2 Biomethanization in Anaerobic Systems
Feedstock and Process Enhancement

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2.1 INTRODUCTION

Anaerobic digestion (AD) has gained popularity in recent times as a technology for the addition of value to biomass and climate change mitigation due to its ability to recover methane from biomass. Methane recovery from biomass is essential because biogas, a product of AD, contains methane that could be used for electricity generation and as domestic cooking gas. With regard to climate change, methane is a potent greenhouse gas that must be captured and reused. Otherwise, when released into the atmosphere, methane causes global warming (and accelerates climate change) with its attendant health, environmental and socioeconomic effects. Hence, understanding and improving AD technology, especially methanization processes, at industrial scale, contributes in no small measure to capturing methane from biomass for reuse in electricity generation and heating, and mitigating climate change.

2.1.1 Fundamentals of Anaerobic Digestion

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Municipalities, industries and agriculture are sources of biomass that could be used as feedstock in AD. These biomass feedstocks include biotonne, food residues from restaurants; process residues from agro-industries; silages from grasses and agricultural residues; and wastewater. Other biomass feedstocks used in AD include energy crops such as maize silage and sunflower. Biomass feedstocks are composed of degradable and nondegradable components. The complex degradable organics are hydrolyzed to simpler compounds such as sugars, amino acids and fatty acid of various carbon lengths. The simpler compounds are further converted to energy-rich biogas through interdependent microbial processes (Schink and Stams, 2012). Biochemically nondegradable fractions remain as digester solids or digestate.

During stable biomass methanization, methane (CH_4) content of the biogas could range between 50% and 75% depending on whether carbohydrate- or lipid-rich substrates were used as feedstock. Other constituents of biogas could include CO_2 (25%–50%), O_2 (< 1%), and N_2 (<1%). Traces of hydrogen sulfide gas (H_2S), hydrogen gas (H_2) and chlorinated hydrocarbons could also be present, depending on the operating conditions (Appels et al., 2008).

2.1.2 Operating Conditions

Biomass could be fed to AD digester in batch, continuous or semicontinuous mode. AD temperature could be mesophilic or thermophilic, and a variety of digester designs are utilizable. For any biomass, AD temperature or digester design, optimum methanization is only possible with the right range of pH; C, N and S ratio; appropriate biomass sizes; and micronutrients (Gustavsson et al., 2011). Determining what ranges of the general factors that constitute optimum condition during methanization is a challenge. This is because biomass characteristics vary widely in composition, and different microorganisms are associated with different substrate types, digester design and temperature. Hence, methanization conditions are optimized for the microbes that produce CH_4 or for the microbes that degrade the fatty acids to CH_4-producing intermediates. Some of the conditions for optimum methanization are discussed next.

2.1.2.1 Temperature

Temperature stimulates changes in the composition, growth rate and metabolism of the microorganisms and also affects substrate degradation rates. Thermophilic temperature, T_t ($45°C \leq T_t \leq 60°C$), can speed up slow and energy-demanding reactions such as the degradation of volatile fatty acids (VFAs) to acetate, CO_2 and H_2. Conversely, mesophilic temperature, Tm ($35°C \leq Tm \leq 45°C$), is required for reactions which are exergonic (energy yielding), e.g., hydrogen-dependent methanogenesis (Kleerebezem and Stams, 2000). Temperature variation $\geq 1°C$/day in both mesophilic and thermophilic AD is detrimental to optimum methanization, but variations $\leq 0.6°C$/day are tolerable. Methanogens are the most sensitive to variations in temperature, and aceticlastic methanogens are more sensitive than hydrogenotrophs (Ahn and Forster, 2002).

2.1.2.2 Process Duration and Substrate Retention Time

Depending on substrate type, loading rate and other process conditions, substrate retention in the digester could range between 5 and 60 days. 5–10 days are recommended for microbial stabilization and substrate methanization in sewage sludge and biowaste. Conversely, longer retention time is required for lipid-like substrates such as grease trap residue, which could begin CH_4 formation not earlier than 10 days (Appels et al., 2008).

2.1.2.3 Volatile Fatty Acids/Alkalinity and pH

The ratio of VFAs to alkalinity during methanization is principally controlled by the concentrations of VFAs and hydrogen carbonate (HCO_3^-). Ammonia (NH_3) and ammonium (NH_4^+) are pH-dependent and interconvertible and could also contribute to alkalinity (Bardi and Aminirad, 2020). The ratio of VFAs to alkalinity is related to C:N since N content is related to NH_3 formation and VFAs derive from C content. Any ratio between 30 and 35 is optimum for maintaining optimum VFAs/alkalinity. For stable pH during AD, a steady HCO_3/VFAs molar ratio of 1.4 should be maintained. pH influences microbial rate of growth, metabolism and dominance, as well as composition of VFAs. For example, acetate-propionate dominance is established at pH 8.0 and acetate-butyrate dominance prevails at $pH < 8$. The phases of

methanization also vary in optimum pH; a pH range of 4.0–8.5 is suggested for acidogenesis; and a narrower pH range of 6.5–7.4 is recommended for methanogenesis (Wainaina et al., 2019).

2.1.3 Phases of Anaerobic Digestion

AD proceeds in four biochemically interdependent phases. Each phase generates intermediate products which become substrates for succeeding stages until the biomass is completely mineralized. These phases include hydrolysis, acidogenesis, acetogenesis and methanogenesis.

2.1.3.1 Hydrolysis

Substrate hydrolysis is essentially a solubilization of macromolecules, and it is the slowest phase in methanization. High-molecular-weight compounds such as lipids, polysaccharides, proteins and nucleic acids are converted into soluble organics such as sugars, amino acids and fatty acids. Hydrolysis could be solely by physicochemical means or aided by enzymes of hydrolytic bacteria. The physicochemical factors include substrate composition and pH, and biochemical factors include the concentrations of the hydrolytic enzymes and enzyme-substrate interactions (Rajagopal and Béline, 2011).

2.1.3.2 Acidogenesis (Acidification)

Soluble organic intermediates derived from the biomass hydrolysis are split during acidogenesis to VFA molecules with carbon length between 1 and 5 (C1–5) by heterogeneous fermentative bacteria (acidogenic bacteria). Other products of this phase include alcohols (KOH), H_2S, NH_3 and CO_2. Acidogenic bacteria are both obligate and facultative anaerobes. During acidogenesis, factors such as pH, interspecies H_2 transfer and inoculum age play significant roles in converting soluble organics into VFAs (Mata-Alvarez, 2003).

