# PRETREATMENT OF SECONDARY SLUDGE WITH COMMERCIAL ENZYMES AND MULTIENZYME EXTRACT FROM SOLID-STATE FERMENTATION BEFORE ANAEROBIC DIGESTION

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**ABSTRACT:** Anaerobic sludge digestion is an efficient and environmentally sustainable technology to produce heat, electricity, and vehicle fuel. Pretreatment of sewage sludge before its anaerobic digestion has been studied to optimize the hydrolysis, limit the steps in the anaerobic digestion process, reduce the sludge particle size, release simpler compounds for the later steps, and boost methane generation. Thermal, biological, physical, chemical methods, or a combination of these can be applied to pretreat the sludge, which will later undergo anaerobic treatment. Enzymatic hydrolysis is conducted by extracellular enzymes produced by the sludge biomass. However, this step of the anaerobic digestion process can be improved by adding exogenous enzymes to accelerate decomposition and using organic compounds to generate biogas. Commercial enzyme mixtures are more efficient in sludge hydrolysis; however, their application increases the cost of sludge treatment. On the other hand, the use of enzyme extracts obtained by solid-state fermentation is low cost, and filamentous fungi synthesize a wide range of enzymes from varied raw materials. Studies are still needed to understand the mechanism of action of enzymes in sludge degradation and obtain the best conditions for applying enzymes in the pretreatment of sewage sludge.

**KEYWORDS:** anaerobic digestion, biogas, enzymatic pretreatment, secondary sludge, solid-state fermentation.

# 1. INTRODUCTION

The activated sludge system for wastewater treatment is widely used throughout the world, due to its efficiency and robustness. The term activated sludge refers to the flocculent microbial mass formed when biodegradable wastewater is subjected to aeration. This process generally has the following system components: an aeration tank, a secondary decanter, and a system for recycling the settled flocs to the aeration tank (Metcalf & Eddy (Inc.), 1991). However, the intense production of biomass in the activated sludge system leads to the disposal of part of this biomass, called excess sludge. This sludge presents environmental risks; therefore, a correct form of treatment is necessary to reduce its environmental impacts.

Anaerobic digestion (AD) is an attractive technology for treating organic waste, generally used to stabilize organic matter in sludge and, thus, prevent or delay the release of chemical substances that can harm the environment (Romano *et al*., 2009). The AD process is divided into three steps (hydrolysis, acidogenesis, and methanogenesis). Due to the high content of solids and complex structure of the sludge, the initial hydrolysis step is a limiting step, as it takes a relatively long time (days), decreasing treatment efficiency and methane production and increasing the operational cost (Lv *et al*., 2010; Neumann *et al*., 2016).

The high cost of sludge management and the interest in alternative energy sources have amplified the attractiveness of pretreatment proposals to hydrolyze sludge, improving biogas production, reducing volatile solids, and increasing the quality of digested sludge. The existing techniques include thermal hydrolysis, physical processes dedicated to cell destruction and lysis, chemical processes, and biological processes based on the use of enzymes, as well as combinations of these pretreatments (Neumann *et al*., 2016).

Biological pretreatment using enzymes accelerates the hydrolysis process, reducing the sludge solubilization time to a few hours (Teo; Wong, 2014). More recent publications report the use of commercial enzymes for prior hydrolysis of sewage sludge, mainly lysozyme, proteases, amylases, and lipases (Roman *et al*., 2006; Yang *et al*., 2010; He *et al*., 2014; Xin *et al*., 2016, 2018; Kang *et al*., 2023). However, the large-scale application of commercial enzymes is not economically viable due to their high cost.

Biotechnological studies conducted by the UFRJ research group (Lima; Cammarota; Gutarra, 2018; Silva; Cammarota; Gutarra, 2022) have demonstrated that the Solid-state Fermentation (SSF) process could be an attractive alternative to obtain enzymes for pre-hydrolysis of sewage sludge. In this process, microorganisms develop on the surface and inside particles of solid raw materials, with little or no water not absorbed into the solid matrix. Furthermore, SSF presents little risk of contamination, requires less electricity, has lower operational costs, and may depend on the use of less complex manufacturing equipment (Pandey, 2003).

The microorganisms usually applied are filamentous fungi, capable of assimilating a wide range of substrates and synthesizing different types of enzymes. By producing enzymatic complexes containing different types of enzymes and using agro-industrial waste without pretreatment as raw material and source of substrates and nutrients, SSF can reduce costs for enzymatic pre-hydrolysis of sludge and facilitate anaerobic digestion.

## 2. SLUDGE GENERATED IN SEWAGE TREATMENT

Sludge is one of the by-products generated in Wastewater Treatment Plants (WWTPs) and can be in solid, semi-solid, or liquid form. Rich in organic matter and nutrients, it is generated in large volumes, has great polluting potential, and can be expensive to manage properly. WWTP sludge is mostly water, with 0.25 to 12% concentration of solids. It is conventionally called a solid phase to distinguish it from the liquid flow (sewage) being treated (Metcalf & Eddy (Inc.), 1991). Different types of sludge can be generated depending on the treatment technology applied; the more complex the flowchart of a WWTP, the greater the variability of the sludge produced. Figure 1 presents details of the treatment levels in a traditional wastewater treatment system and the different sludges generated in the plant.

