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MECHANICAL PRE-TREATMENTS AND PARTICLE SIZE DISTRIBUTION IN ANAEROBIC DIGESTION

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Fossil fuels, due to their ancient origins and finite availability, present uncertainties regarding future supply. Their extraction and use have significant negative impacts on the environment, including contributing to climate change, greenhouse gas emissions, and acid rain, as well as adverse effects on human health. Consequently, reducing reliance on oil and securing a sustainable energy future is crucial. Biofuels emerge as a promising alternative to fossil fuels and are categorized into four generations: First Generation, derived from vegetable oils (biodiesel) and fermentation of simple sugars from sugar and starch crops (ethanol), including biogas; Second Generation, produced from lignocellulosic biomass, agricultural residues, or waste plant material, which are sustainable feedstocks not intended for human consumption; Third Generation, involves algae cultivation that does not require arable land and can fix CO2; Fourth Generation, includes electrofuels and photobiological solar fuels, which do not necessitate the destruction of biomass unlike third-generation biofuels. Among these, biogas, a second-generation biofuel, is produced through Anaerobic Digestion (AD) of various organic materials such as sewage sludge, zootechnical waste, agricultural biomass, and other waste products. This process not only offers a renewable energy source but also addresses waste management challenges. Emphasizing the development and utilization of biofuels, especially second-generation and beyond, can significantly mitigate the environmental and health impacts associated with fossil fuels, while ensuring a more sustainable energy future.

Keywords: fossil fuels, biofuels, first generation, second generation, third generation, fourth generation, anaerobic digestion, biogas, renewable energy, sustainability.

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1. Introduction to Anaerobic Digestion

Fossil fuels, as the name suggests, are very old. Due to this fact and also to the limited amount available of these resources, a future supply is uncertain. Furthermore, the exploitation of these resources has undesirable effects on the environment, regarding climate issues, greenhouse gases emissions and acidic rain formation, in addition to the negative effects on human health. Therefore, it is suitable to reduce the dependence on oil and secure future supply of energy. Along these lines, biofuels intervene as an alternative to fossil fuels; they are mainly grouped in four generations [1]:

- First Generation biofuels are biodiesel from vegetable oils and ethanol from fermentationof simple sugars contained in sugar crops or starch crops and biogas;
- Second generation biofuels are made from lignocellulosic biomass, agricultural residues or waste plant material i.e. sustainable feedstock that is not destined to human consumption;
- Third generation biofuels involve algae cultivation, that do not use arable-land and fix CO_2;
- Fourth generation biofuels, unlike third-generation biofuels, do not require the destructionof biomass and include electro fuels and photobiological solar fuels

Biogas is a biofuel of Second Generation produced through the process of Anaerobic Digestion (AD) starting from different organic materials, such as sewage sludge, zootechnical waste, agricultural biomass and other wastes (Table 1.1).

Biogas consists of methane (50-70%), carbon dioxide (30-50%) and some impurities, such as ammonia, hydrogen sulphide, siloxane and halides. The calorific value of biogas is dependent only on the methane content; biogas containing 55% CH_4 has a calorific value of 21,5 MJ/Nm^3 while pure methane has a calorific value of 35,8 MJ/Nm^3 [2]. For this reason, CO_2 should be removed and earmarked for other aims (Figure 1.1).

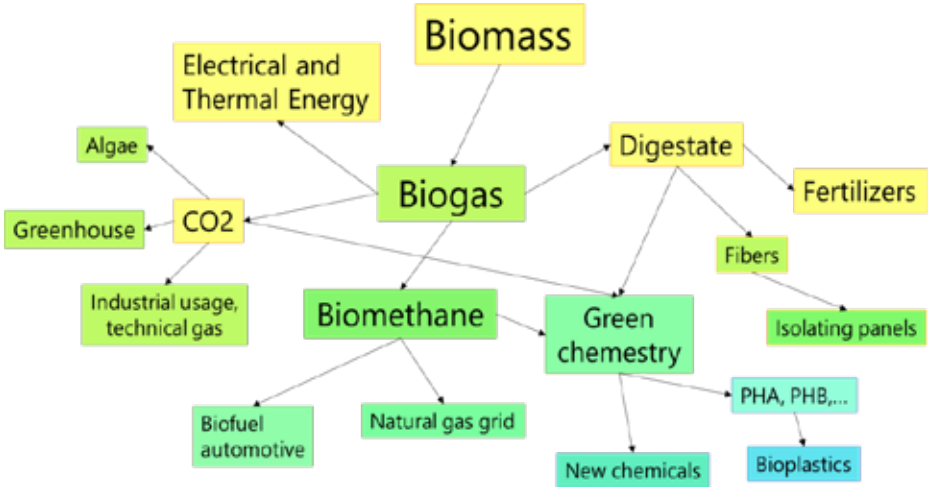

Figure 1.1: Development of biogas production chain [3].

Table 1.1: Industrial Wastes Amenable to AD [4].

Substrate	Example of substrate	Yield in biogas (m^3/tonne of VS)	CH4 in biogas (%)
Sewage		300-550	60-65
	Bovine	300-450	60-65
	Pork	450-550	60-65
Manures		200-550	60-65
	Bovine	200-300	60-65
	Pork	450-550	60-65
	Ovine	240-500	60-65
Dedicated crops		300-650	50-60
	Maize silage	350-550	53-55
	Beetroot	450-550	55-60
	Grass	300-500	53-55
	Clover	300-500	50-55
Agro-industrial byproducts		300-600	50-60
	From juices processing	500-600	55-60
	Brewers grains	300-400	50-55
	Molasses	300-450	50-55
	Straw	450-550	53-55
	From cerealsdistillation	400-500	50-55
Wastes		300-850	50-70
	Organic fraction ofmunicipal solid waste	300-450	50-60
	From food services	650-800	50-60

The number of biogas plants in Europa practically tripled in only 6 years, reaching the value of 17,376 biogas plants in 2015. With 1,550 biogas plants, Italy is second after Germany which has 10,846 (Figure 1.2).

In Germany, for the year 2011 about 18 billion kWh of electricity were generated from biogas, which means suppling around five million houses with electricity [5]. When biogas is converted into electricity in cogeneration units, heat is also produced: with around 18 billion kWh, heat is provided to over 530,000 houses [5]. Moreover, once upgraded to biomethane, biogas is a motor fuel similar to natural gas. Hence, biomethane can be used as fuel in natural gas-powered vehicles without technical modifications. Nearly 200 of about 900 natural gas-filling stations sold fuel containing 5-100% bio-methane [5].

