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# A Comprehensive Review of the Strategies to Improve Anaerobic Digestion: Their Mechanism and Digestion Performance

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**Abstract:** Low and unstable digestion performance is a challenging issue for anaerobic digestion, which prompts researchers to develop new strategies. In addition to traditional approaches such as co-digestion, pre-treatment, and recirculation, some emerging strategies, namely additive processes and microaeration, have also been recognized and developed in recent years. Many studies have evaluated the effect of these strategies on digestion performance. However, their comprehensive analysis is lacking, especially regarding the mechanisms of the different strategies. This review presents a comprehensive overview of research progress on these strategies based on the latest research, considering the five main strategies listed above. Through critical thinking, a summary of their mechanism, reactor performance, and availability of these strategies is presented. The results demonstrate that the contribution of microaeration is mainly to balance the composition and activity of hydrolysis, acidogenesis, and methanogenic archaea. Recirculation and co-digestion mainly balance mass and reaction environments. Pre-treatment, such as removing lignin, reducing cellulose crystallinity, and increasing the substrate-specific surface area, makes the characteristics of the substrate more conducive to the digestion of microorganisms. The mechanism of additive strategies varies greatly depending on the type of additive, such as enhancing interspecies electron transfer through conductive materials, resisting adverse digestion conditions through functional microbial additives, and accelerating nutrient absorption by regulating the bioavailability of trace elements. Although these strategies have different mechanisms for promoting digestion performance, their ultimate effect is to allow the parameters of the reactor to reach an ideal status and then achieve a balance among the substance, microorganisms, and water in an anaerobic reactor.

**Keywords:** anaerobic digestion; biogas production; mechanism; promotion strategies



**Citation:** Li, X.; Wang, Z.; He, Y.; Wang, Y.; Wang, S.; Zheng, Z.; Wang, S.; Xu, J.; Cai, Y.; Ying, H. A Comprehensive Review of the Strategies to Improve Anaerobic Digestion: Their Mechanism and Digestion Performance. *Methane* **2024**, *3*, 227–256. https://doi.org/10.3390/methane3020014

Academic Editor: Patrick Da Costa

Received: 29 December 2023
Revised: 9 March 2024
Accepted: 26 March 2024
Published: 15 April 2024



## 1. Introduction

By 2050, the population is projected to increase to more than 9 billion. Meanwhile, energy consumption will double [1]. The continued excessive consumption of non-renewable energy from fossil fuels continues to raise environmental concerns [2]. Furthermore, population growth will generate more organic waste, which will also cause environmental pollution. Anaerobic digestion (AD) is an effective way to treat organic waste and produce renewable energy. Moreover, AD is also an essential part of circular agriculture. The quality of biogas slurry and digestate is closely related to AD performance.

AD occurs as a result of the activities of bacteria and archaea and involves the following three stages: (i) the hydrolysis of macromolecules (polysaccharides, fats, and proteins) and the subsequent production of monosaccharides, fatty acids, amino acids, and $CO_2$; (ii) the conversion of monosaccharides, fatty acids, and amino acids to volatile fatty acids (VFAs) and $H_2$; and (iii) the conversion of VFAs, $CO_2$, and $H_2$ to methane [3]. Any measure that affects these three stages may change the AD performance. In recent years, AD still faces some enormous challenges. Some examples include the low hydrolysis rate of lignocellulosic agricultural wastes, ammonia inhibition in livestock and poultry manure, and the lack of trace elements in some digestion systems [4–6]. To solve those problems and achieve high performance in AD, conventional strategies have been developed, including pre-treatment, co-digestion, and recirculation. Pre-treatment technology is mainly applied to substrates that are difficult to directly decompose by microorganisms such as crop straw, microalgae, and activated sludge. Co-digestion is the most useful promotion strategy, and its practical effect has been widely confirmed. Recirculation has unique advantages in areas where water is scarce.

Recently, the understanding of the AD process has improved immensely, and additional strategies and technologies have been developed. For example, in the past, AD was considered to require a strict anaerobic environment. However, recent research has demonstrated that supplying a small amount of oxygen can promote the AD process [7]. Previously, electron transports were only supposed to use $H_2$ as a shuttle by syntrophic partners [8]. Nevertheless, it has recently been found that electron transfer can occur directly between different microorganisms, and direct interspecies electron transport (DIET) can be enhanced by conductive materials [9]. The emerging promotion strategies mainly include additive processes and microaeration. There are various types of additives, such as conductive materials, functional microorganisms, enzymes, and trace elements. The microaeration strategy is mainly used in these substrates, which are difficult to hydrolyze.

Although many experiments have proved the actual effect of traditional and emerging strategies on digestion performance, a systematic summary of their promoting mechanisms is still lacking. The objective of this review is to offer a comprehensive overview of the main strategies reported by the latest research (the last 20 years). Through critical thinking, a summary of these promotion strategies is presented, considering their mechanisms, reactor performance, and availability. Finally, conclusions and the outlook for future research are given.

## 2. Strategies to Promote the AD Process

AD produces biogas as renewable energy and is environmentally favorable in terms of low greenhouse gas and odor emissions. AD still faces many problems, which limit the development of biogas projects, such as low hydrolysis efficiency for lignocellulosic biomass, instability for FW (food waste), and ammonia inhibition for livestock manure [10–15]. In order to improve the efficiency of AD, several strategies have been developed. In fact, research interest in strategies to promote AD has increased in the past 5 years (Table 1). More than half of the research papers in the AD field were on this topic of strategies in 2019. In this section, these strategies are discussed, considering their mechanism, availability, and actual effect on reactor performance.

**Table 1.** "Web of science" bibliometric study the topics of "ten items" and "anaerobic digestion".

| | **Time (Year)** | **2015** | **2016** | **2017** | **2018** | **2019** |
|---|---|---|---|---|---|---|
| Published paper number of pre-treatment | Microbial pre-treatment | 64 | 99 | 94 | 142 | 182 |
| | Physical pre-treatment | 19 | 27 | 15 | 27 | 25 |
| | Chemical pre-treatment | 132 | 148 | 154 | 179 | 206 |
| Published paper number of Co-digestion | Co-digestion | 403 | 475 | 575 | 720 | 778 |

**Table 1.** *Cont.*

| | Time (Year) | 2015 | 2016 | 2017 | 2018 | 2019 |
|---|---|---|---|---|---|---|
| Published paper number of recirculation | Recirculation | 34 | 55 | 48 | 57 | 55 |
| Published paper number of microaeration | Microaeration | 2 | 6 | 7 | 7 | 7 |
| Published paper number of additives | Biochar | 13 | 31 | 48 | 67 | 103 |
| | Bioaugmentation and enzymes | 20 | 19 | 17 | 39 | 38 |
| | Trace element | 30 | 28 | 28 | 53 | 63 |
| | Conductive material | 2 | 9 | 17 | 33 | 14 |
| Published paper number of anaerobic digestion | Anaerobic digestion | 1863 | 2149 | 2311 | 2633 | 2573 |
| | The ratio of strategies/ anaerobic digestion | 38.6% | 41.7% | 43.4% | 50.3% | 57.2% |

Note: The data come from "Web of science core collection". Every item was searched with "corresponding item name" + "anaerobic fermentation" as the topic.

### *2.1. Pre-Treatment*

Pre-treatment processes, which can be physical, chemical, or biological, increase the availability of a digestion substrate. However, experimental data on biomasses such as straw and maize stover are usually obtained through laboratory-scale trials; economic and energetic assessments of pre-treatment are rarely reported. The lack of such information prevents the formation of in-depth conclusions on the economic and energetic sustainability of pre-treatment. The effects of physical, biological, and chemical pre-treatments on methane yields from different substrates are shown in Table 2. The actual results rely on the particular mechanism of the pre-treatment method. Tables 2 and 3 clarify the mechanisms, advantages and disadvantages, and tangible effects of different pre-treatment methods on the digestion performances of substrates. As each pre-treatment method has its own limitations, a combined pre-treatment method is expected to achieve synergistic pre-treatment results.

**Table 2.** The comparison of different pre-treatment methods.

| Pre-Treatment Methods | Mechanism | Cost | Advantage | Disadvantage |
|---|---|---|---|---|
| Physical pre-treatment | Break complex structures and increase specific surface area | +++ | Simple principle and operation, no inhibitors generate | High energy consumption |
| Chemical Pre-treatment | Destroy molecular structure, reduce the crystallinity of lignocellulosic, dissolve lignin | ++ | High efficiency | Potential secondary pollution |
| Biological pre-treatment | Production of enzymes capable of decomposing complex organic matter | + | No environment pollution, mild reaction, less energy consumption | Long pre-treatment cycle, complex culture conditions, loss of organic matter, low efficiency |

+: Low; ++: Middle; +++: High. Note: Chemical pre-treatment methods include alkali, acid, thermo-chemical, and oxidative pre-treatment. Physical pre-treatment methods include grinding, microwave, and thermal pre-treatment. Biological pre-treatment methods include fungal, microbial community, and enzymatic pre-treatment.

**Table 3.** The effect of physical, biological, and chemical pre-treatments on methane yields from different substrates.

| Pre-Treatment | Condition | T (°C) | Substrate Type | Methane Yield [a] | Methane Yield [b] | Ref. |
|---|---|---|---|---|---|---|
| Biological Pre-treatment | Secreted enzymes | 37 ± 2 °C | Maize straw | 250.2 [c] | 277.0 [c] | [16] |
| Biological Pre-treatment | Fungi | 37 ± 1 °C | Yard trimmings | 8.5 [d] | 40.0 [d] | [17] |
| Biological Pre-treatment | Fungi | Not given | Corn straw | 131.0 [d] | 239.0 [d] | [18] |
| Biological Pre-treatment | Bacterium | 35 °C | MSW | 97.8 [d] | 221.0 [d] | [19] |
| Biological Pre-treatment | Biogas slurry | 35 ± 1 °C | Rice straw | 174.3 [d] | 233.3 [d] | [20] |
| Biological Pre-treatment | Fungi | 36 °C | Wheat straw | 118.0 [c] | 182.0 [c] | [21] |
| Chemical Pre-treatment | 2% NaOH | 35 ± 1 °C | Corn stalk | 187.0 [d] | 196.0 [d] | [22] |
| Chemical Pre-treatment | 10% CaO | 35 °C | Microalgae | 257.0 [d] | 292.0 [d] | [23] |
| Chemical Pre-treatment | 4% NaOH | 37 ± 0.5 °C | *Pennisetum Hybrid* | 249.3 [d] | 281.4 [d] | [24] |
| Chemical Pre-treatment | 1.6% NaOH | 37 ± 2 °C | Wheat straw | 263.0 [d] | 314.0 [d] | [25] |
| Chemical Pre-treatment | 20 g N/L $NaNO_2$ | 35 °C | Waste activated sludge | 132.0 [d] | 153.0 [d] | [26] |
| Chemical Pre-treatment | 1% urea | 35 °C | Wheat straw | 210.4 [d] | 305.5 [d] | [27] |
| Chemical Pre-treatment | 10.0% NaOH | 37 °C | Dairy cow manure | 292.1 [d] | 361.0 [d] | [28] |
| Chemical Pre-treatment | 3%$H_2O_2$ | 25 ± 2 °C | Corn straw | 100.6 [d] | 216.7 [d] | [29] |
| Physical Pre-treatment | milling | 38 °C | Wheat straw | 127.4 [d] | 250.3 [d] | [30] |
| Physical Pre-treatment | Microwave | 35 °C | Microalgae | 170.0 [d] | 270.0 [d] | [31] |
| Physical Pre-treatment | Microwave | 37 ± 0.5 °C | FW and Sewage sludge | 285.0 [d] | 310.0 [d] | [32] |
| Physical Pre-treatment | Thermal | 37 °C | Algae | 279.0 [e] | 391.0 [e] | [33] |
| Physical Pre-treatment | Thermal | 35 °C | Wheat straw | 404.0 [e] | 615.0 [e] | [34] |
| Physical Pre-treatment | Thermal | 35 °C | Microalgae | 181.0 [d] | 106.0 [d] | [35] |

[a] Before pre-treatment; [b] After pre-treatment; [c] mL methane $g^{-1}$ TS; [d] mL methane $g^{-1}$ VS; [e] mL biogas $g^{-1}$ VS.