To define the most appropriate treatment, it is important to know the variety of sludge that exists, considering its characteristics for adequate disposal and the volume reduction to be achieved. In the primary clarifier, primary sludge is generated, consisting of suspended solids from the sewage. Secondary or biological sludge is generated in biological treatment. Primary sludge is richer in lipids than secondary sludge, which has more nitrogen. However, large concentrations of nitrogenous and phosphorous organic material are commonly found in both primary and secondary sludge. Treatment before final disposal can be done with the primary sludge or secondary sludge separately or with both sludges together (mixed sludge) (Raposo *et al.*, 2011).

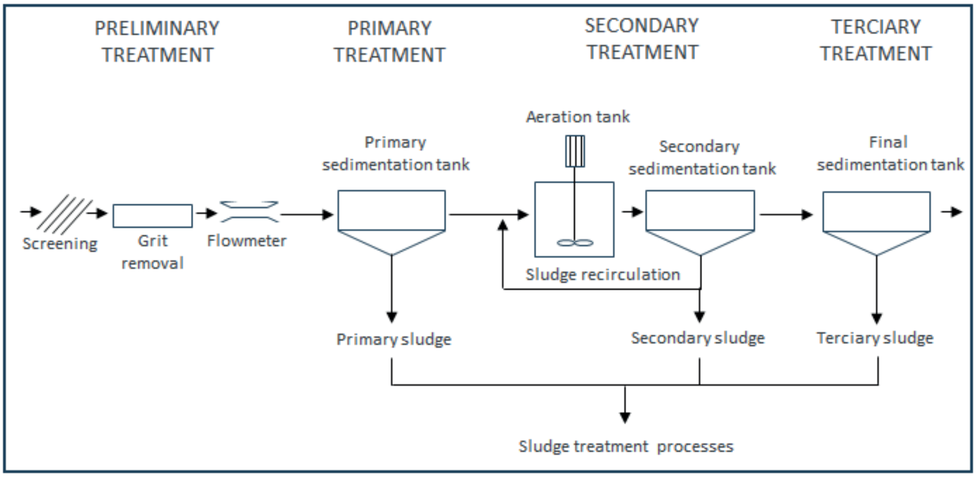


Figure 1 - Stages of a conventional wastewater treatment system and the different types of sludge generated.

Biological sludge from activated sludge systems is basically composed of microorganisms, mostly live bacteria, which grew using sewage as a substrate (Fernández; Sánchez; Font, 2005). Microbiological aggregates are held together in a three-dimensional gelatinous matrix by extracellular polymeric substances (EPS) (Flemming; Wingender, 2002), which are the main components of activated sludge flocs (Ye *et al*., 2011). These biopolymers can be formed by carbohydrates, proteins, nucleic acids, lipids, and humic substances. EPS normally have proteins and carbohydrates as their dominant components, in addition to lipopolysaccharides, glycoproteins, and lipoproteins (Shi *et al*., 2017). Several microorganisms can produce specific EPS, resulting in different exopolymers in the biological floc (Flemming; Wingender, 2002).

Sewage sludge can contribute to the proliferation of disease vectors; may contain heavy metals, persistent organic compounds, and pathogens; and has potential health and environmental risks (Fijalkowski *et al*., 2017). Thus, the final disposal of waste sludge becomes an environmental problem, and WWTPs are constantly looking for alternatives to solve the problem and for more efficient treatments.

The sludge must be removed to prevent it from accumulating in the treatment units or being released together with the final wastewater. Numerous sludge treatment alternatives are currently available. However, AD is certainly the most used, as in addition to being one of the best solutions for stabilizing and partially sanitizing the organic matter contained in sludge (around 2/3 of organic matter can be removed from the sludge), it can also produce renewable energy (through biogas formed in the process) (Khanal, 2008; Iacovidou; Ohandja; Voulvoulis, 2012).

## 3. ANAEROBIC DIGESTION AND BIOGAS PRODUCTION

Anaerobic digestion is a process that occurs in the absence of oxygen, in which several microorganisms convert organic matter into gases, mineral salts, and new cells. The main stages of the AD process are hydrolysis, acidogenesis, acetogenesis, methanogenesis, and sulfetogenesis (Chernicharo, 2007). The initial organic matter is degraded into simpler substances throughout these stages by different groups of microorganisms, which successively decompose the products of the previous stages (Al Seadi *et al.*, 2008). The simplified diagram in Figure 2 illustrates the metabolic routes and microbial groups involved in the AD process.