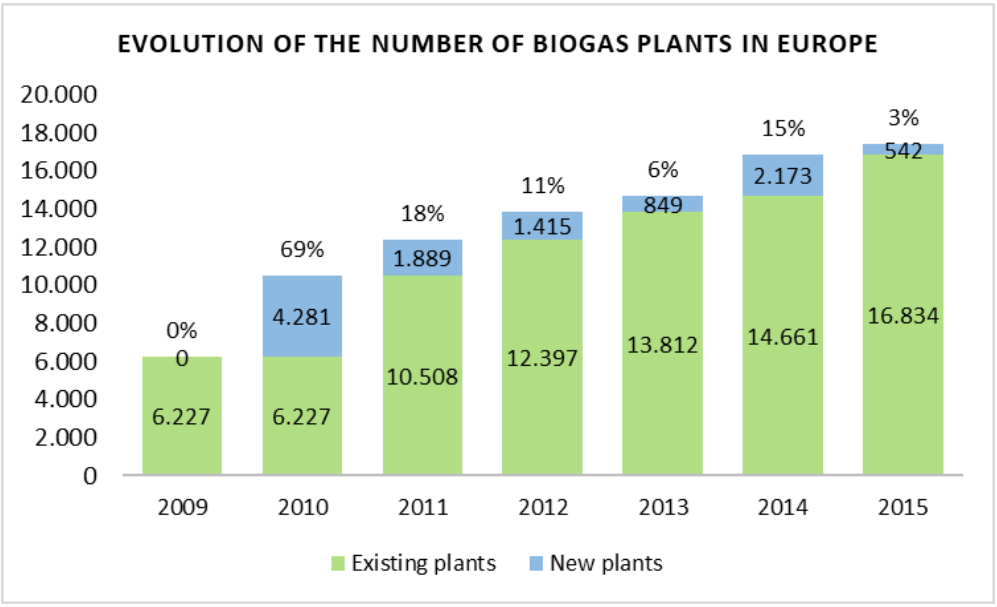

Figure 1.2: Biogas plants [6].

1.1 The microbiology of Anaerobic Digestion

Anaerobic digestion represents a catabolic process, in which bacteria degrade substrate using enzymes. They have a protein nature and can catalyse biochemical reactions thanks to active sites. Furthermore, they can be broadly classified as endo- or exoenzymes; both are produced in the cell, but the second ones are released outside the cell (Figure 1.3). A large and diverse community of bacteria is required to ensure that the proper exoenzymes and endoenzymes for degradation of the substrates are present, since no bacterium produces all of them by its own (Table 1.2).

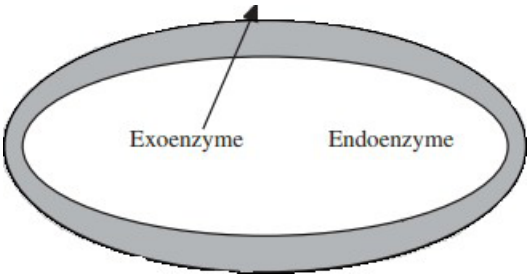

Figure 1.3: Enzymes used by bacteria to degrade substrate [7].

The bacterial activity in AD can be strictly/facultative anaerobic (sludge digestion) or strictly aerobic (sludge stabilization). Aerobes derive biosynthetic energy and produce metabolites onlyin presence of free molecular oxygen, as they produce ATP through aerobic respiration. Strict anaerobes, on the other end, are inactive in the presence of free molecular oxygen. They lack certain enzymes that are essential for bacteria to survive in presence of oxygen, as they removereactive oxygen species. These result from the reduction of molecular oxygen and cause intracellular damages. While the facultative anaerobic term establishes an ideal growth condition for the absence of oxygen, but, if the oxygen partial pressure rises in the medium, it is non-toxic to these microorganisms and can continue to grow. Certain species, called microaerophiles, instead, grow best in the presence of low amounts of oxygen.

Table 1.2: Exoenzymes and substrates [7].

Substrate	Example	Exoenzyme	Bacterium	Product	Yield (m^3CH4/k g) [4]
Polysaccharides	Saccharolytic	Cellulase	*Cellulomonas*	Simplesugars	0,40
Proteins	Proteolytic	Protease	*Bacillus*	Aminoacids	0,50
Lipids	Lipolytic	Lipase	*Mycobacterium*	Fattyacids	0,85

The AD process is composed by different steps (Figure 1.4), as it is briefly hereinafter discussed. Prior to AD the feedstock is prepared by removing contaminants such as grits, metals, and other particles depending on the specific source of the substrate.

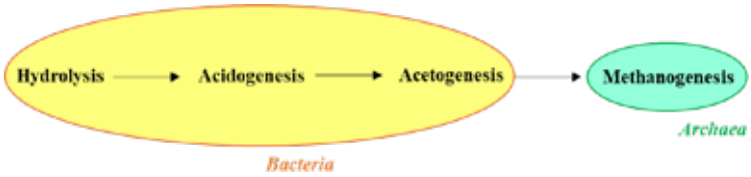

Figure 1.4: Biochemistry of AD.

In each phase one metabolic group of microorganisms is more active than the others: the microorganisms that play the key role in the first three stages of AD (hydrolytic-fermentative bacteria, acidogenic bacteria) are part of the domain *Bacteria*, on the other hand in the last step, aceticlastic methanogens and hydrogenotrophic methanogens are members of the *Archaea* domain (Figure 1.5).

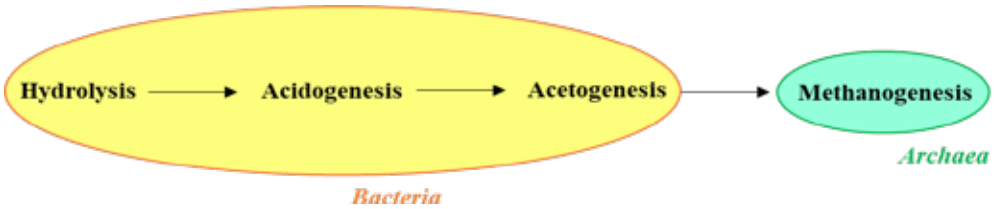

Figure 1.5: Domain of microorganisms in AD steps.

1.1.1 Hydrolysis

The first step of the process, *hydrolysis*, involves the feedstock disintegration operated by facultative and obligatory anaerobic bacteria through the production of exoenzymes (hydrolyses). Carbohydrates (such as cellulose) are broken down into simple sugars; proteins become individual amino acids; while fats, esters of the alcohol, glycerol and three fatty acid chains, have the latter parts removed from the head group. When the substrate is complex, thisstep is the rate-determining step.

1.1.2 Acidogenesis

During the second step, *acidogenesis*, monomers formed in the hydrolysis step are converted into short-chain (C1-C5) organic acids (butyric acid, propionic acid, acetic acid), alcohols, a few organic-nitrogen and organic-sulphur compounds, together with hydrogen and carbon dioxide. It is also commonly known as the first fermentative step. A pH value lower than 5,5 induce solvents productions (such as alcohols and acetone); the cells enter the second phase, known as *solventogenesis*.

