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Two-stage anaerobic digestion using protein-rich synthetic wastewater inoculated with anaerobic sludge

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ABSTRACT

Foam and scum have impacted digestion, causing process disturbances in several industries. The cause of foam and scum is difficult to determine as there are complex compounds in wastewater. Proteins are the primary compound found in food processing industries wastewater. This study investigates the effect of protein concentration on foaming and scum formation using protein-rich synthetic wastewater in a two-stage anaerobic digester inoculated with anaerobic sludge. The protein concentration in the digester was altered using gelatine as a protein source. The foaming tendency, scum production, biogas production, protein, and chemical oxygen demand (COD) removal, total volatile fatty acid (TVFA) concentration, and ammoniacal nitrogen (AN) concentration were measured to comprehend the findings. The results show no foaming and scum in the digester; however, sludge residue was present at high protein concentrations. The Fourier-transform infrared spectroscopy analysis revealed that the residue contained sludge and protein. The biogas production began to decrease at the protein concentration of 12 g/L. The TVFA and AN increased steadily with an increase in protein concentration in Tank 1, while the protein and COD removal percentage was higher in Tank 2.

Keywords: Anaerobic digestion; Scum; Anaerobic sludge, Foaming, Protein

1. Introduction

The two-stage anaerobic digestion system was introduced as a physical separation of two groups of bacteria with the notion of enhancement in process stability and control [1]. Many studies have reported that two-stage AD is an exciting alternative for better overall process performance leading to higher energy yield and degradation rates [2–4]. However, scums and foams are significant drawbacks [5–7].

Scums are slow-degrading organic matter formed on a digester's surface, generally of metastable viscous liquid. Scum formation leads to physical, biological, and economic failures if left untreated. They often cause pipe blockage, reduce reactor surface area and interfere with mixing

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equipment. Some causes of scum formation are improper mixing, temperature fluctuations, and organic overloading [8]. The occurrence of scum on the surface of the digester is not clearly understood, as there are limited resources on the causes of scum formation, especially in a two-stage digestion system [9]. The presence of scum on the surface of the digester is correlated with the high overload of organic matter [8]. Organic matter's three major macromolecules are carbohydrates, proteins, and lipids. Anaerobic degradation of proteins is slower than the breakdown of other biomolecules. In dairy effluent, for example, carbohydrates are thought to be more acidified than proteins [10].

Similarly, it was discovered that proteins were the most abundant remaining components after the anaerobic process of domestic wastewater [11] indicating that they can easily accumulate over time. Besides that, the high organic content of lipids in food processing wastewater (FPW) has also resulted in the accumulation of scum in the digester, causing blockages of pipes, thus requiring frequent cleaning and maintenance [12,13]. From these studies, a complex fraction of organic matter in the wastewater makes the root cause of scum production seemingly challenging to comprehend. Proteins should be researched in depth as it is one of the main substances in wastewater and waste, and it contributes to 20%–40% of chemical oxygen demand in domestic wastewater and up to 60%–90% in food wastewater [14].

Besides that, high organic content in the substrate often triggers foaming to occur in the digester. Foams are a collection of persistent bubbles formed when air or gases are introduced beneath the liquid's surface, which expands to surround the gas in a liquid film known as lamellae [15]. It leads to unstable operation and reduced digester working volume, thus decreasing microbial activity and biogas yield [16]. Studies have shown that the primary causes of foaming incidents in the digester are caused by unstable operating conditions, such as temperature fluctuations, inadequate mixing, and inconsistent loading rates [17,18]. Other studies have suggested that the accumulation of surface-active agents such as proteins, lipids, and extracellular polymeric substances (EPS) in the digester causes foaming to occur [18]. Surface-active substances such as proteins are amphiphilic, which means they have both hydrophobic and hydrophilic components. These compounds are found near the gas/liquid interface, with the hydrophilic side of the molecule oriented toward water and the hydrophobic tail of the structure in the gaseous phase [19]. This reduces the free energy of the system as well as the interfacial tension, resulting in foam stabilization [20]. As an example, increasing the protein concentration in a one-stage AD digester increased the foaming tendency when inoculated with anaerobic sludge and cow manure [21]. Besides that, nitrogen is bound in the form of amino groups in proteins. During AD, nitrogen is released in the form of ammonium. Ammonium can dissociate to ammonia, a potent cell toxin, according to numerous stimuli such as temperature rise and pH shift [22]. The death of microorganisms due to ammonia toxicity can increase the likelihood of excessive foam generation [19]. Thus, this study aims to investigate the effect of protein concentrations on scum and foam production in two-stage AD using protein-rich synthetic wastewater (PRSW) and sewage sludge as inoculum.