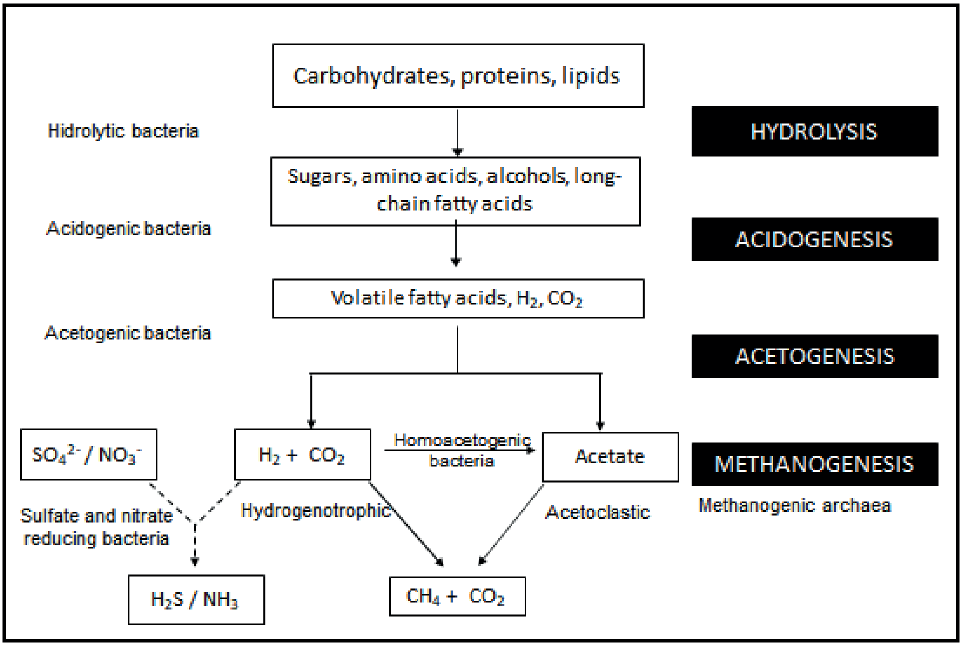


Figure 2 - Steps in the anaerobic digestion of organic matter.

Anaerobic digestion can be applied to stabilize various wastes, such as WWTP sludge, agricultural waste, and animal excrement (Chernicharo, 2007). It is an attractive technology for countries where average ambient temperatures are above 20 °C, as anaerobic microorganisms show low activity at temperatures below this, and heating the reactors is economically unfeasible (Aiyuk *et al.*, 2006).

In the 1950s and 1960s, the high availability of traditional energy sources discouraged the use of biogas in most developed countries. Countries with limited capital and energy resources used biogas in small rural regions to reduce the consumption of firewood, decrease indoor air pollution, and improve soil fertility (Vögeli *et al.* 2014). However, the environmental crisis led to the adoption in 1997 of the Kyoto Protocol, which is an international treaty to reduce the emission of greenhouse gases. Brazil adopted the Clean Development Mechanism, which is the only mechanism in the Kyoto Protocol that admits voluntary participation from developing countries (Bittencourt; Bush; Cruz, 2019). Moreover, the increase in the price of fuels has made methane generated in anaerobic digesters an interesting energy source.

In the last decade, biogas has increasingly been used as an energy source due to the incentive of the carbon credits market. Currently, Brazil has 530 biogas plants (ANEEL, 2023), which use biogas to generate thermal, electrical, and mechanical energy and produce renewable natural gas (biomethane). The main substrates currently employed are sludge from municipal WWTPs, manure, crops and crop residues, waste from the food and feed industries, source-sorted food waste, and slaughterhouse waste (Schnürer; Jarvis, 2019).

The use of biogas from WWTPs is energy efficient, in addition to meeting the requirements for cleaner production (EPA, 2011). Worldwide, WWTP biogas comes mainly from sludge digesters. At WWTP in Ribeirão Preto (São Paulo, Brazil), for example, sewage treatment is conducted in a conventional activated sludge unit, and excess sludge is sent to sludge digesters. Biogas, which was previously burned in the flare, emitting greenhouse gases, is now used to generate electrical energy consumed in the plant itself, reducing its operational cost (Probiogás, 2017).

The composition of biogas can vary depending on the material used in decomposition and the efficiency of the biodigester. The constituents normally present in biogas generated by AD of different organic wastes are represented in Table 1. Biogas is mainly composed of methane and carbon dioxide, with small amounts of other substances, including $H_2S$. Although $H_2S$ is a flammable gas that contributes to the energy potential of biogas, it causes corrosion in storage tanks, metal pipes, and combustion engines. Thus, high concentrations of $H_2S$ result in the deterioration of biogas production infrastructure.

Table 1 - Average biogas composition

| **Gases** | **Composition (%)** |
|---|---|
| **Methane ($CH_4$)** | 50 – 75 |
| **Carbon dioxide ($CO_2$)** | 25 – 45 |
| **Water vapor ($H_2O$)** | 2 (20 °C) - 7 (40 °C) |
| **Nitrogen ($N_2$)** | < 2 |
| **Oxygen ($O_2$)** | < 2 |
| **Ammonia ($NH_3$)** | < 1 |
| **Hydrogen sulfide ($H_2S$)** | < 1 |
| **Hydrogen ($H_2$)** | < 1 |

Fonte: Al Seadi *et al*. (2008).

The energy potential of biogas is established according to the amount of methane contained in the gas. Considering biogas with the standard methane content of 50%, the heating value is 21 MJ/Nm³. Biogas can be used directly or purified and stored for later use. Currently, it can be used to produce heat by direct combustion, electricity by fuel cells or micro-turbines, combined heat and power, or as vehicle fuel (Al Seadi *et al.*, 2008).

## 4. ENZYMATIC PRETREATMENT OF SLUDGE FOR ANAEROBIC DIGESTION

Conventional AD of sludge employs long hydraulic retention times (30 to 40 days) due to the low conversion rates of microorganisms, especially during the hydrolysis step, which is a limitation of this treatment. Applying a pretreatment to sludge to disrupt the cell wall and facilitate the release of intracellular matter, thereby increasing biodegradability, can improve anaerobic digestion. Anaerobic digestion of pretreated sludge has a shorter hydraulic retention time and higher biogas production (Pavlostathis; Gosset, 1986; Pavlostathis; Giraldo-Gomez, 1991).