- Pathways of degradation of sugars:

  - formation of propionic acid via the succinate pathway and the acrylic pathway (degradation of pyruvate);

  - formation of butyric acid via the butyric acid pathway;

- Pathways of degradation of fatty acids: β-oxidation (stepwise, in each step one acetate is set free).

- The pathway of degradation of amino acids is the Stickland reaction (two amino acids per time are split: one act as hydrogen donor, the other as acceptor). During splitting ofcysteine, hydrogen sulphide is released.

With the development of fixed-film bacterial growth in anaerobic digesters, many soluble organic wastes can be digested quickly and efficiently [7].

1.1.3 Acetogenesis

The third step, *acetogenesis*, or the second fermentation, involves the conversion of the volatile acids (VFA) (1.1) [8] and alcohols to acetic acid and hydrogen gas.

$$CH_3\ CH_2COOH + 2H_2O \rightarrow CH_3COOH + CO_2 + 3H_2 \quad \Delta G = +31{,}8\ kJ{\cdot}mol^{-1}\ [8] \quad (1.1)$$

The acetogenic bacteria are obligatory H_2 producers, inhibited by an excessive amount of the product (hydrogen). Then, a syntrophic relationship must exist between the acetogens and hydrogenotrophic methanogens [9]. Syntropy is the phenomenon for which one species lives off the products of another species [9]. Indeed, the second ones constantly remove the products of metabolism of the first ones from the substrate, keeping the hydrogen partial pressure at a low level, suitable for the acetogenic bacteria. Problems can occur when the acetogens create a syntrophic relationship instead with a methanogenic species with other organisms, that compete with methanogens for H_2. In waste water technology, some facultative anaerobes reduce sulphate (SO_4^{2-}) to hydrogen sulphide (Figure 1.6). In this case, the hydrogenotrophic methanogens

form less methane (receive less feed) and are also toxically affected by hydrogen sulphide. Furthermore, many of the monomers (e.g. sugars) can be catabolized by homoacetogenic bacteria to acetate, which then serves as substrate for acetoclastic methanogens converting it to CH_4 and CO_2.

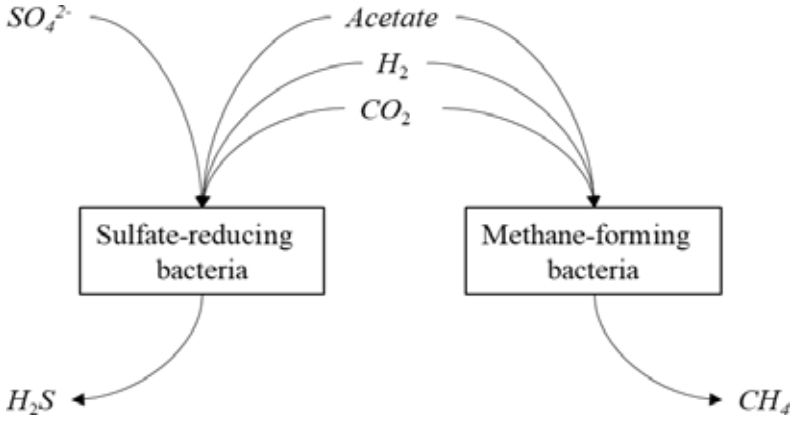

Figure 1.6: Competition between sulphate-reducing bacteria and methane-forming bacteria [7].

1.1.4 Methanogenesis

1.1.4.1 1.1.4.1 Bacteria and reactions involved

Methane-forming bacteria, grouped in the domain *Archaea* (Figure 1.3), that gathers a wide spectrum of microorganisms living in extreme environments, that are prohibitive for all other organisms (methane-forming bacteria, extremely halophilic, thermoacidophile, extremely thermophilic). Because of the long reproduction time, a long retention period is required in the digester (at least 12 days). Methane-forming bacteria grow as microbial consortia, are obligate anaerobes and grow on a limited number of simple substrates. They obtain energy and building blocks for the biosynthesis from hydrogen, 1-C compounds (formate, methanol, carbon dioxide, carbon monoxide and methylamine) and acetate (2-C). Mainly two groups can be distinguished: hydrogenotrophic methanogens and acetoclastic methanogens, converting hydrogen and carbon dioxide, or acetic acid, respectively, to methane. Methane production occurs, therefore, from the degradation of acetate (1.1) and the reduction of carbon dioxide by hydrogen gas (1.2) [7].The second pathways are believed to account for about 27-30% of CH_4 production in AD reactors.

$$CH_3COOH \rightarrow CH_4 + CO_2 \qquad \Delta G = -36\ kJ{\cdot}mol^{-1}\ [10] \qquad (1.2)$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G = -131\ kJ{\cdot}mol^{-1}\ [10] \qquad (1.3)$$

1.1.5 Energetic rationale

If only glucose is considered as substrate of AD (most fermentable substance), the combustion reaction (1.4) would release about 2870 kJ, while the reaction of conversion of glucose to methane through AD (1.3) releases only 390 kJ.

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \qquad \Delta G = -2.870\ kJ \cdot mol^{-1}\ [10] \qquad (1.3)$$

$$C_6H_{12}O_6 \rightarrow 3CH_4 + 3CO_2 \qquad \Delta G = -390\ kJ \cdot mol^{-1}\ [10] \qquad (1.4)$$

Microorganisms take advantage of the energy present in the carbon-carbon bonds, causing a change in the oxidation state of carbon-atoms from 0 in the glucose to -4 in the methane. In the new oxidation state lies energy, which is stored in this way in the product (methane) and is notreleased; as shown clearly by the Gibbs free energy of 1.4, lower than the one of 1.3. This storedenergy can be exploited afterwards, for example by combustion. If this is the case, methane LHV is about 800 kJ/mol and since 3 mol of methane are gained, the amount of energy theoretically obtained is about 2400 kJ/mol, closed to the Gibbs free energy of the reaction 1.3.Though the $|\Delta G|$ is higher in the combustion reaction, the biomass can hold a great amount of water that should be vaporized, but AD process occurs in presence of water. Moreover, the process is driven at low-temperature ranges, under stable continuous operating-mode and without the need of light (ensuring a 24h-production).

1.2 Microbial consortia

The phyla of Bacteria responsible for AD are Firmicutes and Archaea. Firmicutes are the syntrophic-fermenters bacteria responsible for the digestion of VFAs (see chapter 1.1.3). Due to the great availability of VFAs, Firmicutes are quantitatively dominant in the digester. The phylum Firmicutes is majorly composed of two classes: Clostridia (13%) and Bacilli (76%) [11].

