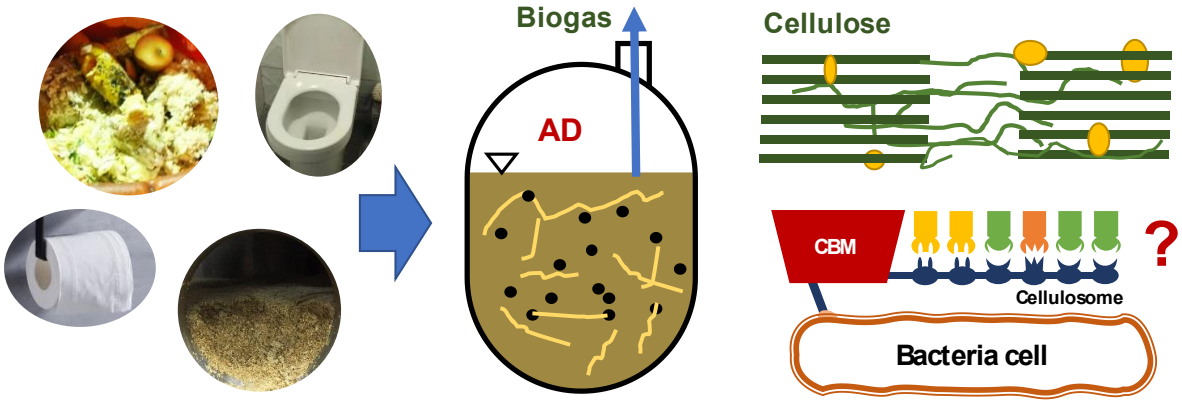
Graphical Abstract



Highlights

- Genomic information of cellulosome-embedded prokaryotes in ADs were commented;
- Substrate-related factors of lignocellulosic biomass in ADs were introduced;
- Cellulosome related subunits were overviewed with corresponding genes;
- Cellulose separation methods to enhance cellulosome cellulolysis were studied;
- Benefits and mechanisms of micro-aeration and enzyme additions were reported.

Genomic Driven Factors Enhance Biocatalyst-related Cellulolysis Potential in Anaerobic Digestion

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Abstract

Anaerobic digestion (AD) is a promising technology to recover bioenergy from biodegradable biomass, including cellulosic wastes. Through a few fractionation/separation techniques, cellulose has demonstrated its potential in AD, but the performance of the process is rather substrate-specific as cellulolysis bacteria are sensitive to the enzyme-substrate interactions. Cellulosome is a self-assembled enzyme complex with many functionalized modules in the bacteria, which has been gradually studied, however the genomic fingerprints of the culture-specific cellulosome in AD are relatively unclear especially under processing conditions. To clarify the key factors affecting the cellulosome induced cellulolysis, this review summarized the most recent publications of AD regarding the fates of cellulose, sources and functional genes of cellulosome, and omics methods for functional analyses. Different processes for organic treatment including applying food grinds in sewer, biomass valorization, cellulose fractionation, micro-aeration, and enzymatic hydrolysis enhanced fermentation, were highlighted to support the sustainable development of AD technology.

Keyword: Anaerobic digestion, cellulolysis, biomass, cellulosome, cellulosome-embedded prokaryotes

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1. Cellulosic Substrates in AD

Climate change has started to demonstrate its dramatic impacts on human society and global environment. Anaerobic digestion (AD) among the biochemical conversion practices to reduce greenhouse gases emissions (*e.g.*, non-point sources CH_4) has become a critical trend toward decarbonized economy (Meckling et al., 2017). AD is a widely used technology for waste management, energy recovery, and biorefinery for various organic wastes and biomass. Lignocellulose has significant potential for the production of biogas and biochemicals due to its high energy value building block components in the substrate, *e.g.*, cellulose. Cellulose is an abundant and valuable resources in food waste (FW); agriculture waste (AW); and primary sludge (PS) from the municipal wastewater treatment system (Li et al., 2021; Zhuang et al., 2020). The predicted global production of FW will reach 2.5 billion tons in fresh weight by 2025 (Liu et al., 2020), of which its compositions differ significantly among regions and sources (*e.g.*, family, catering, and food industry) (Wang et al., 2020a), and carbohydrates contributed from cereal, staple food, and lignocellulose account for approximately 35.5-69.0% of FW by weight (Zou et al., 2020). AW is the largest source of cellulosic biomass, of which its annual production is over 3 billion tons in fresh weight; the global AW is expected to increase by 60% higher than the current value by 2050 (Tripathi et al., 2019). Finally, cellulose in municipal wastewater accounts for 25-30% of total solids before being treated in the sewage treatment works (Ahmed et al., 2019). The cellulosic biomass contributed considerably to the total organic loads, and can increase energy consumption (through aeration) of the overall system if not separated (as PS) from the influent (Ahmed et al., 2019).

With the potential to produce easily digestible glucose, cellulose-rich solids have demonstrated its value in AD (Crutchik et al., 2018). However, the hydrolysis rate of cellulose has often become the rate limiting factor of the AD. The fundamental genomic approach to investigate the impacts is also limited. Based on the recent publications, this paper aims to

provide an overview of the publication within the past five years to expend the technical and economic feasibilities of AD. While the substrate-related factors regarding to this approach have been reviewed elsewhere (Wang et al., 2019), this study will focus more on the bio-related factors such as cellulose and cellulosome characterization; cellulose degrading microbes/genes; genomic foundation of cellulose hydrolysis; and genomic driven AD applications toward more effective and efficient operation.

2. Hydrolysis of Lignocellulosic Biomass in AD

2.1 Characteristics lignocellulosic biomass and their hydrolysis in AD

The average biogas production of different lignocellulosic and cellulosic substrates with different chemical compositions, treatment processes, and biogas productivities have been demonstrated in **Table 1**. Although due to the high quantity in the substrate the hydrolysis of the cellulosic component has served as the main concern of the AD, the rates of cellulolysis can be severely interfered by the other building block components in the plant cell wall micro-structure. Lignocellulosic biomass is composed of cellulose, hemicellulose, lignin, and small amounts of other compounds (such as protein and pectin). Pabón-Pereira et al. (2020; 2012) indicated that cellulosic biomass demonstrated quite different methanogenesis potential, *i.e.*, 0.14 to 0.37 L CH_4·g VS^{-1} under biochemical methane potential (BMP) tests. The conceptual structure of the plant cell wall, with the spatial arrangement and related hydrolyzing enzyme for each building block chemicals has been demonstrated in **Figure 3 and 4**.

Cellulose is the key component of plant cell walls, which exists in fiber forms embedded in a complex matrix of other building block chemicals . The cellulosic long-chain is composed of hundreds to thousands D-glucopyranose units connected with β-1,4-glycosidic linkages . The micro-fibers are attached in line through hydrogen bonds, and the various forms and quantities of the bonding resulted in the unique crystalline and amorphous domains of the biopolymer

(Pensupa et al., 2017). As the arrangement patterns of the cellulose differ significantly from the species and parts of the plants, different crystallinity, hydrophilicity, and accessibility of enzyme to cellulose (Liu et al., 2016) are formed and resulted in different biodegradabilities in the AD. Popovic et al. (2019) reported that the hydrolysis of amorphous cellulose to aqueous glucose has negative change in Gibbs free energy (at 0 to 100°C), indicating that the reaction is thermodynamically favorable, *i.e.*, the chemical reaction could happen without extra energy input. However, the change in Gibbs free energy from crystal cellulose to aqueous glucose could vary from -6.6 to 3.0 kJ/mol depending on the types of crystal forms and reaction temperature, suggesting increased difficulties in hydrolysis of crystalline cellulose. Although the reaction is thermodynamic favorable (Gibbs free energy is negative) the reaction rate was still affected by the physical structure of the substrate and the catalyst factor in the system. During hydrolysis, the crystal cellulose is peeled off layer by layer, and can be accelerated by changing the ultrastructure from cellulose I to cellulose II or cellulose III (Horikawa et al., 2013; Wada et al., 2010).

Hemicelluloses are polysaccharides attached amorphously with the cellulosic fibers. Xylan (a five carbon, C5 sugar) and mannan (a C6 sugar) are the most abundant hemicellulose in hardwood/herbal and softwood, respectively. Different enzyme systems are responsible to the digestion of hemicelluloses and cellulose. Previous BMP tests showed that the hydrolysis rate of hemicellulose was faster than that of cellulose and pure cellulose showed higher methane potential than hemicellulose (Li et al., 2018a). Thermodynamically, hemicellulose hydrolysis is an endotherm and hence extra energy or additional product is needed in the reaction (Tizazu & Moholkar, 2018). Some hemicellulose could also inhibit cellulolysis (Chen et al., 2020). However, pentose and hexose are important carbon and energy sources to be completely utilized in an AD (Chen et al., 2019).

