# Chapter 3

# Trace element enzymes in reactions essential for anaerobic digestion

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## ABSTRACT

Trace elements play a very important role on the performance and stability of biogas digesters from a variety of biomass-containing residues, both natural or synthetic. Degradation of these complex chemical compounds occurs by the interaction of numerous microorganisms carrying out a series of pathways involving fermentative processes that ultimately lead to methane production. The purpose of this study was to provide an overview of the direct relationships existing among trace elements and enzyme activity which regulates the anaerobic digestion processes carried out by these microorganisms. Methanogenesis is one of the most trace-element enriched enzymatic pathways in biology. Trace elements are major key elements in the functioning of multiple enzymes reviewed within this work. Although exact trace-element requirements may differ slightly between pathways depending on composition and the microorganisms involved, there are some general trends characterizing the anaerobic digestion processes. Iron (Fe) is the most abundantly required metal, followed by nickel (Ni), cobalt (Co), molybdenum (Mo), tungsten (W), and zinc (Zn). In order to sustain the


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anaerobic digestion, trace element ions are needed for the correct structural formation and the working of those enzymes. The lack of understanding on metabolic prerequisites of microorganisms and their regulatory networks, above all at full-scale industrial anaerobic digesters, may result in consequent borderline conditions with insufficient microbial activity towards optimized methane production processes.

**KEYWORDS**: anaerobic digestion, enzymes, metals, methanogenesis, trace elements

## 3.1 INTRODUCTION

Anaerobic digestors represent a complex set of interactive actions by numerous types of microorganisms carrying out multiple metabolic pathways involved in the final production of methane, a potential source of renewable energy (Ferry, 2011). Methane and surplus heat are final products of anaerobic digestion and come with other benefits, such as a reduction in input organic loads. Also, bioremediation or biodecontamination of multiple pollutants or undesired compounds which need to be processed to reduce secondary environmental effects thus leading to an improvement in output waste which can then be used safely for other means, such as fertilizers or to meet the requirements for disposal to the environment.

The importance of anaerobic digestion is increasing due to increasing volumes of wastes that need to be processed and the diversity and variety of wastes (Verstraete *et al*., 2002; Tabatabaei *et al*., 2010). To achievement required levels for modern processes, anaerobic digestions need to be optimized so that the increasing amounts of wastes generated by modern society can be processed. Anaerobic digestors are major contributors to the processing of wastewaters, industrial sludges and numerous residues, including toxic and highly polluting wastes. Anaerobic digesters offer a practical solution to the processing of increasing amounts and variety of residues being generated. The same anaerobic processes take place in natural environments such as animal rumen, rice fields, peatlands, soils, and other ecosystems. Different environments, either natural or man-made, can present some differences in the microorganisms involved in the processes of degrading biomass all the way down to methane. The large microbial diversity existing in these microbial communities is well known and has been shown to be fairly stable over time in spite of moderate external or environmental changes.

Depending on the history, biogeography, and operational parameters of biogas reactors, crucial microbial constituents can fluctuate showing different abundance and level of activity as a consequence of, for instance, trace-element deficiency, long-term limitation of organic substrate or short-term overload (Murovec *et al*., 2018; Repinc *et al*., 2018; Sun *et al*., 2015). This variability

builds up the diversity of both microorganisms and their enzymes implicated in the processes leading to methane and the complete mineralization of organic matter (i.e. biomass).

Consequently, anaerobic digestion is a complex process involving many types of microorganisms which complement their metabolic capabilities to mineralize or degrade a huge variety of organic compounds to $CO_2$ and methane to the highest extent possible under anaerobic conditions within the variable environmental conditions present in anaerobic digesters (Ferry, 2011). Different microorganisms carry out different steps of the processes and this explains the existence of the numerous types of metabolisms involved in the whole anaerobic digestion process of chemically highly divergent organic materials of either plant, animal or anthropogenic origin. Obviously, different metabolic pathways are constituted by numerous enzymatic reactions. The individual enzymes participating in each of these steps of the metabolic pathways involved are the final unit pieces of the anaerobic bioprocessing. Many of these enzymes are produced by a number of functionally equivalent microorganisms and require cofactors, trace elements, activators or complements and reaction substrates that need to be available to catalyze a specific chemical reaction. Trace elements are major key elements in the functioning of multiple enzymes. Metalloenzymes are metal-dependent enzymes and a large number of metalloenzymes has been described so far. Anaerobic processes (denitrification, sulfur reduction, methanogenesis and others) contain metal-dependent enzymes (Kapoor *et al.*, 2015). Methanogenesis is one of the most trace-element enriched enzymatic pathways in biology. Although the exact trace-element requirements may differ between the methanogenic pathways, depending on the used substrates, there are some general characteristics on metal requirements. Iron (Fe) is the most abundantly required trace element, followed by nickel (Ni) and cobalt (Co), and trace amounts of molybdenum (Mo) and/or tungsten (W) and zinc (Zn). Fe is generally used for transference of electrons in Fe–S clusters (Glass & Orphan, 2012), Ni can be either bound to Fe–S clusters or in the center of porphyrin (cofactor $F_{430}$) that is a hallmark of methanogens. Cobalt is present in cobamides involved in methyl group transfer, whereas Zn occurs as a single structural atom in several enzymes. Molybdenum (Mo), or tungsten (W), is attached to a 'pterin' cofactor to form 'molybdopterin' or 'tungstopterin', respectively, and involved in catalyzing electron redox reactions. Other alkali metals and metalloids, such as sodium (Na) and selenium (Se), are also essential for methanogenesis (David & Alm, 2010; Dupont *et al.*, 2006, 2010; Glass & Orphan, 2012). In order to sustain metabolic activity of the cell all these ions are required.