1.2.1 Clostridia

Clostridia are a class of Gram-positive, rod-shaped, endospore-forming (see chapter 1.2.2) bacteria. The endospores are the “dormant state” to which the bacteria can reduce themselves under stressful environmental conditions, also for very long periods. Furthermore, Clostridia are anaerobic or aerotolerant and can be viewed as the evolutionary predecessors of Bacilli.

This class of microorganisms includes saprotrophic (decomposing) organisms, living in anaerobic habitats containing organic matter, including soils, aquatic sediments, and anaerobic tissues (such as intestinal tracts of animals). As such, they got a strictly fermentative-type of metabolism by which they convert many simple and complex carbohydrates (e.g. cellulose), as well as CO_2/H_2 or CO, (Table 1.3) but also amino acids, proteins and other organic molecules.

Table 1.3: Substrates clostridia can utilize [12]. BW stays for Biomass Wastes (e.g. agricultural)

andOW for Other Wastes (e.g. sludge).

Feedstock	Substrate	Monomericsubunit	Clostridiagroup
Crops	Molasses	Glucose, Fructose	Solventogenic
Crops	Starch	Glucose	Solventogenic,some Cellulolytic
Crops, BW	Cellulose	Glucose	Cellulolytic,some Solventogenic
Crops, BW	Hemicellulose	Glucose, Xylose, Mannose, Galactose, Rhamnose, Arabinose	Cellulolytic, Solventogenic
Crops, BW	Pectin	Galacturonic acid, Rhamnose	Cellulolytic, Solventogenic
OW	Glycerol		Solventogenic
	Cheese way	Lactose	Solventogenic
OW	Short chain fatty acids (acetic, butyric, lactic)		Solventogenic
OW	Unknown		Cellulolytic,Acetogen
Crops, BW, OW	CO_2/H_2, CO		Acetogen

The products may be gas (such as CO_2 and H_2), alcohols, carboxylic acids, ketones of C2 to more than C8 (Table 1.4). During growth, first the acidogenic phase occurs, then, as the pH decreases due to the accumulation of acids, the culture enters the stationary phase, shifting to solventogenic phase (see Figure 1.2). Indeed, a pH value lower than 5,5 induce solvents productions, such as alcohols and acetone (i.e. *solventogenesis)*. In this phase, strains such as *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium butyricum*, *Clostridiumsaccharobutylicum* and *Clostridium saccharoperbutylacetonicum* produce butanol in highconcentration with acetone (or isopropanol) and ethanol. This process is called Acetone–butanol–ethanol (ABE) fermentation [13].

Costridia encompass as well many acetogens that fix CO_2 (using e.g. H_2) or CO (alone) and use one-carbon compounds (e.g. formate or methanol), some of them are *Clostridium ljungdahlii, Clostridium thermoaceticum*, *Clostridium carboxidivorans.*

Cellulolytic clostridia digest cellulose, such as *Clostridium thermocellum*, *Clostridium cellulolyticum* and *Clostridium phytofermentans*.

Finally, when clostridia grow on amino acids or fatty acids, malodours compounds areproduced.

Table 1.4: Products of clostridia [12].

	Products	Organisms
gas	H_2, CO_2	Acetogens, Solventogenic,Cellulolytic
2-C	Ethanol, Acetic acid	
3-C	Propanone, Propionic acid, Propanol, 1,3-propanediol, Lactate, Acrylate	Solventogenic, Propionic, others
4-C	Butyric acid, Butanol, Acetoin,Succinate, 2,3-butanediol	Acetogens, Solventogenic, others
5-C	Pentanoic acid (Valeric a.)	*C. scatologenes, C. kluyveri*, others
6-C	Hexanoic acid (Caproic a.), Hexanol, 2-hydroxy-4-methylpentanoate	*C. scatologenes, C. kluyveri, C.butyricum*
8-C	Octanoic acid (Caprylic a.)	*C. kluyveri*

1.2.2 Bacilli

Bacilli is a class of Gram-positive, rod-shaped, endospore-forming bacteria. Bacilli are obligate aerobes or facultative anaerobe and can be viewed as the evolutionary successors of the anaerobic Firmicutes Clostridia.

During H_2 production by anaerobic (Clostridia) and facultative bacterial population (Bacilli) in fermentation, various metabolic pathways can be simultaneously present during H_2 production. Figure 1.7 shows some of the alternative metabolic pathways.

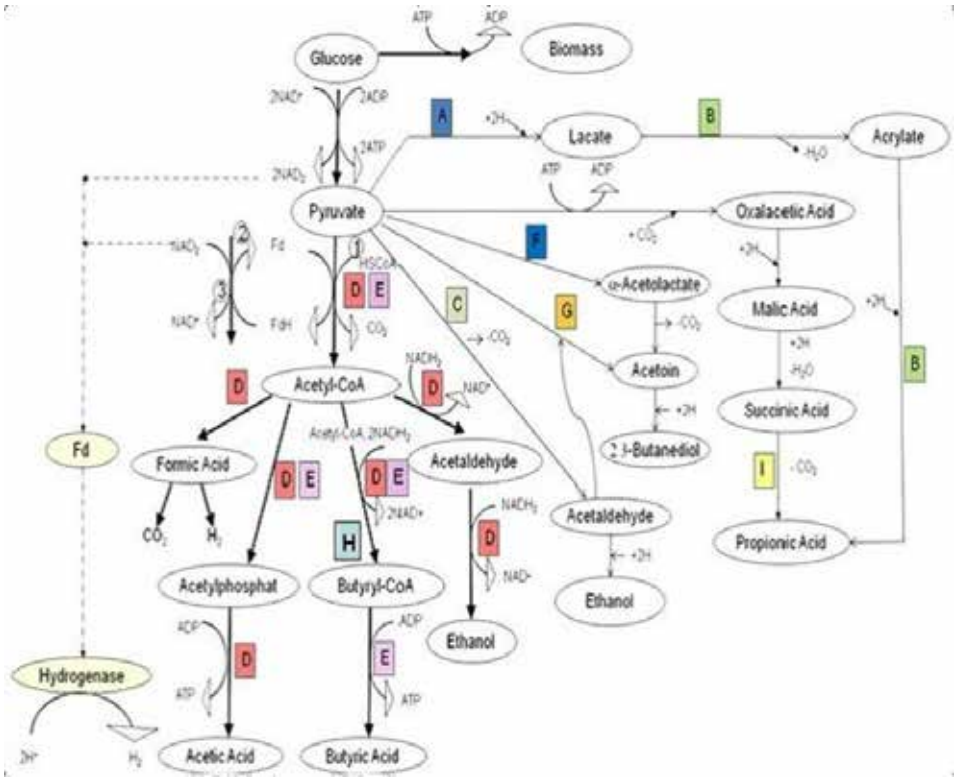

Figure 1.7: Metabolic pathway of glucose by hydrogen-producing bacteria in AD [14]. Letters indicate the organisms that conduct the reaction: A, Lactic acid bacteria (Streptococcus, Lactobacillus); B, Clostridium propionicum; C, Yeast, Acetobacter, Zymomonas, S. ventriculi, E.amylovora; D Enterobacteriaceae (coli-aerogens); E, Clostridia; F, Aerobacter; G, Yeast; H, Clostridia (butyric, butylic organisms); I, Propionic acid bacteria.