Lignin is a three-dimensional biopolymer composed of three monolignols (Islam et al., 2020). It fills up the space among the cellulose, hemicellulose, and pectin, and forms into a crosslinked network structure with various types of linkages, *i.e.*, covalent, hydrogen bonds, and van der Waals forces (Speight, 2020). The massive aromatic nucleus in lignin matrix makes it extremely recalcitrant against chemical and biological degradation (Khan & Ahring, 2021). The hydrolysis of lignocellulosic structure is related to many substrate-related interactions between enzymes and lignin. The biodegradability of lignocellulosic biomass in methanogenesis AD could vary from 0.1% to 76.4%, and the lag phase could range from 0 to 27.4 days (Li et al., 2018a; Ma et al., 2019). Lignin may absorb and/or deactivate the enzymes (Li et al., 2018b). Complete decomposition of lignin is a slow process that could last for centuries (Zabel & Morrell, 2020). Lignin depolymerization in AD are mainly carried out by *Spirochaeta* and *Chitinophaga* (Pandit et al., 2016). The biodegradation process starts from breakages of side chains, functional groups, and/or aromatic nucleus, and complete degradation of lignin (breakage of the aromatic rings) have never been observed anaerobically. With limited energy yield lignin depolymerization often couples with the metabolisms of easily degradable substrates. Some studies found that sulfate reducing bacteria of *Desulfomicrobium*, *Desulfomicrobium*, and *Desulfobulbus* genus could decompose lignin by using sulfate as election acceptor (Wang et al., 2013; Yamashita et al., 2011).

2.2 Enzymatic system for cellulolysis and glycoside hydrolase families

Cellulose degradation in bioconversion process has been mainly carried out by cellulase produced from anaerobic or aerobic microorganisms, such as bacteria and fungi. Cellulases include a few types of enzymes which can be divided into three categories according to the catalytic functions, *i.e.*, exoglucanases (1,4-D-glucan glucanohydrolases and 1,4-Dglucan cellobiohydrolases), endoglucanases (1,4-D-glucan-4-glucanohydrolases), and β-glucosidases

(β-glucoside glucohydrolases) (Bhati & Sharma, 2020; Lynd et al., 2002; Schwarz, 2001). Complete hydrolysis of cellulose relies on the synergetic reactions of the enzymes, *i.e.*, exoglucanases hydrolyze the crystalline cellulose from either the reducing end or non-reducing end of the cellulosic chain (Teeri, 1997; Zhang et al., 2018); endoglucanases randomly break the glycosidic linkages in the amorphous cellulose, releasing large numbers of short-chain cellulose and available free ends for exoglucanases (Brunecky et al., 2017); and β-glucosidases break the glycosidic linkages of di-glucosides, alkyl/aryl glucosides, cyanogenic glucosides, and oligoglucosides for glucose (Thapa et al., 2020). In this process, the main rate-limiting step is the hydrolysis of crystalline cellulose (by exoglucanases), while the number of exoglucanases in most AD system is too low to support the cellulolysis at high efficiency.

In addition to cellulases, hemicellulases also participate in lignocellulose hydrolysis. The related enzymes can be divided into three major types according to the target positions of enzymes on hemicellulose chains. Exohemicellulases, such as β-D-xylosidase and β-D-mannosidase, can break the glycosidic bonds at free ends of the hemicellulose chains; endohemicellulases, such as β-D-xylanase, β-D-mannanase, can break glycosidic bonds at random positions or specific positions; and additional enzymes such as acetyl xylan esterase are required to remove side-chain hemicellulose (Franco et al., 2004; Razeq et al., 2018).

Since both of cellulases and hemicellulases are both responsible catalyzing lignocellulose hydrolysis and share some common features, the enzymes were grouped into Carbohydrate-Active Enzymes (CAZymes) database with some other enzymes with hydrolytic activities for carbohydrates such as starch and chitin. They have been further classified into 169 glycosyl hydrolases (GHs) and 18 carbohydrate esterases (CEs) families (Lombard et al., 2014) based on the nucleic acid or amino acid sequence enzymes' catalytic modules. Some GHs are multifunctional, having high catalytic activity to several kinds of cellulosic substrates. For example, the endoglucanase EG5C-$_1$ from *Bacillus subtilis* BS-5 can hydrolyze both

carboxymethyl cellulose (CMC) and *p*-nitrophenyl-β-D-cellobioside at hydrolytic activities of 4,170 and 2,550 U/μmol, respectively (Zhan et al., 2018). CbXyn10C and Bgxg1 expressed from *Lactobacillus plantarum* also showed hydrolytic activities on xylan, avicel, and CMC (Guo et al., 2019). A total of 39 cellulases and hemicellulases can be related to *Proteiniphilum saccharofermentans* M3/6 for the degradation of arabinan, xylan, mannan, and β-glucans (Tomazetto et al., 2018). The example illustrates the complexity of cellulolysis enzyme system in AD, and the necessity of genomic-based study for understanding such system in the microbes.

2.3 Cellulosomal enzymatic components in AD

AD is an engineered environment with limited electron acceptors, in which the microbes cannot completely oxidize the organic matters for energy. To survive in the critical condition, cellulosomes was evolved in anaerobes for effective uptake of the hydrolysate after cellulolysis. Cellulosome is a function-specific unit composed of catalytic proteins and non-catalytic proteins (Wang et al., 2019). Cellulosomes have been identified on the surface of several anaerobes, *i.e.*, most of which are bacteria and some fungi. In different species, cellulosomes present with specific molecular arrangement with varying degrees of complexity (Artzi et al., 2017). An example of cellulosomes formed by *Clostridium clariflavum* and their assembly structure proposed by Artzi et al. (2015) has been illustrated in **Figure 4**. The non-catalytic proteins are referred to scaffoldins (Ding & Bayer, 2020; Ichikawa et al., 2014), which are large proteins serving as foundation of the cellulosomal complex. The functional domains on scaffoldins are carbohydrate-binding modules (CBMs) and cohesions (Leis et al., 2017), of which CBMs are the protein domains that can tightly bind to the lignocellulose fibers, and cohesions contains GHs and CEs as major catalytic proteins for hydrolysis. The scaffoldins can contain up to hundreds of cohesions regions for various specific types of substrates and thus the fixed catalytic subunits can continuously conduct hydrolysis.

Cellulosomes can consist of more than one scaffoldins binding to each other by cohesin-dockerin. For example, the cellulosome formed by *Clostridium thermocellum* can have at least 8 types of scaffoldins (Hong et al., 2014). ScaA is the scaffoldin with CBM, it has type I cohesin and type II dockerin **(Figure 4)** (Bule et al., 2017). It can anchor on the cell surface by connecting to ScaF or ScaD with type II dockerin-cohesin connection (Bule et al., 2018). ScaE is the cell-free scaffoldin, which can create a large cell-free cellulosome by connecting its type II cohesins with type II dockerins of ScaH/L and ScaA. In addition, free uncomplexed enzymes and cell-bound cellulosome complexes were produced simultaneously. The structure, compositions and corresponding substrates of cellulosomes are regulated and varied by the nature of the growth substrates and the host microbes. Artzi et al. (2015) demonstrated that the cellulosome proteins produced from *Clostridium clariflavum* were regulated by the type of substrates including cellobiose, microcrystalline cellulose, and acid-pretreated switchgrass. The outer membrane receptor protein and sensor may have potential function of carbohydrate-sensing and CAZyme gene regulation (Kougias et al., 2018). A bioinformatics study for screening the cellulosomal modules found that beyond the cellulolysis, 23.6% of the dockerin-containing proteins were related to alternative biological processes such as protein degradation or putative lysozyme activity in the bovine rumen microbiome (Bensoussan et al., 2017). The dockerin-cohesin systems in a wide variety of different biological and cellular processes implied its possible evolutionary directions and potential application for cellulosomes complex. The multi-enzymatic systems could serve as a powerful tool that assists in novel biotechnologies.

In summary, cellulosomes can improve the synergistic efficiency among hydrolytic subunits; prevent a single low-efficiency enzyme from adsorbing cellulose and competition between different enzymes due to the same absorption site; and have a continuous cellulolysis

on long-chain lignocellulose. Its modular nature makes it highly efficient in cellulolysis and feasible to be artificially manufactured through biotechnology for large-scale applications.

3. Genetic Fingerprint of Cellulolysis

3.1 Cellulolysis and methanogenesis of anaerobic microbes

Hydrolytic microbes are key participants of AD. The most predominant bacterial hydrolyzers belong to several phyla, *i.e.*, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Chloroflexi*. The abundances of those hydrolytic bacteria are related to the process conditions such as organic loading, solids retention time (SRT), temperature, and types of substrates (Chen et al., 2016; Nguyen et al., 2019b). Meanwhile, it is difficult to estimate the taxonomic borderline of hydrolyzers, acidogens and acetogens. For example, more than hydrolysis of cellulose, some hydrolyzers such as *Clostridium cellulolyticum* can further oxidize the glucose and produce acetate H_2 and CO_2 (Jia et al., 2018). To understand the niche of those multifunction players may help to design the system with better robustness and stability.