#### 2.1.1. Biological Pre-Treatment

Biological pre-treatment is a safe and environmentally friendly method with unique advantages such as low energy consumption [4,19]. The main biological pre-treatments utilize fungi, microbial consortia, or enzymes. Fungal pre-treatment [typically using white-rot fungi (WRF)] is most commonly used.

##### Microbial Pre-Treatment

Fungi secrete cellulases, hemicellulases, and ligninase that decompose lignocellulosic biomass [mainly crop straw, energy crops, and the lignocellulose fraction of municipal solid waste (MSW)]. In the pre-treatment of lignocellulosic biomass, the dominant roles of fungi are modifying the lignin structure (the guaiacyl/sinapyl ratio) [16,17,36], decreasing the crystallinity of cellulose, increasing the substrate porosity, and changing the hemicellulose structure (xylose/arabinose ratio) [37].

WRF, brown-rot fungi (BRF), and soft-rot fungi (SRF) are used in pre-treatment systems. The degradation mechanisms of SRF remain unclear [37]. BRF can degrade cellulose and hemicelluloses and modify lignin to a small extent; WRF more effectively performs delignification than BRF and SRF because it secretes hydrolases and possesses a ligninolytic system involving lignin peroxidase, manganese peroxidase, and laccase [37]. This unique enzymatic system provides fungi with their ability to degrade lignin (mainly phenolic structures) into $CO_2$ [38]. Recently, some laboratory-scale studies have confirmed that fungi can effectively pretreat lignocellulose. Zhao et al. [17] reported that the methane yields from unsterilized branches increased from 20 L/kg VS to 40 L/kg VS after WRF pre-treatment, demonstrating that fungal pre-treatment can increase the digestibility of branches. Consistent with this result, Mustafa et al. [39] reported that methane production increases linearly with lignin degradation of WRF-pretreated rice straw. However, the degradation activity and ability of pure fungal cultures are insufficient for the pre-treatment systems of large-scale biogas projects. In general, pure cultures of fungi can only degrade substrates with simple structures, such as artificial xylan and pure cellulose [19].

Microbial consortia can overcome the disadvantages of pure fungal cultures and are also applied in pre-treatment of lignocellulosic biomass [18,19]. The organisms in a microibial consortium are usually screened through restrictive culturing. If the targeted microorganisms are cellulolytic bacteria, the consortium can be continuously subcultured on a cellulose-based substrate. The functions of microbial consortia in pre-treatment have been reported at the laboratory scale. Yuan et al. [19] pretreated the lignocellulose in MSW (office paper, newspaper, cardboard, and mixtures) using a thermophilic microbial consortium of *Clostridium straminisolvens* CSK1, *Clostridium* sp. FG4b, *Pseudoxanthomonas* sp. train M1–3, *Brevibacilus* sp. M1–5, and *Bordetella* sp. M1–6. They reported that methane production more than doubled after this pre-treatment. Similarly, Zhong et al. [18] reported a 75.6% increase in methane yield after microbial consortium pre-treatment. Interestingly, the efficacy of microbial consortium pre-treatment often depends on the duration of the pre-treatment. For example, Yuan et al. [19] reported no obvious increase in methane yield when the pre-treatment time exceeded 10 d because large portions of the organic matter were consumed by the microorganisms. The same phenomenon was reported elsewhere [16]. The main drawback of microbial pre-treatment is the loss of organic carbon due to microbial growth. Therefore, optimizing the culture time is crucial for reducing the loss of organic matter and optimizing the pre-treatment results.

The pre-treatment results can be improved by combining microbial pre-treatment with physical or chemical pre-treatment. Microbial pre-treatment mainly obtains good results under the manageable conditions of laboratory-scale research and holds considerable promise for further application, but its application to large-scale projects must be further explored.

### Enzyme Pre-Treatment

To overcome the challenges of controlling microbial pre-treatment, some researchers have attempted to screen and utilize microbial enzymes for pre-treatment. The enzyme pre-treatment method is often performed on two categories of substrates with different characteristics: (i) agricultural waste (mainly lignocellulosic biomass) and (ii) sludge (mainly waste-activated sludge). Other substrates with high natural hydrolysis rates, such as food waste (FW) and organic fraction of municipal solid waste (OFMSW), have achieved limited results and are deemed unsuitable for enzyme pre-treatment [16,40]. Enzyme pre-treatment has outperformed conventional microbial pre-treatment on lignocellulosic biomass. Laccases and peroxidases are often used as lignin degraders and have demonstrated actual pre-treatment effects on lignocellulosic biomass. Schroyen et al. [41] reported a 17% increase in methane yield after laccase pre-treatment of corn stover. Consistent with this result, Frigon et al. [42] obtained methane production increases of 29% and 41%, respectively, during lignin and manganese peroxidase pre-treatment of switchgrass. However, Schroyen et al. [41] reported that increasing the lignin concentration in lignocellulosic

biomass decreases the pre-treatment efficiency of the degraders (laccases and versatile peroxidases). In fact, AD is strongly inhibited by phenolic compounds released from the lignin degradation process, indicating that substrate choice is a crucial determiner of methane yield from lignin degradation [40,43]. Cellulases, hemicellulases, amylases, and pectinases have been widely tested as carbohydrate degraders on agricultural waste, obtaining favorable methane yields [40]. Nevertheless, for a given substrate, the enzymes, incubation time, temperature, and pH must be carefully selected to avoid negative results [44]. Moreover, the high cost of existing commercial enzymes limits their applicability to AD of lignocellulosic biomass. Recently, an enzyme production technology based on solid-state fermentation has obtained cheap enzymes for pretreating lignocellulosic biomass [12,45]. This low-cost, environmentally-friendly technology achieves a good pre-treatment effect on organic wastes as the main culturing substrates. Enzymes produced through solid-state fermentation can potentially be applied in large-scale projects.

Sludge, a by-product of wastewater treatment plants (WWTPs), is another common substrate for enzyme pre-treatment. Sludge can be divided into primary sludge and waste-activated sludge (WAS). WAS consists of flocs and is rich in microbial biomass and extra-cellular polymeric substances composed of carbohydrates and proteins [40]. The composition of sludge is ideally suited for pre-treatment with carbohydrases and proteases. Yu et al. [46] reported a 23% increase in biogas yield after amylase and protease pre-treatment at 37 °C. Yin et al. [47] reported a threefold and twofold increase in the hydrolysis rate and methane yield, respectively, after WAS pre-treatment with fungal mash (which is mainly enriched in carbohydrases). Nevertheless, the activity lifetime of enzymes in sludge is short (generally less than 24 h) because the enzymes are degraded by endogenous proteases and inhibited by unknown compounds in sludge [40,48]. The short enzyme lifetime largely increases the cost of obtaining the desired effects. Recently, WAS pre-treatment with garbage enzymes has improved the solubilization and biodegradability of WAS in AD [49–51]. Garbage enzymes are by definition crude enzymes that digest fruit wastes. Garbage enzymes can be produced at low cost, are environmentally friendly, and achieve pleasant pre-treatment results. Therefore, garbage enzyme pre-treatment is potentially applicable to sludge in large-scale biogas projects.

#### 2.1.2. Chemical Pre-Treatment

Chemical pre-treatment methods include (but are not limited to) alkalis, acids, thermochemical, and electro-chemical methods [22–28,52–56]. Like microbial pre-treatment, chemical pre-treatment is commonly applied to lignocellulosic biomass because it effectively breaks the ester bonds between polysaccharides and lignin [23]. Alkali treatment is the most frequently employed chemical pre-treatment method, as it effectively reduces the crystallinity of cellulose, removes lignin, and increases the surface area and porosity, thus improving the digestibility of the substrate. Panels a and b of Figure 1 show the mechanisms of the reaction between lignin, lignin complex, and $OH^-$, respectively. $OH^-$ ions can partially resolve and separate lignin and hemicellulose by breaking the ester–ether bond between lignin and polysaccharides and weakening the hydrogen bonds between hemicellulose and cellulose [30]. Two commonly used alkalis, CaO and NaOH, are highly effective pre-treatment agents. Solé-Bundó et al. [54] reported an 11.99% increase in methane yield after pre-treatment using 10% CaO. Likewise, Mancini et al. [25] showed that alkaline pre-treatment enhances the methane yield from wheat straw by 155%. In addition to CaO and NaOH, Yao et al. [27] suggested that urea loosens the constituent cell wall polymers of wheat straw lignocellulose. Although lignin dissolution increases the susceptibility of lignocellulose to digestion, excessively high lignin contents inhibit the reaction system. Koyama et al. [53] reported that the hydrolysis step can be inhibited by dissolved lignin during methanogenesis and acidogenesis. The hydrolysis efficiency decreased by 25% at a dissolved lignin concentration of 1.0 g $L^{-1}$ [53]. Acid can also change the biodegradability of lignocellulosic biomass by dissolving the hemicellulose. The commonly used acids are $H_2SO_4$, HCl, $H_2O_2$, and $CH_3COOH$. However, at low concentrations, acids cannot

effectively pretreat lignocellulosic biomass because they weakly act on the lignocellulosic structure. Inorganic acids are more impactful during acid pre-treatment than organic acids, which have mild chemical properties. Song et al. [29] compared the effects of $H_2SO_4$, $H_2O_2$, HCl, and $CH_3COOH$ on rice straw pre-treatment. The biogas yields increased in the order of 3% $H_2O_2$ > 2% $H_2SO_4$ > 2% HCl > 4% $CH_3COOH$. In general, pre-treatment using low concentrations of organic acids achieves poor biogas production results, whereas pre-treatment with high organic acid levels will remove large amounts of dry matter, which is detrimental to AD [30,57].

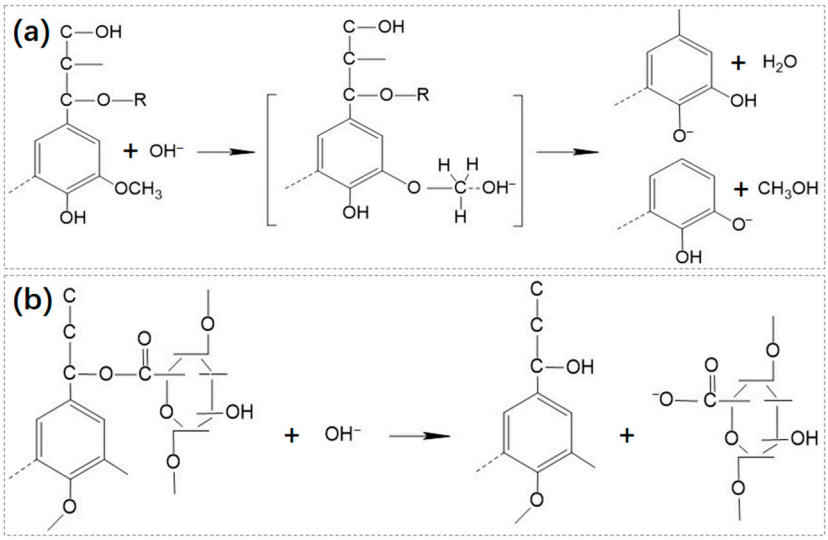


**Figure 1.** Schematic diagram of reaction mechanism between lignin, lignin complex, and $OH^-$. (**a**) The reaction mechanism between lignin and $OH^-$. (**b**) The reaction mechanism between lignin complex and $OH^-$. This picture was adapted from Yu et al. [48], with permission from Elsevier, copyright 2018.