In general, an enzyme accelerates the rate of a chemical reaction, decreasing the thermodynamic threshold for the reaction to occur. Similarly, metalloenzymes catalyze chemical reactions if the adequate trace-element atom is available in the process. Depending on the enzyme, the trace-element atom is required as a redox element, a cofactor for the enzyme, for the proper configuration of the enzyme to

reach its fully functional three-dimensional structure through correct protein folding. This chapter attempts to understand the interaction of trace elements with enzymes so that these biocatalyzers achieve optimum activity and so the anaerobic digestion process develops its maximum potential.

The anaerobic process is completed by the interactive action of microorganisms each performing its characteristic metabolism. Each metabolic pathaway involves the activity of different enzymes tightly regulated to perform serially from specific substrates to final defined products. In response to environmental conditions, short-term adjustments in the anaerobic digestion process conduce to determined levels of gene expression which may induce variants of specific enzymes. Slightly different enzymes have been considered as the initial step of process heterogeneity (Delvigne *et al*., 2014), the initiator of microbial diversity and process variability. This in turn results in ongoing redistribution of organic matter, trace elements and secondary metabolites between various microorganisms within a microbial community, generating fluctuations in the activity of the whole microbial community that ultimately results in a modified community structure over time, as reported before (Repinc *et al*., 2018; Sun *et al*., 2015).

This chapter deals with the importance of trace elements for the enzymes, which represents the minimum functional units involved in the process, so that the biocatalytic processes can be optimally carried out during anaerobic digestions. Microbial growth requires nutrients in the form or organic and inorganic compounds to produce energy and biomass for growth. Energy can be used to support maintenance functions by prokaryotes in the cells as well as to contribute to building the biomolecular blocks required for growth through a progressive increase in biomass and final cell division into daughter cells. The capability of microorganisms (i.e. Bacteria and Archaea) to self-maintain and to obtain and process the substances available in their surroundings are the basis for the optimization of major biological processes required in the regeneration of industrial and society residues, such as in anaerobic digesters.

## 3.2 MAJOR PATHWAYS AND TRACE-ELEMENT REQUIREMENTS IN ANAEROBIC DIGESTION

During anaerobic digestions of chemically complex substrates there are many microbial metabolic pathways involved in the production of methane, such as wastewater treatment plant sludge, paper mill sludge, organic fraction of municipal solid waste, biogas energetic plants, fats and oils, dairy wastewaters, spoiled food and drinks, and waste streams from food and pharmaceutical industries (Ferry, 2011). Many of the steps involved in anaerobic digestion require the availability of specific trace elements to maintain the process and at a rather high rate under the complex environmental conditions of industrial scale reactors.

In order to simplify the complexity of reactions participating in methane production and the mechanisms of trace-element involvement in anaerobic digesters, the following major pathways need to be introduced. First, the bundle of hydrolysis, acidogenesis, acetogenesis where the organic load mostly formed by complex organic molecules, that is, polymers, are degraded into smaller molecules easily taken up by most microorganisms. This represents the basic organic carbon processing routes summarized in Section 3.2.1. Second, the nitrogen cycle is of critical interest in anaerobic systems, above all because some major pathways of nitrogen processing are exclusively performed in anaerobiosis. This is the case for denitrification, dissimilatory reduction of nitrate to ammonia, and anaerobic ammonia oxidation processes, essential for the removal of large amounts of nitrates or ammonia during anaerobic digestion, without methane production (Bothe *et al*., 2007). Third, sulfate reduction represents another central microbial metabolism in anaerobic systems, especially as it interacts with various trace elements that precipitate as sulphides and thus become unavailable to microbes due to the production of $H_2S$ by dissimilatory sulfate-reducing bacteria (DSRB) which are common competitors of methanogenesis (Barton & Fauque, 2009; Lens & Kuenen, 2001; Muyzer & Stams, 2008). Last, the methanogenic pathway, or methanogenesis, represents the final steps for the production of methane that is carried out exclusively by methanogenic Archaea under strict anaerobic conditions.

### 3.2.1 Organic carbon processing

A major series of preliminary steps during anaerobic digestions is the hydrolysis of complex organic matter down to small molecules or monomers that can be taken up by microorganisms as building blocks for biosynthesis to produce biomass and turnover the biomolecules required to maintain the cellular biological machinery.

Cells incorporate trace elements using specific transporters and sensors which are often discovered as metal-resistance genes (Waldron & Robinson, 2009). Their novelty suggests that there is a broad field of research to be studied in the future years. Cells have evolved mechanisms to recover the required trace elements from the environment to meet their needs. Another point that has been demonstrated is that the removal of trace elements from a bacterial medium, for instance by metal chelation, leads to the inhibition of bacterial growth, a sign of critically reduced metabolic activity (Corbin *et al*., 2008). Thus, bacteria require the presence of certain concentrations of trace elements for proper functioning at the cellular level within the biogas production environment. This is ultimately due to the metallic prerequisites for the correct functioning of multiple enzymes.

A large fraction of biomass supplied to anaerobic digestion is represented by residual plant biomass from crop or food industries. Thus, the residues to be

processed contain a highly divergent fraction of lignin, hemicellulose and cellulose, all randomly intertwined within plant cell walls for structural and antimicrobial effects. These polymers are the most abundant components of litter and need to be depolymerized, that is, chemically broken down in order to be metabolized (Cooke & Whipps, 1993; Fioretto *et al*., 2005). Microorganisms experience great difficulties in degrading lignin compounds under the anaerobic conditions of biogas digesters, hence the extent of lignin derivatives in the form of aromatic compounds increases over time. The process of lignin and cellulose decomposition in biological systems has attracted attention because their hydrolysis frequently limits plant biomass degradation and consequently, the processing of wastewaters, industrial organic sludge and residues containing a large proportion of plant biomass. Usually, anaerobic reactors must perform the whole digestion of input residues down to the end products of fermentations which represent the basic substrates used for methanogenesis (Acetate, $CO_2$ and $H_2$ and one- or two-C molecules such as methylamines, methanol, formate, as some examples) (Murovec *et al*., 2018). The complex metabolism results in a number of metabolites (Murovec *et al*., 2018) produced by highly active interactions between different groups of microorganisms which center their ecological niche in maintaining degradation of specific type of chemical bonds between or within complex molecules. The sequential decomposition of major polymers involves many and diverse sets of microorganisms (Guo *et al*., 2015; Madigan *et al*., 2003). Figure 3.1 shows a simplified and generalized scheme of the processes involved in anaerobic decomposition of complex polymers (e.g. hemicellulose, xylan and cellulose) down to methane through small organic acids and substrates adequate for methanogenesis.