1.3 Feedstock flexibility

In absence of essential nutrients, the microorganism's growth stops. Therefore, it is often needed to feed possibly essential nutrients together with the substrate. The essential nutrients can be divided into macronutrients (carbon source and nitrogen source) and micronutrients (trace minerals). As carbon source, for example, the sugar is consumed very fast, unlike lignocellulose, that, indeed, is made up of three types of polymers (i.e. cellulose, hemicelluloseand lignin) strongly interlinked by covalent bonds and non-covalent forces. Moreover, intermediate products of the metabolism can also have a limiting or even inhibiting effect.

In general, every biomass can be used as substrate if it contains carbohydrates, proteins, fats, cellulose, and hemicellulose as main components. Different wastes have been already utilized; the form ranges from solid, semi-solid and liquid, such as manure or by-products of

industry, agricultural farms, disposal plants, etc. The use of more than one substrate (co-digestion) for biogas production has some advantages including: faster degradation rate, cost-effectiveness in terms of product formation, optimization of moisture and nutrient contents and concentration reduction of inhibitory compounds [14]. For example, nowadays in most agricultural biogas plants liquid manure is fermented quite often combined with different co-substrates, such as energy crops [14]. The addition of solid-organic waste to liquid manure can increase the yield of biogas (per digester volume); this thanks to the higher content of readily-degradable organicsubstances. The same approach is valid for sewage sludges, where co-substrates consist in thiscase of floated materials, such as fat remains from pig slaughter.

1.4 Pre-treatment

Since the anaerobic biological process is so slow, its economic viability becomes questionable and hence institutions hesitate to invest in any form of this potentially sustainable process [16].Generally, hydrolysis has been identified to be the key step in determining the rate of the digestion process, due to the presence of hardly degradable matrices in the substrates. Based onthis, different pre-treatment methods aimed at improving the hydrolysis have been carried out [17]. The problem lies in lignocellulose materials and it specificity regards the behaviour of the lignin as a barrier preventing the biodegradation. Pre-treating the substrate before it enters the anaerobic reactor means breaking lignin and hemicellulose and reducing the crystalline structure of cellulose. In this way, the available surface area exposed to the attack of microorganisms is higher and a faster digestion is promoted. The pre-treatments currently applicable are classified according to different categories:

- mechanical (grinding, ultrasound, etc.)
- thermal
- chemical (through use of alcohols, acids, ozone, etc.)
- biological (by specific microorganisms or by specific enzymes)The classification is hereinafter more extensively illustrated.

1.4.1 Mechanical pre-treatment methods

The mechanical pre-treatment leads to smaller particles or to squeezed substrate pieces. Indeed, particle size can affect the rate of anaerobic digestion as it affects the availability of a substrate (i.e. the surface area) to hydrolysing enzymes. This is particularly true with plant fibres; AD of pre-treated and not pre-treated sisal fibre waste was carried out and the methane yield increased by 23% (from 0,18 m^3 CH_4/kgVS to 0,22 m^3 CH_4/kgVS) when the fibre size was reduced to 2 mm (from 100 mm) with a shearing pre-treatment. [18]. Also, the viscosity in the digester decreases consequently to a reduction of the particle size, making mixing easier, as the system results to be more homogeneous.

1.4.1.1 Shearing

Knife mills or shredders can be used to, respectively, cut or shred the substrate. Knife mills are usually used for wet feedstock, that is continuously cut until it is small enough to pass through a sizing screen [19]. Shredders are limited to biomass with a moisture content of under15% [20], but many industrial shredders use substrates with much higher moisture contents.

1.4.1.2 Ball milling

In this pre-treatment, glass beads (grinding media) are thrown against the cells.The acceleration of the beads is obtained:

- by shaking the entire container (Figure 1.8 and 1.9);

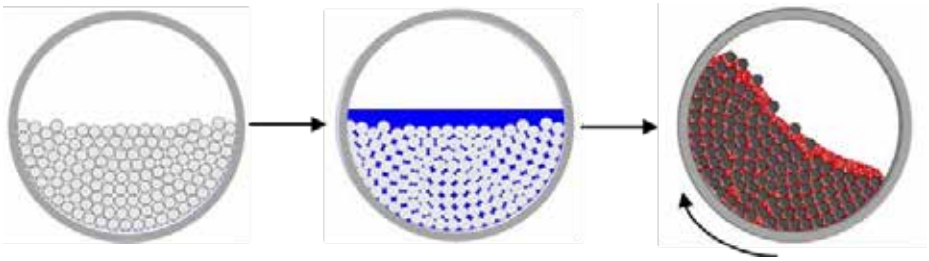

Figure 1.8: Bead milling in which the entire container is shaken [21].

Figure 1.9: Example of bead milling machine with 2 containers [22].

- by a spinning agitator within the container (Figure 1.10).

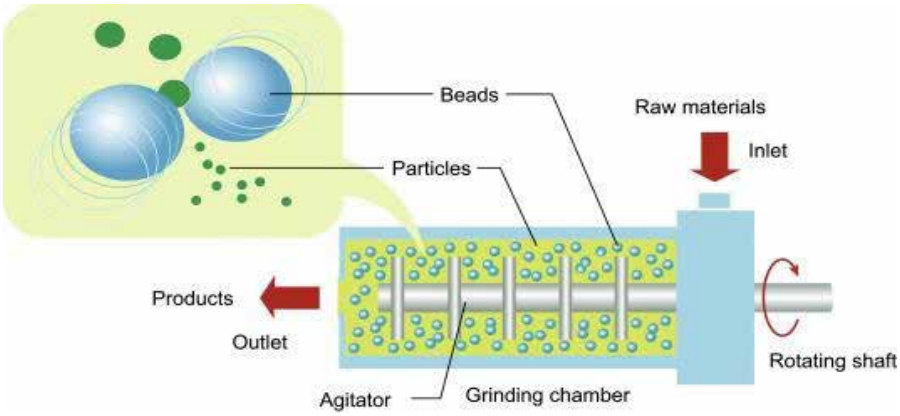

Figure 1.10: Bead milling in which a spinning agitator accelerates the beads [23].

1.4.1.3 High-pressure homogenization

The pressure is increased up to several hundred bars and then the suspension is homogenized under strong depressurization. The French press is an example of high pressure homogenizer. It works applying first a pressure of 83 MPa on the sludge and then releasing it quickly by opening a needle valve. The decrease of the pressure creates a very high shear force on the particles, making them explode.