2.1.3.3 Acetogenesis

In this phase, acetate-producing bacteria (acetogens or acetogenic bacteria) degrade the VFAs produced in acidogenesis to acetate, CO2 and H2 (Equations 2.1 and 2.2). The H2 concentration must be kept low to avoid inhibition. Therefore, most of the acetate is produced by syntrophic feeding relationship between VFAs-oxidizing bacteria and H_2-utilizing microorganisms (Scholten and Conrad, 2000). Certain species of the *Clostridium* and *Pseudomonas* genera are capable of producing acetate as sole product from CO_2 and H_2 using the reductive acetyl-CoA pathway as shown in Equation 2.3. These species are called homoacetogens, and the process is referred to as homoacetogenesis (Ragsdale and Pierce, 2008)

$$\text{Propionate} + 3H_2O \rightarrow \text{Acetate} + HCO_3^- + H^+ + 3H_2 \qquad \Delta G_0 = +76.1 \text{ kJ/mol} \quad (2.1)$$

$$\text{Butyrate} + 2H_2O \rightarrow 2\text{Acetate} + H^+ + 2H_2 \qquad \Delta G_0 = +48.3 \text{ kJ/mol} \quad (2.2)$$

$$4H_2 + 2HCO_3^- + H^+ \rightarrow \text{Acetate} + 4H_2O \qquad \Delta G_0 = -104.6 \text{ kJ/mol} \quad (2.3)$$

2.1.3.4 Methanogenesis

Methanogenesis completes the mineralization of biomass to CO_2 and CH_4. Important biomass intermediates for methanogenesis include acetate, CO_2, H_2, and methylated compounds such as methanol, methylamine and dimethylsulfide. Other biomass intermediates include carbon monoxide (CO) and formate ($HCOO^-$). Methanogenesis complements acetogenesis by the utilization of acetogenic substrates such as formate and H_2, thereby making VFAs degradation energetically favorable and thermodynamically feasible (Schink and Stams, 2012). Figure 2.1 summarizes the flow of carbon intermediates between the different phases of methanization. Part of the energy generated from degrading the substrates is retained in the C-H bond in CH_4 and is not available to the microorganisms involved in AD. The retained energy is the important resource in CH_4. Retaining the energy of degradation of VFAs in CH_4 is also responsible for lower energetics and slower degradation of substrates during AD when compared with aerobic digestion (Kleerebezem and Stams, 2000).

2.1.4 Biomass Degradation to Volatile Fatty Acids

VFAs are produced during acidogenesis as degradation products of long-chain fatty acids (LCFA). The carbon length of short chain VFAs could range between 1 and 5, and these include formate, acetate, propionate and butyrate. Others VFAs encountered in AD include valerate and the isoforms of butyrate and valerate. The most

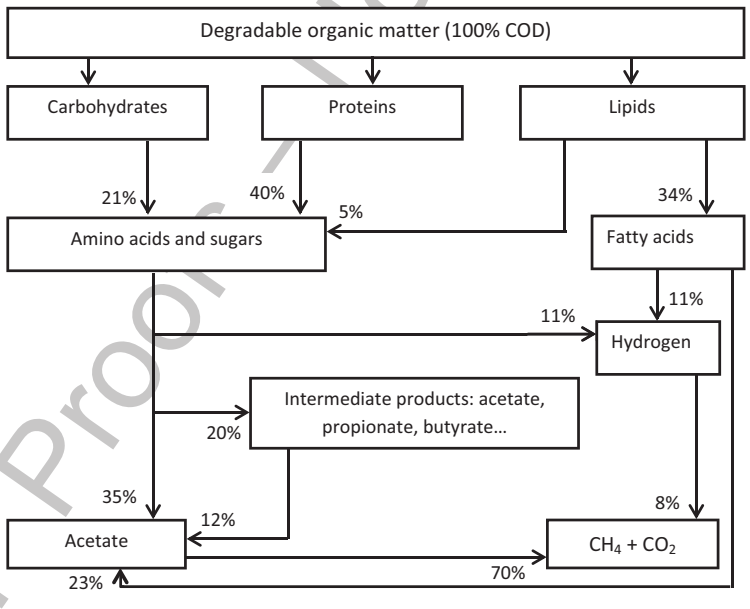

FIGURE 2.1 Bioconversion of substrates in anaerobic digestion with flow of substrate intermediates expressed as percent of chemical oxygen demand (COD). (Modified by Ezebuiro, (2014) from Siegrist et al., 1993.)

important VFAs are acetate, propionate and butyrate because they are the regular intermediate products from the beta-oxidation of LCFA. Small amount of propionate is produced by beta-oxidation of LCFA with odd number of carbon atoms. Oxidative degradation of branched-chain amino acids (valine, isoleucine, threonine and methionine) also produces propionate. Butyrate is produced from degradation of LCFA with even number of carbon and from carboxylation of propionate. Acetate is a common intermediate in the degradation of VFAs (Meegoda et al., 2018). VFAs are activated and degraded to CH_4 and CO_2 by different processes and pathways that form the focus of the next subsection.

2.1.4.1 VFAs Activation

VFAs metabolism to intermediates that are utilizable by CH_4-forming microorganisms begins with activation. Activation requires biochemical energy and involves transfer of phosphate group (PO_3^{4-}) from adenosyl triphosphate (ATP) to the VFA to form phosphorylated VFA (acyl-phosphate) as shown in Equations 2.4 and 2.5:

$$\text{VFA} + \text{ATP} \rightarrow \text{Acyl} - \text{Pi} \tag{2.4}$$

$$\text{Acyl} - \text{Pi} + \text{SH} - \text{CoA} \rightarrow \text{Acyl} - \text{CoA} + \text{ATP} \tag{2.5}$$

The phosphorylated VFA further reacts with coenzyme A (SH-CoA) to form an acyl-CoA. The activated VFA enters the appropriate degradation pathway depending on the VFA involved. During propionate degradation, the 3-Carbon backbone is either decarboxylated to form 2-Carbon acetate or carboxylated to a 4-Carbon molecule that can undergo beta-oxidation. Nutrient availability, process conditions and microbial composition are the major determinants of the prevalent pathway for propionate degradation during methanization (Ziemiński and Frac, 2012). Two important pathways that are commonly reported include the methylmalonyl-CoA (MMC) and butyryl-CoA pathways.

The MMC pathway produces a complete degradation of propionate to CH_4. MMC involves the carboxylation of propionate to the 4-C succinate, which is beta oxidized to acetate. MMC pathway is common to syntrophic relationship between VFAs-oxidizing bacteria species of *Syntrophobacter*, including *S. pfennigii* and *S. fumaroxidans*, and H_2-utilizing methanogens of the orders *Methanobacteriales* and *Methanomicrobiales* (Li et al., 2012). An important determinant of the acetate-yielding intermediates in the MMC pathway is the concentration of Co. Succinate is produced and converted to acetate when Co is optimum, and hydroxypropionate is produced and converted to acetate in the absence of Co. MMC pathway involving succinate intermediate could be more energy saving than the alternative pathway involving hydroxypropionate (Scholten and Conrad, 2000).