#### *3.2.1.1 Complex polymers*

Although most polysaccharides are decomposed by extracellular enzymes that do not show trace-element requirements, the microorganisms involved in this decomposition require basic sets of trace elements at the level of their cellular metabolism. Microorganisms require trace elements for many of their enzymes to work properly and an important set of metalloenzymes, central to the metabolism of cells, are those involved in redox processes where Fe, Ni, Co and Zn are typically required (Garuti *et al*., 2018).

The processes of lignin and cellulose enzymatic degradation have also been reported to be influenced by trace elements. For instance, Berg *et al*., (1995) reported that high concentrations of Mn were essential for the correct functioning of some lignin-degrading enzymes, such as Mn peroxidases, which are also directly involved in the oxidative decomposition of lignin. Other authors (Quinlan *et al*., 2011) have observed that Cu greatly enhances the oxidative degradation of cellulose by some oxidative metalloenzymes. Research has also shown that divalent trace element salts (i.e. Ca, Mg) participate in the formation

of metal-lignin complexes which enhances the degradation of lignocellulosic materials (Liu *et al.*, 2010) because the trace element bound improves the accessibility of enzymes to target substrate sites, a major limiting factor for lignin biodegradation. Recent investigations have shown that supplement of Ba (10 mM) greatly enhances the activity of cellulases and esterases (Muñoz *et al.*, 2016), enzymes that complement the enzymatic decomposition of cellulose and lignin. Besides fermentations that produce low-molecular weight end products (i.e. small organic acids, ethanol, $CO_2$, $H_2$) from sugars, a major route for the production of acetate in anaerobic systems is the anaerobic oxidation of fatty acids, which is often performed by syntrophic relationships with methanogenic Archaea and sulfate-reducing bacteria depending on the existing conditions. The process and the syntrophic relationship have been reported to require Mg (0.15% w/v; Stieb & Schink, 1985).

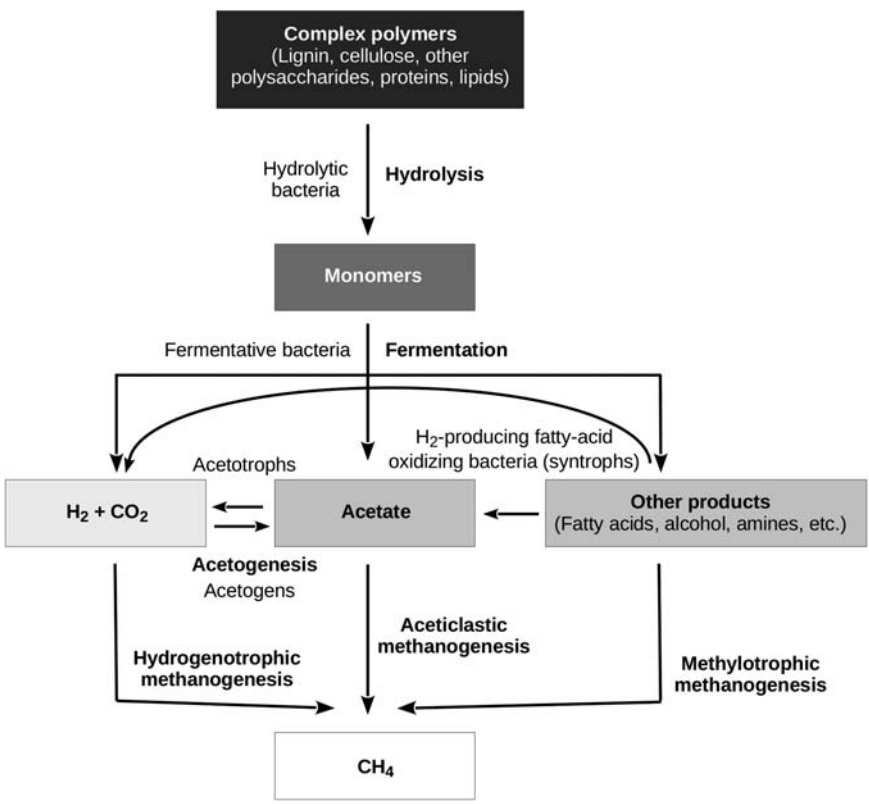


**Figure 3.1** A model of serial decomposition of complex organic matter (i.e. plant biomass) to end products of fermentation and the formation of methane during anaerobic digestion.

Simultaneously to the fermentation process carried out under anaerobic conditions, and responsible for breaking down polymers into small fermentation products, the bacteria involved in these processes require the use of electron-transport systems which also require specific trace elements to maintain their function. Typically, the requirement for Fe-dependent hydrogenases and redox transformations involving cytochromes (Lehninger, 2000) during the growth of anaerobic bacteria could represent a major limitation to good performance in anaerobic bioreactors.

### 3.2.2 Nitrate and sulfate reduction

The sporadic inclusion of substrates that contain elevated concentration of oxidized forms of nitrogen or sulfur into anaerobic digesters results in their reduction to ammonia or hydrogen sulfides, and trace-element precipitates with a concomitant reduction in methane production. Enzymes involved in nitrate reduction to either $N_2$ (denitrification) or ammonia (DNRA, Dissimilatory Nitrate Reduction to Ammonium) or sulfate reduction to sulfide represent complex enzymatic pathways that all contain numerous metalloenzymes.