Thermo-chemical and electro-chemical pre-treatment methods are also efficient. Passos et al. [28] reported that pre-treatment with 10% NaOH at 100 °C increases the methane potential of dairy cow manure by 23.6%. Solé-Bundó et al. [23] similarly found that thermo-chemical pre-treatment increases the methane yield of microalgae by 15% from that of the untreated control. Electro-chemical pre-treatment is often applied to WAS prtreatment. Electrolysis in conjunction with alkali treatment can disrupt the microbial cells in WAS gels and release their biopolymers (proteins and polysaccharides), thus enhancing the breakdown/solubilization of sludge flocs [55,56]. However, a technical and economic analysis showed that thermo-chemical pre-treatment offers zero advantage over no pre-treatment [28].

The properties of the substrate should be considered when selecting chemical pre-treatment methods. The selected chemical reagents should benefit the subsequent processing; for example, KOH can be used as a fertilizer. Chemical pre-treatment converts alkalis to salts, which embed within the biomass where they cannot be recovered [58]. Thus, the choice of base for the pre-treatment is crucial because $Na^+$ ions in the biomass can hamper microbial activity during the AD process; methanogens, in particular, are highly sensitive to $Na^+$ [3,6,59]. Moreover, Na is environmentally harmful because its disposal causes soil degradation [59,60]. KOH is approximately three times more monetarily expensive than NaOH but is less toxic to microbial activity [3,61]. Although chemical pre-treatment effectively enhances the AD efficiency, it is severely disadvantaged by secondary pollution and high economic input and is therefore not preferred.

#### 2.1.3. Physical Pre-Treatment

Physical pre-treatment methods include milling, radiation (microwave and ultrasound), and thermal pre-treatments [31–35,62–65].

Milling pre-treatment reduces the substrate particle size, decreases the crystallinity of the lignocellulose substrate, and increases the surface area, increasing the ease of digestion and shortening the digestion time [30]. Milling usually precedes other pre-treatment methods to obtain a synergistic pre-treatment effect. Mustafa et al. [39] reported that a combined milling and fungal pre-treatment improves methane production from rice straw by 165%. In general, this method is economically infeasible because it demands high energy to obtain the desired particle size (a few millimeters at the laboratory-scale level) [66]. Therefore, this method is not recommended for large-scale projects [67]. In addition, the energy consumption depends largely on the type of material. Mönch-Tegeder et al. [34] reported that the energy consumption of milling pre-treatment is negligibly important in a large-scale biogas plant using horse manure with a particle size of 3 mm, as milling boosts the methane production by 26.5% [34]. Thus, the availability of milling pre-treatment mainly depends on the type and milling degree of the digestion substrates [37].

Microwave pre-treatment is often applied to sludge and microalgae [31,32,63–65]. Microwave pre-treatment can disrupt the cell walls of the microbial cells in sludge, which encloses the cells within an extra-cellular polymeric floc matrix. The intracellular organics released from the disrupted cells are easily digested [65]. Although microwave pre-treatment cannot induce cell wall lysis in microalgae, microwave-pretreated algal cells are more susceptible to microbial attack, and hence more biodegradable, than untreated cells [31]. Ultrasound pre-treatment is also commonly used because it enhances the solubilization of organic matter [62]. Liu et al. [68] reported that the maximum volatile fatty acid (VFA) yield and highest percentage of $H_2$ in the biogas increased by 65.3% and 59.1%, respectively, after microwave pre-treatment under the optimal sonication conditions. However, the high installation costs of microwave and ultrasound are impractical for sizeable biogas projects. Thus, radiation is the only feasible auxiliary process of other pre-treatment methods.

Thermal pre-treatment is a physical pre-treatment that heats the lignocellulosic biomass at a specific pressure and temperature (50 °C–240 °C) [34]. Marsolek et al. [33] reported a 35.8% increase in algal biogas yield during AD after pre-treatment at 90 °C for 12 h. Passos et al. [28] compared the effects of microwave, ultrasound, and thermal pre-treatments of microalgae. All three methods improved the solubilization of organic matter, but whereas thermal and microwave pre-treatments increased the methane yield by 72% and 21%, respectively, ultrasound pre-treatment exerted no significant effect. These different results of different physical pre-treatments can be attributed to the broad range of differences among the properties of substrates.

Physical pre-treatment, especially milling, is necessarily combined with other pre-treatment methods. Prior to use, pre-treatment must be assessed for its energy input and output efficiency to determine its potential application value.

### *2.2. Co-Digestion*

Numerous studies have demonstrated the advantages of co-digestion of multiple raw materials over traditional mono-digestion. Co-digestion is the most commonly used strategy for enhancing AD performance. Unlike mono-digestion, co-digestion involves multiple substrates and hence avoids common obstacles in mono-digestion, namely C/N imbalance, low buffering capacity, and lack of trace elements.

#### 2.2.1. C/N Ratio: The Most Important Parameter in Co-Digestion

The commonest co-digestion substrates are livestock manure and crop straws. As shown in Figure 2, the C/N ratio of lignocellulosic biomass often exceeds 60, leading to low buffer capacity and high accumulation of VFAs and consequent instability of the system. Conversely, the C/N ratio of livestock manure is often less than 20, which inhibits ammonia

and lowers the biogas production efficiency [14,15]. Therefore, co-digestion of livestock manure and lignocellulosic biomass can effectively improve the overall decomposition efficiency [69]. The optimal ratio of lignocellulosic biomass to livestock manure in co-digestion typically ranges from 1:2 to 2:1 (based on total solids) [69–71]. Although co-digestion can improve the digestion efficiency, it is ineffective when the substrates are seasonable or site-specific, especially for livestock manure and lignocellulosic biomass [72]. In recent years, digestion substrates have gradually diversified to include food industry wastewater, FW, energy crops, microalgae, vegetable waste, dairy wastewater, slaughtering waste, and OFMSW. Co-digestion with more than two materials is also trending. The AD process requires a proper C/N ratio. The optimal C/N ratio falls within the range 10:1–25:1. Above the maximum feasible C/N ratio (100:3), nitrogen is not viably utilized by the organisms and the methane production efficiency reduces. Conversely, if the C/N ratio is excessively low, toxic ammonia is formed and the pH increases [73]. The C/N ratio is an important indicator during research on co-digestion of various materials. Although some studies have confirmed that C/N ratios around 25 maximize the digestion efficiency, inconsistent results have been reported. For example, Zheng et al. [35] reported that co-digestion of cow manure and switchgrass (a typical lignocellulosic biomass) is maximized at a C/N ratio of 29.4. Wang et al. [74] optimized the C/N ratio of co-digested dairy manure (DM) and poultry manure (CM) with wheat straw for methane production. They reported a maximum methane potential at a DM/CM ratio of 40.3:59.7 and a C/N ratio of 27.2:1.0. However, Latha et al. [71] reported that a C/N ratio of 12.9 maximizes the methane yield of co-digested FW and sludge. The obvious differences among the optimal C/N ratios might be explained by the large differences in the characteristics (such as biodegradability and trace element contents) of the raw materials [75,76]. Therefore, further research on the C/N ratio should consider the properties of specific substrates.

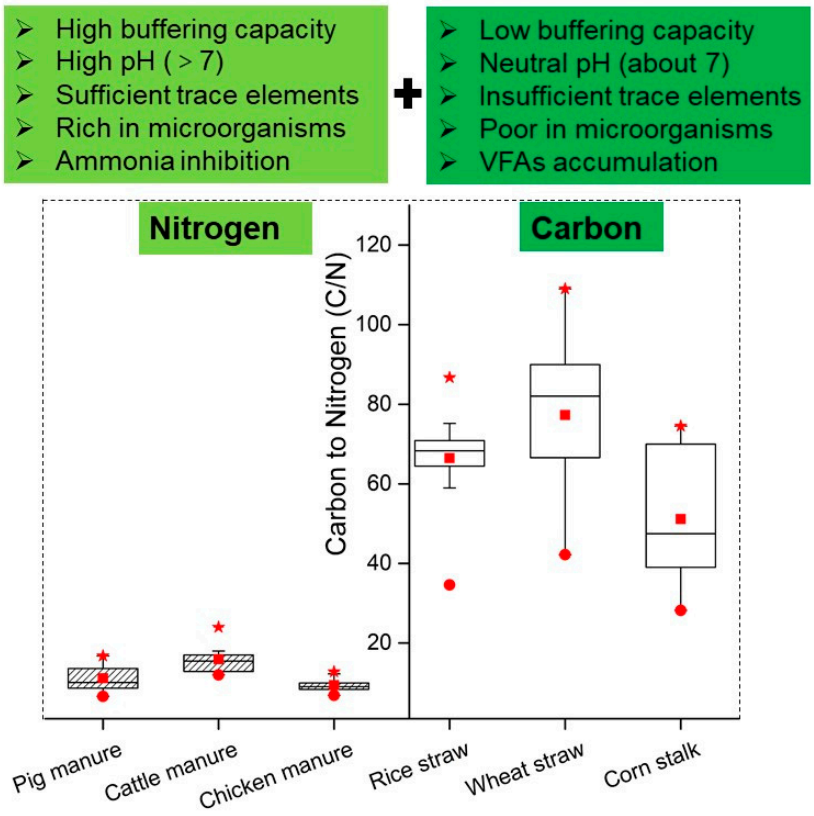


**Figure 2.** The principle of co-digestion using carbon-rich substrates (using crop straw as an example) and nitrogen-rich substrates (using livestock manure as an example). The carbon and nitrogen reflect total carbon and total nitrogen, respectively. For every substrate, $n$ = 10. Note: Red stars indicate outliers.

#### 2.2.2. Mechanism by which Co-Digestion Promotes Digestion Efficiency

Co-digestion technology promotes nutritional balance and increases the buffering capacity of a digestion system. These effects are ultimately reflected in the microbial community structure and the absolute number of microorganisms. Zhang et al. [74] reported that the dominant bacteria (*Aminobacterium* and *Proteiniphilum*) and methanogenic archaea (*Methanobacterium* and *Methanosarcina*) in co-digestion of horse manure and FW notably differ from those in mono-digestion of FW. Zhang et al. [74] reported higher relative abundances of hydrogenotrophic methanogens, acetoclastic methanogens, and bacteria in co-digestion of blackwater with kitchen organic waste than in mono-digestion of blackwater. Consistent with these findings, Mu et al. [75] reported higher absolute abundances of bacteria and archaea in co-digestion of sewage sludge and FW than in mono-digestion of FW. In general, methanogenic archaea are more sensitive to changes in the digestion conditions than bacteria. Therefore, when the digestion system is stressed (mainly by high concentrations of VFAs and ammonia), the methanogenic archaea undergo more evident changes than bacteria. For example, Capson-Tojo et al. [77] reported that gradually increasing the ammonium concentration induces a change from the acetoclastic methanogenesis pathway to the hydrogenotrophic methanogenesis pathway. Methanogenic archaea include acetoclastic methanogenesis archaea, mixotrophic methanogenesis archaea, and hydrogenotrophic archaea. As the ammonium concentration increases, the dominant methanogenic archaea alter from *Methanosaeta* (strict acetoclastic methanogenesis archaea) to *Methanosarcina* (mixotrophic methanogenesis archaea) and eventually to *Methanoculleus*, *Methanobacterium*, or *Methanobrevibacter* (hydrogenotrophic archaea) [77]. The acetoclastic methanogenesis pathway is generally considered as the main pathway in co-digestion, but the methanogenesis pathway will change under stressful conditions of the digestion system. Co-digestion environments enable a more efficient community structure of methanogenic archaea than mono-digestion environments [78–80]. Therefore, provided that the types and amounts of raw materials are sufficient, co-digestion is the preferred strategy for promoting the efficiency of AD.