The butyryl-CoA pathway is independent of the concentration of Co. It is found in the syntrophic relationships between members of the genera *Smithella* and *Syntrophomonas*, and aceticlastic methanogens of the genera *Methanosaeta* and *Methanosarcina*. It involves conversion of propionate to hydroxyl-butyryl-CoA and β-oxidation of hydroxyl-butyryl-CoA to acetate (Kleerebezem and Stams, 2000).

2.1.4.2 VFAs Accumulation and Anaerobic Digester Failure

Accumulation of VFAs inhibits further biomass degradation and induces digester failure. H_2, formate and acetate are important intermediates generated from long-chain VFAs oxidation that must be removed or kept at very low concentrations for VFAs degradation to be thermodynamically feasible. The removal of VFAs degradation intermediates controls VFAs degradation rate and bioenergetics. The accumulation of H_2 or formate in AD reactors inhibits acetate, propionate and butyrate oxidation, whereas the accumulation of H_2, formate, acetate or butyrate inhibits propionate oxidation (Li et al., 2012). Furthermore, reactor acidification, especially due to propionic acid accumulation during methanization, induces loss of HCO_3^- buffer as gaseous CO_2, and this partly accounts for high CO_2 content in biogas during conditions of failed methanization (Wainaina et al., 2019).

2.1.4.2.1 Suppression of Methanogens as Cause of Digester Failure

Suppression of methanogens that utilize the intermediate products of VFAs degradation results in VFAs accumulation and digester failure. Suppression of methanogens could be due to presence of inhibitory substances or as a result of competition for H_2 by sulfate-reducing bacteria (SRB). It could also be as a result of process modification to generate and recover VFAs as industrial raw materials. Methanogenic suppression is generally accompanied by low pH and decline in CH_4 content in biogas. Accumulation of propionic acid and, to a lesser extent, the iso-forms of valeric and butyric acids generally precedes methanogenic suppression (Magdalena et al., 2019).

2.1.4.2.2 Dominance of Unionized VFAs Species as Cause of Digester Failure

Digester failure is more often associated with conditions that induce accumulation of unionized forms of VFAs, and the most important condition is pH. pH ranges that keep the VFAs in ionized state reduce the risk of digester failure in spite of relatively high VFAs concentrations. A pH range of 7.8–8.2 for AD is recommended to maintain VFAs in ionized forms and eliminate SRB competition. Methanization pH is particularly important for propionic acid because it is usually in the highest concentration when VFAs accumulate, and 7.0–8.5 is an appropriate methanization pH range for keeping propionic acid concentration low and degradable (Li et al., 2012). It has been established that accumulation of propionic acid is related to the following biochemical conditions:

- Similarity in the enzymes (Kinases) of propionate and acetate degradation, with acetate-Kinase having better kinetics (Ingram-Smith et al., 2005); and
- Joint inhibition by butyric- and acetic acids, with butyric acid being about 2.4 times more inhibitory to propionic acid degradation than acetic acid (Amani et al., 2010).

2.1.4.3 Monitoring VFAs Accumulation and Digester Failure

Deviation from regularly observed VFAs levels or established VFAs degradation rates and CH_4 yields is the most reliable indicator that the microorganisms are responding to inhibitory influences. However, there is no consensus on VFAs concentrations that constitute the "critical levels." It is suggested that VFAs concentration ≥ 50 mmol/L

is ideal for stable methanization, and ≥ 200 mmol/L could induce instability in AD (Batstone et al., 2000; Voss et al., 2009; Zhang et al., 2010). Fatty acid/alkalinity (FOS/TAC) is also an important parameter for monitoring process stability during methanization. An FOS/TAC value of 0.15–0.45 indicates stability, whereas values above 0.6 are an indication of the onset of instability (Voss et al., 2009).

2.1.4.4 Other Biochemical Factors That Affect VFAs Degradation

Factors that affect the growth of the desired microbial population are capable of impeding VFAs utilization. These are discussed.

2.1.4.4.1 Methanogen and Acetogen Ratio (M/A)

The methanogen and acetogen ratio (M/A) of the microbial population participating in syntrophic feeding relationship is an important parameter for VFAs degradation. Considering that methanogens are slow growing anaerobes, relatively high proportion of methanogens is required at the start-up of methanization compared to fast growing acetogens. In a study, Amani et al. (2011) indicated that utilization efficiency for propionic, butyric and acetic acids increased from 10% to almost 60% by increasing M/A from 1:1 to 2:1. Conversely, at M/A of 3:1, the advantage for VFAs degradation was lost, acidification ensued and the accompanying low pH resulted in acetate accumulation. This was because there were less propionic- and butyric acid oxidizing bacteria than were required to carry out degradation of the accumulating VFAs to acetate.

2.1.4.4.2 Inoculum Structure

VFAs must be in contact with microorganism or with microbial enzymes in the reactor in order to be degraded. This requires that the diffusion distance between the microorganisms and the substrates must be short. Short diffusion distance allows for rapid transfer of metabolites and increases degradation energetics and kinetics. Short diffusion distance exists between VFAs and microorganism when the microorganisms are in clusters (e.g. biofilms). This is particularly important for H_2 and formate metabolism (Kleerebezem and Stams, 2000).

2.1.4.4.3 Microbial Competition for Carbon Sources

Most competition during methanization is between microorganisms that use H_2 generated during VFAs degradation to reduce CO_2 to CH_4 (MPB) and microbes that reduce SO_4^{2-} to H_2S (SRB). SRB possess stronger kinetic affinity for H_2 than MPB. MPB produce CH_4 (Equation 2.6), and SRB produce H_2S (Equation 2.7) with H_2 generated during VFAs degradation. The use of H_2 by SRB to produce H_2S is at the expense of methanogenesis and results in increased concentration of H_2S in biogas and toxicity of sulfide ion (HS^-) to MPB (Chen et al., 2008).

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \qquad \Delta G_0 = -135.5 \text{ kJ/mol} \qquad (2.6)$$

$$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O \qquad \Delta G_0 = -151.9 \text{ kJ/mol} \qquad (2.7)$$

Just like methanogens and acetogens, SRB grow on propionate, butyrate and acetate. SRB have higher affinity for and derive more energy from H_2 and propionate

compared to acetate and butyrate. Equations 2.8 and 2.9 show the energetics advantage of SRB over acetogens respectively in propionate conversion to acetate.

$$\text{Propionate} + 0.75SO_4^{2-} \rightarrow \text{acetate} + HCO_3^- + 0.75HS^- + 0.25H^+ \qquad \Delta G_0 = -37.7\ \text{kJ/mol} \tag{2.8}$$

$$\text{Propionate} + 2H_2O \rightarrow \text{acetate} + CO_2 + 3H_2 \qquad \Delta G_0 = +76.1\ \text{kJ/mol} \tag{2.9}$$

Identifying factors that enable SRB to outcompete other microbes of methanogenic significance is important for methanogenesis. Two factors have been widely reported and these include Chemical Oxygen Demand/SO_4^{2-} and SRB/MPB; and temperature. Chemical Oxygen Demand (COD) and SO_4^{2-} ratio and the start-up ratio of SRB and MPB influence aceticlastic methanogenesis. Operational AD temperature also plays a significant role during H_2 utilization by SRB and MPB. Colleran and Pender (2002) reported that H_2-dependent reduction of SO_4^{2-} prevails over H_2-dependent CH_4 formation at 35°C–37°C; conversely, H_2-dependent CH_4 formation prevails over H_2-dependent SO_4^{2-} reduction at 55°C. This might suggest that processes involving MBP are reasonably optimized by temperature in thermophilic methanization, while mesophilic methanization requires optimization measures for MPB for enhanced H_2 utilization.