Enzymes involved in denitrification and DNRA contain nitrate, nitrite, nitric oxide, nitrous oxide reductases that require Mo-bis-molybdopterin guanine dinucleotide cofactor and at least one 4Fe-4S cluster, copper (CuNIR, nitrite reductase) or iron (heme cd1 NIR, nitrite reductase), iron (heme cNOR, nitric oxide reductase) and copper (NOSZ, nitrous oxide reductase) (Kielemoes *et al.*, 2000). Besides, DNRA pathway contains an additional complex set of enzymes requiring Mo, Fe, Fe-S clusters.

Enzymes accomplishing S reduction are complex structures with large molecular mass and possess at least two different polypeptides in an a2b2 tetramer containing [4Fe–4S] centers and siroheme in sulfate oxidation. The tetraheme cytochrome c3 represents the constitutive enzyme group in elemental sulfur reduction. Both groups are dependent also on the three classes of hydrogenases that contain [Fe], [NiFe] and [NiFeSe] that are essential for effective sulfate oxidation to hydrogen sulfide (Glass *et al.*, 2014).

The existence of N and S reduction pathways that rely heavily on the uptake of trace elements from the pool present in the anaerobic system represent an obstacle to simple systematic saturation of the anaerobic digestion processes by trace-element augmentation. Microorganisms performing various enzymatic reactions, thus, constantly compete for trace elements between themselves and with environmental conditions that may lead to sequestration of trace elements through either polyvalent aromatic compounds and siderophores, or precipitation with sulfur.

### 3.2.3 Methanogenesis

Methanogenesis is the final and most critical pathway of the anaerobic digestion because it is the process leading to the production of methane. Methanogenesis

has been classified in three metabolic groups according to the use of acetate, hydrogen and $CO_2$ or methyl-groups (methanol, methylamines, formate, etc.), namely aceticlastic, hydrogenotrophic and methylotrophic pathways, respectively. All three pathways end in the final common steps that result in the release of methane, which is governed by the methyl coenzyme M reductase and the Zn-containing heterodisulfide reductase, both common to all methanogenic pathways. Extensive studies on the whole methanogenic pathway implicated enzymes that required novel cofactors and additional trace-element requirements. Figure 3.2 shows the enzymes characteristics of the methanogenic pathways with indications of major trace-element requirements. Trace-element requirements of the three general methanogenic pathways are relatively overlapping therefore unified and simplified observations are presented in this chapter rather than small differences between the three routes or specific differences in particular species traits.

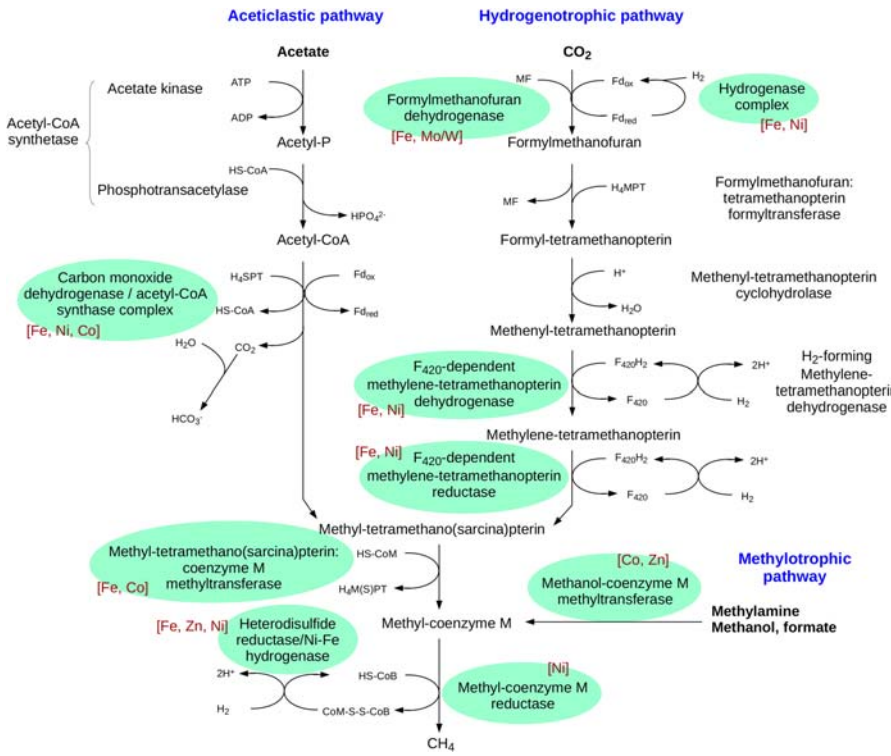


**Figure 3.2** Representation of the enzymatic steps involved in the three methanogenic pathways with indication of the enzymes and their trace element requirements.

The trace element requirements of the enzymes involved in the methanogenesis have been reviewed previously (Glass & Orphan, 2012) providing the basis for the requirements of different trace elements as inhibitors or activators of the corresponding enzymes according to their depletion or supplementation, respectively.

Methanogenesis has revealed the existence of unique trace-element-requiring enzymes (Zerkle *et al*., 2005) which has required intensive work in the field (Ferry, 2010; Thauer, 1998). Generally, Fe, Ni, Co, Mo or W and Zn are the most important trace elements required in the process (following order of abundance). Along the methanogenesis pathway, redox reactions represent some of the critical steps. The presence of Fe is typically observed forming part of the Fe-S clusters which are involved in electron transfer and are a common feature in most enzymes in the pathway. Ni binds to some Fe-S clusters and to the porphyrin ring forming the unique methanogenesis cofactor $F_{430}$ (see below and Figure 3.3).