1.4.1.4 Rotor-stator degradation

The rotor-stator homogenizer is composed of a rotating metal shaft (*rotor*) inside a stationary open-ended tube (*stator*), this is shaped with slots in the end (Figure 1.11).

Figure 1.11: Rotor and stator [24].

The rotation of the rotor creates a suction effect which draws the sample into the space between the rotor and stator, here the sample is subject to high shear forces due to the small space and the homogenization occurs (Figure 1.12).

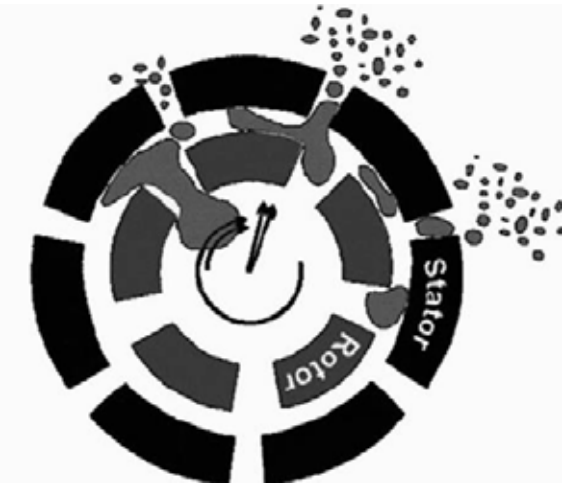

Figure 1.12: Rotor-stator shear by spinning shaft, inside view [25].

1.4.1.5 Ultrasonification

Ultrasound can be applied either *indirectly* through a bath or *directly* by a probe. Direct sonication, as the probe is in direct contact with the sample, guarantees more efficient operation. Indeed, the treatment is based on the utilization of acoustic waves, which are propagated by the probe into the liquid in contact with it. In particular, acoustic waves are compression-rarefaction waves, that, when propagated into a liquid at high intensities, generate alternative high- and low-pressure cycles. During the low-pressure cycle, the liquid reaches the vapour pressure, creating small bubbles. During the high-pressure phase the bubbles can no longer keep the balance between the pressure and the viscosity forces and implode violently. The violent implosion of these might disrupt the nearby substrate's particles' walls, that could release intercellular matter that can be then more easily degraded by microorganisms during AD. Cavitation is therefore the phenomenon behind the ultrasonification and it is always accompanied by intense local heating, that in some case can reach up to 5000 K (Figure 1.13).

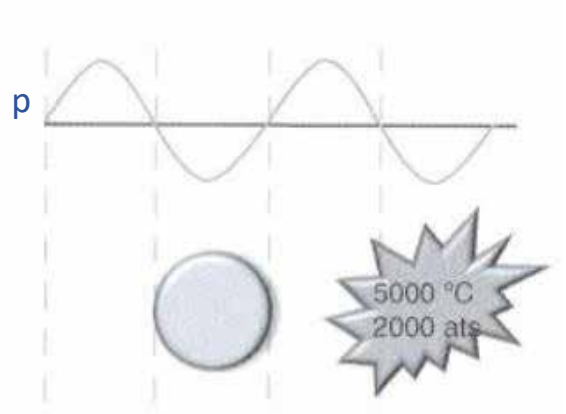

Figure 1.13: Cavitation phenomenon [26].

The ultrasonic equipment is composed by four components: generator, converter (or transducer), booster and probe (or horn or sonotrode) (Figure 1.14).

Figure 1.14: Ultrasonic equipment [27].

The generator transforms AC line power in high frequency electrical energy (20 - 100 kHz), which is received from the converter and further transformed into vibration (electrical energy is converted into mechanical energy), thanks to the piezoelectric material of which the converter is made. The booster increases the amplitude of the waves and the probe finally propagates the acoustic waves into the medium. Several studies have shown that the rate of bacterial degradation can accelerate up to 4 times compared to conventional treatment [28].

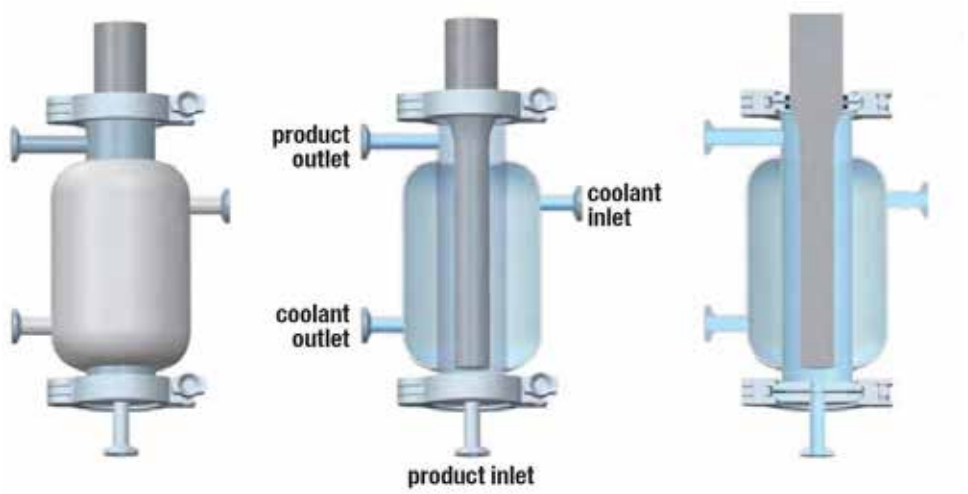

Figure 1.15: Continuous sonication [29].

1.4.2 Thermal

This is one of the most applied at industrial scale for AD of various substrates. Thetemperature range goes from 50-250 °C, as sludge solubilisation was seen at temperatures as low as 50°C [30]. The retention time clearly increases with the decreasing of the temperature, as the kinetic of the any reaction is favoured by the temperature. Reported retention time variesfrom 0 minutes to 72 hours [31]. Thermal pre-treatment removes pathogen, improves dewatering and reduces viscosity of the digestate, but inhibitory compounds can be formed, especially for long thermal treatment times. Regarding this, one of the most known phenomenais the Mallaird reaction between carbohydrates and amino acids. It occurs at temperature valueshigher than 150°C or at lower temperatures (<100°C) for a longer time and results in the formation of complex substrates difficult to biodegrade [30]. The effect of the treatment depends anyway on the substrate type and temperature range [30]. Finally, even though the thermal pre-treatment requires a great amount of energy, the energy requirements during the pre-treatment step can be covered by the extra methane production with a positive net-back [32].

1.4.3 Chemical pre-treatment methods

Chemical pre-treatments consist in the addition of chemical substances, like alkalis, strong acids or oxidants to achieve the destruction of the organic compounds, improve the hydrolysis rate and enhance the biogas production. AD generally requires an adjustment of the pH towards a greater alkalinity, thus alkali pre-treatment is the preferred chemical method [33].