2.1.4.4.4 Inhibitory Substances

A substrate or its intermediate is inhibitory when it affects growth of microbes, adversely changes the microbial population, kinetics or energetics of a particular phase with reference to the generation of a choice product. NH_3, SO_4^{2-} and HS are dominant inhibitory intermediate products of substrate degradation that lead to reduction in the rate of CH_4 production and cause accumulation of VFAs (Chen et al., 2008). NH_3 is produced from degradation of urea and protein-rich compounds. NH_3 is protonated to ammonium ion (NH_4^+) in low pH solution, and both species form the total ammonia nitrogen (TAN) (Meegoda et al., 2018). NH_3 is capable of diffusing across bacterial and archaeal membranes and causing adjustment in intracellular proton balance. Other toxic intracellular effects of NH_3 include interferences with process bioenergetics and enzyme inhibition (Gallert and Winter, 2008).

NH_3 toxicity causes digester instability due to the accompanying fluctuations in process pH. The ratio of NH_3/NH_4^+ is dependent on process temperature and pH, and total ammonia nitrogen ≤ 5.0 g/L with an NH_3 concentration of about 1.0 g/L (pH 7.9; 55°C) is toxic to both acetate-producing and acetate-utilizing microorganisms. Higher temperature generally increases toxicity of NH_3, but this can be attenuated by adding Cu and Co to anaerobic digesters (Bardi and Aminirad, 2020).

2.1.5 Enhancing Methanization through Metal-Based Biocatalysis

Generally, substantial amount of the energy generated during methanization is trapped in CH_4 and is unavailable for biochemical work. Biocatalysis has evolved in AD to counteract the low energy exchange associated with methanization in general and

VFAs degradation in particular. Low AD energetics implies that some of the AD reactions cannot go on spontaneously and must be catalyzed. The incorporation of trace elements (TEs) into the enzymes of microorganisms that are involved in AD reactions with low energy exchange ensures that the rate of these reactions meet thermodynamic requirements. Enhancing the activities of these metal-containing enzymes or metalloenzymes (MEs) that are associated with AD is an important aspect in methanization process enhancement and is referred to as biocatalysis. Different phases of AD have different MEs that perform varying functions. These are discussed.

2.1.5.1 TEs and MEs of Hydrolysis

Hydrolysis is a solubilization reaction of the type shown in Equation 10 in which R is an organic compound, and the transfer of the OH^- from H_2O in an aqueous solution could be influenced by pH, a catalyst, an enzyme or all three.

$$RX + H_2O \rightarrow ROH + HX \tag{2.10}$$

Hydrolytic enzymes are called hydrolases and could hydrolyze AD substrates inside (intracellular) or outside (extracellular) the cell of the microorganism. Hydrolases could also be both intra- and extracellular depending on the type of reaction that is catalyzed. However, the important hydrolases of AD are extracellular enzymes and include lipases, glucosidases and proteases (Burgess and Pletschke, 2008).

The active sites of hydrolases generally consist of charged amino acid residues but might also contain one or two divalent metal ions such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Co^{2+} (Heikinheimo et al., 2001). The hydrolytic potential of the MEs varies with the number of coordinating divalent metal ions, and the hydrolytic effect of two divalent metal ions is twice as high as that of a single divalent metal ion. This explains the presence of two divalent metals ions participating in the hydrolytic mechanisms of MEs such as ureases, alkaline phosphatase and inorganic pyrophosphates among others.

2.1.5.2 TEs and MEs of Acetogenesis

The predominant pathway for growth and metabolism in acetogens is the acetyl-CoA pathway. Acetyl-CoA pathway involves metabolic processes that reduce CO_2 or CO to form acetate by using H_2 as electron donor. In the reverse direction, acetate is oxidized to its precursors. Acetyl-CoA pathway is present in both acetogens and methanogens involved in the conversion of acetate to CH_4 and reduction of CO_2 to acetate using H_2 (Drake et al., 2008). The principal enzymes of the acetogenic and methanogenic acetyl-CoA pathway are MEs and include hydrogenases, FDH, MeTr and CODH/ACS.

2.1.5.2.1 Hydrogenases

During CO_2 reduction to methane, hydrogenases provide redox electrons by oxidation of H_2. Some hydrogenases contain metals, while others do not. The metals of importance in hydrogenases include Ni and Fe (Figure 2.2a); other components include amino acid residues and sulfur (S). Ni-Fe complex is necessary for substrate binding, and Fe-S complex (Figure 2.2b) is required as conduit for electron transfer (Shima et al., 2002).

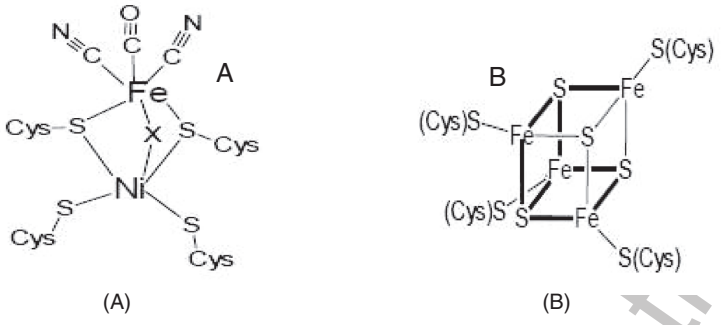

FIGURE 2.2 Ni-Fe catalytic center in hydrogenases (a); Fe-S cluster in hydrogenases (b) (Shima et al., 2002).

2.1.5.2.2 Formate Dehydrogenases (FDH)

These MEs contains Se, W or Mo at the active site, and the TEs found at the active site depend on the microbial species, the growth substrates or bioavailability of the TEs in the growth medium. FDH catalyzes thermodynamically unfavorable CO_2 reduction to formic acid (HCOOH). Redox electrons for the biocatalysis are obtained from reduced nicotinamide adenine dinucleotide phosphate (NADPH), H_2 or pyruvate. When CO is the substrate instead of CO_2, FDH is associated with oxidation of CO to CO_2 and formic acid (Reda et al., 2008; Seravalli and Ragsdale, 2000).