2. Materials and methods

2.1. Protein-rich synthetic wastewater (substrate)

The substrate used in this research was synthetic wastewater based on food processing wastewater characteristics. The high protein content in this mixture was suitable for investigating the effect of protein on scum and foam. The PRSW was prepared with yeast extract (marmite) and meat extract (Bovril) as they act as a good micronutrient nutrient source for archaea. Previous studies on anaerobic digestion also used yeast extract as the synthetic wastewater medium and have proven that yeast extract is a good bio-stimulant for anaerobic microorganisms [23,24]. The meat (470 g) and yeast (470 g) extracts were mixed in a beaker of 1 L of distilled water as stock solution. The estimated chemical oxygen demand (COD) of the stock solution was 30,000 mg/L with a pH of 4.5–5. In order to alter the protein concentration further without the addition of other compounds, gelatine was used. Besides that, gelatine is the primary compound found in food processing wastewater [6]. The exact composition of meat and yeast extract from the manufacturer is shown in Table 1.

2.2. Anaerobic sludge (inoculum)

Anaerobic digestion of wastewater with sewage by microbial anaerobic digestion allows energy recovery in biogas (methane) while simultaneously lowering the proportion of organic substrates and eliminating pathogens [25]. The anaerobic sewage sludge used in this study was obtained from an anaerobic sludge tank, sampling point digester 3 of the municipal sewage treatment plant Indah Water Konsortium, located in Kuala Lumpur. The digester in the Konsortium runs at mesophilic temperature 35°C–38°C with a flow rate of 300–400 m^3/d.

2.3. Digester configuration and operating conditions

The digester was configured as an up-flow anaerobic two-stage reactor with a total working volume of 30 L,

Table 1
Ingredients of meat and yeast extract from the manufacturer (per 100 g basis)

Ingredients	Meat extract	Yeast extract
Protein, g	13.3	38.4
Carbohydrates, g	24.4	19.2
Sugars, g	0.7	0.5
Total fat, g	0.1	0.1
Saturated fat, g	0.1	–
Sodium	3,510 mg	4.3 g
Potassium, mg	1,200	–
Fiber, g	–	3.1
Thiamin, mg	–	5.3
Riboflavin, mg	–	7.0
Niacin, mg	–	160
Folic acid, μg	–	2,500
Vitamin B12, μg	–	15

comprises two cylindrical Plexiglas vessels, and is divided into two parts [13]. The first stage is a 10 L acidogenic reactor (AR) with 20 cm diameter × 44 cm height dimensions. Meanwhile, the second-stage is a methanogenic reactor (MR) measuring 30 cm in diameter, 46 cm in height, and holding a volume of 20 L. The headspace height of both reactors is 3 cm. The substrate was fed using a peristaltic pump (Longerpump, BT600-2J). The sampling process from Tanks 1 and 2 was done every day after 24 h of feeding. The system used a heating belt and a submerged heater (Eco Aquarium Heater) to maintain temperatures between 35°C–38°C in Tanks 1 and 2, respectively.

2.4. Experimental procedure for the effect of protein on foaming and scum production

The experimental procedure began with the acclimatization of the inoculum. The raw anaerobic sludge was first acclimatized in a 1 L Scott bottle (working volume of 0.8 L) with water at a 1:1 ratio before introducing PRSW for 7 d to allow the inoculum to degrade the previous substrate [26]. Synthetic wastewater at a low COD concentration (2,000 mg/L) was introduced for the sludge to adapt. The inoculum was fed repeatedly with synthetic wastewater (2,000 mg·COD/L) until it achieved a 90% percentage removal before higher COD concentrations were introduced (3,500 and 5,000 mg/L). Once the percentage removal of 90% was achieved at a COD concentration of 5,000 mg/L, the sludge was transferred to the two-stage digester. In the two-stage digester, the substrate was semi-continuously fed intermittently with synthetic wastewater to allow the inoculum to adjust to the new system. The hydraulic retention time (HRT) was gradually decreased at 0.8, 0.6, and 0.5 [resultant organic loading rate (OLR) of 0.18, 0.26, and 0.75 g·COD/L·d, respectively] to allow inoculum adaptation. The experiment was initiated after the inoculum achieved a steady biogas production and COD removal of 90%.

