from 99 % DL-methionine (yield 0.8)	Nihongi, JPN	250,000	n.a.	Nisso production stopped 2006
Unisplendour Tianhua Methionine Co., Ltd & Cheman Co. Ltd, CHN	99 % feed-grade DL-methionine	Chongqing, CHN	(60,000)	0	2010- Q4 2013
		Xiang, CHN		25,000	
Sumitomo Chemicals Co., Ltd, JPN	MHA converted from 99 % DL-methionine (yield 0.8)	Dalian, CHN	20,000	<10,000	2010
		Niihama, JPN	140,000	10,000	Q1 2010
Others			300	n.a.	
JSC Volzhskiy Orgsynthese, RUS	99 % feed-grade DL-methionine	Volzhskiy, RUS	>23,000	23,000	
Total (June 2014)			1,072,000	700,000	

Data from CCM (2014), FeedInfo (2014), and own investigation (see below). Data in brackets—plant not yet or no longer in operation

n.a. data not available, *MHA* methionine hydroxy analog

upon a patent, where genetically engineered *E. coli* strains produced about 6.5 g/L L-methionine from glucose and sulfate (Brazeau et al. 2013). It is so far the only commercial L-methionine fermentation plant. A request from the company CJ Europe GmbH to the European Community (EFSA 2013) for authorization of their GMO products L-methionine and L-methionine, feed grade as a feed additive for all animal species (EC 2014b) indicates the early marketability of the products. However, assuming yield and glucose price, the process seems to have no economic advantage over synthetic methionine production.

- **Aventis S.A.** (formerly Rhone Poulenc, since 2002 Adisseo, see above)—Aventis, one of the major DL-methionine manufacturers and a member of the methionine cartel fined in 2002, revealed the cartel's existence and was therefore granted immunity from fines (Pieters 2002). In Q1 2002, Aventis sold its animal nutrition business to CVC-Capital Partners, London, which became autonomous under the name Adisseo (Anonymus 2002).
- **BASF** (Germany; www.animal-nutrition.basf.com)—BASF has several feed additives (vitamins, organic acids, carotenoids) in their portfolio; however, no amino acids have been produced so far. For 10 years, BASF has been filing patents regarding the fermentation of L-methionine using GMOs of *C. glutamicum* (Kröger et al. 2003). Sauer et al. (2006) and Zelder et al. (2007) claimed a process starting from reduced homolanthionine, including a reduced citrate dehydrogenase to produce fine chemicals of the aspartate family, especially methionine. However, the same working group (Zelder et al. 2013) owns a patent, assigned to Evonik Degussa GmbH.
- **ChemChina-BlueStar/Adisseo Nutrition Group Ltd.** (China/France; www.chemchina.com.cn/en/)—In 2006, the French company Adisseo (see above) became a member of China's BlueStar-Group, since 2004 a subsidiary of ChemChina. In 2013, Adisseo confirmed the start-up of its Chinese methionine unit in Nanjing according to plan, which mirrors its sister plant in Burgos, Spain. Feed-grade DL-methionine is produced by subsidiary Adisseo-France (formerly Aventis) under the brands Rhodimet® AT88 (liquid) and Rhodimet® NP99 (powder). Newer products are Smartamine® and Metasmart®, both rumen-protected products for dairy cows to increase the methionine content in milk. The entire process is now fully operational and delivers Rhodimet® AT88 on specification with the same quality standard as the plant in Burgos, Spain. The production capacity in 2013 was 70,000 tons/year and will be expanded to maximum 140,000 tons/year by 2016. In 2014, most of the production in China will be reserved for the domestic market (BlueStar 2014).