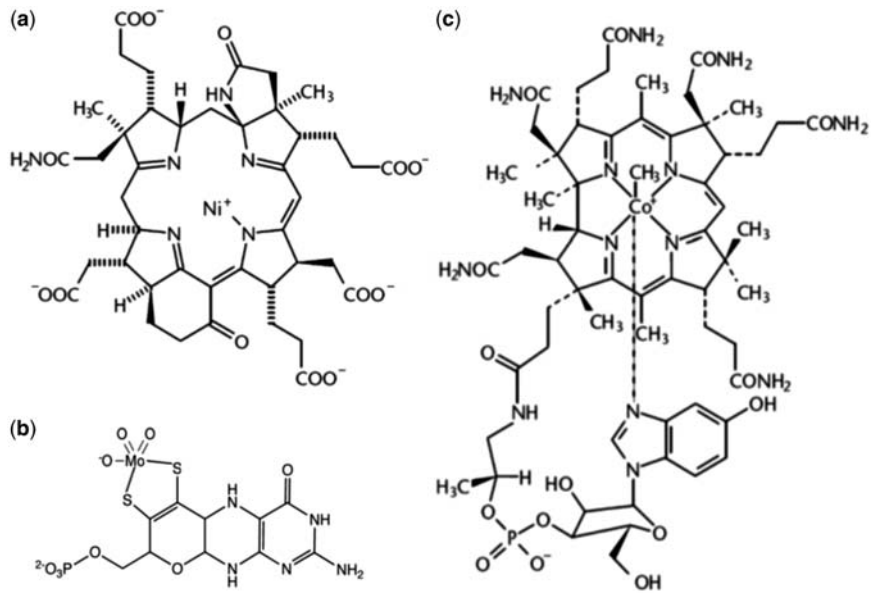


**Figure 3.3** Additional examples of unique trace-element requirements in methanogenesis. Trace-element-containing cofactors unique to the methanogenic pathways: A – factor $F_{430}$; B – molybdenum-pterin (replace Mo with W to visualize the tungsten-pterin structure); C – 5-hydroxybenzimidazolylcobamide (Factor III).

All the methanogenic species described so far belong to the Archaea domain (Madigan *et al*., 2003). Most methanogenic Archaea are able to perform only one of the three pathways although the exception is represented in the

Methanosarcinales; for example, *Methanosarcina acetivorans* (Galagan *et al*., 2002) is able to carry out methanogenesis using the three described pathways metabolizing a broad range of substrates. Other unexpected results are, for instance, the report of methane production and high-density growth by *Methanocaldococcus jannaschii* from starch and organic supplements (Mukhopadhyay *et al*., 1999), which contrasts with the typical one- or two-C molecules typically used by methanogens and suggests that further research is needed on the physiology of methanogens.

The first enzyme in the hydrogenotrophic pathway from $CO_2$, formylmethanofuran dehydrogenase binds with up to 9 $Fe_4S_4$ clusters including their links to polyferredoxin and to a Mo or W-pterin (Figure 3.3) subunit depending on the species (Vorholt *et al*., 1996). Besides, the formyl methanofuran dehydrogenase is intimately related to four different energy-converting hydrogenases containing multiple $Fe_4S_4$ clusters and a Ni atom in addition to its dependency to polyferredoxins, which contain multiple (additional 6–14) $Fe_4S_4$ clusters forming a highly evolved electron-transfer system from $H_2$ (Daas *et al*., 1994; Ferry, 1999).

Down the hydrogenotrophic pathway, a multi-aggregated hydrogenase containing Ni and Fe in its active site and four $Fe_4S_4$ clusters (Fox *et al*., 1987) is in charge of reducing cofactor $F_{420}$ with $H_2$. Under limitation of specific trace elements (i.e. Ni), this hydrogenase can be partially replaced by others with lower or different trace-element requirements, but some trace elements are always required in this step (Afting *et al*., 1998; Shima *et al*., 2008; Thauer *et al*., 2010).

In the aceticlastic pathway, a carbon monoxide dehydrogenase/acetyl-CoA synthase complex actively participates in the process releasing a $CO_2$ molecule, transferring a methyl group down the pathway. This enzymatic complex contains multiple $Fe_4S_4$ clusters, Ni and Co.

Both the aceticlastic and hydrogenotrophic pathways arrive at a common enzymatic reaction catalyzed by the methyl-tetrahydromethanopterin-coenzyme M methyltransferase (or methly-tetrahydrosarcinapterin-coenzyme M methyltransferase in *Methanosarcina* species) that transfers a methyl group to coenzyme M. This methyltransferase contains Fe atoms and cobamine cofactors containing Co (Gartner *et al*., 1993). In addition, the synthesis of methyl-coenzyme M needs multiple Co requiring methyltransferases in methylotrophic pathway.

A final and common enzyme for all methanogenic pathways is the methyl coenzyme M reductase which releases methane from the methyl-coenzyme M generating the coenzyme M-coenzyme B heterodisulfide. The methyl coenzyme M reductase contains two coenzyme $F_{430}$ (Ermler *et al*., 1997), unique to methanogens, with a Ni atom each (Figure 3.4) and the cellular level of $F_{430}$, depends on Ni availability (Diekert *et al*., 1981; Lin *et al*., 1989) which could be reflected in potential Ni-limiting growth and methane production.