Anaerobic hydrolytic fungi belong to phylum *Neocallimastigomycota*. Compared to the hydrolytic bacteria, hydrolytic fungi have rhizoid system which provides ability of mechanical disruption of the lignocellulose tissues through invasive growth (Dollhofer et al., 2015). *Neocallimastigomycota* are also the only fungi forming cellulosomes, of which the genes encoding cellulosomal enzyme may be obtained from hydrolytic bacteria through horizontal gene transfer (Murphy et al., 2019). Although with some imperfection such as low abundance in system and easy to be wash out, anaerobic fungi have great potential in engineering application. Fungal bioaugmentation for enhancing cellulolysis in AD increased the methane yield by 15-33% for treating cereal crops/straws and cow manure (Akyol et al., 2019). Using immobilized fungi in AD increased the methane yields of batch and continuous flow system by 50% (from around 250 ml/g VSS increased to around 375 ml/g VSS) and 13-31% (from

around 600 (Day 0 to Day 10) and 4,200 ml/d (Day 70 to Day 90) increased to around 680 and 5300 ml/d, respectively), respectively (Guo et al., 2021).

3.2 Genome-wide analysis of cellulosome system and cellulosomics

Fundamental cellulosome study has been conducted after a comprehensive and time-consuming procedure. The most accurate way to study the enzyme system is by cultivating specific microbes for pure enzyme analysis. However, only a small fraction of anaerobic bacteria can be grown in the laboratory. The current understanding of cellulosomes were thereafter limited only within a finite number of bacteria strains. With the rapid development of novel sequencing technology, nevertheless, new perspectives for deciphering the complex and ingenious cellulolysis system are constructed. The gene-centric cellulosome systematic study approach can now screen and discover unknown CAZymes, dockerin-cohesin systems, signal/regulation system, and other cellulosome-relative active substance (Smith et al., 2017). Metagenome, whole-genome, and transcriptome sequencing approaches have been widely used to investigate the cellulolytic microbes and cellulosome relative proteins.

Metagenomics and transcriptomics study provide powerful tools in cellulolysis research. AD is a mix-culture microbial system and metagenome information can provide immediate insights of the major participants and the potential metabolism pathway of those microorganisms. Transcriptomics analysis focuses on mRNA analysis of microbes, which is an index of gene expression (Chen et al., 2017). It is the tool to compare different gene expressions among distinct cell populations, or the gene expressions of a cell population response to different environmental conditions. It is also a useful method to identify the metabolism pathways, the cell signaling system, and the gene regulation models in biological systems. For cellulosome study, transcriptomics has been applied to observe the changes in gene expression of an engineered *C. thermocellum* strain as a response to change carbon

sources from xylose to cellobiose (Tafur Rangel et al., 2020). Kougias et al. (2018) conducted a shotgun metagenomics analysis on the AD treating pig manure and meadow grass. A total of 151 genomes were recovered by binning process, while 80 of which were completely new and cannot be found in existing database. Uncultured members of *Bacteroidetes* and *Firmicutes* were indicated to play important roles for polysaccharide degradation, among which the *Firmicutes* species, the number and functional roles of CBMs of cellulosomes were different. Wilkens et al. (2017) identified 19,335 CAZymes genes from four full-scale mesophilic AD treating sludge in municipal wastewater treatment plants by metagenomic sequencing, A total of 10,374 genes belonged to 133 GH families and 2,064 belonged to 17 CE families. However, only 54% of the CAZymes genes were annotated with its function and the others can only be classified into one enzyme family. The results indicated that our knowledge of the CAZy subfamilies are limited, while more comprehensive database is to be developed especially for the AD treating lignocellulosic biomass.

Potential RsgI-like carbohydrate sensing proteins were identified with a group of upregulated xylose hydrolytic enzymes. In addition, the candidate ABC transporters for xylose transport and assimilation were also revealed in this transcriptomics study. By transcriptomic analysis, Raman et al. (2011) observed in later stages of cellulose fermentation with *C. thermocellum* ATCC 27405 that several cellulosomal catalytic subunits (*i.e.*, mannanases, xyloglucanase, pectate lyase, etc., belongs to GH families) were significant upregulated for degrading non-cellulolytic substrates. Increasing expression of non-cellulolytic enzymes in the free cellulosomes during the latter stage of growing might be applicable for exposing the cellulose from untapped resources. The protease inhibiting components were also expressed progressively, which might protect the cellulosome from protease driven degradation.

At present, the sequencing technologies have been updated rapidly. The third-generation sequencing platforms such as PacBio Sequel and Oxford Nanopore are able to generate more

accurate sequence reads with enhanced information like DNA methylation signals. The decreasing sequencing costs would also allow researchers to pay more attention on cellulosomics. The extension of the genetic information database coupling with more analytical tools will definitely help to understand and utilize the cellulosome system in AD.

4. Adjustment of Reaction Kinetics in Considering Cellulolysis

4.1 Micro-aeration for enhancing biodegradability of lignocellulosic biomass in AD

Microaerobic digestion has gained increased attention in recent years. This process refers to the addition of a small well-controlled amount of air or oxygen into the AD. Although oxygen has long been considered as an inhibitor to obligate anaerobes, many studies have demonstrated improved digestibility and stability of AD processes under microaerobic conditions (Botheju & Bakke, 2011; Nguyen & Khanal, 2018). The merits of this operational approach include improved methane production, enhanced enzymatic hydrolysis, release of VFA accumulation, and mitigation of hydrogen sulfide, which facilitate the overall performances of the AD systems (Charles et al., 2009; Fdz-Polanco et al., 2009; Johansen & Bakke, 2006; Lim & Wang, 2013; Nguyen et al., 2019a). Specifically, an oxidation-reduction potential (ORP)-based oxygenation approach, where ORP level varies linearly with the logarithmic dissolved oxygen (DO) concentration, allows for a more precise control of oxygen/air injection far below 0.1 mg/L into a digester (Khanal & Huang, 2003). Given these advantages, several research groups have applied such microaerobic strategies for digestion of lignocellulosic biomass such as corn/wheat/rice straw, napier grass, cow manure, etc. (Fu et al., 2016a; Fu et al., 2016b; Nguyen et al., 2019a; Tsapekos et al., 2017; Wang et al., 2020c). Fu et al. (2016a; 2016b) reported simultaneous improvements in methane yield and VS removal efficiency in batch thermophilic digestions of corn straw either by air pretreatment or by direct air injection. While pretreatment obtained 16.24% higher in methane production, direct

328 treatment achieved 28.45% higher in methane production and 29.43% higher in VS removal,
329 respectively. Nguyen et al. (2019a) developed an ORP-based intermittent microaerobic AD
330 system to treat napier grass and achieved a stable methane yield of 80.8±9.6 N CH_4 mL/VS g
331 when picomolar oxygen was applied by setting the ORP + 25 mV above the strict anaerobic
332 baseline every other day.

333 The underlying microbial mechanism is that the supply of insufficient oxygen creates a
334 unique ecological niche suitable for both anaerobes and aerobes, promoting a new balance
335 between hydrolytic bacteria, fermentative bacteria, methanogens, as well as facultative bacteria
336 (Nguyen & Khanal, 2018; Nguyen et al., 2019a; Wu et al., 2021). The facultative oxygen
337 scavenger may rapidly consume the dissolved oxygen in system, shielding the surrounding
338 anaerobic cells from direct oxygen attack. Moreover, facultative anaerobes perform
339 thermodynamic advantages over strictly anaerobes, making them grow faster and
340 predominance under microaerobic conditions (Nguyen et al., 2019a; Rittmann & McCarty,
341 2001). Wu et al. (2021) unveiled the metabolic pathway of an ORP-based microaerobic
342 digestion of lignocellulose by genome-centric metagenomics. It confirmed that the cellulolytic
343 facultative *Proteiniphilum* strains repeatedly dominated in microaerobic system, which
344 encoded rich extracellular enzymes in lignocellulose hydrolysis, fermentative pathway to
345 formate, acetate or propionate, as well as complete aerobic respiration pathway to CO_2.
346 Meanwhile, the high oxygen affinity bd-type terminal oxidases among oxidative
347 phosphorylation pathway in *Proteiniphilum* strains were assumed to closely associated with
348 such picomolar oxygen conditions. Moreover, many microbial community analyses also
349 reported that microaeration led to higher abundance and activity of hydrolytic bacteria,
350 especially the phylum Firmicutes (Fu et al., 2016a; Lim et al., 2014). In addition, the strictly
351 anaerobes (especially the methanogens), as well as facultative bacteria, both have been widely
352 found to possess various antioxidant enzymes (e.g., superoxide dismutase (SOD), superoxide

353 reductase (SOR), etc.), overcoming the oxidative stress under microaerobic circumstances
354 (Imlay, 2002). Microaeration triggers a synergistic relationship between hydrolytic facultative
355 heterotrophs and anaerobic methanogens, which facilitate the energetic conversion from
356 intermediates to methane in order to maintain an overall stability of AD processes (shown in
357 **Figure 4**, by Nguyen et al. (2019a) and Wu et al. (2021)).