2.1.5.2.3 Methyltransferase (MeTr)

MeTr is associated with transfer of $-CH_3$ and is unique to microorganisms that employ the acetyl-CoA pathway for acetate metabolism. It has either of two types of active-site complexes: cobalamin or cobamide, and both are activated by Co. The active site of MeTr is capable of accepting $-CH_3$ groups when the Co is in Co^+ state to form an enzyme-bound CH_3-Co^{3+} complex. The $-CH_3$ group is transferred to coenzyme M (SH-CoM) to form CH_3-S-CoM, and the active Co^+ state is regenerated. Temperature range between 35°C and 40°C and pH range between 7.5 and 7.7 are optimum for MeTr-dependent biocatalysis. The enzyme could be stable at temperatures up to 45°C, and activity-decline sets in around 70°C (Caspi et al., 2008).

2.1.5.2.4 CODH/ACS Complex

CODH/ACS complex is the main enzyme for the metabolism of acetate in both acetogens and methanogens. It comprises two sub-units: CODH and ACS. CODH/ACS complex is involved in the metabolism of H_2, CO_2, CO and VFAs. During CO_2 metabolism, one molecule of CO_2 is reduced to CO by CODH, and this becomes the carbonyl group of acetate. Another molecule of CO_2 is reduced to formate ($HCOO^-$) and further to $-CH_3$ group, and this forms the $-CH_3$ group of acetate. The ACS unit

condenses the CO and $–CH_3$ with CoA to form acetyl-CoA. Structurally, the active sites of the CODH/ACS complex in anaerobes contain Ni ions linked to the Fe-S electron conduit (Lindahl, 2004).

CODH/ACS complex may additionally contain Mo and Cu to detoxify reactive oxygen species (Gnida et al., 2003). The biochemical and kinetic properties of CODH/ACS complex depend on whether it is involved in acetogenesis or methanogenesis. Optimum temperature and pH for CODH are 60°C and 6.3, respectively, for acetogenic CO_2 reduction, and about 70°C is optimum for ACS in aceticlastic methanogenesis. This explains the observation that in extreme thermophilic AD (≥60°C), CO_2 and H_2 are converted to acetate instead of CH_4 (Iranpour et al., 2005). It also explains reports that CH_4 production in thermophilic AD is optimum compared to mesophilic AD that require TEs supplementation for optimum CH_4 production (Uemura, 2010).

2.1.5.3 TEs and MEs of Methanogenesis

CH_4 production is possible through three different routes by different methanogens, and these include hydrogenotrophic methanogenesis where CH_4 is formed from H_2 reduction of CO_2. Others include aceticlastic methanogenesis involving the cleavage of acetate into CO_2 and CH_4 and methylotrophic methanogenesis comprising redox reactions using methylated substrates such as methanol, methylamines and dimethylsulfide to form CH_4. Some MEs that are common to both acetogenesis and methanogenesis such as MeTr and CODH/ACS have been discussed. However, MEs associated with CH_4 formation from acetate or H_2 reduction of CO_2 are unique to methanogens (and to some extent, are also involved in sulfate reduction). These unique MEs of methanogenesis and their TEs relations are discussed next.

2.1.5.3.1 Formyl-Methanofuran Dehydrogenase (FMD)

FMD is the methanogenic analog of FDH. It is also called methanofuran reductase (MR) (Ragsdale and Pierce, 2008). Some important features of FMD include: the presence of Mo or W at the active site (depending on which of the TEs is bio-available in the substrates), but it is more active with Mo than with W.

2.1.5.3.2 Methyl-CoM Reductase (MCR)

MCR is only found in methanogens and catalyzes the final stage of CH_4 production from the reduction of $–CH_3$. This involves the reaction between methyl-coenzyme-M (CH_3-S-CoM) and coenzyme-B (SH-CoB) in the presence of MCR to form CH_4, SH-CoM and SH-CoB. Two forms of MCR known as MCR-I and MCR-II exist: MCR-I functions optimally in the pH range between 7.0 and 7.5 and has a maximum reaction rate of approximately 6 µmol/min/mg. The maximum reaction rate for MCR-II is approximately 21 µmol/min/mg and the optimum pH range is 7.5–8.0. Notwithstanding the form of existence, the general feature of MCR includes a reactive co-factor known as F_{430}, which is dependent on its active Ni-complex for catalysis. Ni-complex in MCR is similar to the Co-complex in MeTr, and the active form of Ni in MCR is the Ni^+ state, which forms CH_3-Ni^{3+} when it receives CH_3 group from MeTr (Caspi et al., 2008).

2.2 CONSIDERATIONS FOR THE USE AND APPLICATIONS OF METAL-BASED BIOCATALYSIS IN AD PROCESSES

2.2.1 Considerations for Use of Metal-Based Biocatalysis in AD Processes

The biocatalytic potentials of TEs have resulted in the deployment of the technology in many aspects of AD. The success of such deployment depends on certain considerations. The three most important considerations include inoculum composition and feeding mode, composition of the biomass feedstock and interaction effects of the TEs with biomass VFAs.

2.2.1.1 Inoculum Composition and Feeding Mode

The efficiency or otherwise of TEs-based biocatalysis is influenced by the concentration of TEs in the biomass and in the inoculum (seeding sludge) used. The inoculum is a significant reservoir of TEs, and the composition of the inoculum is strongly influenced by the digested biomass and its source. Fe-rich sludge used as inoculum may result in limited AD improvement or even a decline in process stability and methane production relative to a control system compared to Fe-limited inoculum (Yazdanpanah et al. 2018). Figure 2.3 shows the TEs composition of inoculums from digested sewage sludge obtained from two sources and digested maize silage-based feedstock.

The TEs contents of the DS-1 and DS-2 varied widely, and this could be attributed to the different sources of the sludge and the digestion process. The MSF-D in Figure 2.3 has significantly elevated TEs contents because TEs were supplemented to the digester during the AD process. Continuous feeding operation in AD, which is usually accompanied by loss in seeding sludge, may result in reduced CH_4 yield due to loss of TEs during digestate removal. Conversely, batch feeding operations have the advantage that the starting TEs concentration and configuration remain in the

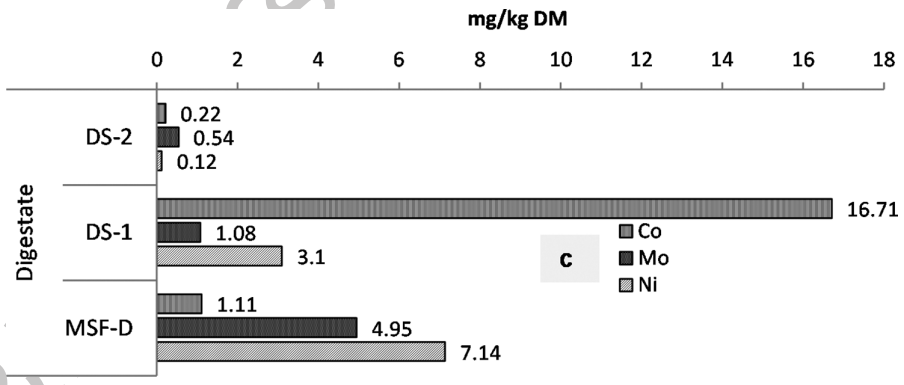

FIGURE 2.3 Nickel, molybdenum and cobalt contents of digested sewage sludge (DS-1 and DS-2: digested sludge 1 and 2; MSF-D, digestate from maize silage-based feedstock). (Adapted from Ezebuiro and Koerner, 2017.)

digester all through the methanization period. Hence, even if the TEs are inadequate, this is not compounded by removal of biomass and inoculum during feeding.