The experiment began by feeding the digester with synthetic wastewater at an initial COD of 2,800–2,900 mg/L, which contains a protein concentration of 3 g/L (without the addition of gelatine). The following protein concentrations were adjusted by adding gelatine accordingly. The system's temperature was at mesophilic temperature. The pH of the substrate was kept constant at pH 7, and the system's alkalinity was monitored throughout the experiment [26]. The experiment was conducted for 4 d, according to our observation. The protein concentration examined in this research was 3, 6, 9, 12, and 15 g/L. The inoculum in the system floated on the third day after feeding at the highest protein concentration (15 g/L). Thus, the experiment was halted at this protein concentration to avoid further digestion problems.

2.5. Analytical methods

Throughout the experiment, various characterization and analyses were performed to obtain the results of this study. The oil and grease, COD, ammonia, and total volatile fatty acid (TVFA) analyses were measured according to the APHA Standard Methods for Examining Water and Wastewater (2005). pH measurements were taken with a digital pH meter (HM Digital). The protein concentration was determined using the Bradford Assay Protocol. The parameter used to investigate the foaming in the physiochemical test was a foaming tendency. The foaming tendency analysis was calculated from the volume of foam (mL) right after aeration, divided by airflow rate (mL/min) [27]. The total biogas production was collected in the gas bag and later measured using the water displacement method to avoid pressure build-up [28]. The biogas was collected in the measuring cylinder displacing water, and the reading was taken daily. The foaming test for the solution was determined using the aeration method, where the foaming tendency (mL-foam/(mL-air·min)) was calculated from the volume of foam (mL) right after aeration divided by air flow rate (mL/min) [29]. The scum production was calculated using the circumference and volume of the digester over time [8].

3. Results and discussion

3.1. Scum and foam production

It is common in a digester that has high concentrations of surface-active agents such as proteins and lipids to produce foaming [19,30,31]. Proteins have been associated with foam in many biogas plants. In this research, the foaming tendency test revealed that foaming was not present in the collected samples. Also, there was no observable foam on the surface of Tanks 1 and 2 digester. The absence of foaming in the digester could be due to the weak interaction at the water-air interface [32]. A stable foam requires a protein with hydrophobic and hydrophilic groups on its surface and flexibility. Gelatine is created by acid or alkaline hydrolysis of collagen. The source of collagen, as well as its age and kind, determine its final qualities. Gelatine molecules can take a wide range of conformations in an aqueous solution under regulated temperature, pH, and solvent quantity, which could ultimately influence foam stabilization [33,34]. Gelatine's complex conformations could have contributed to the digester's absence of foam, demonstrating that protein-induced foam depends on the source of protein.

Besides that, scum is also a common operational problem in the digester. Typically, scums are found in a digester that treats high-strength wastewater, such as food waste and domestic wastewater, with a high concentration of organic substances [8,35]. However, when using a two-stage digester in this study, scum was absent in both Tanks 1 and 2 for all protein concentrations. The absence of scum in the digester supports previous findings that it is more correlated to lipid inhibition from the accumulation of long-chain fatty acid (LCFA) [36,37]. Furthermore, in a two-stage digester, the production of scum is still widely not understood due to the complexity of the process and the presence of various organic substances. The presence of multiple organic substances in the substrate makes it difficult to comprehend the role of protein in scum production since it has a hydrophobic characteristic and is a slow degrading substance compared to carbohydrates [10,38]. Thus, it can be ruled out that protein does not produce scum on the surface of the digester.

Although there was no scum and foam in the digester, there were traces of sludge flotation in Tank 1 when the two-stage digester was fed with high protein concentrations (12 and 15 g/L), as shown in Fig. 1a. One reported cause of

sludge flotation is a high protein concentration, as studies have found that protein-grown sludge granules have poor sludge properties, such as low density, compactness, and settleability [39,40]. The residue was present in Tank 1 and was carried into Tank 2. Besides that, the residue was also present in the effluent of the digester, as shown in Fig. 1b. From observation, the sludge clumped together and surrounded by a thin film. The presence of the residue required frequent removal as it caused blockage of pipes and disrupted the effluent flow.