Recent research shows that sludge pretreatment contributes to better development of the AD process. Different pretreatment techniques include thermal hydrolysis; physical processes for cell destruction and lysis; chemical processes with acids, alkalis, and oxidants; and biological processes using enzymes. However, these technologies are not economically viable, limiting their expansion and implementation (Neumann *et al.*, 2016).

Enzymes are molecules composed of polymers of amino acids (proteins) covalently linked by peptide bonds, with the molecular structure formed by hydrogen bonds, hydrophobic interactions, disulfide bonds, van der Waals forces, and ionic bonds. Enzymes are biocatalysts whose catalytic activity, stability, and specificity depend on their three-dimensional structure (Antranikian; Vorgias; Bertoldo, 2005; Olempska-Beer *et al.*, 2006).

In anaerobic digestion, sludge microorganisms produce and secrete hydrolytic enzymes to convert macromolecular structures into soluble matter, such as simple sugars, amino acids, glycerol, and fatty acids, facilitating transport across the cell membrane (Mshandete *et al.*, 2007).

The addition of hydrolytic enzymes favors the decomposition of insoluble organic molecules in activated sludge flocs and their conversion to simpler molecules (Wawrzynczyk *et al.*, 2008), leading to benefits such as increased methane production (Davidsson; Jansen, 2006; Dursun *et al.*, 2006) and sludge stabilization (Yang *et al.*, 2010). Figure 3 shows a generic scheme of enzymatic sludge hydrolysis followed by biogas/methane production in anaerobic digestion. Insoluble organic substances (polymers and macromolecules) from the EPS matrix, from the composition of membranes and cell walls or inside cells, are hydrolyzed to substances with lower molecular mass, soluble and readily biodegradable. These substances then serve as substrates for anaerobic microorganisms that convert them to methane in anaerobic digestion.

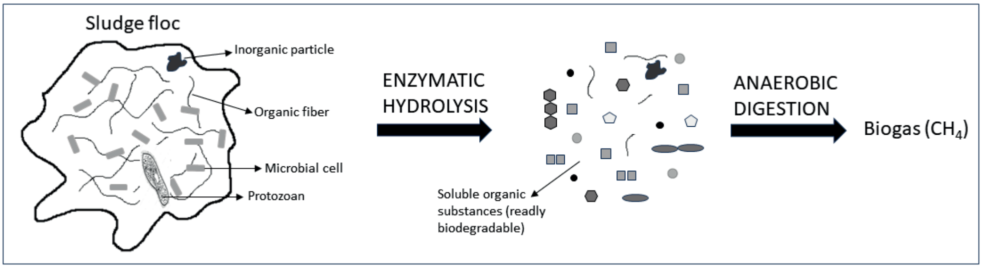


Figure 3 - Scheme of enzymatic sludge hydrolysis followed by biogas/methane production in anaerobic digestion.

Higuchi *et al*. (2005) reported that α-amylase can increase the efficiency of sludge lysis and that this enzyme may be associated with the cells or EPS of the flocs responsible for the hydrolysis of organic solids in the sludge. The use of cellulase can improve the efficiency of sludge hydrolysis. Nouha *et al*. (2018) found that *Acetobacter xylinum* bacteria synthesize cellulose for sludge flocculation. Furthermore, the sludge may contain large amounts of cellulose from other sources, mainly in municipal WWTPs and in paper and textile industries (Parmar; Singh; Ward, 2001), and hemicellulose may also be present in the sludge. Thus, the degradation of hemicellulose by hemicellulase enzymes is interesting, as it results in easily assimilable monosaccharides, such as xylose, glucose, galactose, arabinose, and mannose (Parawira, 2012).

Proteins and carbohydrates are the main components of activated sludge and its EPS, with proportions that vary with the origin of the activated sludge (Houghton; Quarmby; Stephenson, 2001; Liu; Fang, 2002). Yasunori (1994) showed that lysozyme acts in the hydrolysis of sludge by comparing the reduction in volatile suspended solids (VSS) of a concentrated sludge inoculated with bacteria that secrete lysozyme with that obtained with the same sludge without any inoculation. The VSS reduction in the inoculated sludge reached 62% after five days, while in the uninoculated sludge, it was less than 10%. The lysozyme secreted by the inoculated bacteria probably acted on the polysaccharides in the cell wall of the sludge bacteria.

Recent works have demonstrated that the application of commercial enzymes is an efficient option for the biological pretreatment of sludge, accelerating the hydrolysis step of sludge digestion. The enzymes used include amylases, cellulases, proteases, and lyzosyme (Yang *et al*., 2010; Rashed *et al*., 2010; Luo *et al*., 2012; Xin *et al*., 2016, 2018; Zhang *et al*., 2022; Kang *et al*, 2023). Different enzymes and their mixtures have varying effects on sludge hydrolysis. Therefore, selecting the appropriate enzymes is an important step to maximize the impact of pretreatment in anaerobic digestion.

Yang *et al*. (2010) evaluated the effect of different commercial protease and α-amylase mixtures on the hydrolysis of secondary sludge. The addition of exogenous enzymes increased the biological hydrolysis of the sludge, with amylase exhibiting higher hydrolysis efficiency (54.2%) than protease (39.7%). However, a greater reduction of VSS (68.4%) and solubilization of organic matter were obtained with a 1:3 protease: amylase mixture. Luo *et al*. (2012) evaluated the individual action of the α-amylase enzyme in sludge pre-hydrolysis. They obtained a 32.7% reduction in VSS and an 11-fold increase in carbohydrate solubilization compared to sludge without enzyme addition.

Rashed *et al*. (2010) studied the effect of six commercial enzyme mixtures on different combinations of primary sludge, secondary sludge, and digested sludge. The commercially available enzymes were Alcalase, Caresyme (imiglucerase), Celluclast (cellulase complex), Lipolase, Termamyl, and Viscosyme (multi-enzyme complex containing a wide range of enzymes, including arabinase, cellulase, β-glucanase, hemicellulase, and xylanase). The VSS reduction in their study depended on enzyme and sludge combinations; nevertheless, the authors demonstrated that enzymatic pretreatment can be used successfully to treat sludge mixtures produced in WWTPs.

Sludge dewaterability is essential for treatment and disposal of waste-activated sludge from WWTPs, and enzymatic biological conditioning is a promising method to increase its dewaterability. Kang *et al*. (2023) investigated the ideal conditions of pH, temperature, bioenzyme dosage, and treatment time for five biological enzymes (α-amylase, cellulase, acidic protease, neutral protease, and alkaline protease). α-Amylase and neutral protease performed well in condition optimization experiments. After biological enzymatic conditioning of the sludge, with 15.5 g/L TSS (total suspended solids) and 80.2% water content, the concentration of proteins, polysaccharides, and soluble chemical oxygen demand increased in the liquid phase. Sludge water content decreased while capillary suction time increased. The optimal conditions for α-amylase were pH 6, 45 °C, dosage of 30 mg/g TSS, and 3 h treatment time. Under these conditions, the water content reached its lowest value (68.7%). The optimal conditions for neutral protease were pH 6.5, 40 °C, dosage of 30 mg/g TSS, and 2 h treatment time, with water content of 69.8%.

Xin *et al*. (2016) studied a mixture of hydrolytic enzymes (lysozyme, α-amylase, protease, and cellulase) in sludge digestion. Soluble chemical oxygen demand, proteins, and carbohydrates in solution substantially increased to 6000–9000 mg/L, 1500–3000 mg/L, and 550–700 mg/L, respectively, after 180 min of sludge digestion with the enzyme mixture. In a later study, Xin *et al*. (2018) evaluated AD after enzymatic hydrolysis of sludge using the same mixture of enzymes and proportions. The authors proved that enzymatic catalysis improved biodegradability, obtaining 100 mL biogas/g VSS, greater yield than AD of only sludge.

The various published studies prove that enzymatic pretreatment of sludge accelerates the hydrolysis process, increasing the degree of sludge disintegration in a short operating time (Xin *et al*., 2016; Luo *et al*., 2012; Yang *et al*., 2010; Rashed *et al*., 2010), and contributes to better performance of AD (Xin *et al*., 2018).

## 5. FERMENTATIVE PROCESSES FOR ENZYME PRODUCTION

Fermentative processes are used to convert raw materials into products or to physically and/or chemically modify the material through the action of microorganisms. They are widely used for the production of bioproducts, such as enzymes. These processes are classified into submerged fermentation (SF) and solid-state fermentation (SSF). The difference between the two fermentations is the amount of free water in the cultivation medium. In SF, for the production of enzymes, the solids content usually does not exceed 50 to 100 g/L, while in SSF, the solids content varies from 20 to 70% of the total mass. Thus, SF uses abundant free water and a lower content of solids, while SSF occurs with a low amount (or absence) of water not absorbed into the solid material (Mitchell; Berovic; Krieger, 2002; Singhania *et al*., 2009).

### 5.1. SOLID-STATE FERMENTATION

In SSF, the water content is sufficient for the microorganisms to grow. Generally, the only component necessary to add to the medium is water, with or without the addition of minerals, nitrogen, and easily assimilated carbon sources, depending on the characteristics of the raw material (Pandey, 2003).

SSF using filamentous fungi was primarily introduced to produce enzymes and other products in the textile and food industries. An example is the use of enzymes produced by *Aspergillus oryzae* in Eastern countries to manufacture soy sauce (Shoyu), miso, and sake (Machida; Yamada; Gomi, 2008). Later, SSF became an interesting process for regions with large agro-industrial production, which generates different types of residual biomass, which is a lower-cost raw material that can be used as a support medium and/or source of nutrients in the cultivation medium of different biotechnological processes (Pandey, 2003; Singhania *et al*., 2009).

In addition to the production of enzymes, SSF is used in the production of aromas, bioactive products, biopesticides, organic acids, and fermented foods, as well as in the bioremediation and biodegradation of compounds and biological detoxification of agro-industrial waste (Pandey, 2003;).

The main parameters evaluated during fermentation processes are aeration rate, temperature, moisture and water activity, granulometry, and heat and mass transfer. These parameters are extremely important for microbial growth and, consequently, for the fermentation process (Mitchell; Berovic; Krieger, 2002). The low conductivity of the solid substrates used in SSF, combined with the low amount of water in the process, forms temperature, moisture, substrate, and product gradients. The formation of temperature gradients is one of the main problems of SSF, which is not observed in SF due to the use of a large amount of water and agitation (Te Biesebeke *et al*., 2002). In SF, the main operational difficulty is the transfer of oxygen to microorganisms due to the low solubility of oxygen in water. In SSF, oxygen transfer is generally not limiting, as the liquid phase around the solid substrate allows oxygen transfer, which can also occur directly from the gas phase in the case of filamentous fungi (Schutyser *et al*., 2003).