358 In application, microaeration significantly relies on the appropriate oxygen injection into
359 the digester, which refers to aeration type (air or pure oxygen), method (direct inject,
360 electrolytic aeration, etc.), frequency (one time, intermittently, or continuously), and
361 application stage (pre-, post-, or simultaneous treatment) (Girotto et al., 2018; González-
362 González & Cuadros, 2015; Rafieenia et al., 2017). While excess aeration resulted in an
363 overwhelming deterioration to AD process (Xu et al., 2014), inadequate aeration also requires
364 a new microbial balance in system, which influence the system stability (Zhu et al., 2009).
365 Thus, for widely use of microaerobic approach in cellulose digestion, fully understanding of
366 cellulose conversion pathway under different microaeration control process are necessary.

367 The biological process control and oxygen uptake is a crucial parameter for metabolic
368 pathway and system optimization. The oxygen transfer efficiency (OTE) decreases during the
369 aeration process because of changing sludge conditions can be quantified by the α-factor, the
370 ratio of OTE in the process condition to that in clean water (Metcalf et al., 2014). When
371 microaeration is performed as a pretreatment method, the α-factor should be carefully
372 monitored because the excess DO could reduce the methane potential, and insufficient oxygen
373 could potentially fail to produce the beneficial effect of microaeration (Girotto et al., 2018).
374 The intensity of microaeration measured in terms of aeration or oxygen flow rate is the key
375 control parameter determining the effects of microaeration (Nguyen & Khanal, 2018). In the
376 microaeration system, high mixed liquor suspended solids (MLSS) could systematically

decrease the α-factor, and ultra-fine bubble diffusers could suffer from greater oxygen transfer depression (*i.e.*, fouling, viscosity, and bubble coalescence) (Garrido-Baserba et al., 2017).

4.2 Pretreatment to improve biodegradability of lignocellulosic biomass before AD

Integrating pretreatment techniques with AD to improve the hydrolysis efficiency has attracted significant attentions for the valorization of lignocellulosic biomass (Xu et al., 2019). In biorefinery, pretreatment process has been developed mainly to decompose, migrate, and modify the building block chemicals to facilitate the enzyme-substrate interactions (Liu et al., 2016). The key mechanisms applied in pretreatment process design include biomass depolymerization (Li et al., 2020), reduction and form-shifting of the crystalline cellulose in the substrate(Ling et al., 2020), breakage of the lignin obstruction (Sabeeh et al., 2020), removal of inhibitors (Koupaie et al., 2019), creation of more accessible binding sites for hydrolytic enzymes (Kainthola et al., 2019a), and ease the growth of methanogens (Zhen et al., 2017). Pretreatment is a substrate- and product-specific unit operation, for which significant amount of energy, chemicals, and/or other resources (*e.g.*, space) are invested for waste valorization (Zhang et al., 2020). Based on the decomposition principles and process conditions, the modern pretreatment can be classified into three categories, *i.e.*, physical, chemical, and biological processes, which summarized in **Table 2** and discussed in the following sections.

Physical pretreatment aims at increasing the surface area and biomass porosity to increase the accessibility of enzyme (Leu & Zhu, 2013) and/or anaerobic microorganism (when cellulosome is of concern) to the substrate surface (Wang et al., 2019). It can also reduce the viscosity of the slurry substrate for better agitation even under high solid loads (Lu et al., 2020). The techniques involve in physical pretreatment include mechanical, thermal, ultrasonic, and microwave process (Wang et al., 2020b). The mechanical pretreatment such as milling, grinding, and drum rotating are developed to reduce the particle size of substrates and/or

improve the mixing between the treated solids and liquid (Carrere et al., 2016). Kang et al. (2019) reported an increased biodegradability index by 13.5% when the size of *hybrid Pennisetum* biomass decreased from 0.83 mm to 0.25 mm. When the size of wheat straw reduced from 50 mm to 2 mm, the methane yield increased from 285 to 334 mL/g VS (Menardo et al., 2012). The methane yield also increased 53% when the particle size of *Laminariaceae spp.* Biomass was reduced to 76-836 µm (Tedesco et al., 2014). However, previous studies also indicated that, in some occasions, further reduction substrate particle size could result in over production of VFAs which inhibited the biogas production (Izumi et al., 2010). Thermal pretreatment is typically carried out by pressurized vessel, in which the substrate can be swelled under pressure and heat (Banu & Kavitha, 2017; Zhou et al., 2015). Depends on the process condition, thermal pretreatment may induce different chemical reactions such as hemicellulose hydrolysis, hydrogen bonds breakage in crystalline celluloses, and/or changes in lignin phase (Veluchamy & Kalamdhad, 2017). Hydrothermal pretreatment coupled with AD was proven to complete removal of hemicellulose, for which the cellulose-enriched biomass can be used for biomethane production (Kaur et al., 2019). Biomass treated under mild and eco-friendly hydrothermal condition reduced the formation of inhibitors prior to AD (Dasgupta & Chandel, 2019). Passos et al. (2018) reported a 96% increase of methane yield when coffee husk and microalgae were pretreated at 120°C for 60 min. The methane yield was increased by 57.7% (344 mL/ g VS) when the wheat straw was pretreated at 180°C for 1 h (Rajput & Visvanathan, 2018). Microwave treatment is another type of thermal pretreatment of which electromagnetic radiation was applied for substrate decomposition at a wide range of frequency and wavelength (Marin et al., 2010). This pretreatment process can effectively increase the methane yield of AD (Wang et al., 2021), while some negative impacts were reported when it was applied on low lignin or lignocellulose substrate such as food or vegetable/fruit wastes (Atelge et al., 2020). Ultrasonic pretreatment mechanically disrupts the fiber, cell structure and floc matrix through

427 the shear force by the vibration probe (Elliott & Mahmood, 2007). Pilli et al. (2016) applied
428 ultrasonic pretreatment (750 w, 20 kHz) on PS and reported an increase of 17.3% higher
429 methane yield (273 mL/g VS) than the control study. Ormaechea et al. (2018) found that
430 ultrasonic pretreated cattle manure reached to 460 mL/g VS, which was 58.6% higher than the
431 control. Microwave and ultrasonic pretreatment are effective but both require high energy and
432 maintenance fee when large scale application is of concern (Hassan et al., 2018).
433 Chemical pretreatment is a common approach to reduce biomass recalcitrant of
434 lignocellulosic biomass in biorefinery (Islam et al., 2020). Acids, alkaline and oxidants have
435 been applied under various conditions to destroy or fractionate the complex structure and to
436 improve biodegradability of the substrate (Nguyen et al., 2021). Alkaline pretreatment is a
437 widely applied strategy for low recalcitrant biomass which can be conducted at ambient room
438 temperature and pressure with modest energy consumption and without producing harmful by-
439 product for downstream process (Behera et al., 2014; Shah, 2018). Alkaline also has the
440 function of swelling, and solubilization of COD and other macromolecules, such as
441 carbohydrates and protein to increase the digestibility of substrate (Nguyen et al., 2021). You
442 et al. (2019) reported an increase of 38.5% methane yield when conducting the co-digestion of
443 NaOH and CaO pretreated corn stover and swine manure. Zhu et al. (2020) tested the AD
444 performance of wheat straw by using 2.0% alkaline liquor and 1:3 synthetic urine, they
445 obtained increase in the methane yield by 68% and 56%, respectively. Acid pretreatment is
446 another effective method to break down the lignocellulosic structure (van der Waals force,
447 hydrogen bonds and covalent bonds) and for higher AD performance (Mussoline et al., 2013;
448 Wada et al., 2010). Dilute acid (DA) pretreatment assists the higher reaction rates of AD and
449 improves the susceptibility of cellulose to hydrolysis (Amin et al., 2017). In a study conducted
450 by Song et al. (2014), the biodegradation of corn straw was effectively accomplished in all acid
451 pretreatments. Among the studied acids (CH_3COOH, HCl and H_2SO_4), the corn straw

pretreated with 2% H_2SO_4 obtained the highest methane yield of 175.6 mL/g VS ,which was 74.6% higher than that of untreated straw. Syaichurrozi et al. (2019) pretreated *Salvinia molesta* biomass by 4% H_2SO_4 and generated 81.8% higher biogas production with shortened lag phase. Saha et al. (2018) achieved a 10.0% increase in methane yield after 0.2 M acetic acid pretreatment of mixed fruit waste. However, growth inhibitors could be produced during DA pretreatment, such as furfural and hydroxymethylfurfural (HMF) which hinder the AD (Jönsson & Martín, 2016).