Chemical pre-treatments are not suitable for easily biodegradable substrates, due to their accelerated degradation and subsequent accumulation of VFA [34]. On the opposite, the effect is clearly positive on substrates rich in lignin [35].

1.4.3.1 Alkali pre-treatment

The alkali treatment causes high pH values in the substrate. It is important to underline that high salts concentration can cause bacterial cells to dehydrate due to osmotic pressure [36]. Moreover, light metal ions (i.e. sodium) are required for microbial growth and, consequently, affect specific growth rate. While moderate concentrations stimulate microbial growth, excessive amounts slow down the growth, and even higher concentrations can cause severe inhibition or toxicity [36]. This pre-treatment may be useful for acidic and lignin-rich substrates that could otherwise not be anaerobically digested [20]. About 55% of the dry matter can be decomposed, especially when using NaOH to saponify [37]. Other chemicals can be used, such as lime or ammonia, but the high costs of alkalis in general make this technology economically unattractive (Table 1.5).

Table 1.5: GER of some Alkalis (SimaPro Database).

Chemical	GER (MJ/Kg)
Soda, powder	5,99
Ammonia, liquid	43,6
Ammonia, partial oxidation, liquid	49,5
Ammonia, steam reforming, liquid	41,8

1.4.3.2 Acid pre-treatment

Acid treatment is the most widely used conventional pre-treatment method for lignocellulosic feedstocks on industrial scale [38], even if the corrosive and toxic nature of most acids requires a suitable material for building the reactor [39]. Acid pre-treatment can be used in combination with heat, but at high temperatures from 60°C to 160°C (temperatures higher than 160°C in combination with acids show a drop in methane production) [20], that make the process not anymore energetically sustainable.

1.4.3.3 Oxidative pre-treatment

Oxidative pre-treatment is based on the use of radicals. The radicals are formed during the oxidation reaction, that is the reduction of the oxidizing agent, such as oxygen, ozone (O_3) and hydrogen peroxide (H_2O_2). By using ozone, the substrate can become permeable and water-insoluble macromolecules can decompose themselves into smaller-water soluble molecules.

The disadvantages include the formation of toxic substances for the methanogens (e.g. formaldehyde) and the increased amount of free carbon, that cannot be degraded during AD.

Table 1.6: GER of some oxidizers (SimaPro Database).

Chemical	GER (MJ/Kg)
Ozone, liquid	174
Hydrogen peroxide, 50% in H_2O	23,8

1.4.4 Biological pre-treatment methods

1.4.4.1 TSAD

Also called pre-acidification, two-stage digestion or dark fermentation coupled to methanogenic reactor, it consists of separating the first two steps of the process (*hydrolysis* and *acidogenesis*) from the second two (*acetogenesis* and *methanogenesis*). The pH value in the first digester lies between 4 and 6, inhibiting methane production (that occurs at pH values higher then 6,5) and causing a volatile fatty acids accumulation, but the enzymes responsible for the degradation of cellulose, hemicellulose and starch work very well under these conditions. CO_2 is also formed in the first digester and at low pH it is released in the gas produced in the pre-acidification step. Therefore, in the following stage the methane concentration is higher.

1.4.4.2 Enzymatic pre-treatment method

A mixture of enzymes, different from the ones already present (produced by the microorganisms of AD), is added. The addition can occur in the digester in which AD takes place, in the first step of a two-step AD process (see chapter 1.3.4.) or in a dedicated vessel forthe enzymatic pre-treatment. There is some evidence to suggest that enzymes added directly tothe biogas reactor are degraded quickly after addition [20]. Several batch AD studies have indicated that the addition of enzymes to the first stage of a two-stage AD process leads to slightly higher substrate solubilisation and to higher biogas yields [20]. The mixture of enzymesmay include different types that are able to specifically degrade: cellulose, hemicellulose, pectinand starch.

1.4.5 Shear stress induced on bacteria

Pre-treatments can impact not only the substrate, but the microorganisms of AD as well in case of wet AD processes, where fresh feedstock is slurried with the addition of water. In this case, dewatering is the first stage of postdigestion processing; the dewatered solid fraction is destined to be fertilizer and the water is recycled to supply the plant. In this case, microorganisms of AD are treated together with the substrate. Clearly, the wanted effect for the substrate (its breakage) is not desirable for the microorganisms as well; which should not be damaged in order to be able to drive the different phases of the AD process. The domain (see Figure 1.3) and the evolution underwent by the specific type of microorganism determine how easy AD microorganisms can be broken.

The Gram-positive bacterial cell is contained in a cell membrane, that is still surrounded by the cell wall. The membrane's function is not to protect the cell from osmotic shock, but it is rather elastic, interactive and semi-permeable, as it is mostly formed of phospholipids and proteins. Here, concentration gradients are actively maintained through transport systems, such as electron transport chain to produce ATP. Between the cell membrane and the cell wall a periplasmic space is located. The cell wall is thin and rigid; it is made up of peptidoglycan, that gives a structure to the cell envelope. It gains its strength from its chemically bonded nature. The peptidoglycan layer is composed of N-acetyl-glucosamine and N-acetyl-muramic acid residues sticked by ß-(1-4)-glycosidic bonds. The chains are crossed-linked by a tetrapeptide through the NAM residue. The peptides are further cross-linked, and the result is a rigid strict structure, that provides shape, tensile strength and protection from osmotic shock. Gram-positive bacteria, such as Bacillus, possess a thick cell wall composed of 50-80% peptidoglycan together with teichoic acid. On the other hand, Gram-negative bacteria have a relatively thin cell wall consisting of a few layers of peptidoglycan (even though this is the major responsible for cell resistance) but surrounded by a second lipid-protein bilayer membrane, that comprises proteins, lipopolysaccharides and phospholipids. The Firmicutes, such as Clostridia and Bacilli (see Chapter 1.2), and Actinobacteria are classified as Gram-positive bacteria.

Methane-forming bacteria, classified in the domain Archaea (see Figure 1.3, Chapter 1), present a different cell wall. The cell wall lacks muramic acid, and the cell membrane does not contain an ester lipid as its major constituent [7] (Figure 1.16).