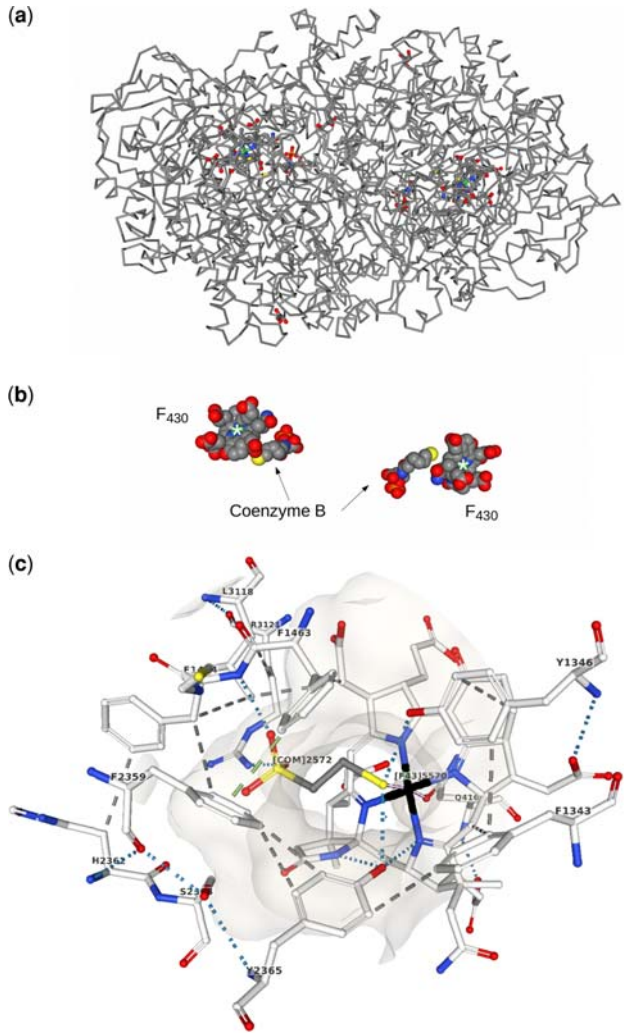


**Figure 3.4** 3D structure of the enzyme methyl-coenzyme M reductase from *Methanosarcina barkeri* (Accession Number 1E6Y) at 1.6 Å resolution: A – general 3D-structure of the enzyme showing the location of cofactors and trace elements; B – location of coenzyme B and coenzyme F430 within the whole enzyme structure (The Ni atoms are indicated with a white asterisk in the center of the F430 molecules); C – a single-ligand pocket view showing the interactions with the amino acidic structure ([COM] - Coenzyme M; [F43] – Factor F430 showing the central Ni atom in black; hydrogen bonds – dotted links; hydrophobic contacts – grey dashed links; trace-element interactions – white-centered dashed links). A and B are at the same scale so they can be superimposed. C is magnified to visualize interactions of amino acid residues, cofactor and trace element.

All methanogenic pathways use the heterodisulfide reductase, which contains Zn and multiple $Fe_4S_4$ clusters to catalyze the reduction of the heterodisulfide (i.e. coenzyme M-coenzyme B) to recycle the coenzyme M and coenzyme B molecules. This enzyme is tightly linked to a methyl-viologen hydrogenase which also contains Ni and multiple types of Fe-S clusters (Hedderich *et al*., 2005; Thauer *et al*., 2008) besides its relationship to additional polyferredoxins containing 12 $Fe_4S_4$ clusters (Reeve *et al*., 1989). Peculiarities of subunits and their relationships to different hydrogenases exist in different methanogens and all these associated reactions sum up more trace element requirements (mainly Fe, Ni and Zn) (Glass & Orphan, 2012).

## 3.3 MAJOR ENZYMES INFLUENCED BY TRACE ELEMENTS

As summarized above, numerous trace-element-dependent enzymes participate in the metabolic pathways that lead to the production of methane during anaerobic digestion. In this section, the interactions taking place within some enzymes with trace element atoms are reviewed in order to present the essential participation of trace elements to maintain the enzymatic activity and structure required in the described pathways. Key enzymes of the methanogenic pathways are presented as examples of those essential interactions to understand the importance of trace elements and the mechanisms involved in trace element requirements during anaerobic digestion. An exhaustive list and evaluation of all the potential enzymes and trace elements involved in the anaerobic processing of complex organic loads is not the aim of this chapter.

### 3.3.1 Methyl-coenzyme M reductase

Methyl-coenzyme M reductase is a nickel tetrahydrocorphinoid-containing enzyme (coenzyme $F_{430}$; Figure 3.4) involved in the biological synthesis and anaerobic oxidation of methane, specifically involved in the latter, and a common step of methane production. Methyl-coenzyme M reductase catalyzes the conversion of methyl-coenzyme M and coenzyme B to methane and the heterodisulfide of coenzyme M and coenzyme B (DiMarco *et al*., 1990; Wongnate & Ragsdale, 2015). This is the rate-limiting step and so, a main candidate to define a reduction of methane production as a result of potential scarcity of trace elements (i.e. Ni).

Structures of methyl-coenzyme M reductases have been solved and their general structures have been well described, in brief, by Wongnate & Ragsdale (2015): 'The crystal structures show that MCR is a dimer of heterotrimers ($\alpha_2\beta 2\gamma_2$) with a molecular mass of 270 kDa (Ellefson & Wolfe, 1981). The three subunits ($\alpha\beta\gamma$) tightly associate to form two 50 Å hydrophobic channels (one in each heterotrimer) (Ermler *et al*., 1997) ending in a pocket that accommodates a

redox-sensitive nickel tetrapyrrole cofactor (coenzyme $F_{430}$), which plays an essential role in catalysis (Goubeaud *et al*., 1997; Becker & Ragsdale, 1998)'. The mechanisms involved in this enzyme activity have been studied (Wongnate & Ragsdale, 2015) and the enzyme requires strict anaerobic conditions to show activity. The structure envisions the complexity of this enzyme forming two symmetric active sites and showing the location of the substrates (methly-coenzyme M and coenzyme B) and the Ni-containing cofactor (coenzyme $F_{430}$). Figure 3.5 shows the three-dimensional structure of a methyl-coenzyme M from a methanogenic archaeon.

### 3.3.2 Heterodisulfide reductase

The function of this enzyme is to recycle the oxidized coenzyme M-coenzyme B compound resulting from the activity of the methyl-coenzyme M reductase to regenerate the reduced forms of coenzymes M and B. This is a required step to maintain the pathway by providing reduced forms of these coenzymes. Heterodisulfide reductase forms a complex with Ni and Fe-dependent hydrogenase, methyl-viologen hydrogenase. The visualization of these coenzyme and trace-element interactions within the heterodisulfide reductase/[NiFe]-hydrogenase tridimensional structure is shown in Figure 3.5. The high number of different types of Fe–S clusters ($Fe_4S_4$, $Fe_3S_3$, $Fe_2S_2$, etc.) present in the molecule and coordinated in the Cys-Cys-Gly (CCG) motifs (Wagner *et al*., 2017) is worthy of note. For instance, in *Methanothermobacter marburgensis*, a novel type of $[Fe4\text{-}S4]^{3+}$ was reported (Hamann *et al*., 2007) and the N-terminal CCG domain could be linked to a Zn site suggesting an additional interaction enzyme-metals. This is another example of the extensive requirement of trace elements in enzymes of the methanogenic pathways.