In addition to acid and base, oxidants such as hydrogen peroxide (H_2O_2) has been commonly used in pulping and bleaching industries for lignin removal. H_2O_2 can break down the structure of lignocellulose through releasing hydroxyl radicals (*e.g.*, HO· and HOO·) and molecular oxygen at low concentration via strong oxidation ability (Mao et al., 2015). Unlike alkaline or DA pretreatment, peroxide pretreatment can be used at relatively mild concentration, temperature and atmospheric pressure, while effectively removing lignin from various lignocellulosic agricultural residues (Cabrera et al., 2014). When *Miscanthus floridulus* was treated by 0.8% H_2O_2 for 24 h, the methane yield of 279 mL/g VS was achieved, which was 49% higher than the control assay (Katukuri et al., 2017). Liu et al. (2018) demonstrated that peroxidation through H_2O_2 was a useful method to disintegrate sludge, leading to higher sCOD and increased methane production by 19.89% when microwave-H_2O_2 pretreatment applied.

Biological pretreatment is considered as environmental-friendly and inexpensive technique for AD as this process does not require high energy and chemical inputs for operation (Zhao et al., 2018). Previous researches have tried to improve the AD process by using anaerobic or aerobic predigestion or enzyme addition (Atelge et al., 2020). The anaerobic pretreatment predigests the substrate under mesophilic or thermophilic environments (Neumann et al., 2016). For anaerobic pretreatment of sewage sludge, temperature phased anaerobic digestion (TPAD) is a commonly applied choice for which a primary or hyper thermophilic digester is applied

with a secondary mesophilic digester (known as two-stage AD) (Ariunbaatar et al., 2014). A recent study performed TPAD on wastewater sludge digestion found a methane release rate of 3.55 L CH_4 /L day at 45°C along with a 77% reduction in VS (Hameed et al., 2019). For fungal pretreatment, several classes including brown-rot, white-rot, soft-rot and basidiomycete fungi, have been used in pretreatment to increase feedstock solubilization (Shirkavand et al., 2017). Enzymes such as peroxidase and laccase excreted by fungi can directly degrade the lignin and increase the digestibility of substrate in AD (Zheng et al., 2014). The pretreatment of rice straw by wood-decay fungus *Pleurotus ostreatus*, *Phanerochaete chrysosposrium*, and *Ganoderma lucidum* increased 64%, 122% and 88% of gas production, respectively (Kainthola et al., 2019a; Kainthola et al., 2019b). *Trichoderma reesei* and *Aspergillus* are investigated in many studies for its capability to excrete cellulase to degrade the insoluble cellulose that is found in large portions in the sludge, food waste and other lignocellulosic biomass (Gerhardt et al., 2007; Nguyen et al., 2021). The effects of lysozyme pretreatments on WAS hydrolysis and degradability was evaluated, and Chen et al. (2018) found that lysozymes can increase the sCOD concentration in the sludge by 2.23 times and 2.15 times higher than protease and α-amylase. The sludge flocculation disintegration was also improved. Comparing with the non-biological pretreatment, biological pretreatment generates no toxic compounds (*e.g.*, phenolic compounds), production of potential useful by-products, milder operation condition and low cost for waste disposal which is more compatible with AD (Wagner et al., 2018; Wu & He, 2013).

While demonstrated to be effective in biomass fractionation, the increased capital and operational costs of different pretreatment processes have hindered the large-scale application of biorefinery. Most of the pretreated substrates are free from microorganisms and ready to be utilized by pure enzyme or single strain. The pretreatment techniques need to apply with specific targets with the production extremely high value products to achieve a more

economical operation of AD. **Figure 6** illustrates the trades of various parameters applied in AD among increased costs from pretreatment, changed methane yield, and extended time of operation. The overall operational costs of enzymatic pretreatment are lower in the report due to its extremely low energy and chemical consumption in physical and chemical pretreatment processes. However, the significant limitation on the application of the biological pretreatment are of its long reaction time and more sensitive responses to the ambient environment (Sindhu et al., 2016). The requirement of long SRT operation reflected into large land requirement has prevented a wide application of biological pretreatment in urbanized areas, while also increase the difficulty of process control and operation.

5. Perspectives

AD has demonstrated to be a useful selection for the conversion of pretreated lignocellulosic substrate and high strength cellulosic waste. With the advances of new generation genomic tools, the dynamic changes of the microbial ecology have been gradually clarified, which has been demonstrated in the example of cellulosome-induced-cellulolysis in this review. Co-digestion of different feedstock, *i.e.*, FW, AW, and/or cellulosic sludge, with activated sludge have been attracting significant attentions in large-scale practices in recent years. However, recent publications on the optimization of substrate-sludge combinations for specific AD process may not represent the total production rates of various waste sources. More advanced technique and understanding will therefore soon become mandatory for a broad range of AD research, *i.e.*, from process design/operation to genetic modification. By clarifying the dockerin-cohesin systems in AD, more effective hydrolyzing tools may be synthesized from wastewater, which benefits cellulose valorization in multi-substrate treatment.

For process upgrade, microaeration offers significant benefits to cellulolysis-methanogens interactions in AD, while innovative gas diffusion process, real-time monitoring principles,

and control techniques are to be investigated in order to optimize the αOTE in the high solid loading environment. The potential residual oxygen in the solid layers and headspace in large scale operation needs to be carefully designed with minimum energy inputs, gas flow, and fire risks. In addition, pretreatment techniques of lignocellulosic biomass has demonstrated its role before bioconversion. Physical, chemical, and biological processes have claimed their niches in different beneficial areas among conversion efficiency, product values, and costs, respectively. While harsh pretreatment may better facilitate the biomass recalcitrant for bioconversion, the "bacteria-free" nature of pretreated substrate leads to an AD (multi-culture) over saccharification (cellulase)/fermentation (single culture) discussion on how to utilize the easily degradable substrates for most valuable products. Future development of the pretreatment/AD processes may consider the joint mechanisms applying the energy and/or recycled bioconversion metabolites (*e.g.*, waste heat, ammonia, organic acid, and other fermentation products) for the development of integrated processes. Separation techniques for treated solid digestate are as importance as conventionally believed for balancing the product values from AD with various critical SRTs to be investigated.

6. Conclusions

Hydrolysis serves as the rate limiting factor of AD receiving lignocellulosic and cellulosic needs to be accelerated by various approaches toward a sustainable bio-economy. This critical review summarized the key biological factors when applying AD for the valorisation of cellulosic biomass waste, including biodegradabilities of lignocellulose biomass, functional microorganisms of cellulolysis, their functional enzyme category, and cellulosomes. The genetic fingerprint of cellulolysis, engineering application of micro-aeration with its inherent genetics changes and pretreatment methods for lignocellulose biomass are also reviewed.

Acknowledgement

The authors thank for the financial support from the Hong Kong Research Grant Council, General Research Fund (RGC/GRF15212319); Innovation and Technology Commission (GHP/042/18GD); and the Research Institute for Sustainable Urban Development (RISUD, PolyU 1-BBW6) of the Hong Kong Polytechnic University. The authors also thank Mr. W.S. Lam in the Water and Waste Analysis Laboratory and Dr. Hang Liu in the University Research Facility in Chemical and Environmental Analysis (UCEA) for sample analysis.

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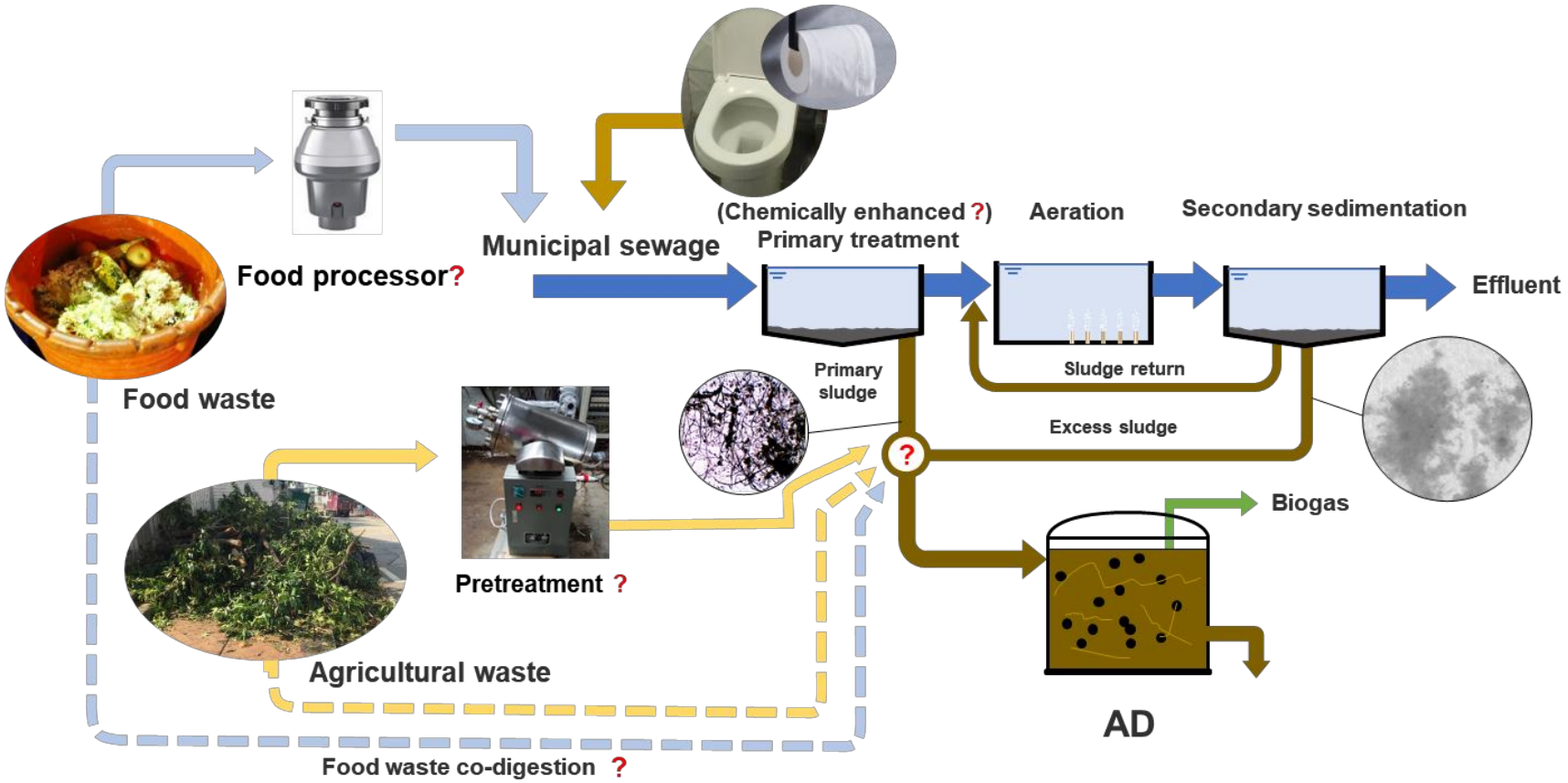