Fourier-transform infrared spectroscopy (FTIR) analysis was carried out to analyze the residue (Fig. 2). Three samples were analyzed, which were 15 g/L protein wastewater (sample PRSW), sludge residue from Tank 1 two-stage reactor (sample residue), and anaerobic sludge (sample sludge). The most dominant peak for the analysis results are 3,396, 3,342, and 3,403 cm^{-1} for PRSW, residue, and sludge samples, respectively. The peak is correlated to polysaccharides structure (stretching of O–H bonds), a hydroxyl functional group. The peaks 2,884 and 2,817 cm^{-1} in sample PRSW, 2,918 cm^{-1} in sample residue, and 2,885 cm^{-1} in sample sludge indicate aliphatic C–H stretching [41]. The broad shortened peaks of 2,130; 2,123 and 2,130 cm^{-1} for samples PRSW, residue, and sludge, respectively, indicate the presence of alkynes C triple bond C stretching. The peak at 1,644 cm^{-1} (sample PRSW), 1,646 cm^{-1} (sample residue), and 1,645 cm^{-1} (sample sludge) shows the existence of amides I, stretching of C=O and C–N bonds which are protein secondary structures. The peaks of 1,440 cm^{-1} (sample SW), 1,461 cm^{-1} (sample residue), 1,440 cm^{-1} (sample sludge), and 1,362 cm^{-1} (sample sludge) represent the existence of amides III (C–N stretching). Besides that, the peak of 724 cm^{-1} in sample SW, 722 cm^{-1} in sample residue, and 719 cm^{-1} in sample sludge indicate amide IV. The main components of the samples classified in the FTIR spectra are proteins and polysaccharides [41]. From the results obtained, it can be concluded that the floating matter in the digesters is the anaerobic sludge residue.

3.2. Percentage of protein removal

The protein removal efficiency for a two-stage anaerobic reactor was investigated at different protein concentrations. Figs. 3 and 4 show the results of protein removal in Tanks 1 and 2, respectively. The highest percentage of protein removal was at protein concentrations of 3 and 6 g/L, achieving a 100% reduction in both Tanks 1 and 2. This result indicates that the protein was digested efficiently in the first three phases of the anaerobic digestion process. At a substrate concentration of 9 g/L, the protein removal efficiency slightly reduced to 93.5% in Tank 1 but stayed at 100% in Tank 2, indicating good performance. However, at higher substrate protein concentrations of 12 and 15 g/L, there were signs of reduction in digestion performance as the percentage of protein removal reduced to 34.3% and 37.2%, respectively, in Tank 1 and 71.9% and 46.9% in Tank 2. The reduction in protein removal indicates anaerobic

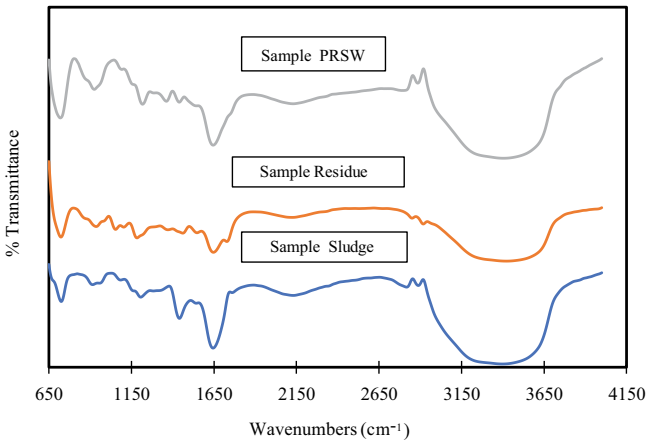
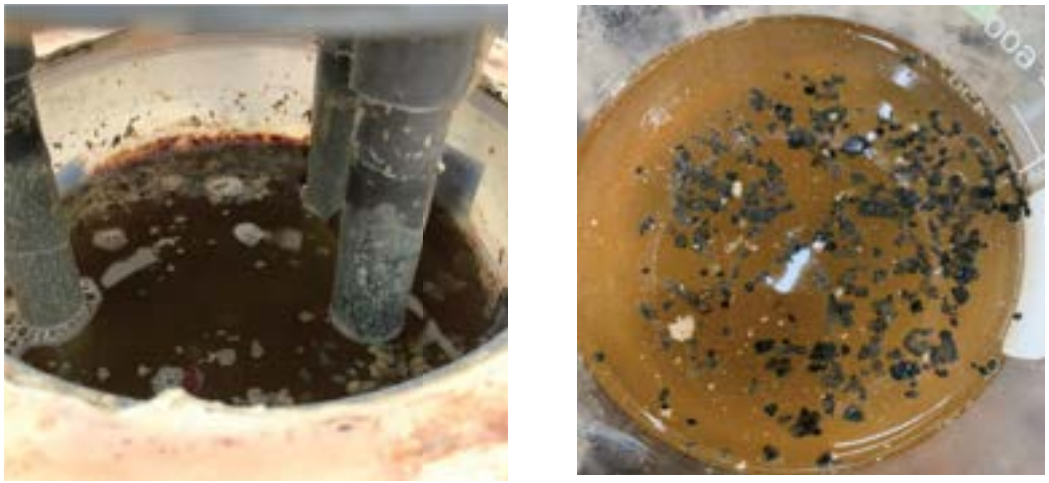


Fig. 2. Fourier-transform infrared spectroscopy analysis of the sludge residue, protein-rich synthetic wastewater, and anaerobic sludge.



(a) **(b)**

Fig. 1. Sludge flotation observed: (a) sludge residue on the surface of Tank 1 and (b) sludge residue present on the effluent of the digesters.

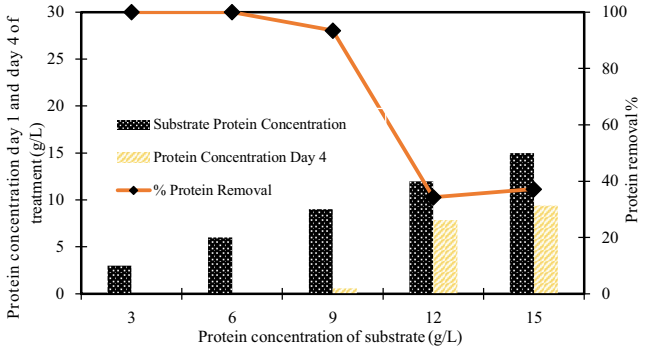


Fig. 3. Protein removal in Tank 1 (acidogenic reactor).

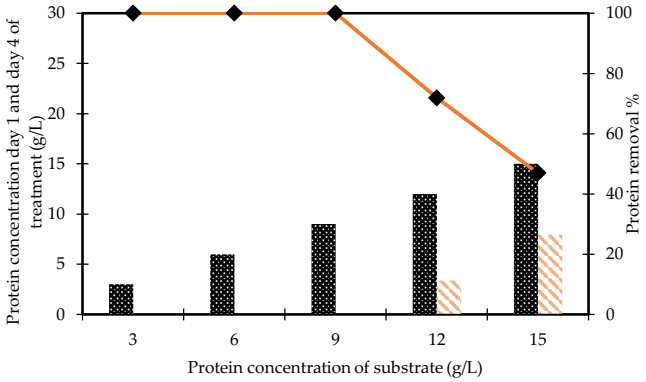


Fig. 4. Protein removal in Tank 2 (methanogenic reactor).

process inhibition, possibly due to the high concentration of volatile fatty acid (VFA) reducing the microbial activity.

The reported high protein degradation suggests that a significant portion of the protein was indeed converted into soluble nitrogen compounds. However, there are limitations to the type of amino acid chains that can be detected in the Bradford Assay Protocol method. The Bradford Assay Protocol detects basic amino acid chains such as lysine and histidine, whereas the highest concentration of amino acids found in the degradation of gelatines are glycine, proline, glutamic acid, and alanine [42]. The high percentage of protein removal could have left out these types of amino acids that may have still been present in the digester.

3.3. Ammoniacal nitrogen concentration and TVFA concentration

This study observed that at high protein concentrations (12 and 15 g/L), the ammoniacal nitrogen (AN) concentration increased above 200 mg/L. In Tank 1, the AN concentration was 278.6 and 219.4 mg/L on day 4, respectively, as shown in Fig. 5. Meanwhile, in Tank 2 (Fig. 6), the AN concentration was 219.2 mg/L for 12 g/L protein and 260.12 mg/L for 15 g/L protein. AN concentration of 50–200 mg/L benefits anaerobic processes because AN is required to synthesize amino acids and nucleic acids, which are essential for bacterial growth. It also assists in maintaining a neutral pH at a mesophilic temperature between 7.2 and 7.5. Most anaerobic digestion reactors are sensitive to ammonia, especially in the methanogenesis reactor. A study conducted with food

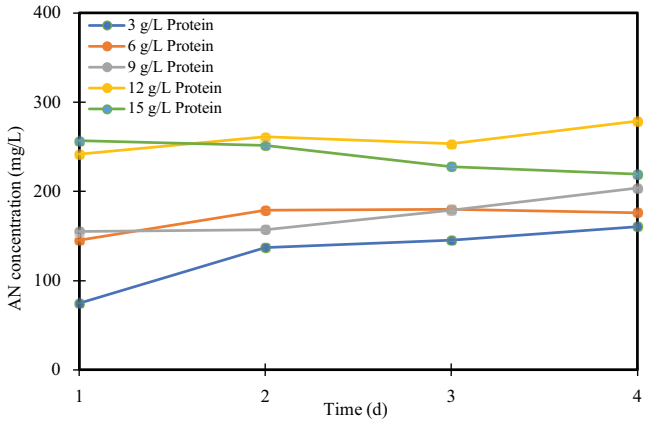


Fig. 5. Ammoniacal nitrogen concentration in Tank 1 (acidogenic reactor).

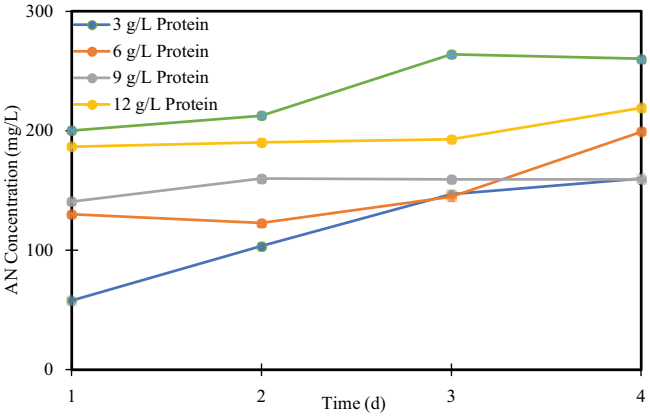


Fig. 6. Ammoniacal nitrogen concentration in Tank 2 (methanogenic reactor).

waste revealed that ammonia at a concentration of 2 g/L is sufficient to cause disturbances in the process when pH is not controlled [43], which was the case for this study. While hydrolysis and acidification were less impacted, ammonia nitrogen disruptions in AD processes were primarily due to the suppression of methanogenesis. High ammonium concentrations' inhibitory effects on AD would cause VFA build-up and a drop in pH. These elements would inhibit *Methanosaeta* action and the acetoclastic pathway [43]. The reactor system in this study may have experienced this inhibition, resulting in lower AN concentration's than theoretically predicted from protein breakdown [44].

It was observed that the TVFA increased higher in Tank 1 (Fig. 7) compared to Tank 2 (Fig. 8) due to the production of organic acids by the acidogenic bacteria [45]. Even though the process was slowed down at higher protein concentrations in both tanks due to AN inhibition [46], it was observed that the TVFA increased over time. Several researchers proposed that higher concentrations reduce the performance of anaerobes that utilizes propionic acid. Thus, propionic acid starts to accumulate. Propionic acid accumulation further inhibits the methanogens and increases the TVFA concentration in the reactor, causing an imbalance in the reactors due

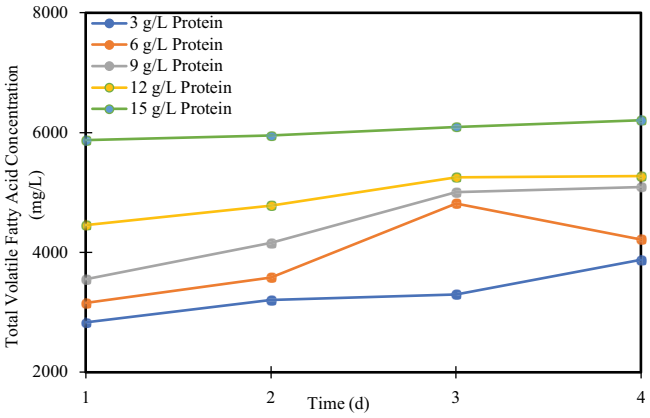


Fig. 7. Total volatile fatty acid concentration in Tank 1 (acidogenic reactor).

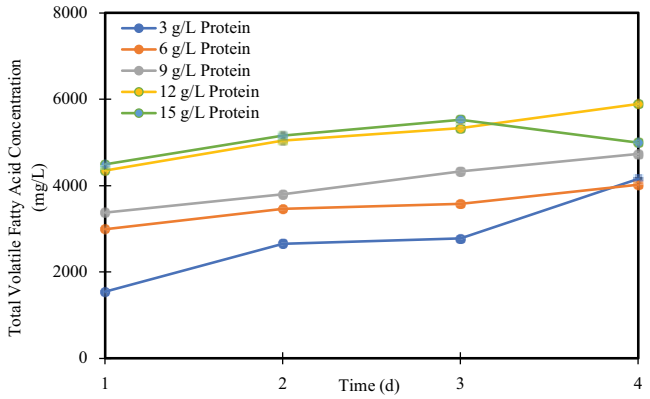


Fig. 8. Total volatile fatty acid concentration in Tank 2 (methanogenic reactor).

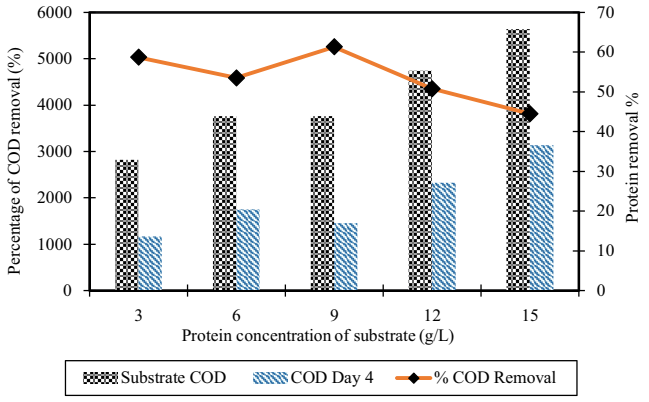


Fig. 9. Percentage of chemical oxygen demand removal Tank 1 (acidogenic reactor).

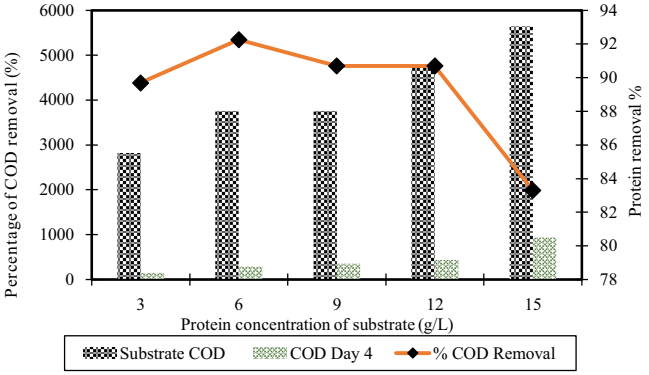


Fig. 10. Percentage of chemical oxygen demand removal Tank 2 (methanogenic reactor).

to acidification and reduced buffer capacity and the reduction of pH [46–48]. Additionally, the presence or build-up of intermediates, such as acetic acid, during the anaerobic degradation of gelatine may slow down the rate of gelatine hydrolysis in a mesophilic, anaerobic environment [49].

3.4. COD removal efficiency

For COD removal in Tank 1, the highest COD removal was at 9 g/L protein concentration of substrate at 61%, followed by 3, 6, 12, and 15 g/L, as shown in Fig. 9. Whereas for Tank 2 (Fig. 10), the highest COD removal was at 6 g/L protein concentration with a percentage of 92%. The COD removal percentage seemingly increased, above 53%, when treated with 3–12 g/L substrate protein concentration; however, it reduced when treated with 15 g/L protein concentration. Heat treatment to cheese whey powder to precipitate the protein before it was further hydrolyzed for biohydrogen production was used to increase the tendency of the protein to degrade [50]. It was also observed that the VFA was high even when the COD removal was above 90% (Fig. 10). High VFA and low COD concentrations in the effluent imply incomplete organic matter breakdown and a potential imbalance in the anaerobic digestion process. Besides that, the standard COD analysis method can be influenced by many factors such as digestion time and reagent strength. It is also possible that the soluble COD was easily detectable as compared to slowly biodegradable COD [51]. For the microbial activity to be at its peak, variables like temperature, pH, HRT, and OLR must be carefully managed [52]. Process imbalances and insufficient organic matter decomposition might occur when operating conditions differ. For instance, microbial activity may be hindered if the temperature is too low or the HRT is too brief [53,54].

3.5. Cumulative biogas production

The biogas accumulation increased steadily with an increase in protein concentration increment up to 12 g/L protein in the substrate (Fig. 11). The highest biogas accumulation was at a substrate concentration of 12 g/L at 21,100 mL on day 4, while the lowest biogas accumulation was substrate concentration of 15 g/L at 8,120 mL. Biogas accumulation was drastically decreased when fed with a protein concentration of 15 g/L. As discussed previously, this could be due to the high concentration of VFA in the digesters. According to current research, methanogen inhibition and organic matter hydrolysis in alkaline circumstances can increase the benefits of VFA generation compared to acidic or near-neutral environments [55]. In this instance, the high

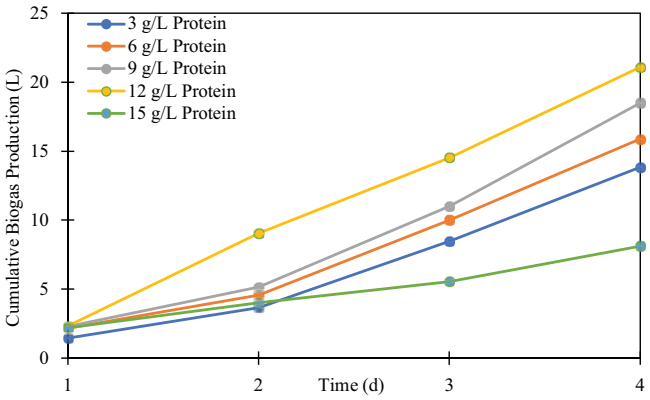


Fig. 11. Cumulative biogas production.

concentration of VFA reduced the pH of the digester, thus causing disturbance to the methanogens. Additionally, it has been noted that wastewaters containing protein have lower biogas yields, foaming, and poor effluent quality [56]. The combination of protein and carbohydrates is the most effective anaerobic digestion. Additionally, it was noted that anaerobic digestion based only on the protein was the least effective because the rates of organic matter removal and methanation declined with increasing protein concentration due to propionic acid build-up, a low C/N ratio, and reduced microbial activity [22,24].

The low cumulative biogas production and poor biogas yield coefficient indicate low process efficiency and ineffective biogas generation. Several circumstances may have caused the discrepancy. Proteins' complex metabolic properties, such as their high molecular weight or interactions with carbohydrates, and the high protein concentration in the substrate could lead to an incomplete breakdown. Due to this partial breakdown, fewer proteins are converted into biogas [57]. The high VFA concentrations in the digester suggest that ineffective protein breakdown can lead to a build-up of VFAs. Acetic acid, propionic acid, and butyric acid are examples of VFAs that can build up and hinder methanogenesis while upsetting the equilibrium of the microbial community in the anaerobic digestion system [53], which could be the case for this study [58]. By archaeal community analysis, *Methanosphaera* was the dominant species found present in a previous study of the same system [2] The majority of methane produced—nearly 70% of it—is created by acetoclastic methanogens. They primarily fall under two groups: *Methanosarcina* and *Methanosaeta*, which in this study is lacking. The limited acetoclastic methanogens could result in lower biogas yield.

4. Conclusions

Even at high protein concentrations, foam, and scum were absent in two-stage AD fed with PRSW. The absence of foam is related to the weak interaction of protein and complex configuration of gelation, while the absence of scum is due to the lack of lipidic materials in the digester. The COD and protein removal were higher in Tank 2 compared to Tank 1, while the TVFA were higher in Tank 1 compared to Tank 2. The protein concentration toxicity was observed at almost all the removals when fed as a single substrate. The highest biogas production was noted at 12 g/L with a cumulation volume of 21,000 mL, and the increase in protein concentration of 15 g/L led to a reduction of gas production to a volume of 8,120 mL.

Author contributions

Conceptualization, SNSSA, RO, HCM, AIMI, and HAT; methodology, SNSSA, RO, HCM, AIMI, HAT, and LCA; validation, RO, HCM, AIMI, and HAT; formal analysis, SNSSA, and RO; investigation, SNSSA; resources, RO and HCM; data curation, SNSSA; writing—original draft preparation, SNSSA; writing—review and editing, RO; visualization, SNSSA; supervision, RO, HCM, AIMI, and HAT; project administration, RO; funding acquisition, RO. All authors have read and agreed to the published version of the manuscript.

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Institutional review board statement

Not applicable

Informed consent statement

Not applicable

Data availability statement

The data from this study can be obtained from the corresponding author upon request.

Conflicts of interest

The authors declare no conflict of interest.

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