Compared to SF, SSF has advantages such as the wide use of waste as culture media, lower risk of contamination, low energy demand and operational costs, less complex equipment, and higher yields. The disadvantages are greater difficulty monitoring and controlling the process; furthermore, not all microorganisms can develop under greater water restrictions.

## 5.2. MICROORGANISMS IN SOLID-STATE FERMENTATION

Due to the low quantity of water, a limited number of microorganisms can perform SSF. The most commonly used microorganisms in SSF are filamentous fungi as well as some yeasts and bacteria (Pandey, 2003; Singhania *et al*., 2009).

Filamentous fungi are eukaryotic and multicellular organisms with elongated and branched cells (hyphae), primarily reproduce by the production of spores, easily disperse through air and water, among others. Filamentous fungi have varied morphology, physiology, and biochemistry. They grow in SSF conditions because these are similar to their natural habitat. The hyphae penetrate into the agro-industrial residue (substrate/support), allowing filamentous fungi greater accessibility to nutrients and making them the most applied microorganisms in SSF (Richards *et al*., 2006; Santos *et al*., 2004).

Filamentous fungi are heterotrophic and can metabolize a huge variety of organic materials and convert them to a range of enzymes (lipases, amylases, cellulases, xylanases, mannanases, proteases, among others). Therefore, filamentous fungi are widely used to produce enzymes and catalyze the hydrolysis of different molecules (Corbu *et al*., 2023).

Some species of filamentous fungi of the genus *Aspergillus* have GRAS (Generally Recognized as Safe) status by the Food and Drug Administration (FDA, USA). These microorganisms are considered safe due to their historical use in the food industry (cheese and shoyu) and the traditional beverage industry (sake), as well as in the production of enzymes (Iwashita, 2002).

Several species of Aspergillus, such as *Aspergillus niger*, *A. nidulans*, *A. oryzae*, *A. versicolor*, and *A. awamori*, can synthesize different extracellular enzymes, according to their needs, which have applications in several biotechnological areas (Cui *et al*., 1998; Souza-Motta *et al*., 2005).

The following are some examples of enzyme production by SSF using filamentous fungi of the genus *Aspergillus*. Vishwanatha, Appu Rao, and Singh (2010) reported the production of nearly 40,000 U/g of protease by SSF after 120 h. The authors used *A. oryzae* (MTCC 5341) and wheat bran with 4% defatted soy flour, and 2% skim milk as the substrate.

Pirota *et al*. (2013) indicated that *A. oryzae* (P6B2) produced xylanase (2,830 U/g) in the fungal cultivations carried out for 72 h using wheat bran as the solid substrate in a bioreactor at 35 °C and initial moisture of 80%. Francis *et al*. (2002) demonstrated that *A. oryzae* is also capable of producing α-amylase, using spent brewing grains as the sole carbon source; a maximum production of 6,870 U/g of dry substrate was achieved with 70% initial moisture and 30 °C after 96 h.

The effects of operating conditions to produce cellulase by *A. oryzae* were evaluated by Pirota *et al.* (2016), with wheat bran as substrate in a bioreactor with online monitoring and control of airflow and relative humidity of the incoming air. The authors found the highest production of FPase (0.4 IU/g), endoglucanase (123.6 IU/g), and β-glucosidase (18.3 IU/g) at 28 °C, using an initial moisture content of 70%, with an inlet air humidity of 80% and an airflow rate of 20 mL/min for 72 h. López *et al.* (2013) reported the variety of enzymes that *A. awamori* (IOC-3914) can secrete, obtaining a crude enzyme solution with endoamylases (22.8 U/mL), exoamylases (14.1 U/mL), proteases (1.9 U/mL), xylanases (12.3 U/mL), and cellulases (1.2 U/mL) after 96 h of SSF at 30 °C and moisture of 70% with babassu cake as the substrate.

Dias *et al.* (2018) applied the fungus *A. niger (*SCBM1) in biomass sorghum supplemented with peptone. Maximum production levels were achieved for xylanase and exoglucanase (300 and 31 U/g, respectively) after 72 h of fermentation, for *β*-glucosidase and endoglucanase (55 and 41 U/g, respectively) after 120 h, and for *β*-xylosidase (65 U/g) after 144 h.

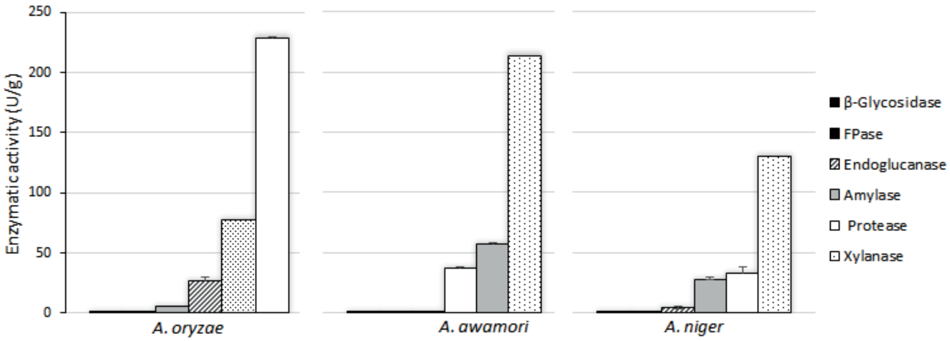
Amylolytic enzymes were produced by SSF, using the fungus *A. niger* and groundnut oil cake medium for 144 h at 37 °C. After the addition of a nitrogen source, a high yield of enzyme production (320 U/mg protein) was obtained (Suganthi *et al*., 2011).