Figure 1 Possible strategies of AD management for treating lignocellulosic biomass waste from different sources

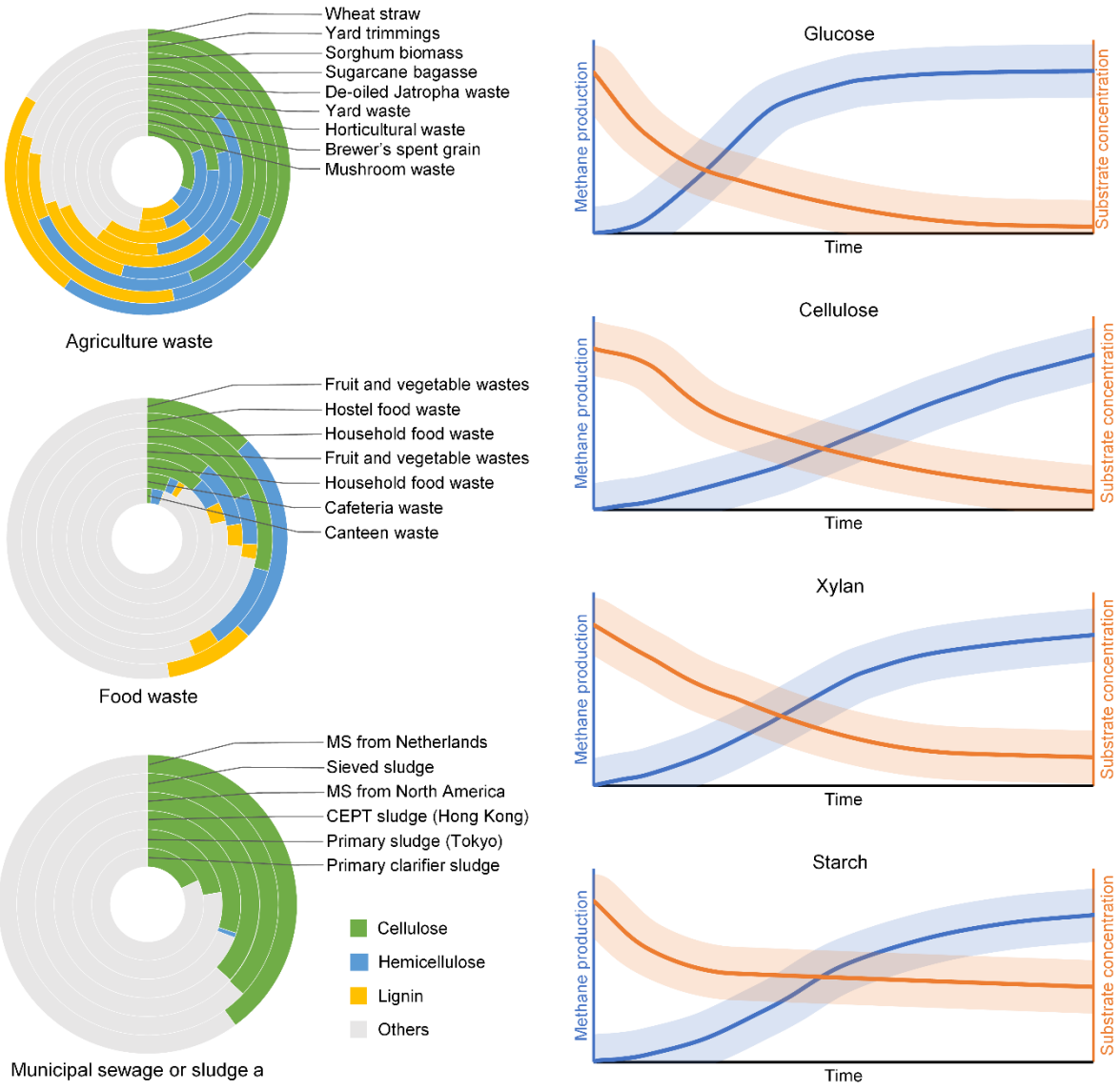

Figure 2 Compositions of lignocellulosic biomass waste from different sources and the kinetics of AD treating individual components

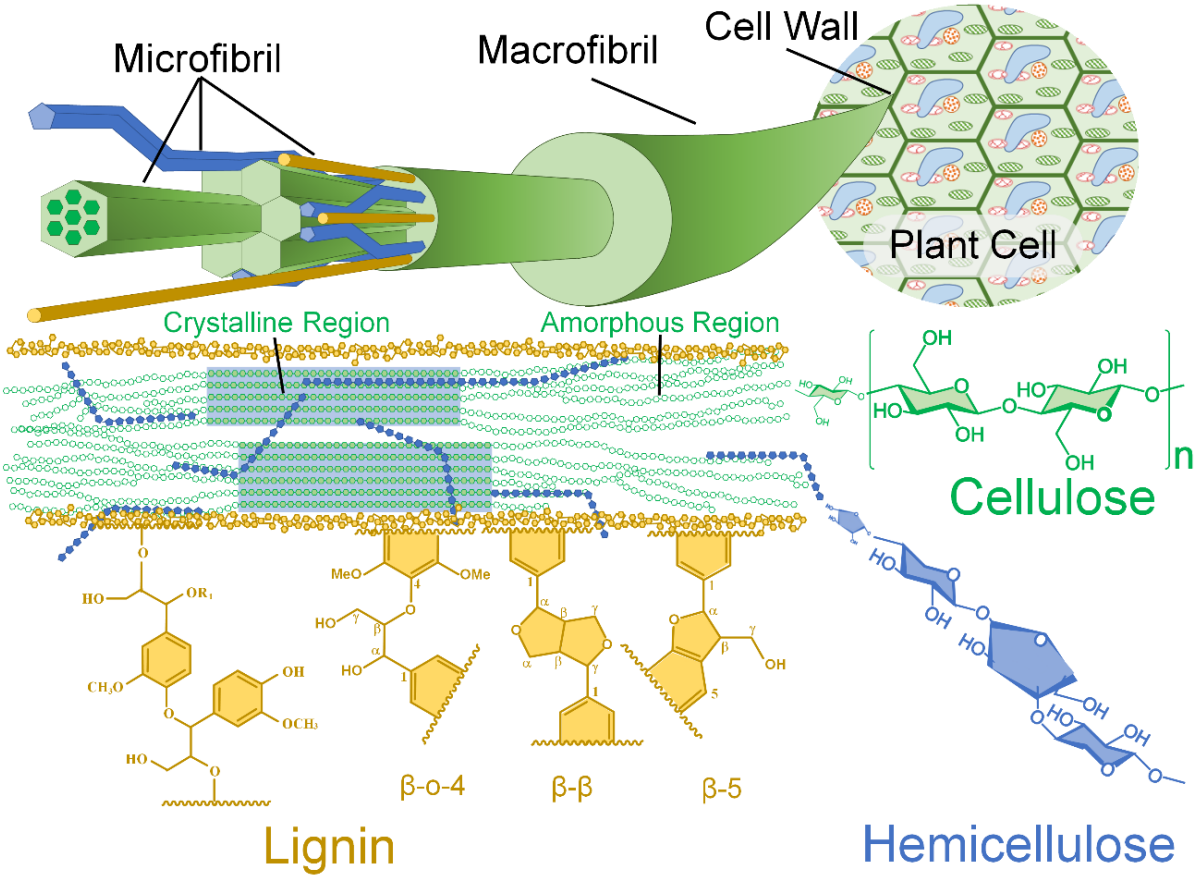

Figure 3 The conceptual structure of the plant cell wall with the spatial arrangement