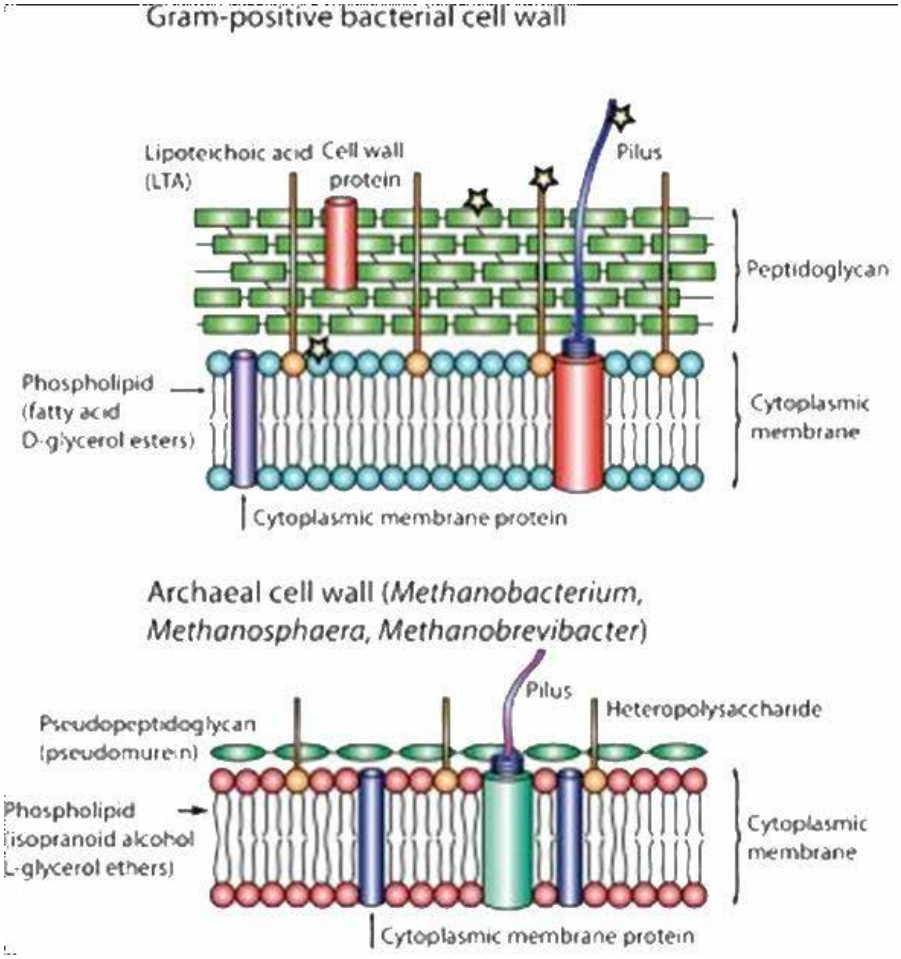

Figure 1.16: Comparison between cell wall of bacteria and cell wall of methanogens [40].

It is possible to conclude that methanogens will be more affected by shear stresses, due to theconstitution of both cell membrane and cell wall, that lack of certain components responsible for strength and resistance, that, on the opposite, are found in the composition of membrane and wall of Gram-positive bacterial cells.

1.5 In situ laser back-reflection

The optical-based method FBRM (focused beam reflectance method) is based on the detection of backscattered laser light and the time of flight (TOF) of the beam. However, only elongated-shape particles can be efficiently analysed and not spherical-shaped particles (bacteria).

It is desirable now to focus on a three-dimensional optical reflectance method (3D-ORM) probe of Sequip S+E GmbH. It is equipped with an excitation beam that can be moved in three dimensions, opening the doors to the analysis of spherical bacteria. Furthermore, the probe is heat-sterilisable (internal electronic parts can be removed for this purpose) and can be used for *in situ* measurement. Additionally, information about the cell size distribution are returned. The components of the probe are briefly hereinafter discussed (Figure 1.17).

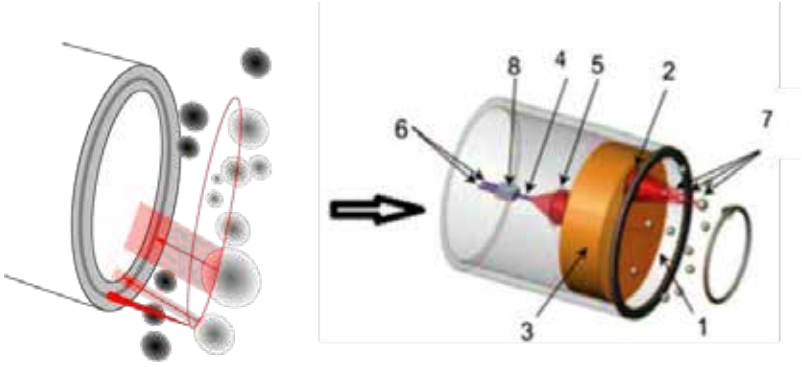

Figure 1.17: Schematic representation of the Sequip-probe [41]. A highly efficient focusing length (2) allows to scan precisely microbial cells, that enter the multiple focal planes surrounding the probe. The laser beam has an intensity inferior to 5 mW. The inlet of the laser beam into the probe is accomplished by a 12,8 µm single-mode fibre (6). The separation of incoming and outgoing signals is realized with a fibre optic coupler (8). Thanks to the rotating optical system (3) and the dynamic focus (7), the imaging of a spiral path with the diameter of 8.5 mm takes place. The coupling out of the reflected signals from the probe is realized with the incoming single-mode fibre (6). The small cross section of such fibre (4) ensures that only particles directly or close to the focal point are detected.

1.6 Probability distribution

A probability distribution is a mathematical model which connects the values of a variable tothe probability that such values can be observed. Probability distributions are used for modelling the behaviour of a phenomenon of interest in relation to the total amount of cases inwhich the experimenter observes a stated sample. The variable of interest is seen as one randomvariable whose probability law expresses the degree of uncertainty with which his value can beobserved. Furthermore, in continuous distributions the variable is expressed on a continuous scale.

Probability distributions are expressed by a mathematical law called probability density functions (PDF). The probability density functions of interest for this work are the following ones.

- The normal distribution, where the *mean* **a** and the *variance* $\mathbf{b^2}$ (or standard deviation **b)** of a random variable (*x*) are the parameters of major interest as they express respectively the central tendency and the variability of the random variable.

$$y = \frac{1}{\sqrt{2\pi}b} e^{-\frac{(x-a)^2}{2b^2}}$$

- The Cauchy distribution

$$y = \frac{1}{\pi b\left(1+\left(\frac{x-a}{b}\right)^2\right)}$$

- The Gamma distribution

$$y = \frac{1}{b^a \Gamma(a)} x^{a-1} e^{-\frac{x}{b}}$$

- The logistic distribution

$$y = \frac{1}{b}\left(e^{\frac{x-a}{2b}} + e^{-\frac{x-a}{2b}} \right)^{-2}$$

- The log-normal distribution is the probability distribution of a random variable whose logarithm has a normal distribution.

$$y = \frac{1}{x\sqrt{2\pi}b} e^{-\frac{(\ln x-a)^2}{2b^2}}$$

- The log-logistic distribution is the probability distribution of a random variable whose logarithm has a logistic distribution.

$$y = \frac{\frac{b}{a}(\frac{x}{a})^{b-1}}{(1+\left(\frac{x}{a}\right)^{b})^{2}}$$