2.2.1.2 Composition of the Biomass Feedstock

Stimulatory influences of TEs have been reported for AD from both mono-substrates and complex substrates. However, the degree of stimulation is directly related to the inherent concentration of TEs in the biomass. Figure 2.4 shows the Ni, Co and Mo composition of AD substrates based on biomass complexity. In many instances, Se concentrations in biomass are so low that they occur below the detection limit of the measuring equipment (Ariunbaatar et al., 2016; Ezebuiro and Koerner, 2017).

Simple substrates predominantly comprise a biomass type such as grass specie or leaf type or a digested form of it as in the dung. In comparison to simple substrates such as LG and SG or ML (Figure 2.4a) that may constitute the original feedstock, dungs are generally known to have lower TEs contents. This could be due to assimilation of the nutrients by the animals for growth and metabolism. Hence, TEs supplementation is often beneficial for digesters using simple substrate during AD. Complex substrates may combine two or more simple substrates and, as a result, may have better TEs composition and configuration.

In Figure 2.4b, the complex substrates contain more TEs and reflect more variation in the TEs. For example, Ni content varies widely between 0.12 and 3.62 mg/kg DM compared to Mo and Co, and the MSF has the highest content of Ni, Co and Mo among the complex substrates shown. Such wide range of TEs in substrates are common place in reports relating to AD substrate characteristics (Hullebusch et al., 2005; Zandvoort et al., 2003). The implication of such variation in TEs is that specific TEs may need to be supplemented to produce a more balanced TEs mixture because they are insufficient in the biomass.

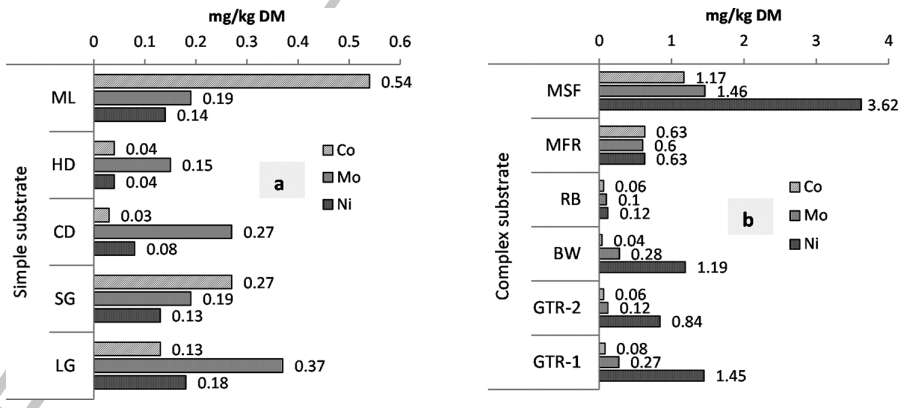

FIGURE 2.4 Nickel (Ni), molybdenum (Mo) and cobalt (Co) contents of selected AD substrates including (a) Simple Substrates: LG, lawn grass; SG, spear grass; CD, cow dung; HD, horse dung; ML, mixed leaves; (b) Complex Substrates: MSF, maize silage-based feedstock; MFR, mixed fruit residue; RB, restaurant biowaste; BW, blackwater; GTR, grease trap residue. (Adapted from Ezebuiro and Koerner, 2017.)

2.2.1.3 Interaction Effects of TEs with VFAs from Biomass Degradation

The biochemical efficiency of TEs is enhanced when an individual TE is in interaction with another TE (TE*TE) or biomass degradation intermediate such as VFAs (VFAs*TE) (Ezebuiro, 2018; Higuchi et al., 2005). Generally, when two or more TE ions interact in a mixture, the individual influences of one or all the interacting ions could weaken (antagonism) or strengthen (synergism) the AD process under consideration. TEs mixtures of Zn, Cu and Ni; Ni and Cu; and Ni, Mo and Co have been established as synergistic in AD (Bardi and Aminirad, 2020; Ezebuiro and Koerner, 2017). Regarding VFAs and TEs interactions and their effects, digester VFAs level is a significant consideration for the effects of TEs on methane production processes and VFAs metabolism. Figure 2.5a shows the significant TE*TE and VFAs*TE for