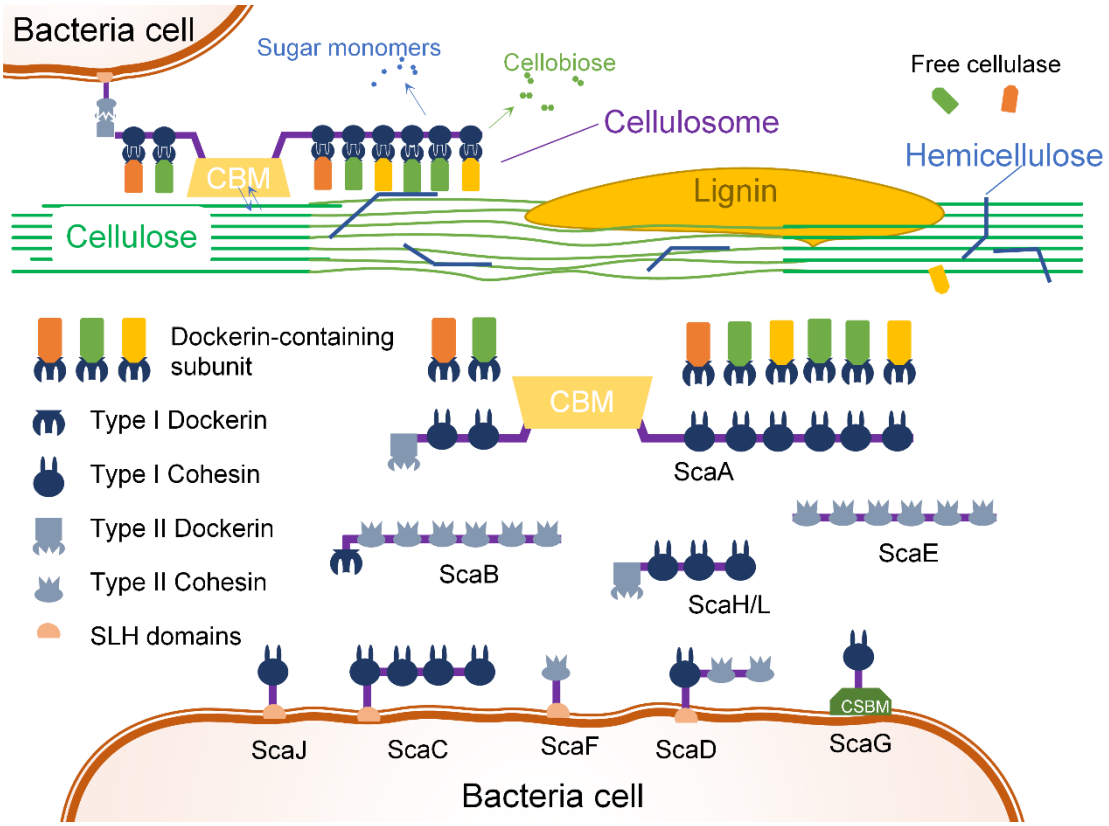

Figure 4 Components of cellulosomes formed by *Clostridium clariflavum* and their poteintail assembly structure

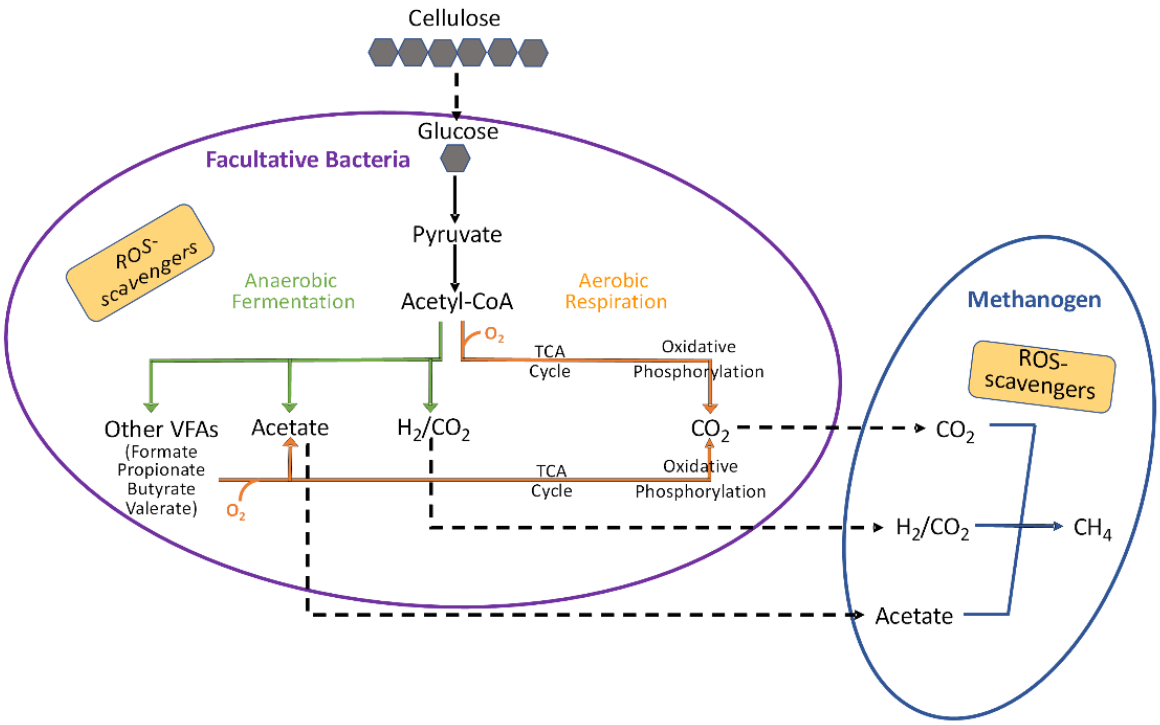

Figure 5 Synergistic relationship between hydrolytic facultative heterotrophs and anaerobic methanogens under microaerobic digestion of cellulose, by Nguyen et al. (2019) and Wu et al. (2021).