### *2.3. Recirculation*

Recirculation is the process by which digestates, sludge residue, and leachate are returned to the reactor. Figure 3 shows the four common recirculation types: liquid digestates recirculation, sludge recirculation, leachate recirculation, and methanogenic effluent recirculation. Recirculation reduces the discharge of digestate, improves the buffering capacity and alkalinity of the system, increases the stability of the digestion system, changes the dominant microorganism and methanogenic pathways, and increases the mass transfer, thereby increasing the hydrolysis rate, acidification efficiency, and methane yield [81–85]. Table 4 describes the effects of different recirculation types on digestion systems with different substrates, reactor types, recirculation types, and recirculation rates. Wu et al. [82] found that digestate recirculation improves the methane yield, OLR, and systematic hydrolysis rate of FW. Recirculation also increases the alkalinity of the system, maintaining an ideal pH for methanogens. Pezzolla et al. [81] reported that recirculation prevents VFA accumulation, thus improving the stability of the digestion system. In contrast, Qian et al. [85] reported rapid VFA accumulation and a high VFA concentration peak within a short time. These contrasting findings possibly reflect differences among the frequencies and rates of recirculation systems. At higher circulation frequencies, Qian et al. [85] reported obvious increases in hydrolysis, acidogenesis, and mass transfer, whereas Ni et al. [84] demonstrated a decreased methane yield, which they attributed to the considerable increase in viscosity (from 30 to 1000 mPas) and decreased mass transfer under excessive recirculation conditions. Excessive recirculation may also cause accumulations of heavy metals (mainly Mn, Pb, Zn, and Cu) and ammonia in the digestate [82,84], which disrupt the metabolic balance between methanogens and bacteria.

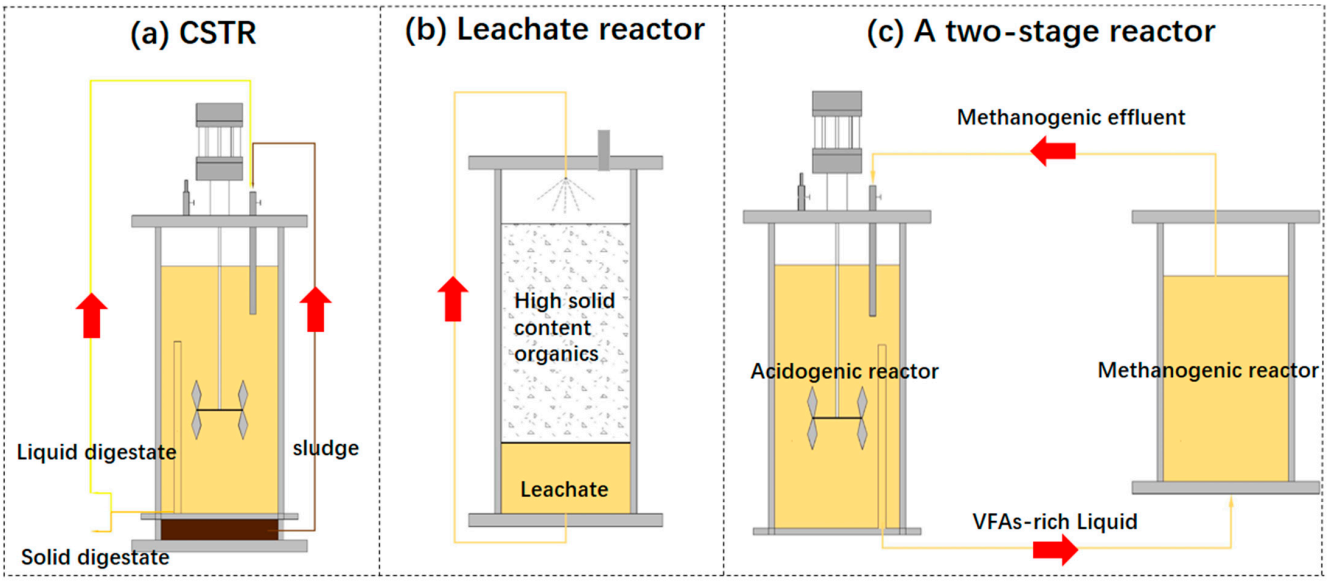


**Figure 3.** Four recirculation types for three common reactors. (**a**) Liquid digestate or sludge recirculation. (**b**) Leachate recirculation in leachate reactor with high-solid-content organics as the substrate. (**c**) The digestate in methanogenic reactor recirculates to acidogenic reactor in a two-stage reactor. The picture was adapted from Zamanzadeh et al. [83] and Pezzolla et al. [81].

**Table 4.** The effect of recirculation on reactor performance.

| Substrate | Reactor Type | Recirculation Type | Conclusions | Ref. |
|---|---|---|---|---|
| Vegetable waste | Two-stage reactor | Recirculation rates from 0 to 1.4 [a] | pH was significantly increased in acidogenic reactor. Biogas production rates increased more than 3 times. | [83] |
| Corn stover | CSTR | Liquid fraction of the digestate total recirculation | Methane and biogas production were increased significantly by 2.3% and 10.8% due to increased process stability. | [68] |
| FW | Integrated two-phase reactor | Leachate recirculation rates [b] at 0%, 25%, 50%, or 75% of collected leachate | Enhance the hydrolysis efficiency and methanogenic reaction, 50% recirculation obtained optimal effect. | [86] |
| Wastewater | CSTR and AnMBR | Sludge recirculation | COD removal rate reaches its highest, at 96.7%, when sludge recirculation rate is 2. | [87] |
| FW | CSTR | Recirculation liquid fraction of the digestate, recirculation rate is 2 [c] | The methane yield of recirculation and no-recirculation was similar. | [83] |
| Pig slurry and straw (3:1, *w*/*w*) | Leachate reactor | Recirculation of all leachate | A better system stability was obtained because recirculation avoided the accumulation of VFAs. | [81] |
| Pig manure | CSTR | Liquid digestate | Recirculation operation could improve the bioenergy production under OLRs below 5 g VS $L^{-1}$ $d^{-1}$. However, OLRs more than 6 g VS $L^{-1}$ $d^{-1}$ recirculation decreased mass transfer characteristics and increased heavy metal accumulation. | [84] |

**Table 4.** *Cont.*

| Substrate | Reactor Type | Recirculation Type | Conclusions | Ref. |
|---|---|---|---|---|
| OFMSW and Corn Straw | Leachate reactor | Leachate recirculation rates are 0.3, 0.6, 1.2, 2.4, and 4.8 [d] | High recirculation rate positively contributed to the hydrolysis and acidogenesis rate due to its inoculation effect and mass transfer enhancement. Highest methane yield was obtained when recirculation rate was 0.3. | [85] |

[a] The recirculation rate is the ratio of the returned flow rate to inlet flow rate. [b] The recirculation rate is ratio of the recirculation volume to the total volume. [c] The recirculation rate is 200 mL digestate to 100 mL feed. [d] Leachate recirculation rate is the ratio of daily leachate recirculation volume to total leachate volume.

Recirculation can alter the metabolic pathways and structure of a microbial community. For example, stable carbon isotope analysis indicates that the hydrogenotrophic methanogenesis and acetoclastic methanogen pathways dominate in the presence and absence of digestate recirculation, respectively [84]. Zamanzadeh et al. [83] reported that recirculation can change the dominant bacterial phylum in mesophilic FW AD from *Chloroflexi* to *Firmicutes*. The effect of recirculation on the microorganisms and dominated methanogenesis pathways ultimately originates from changes in the substances of the digestion system, such as nutrient enrichment, increased contents of buffer substances, and increased numbers of microorganisms.

In summary, recirculation can either benefit or inhibit the AD and requires proper control of the extent and frequency of recirculation to achieve good results. Different substrates often require different recirculation strategies (mainly recirculation types, recirculation ratios, and recirculation frequencies). The selected strategy is particularly important in anaerobic digestions of FW and livestock manure, which are prone to ammonia accumulation.

### *2.4. Microaeration*

Microaeration (also called micro-oxygenation, limited aeration, and microaerobic conditions) is the process of supplying small volumes of air or oxygen (typically 0.005–5 L $O_2/L_{reactor}/d$) to the anaerobic reactor [88–90]. Traditional systems should prevent the entry of oxygen or air because oxygen inhibits the growth and metabolism of methanogens, which are obligate anaerobes [7]. In recent years, scientists have discovered that microaeration can resolve the low hydrolysis rates, toxicity of high-concentration hydrogen sulfide, and instability at high OLRs, which degrade the performance of anaerobic digestion systems [13,91–93].

#### 2.4.1. Digestion Performance under Microaerobic Conditions

Different microaerobic conditions elicit different effects in reactors (see Table 5). The microaerobic status depends on the $O_2$ utilization rate, which is related to the inoculated species (source and adaptation to substrate), substrate (type and organic loading rate), the microaerobic method (air/$O_2$, single/continuous, or injection phase), and the $O_2$ transfer rate (itself related to the reactor configuration: one/two-staged or batch/continuous stirred-tank reactor) [7].

**Table 5.** The oxygen dosing for different purposes.

| Objective | Reactor Type | Substrate | Oxygen Dosing Rate Equivalent (L $O_2/L_{reactor}/d$) * | Results | Ref. |
|---|---|---|---|---|---|
| Enhance hydrolysis | CSTR | FW and brown water | 0.005 and 0.007 | Bacterial diversity and concentration of VFAs increased. | [61] |
| Enhance hydrolysis | CSTR | Primary sludge | 0.21 | Hydrolysis rate increased by 50–60%. However, methane yield, VFAs, and sCOD decreased due to aerobic substrate consumption. | [91] |

**Table 5.** *Cont.*

| Objective | Reactor Type | Substrate | Oxygen Dosing Rate Equivalent (L $O_2$/$L_{reactor}$/d) * | Results | Ref. |
|---|---|---|---|---|---|
| Enhance hydrolysis | CSTR | Primary sludge | 0.5 | Hydrolysis of carbohydrates and protein was enhanced accompanied by increased solubilization of COD. | [94] |
| Enhance hydrolysis | Leach bed reactor | Synthetic FW | 2.1, 4.4 and 6.5 | Middle aeration rate was best: increased hydrolysis. | [95] |
| Enhance methane yield | Batch reactor | Corn straw | 0.003–0.021 | At lower micro-aeration intensity, enhanced methane yield, diversity of phylum Firmicutes, and VS removal were obtained. | [96] |
| Enhance methane yield | Batch reactor | Long-chain fatty acids | Not given | A significant increase in methane yield. | [97] |
| Remove $H_2S$ | Sludge reactor | Waste-activated sludge | 0.01 | 98% $H_2S$ removal from biogas. | [98] |
| Remove $H_2S$ | UASB | Synthetic brewery | 0.08 | 73% $H_2S$ removal. | [99] |
| Remove $H_2S$ | UASB | Wastewater | 0.03 mol $O_2$ $m^{-3}$ | The highest $H_2S$ removal efficiency was 91.2% and obtained for an $O_2$:S ratio of 0.5. | [92] |
| Control VFA accumulation and improve effluent quality | CSTR | Waste-activated sludge | 0.03 | 3.5 times lower VFAs and 33% lower sCOD were obtained. Compared with anaerobic conditions, microaerobic conditions have lower foaming and better dewaterability. | [100] |
| Overcome overloading and improve reactor stability | CSTR | Waste-activated sludge | 0.01 | Overcame hydraulic overloading, promoted growth of hydrogenotrophic bacteria. | [93] |
| Produce VFAs | Batch | Batch reactor | 0.09 and 1.9 | Highest VFA production was obtained with 15 mL $O_2$/g VS and 3 days' incubation time using cattle manure as inoculum. | [11] |

* Calculated from reported microaeration intensity with assumption $O_2$ = 21% (*v*/*v*) of air.