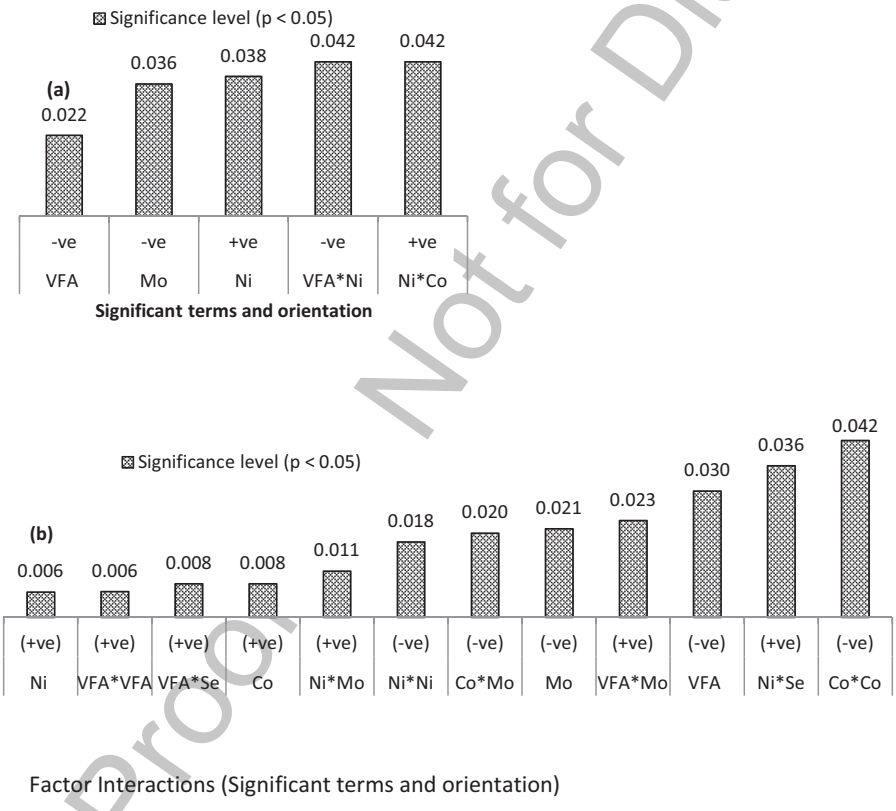

FIGURE 2.5 Significant TEs and VFAs interactions that influence (a) methane production. (b) VFAs degradation rate due to Ni, Co, Se and Mo supplementation to VFAs levels between 28 and 213 mmol/L at 37°C (Adapted from Ezebuiro et al., 2018). NOTE for Figure 2.5a and b: The factor is either an individual factor concentration (Ni, Co, Se, Mo or VFAs); or a combination of any of the individual factors (e.g. Ni*Mo, VFA*TE). The effect of a model term (also called factor effect) on a response may be positive (+ve) or negative (-ve) and the significance of the effect is given by the *p values*. The effect (+ve or –ve) of a model term on a response is significant when its *p value* is less than 0.05.

methane production, and Figure 2.5b shows for VFAs degradation rate due to Ni, Co, Se and Mo supplementation to VFA levels between 28 and 213 mmol/L at 37°C.

2.2.2 Applications of Metal-Based Biocatalysis in AD Processes

The most common applications of TEs in AD operations are in the improvement of hydrolysis and acidification to increase VFAs yield, increase in conversion of short chain fatty acids to acetate, and enhancement of process stability and production of methane from biomass.

2.2.2.1 Improvement of Hydrolysis and Acidification Rate

TEs supplementation has been shown to increase biomass hydrolysis and acidification. Hydrolysis is a limiting step during methanization in AD. The amount of VFAs available for metabolism is dependent on the rate of hydrolysis and acidification. Hence, increasing hydrolysis and acid formation potential of biomass ensures availability of intermediate substrates for the succeeding AD phases of acetogenesis and methanogenesis. Fe, Mo and Co supplementations have been specifically identified as having stimulatory effects on hydrolysis in digesters converting food waste to methane (Yazdanpanah et al., 2018). Similarly, hydrolysis and acidification of waste activated sludge can be improved by addition of potassium ferrate (He et al., 2018). HAR improvement is important not only for methanization but also for the targeted production of short chain VFAs such as acetate, propionate and butyrate. These intermediates form the precursors for many industrial products and have received increased attention lately with regard to AD process manipulations for enhanced production (Magdalena et al., 2019; Wainaina et al., 2019).

It is important to highlight that during hydrolysis, the accumulation of organic acids prevents further biomass hydrolysis. This challenge has been shown to be eliminated by exploiting the interaction effects of TEs and relationships between TE and VFA levels. Reported positive interactions at pre-acidification levels ≥ 200 mmol/L VFAs include Co*Se, while a negative influence on HAR has been reported for Co*Mo at the same acidification level. Similarly, the presence of Ni and Mo in TEs mixture and VFA*Mo improves hydrolysis of maize silage feedstock at pre-acidification levels of 10 and 120 mmol/L VFAs. It has also been shown that optimum concentrations and configurations of TEs are required for HAR at different acidification levels. Figure 2.6a–c shows optimum configuration of Ni, Co, Se and Mo supplementation for the enhancement of the HAR of maize silage biomass at different levels of pre-acidification during methanization. The process efficiencies at 10, 120 and 200 mmol/L VFAs pre-acidification levels as expressed by desirability function are 87%, 43% and 88%. Similarly, the relative HARs (compared to a baseline condition of no TEs supplementation) at the corresponding pre-acidifications levels are 82%, 13% and 85%. Such advanced analysis enables the computation of specific TEs requirements for the enhancement of biomass HAR at different levels of process acidification. This is particularly important considering that acid accumulation prevents biomass hydrolysis.