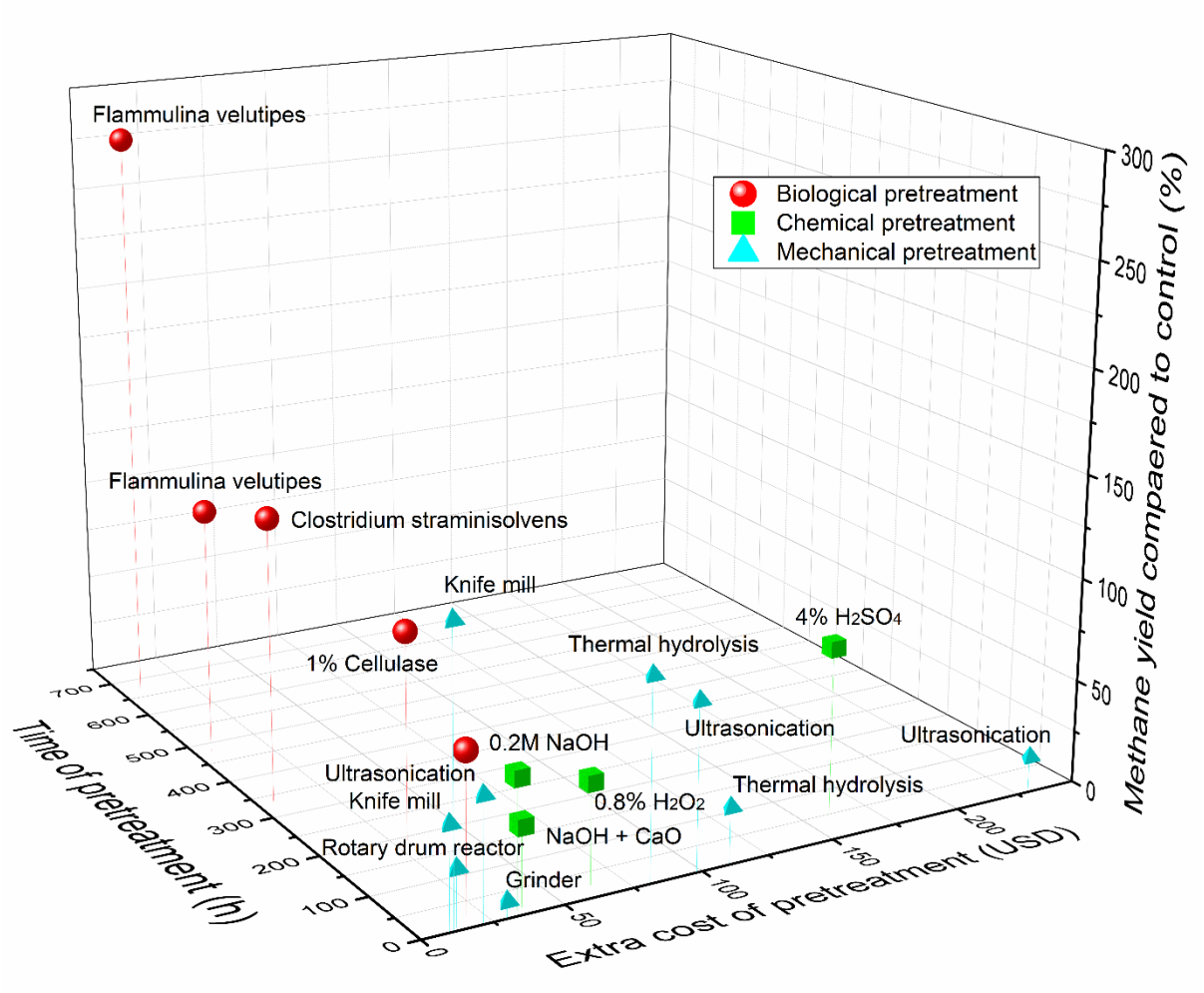

Figure 6 Extra cost and time consumption of pretreatment in AD and methane yield changes compared to the ADs without pretreatment

Table 1 Composition of reported lignocellulosic and cellulosic components in the waste biomass treated in AD

Type	Type of biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Treatment	Production of Anaerobic digestion	Reference
Agriculture waste	Wheat straw	36.9	23.0	24.0	Fungal pretreatment for ethanol fermentation	123 ± 5 mg ethanol /g dry wheat straw	(Salvachúa et al., 2011)
	Sugarcane bagasse	33.1±4.2	20.8±2.7	16.3±0.8	Two-stage AD	195 L CH_4/kg VS	(Kan et al., 2017)
	Brewer's spent grain	18.6±2.4	26.1±3.3	7.8±0.4	Two-stage AD	400 L CH_4/kg VS	(Kan et al., 2017)
	Sorghum biomass	43.8	24.6	9.2	Saccharification and acid hydrolysis	260 g ethanol /kg dry biomass	(Koradiya et al., 2016)
	Mushroom waste	31.2	6.9	14.0	anaerobic biohydrogen fermentation	10.08 L H_2/kg VS	(Lay et al., 2012)
	Horticultural waste	24.6±3.1	15.0±1.9	20.6±1.5	Two-stage AD	39 L CH_4/kg VS	(Kan et al., 2017)
	Yard trimmings	30.8±0.9	15.9±0.5	32.9±0.2	Fungal pretreatment	40 L CH_4/kg VS	(Zhao et al., 2014)
	De-oiled Jatropha waste	14.1	24.2	30.4	Heat pretreatment	13.1 L H_2 and 34.5 L CH_4 per kg VS	(Kumar et al., 2015)
	Yard waste	20.9±1.1	27.1±1.4	12.5±0.6	Co-digestion AD	34.9 – 44.6 L CH_4/kg VS	(Zhang et al., 2018)
Food waste	Household food waste	18.3±0.2	7.6 ± 0.4	2.2 ± 0.2	High solids fermentation for ethanol production	95.1±1.8 g ethanol /kg dry biomass	(Matsakas et al., 2014)
	Canteen waste	1.4±0.1	4.0±0.2	0.3±0	Co-digestion AD	34.9 – 44.6 L CH_4/kg VS	(Zhang et al., 2018)
	Cafeteria waste	5.7	2.4	1.8	Co-digestion AD	389±53 L CH_4/kg TS	(Xing et al., 2020)
	Household food waste	12.0±0.7	5.6±0.6	3.9±0.2	High solids simultaneous saccharification and fermentation for ethanol production	147.7±12.7 g ethanol /kg dry biomass	(Loizidou et al., 2017)
	Hostel food waste	29.2±3.8	11.2±1.2	3.4±0.8	Co-digestion AD	335 L CH_4/kg VS	(Panigrahi et al., 2020)

	Fruit and vegetable wastes	12.8	24.4	10.3	Acid hydrolysis followed by mesophilic dark fermentation and AD	80.5 L H_2 and 218.0 L CH_4 per kg VS	(Rodriguez-Valderrama et al., 2020)
	Fruit and vegetable wastes	11.1±0.1	11.4±0.3	3.8±1.4	AD	285 L CH_4 /kg VS	(Edwiges et al., 2020)
Municipal sewage (MS) or sludge (S)	MS from Netherlands*	40.0	-	-	-	-	(Ruiken et al., 2013)
	MS from North America*	36.8±0.7	-	-	-	-	(Ahmed et al., 2019)
	Sevied sludge	37.0±1	-	-	-	-	(Gupta et al., 2018)
	Primary clarifier slduge	18.0±0	-	-	-	-	(Gupta et al., 2018)
	Primary sludge (Tokyo)	22.4±6.6	-	-	-	-	(Honda et al., 2002)
	CEPT sludge (Hong Kong)	30.0±1.2	0.8±0.2	-	-	400 L CH_4 /kg VS	(Jing et al., 2019)

* represents the composition of the total solid.

Table 2 summary of pretreatment of lignocellulose biomass for anaerobic digestions

Pretreatment	Substrate	Composition	Methane and biogas yield	Reference
Mechanical pretreatment				
Knife mill	Giant reed stem	TS 89.6% VS 84.7%	212.8 ml/g VS	(Dell'Omo & Spena, 2020)
Knife mill	Wheat straw	TS 92.7% VS 84.6%	250 ml/g VS	(Dell'Omo & Spena, 2020)
Ultrasonic	Primary sludge	TS 3.22% VS 2.31%	272.6 ml/g VS	(Pilli et al., 2016)
Ultrasonic	Fruit and vegetable waste	TS 18.7% VS 14.2%	396 ml/g VS	(Zeynali et al., 2017)
Ultrasonic	Cattle manure	TS 8.4% VS 4.0%	460 ml/g VS	(Ormaechea et al., 2018)
grinder	Hybrid Pennisetum	*TS 29.1%* *VS 26.0%* *Cellulose 9.9%*	291.9 mL/g VS	(Kang et al., 2019)
Rotary drum reactor	MSW	TS 40.4% VS 30.6% 37.4% cellulose	261-320 ml/g VS	(Gikas et al., 2018; Zhu et al., 2009)
80°C, 30 min thermal	Saline WAS	TSS 1.95% VSS 1.42%	113 ml/g VSS	(Biswal et al., 2020)
120°C, 60 min thermal	Coffee husk /microalgal	TS 89.4% VS 70.1%/ TS 2.36% VS 1.9%	196 ml/g VS	(Passos et al., 2018)
Chemical pretreatment				
0.8% H_2O_2, 24 h	*Miscanthus floridulus*	TS 95% VS 88.2% Cellulose 34.2%	279 ml/g VS	(Katukuri et al., 2017)
2M KOH	whole poultry carcasses	TS 0.16% VS 0.1%	607 ml/g VS	(Arias et al., 2018)
0.2M NaOH 10h	Mango leaves	TS 89.9% VS 80.2%	644 ml biogas/ g VS	(Abudi et al., 2020)

NaOH and CaO (0.075 g NaOH + 0.05 g CaO)/g TS	Corn stover and swine manure	Corn stover: TC 29.9% Reduced sugar 15.2% Swine manure: 0.13%	322 ml/g VS	(You et al., 2019)
4% Sulfuric acid	Salvinia molesta	TS 88.36% VS 48.2% Cellulose 26.24%	24.1 ml/g VS	(Syaichurrozi et al., 2019)
Biological pretreatment				
30 U/g cellulase, 8 days	Rice straw	TS 85.6% VS 74.9% Cellulose 38.3%	150 ml biogas/g VS	(Dai et al., 2018)
1% cellulase, 24h	microalgae	TS 0.69% VS 0.65%	537 ml/g VS	(Córdova et al., 2018)
A Niger, 7 days	Sugarcane bagasse	Cellulose 31.4%	140 ml/g VS	(Braga et al., 2018)
White rot fungus (C. subvermispora) 30 days	Yard trimming	TS 94.35% VS 93.3% Cellulose 29%	44.6 ml/ g VS	(Zhao et al., 2014)
Flammulina velutipes, 20 days	Tall Wheat Grass	Cellulose 46.24%	398 ml/ g VS	(Lalak et al., 2016)
Flammulina velutipes, 28 days	tall wheat grass	Cellulose 46.24%	181 ml/g VS	(Kasprzycka et al., 2018)
Clostridium straminisolvens (CSK1),14 days	Cotton stalk	TS 94.3% VS 89.7% Cellulose 39.22%	118 ml/ gVS	(Yuan et al., 2016)

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Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: