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Review

Recent Approaches for the Production of High Value-Added Biofuels from Gelatinous Wastewater

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Abstract: Gelatin production is the most industry polluting process where huge amounts of raw organic materials and chemicals (HCl, NaOH, Ca^{2+}) are utilized in the manufacturing accompanied by voluminous quantities of end-pipe effluent. The gelatinous wastewater (GWW) contains a large fraction of protein and lipids with biodegradability (BOD/COD ratio) exceeding 0.6. Thus, it represents a promising low-cost substrate for the generation of biofuels, i.e., H_2 and CH_4, by the anaerobic digestion process. This review comprehensively describes the anaerobic technologies employed for simultaneous treatment and energy recovery from GWW. The emphasis was afforded on factors affecting the biofuels productivity from anaerobic digestion of GWW, i.e., protein concentration, organic loading rate (OLR), hydraulic retention time (HRT), the substrate to inoculum (S_0/X_0) ratio, type of mixed culture anaerobes, carbohydrates concentration, volatile fatty acids (VFAs), ammonia and alkalinity/VFA ratio, and reactor configurations. Economic values and future perspectives that require more attention are also outlined to facilitate further advancement and achieve practicality in this domain.

Keywords: gelatinous wastewater; protein degradation; anaerobic digestion; energy recovery; biofuels

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1. Introduction

The gelatin production in the world exceeds 375,000–400,000 tons per year which has a considerable share in the economy of countries [1]. Each ton of raw materials, mainly bones, produces 300 m^3 of gelatinous wastewater [2]. The gelatin industry generates huge quantities of heavily polluted wastewater containing proteins, carbohydrates, and lipids [3–6]. The gelatin processing end-of-pipe effluents have high organic contents (i.e., biochemical oxygen demand (BOD), chemical oxygen demand (COD), suspended solids (SS), and ammonia nitrogen (NH_4-N) [3,4]. Moreover, the gelatinous wastewater (GWW) contains high concentrations of sulfate (SO_4^{-2}), calcium, and phosphorus [4,7,8]. Dicalcium phosphate is mainly produced during the de-mineralization process of bones [2,9]. The main serious problem from gelatinous wastewater (GWW) is the odor, nuisance, and that it is highly obnoxious to the habitation [2,10]. The GWW could be alkaline or acidic based on the manufacturing process and the utilized raw materials [9]. GWW is greasy and fibrous containing bovine bone pieces and hairs, resulting in high quantities of particulate organic matters [11,12]. The particulates mainly consist of animal crushed

bones, skin, and hair, which is highly obnoxious [9]. The COD of GWW ranged from 9691.2 to 23,016 mg/L due to the usage of raw materials rich in proteins, lipids, and carbohydrates. The bovine hide is soaked in a lime tank at a pH exceeding 12.0 for 10–12 weeks, resulting in high residual values of calcium in the wastewater streams ranging from 3175.3 to 5210 mg/L and alkaline pH of 11.1–12.4. Sodium hydroxide is added during the manufacturing process resulting in alkalinity of 5432–6527 mg $CaCO_3$/L [3,5]. Sulfate (SO_4^{-2}) (1000–1496 mg/L) and NH_4–N (163.2–190 mg/L) are produced in the GWW due to the ammonification and acidification processes [3,6]. The discharge of such wastewater into sewerage networks or water streams causes severe pollution problems for drinking water [13–15]. This wastewater should be treated prior to discharging it into the environment due to its negative impact on the water streams [12,16,17]. However, simultaneous treatment and biofuels production from GWW save energy and chemicals and produces less excess sludge [18].

2. Gelatin Compositions, Properties, Manufacturing Processes, Utilization, and Applications

Gelatin is a slightly yellow, translucent, brittle solid matter, nearly tasteless, and odourless [7,18,19]. The gelatin is mainly extracted from the collagen of animals' connective tissue (Figure 1). Collagen represents 25–35% of the total protein in the whole animal body. Gelatin is a high molecular weight protein composed of eighteen types of amino acids (Figure 1). Amino acids contain oxygen, carbon, nitrogen, and hydrogen, forming proteins [20]. They contain an amino group (NH_2) and a carboxyl group (COOH–). Gelatin is composed of 98–99% protein and could be varied based on the source of the original raw materials and manufacturing processing technique [8,21]. The amino acids of gelatin are 22% for glycine, 12% for proline, 12% for hydroxyproline, 10% for glutamic acid, 9% for alanine, 8% for arginine, 6% for aspartic acid, 4% for lysine, 4% for serine, 3% for leucine, 2% for valine, 2% for phenylalanine, 2% for threonine, 1% for isoleucine, 1% for hydroxylysine, <1% for methionine, and histidine and <0.5% for tyrosine.

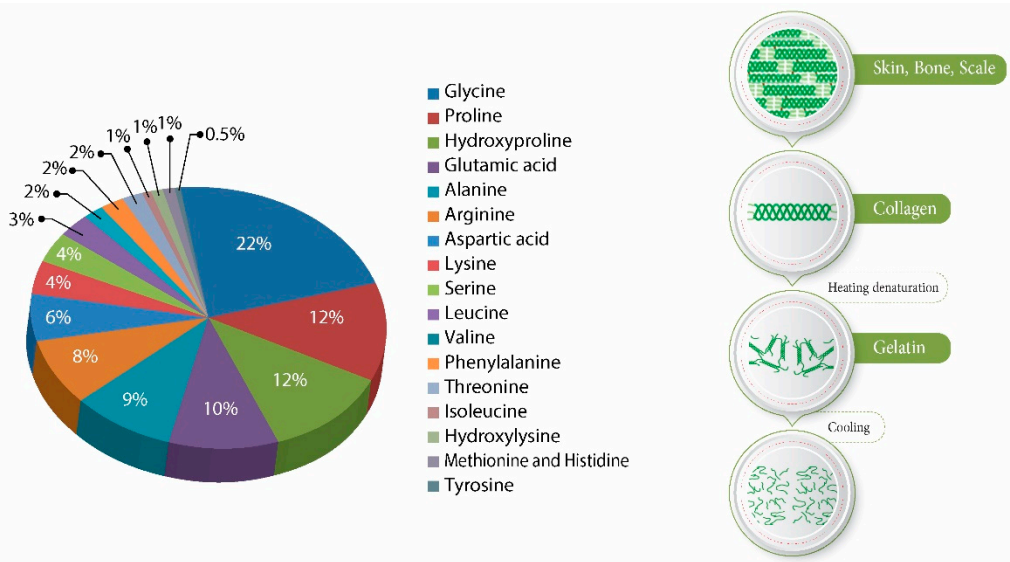

Figure 1. Gelatin composition and general overview of its production process.

The schematic diagram (Figure 2) shows the steps of the manufacturing processes of gelatin from the animal's bones and skin. Gelatin is mainly derived from the skins of pigs and cattle. The raw skin is dried, acidified followed by alkaline processes to extract the collagen [18]. The manufacturing processes of gelatin from animals are comprehensively discussed by [7]. Gelatin applications in the industries are presented in Figure 3.

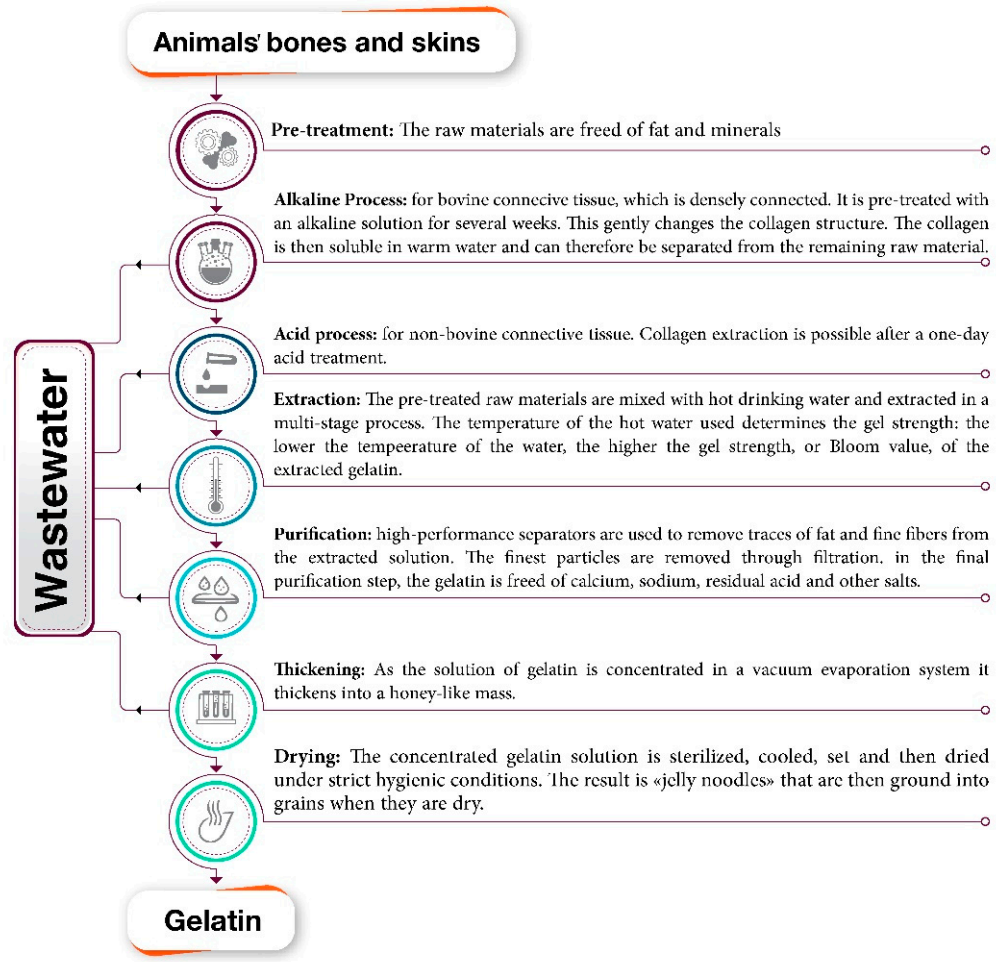

Figure 2. Steps of the gelatin manufacturing processes from animals.

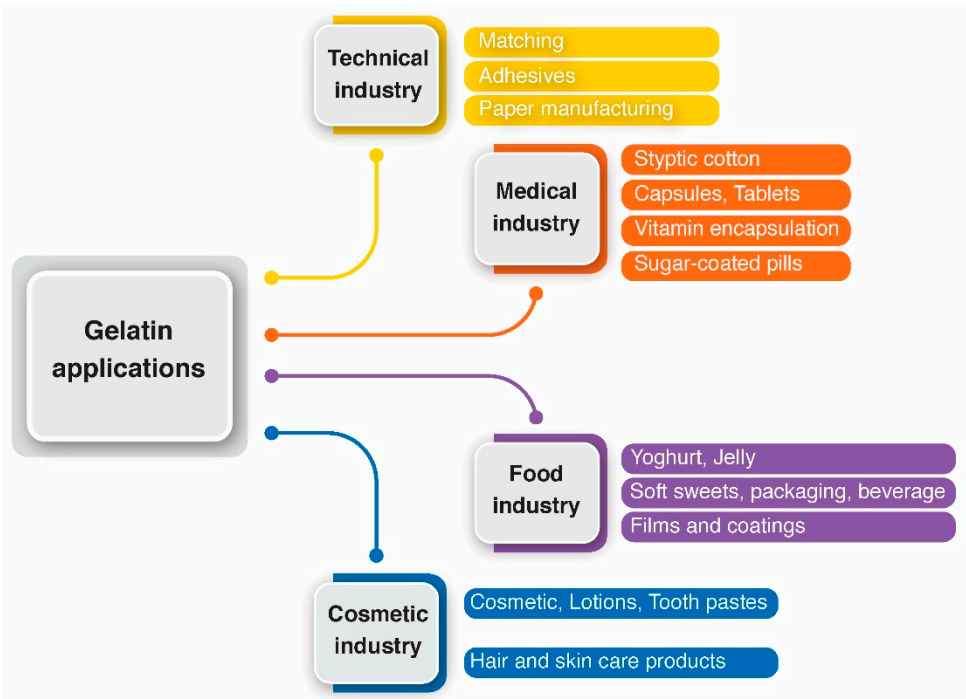

Figure 3. Gelatin applications in the industries.

3. Sustainable Solutions for Simultaneous Treatment and Energy Recovery from Gelatinous Wastewater (GWW)

Several treatment processes were applied to treat GWW, i.e., the coagulation process, which removed only 50% of total organic carbon (TOC). The effluent quality does not comply with discharge standards [2]. The authors further investigated the efficacy of electrocoagulation and electrooxidation for the treatment of GWW. They observed that electrocoagulation using aluminum as anode improved the removal efficiency of TOC by 10% only. Those technologies consume energy and chemicals, produce voluminous sludge, and need particular operation and maintenance skills. Fortunately, GWW enjoys high biodegradability and is suitable for biofuels (H_2 and CH_4) production via the anaerobic digestion process [22]. This approach is promising from an environmental and economic perspective [23,24]. However, anaerobic digestion of protein-rich wastewater often results in scum layer formation and causes sludge washout, which adversely affects the process performance. This problem has significantly hindered the application of the anaerobic process to the treatment of wastewaters from gelatin industries [8,18,19]. Additionally, proteins are degraded more slowly than carbohydrates. Carbohydrates presented in gelatin wastewater lower the protein degradation rate [3,4]. These problems could have been overcome by the intentional discharge of the excess sludge from an anaerobic digester and using a two-stage system for the treatment of gelatinous wastewater [25,26].

3.1. Energy Recovery Pathway from Biodegradation of Gelatinous Wastewater (GWW)

The biodegradability accounted for GWW exceeds 0.6 based on BOD/COD ratio that strongly indicates the suitable anaerobic digestion process for the valorisation of such wastewater. The gelatinous wastewater (GWW) is initially hydrolyzed by proteolytic enzymes, mainly protease, into peptides and amino acids, as shown in Figure 4. Amino acids are individually biodegraded by anaerobes (spore–forming rods) and facultative bacterial species (*Cocci*). The most relevant proteolytic bacteria are *Clostridium* species that have the capability of converting individual amino acids into volatile fatty acids (VFAs) [27,28] (Table 1). The metabolic pathway of anaerobic biodegradation of alanine and glycine is presented in Equations (1) and (2).

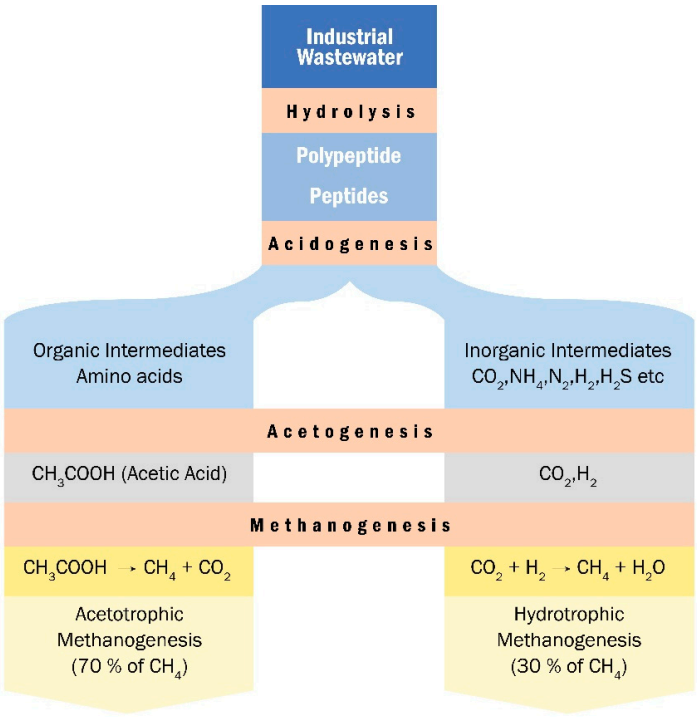

Figure 4. The anaerobic biodegradation of gelatin-rich protein.

Table 1. The anaerobic conversion of individual and pairs amino acids into volatile fatty acids.

1. Conversion of individual and pairs amino acids into volatile fatty acids	
$3\text{Alanine} + 3H_2O \rightarrow 2\text{Propionate}^- + \text{Acetate}^- + HCO_3^- + 3NH_4^+ + H^+$	(1)
$4\text{Glycine} + 4H_2O \rightarrow 3\text{Acetate}^- + 2HCO_3^- + 4NH_4^+ + H^+$	(2)
2. Conversion of individual and pairs amino acids into volatile fatty acids	
$R_1CHNH_2COOH + 2R_2CHNH_2COOH + 2H_2O \rightarrow R_1COOH + 2R_2CH_2COOH + 3NH_3 + CO_2$	(3)
3. Oxidative deamination	
$\text{Alanine} + 3H_2O \rightarrow \text{Acetate}^- + NH_4^+ + HCO_3^- + 2H_2 + H^+$	(4)
$\text{Valine} + 3H_2O \rightarrow \text{Isobutyrate}^- + NH_4^+ + HCO_3^- + 2H_2 + H^+$	(5)
$\text{Leucine} + 3H_2O \rightarrow \text{Isovalerate}^- + NH_4^+ + HCO_3^- + 2H_2 + H^+$	(6)
4. Reductive deamination	
$\text{Glycine} + H_2 \rightarrow \text{Acetate}^- + NH_4^+$	(7)
Sum	
$\text{Alanine} + 2\text{Glycine} + 3H_2O \rightarrow 3\text{Acetate}^- + 3NH_4^+ + HCO_3^- + H^+$	(8)
$\text{Valine} + 2\text{Glycine} + 3H_2O \rightarrow \text{Isobutyrate}^- + 2\text{Acetate}^- + 3NH_4^+ + HCO_3^- + H^+$	(9)
$\text{Leucine} + 2\text{Glycine} + 3H_2O \rightarrow \text{Isovalerate}^- + 2\text{Acetate}^- + 3NH_4^+ + HCO_3^- + H^+$	(10)

Biodegradation of pairs of amino acids by *Clostridia* species occurs following oxidation-reduction reactions [29] Equations (3)–(10). Amino acids conversion is discussed based on the Stickland reaction (Figure 5), where each amino acid (alanine, valine, and leucine) is oxidized (dehydrogenated), and the others (glycine and proline) are mainly reduced (hydrogenated) to complete the biodegrading cycle (Equations (3)–(10)).

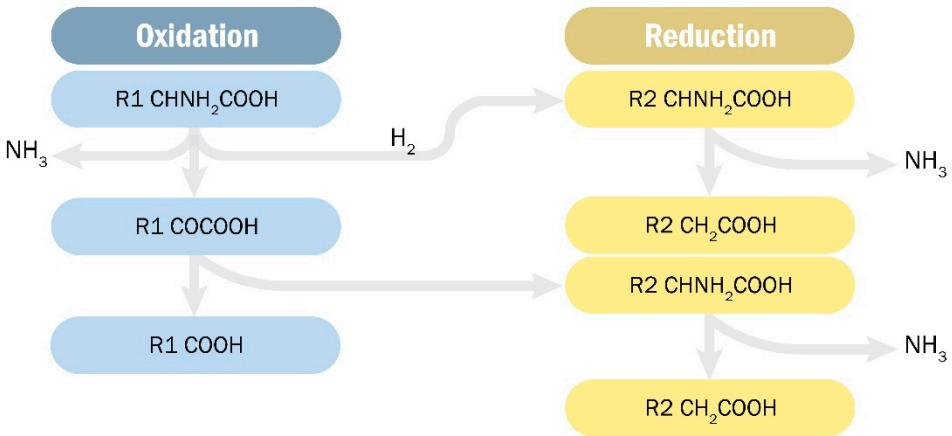

Figure 5. Schematic of the Stickland oxidation-reduction reaction.

Bovine serum albumin (2–4 g/L) was anaerobically converted into amino acids at a pH of 8.0 and incubation time of 3.0 days [30]. The stoichiometric coefficients in their study for VFAs (acetate, butyrate, and valerate) production were comparable with the theoretical values except for propionate produced from the metabolic pathway of proline and arginine. The VFAs were ±15% and ±12% of the theoretical values. This was not the case for stoichiometric coefficients of iso–butyrate and iso–valerate, which accounted for 10% and 15% of the theoretical values, respectively, thereby validating the overall catabolic pathway reaction of anaerobic biodegradation of proteinaceous compounds into volatile organic acids. The fermentation of the amino acids occurred at a pH value of 6.3 [31]. However, protein hydrolysis under anaerobic conditions is the rate-limiting step that adversely affects the overall degradation process [32,33]. Long retention time and imposed low organic loading rate would enhance the hydrolysis step and minimize the limitations of protein degradation. The anaerobic degradation of gelatin was increased from 84.1 to 94.3% when

increasing the HRT from 4 to 24 h. However, the anaerobic conversion efficiency dropped from 98.9 to 89.6% when increasing the gelatin concentration from 2 to 30 gCOD/L [32]. The peptides and amino acids are further acidified by anaerobes (acidogenesis) into volatile fatty acids (VFAs), hydrogen gas, ammonium ions, and reduced sulfur. The VFAs are converted into acetate, H_2, and CO_2 by acetogens, as shown in Figure 4. The fermentation by–products of gelatin resulted from continuous anaerobic culture were mainly acetate (HAc) followed by propionate (HPr) and valerate (HVa) [31]. Fermentation of gelatin using mixed culture anaerobes produced acetate, carbon dioxide, and hydrogen. The iso-valerate and iso-butyrate concentrations were quite low (<1 mM). Increasing the incubation time of gelatin transformed the acidogenesis by-products into methane and carbon dioxide [34,35] (Figure 4).