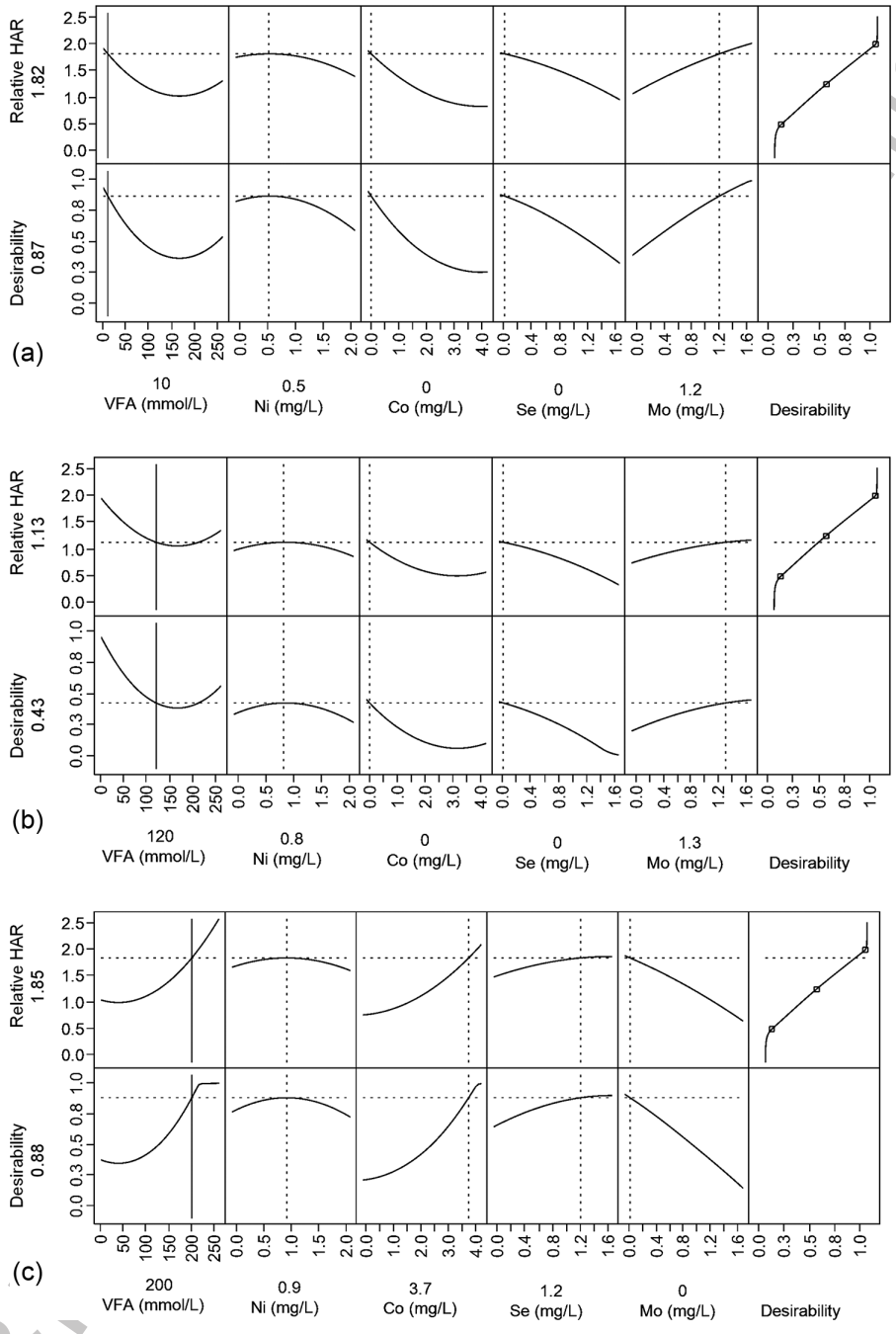

FIGURE 2.6 Optimum Ni, Co, Se and Mo configuration for the enhancement of hydrolysis and acidification rate in maize silage-based feedstock at a pre-acidification level of (a) 10 mmol/L; (b) 120 mmol/L; and (c) 200 mmol/L VFA operated at 37°C. (Adapted from Ezebuiro and Koerner, 2017.)

2.2.2.2 Enhancement of Process Stability and Production of Methane from Biomass

Over the years, researchers have improved methane production by supplementing TEs. In various laboratory and pilot studies, methane production has been increased by up to 80% from baseline production figures as a result of TEs supplementation. In such systems, methane production enhancements are as a result of improved competition of methane producing archaea without affecting archaeal diversity (Chan et al., 2019). AD digesters using simple substrates such as silages from grasses benefit strongly from TEs supplementation because such biomass substrates are known to be deficient in TEs and have poor TEs balance in the digestion mixture (Pobeheim et al., 2010). Other methanization processes such as process stability and VFAs degradation rate have been reported to benefit from the biocatalytic potentials of TEs (Ortner et al., 2014).

Improving methane production based on TEs supplementation is sensitive to organic loading rate and VFAs concentration of the methanization digester. Figure 2.7 shows the optimum TEs configurations for methane production and the associated process efficiencies (as depicted by response surface modeling and desirability function) for different concentrations of digester VFAs. The percent increase in methane production (expressed as relative methane production) and corresponding process efficiency (expressed as desirability) at the shown TEs configuration considered to be optimum for a VFAs concentration include: 55% and 99% at 23 mmol/L VFAs (low organic loading rate); 32% and 89% at 125 mmol/L VFAs (medium organic loading rate); and 19% and 73% at 200 mmol/L VFAs (high organic loading rate).

2.3 CONCLUSION

Biomethanization from biomass is an anaerobic process that involves many stages and requires the understanding of many factors for optimum performance of the process. The need to understand the biochemical basis of the competition for intermediate products of biomass degradation such as VFAs has led to probes into biomass characteristics and biocatalysis. While biomass differs considerably in characteristics, biocatalysis for biomethanization enables the speeding of biochemical processes of biomass degradation to maximize methane yield. A special field of biocatalysis that has gained attention over the years is TEs-based enhancement of metalloenzymes-mediated AD processes. With this advanced response-specific enhancement technique, optimum TEs configurations could be developed and applied to targeted AD responses with increased efficiency. This precise science can be directly applied to the bioenergy industry to increase biomethane production from biomass and as well increase the share of electricity from biomass. It is anticipated that further research into the development of TEs formulation for specific bioenergy processes will further enhance the economics and spread of biomass methanization, especially in regions where conventional energy supply is inadequate.

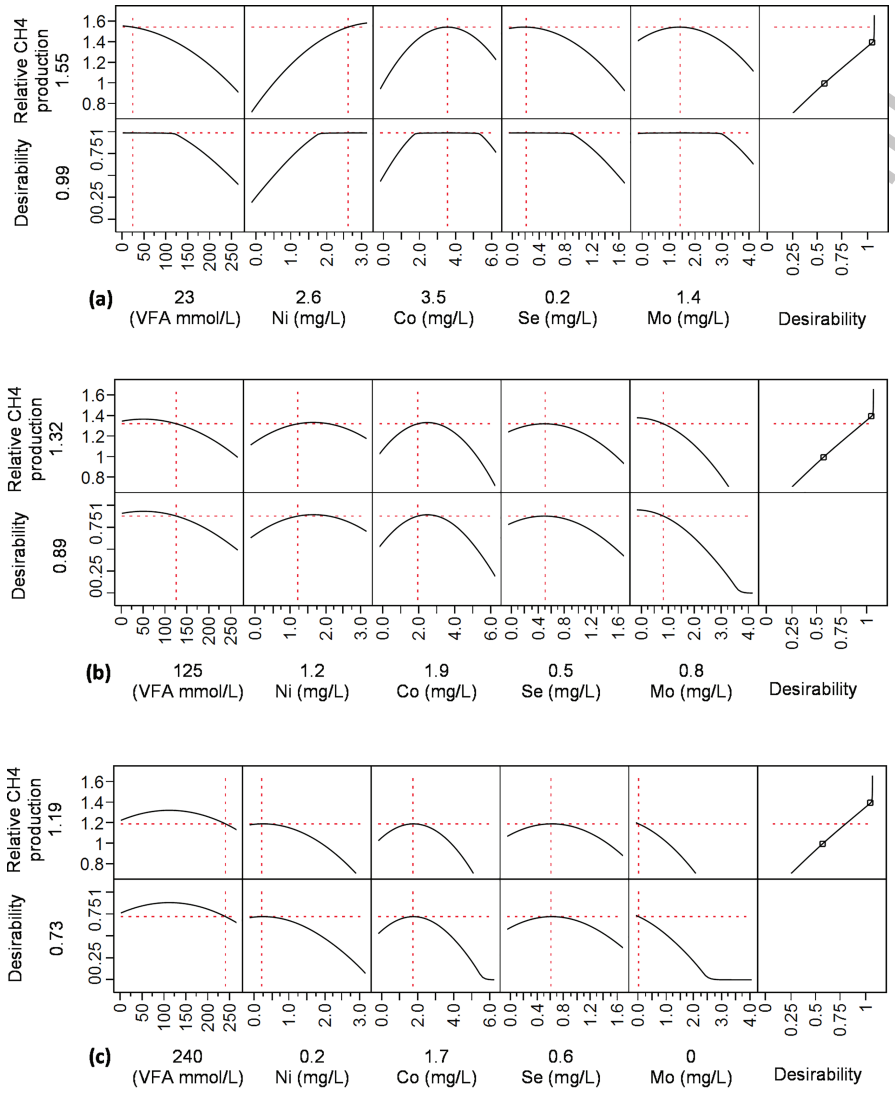

FIGURE 2.7 Optimum TEs configuration for CH_4 production at 37°C due to Ni, Co, Se and Mo supplementation to VFA levels (a) 23 mmol/L; (b) 125 mmol/L; and (c) 240 mmol/L.

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