### 3.3.3 Formylmethanofuran dehydrogenase

The enzyme formylmethanofuran dehydrogenase catalyzes the first step of the hydrogenotrophic pathway of methanogenesis. It reduces $CO_2$ and contains multiple $Fe_4S_4$ clusters (e.g. 46 in *Methanothermobacter wolfeii*; Wagner *et al*., 2016) (Figure 3.6). The enzyme catalyzes the reduction of $CO_2$ and methanofuran to form formylmethanofuran. The crystal structure of a formylmethanofuran dehydrogenase revealed two active sites separated by a 43 Å long tunnel responsible for the transference of the formyl group. The numerous $Fe_4S_4$ clusters apparently couple the four tungsten redox centers present in the enzyme from *M. wolfeii* (Wagner *et al*., 2016) forming a spiral along the protein. The case of *Methanobacterium thermoautotrophicum* is interesting because it contains a molybdenum formylmethanofuran dehydrogenase and a tungsten formylmethanofuran dehydrogenase (Hochheimer *et al*., 1996).

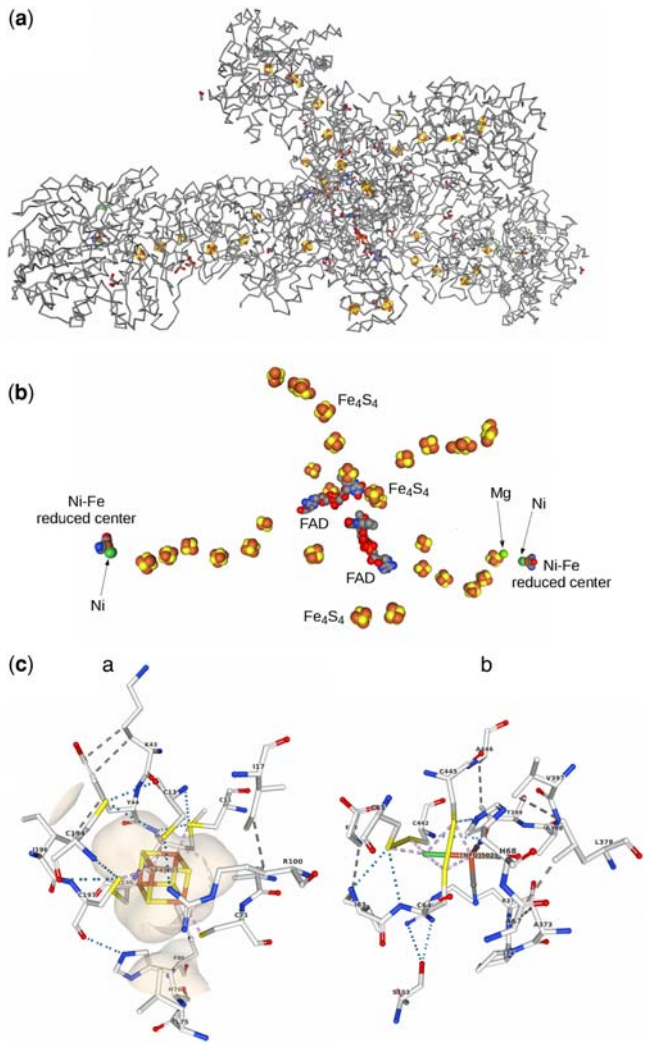


**Figure 3.5** 3D structure of the enzyme heterodisulfide reductase/[NiFe]-hydrogenase complex from *Methanothermococcus thermolithotrophicus* (Accession Number 5ODC) at 2.3 Å resolution: A – general 3D-structure of the enzyme; B – location of flavin (FAD), Fe4S4 clusters and Ni-Fe reduced centers within the whole enzyme structure; C – ligand pocket views showing (a) the interactions of a Fe4S4 cluster ([SF4] showed by the central cube) and (b) a Ni-Fe reduced active center ([NFU]) with the amino acidic structure. [SF4] – Fe4S4 cluster which constitutes the central cube in (C a); hydrogen bonds – dotted links; hydrophobic contacts – grey dashed links; trace-element interactions – white-centered dashed links). A and B are at the same scale so they can be superimposed. C is magnified to visualize interactions of amino acid residues and an Fe4S4 cluster.