Ahmed (2018) reported the use of SSF with *A. niger* and wheat bran as a substrate with different nitrogen sources added to produce protease. Specific activity of 3.5 U/mg protein was obtained with an initial moisture of 67% at 40 °C for 8 days.

## 6. ENZYMATIC HYDROLYSIS AND ANAEROBIC DIGESTION OF SLUDGE

Secondary sludge pretreatment using commercial enzymes and enzymes produced by SSF can increase the efficiency of sludge stabilization by anaerobic digestion. Silva (2020) treated different sludge samples with commercial enzymes – amylase (Termamyl – Novozyme), cellulase (Celluclast – Novozyme, containing β-glycosidase, endoglucanase, and FPase activities), protease (Alkalase – Novozyme), and hemicellulase (Sigma, xylanase) – and with enzymatic extracts obtained by SSF with filamentous fungi of the genus *Aspergillus* (*A. oryzae, A. awamori, and A. niger*).

Babassu cake (agroindustrial waste) was used as the basal medium for SSF, which was conducted at 65% initial moisture and 30 °C for 72 h. The fungi produced the enzymes of interest, with the highest production of protease and endoglucanase by *A. oryzae*. *A. awamori* produced the most amylase and xylanase. Despite not showing the highest production of any enzyme, *A. niger* had good production of amylase, xylanase, and protease (Figure 4). With the exception of endoglucanase, cellulases showed low production, which is in accordance with the low concentration of cellulose (6.94%) in the babassu cake (Castro; Castilho; Freire, 2016).



* All enzymes present values in U/g, with the exception of FPase in FPU/g.

Figure 4 - Enzymatic activities (U/g)* in the enzyme preparations obtained in SSF (72 h) with babassu cake as basal medium and the filamentous fungi *A. oryzae*, *A. awamori,* and *A. niger* (adapted from Silva, 2020).

## 6.1. PRE-HYDROLYSIS OF SLUDGE WITH COMMERCIAL ENZYMES

Pre-hydrolysis (rotary shaker at 100 rpm, for 5 h at 50 °C) and AD (in an oven at 35 °C until the stabilization of biogas production) were evaluated by VSS reduction and biogas/methane yield, respectively. Commercial enzymes were evaluated individually in the hydrolysis of sludge (5 g VSS/L) with enzymes (1 U/mg TSS). Only protease (16%) and lysozyme (15%) obtained a statistical reduction of VSS greater than that obtained in the Control assays (without enzyme, 4%). Hemicellulase (10%) and amylase (9%) presented statistically similar values to those of the Control (Silva, 2020).

Other authors have found higher VSS reduction values for amylase (33%, Luo *et al.*, 2012) and amylase and protease (54% and 40%, Yang *et al.*, 2010), probably due to the different concentrations of enzymes (0.36 U/g TSS amylase and 0.30 U/mg TSS amylase and protease, respectively), specificity of the commercial enzyme preparations and different compositions of the sludge used in the tests. The composition of the sludge is influenced by the characteristics of the treated wastewater, which has different organic matter content, C/N ratio, and nutrients (Mtshali; Tiruneh; Fadiran, 2014). Likewise, the chemical structure of EPS depends on the different environmental conditions in which the microbial cells grew (Nouha *et al.*, 2018).

Anaerobic digestion of sludge after hydrolysis with separate commercial enzymes led to VSS reduction and biogas yield greater than in the Control test. However, the biogas production was quite low.

## 6.2. SIMULTANEOUS HYDROLYSIS AND ANAEROBIC DIGESTION OF SLUDGE WITH MULTIENZYME EXTRACTS FROM SSF

In the evaluation of multienzyme extracts from SSF, a total enzyme concentration of 0.2 U/mg TSS was applied immediately before sludge incubation (22 g VSS/L) for anaerobic digestion. After 20 days at 35 °C, the sludge added of the extract containing the mixture of enzymes from *A. oryzae*, *A. awamori,* or *A. niger* showed twice as much VSS reduction as the Control (with sludge only). The biogas volume was four times greater in the mixture of sludge and enzymatic extract from the fungi *A. oryzae* and *A. niger*, compared to that obtained in the Control (Figure 5). In the mixture of sludge and *A. awamori* enzymatic extract, the biogas yield increased by approximately 1.7 times, compared to the Control (Silva, 2020).

The addition of enzymes to the sludge contributed to better hydrolysis, releasing a greater amount of organic matter that is easily assimilated by microorganisms and consequently more efficient in methane production. Among the mixtures, the one containing the enzymatic extract from *A. niger* obtained the highest concentration of methane in the biogas generated in anaerobic digestion.