Microaeration directly influences the growth and metabolism of microorganisms. Bacteria, which mainly partake in the hydrolysis and acidification stages of anaerobic digestion, usually contribute 80% of the total bacterial and archaeal DNA in a digestion system. Among the dominant bacterial phyla (*Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*), *Bacteroidetes* and *Firmicutes* hydrolyze hemicellulose, cellulose, and other polysaccharides, whereas the *Proteobacteria* utilize glucose and VFAs [7]. As proven in previous studies, hydrolysis is the rate-limiting step during strict anaerobic AD of high-solid-content organic substrates and lignocellulosic biomass [11,58]. Microaeration encourages the formation of diverse hydrolytic and fermentative bacterial communities with high activity [101]. For example, Fu et al. [101] reported that a high percentage of *Firmicutes* emerges under microaerobic conditions, which are linked to high hydrolytic rates. An abundant hydrolytic bacteria community can produce numerous extra-cellular hydrolytic enzymes (e.g., amylases, cellulases, and proteases) that hydrolyse proteins, carbohydrates, and other complex organic substrates. In one study, microaeration treatment increased the *Firmicutes* population in organic wastewater/FW from 58% to 72% [61]. The increased

activity of *Clostridia* and *Bacilli* classes (within the phylum *Firmicutes*) under microaerobic conditions increases the butyric and acetic acid concentrations by threefold, consequently boosting the methane yield [95].

Previous studies have shown that 30% of the protein released during AD is contributed by archaea, which comprise less than 4% of the microbial population [102]. The enhancement of hydrolysis and acidogenesis under microaerobic conditions provides additional substrates for methanogens, increasing their activity [96]. Microaeration also directly affects the methane-producing step by changing the main methanogenic pathway. The shift between hydrogenotrophic and acetoclastic pathways largely depends on the balance between symbiotic acetic acid-oxidizing bacteria (which convert acetate to $CO_2$ and $H_2$) and homoacetogenic bacteria (which reduce $CO_2$ to acetate) [103]. Under microaerobic conditions, the traditional syntrophic collaboration between symbiotic acetic acid-oxidizing bacteria and hydrogenotrophic methanogens can be substituted by synergistic collaboration between facultative bacteria ($CO_2$ and $H_2$ generators) and hydrogenotrophic methanogens ($H_2$ and $CO_2$ consumers). Under such conditions, the changes in VFA, $CO_2$, and $H_2$ concentrations can affect the archaeal communities and hence change the dominant methanogenic pathway [104].

Moreover, the microaerobic conditions can regulate the activity of sulfide/sulfur-oxidizing bacteria (SOB) and sulfate-reducing bacteria (SRB), preventing hydrogen sulfide ($H_2S$) poisoning of the reaction system. Produced via sulfate conversion by SRB, $H_2S$ is highly toxic and corrosive when dissolved in the liquid phase. Specifically, dissolved sulfide ($H_2S + HS^-$) inhibits methanogens and corrodes the reactor equipment. Furthermore, SRB compete with methanogens for the substrates (acetate, $H_2$, and other intermediates). As SRB have higher substrate affinities for acetate and $H_2$ and a higher growth rate than methanogens, the $H_2S$ content rises and the methane yield is low [7,105]. Meanwhile, SOB convert sulfide ($HS^-$) to elemental sulfur ($S^\circ$), thiosulfate ($S_2O_3^{2-}$), and sulfate ($SO_4^{2-}$). Both SOB and SRB exist in AD systems with sulfate-rich substrates. As shown in Table 5, $H_2S$ can be effectively removed by controlling the anaerobic condition with small amounts of aeration [92,98,99].

Although microaerobic technology has been extensively researched and developed, our knowledge of this technology remains limited. The oxygen or air supply must be optimized for different purposes. For example, to increase the hydrolysis efficiency, the oxygen concentration must be raised beyond the level required for $H_2S$ removal. Under digestion processes with different substrates, reactor types, OLRs, and stirring speeds, the effect of microaerobic conditions requires more extensive investigation. Microaeration is a promising technology for large-scale biogas projects, especially on substrates that are difficult to hydrolyze.

#### 2.4.2. Co-Existence and Synergistic Interaction Mechanisms between Bacteria and Archaea under Microaerobic Conditions

Under microaerobic conditions, the use and partial reduction of oxygen by facultative microorganisms produces reactive oxygen species (ROS) such as •OH, $H_2O_2$, and $O_2^-$. These ROS are highly oxidative and can damage proteins, DNA, and the cell membranes of microorganisms [7]. Aerobic and facultatively aerobic bacteria, which generate ROS-neutralizing anti-oxidative enzymes (AOEs), grow well in aerobic environments. According to recent studies, several strict anaerobes (mainly methanogenic archaea) can adapt to the oxidative conditions of microaerobic environments by similarly generating AOEs [106]. These adaptive responses endow strictly anaerobic microorganisms with high tolerance to microaerobic environments.

In an AD system, strictly anaerobic archaea and facultative bacteria tend to form bioflocs with facultative bacteria occupying the outer layer and archaea occupying the inner core (Figure 4). Hydrolytic and fermentative bacteria (facultative bacteria) with high AOE activity can scavenge ROS, reducing the ROS and oxygen concentrations within the bioflocs layer and thus protecting the inner-core anaerobes from oxidative damage [107].

The facultative bacteria and anaerobic archaea exhibit a similar synergetic relationship within aggregated bioflocs (from outer to inner sections) in the substrate flow (Figure 4). The hydrolytic bacteria in bioflocs break down complex organics into diverse intermediates, providing substrates for fermentative bacteria and then for methanogenic archaea. Because of this synergetic relationship, the remaining oxygen (represented by the oxygen reduction potential) decreases to a tolerable level for methanogenic archaea (Figure 4c). Therefore, the facultative bacteria in the outer layer may provide physical and biological barriers to oxygen, thereby protecting the strict anaerobic microorganisms and allowing their effective functioning within the microaerobic environment. Previous research has also shown that when the amount of aeration exceeds the oxygen utilization rate and anti-oxidation abilities of facultative bacteria, the high ROS concentration can damage the phospholipid membranes and DNA of strictly anaerobic microorganisms. Insufficient oxygen dosing can also degrade the AD performance by changing the delicate balance between facultative bacteria and anaerobic archaea [108]. Within an insufficient oxygen concentration environment, anaerobic archaea are relieved of ROS and oxidative damage but the efficiency of facultative bacteria decreases, undoubtedly affecting the growth and metabolism of anaerobic archaea (Figure 4d).

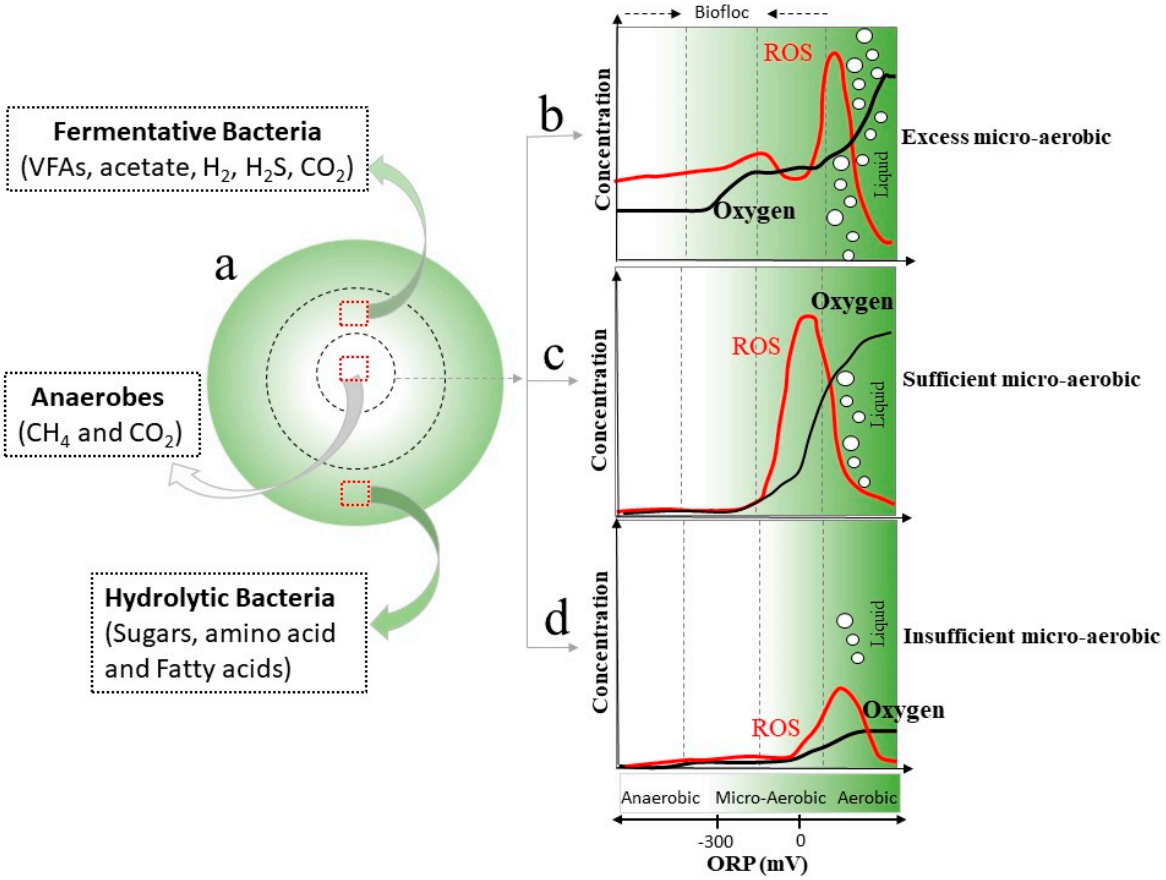


**Figure 4.** Anti-oxidative mechanism of microorganisms in different microaerobic conditions. The figure is adapted from Nguyen et al. [7], with permission from Elsevier, copyright 2017. (**a**) The microorganism and products across bioflocs. (**b**) The change in oxygen and reactive oxygen species (ROS) across the bioflocs under excess microaeration condition, (**c**) under sufficient microaeration condition, (**d**) under insufficient microaeration condition. Note: Distribution of microorganisms in bioflocs: strict anaerobes, hydrolytic bacteria, and fermentative bacteria aggregate in the inner, middle, and outer layers, respectively.

### 2.5. Additives

Trace elements and biochar and biological additives (bioaugmentation and enzymes) are the main additives of AD. The prominent feature of additives is their ability to deliver the desired results in small quantities. The mechanisms of AD stimulation by different types of additives are shown in Figure 5.