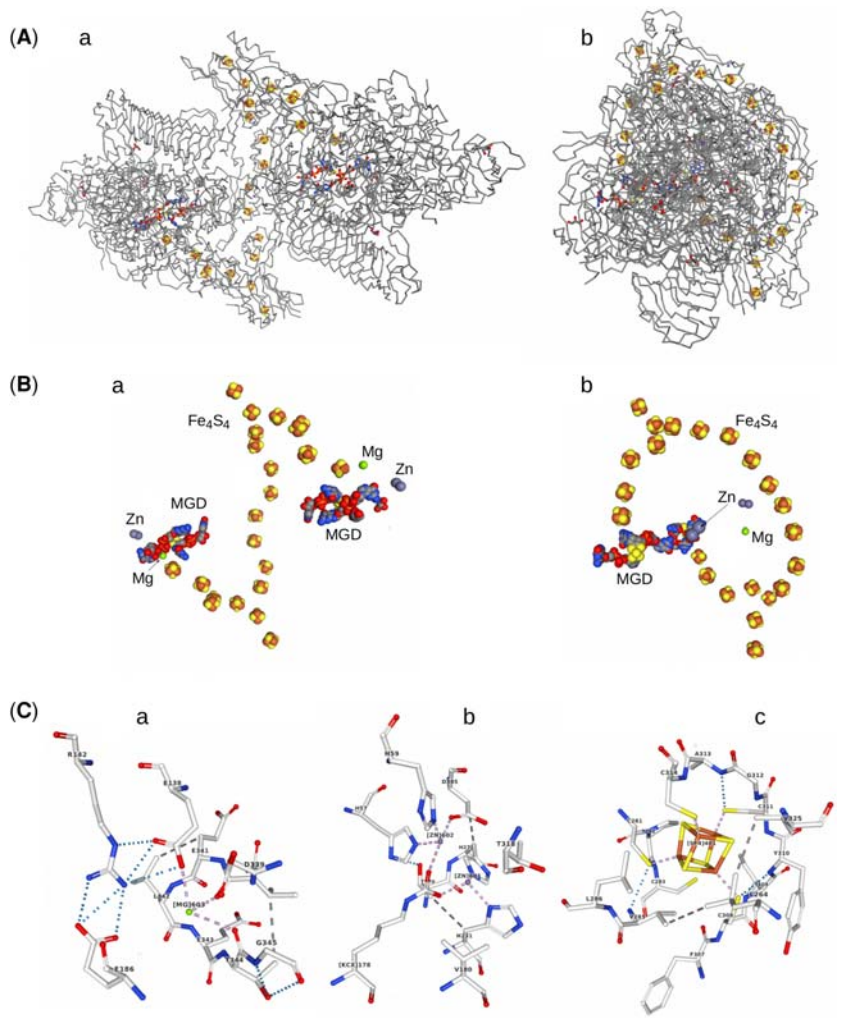


**Figure 3.6** 3D structure of the enzyme formylmethanofuran dehydrogenase from *Methanothermobacter wolfeii* (Accession Number 5T5I) at 1.9 Å resolution: A – general 3D-structure of the enzyme in two different perspectives (a) and (b); B – location of $Fe_4S_4$ clusters, Zn and Mg atoms and molybdopterin guanine dinucleotide (MGD) within the whole enzyme structure using the same perspectives that in A(a) and (b); C – ligand-pocket views showing (a) the interactions of a Mg atom (center of figure), (b) two Zn atoms (b) and (c) a $Fe_4S_4$ cluster (central cube) with the amino acidic structure (hydrogen bonds – dotted links; hydrophobic contacts – grey dashed links; trace-element interactions – white-centered dashed links). A and B are at the same scale so they can be superimposed. C is magnified to visualize interactions of amino acid residues and trace elements.

## 3.4 PERSPECTIVES

Metabolic pathways, including methanogenic pathways and all required diverse groups of microorganisms to fully mineralize biomass, represent highly coordinated networks of interactive features leading to a final product or products. This adds complexity to an already highly complex scenario of interactive behavior whose regulation is barely understood. These regulatory mechanisms remain to be studied in detail within a microorganism and between the microorganisms forming a whole natural, highly complex, community. Metabolism and its regulation is a field requiring intense research that will, eventually, drive us to a in-depth understanding on how microorganisms function and respond to their surroundings and environmental factors. The requirements of different trace elements can limit growth and responsiveness of microorganisms to their community partners, as well as reduce the functional viability of partial or full metabolic pathways. Herein, we have presented an overview of trace element–enzyme interactions which need to be comprehended in order to fully explain microbial and enzymatic response during optimization of methane-producing processes. Future research should be based on the conclusion that trace elements can (and often do) actually limit biomass degradation and methane production during anaerobic digestions.

Further research is required on trace-element interactions and enzymes both in the methanogenesis as well as in many other pathways that directly or indirectly influence methane production during anaerobic digestions of biomass (nitrogen and sulfur metabolisms). This advancement requires multidisciplinary investigations due to the multifactorial problem of the availability of trace elements in nature and complex systems (i.e. anaerobic reactors). Interactions between trace elements and other compounds (e.g. divalent cations, aromatic compounds and humic acids, microbial siderophores) lead to different levels of availability of trace elements for uptake and integration into biomass of microorganisms. Such cases force microorganisms to respond accordingly, increase their energetic expenditure and invest resources to fine-tune their protein makeup to their current environmental conditions. The lack of understanding of metabolic prerequisites of microorganisms in full-scale industrial anaerobic digesters may result in consequent borderline conditions with insufficient microbial activity towards methane production (Garuti *et al*., 2018; Murovec *et al*., 2018; Repinc *et al*., 2018).

A few future options such as nanotechnology, synthetic biology and chemical engineering should be considered. Nanotechnology (Duhan *et al*., 2017) that is used for specific delivery of particular elements or amelioration of limiting compounds contains valuable applied points to be considered. However, currently available solutions cannot be realized within the cost-benefit margins needed for implementation in actual bioreactors when applied to large-scale processes.

Synthetic biology has the potential to generate fully known microorganisms with a highly specific role in anaerobic bioreactors and is an additional alternative, perhaps in the next decade. However, this potential alternative requires precise and integrated knowledge of all processes involved and is still currently outwith our grasp.

The exploration of the fine boundary between the thresholds of methane-limiting trace element concentrations and trace element growth inhibition should be considered above all, because the chemical reactions governing the availability of trace elements in complex systems are not fully understood. The potential toxicity of some trace elements in presence of other elements or organic compounds or under specific conditions further shows the multifactorial complexity that needs to be considered when fine tuning trace-element requirements in complex systems is attempted.

Overall, our current understanding on trace-element limiting methane-production during anaerobic digestion processes is in demand of further research from several perspectives to close the existing gaps in knowledge resulting from the high complexity of anaerobic digesters. This is observed from the huge microbial diversity and variability of enzymes and metabolisms, the regulatory mechanisms involved both in controlling microbial dynamics and enzyme-encoding gene expression, as well as enzyme activity in response to the availability of the trace elements required during the whole biodegradation of biomass to ultimately generate methane.

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