- **DSM**, formerly Gist Brocades (NL; www.dsm.com/markets/anh/en_US/home.html)—DSM is one of the world's leading suppliers of feed additives, such as vitamins, carotenoids, eubiotics and feed enzymes (e.g., proteases). In 2014, DSM announced the opening of a new animal nutrition center in Bazhou (Beijing), China, focused on swine and poultry nutrition. DSM's major quest in animal nutrition is to reduce feed costs by adding special proteases (Ronozyme® ProAct®) to the feed, providing higher digestibility of the proteins (DSM 2014). So far, no amino acids are in the portfolio.
- **DuPont-Danisco Animal Nutrition**, formerly Danisco and Genencor (USA; http://animalnutrition.dupont.com/)—An older Genencor patent provides methods for the fermentation of L-methionine using a genetically engineered *E. coli* and a reduced sulfur source such as sulfide or methylmercaptane=methanethiol (Lievense 1993). Since 2011, Genencor and Danisco were integrated by DuPont and named as Danisco animal nutrition. Betaine from non-genetically modified sugar beet as Betafin® should replace some methionine due to its methyl-donor function (Dupont 2013).
- **Evonik** (Germany; www.evonik.de)—In Q3 2014 Evonik industries (formerly Degussa) will start-up a new DL-methionine plant in Singapore increasing the global capacity by 150,000 tons/year. The Evonik brands of methionine are MetAMINO®, synthesized and Mepron®, a rumen-protected (retard) product of DL-methionine for dairy cows. A new methionine product AQUAVI® is launched for aquaculture of shrimps and crustaceans, mainly in China (Evonik 2014a). The subsidiary for pharmaceutical products is Rexim® with 3000 tons/year production capacity in Nanning, China for pharma grade L-methionine. The biotechnological route to L-methionine is also object of Evonik's research activities (Zelder et al. 2013). In Fall 2013, Evonik called for research proposals (ECRP) concerning DL-methionine synthesis without using the toxic hydrocyanic acid. Some 100 German universities were asked to participate. In Spring 2014, three winners out of 15 proposals were awarded. Evonik is now negotiating about a research partnership with the awarded winners (Evonik 2014b).
- **Hifeed** (China; http://www.hifeedholding.com)—China's leading feed company has started feed-grade (99 %) DL-Met production in the year 2000 at Wuchuan, Guangdong, Hifeed is also supplier to Ajinomoto (see above).
- **Jilin City**, China (http://english.jl.gov.cn)—The National Economic and Technological Development Zone of the city Jilin in the north east of China is projecting a 100,000 tons/year DL-methionine plant at the Jilin chemical industry park. The proposal has been submitted (Jilin 2013).
- **Jingang Chemical Co., Ltd.** (Dalian, China; http://en.jingang-group.com/)—Jingang decided to cooperate with Sumitomo, to build a 20,000 tons/year capacity DL-