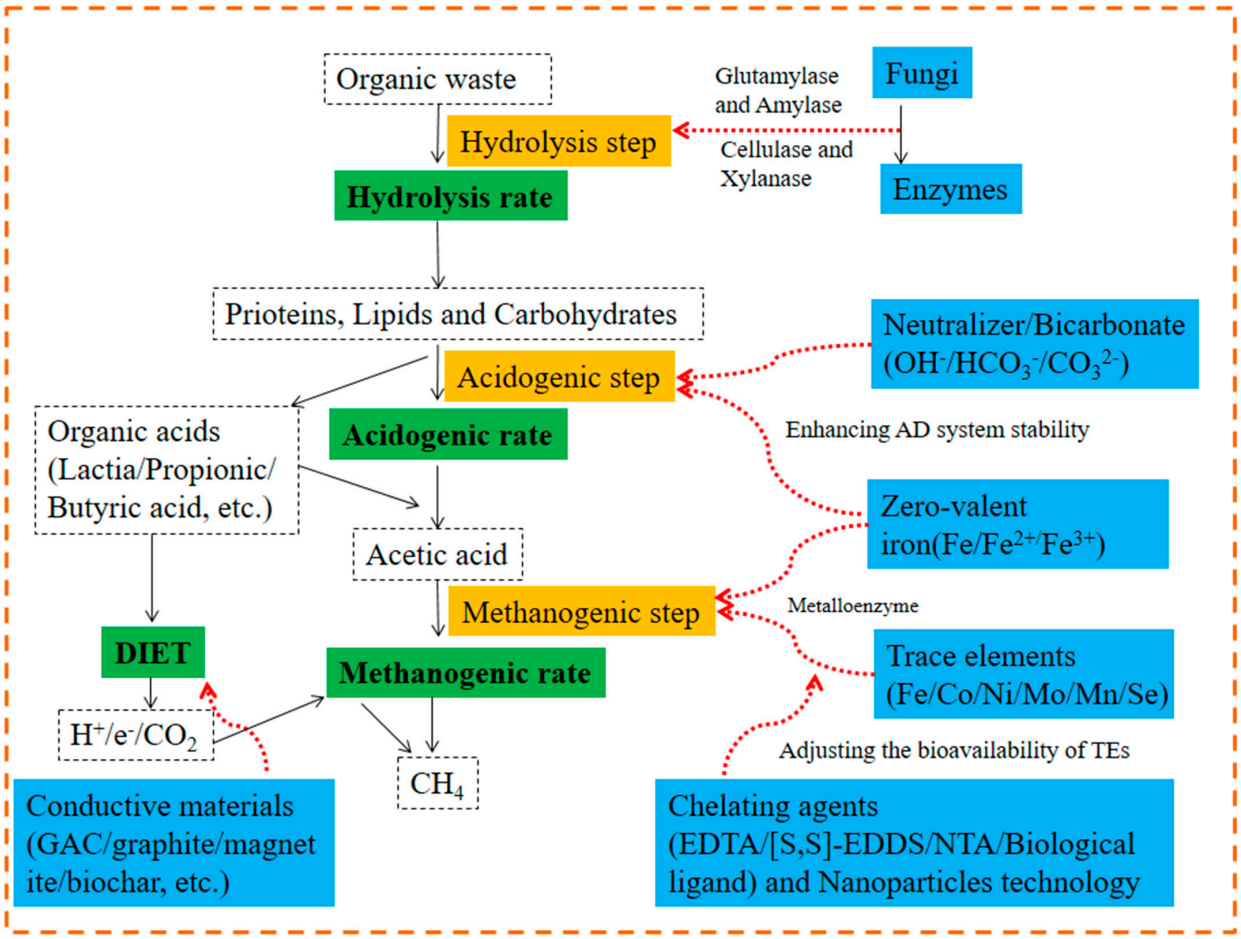


**Figure 5.** The mechanisms of different additives for AD. GAC: Granular Activated Carbon; DIET: direct interspecies electron transfer; TEs: trace elements. This picture was adapted from Ye et al. [109].

#### 2.5.1. Conductive Materials

Conductive materials are broadly divided into carbon-based conductive materials (activated carbon (AC), biochar, carbon cloth, carbon nanotubes, graphene, and graphite) and non-carbon-based conductive materials (magnetite, hematite, and stainless steel) [9]. In recent years, conductive materials have been applied to digestion systems, especially to those in laboratory-scale research.

##### Mechanism of AD Promotion by Conductive Materials

Electron transfer through interspecies hydrogen transfer between acetogens and methanogens using $H_2$ as a shuttle was previously accepted as the main methane-producing pathway [8]. However, direct interspecies electron transport (DIET) has recently emerged as another methane generation route [107]. DIET is an electron transfer process between microbes that avoids the use of mediating diffusive electron transporters. Three types of DIET mechanisms mediated by different agents—membrane-bound electron-transport proteins, conductive pili, and conductive materials—have been identified (see Figure 6) [9,110]. Electronic transfers through membrane-bound electron transport proteins (such as cytochromes) and conductive pili are inherent processes in microbial species and are challenging to reg-

ulate. According to recent studies, conductive materials can also provide carriers for DIET [8,111–119]. Lin et al. [111] reported a 25.0% enhancement of methane yield and a 29.1% improvement in the methanol degradation constant after adding 1.0 g/L graphene to AD. Microbial analyses revealed that *Geobacter* and *Pseudomonas* bacteria and *Methanobacterium* and *Methanospirillum* archaea participate in DIET. Park et al. [9] found that AC supplementation increases the methane yield and production rate by 31% and 72%, respectively, from those of the control treatments. A metagenomics analysis further affirmed that AC addition enhances the $CO_2$ reduction pathway. Dang et al. [115] found that hydrogenotrophic methanogens (*Sporanaerobacter* and *Methanosarcina*) are enriched on carbon cloth surfaces and that *Sporanaerobacter* can participate in DIET along with *Methanosarcina.* Enrichment of *Methanosarcina* was also reported in other studies. Non-carbon-based conductive materials can also play an active role in DIET. Wang et al. [8] reported that magnetite clearly reduces VFA accumulation and accelerates the methane yield by 26.6%. It also improves acetate-dependent methanogenesis in AD of high-solid-content sewage sludge. In addition, the expressions of both cytochrome and pili genes are reduced, indicating that magnetite can substitute the roles of these components in effective electron transfer between acetogens and methanogens. Similarly, Zhao et al. [112] reported that ferrosoferric oxide (as a conductive material) considerably increases the digestion performance of WAS. They found that ferrosoferric oxide enhances DIET between *Syntrophomonas* and *Methanosaeta*, indicating enhancement of the aceticlastic methanogenesis pathway.

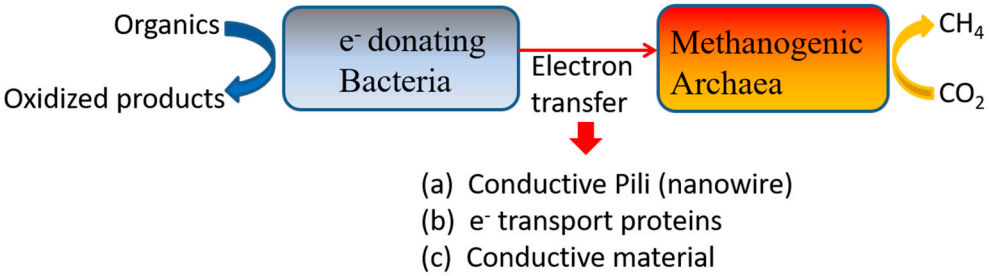


**Figure 6.** Three mechanisms of direct interspecies electron transfer (DIET) between organic-oxidizing bacteria and methanogenic archaea. This picture was adapted from Park et al. [9].

The enhancement of DIET with conductive materials faces several challenges. First, as DIET does not participate in the direct decomposition of complex organics, DIET enhancement should be combined with other strategies that improve the degradation of complex organics. Second, inexpensive and efficient conductive materials should be developed.

#### 2.5.2. Bioaugmentation and Enzymes

Bioaugmentation and enzymes are added as enrichment agents, not as pre-treatment agents, to the digestion process. In general, bioaugmentation defines the application of functional microorganisms to the reactor to improve the reactor performance. Functional microorganisms accelerate the decomposition and transformation of organic macromolecules and VFAs; alternatively, they resist toxic substances (mainly organic acids and ammonia nitrogen) [120]. The actual effects of bioaugmentation on reactor performance, especially at the laboratory scale, have been extensively investigated in recent years. Alternatively, hydrolytic microbes can enhance the rate and extent of hydrolysis. For example, a lignocellulolytic microbial consortium considerably improved the biogas yield during AD of fiber-rich swine manure, improving the cellulose and hemicellulose digestion efficiencies from 15% to 30–62% and from 23% to 31–75%, respectively [121]. Weiss et al. [122] reported a 53% increase in methane yield after adding hemi-cellulolytic bacteria to an AD reactor. Similarly, Martin-Ryals et al. [123] showed that adding cellulolytic microorganisms increases the soluble chemical oxygen demand production and methane yield of the acid phase by 25% and 15%, respectively. Pure bacterial cultures with high hydrolytic abilities have also been added to digestion systems. Lü et al. [124] showed that inoculation with

*Coprothermobacter proteolyticus* enhances hydrolysis of the constituent polysaccharides and proteins. In addition to bacterial consortia, methanogen consortia produce effective results. Li et al. [125,126] reported that a methanogen consortium can restructure the methanogen community by increasing the populations of *Methanothrix* (acetoclastic methanogens) and *Methanolinea* (hydrogenotrophic methanogens), thereby accelerating VFA degradation and increasing the methane yield.

Bioaugmentation is an important method of relieving ammonia inhibition. Hydrogenotrophic methanogen and syntrophic acetate oxidizing bacteria (SAOB) are the most common biological additives used to relieve ammonia inhibition [127–131]. Tian et al. [128] and Fotidis et al. [129] found that the methane yield increased by about 30% after adding *Methanoculleus bourgensis* at high ammonia levels (i.e., 5-11g $NH_4^+$-N $L^{-1}$) [128,129]. Moreover, Tian et al. [130] also showed that another hydrogenotrophic methanogen (*Methanoculleus thermophilus*) could create thermodynamically favorable environments for the process of acetate oxidation and then stimulated the growth of SAOBs, such as *Thermacetogenium phaeum*. Yang et al. [131] explored the effect of seven strains of bacteria and archaea (SAOBs, facultative aceticlastic methanogen, obligate aceticlastic methanogen, and hydrogenotrophic methanogen) on digestion performance and found that together, SAOB *Syntrophaceticu schinkii* and hydrogenotrophic methanogen (*Methanobrevibacter smithii*) were the optimal choice considering the methane yield.

Besides microorganisms, enzymes can also be directly added into AD system. For example, lipase (0.33% *v*/*v*) addition in AD of sewage sludge considerably improved the methane yield from 365 to 452 mL $CH_4$ $g^{-1}$ VS [132]. Sometimes, the effect of the enzyme was better than bioaugmentation. This may be due to the following reasons. (i) Enzymes can maintain high activity under a wide range of environmental conditions (e.g., pH, salinity, and temperature); (ii) enzymes can work in the presence of microbes, inhibitors of microbial metabolism, and predators; and (iii) enzymes have higher solubility, mobility, and more efficiency than microorganisms [120].

In general, bioaugmentation and enzymes are very effective strategies to improve the efficiency of AD systems. Microbial consortia are more effective than a single strain. However, bioaugmentation and enzymes must be added above certain threshold levels to achieve the desired results. The cost of bioaugmentation and enzymes is an obstacle to its application in large-scale biogas projects.

#### 2.5.3. Trace Elements

Relationship between Trace Elements (TEs) and AD

Metal elements could be classified based on their concentration in the cells into major elements (K, Mg, Ca) and TEs (Mn, Fe, Co, Cu, Mo, Ni, Se, and W) [133]. Metal concentration in the cells ranges from $10^{-7}$ to $10^{-3}$ M for the major elements, and from $10^{-6}$ to $10^{-15}$ M for TEs [134].

The mechanism of TEs in the AD system includes the following aspects. (i) TEs could change the metabolic environment to a more favorable one, such as by decreasing oxidative–reductive potential (ORP), increasing buffer capacity, and changing sludge properties. (ii) TEs play a role as the co-factor of several key enzymes [120,135]. AD is a complex biochemical reaction involving a considerable number of metalloenzymes. TEs are involved in the formation of metalloenzymes and play an important role in microbial metabolism [120]. As shown in Figure 7, whether it is the acetoclastic pathway or the $CO_2/H_2$ pathway, there are a large number of TEs involved in the composition of important metalloenzymes, especially the formation of intermediates such as methyl-tetrahydrosarcinapterin ($CH_3$-$H_4$SPT) or methyl-tetrahydromethanopterin ($CH_3$-$H_4$MPT). TEs have the function of generating the active site and co-factor in the metalloenzyme. For example, at least one binding element of Mo or W was needed for the formation of two isoenzymes of formylmethabofuran dehydrogenase, which demand at least one binding element of Mo or W in the process of initiating the $CO_2/H_2$ pathway [136]. In the acetoclastic pathway, the first metalloenzyme is carbon monoxide dehydrogenase/acetyl-coenzyme

A synthase (Cdh) complex enzyme, and the structure of its subunit CdhA contains Fe and Ni [137]. In addition, the cofactor 5-hydroxybenzimidazolylcobamide (factor III, with Co) and the crystal structure of methyl coenzyme M reductase (Mcr) of $CH_3$-$H_4$M(S)PT-coenzyme M methyltransferase (Mtr) has been revealed with two Ni-containing cofactors $F_{430}$ as the active sites [138]. As shown in Figure 8, the TE requirement trend in methanogenesis is Fe >> Ni > Co > Zn = Mo/W, without taking consideration of the substrate medium [139]. In fact, Cai et al. [140] found that the demand trend for TEs did not follow this law. Therefore, although those pathways have been understood well, further research is also required to assess the possible utilization mechanism for TEs, considering some specific factors (such as ammonia concentration, pH of digestion system, and bioavailability of TEs). As shown in Figure 7, Fe, Co, Ni, Zn, W, and Mo have a direct relationship to related enzymes in the methanogenesis stage. Besides these direct TEs, some studies have proven that Se and Mn, which have no direct relationship to the metalloenzymes, also had positive effects on the AD system. The further mechanism of this phenomenon needs to be revealed [6,14,140,141].