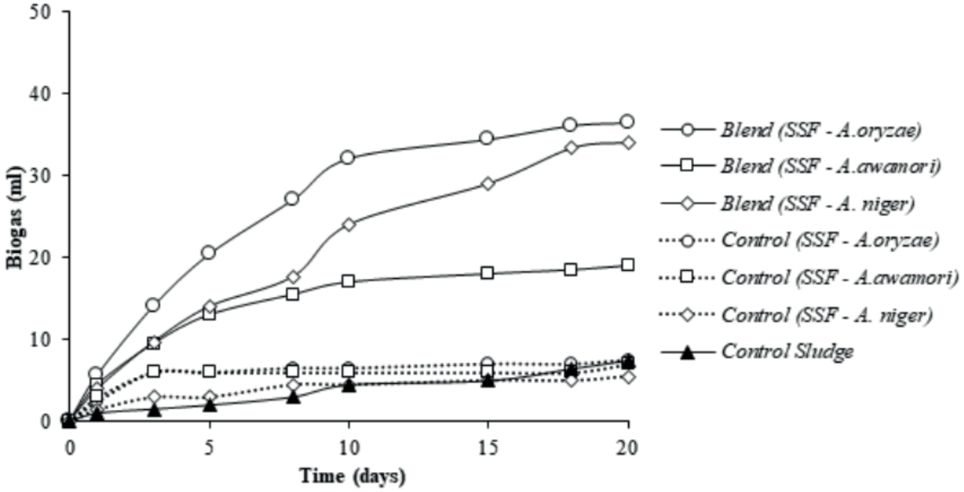


Figure 5 - Biogas production (mL at 35 °C) from secondary sludge (~22 g VSS/L) without (Sludge Control) and with enzymatic extracts from SSF with fungi (*A. oryzae, A. awamori*, and *A. niger*) at a concentration of 0.2 U/mg TSS. Controls (SSF – fungus) refer to tests with enzyme extracts and inoculum.

## 6.3. ANAEROBIC DIGESTION OF SLUDGE WITH MULTIENZYME EXTRACT FROM SOLID-STATE FERMENTATION AND COMMERCIAL ENZYMES

The use of enzymes produced by SSF, in addition to having a lower cost than commercial enzymes, have environmental benefits providing applicability to solid waste. Thus, Silva (2020) carried out a comparison of the enzymatic extract produced by the fungus *A. niger* and a mixture of commercial enzymes in the same proportions found in the SSF extract (total concentration of 0.2 U/mg TSS) as a way to evaluate the action of this extract and possible cost reduction.

Table 2 shows a comparison of the main results of VSS reduction, $CH_4$ molar concentration, and biogas/methane yields for the tests with sludge with or without enzymes. VSS reductions of 32, 23, and 16% were obtained after 12 days of AD at 35 °C of sludge with commercial enzymes blend, SSF blend, and sludge alone (Control), respectively. The biogas volume in the mixture of sludge and SSF blend, after discounting the volume of biogas produced by its enzymatic extract Control, reached 62 mL after 12 days at 35 °C. The biogas volume from the mixtures of sludge with commercial enzyme blend, also discounted from the enzymatic extract Control, reached 52.2 mL, a value close to that shown by the sludge control (53 mL). Thus, the mixture of sludge and SSF blend obtained about 1.2 times more biogas production than both the sludge Control and a mixture of sludge and commercial enzymes blend.

Table 2 - Comparison of the main results of VSS reduction, % molar methane, and biogas/methane yields after anaerobic digestion at 35 °C in tests of sludge with or without added enzymes

(adapted from Silva, 2020).

| Condition | AD time (days) | VSS reduction (%) | Biogas (mL) | %Molar $CH_4$ | Biogas yield[1] | Methane yield[1] |
|---|---|---|---|---|---|---|
| Control (sludge) | 27 | 17.1±2.3 | 56.0±2.0 | 33.5±2.7 | 73.1 | 24.5 |
| | 12 | 16.3±2.3 | 53.0±2.0 | 33.5±3.2 | 69.2 | 23.2 |
| SSF blend (*A. niger*) | 27 | 24.3±4.2 | 76.0±6.0 | 48.1±6.1 | 80.3 | 49.5 |
| | 12 | 22.8±4.2 | 69.7±1.5 | 41.7±7.7 | 81.8 | 34.1 |
| Commercial enzymes blend | 12 | 32.2±3.3 | 61.7±1.2 | 52.4±0.8 | 68.1 | 32.3 |

[1] Biogas yield and methane yield (mL biogas or $CH_4$/g VSS added) of the mixtures calculated by discounting the volume of biogas or methane from the enzymatic extract Control.

The action of the enzymatic SSF blend in sludge of the same batch (~ 17 g VSS/L and 0.2 U/mg TSS) and different AD times (12 and 27 days) showed similar values of VSS reduction (~ 23%). However, with a longer duration of anaerobic digestion, the methane yield increased by approximately 1.4 times. In this case, the best condition should be chosen by the WWTP depending on whether they desire a higher methane yield or shorter retention time.

## CONCLUSIONS

The addition of enzymes to secondary sludge can be a viable alternative to accelerate the hydrolysis process and increase the performance of anaerobic digestion. Enzymes alone or in mixtures can have different effects on sludge hydrolysis.

Commercial enzyme mixtures are more efficient in sludge hydrolysis, but their application increases the cost of sludge treatment. However, enzyme extracts obtained by SSF are low cost, and filamentous fungi can synthesize a wide range of enzymes from varied raw materials. Therefore, this technology is an attractive alternative to promote the AD of secondary sludge.

Studies still need to be carried out to understand the mechanism of action of enzymes in sludge degradation and obtain the best conditions for applying enzymes for pretreatment of sewage sludge.

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