methionine plant in Dalian, China (Sumitomo 2009a); 80 % of the production contributes to Sumitomo and 20 % to Jingang-group.

- **Jirong Amino Acid Co., Ltd.** (Jinzhou, China; www.jirongpharm.com)—The producer of food grade L-methionine and other L-amino acids for pharma applications with an annual output of 500 t is planning to build a new plant in the near future.
- **JSC** (Volzhskiy Orgsynthese), Russia (www.zos-v.ru/en/; http://met.zos-v.ru/en/)—JSC is the only Russian methionine producer of 25,000 tons/year capacity at Volzhskiy near Volgograd situated on the river Volga. Since 2005 GOST-certified feed-grade 99 % DL-methionine is produced and mainly exported.
- **Kyowa Hakko Bio Co. Ltd** (www.kyowahakko-bio.co.jp/english)—Kyowa Hakko Bio, since 2008 a subsidiary of Kyowa Hakko Kirin, is the world's biggest amino acid producer (L-glutamic acid >1 million tons/year). Research on metionine fermentation has been doing in the early 1970s resulting in a methionine overproduding strain ATCC® 21608™ (Nakayama et al. 1973); however by the authors knowledge, an own methionine manufacturing plant is not implemented.
- **Metabolic Explorer (MetEx) and Roquette** (France, see below; www.metabolic-explorer.com)—Metabolic Explorer and Roquette have decided to terminate their previous agreements and to enter into a new agreement on the joint industrial development of L-methionine technology with the assistance of Roquette. The financial terms of this new agreement are confidential. The next step in the regulatory and approval procedures is to obtain the formal authorization from the US Food and Drug Administration (FDA), whose decision is expected by end of 2014. In the future, the construction of the plant at Bio-XCell industrial park in Nusajaya, Johor (Malaysia) will be resumed by Technip, France (MetEx 2014). MetEx owns numerous patents on genetically engineered *E. coli* with respect to L-methionine over-production, especially the energy balance (NADP provision, increasing yield) and so decreasing costs (Figge et al. 2009; Bestel-Corre et al. 2012; Dischert and Figge 2013a; Dischert et al. 2013).
- **Novus** (USA/Japan) www.novusmethionine.com—Novus is privately owned by Mitsui&Co. (USA) and Nippon Soda Co., Ltd. in Tokyo, Japan. They offer four methionine delivering feed products under the brand ALIMET®, an 88 % methionine source, MHA® a feed supplement, both based on the naturally occurring Met-precursor, HMTBa, which is readily converted to L-methionine (yield, 84 %), when entering the tissue of the animal, yielding 84 % L-methionine; Mera™Met, the calcium salt of HMTBa and MFP™, a dried methionine formulation (Novus 2012). The production of HMTBa takes place at the Nihongi Plant (Niigata, Japan). Novus Headquarter is in St. Louis, Missouri, USA. In 1991, Novus joined Nippon soda (Nisso, Japan), one of the oldest DL-Met manufacturers, producing since 1961, and became one of the three biggest Met-producers worldwide at the end of the last century. Nisso itself exited methionine production in 2007 (Cohen 2007).
- **Roquette** (France) www.roquette.com—In 2005, Roquette signed a worldwide, exclusive industrial licensing agreement with Metabolic Explorer (MetEx) on L-methionine production, which was in 2013 terminated and restarted under revised conditions (see MetEx). Actual Met-products are: Nutralys®, a pea protein extracted from dry yellow pea, highly purified and GMO-free and Tubermine® potato protein rich in lysine, methionine, tryptophan, and threonine.
- **Sumitomo Chemical Co. Ltd.** (Japan; www.sumitomo-chem.co.jp/english)—Sumitomo, Japan is one of the biggest methionine producers in Asia with a capacity of 140,000 tons/year. Feed products are Sumimet™-P (DL-methionine feed additive) and Sumimet™-L, the methionine hydroxy analog (MHA). Since 2009, the capacity at Niihama, Japan is expanding by nearly 40,000 tons/year, starting operation in 2010 to achieve total 140,000 tons/year in 2015 (Sumitomo 2009b). In 2014, the output was <10,000 t (FeedInfo 2014).
- **Unisplendour (UNIS) Chemical Co., Ltd.** (China; http://www.unischem.com/en/index.aspx)—Chongqing Unisplendour Chemical Co., Ltd. (CEC) was founded in 2000. DL-Methionine production by chemical synthesis started in 2010 (as demonstration plant) and 2011 (as production plant). The desired capacity of 60,000 t/year was reached in 2013. However, production is stopped since 2012 (FeedInfo 2014).
- **Wacker chemical AG** (Germany; http://www.wacker.com/)—Wacker is the world's leading L-cysteine producer. Wacker is also studying methionine fermentation, obviously as a precursor for their cysteine process. Maier et al. (2004) have filed a patent about it. In an example, a genetically engineered *E. coli* produced up to 4.8 g/L L-methionine in a glucose-controlled fed-batch process supplied with 10 g/L tryptone and 5 g/L yeast extract and thiosulfate as sulfur source. Currently, there are no published activities concerning L-methionine fermentation. In a new approach, L-methionine serves as a precursor for the chemical L-cysteine synthesis (Dassler et al. 2014)

Trends and prospects

Methionine is of major industrial importance. The synthetically produced feed-grade DL-methionine is mainly used in animal feed. Food-grade L-methionine, mainly used in human

nutrition and medicine, amounts to only 5 % of the whole Met market, but due to the higher price, the monetary value is comparable. A third quality should serve the animal feed market in organic farming, where legislation prohibits or limits the use of synthetically produced additives. Thus, companies are trying to develop an economical process for the production of L-methionine from natural sources without using GMOs. Currently, no plant is running on a commercial base. Several fermentation studies from more than three decades have shown that methionine concentrations higher than 5 g/L are hardly achievable using conventional means. Many of the published data are rather questionable and need to be reviewed. Genetic engineering should be able to exceed these results. Currently, there is only one company (MetEx) which could succeed in the next years even though the scientific and technical efforts are extensive. However, the aim to supply the organic farming market with "eco"-methionine is not yet realized.

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