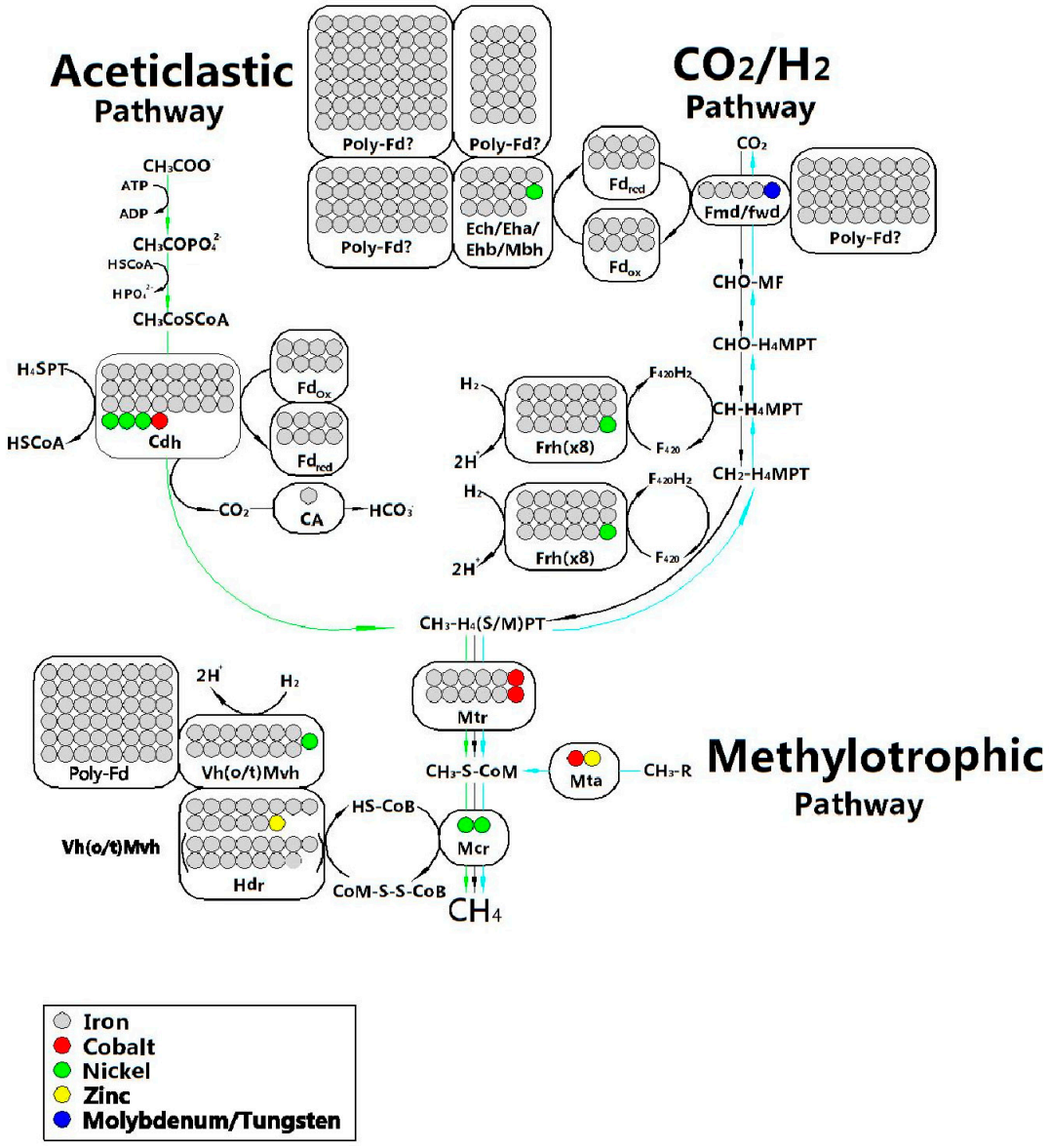


**Figure 7.** Metal content of metalloenzymes in the three pathways of methanogenesis: $H_2/CO_2$, aceticlastic, and methylotrophic (adapted from Glass and Orphan [139]). Question marks mean that enzyme may not be present in all methanogens. For simplicity, only metalloenzymes are labeled.

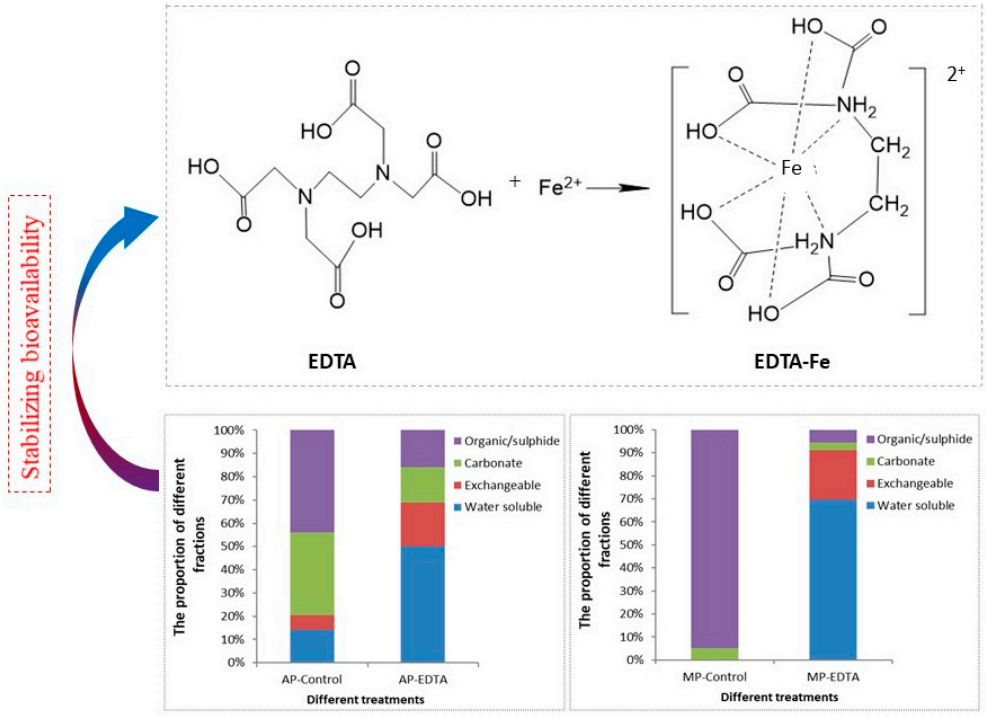


**Figure 8.** The mechanism of adding ligand as an additive to stable bioavailability of trace elements (using Fe as an example). In this picture, the ligand is EDTA and the trace element is Fe. Pictures are drawn from previous published data from Cai et al. [142]. Note: AP means acidogenic phase and MP means methanogenic phase. The water-soluble fraction of trace element has higher bioavailability than other fractions.

### The Requirements for TEs in AD System

TEs are broadly used as additives in AD to relieve the accumulation of VFAs and increase methane yield [141,143]. As shown in Figure 5, the main role of TEs is promoting the conversion of VFAs, $H_2$, and $CO_2$ to methane. In previous studies, the optimal demand for TEs has been studied and summarized [2,120,133,142,144]. The concentration of TEs required in AD differs significantly and depends on the substrate type, digestion mode (mono- or co-digestion), and operating temperature. Cai et al. [135] reported that the concentration of TEs in livestock manure was usually higher than that in FW and lignocellulosic biomass. Therefore, the AD system of kitchen waste and lignocellulosic biomass is more prone to a lack of TEs than the AD of livestock manure [2,145]. The substrates with low concentrations of TEs can be co-digested with livestock manure to prevent the deficiency in TEs. Operating temperature is also an important factor affecting the demand for TEs. Takashima et al. [146] reported that the required concentrations of TEs in thermophilic glucose digestion were significantly higher than those required for mesophilic digestion, which implied an increase in TE requirements at thermophilic temperatures.

### The Bioavailability of TE and the Possibility to Regulate

Based on the definition, bioavailability is the degree to which elements are available for interaction in biological systems [147,148]. The concept of TE bioavailability in AD has been poorly understood in the past [133]. Although TEs are available in the AD systems, the stimulating effects of adding TEs still can be obtained, which indicates that part of those TEs may be present in non-bioavailable form [149]. Therefore, it is vital to understand how TE speciation affects their bioavailability to reduce the amount of TEs supplemented and maximize methanogenic activity. The pH and anions ($S^{2-}$, $CO_3^{2-}$, and $HPO_4^{2-}$) have the capacity to form or dissolve precipitates of TEs. Therefore, they are the two most important factors affecting the bioavailability of TEs [135,150]. For example,

Marcato et al. [147]. reported that Zn gradually dissolved from the slurry into solution when the pH decreased below about 6.0, and dissolved Zn accounted for 40% of the total Zn at pH 2.7. When sulfur-rich materials were used as AD substrates, a large amount of $S^{2-}$ would be produced. The solubility constants for complexes between most TEs and $S^{2-}$ are low and the formation of those complexes is likely to reduce the bioavailability of TEs [151]. Similarly, the concentration of $CO_3^{2-}$ and $HPO_4^{2-}$ can also affect the bioavailability of TEs by forming complexes with TEs.

To regulate the bioavailability of TEs, chelating agents (Nitrilotriacetic acid (NTA), Ethylenediamine-N,N′-disuccinic acid ([S, S]-EDDS), Ethylenediaminetetraacetic acid (EDTA), soluble microbial products (SMPs), extracellular polymeric substances (EPSs), and Yeast extract) have often been used in AD systems [133]. Thanh et al. [152] reported that the Fe-EDDS complex was effective in controlling the change in sulfide levels in the submerged anaerobic membrane bioreactor and then increased methane yields by 9.46%. Zhang et al. [153] found that the addition of EDDS improved the bioavailability of TEs for microbial uptake and then amplified the stimulatory effects of TEs on the AD of FW. The optimum supplementation dose of TEs could be reduced by 50% when EDDS was added at a concentration of 20 mg/L. Similarly, Vintiloiu et al. [154] showed that if TEs werecomplexed with EDTA before their supplementation, the concentration could be reduced by 75% compared to the dose required of non-complexed TEs. Cai et al. [142] explored the effect of EDTA and Fe on the acidogenic phase (AP) and methanogenic phase (MP) and found that EDTA could effectively improve the bioavailability of Fe by increasing the ratio of water-soluble and exchangeable fractions in both the AP and the MP (as shown in Figure 8). EDTA improves the bioavailability of TEs based on the chelation effect. Other ligands such as EDDS, NTA, SMPs, and EPS also have a similar effect on TE bioavailability due to chelation, binding, or adsorption [142]. Yeast extract, SMPs, and EPS are organic ligands produced by microorganisms and they are difficult to obtain in large quantities at low cost. Therefore, theseorganic ligands are not suitable for application in practice.

Although the supplementation of TEs plays a positive role in AD systems, excessive addition will lead to toxic effects and the influence of ligands on the environment should also be taken seriously [155,156].