3.2. Recent Trends for Biofuels (H_2 and CH_4) Production from Gelatinous Wastewater

A limited number of literatures was published for the simultaneous treatment and biofuels production from gelatinous wastewater [8]. *Thermobacteroides proteolyticus* [36], *Methanobacterium sp., and Methanosarcina MP* [37] were identified to be the most thermophilic anaerobes degrading GWW for CH_4 production [38]. Mono–culture bacterium of *T. proteolyticus* is efficient for H_2 production from GWW rich proteins. This is not the case for co–cultures of *T. proteolyticus* and *Methanobacterium* where CH_4 was mainly produced via the acetate fermentation process. The CH_4 was 190 umoles by co–culture of *Thermobacteroides–Methanobacterium*, which was increased up to 315 umoles by adding *Methanosarcina* onto the reaction medium. Likely, the H_2 generation by co–culture of *Thermobacteroides–Methanobacterium* was ten times higher than those obtained from mono–culture of *T. proteolyticus*. Furthermore, Jain and Zeikus [34] found that amino acids of gelatin, except for proline, were utilized by 60 to 91% using mono–culture of methanogenesis of *C. collagenovorans* or co–culture with *M. barkeri*.

The gelatin degradation efficiency and alcohol production were increased from 84.1 to 89.6% and from 0.15 to 0.33 g/gVSS·d at increasing the HRT from 4 to 24 h, respectively. Nevertheless, the conversion efficiency of gelatin was dropped from 65.2 to 51.9% at an increase in influent substrate concentration from 2 to 30 gCOD/L [32], which was not the case for the VFA and alcohol production. The latters were substantially increased from 0.10 to 0.58 g/gVSS·d at increased gelatin concentration (4–30 gCOD/L). A total of 4.5–7.8% of total COD in GWW was converted into biofuels of H_2 and CH_4, and the excess sludge was 0.320 ± 0.014 gVSS/gCOD.

Magnetite/graphene oxide (MGO) nano–composite (100 mg/L) was successfully utilized to increase the hydrogen yield and productivity; i.e., 112.4 ± 10.5 mL $H_2/gCOD_{removed}$ from gelatinous wastewater by immobilization on the anaerobes (mixed culture bacteria) [6]. This was due to the increase in acetate (HAc) from 102 ± 6.8 to 125.3 ± 6.3 mg/gVSS and butyrate (HBu) from 31.1 ± 1.5 to 48.8 ± 3.5 mg/gVSS. Apparently, supplementation of anaerobes with MGO augmented hydrogenase enzyme (HE) activity 9–fold, and the hydrogen producers (*Proteobacteria, Firmicutes, Clostridia, and Bacilli*) were highly elongated from 1.8–2.9 to 2.5–5.1 um. Up–flow anaerobic sludge blanket (UASB) reactor treating proteinaceous wastewater at an OLR of 32 gCOD/L·d and HRT of 9 h achieved 84% COD removal efficiency [39]. In total, 78% of the total COD was converted into CH_4, and 16% of the protein content was not utilized by methanogenesis resulting in a sludge yield of 0.079 gVS/gCOD. The hydrogen production and yield from anaerobic co–digestion of organic fraction of municipal solid waste and paperboard mill sludge were increased up to 1082.5 ± 91.4 mL and 144.9 ± 9.8 mL/$gVS_{removed}$, respectively, due to the supplementation of 20% gelatin solid waste (GSW) rich Ca^{+2} ions [40].

Sequential dark-photo fermentation of wastewater-rich protein is an excellent substrate for anaerobes for increasing the hydrogen yield and productivity where the protein is efficiently hydrolyzed into organic acids and nitrogenous compounds required for the metabolism of photosynthetic bacteria [41]. Meky et al. [21] employed a circular baffled reactor for dark– and photo–fermentation of gelatinous wastewater. The first two com-

partments were dark–fermentation followed by two chambers supplied with fluorescent tubes for photo–fermentation by the purple non–sulfur bacterial family *Rhodospirillaceae*. The module provided a maximum hydrogen yield (HY) of 0.4 L/gCOD and COD removal efficiency of 82% at HRT of 24 h and pH value not exceeding 6.6. The hydrogen yield and productivity were highly deteriorated at a pH value of 5.5 due to a drop in the ammonification process. Breure and Andel [31] found that the hydrolysis and acidification of gelatin in a continuously stirred tank reactor (CSTR) was inhibited with the addition of glucose in the feedstock. Glucose is the preferred substrate for anaerobes, particularly clostridia, and it is easily biodegraded compared to proteins. This hypothesis is confirmed by Breure et al. [42], who observed that the protein fermentation was retarded with supplementation of carbohydrates in the feedstock. Breure et al. [43] comprehensively investigated the impact of the anaerobes adaptation on the fermentation of co–substrates (glucose + gelatin). Anaerobes community was initially fed by glucose at 30 °C, and after attaining a steady–state, the substrate was regularly switched to gelatin. The protein degradation efficiency was < 30% while the glucose was completely metabolized. The authors conducted the experiments in another way where the anaerobes were initially adapted for the degradation of gelatin followed by the addition of glucose, resulting in high protein degradation and CH_4 production. The major portion of the protein was converted (85%) into carbon dioxide and methane in a lab-scale up-flow reactor [31].

4. Factors Affecting Biofuels Productivity from Gelatinous Wastewater

Figure 6 demonstrates different factors affecting biofuels (i.e., H_2 and CH_4) production and yield generated from the anaerobic digestion of GWW. Furthermore, the drawbacks associated with the anaerobic digestion of GWW and sustainable solutions for improving the anaerobic digestion process for more energy recovery are presented in Figure 6.

4.1. Gelatin Concentration

Fang and Yu [32] found that gelatin degradation decreased from 65.2 to 51.9% with the increase in influent gelatin concentration from 2 g/L to 30 g/L due to the growth retardation of *C. collagenovoransa* at 100 mM acetate in the fermentation medium. The propionate, acetate, iso–butyrate, and iso–valerate were progressively produced, and the growth of *C. collagenovoransa* augmented when increasing the gelatin concentration. However, the growth of *C. collagenovoransa* was negatively affected at 100 mM acetate formation. The methane production rate and yield increased at increasing gelatin concentration from 0.5 to 2.5% and decreased due to metabolic inhibition at higher gelatin concentration above 5%.

4.2. Organic Loading Rate (OLR)

The biological processes mainly depend on OLR, where a high load would encourage hydrogen producers, while moderate and low loads would enhance the bio–methanization process [44,45]. At high OLR (>20 gCOD/L·d), the accumulation of solids increases in the bioreactor and the discharge of excess sludge is increased, resulting in a short sludge residence time (SRT) favourable for H_2 productivity. The lower imposed loading rates (<20 gCOD/L·d) encourage the growth of methanogens degrading volatile fatty acids (VFAs), resulting in a higher CH_4 yield and productivity [46]. Anaerobes degrading wastewater-rich protein enhance the protease enzyme activities at high OLR, as reported earlier [47]. The dominance of methane-producing species (*Methanospirillum hungatei, Methanobrevibacter ruminantium, and Methanobrevibacter arboriphilus*) was detected and identified at low OLRs of 3–7 gCOD/L·d [35]. H_2 producers (*Clostridium hungatei*) were the major fraction in the anaerobic reactor operated at OLR exceeding 21.0 gCOD/L·d [35]. Nevertheless, Mahmoud et al. [48] found that *Methanobacterium* and *Methanosaeta* genus were detected in the up–flow anaerobic separation gas reactor treating polyester wastewater at low OLR, and the *Firmicutes* were presented in the sludge due to H_2 production. Therefore, biofuels (H_2 and CH_4) productivity could occur at low imposed OLR and depends on the substrate type. *Methanobacterium* with an average of 56.4% of the archaeal sequences,

44.3% of *Proteobacteria*, 28.9% of *Firmicutes*, 8.9% of *Chloroflexi*, 5.7% of *Actinobacteria*, and 5.6% of *Bacteroidetes* were detected in the anaerobic reactor treating petrochemical wastewater [46]. Meky et al. [21] found that 41% of *Clostridiales* (*Clostridiaceae1 and Clostridiaceae–4*) were dominant for the fermentation of gelatinous wastewater incorporation with 5.2% of *Anaerolineales* and 9.3% of *Bacteroidales*. The methanogens (*Methanosarcina* and *Acetobacterium)* were abundant in the hybrid anaerobic reactor treating hypersaline wastewater at low imposed OLR [45].

The effect of OLR on the hydrogen production from gelatinous wastewater using an up-flow multistage anaerobic sponge reactor (UMASR) was investigated [5]. The authors showed that HPR and HY were increased from 0.3 to 1.2 L/L·d and from 54.6 to 130.5 mL/$gCOD_{removed}$ at increasing the OLR from 10.4 to 20.9 gCOD/L·d, respectively. However, the reactor efficiency was deteriorated by increasing the OLR up to 88.6 gCOD/L·d, where the HPR and HY dropped to 0.48 L/L·d and 33.7 mL/$gCOD_{removed}$, respectively, due to the accumulation of VFAs in the bulk liquid. It was not the case for COD, proteins, lipids, and carbohydrate degradation, where the conversion efficiency was linearly decreased at increasing the OLR from 10.4 to 88.6 gCOD/L·d.

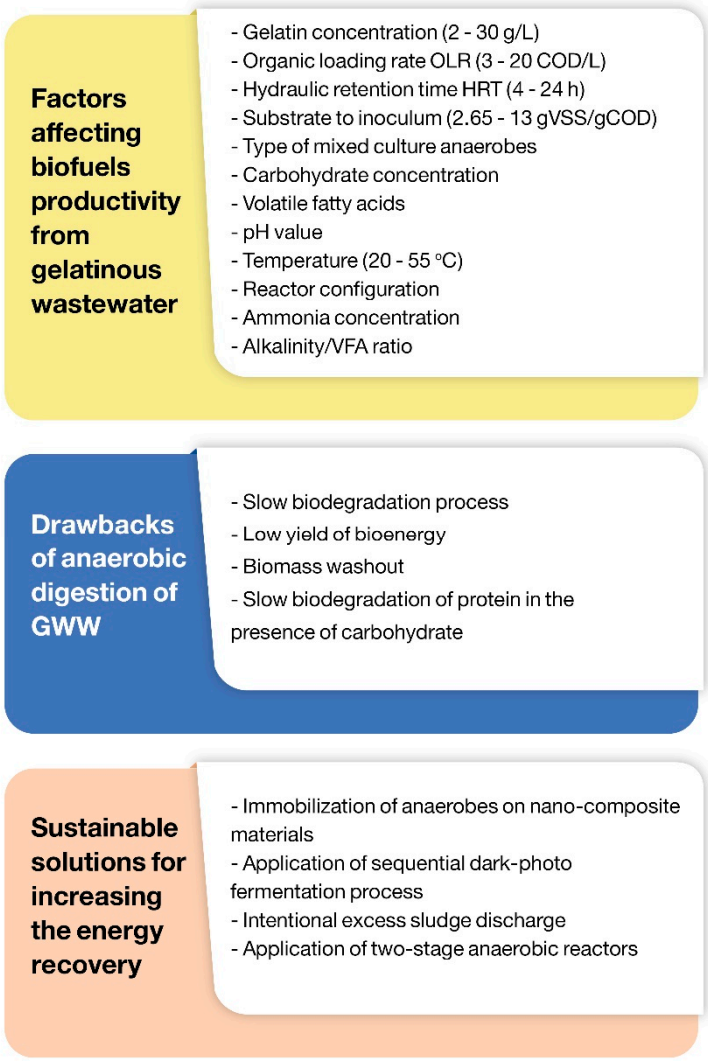

Figure 6. Factors affecting biofuels productivity from gelatinous wastewater, barriers of application of anaerobic digestion process for GWW, and the sustainable solutions for improving the energy recovery.

4.3. Hydraulic Retention Time (HRT)

Fang and Yu [32] observed that gelatin degradation slightly increased from 84.1% to 89.6% with the increase in HRT from 4 h to 24 h. The effect of HRT on hydrogen production rate (HPR), hydrogen yield (HY), COD removal, and protein and carbohydrate degradation during gelatinous wastewater treatment by anaerobic sequential batch reactor (An–SBR) was significant [5]. The HPR of 0.67 ± 0.07 and 1.17 ± 0.14 L/L·d was achieved at HRTs of 36 and 24 h in an anaerobic sequencing batch reactor (AnSBR) treating gelatinous wastewater. A drop in HPR and HY occurred while reducing the HRT from 24 to 6 h due to the bacterial washout. Similarly, COD, protein, carbohydrate, and lipids removal increased at increasing the HRT from 6 to 48 h. The COD, protein, and carbohydrate removal were increased from 37.4 to 60.2%, from 27.5 to 52.5%, and from 71.7 to 100% at increasing the HRT from 6 to 48 h, respectively. The high organics removal efficiency of carbohydrates, proteins, and lipids was due to an increase in the secretion of lipase, protease, and α-amylase activities in the reaction medium [5,49].

4.4. Substrate to Inoculum (S_0/X_0) Ratio

The substrate to inoculum (S_0/X_0) ratio is essential for accomplishing energy production from wastewater where H_2 and/or CH_4 production at low S_0/X_0 ratio is relatively low due to substrate limiting conditions which adversely affects the anaerobic activities [50,51]. Moreover, a high S_0/X_0 ratio would inhibit the anaerobic activities due to the accumulation of metabolite by-products in the reaction medium and subsequently suppress H_2 and/or CH_4 production. Therefore, it is necessary to control such parameters to avoid the deterioration of the process and gain more benefits from the economic and environmental points of view. The specific metabolite by-products are calculated based on Equations (11) and (12).

$$\text{Specific metabolite} - \text{by products}\left(\frac{\text{mg}}{\text{gVS}}\right) = \frac{\text{Metabolite concentration}\left(\frac{\text{mg}}{\text{gVS}}\right) \times \text{ working volume (l)}}{\text{Sludge}\left(\frac{\text{gVS}}{\text{l}}\right) \times \text{Sludge volume (l)}} \tag{11}$$

The S_0/X_0 ratio is calculated based on the following equation,

$$\frac{S_0}{X_0} ratio = \frac{T\ COD\ \left(\frac{g}{l}\right)(wastewater) \times V_{g(l)}}{VS\ \left(\frac{g}{l}\right)\ (inoculum\ sludge) \times V_{s(l)}} \tag{12}$$

where V_g and V_s are the volume of gelatinous wastewater and inoculum sludge, respectively. Mostafa et al. [6] investigated the effect of the initial substrate to inoculum (S_0/X_0) ratios of 0.25, 0.5, 1, 1.5, 2, 3, 5, and 7 gCOD/gVS on H_2 production from gelatinous wastewater. The authors found that the hydrogen potential (P) and hydrogen yield (HY) were increased from 20.2 to 45.1 mL and from 37.2 to 79.2 mL/$gCOD_{removed}$ at increasing the S_0/X_0 ratio from 0.25 to 1.0 gCOD/gVS, respectively. This was mainly due to a higher conversion of *COD*, carbohydrate, proteins, and lipids at an S_0/X_0 ratio of 1.0 gCOD/gVS. However, the P and HY dropped at S_0/X_0 ratios that exceeded 1.0 gCOD/gVS due to biomass metabolic by-products stress and substrate shock load.

Biofuels productivity and metabolite by-products are mainly S_0/X_0-dependent, as reported earlier by [51]. The CH_4 productivity was increased from 97 ± 7 to 290 ± 18 mL at lowering S_0/X_0 ratio from 13.23 to 5.29 gVSS/gCOD, respectively. Nevertheless, the CH_4 productivity was highly dropped to 12 ± 2 mL at S_0/X_0 ratio of 2.65 gVSS/gCOD. High and low substrate loading conditions suppress the methanogenesis and create unfavorable reactions for the biodegradation process. Moreover, the authors found that the CH_4 productivity was dropped at S_0/X_0 ratio of 8.82 and 13.23 gVSS/gCOD due to the excessive ammonia concentration of 493.9 ± 44.3 and 515.5 ± 47.9 $mgNH_4$–N/L in the reaction medium. The H_2 productivity was increased from 19 ± 2 to 48 ± 3 mL when reducing the S_0/X_0 ratio from 13.23 to 3.78 gVSS/gCOD, respectively. However, the H_2 productivity dropped from 48 ± 3 to 27 ± 2 mL when decreasing the S_0/X_0 ratio from 3.78

to 2.65 gVSS/gCOD. The authors concluded that H_2 and CH_4 producers were optimized at S_0/X_0 ratio of 3.78 and 5.29 gVSS/gCOD, respectively.

4.5. Type of Mixed Culture Anaerobes

Breure et al. [43] studied the effect of the mixed culture's adaptation technique on the fermentation of gelatin. In one series of experiments, glucose was fed to the mixed culture. After reaching a steady–state, the carbon substrate was switched to gelatin, and the mixed culture growth was ceased. However, when gelatin was added to the medium as a second carbon substrate, it was found that hydrolysis and fermentation of the protein proceeded to a limited extent (< 30%). However, glucose continued to be completely metabolized. In the second series of experiments, mixed culture was adapted to gelatin degradation. After reaching a steady–state, glucose was added to the medium as a second carbon substrate. Following the new steady state's establishment, it was found that gelatin's hydrolysis was not inhibited, but its fermentation was adversely affected. It is concluded that anaerobic bacterial populations can lose their ability to degrade protein substrate, depending on the adaptation procedure. Co–culture of *Clostridium collagenovorans* and *Methanosarcina barkeri* was attempted to degrade gelatin into methane and carbon dioxide [34]. The amino acids in gelatin, except for proline, were converted into acetate and carbon dioxide as the main products in the mono–culture of *C. collagenovorans*. Moreover, hydrogen, isovalerate, and isobutyrate were detected in trace amounts (<1 mM). It was not the case for co–culture with *M. barkeri*, where gelatin was transformed into methane and carbon dioxide, and acetate was the intermediate compound. The authors recommended using a co–culture that was stable and did not require pH control and exogenous growth factor. The *C. collagenovorans* species can ferment the amino acids of the gelatinous protein to acetate and H_2 as the major by–products. Methanogenic species subsequently utilized these intermediates' metabolites, but not by the obligately syntrophic acetogenic species [52]. *C. collagenovorans* readily degraded various proteins of animal origin as it contains both collagenase and protease activity [34,53]. Nevertheless, the thermophilic methanogenesis of gelatin using triculture of *Thermobacteroides proteolyticus*, *Methanobacterium* sp., and *Methanosarcina* sp. could not convert all of the acetates into methane even after 20 days [38].

The mixed culture bacteria immobilized on magnetite/graphene oxide nano–composite was employed for H_2 harvesting from gelatinous wastewater [6]. The microbial analysis showed that *Proteobacteria, Firmicutes, Clostridia,* and *Bacilli* were dominant. Meky et al. [21,41] showed that *Clostridiaceae_1* and *Rhodospirillales bacterial* families isolated from activated sludge were predominant for H_2 production from gelatinous wastewater using dark and photo–fermentation processes. Mixed culture bacteria were inoculated onto an anaerobic sequencing batch reactor and up–flow multi–stage anaerobic sponge reactor to treat gelatinous wastewater [5]. The latter provided COD, carbohydrates, and proteins removal efficiencies of 60.2, 100, and 52.5%, respectively, at an HRT of 48 h.

4.6. Carbohydrate Concentration

Breure et al. [42] investigated the hydrolysis and fermentation of gelatin in the presence of carbohydrates. It was shown that gelatin degradation was progressively retarded with increased concentrations of carbohydrates. Moreover, the carbohydrate was completely fermented at all dilution rates. The cultures of gelatin-degrading anaerobes were grown in a chemostats module at different dilution rates (pH = 7 and T = 30 °C). Hydrolysis and fermentation of the gelatin in the presence of glucose were assessed [42]. The major fermentation by–products of the acidogenic fermentation of gelatin were acetate, propionate, and valerate. However, butyrate fermentation type dominated after the introduction of carbohydrate as a second substrate. Moreover, the concentration of VFAs was also increased due to the addition of glucose compared to the mono–fermentation of gelatin. It strongly indicates that glucose addition would inhibit gelatinous protein degradation, as anaerobes prefer to digest readily biodegradable organics. The carbohydrate degradation is mainly performed by mixotrophic microorganisms, which would also biodegrade protein-

rich gelatinous wastewater. The retardation of gelatin degradation by carbohydrates is primarily because sugars are the preferred substrates for microbes. Therefore, mixed culture bacteria are preferable for the degradation of wastewater containing a mixture of organics. The protein degradation was not affected at high influent concentrations of VFAs in the feedstock. Therefore, anaerobic treatment of wastewater containing proteins and carbohydrates should be conducted in a two–stage system, where carbohydrates will be initially digested at low pH (5–6) into VFAs. Then, proteins will be fermented at neutral pH in the second stage (methanogenesis). This will avoid the process inhibition owing to the presence of sugars in the feedstock [54]. The VFAs will be further converted into methane and carbon dioxide under the methanogenesis stage.

4.7. Volatile Fatty Acids Concentration

Breure et al. [42] investigated whether high concentrations of VFAs in the reactor inhibit gelatin degradation or not. After reaching steady–state conditions, a mixture of acetate, propionate, and butyrate was added. The results showed that gelatin degradation was not as severely inhibited under conditions where no volatile fatty acids were added. It can be concluded that high concentrations of VFA do not cause inhibition of gelatin degradation. Moreover, the formation of VFAs in the fermentation of glucose or lactose does not cause a severe inhibition of protein degradation [55].

The gelatin–based proteinous compounds are anaerobically biodegraded into H_2 gas and volatile fatty acids (VFAs) under dark conditions, which are further utilized for H_2 generation by photosynthetic microbes. Meky et al. [41] showed that acetate (HAc) and propionate (HPr) were increased up to 247 and 217 mg/L in the treated effluent of the anaerobic reactor treating protein–rich wastewater due to the presence of *Clostridiaceae_1*. Those VFAs fractions were further photosynthetically biodegraded by *Rhodospirillaceae* for biohydrogen generation. The protein and carbohydrate degradation by anaerobes immobilized on magnetite nanoparticles into VFAs and H_2 were significantly improved 1.4– and 2.1–fold as reported by [56]. The magnetite nanoparticles addition enhanced the microbial proteases' enzyme activities and ensured the hydrolysis of wastewater–rich proteins [57]. Extracellular proteases are mainly required to improve the absorbance of VFAs by the anaerobes culture bacteria. Tawfik et al. [58] found that the protein biodegradation into VFAs and H_2 gas by anaerobes (34.53% for *Proteobacteria*, 27.55% for *Firmicutes*, 10.19% for *Chloroflexi*, 9.44% for *Actinobacteria*, 6.64% for *Planctomycetes*, and 3.82% for *Bacteroidetes*) immobilized on graphene/hydroxyapatite nanoparticles was increased by a value of 13%. These microorganisms are surely involved in the production of VFAs and H_2 gas formation. *Proteobacteria* are mainly responsible for converting protein into VFAs, which were highly abundant due to the presence of graphene/hydroxyapatite nanoparticles [59]. Xiang et al. [60] reported that *Proteobacteria* utilized proteins to generate VFAs under mesophilic anaerobic digestion conditions. Likely, He et al. [61] found that *Proteobacteria* were utilized for acetate production from anaerobic degradation of protein–rich wastewater resulting in quite high HAc/HBu ratio in the reaction medium process.

4.8. pH Value

The pH variations affect the bacterial growth rate causing drastic shifts in different species of heterogeneous populations. Microbial metabolism is highly influenced by pH variations that influence substrate biodegradation, synthesis of proteins cells, and the release of metabolic by–products from bacterial cells [62]. Moreover, variations of pH would affect bacterial cell morphology and its structure.

The pH value affects the acidogenesis and methanogenesis processes [35,63]. The production of VFAs during the acidogenesis process results in a significant drop in pH value and creates favorable conditions for hydrogen production. However, VFAs are subsequently utilized by methanogens for CH_4 production. The alkalinity is increased due to the release of CO_2 in the reaction medium, and a drop in alkalinity/VFA ratio occurred. Breure et al. [31] tested four different pH values (5.3, 6.0, 6.3, and 7.0) to determine the

optimal pH value for gelatin hydrolysis and acidification. The results revealed that the optimum pH value was > 6.3. The hydrolysis of gelatin increased from 65 to 78% when increasing the pH values from 5.3 to 7.0. Likely, the hydrolytic activities of the anaerobes substantially increased from 264 to 548 mg C gelatin hydrolyzed/L·h when increasing the pH from 5.3 to 7.0. The effect of pH values on acidification and hydrogen production from gelatinous wastewater was investigated [64]. Gelatin degradation efficiency increased with pH, i.e., from 60% at pH 4.0 to 97.5% at pH 7.0. The optimum pH for the overall acidogenic activity was found to be 6.0 and close to 5.9. The VFAs in the treated effluent were low at a pH value of 4.0 and increased to 1560 mg/L at pH 7.0. The HAc was the principal metabolic by-product and increased from 14.8 to 35% when pH was increased from 4.0 to 7.0. Similar trends were observed for H_2 production and COD and protein conversion.

Elreedy et al. [51] found that decreasing the initial pH value of wastewater from 7.0 to 5.0 enhanced the H_2 productivity 1.44–fold, attaining the maximum value of 79 ± 6 mL. This is mainly due to the positive effect of the acidic pH value of 5.0 on the hydrogenase enzyme activity, as reported by [65–67]. Further reduction in pH values less than 5.0 (4–4.5) would inhibit the growth of H_2 producers and reduce the hydrogenase activities.

4.9. Temperature

Temperature affects the maximum gelatin utilization rates by acidogenesis and methanogens, where low operational temperature resulted in a reduction in the maximum specific growth and substrate utilization rate of anaerobes. Furthermore, the discharge of the excess sludge is quite low due to the accumulation of the solids in the sludge bed of the fermenter [22,68].

Temperature is one of the important parameters affecting the activity of the anaerobes, where the metabolic activities increased at high temperatures. The acidification of gelatinous wastewater was slightly affected by the temperature [64]. The protein degradation, formation rate of VFAs and alcohols were slightly increased with increasing the temperature. HAc was the main intermediate product (20–27%) from acidification of gelatinous wastewater at a temperature (20–55 °C) with an average value of 24%. However, the correlation coefficient between temperature and HAc production was quite low. The HPr and HBu were 12–18% and 10–15%, with an average value of 15 and 13%, respectively. It indicates that temperature did not significantly affect the acidification of gelatinous wastewater [64]. However, the protein degradation and hydrogen gas were linearly increased with increasing the temperature. Gelatin biodegradation and acidification efficiency were slightly increased from 0.370 to 0.443 g/gVSS·d and from 56.4 to 72.6% at increasing the temperature from 20 to 55 °C [64].

4.10. Reactor Configuration

The anaerobic module could be a suspended growth system or attached biofilm reactor. The latter performed well at long sludge residence time (SRT) and absorbed the organic shock loading, which could be applicable for energy production from high-strength wastewater such as GWW. Mostafa et al. [5] compared the efficiency of an up–flow multistage anaerobic sponge reactor (UMASR) and an anaerobic sequencing batch reactor (AnSBR) for hydrogen production from gelatinous wastewater. The UMASR and AnSBR were attached and bacterial growth processes suspended, respectively. Both units were operated at HRTs of 48, 36, 24, 12, and 6 h. UMASR provided HPR of 0.30 ± 0.01 L/L·d and HY of 54.6 ± 2.4 mL/$gCOD_{removed}$, which were higher than those achieved in AnSBR (0.13 ± 0.01 L/L·d and 19.6 ± 1.7 mL/ $gCOD_{removed}$) at HRT of 48 h and OLR of 10.4 ± 0.4 gCOD/L·d. The UMASR was superior for HPR and HY due to a higher production of VFAs, i.e., acetate (HAc) and butyrate (HBu) at longer SRT. However, the AnSBR achieved a higher COD removal efficiency of 60.2 ± 4.4% due to the accumulation of active biomass in the attached culture reactor, which contributed to higher substrate degradation efficiency. AnSBR exhibited superiority in carbohydrates and proteins removal efficiencies of 100 and 52.5 ± 2.4%, respectively. Maree et al. [3] found that

the combined anaerobic–aerobic system showed better performance for GWW treatment than the anaerobic system alone. AnSBR treating GWW achieved COD removal efficiency close to 70% at OLR of 0.05–0.35 gCOD/gVSS·d and volumetric loading rate (VLR) of 0.14–0.37 gCOD/L·d. The module was operated at a cycle of 0.5 h feeding, 22 h reaction, 1 h settling, and 0.5 h decanting [4].

Meky et al. [21,41] investigated a dark-photo circular baffled reactor (DP–CBR) for the hydrogenation of gelatinous wastewater at 21 ± 10 °C. The standalone reactor achieved a fairly high H_2 yield of 0.4 L/gCOD and minimized the negative impact of ammonia generation at HRT of 24 h and pH value of 6.5. The UASB reactor was used for the treatment of wastewater–rich protein; however, the reactor failed at a high imposed OLR of 15.6 gCOD/L·d due to the sludge washout from the bioreactor [68]. Intentional sludge discharge is recommended avoiding the biomass washout (Figure 6). An anaerobic baffled reactor was employed for simultaneous treatment and H_2 production from industrial wastewater [69–71]. The module achieved a H_2 yield of 0.17 L/$gCOD_{removed}$ at an OLR of 8 gCOD/L·d. Based on these results, a dark–photo circular baffled reactor (DP-CBR) is recommended for application at the industrial scale for the valorization of GWW (Figure 6). However, further investigation for the excess sludge production from such a module is required.

4.11. Ammonia Concentration

Gelatinous wastewater is rich in protein, mainly hydrolyzed in biological reactors, and ammonia is one of its by–products. The proteins are partially hydrolyzed in sewers of the factory and produce ammonia in the end–of–pipe effluent. Moreover, the ammonification process occurs in the anaerobic reactor due to a chemical reaction in which NH_2 groups are converted into ammonium (NH_4^+) or the N–org is transformed into ammonia Equations (13) and (14).

$$RCHNH_2COOH + HOH \rightarrow RCOOH + CO_2 + NH_4 \quad (13)$$

$$CO(NH_2)_2 + HOH \rightarrow 2NH_3 + CO_2 \quad (14)$$

NH_3–N was increased in the treated effluent during the acidification of GWW in a UASB reactor [32]. The effluent NH_3–N concentration increased with increasing the gelatin concentration and decreased with the increase in HRT. However, the NH_3–N in the treated effluent did not exceed 5.0 g/L; still, it is toxic for anaerobes [31]. Acidogenic conditions prevailed in the reactor, which minimize the release of toxic ammonia (NH_3–N) in the treated effluent. However, in the methanogenesis process, the pH levels of the reaction medium tend to rise, which is preferable for ammonia (NH_3–N) production and may result in process failure. Thus, controlling the NH_3–N is critical and necessary. Mistry and Patel [4] evaluated the effect of NH_4–N concentration on the COD removal efficiency from GWW using an AnSBR. The NH_4-N concentration in the range of 150–250 mg/L did not have any adverse effects on COD removal and biofuels (H_2 and CH_4) production.

4.12. Alkalinity/VFA Ratio

Mistry and Patel [4] observed that in anaerobic fermentation of gelatin, the feed Alkalinity/VFA ratio ranged from 2 to 5, whereas the Alkalinity/VFA ratio in effluent increased to about 5–10 with as high as 30–35. It indicates that there was no acid accumulation, and all the VFAs were consumed by methanogens. The results of Alkalinity/VFA were thus consistent with COD removal. It may further be noted that Alkalinity/VFA ratio in effluent on 25th and 26th days was dropped suddenly along with a drop in the temperature (14–15 °C) compared to other days with an average temperature of 30–35 °C. The Alkalinity/VFA ratio in the effluent on these two days dropped to 1.16. It is known that the optimum temperature for methanogens is around 35 °C. Thus, a reduction in Alkalinity/VFA ratio may be due to the accumulation of VFAs at a lower temperature, when methanogenesis may be temporarily inhibited or slowed.

4.13. Effect of Combined HRT and OLR

The most critical combined parameters affecting the hydrogen productivity from wastewater are the HRT and OLR. Increasing the OLR and reducing the HRT would encourage the H_2 producers to achieve a high yield and good effluent quality effectively. The long HRT and low OLR are preferable for the biomethanization process. The paperboard mill industry was utilized for H_2 production using an up–flow intermitted stirring tank reactor at different HRT and OLR. The results showed that the H_2 productivity significantly increased from 0.17 ± 0.03 to 0.36 ± 0.04 L H_2/L·d when reducing HRT from 48 to 6 h and increasing OLR from 2.19 to 17.78 gCOD/L·d [72]. At a high OLR of 17.78 gCOD/L·d, the production of VFAs and hydrogenase enzyme activities are quite high, resulting in a high yield of H_2 energy. Farghly and Tawfik [62] found that H_2 productivity from paper mill industry wastewater was highly increased up to 0.46 L H_2/L·d at an HRT of 12 h and OLR of 3 gCOD/L·d. However, lower H_2 production of 0.004 L H_2/L·d was obtained at a longer HRT of 36 h and lower OLR of 0.75 gCOD/L·d.

5. Economic Values of Energy Recovery and the Pretreated Effluent of Gelatinous Wastewater

Treatment of gelatinous wastewater by chemical coagulation (CC) and electrochemical coagulation (EC) was investigated by Arturi et al. [1]. Both technologies were only efficient for removing particulate organics, i.e., 73.6% by EC and 55.6% by CC resulting in a high yield of excess sludge with low settling properties, which needs further treatment. Moreover, these technologies were very poor for removing soluble organic matter and consuming high quantities of energy and chemicals. The effluent quality is not mainly complying with discharge into the environment. Lakshmi Kruthika et al. [2] achieved a removal efficiency of 60% of TOC from GWW by electrocoagulation, and the efficiency was deteriorated due to the scaling of the aluminium electrodes in the presence of dissolved calcium in the influent. Accordingly, anaerobic technology is recommended for energy recovery and treatment of gelatinous wastewater from economic and environmental points of view. Meky et al. [21] assessed the economic analysis in terms of capital, annual costs, gas purification, nutrients, lighting, pumping, and revenues based on H_2 energy productivity and pollutants removal of the anaerobic digestion of 600 m^3/d GWW with influent COD concentration of 2000 mg/L. The dark-photo circular baffled reactor (DP–CBR) provided a payback period of 3.4 yrs and a net profit of 22,638 $/year at pH value of 6.5 and HRT of 12 h. Moreover, the anaerobically pretreated effluent of GWW is very rich in ammonia, phosphorous, and calcium which is necessary for the production of microalgae species and resulting in an effluent quality free from nutrients. Blanco et al. [73] successfully cultivated *Chlorella vulgaris* microalgae in bubble column photobioreactors (PBRs) fed with anaerobic effluent of GWW. The authors found that the addition of anaerobic effluent of GWW into PBRs significantly increased the biomass productivity of microalgae by 57.5% compared to the control samples indicating the economic importance value of using such substrate. Ali et al. [74] and Bakr et al. [75] employed an immobilized biomass reactor to polish the anaerobic effluent of industrial wastewater. The module achieved an effluent quality complying with safely reuse and discharge into the water streams. Likely, a good quality effluent in terms of COD, TSS, NH_4–N, and NO_x–N was achieved using a down–flow hanging luffa system treating anaerobic effluent [76,77].

6. Conclusions

Classical chemical and biological treatment processes of gelatinous wastewater are costly to be applied due to the consumption of energy and chemicals. Moreover, the excess sludge production is quite high, and its disposal is economically unacceptable. Fortunately, the gelatinous wastewater is rich in protein which can be easily biodegraded by anaerobic bacteria for energy recovery in terms of H_2 and CH_4. However, the energy yield from anaerobic digestion of such wastewater mainly depends on substrate concentrations, OLR, HRT, and ammonification process. Moreover, the presence of carbohydrate in GWW

adversely affects the degradation of protein-rich gelatinous effluent. Therefore, two-stage anaerobic reactors are preferable for simultaneous treatment and energy recovery from the degradation of GWW, where the carbohydrate and protein will be degraded in the first and second stages, respectively. Another approach is applying dark–photo–fermentation of GWW, where the hydrolysis of protein into nitrogenous compounds in the first step occurs and enhances the photo–degradation of organic acids and subsequently increases the bioenergy production, particularly hydrogen. The use of gelatin sludge incorporation with chitosan for the adsorption of pollutants from wastewater could be a great option for the valorization of such waste. Immobilization of anaerobes on nano–composite materials is a novel approach for gelatin degradation and biofuels production; however, its application at a full scale is still questionable.

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