#### 2.5.4. Biochar

Biochar is produced from biomass pyrolysis under anaerobic conditions and high temperature [157]. As shown in Figure 9, several inherent characteristics of biochar, such as richness in functional groups, a large surface area, porosity, and conductivity, are beneficial for enhancing AD performance. Because of these characteristics, biochar has the functions of adsorbing toxic substances, enhancing the DIET process, and immobilizing microorganisms. Different sorption mechanisms exist because of the co-existing non-carbonized and carbonized fractions in the biochar surface. The adsorption process is facilitated by hydrogen bonding, ion exchange, electrostatic attraction, and hydrophobic effect [158]. Figure 9a,b show the adsorption mechanism of inorganic and organic pollutants, respectively. As Figure 9a shows, there are four adsorption routes for inorganic pollutants, including ion exchange, anionic metal attraction, cationic metal attraction, and precipitation. The existence of charged functional groups and metal ions provides the possibility of anionic and cationic metal attraction and ion exchange, respectively. As Figure 9b shows, electrostatic, polar organic, and non-polar organic attractions are main adsorption pathways for organic pollutant adsorption. Electrostatic attraction is possible because of potential metal exchange with alkali metals (such as $K^+$ and $Na^+$), which are available on the biochar surface [158]. The biochar surface is normally negatively charged because of the dissociation of oxygen-containing functional groups. This enables the electrostatic attraction of positively charged organic pollutants [158–160]. However, biochar becomes weakly polar due to the loss of the oxygen- and hydrogen-containing functional groups when the pyrolysis temperature is higher than 450 °C [160]. This affects the adsorption

effect of the biochar on polar organic pollutants. Furthermore, hydrophobic sites that existed on the biochar surface can attract non-polar organic pollutants.

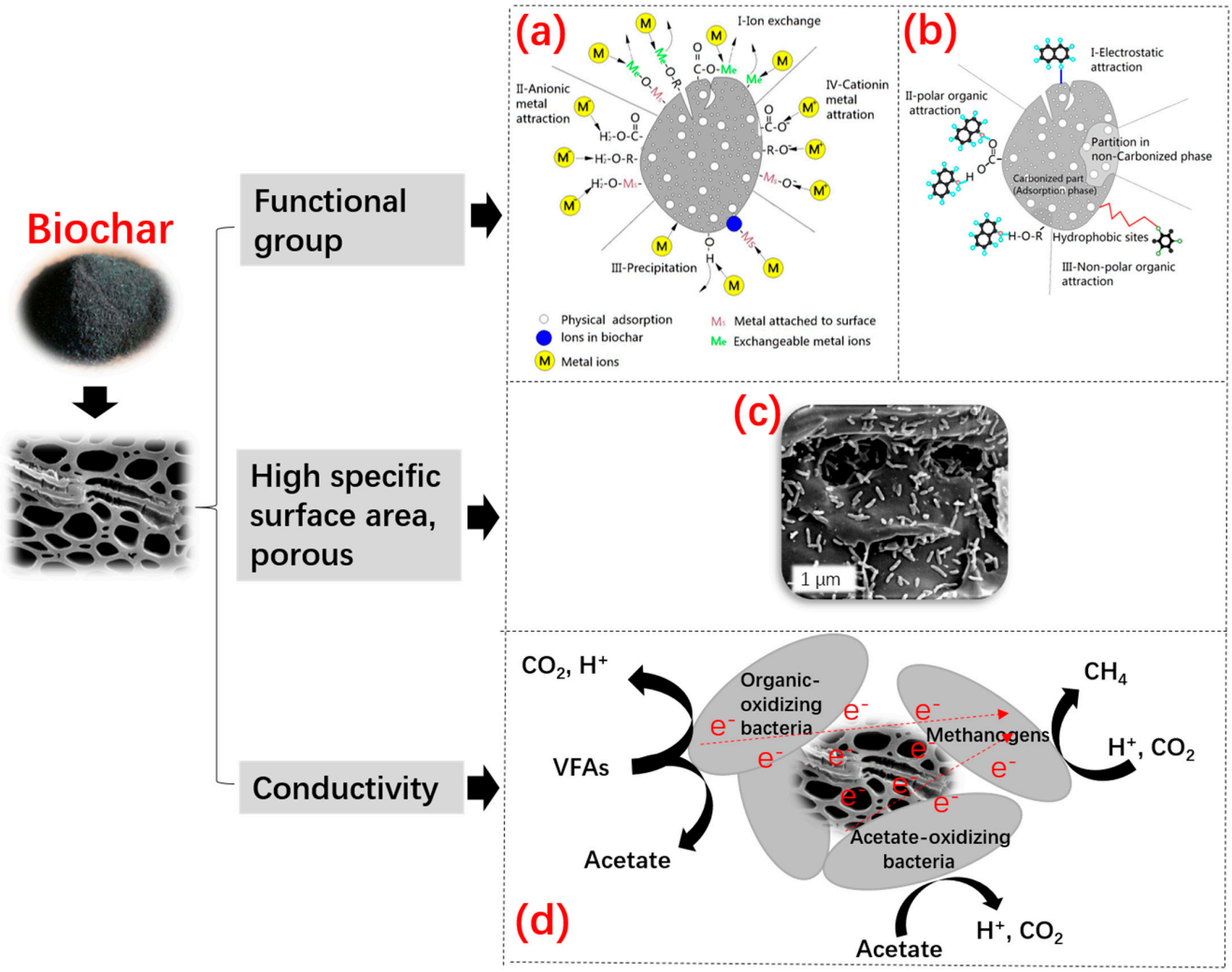


**Figure 9.** The mechanism of biochar in anaerobic digestion considering its three basic characteristics for (**a**,**b**) adsorption mechanism of inorganic pollutant and organic pollutant on biochar surface, (**c**) the function of biochar on immobilization of microorganisms, and (**d**) biochar-mediated direct interspecies electron transfer (DIET) (adapted from Ahmad et al. [160] and Baek et al. [161]).

In recently published work, it is suggested that biochar could work as an electron carrier to favor DIET, thereby accelerating the yield of methane [162]. The mechanism of DIET is shown in Figures 6 and 9d. The ammonia inhibition can be relieved by its adsorption, which is augmented by the large biochar surface area and functional groups [158,163]. In addition, as Figure 9c shows, the abundant pores in biochar provide a microenvironment to immobilize microbes, resulting in an improvement in AD performance [163,164]. The alkaline nature of biochar facilitates the decrease in $CO_2$ with $H_2S$ level, which will increase the methane yield [165]. Wang et al. [162] reported that biochar addition could reduce the concentration of VFAs, which might be due to the buffering capacity of biochar. Furthermore, the addition of 20 g/L biochar to a wastewater AD system could reduce the environmental risk of antibiotic resistance genes by adsorbing antibiotics [157].

Although biochar has a good effect on AD, its application in large-scale biogas projects remains limited because it is difficult to reuse. This results in a high cost.

## 3. Conclusions and Recommendations for Future Research

### *3.1. Conclusions*

Given the advantages and disadvantages of pre-treatment, combining biological pre-treatment with physical pre-treatment or chemical pre-treatment is the most promising pre-treatment model because of the complementary advantage. Co-digestion and recirculation have multiple positive effects on balancing water, substance, and microorganism. With the diversification of digestion feedstock, co-digestion should be applied first when the type and amount of raw materials are sufficient. Microaerobic technology can achieve a delicate balance between bacteria and methanogens and its advantage is obvious, especially when the digestion substrate is difficult to hydrolyze (mainly lignocellulosic biomass). Additive technology can be a complementary strategy to other strategies. Cheap, reusable, and effective additives are still scarce, which limits their application in large-scale biogas projects. To obtain sustainable development, the potential impact of those strategies on the environment and agriculture must be considered.

### *3.2. Recommendations for Future Research*

A large amount of literature has reported the effects of different strategies on the AD system, and many excellent results have been obtained. Nevertheless, it still needs further research to accelerate the application of these strategies in large-scale project.

Both pre-treatment and additive strategies involve additional substance or energy; therefore, reasonable models should be developed considering practicality, economy, and sustainability of the entire biogas industry chain. Some pre-treatment and additive strategies remain at the laboratory level, such as microbial or enzyme additives and microbial pre-treatment. The initial investment and additional operating costs cannot be ignored when these strategies are considered for application in large-scale projects. Therefore, larger-scale experiments should be carried out to evaluate their economy and practicality in further research. The characteristics and functions of biochar obtained under different conditions, such as hydrolysis temperatures and raw materials, should be further explored to achieve the ideal effect considering different purposes. The bioavailability of TEs has not been taken seriously, and the change in TE bioavailability under various digestion conditions still needs to be further explored. The value of adding biochar and TEs for the utilization of biogas digestate also requires further evaluation.

Co-digestion and recirculation are very practical strategies. The most important point of the two strategies is promoting the balance of substances in the environment of the reactor. Both strategies should adapt to local conditions and consider the match of substrates.

The substrates of AD are gradually diversifying. Besides traditional digestion substrates (mainly livestock manure), lignocellulosic biomass (crop straw, microalgae, and energy grass) has also attracted broad interest from researchers. The hydrolysis stage of lignocellulosic biomass is a speed-limited step in AD. Therefore, microaerobic technology should be applied first when digestion substrates are difficult to hydrolyze. To obtain optimal condition for hydrolytic fermentation bacteria and methanogenic archaea, the two-stage digestion mode has obvious advantages and should be studied further, especially for microaerobic control under different operating conditions, including different substrates, reactor types, and temperatures.

**Author Contributions:** Conceptualization, X.L. and Z.W.; methodology, Y.H. and Y.W.; software, S.W. and Z.Z.; validation, Y.C. and S.W. (Shilei Wang); formal analysis, Y.C. and S.W. (Songtao Wang); investigation, Z.W., Y.H. and Y.W.; resources, S.W. (Songtao Wang); data curation, Z.Z.; writing—original draft preparation, X.L. and Y.H.; writing—review and editing, Z.W. and Y.C.; visualization, J.X.; supervision, H.Y.; project administration, S.W. (Shilei Wang); funding acquisition, H.Y. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was supported by the National Natural Science Foundation of China (52200178; 32300092), National Engineering Research Center of Solid-state Brewing (GFGS-2023000501; 2023HX02), Henan Province Science and Technology Research (222102310142), the Key Research Project of the Higher Education Institutions of Henan Province, China (22A530010), and the Key Program for Collaborative and Innovation of Nanyang, Henan Province, China (21XTCX21001).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The authors confirm that the data supporting the findings of this study are available within the article. Interested parties can request data from the corresponding author.

**Conflicts of Interest:** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Abbreviations

WRF, white-rot fungi; BRF, brown-rot fungi; SRF, soft-rot fungi; AC, activated carbon; AD, anaerobic digestion; C/N, carbon-to-nitrogen; COD, chemical oxygen demand; CSTR, continuously stirred tank reactor; DIET, direct interspecies electron transfer; EDTA, ethylenediaminetetraacetic acid, EPS extracellular polymeric substances; IHT, interspecies hydrogen transfer; ISR, inoculum substrate ratio; OLR, organic loading rate; ORP, oxidation reduction potential; sCOD, soluble chemical oxygen demand; SMPs, soluble microbial products; SOB, sulfide/sulfur-oxidizing bacteria; SRB, sulfate-reducing bacteria; [S, S]-EDDS, ethylenediamine-N,N′-disuccinic acid; TEs, trace elements; TS, total solids; UASB, up-flow anaerobic sludge bed reactor; VFA, volatile fatty acid; VS, volatile solids; FW, food waste; WAS, waste-activated sludge; WWTP, wastewater treatment plants; AOEs, anti-oxidative enzymes; OFMSW, Organic Fraction of Municipal Solid Waste; MSW, municipal solid waste; SAOB, syntrophic acetate oxidizing bacteria; CA, carbonic anhydrase; Ech/Eha/Ehb/Mbh, energy-converting hydrogenase; Cdh, carbon monoxide dehydrogenase/acetyl-CoA synthase; Fd, ferredoxin; Frh, $F_{420}$-reducing hydrogenase; Fmd/Fwd, Mo/W formylmethanofuran dehydrogenase; Hdr, heterodisulfide reductase; Hmd, Ni-free Fe hydrogenase; Mta, methanol-coenzyme M methyltransferase; Mcr, methyl coenzyme M reductase; Vh(o/t)/Mvh, Ni-Fe hydrogenase; Mtr, $CH_3$-$H_4$M(S)PT-coenzyme M methyltransferase.

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