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The effect of pretreatment on VFA production from tofu and tempeh wastewater through anaerobic digestion batch

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Abstract: Tofu and tempeh, derived from soybeans, are widely consumed for their nutritional value and high protein content. However, the production of these foods generates nutrient-rich wastewater that poses environmental challenges while offering opportunities for valorization. This study investigates the production of volatile fatty acids (VFAs) and acetic acid from tofu and tempeh wastewater *via* batch anaerobic digestion, utilizing various pretreatment methods. The pretreatments included adjustment of the pH to 6 and inoculum treatments with and without heat shock under mesophilic and thermophilic conditions. Results demonstrated that the highest average total VFA concentrations of 10.08 and 9.79 g L^{-1} were achieved for tempeh at T3 (tempeh wastewater + pH 6 + thermophilic + heat shock) and tofu wastewater at TF3 (tofu wastewater + pH 6 + thermophilic + heat shock), respectively. The highest acetic acid concentrations were observed under mesophilic conditions, reaching 77.32% for tempeh wastewater at T7 (tempeh wastewater + unadjusted pH + mesophilic + heat shock) and 92.40% for tofu wastewater at TF10 (tempeh wastewater + pH 6 + mesophilic + non-heat shock). Notably, increased VFA production was associated with reduced cumulative methane yields, such as 3.65 mL g^{-1} volatile solid (VS) for tempeh at T3 and 25.23 mL g^{-1}-VS for tofu wastewater at TF3. These findings indicate the effectiveness of the pretreatment strategies in enhancing VFA and acetic acid production, suggesting significant potential for industrial applications. Further research is

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recommended to optimize production processes and explore the broader utilization of VFAs and acetic acid in the bioeconomy, promoting sustainability.

Key words: volatile fatty acid; wastewater; sustainability; methane; anaerobic digestion; resource recovery

Introduction

Soy products are the most prevalent protein sources in the human diet. However, they produce substantial amounts of wastewater, approximately 10 L per liter of raw material. The treatment costs associated with soy and dairy are also high, amounting to 130 US$ per cubic meter of effluent-treated water.[1] The by-products of tofu and tempeh production are solid waste and wastewater. The solid wastes from the tofu and tempeh industries are distinct owing to the varying production methods. Solid waste from the tofu industry is commonly referred to as 'tofu dregs or okara', which is more abundant than solid waste from the tempeh industry (banana leaves, plastic, and soybean skins). For every kilogram of soybeans processed into tofu, approximately 1.2 kg of soybean residue is produced.[2] Solid waste from tamari and tofu does not pose environmental harm, as most of it is sold to other industries or farmers for direct use in human food production and animal feed.[3]

Tofu wastewater is formed from the production process (soaking, washing soybeans, washing the equipment used for the production process, filtering, and pressing tofu in the molding process).[4] However, tempeh wastewater in the process of production is from washing, boiling, soaking, and mixing.[5] The quantity of wastewater from tofu process production is tremendous; in the case of the small-scale industry, with a production capacity of 150 kg of soybeans per day producing 147 kg of tofu, the by-product generated is 71.6 kg of solid waste consisting of 60 kg pulp, 11.6 kg soybeans skin, and 637.3 L wastewater.[6] Tofu and tempeh wastewater still have high protein and nutrition that can be utilized, such as for bioenergy and as an acid solution source owing to the effect of acid solution used in the coagulation process, such as acetic acid (CH_3OOH) and calcium sulfate ($CaSO_4$).[7] The types of acid solutions used for coagulation and the method used in the production process of tofu impact the characteristics and quality of wastewater like biological oxygen demand, chemical oxygen demand (COD), total suspended solids (TSS), and pH value.[8] The characteristic of tofu and tempeh wastewater is provided in Table 1.

Wastewater from tofu and tempeh processing is characterized by a high organic loading with high biodegradable compounds, which promotes intensive microbial metabolism, leading to acidification and the production of malodorous byproducts.[16] Moreover, the physicochemical characteristics of the liquid effluent from the tempeh production process indicate that nitrate concentration remains within regulatory limits, whereas free ammonia originating from soybean soaking wastewater exceeds the permissible threshold, thereby posing a potential risk to the aquatic ecosystem. In addition, proteins present in tofu wastewater are largely transformed into ammonium and phosphate ions, which function as key nutrients supporting microbial energy metabolism, cell membrane integrity, carbohydrate and amino acid biosynthesis, and cell proliferation.[17–19]

However, there are some obstacles for industry owners, especially in micro, small, and medium enterprises in managing wastewater, namely a lack of knowledge in waste management, the narrow space to manage their wastewater, financial and technical issues, including a lack of training and campaigns from stakeholders linked to industrial activities. Moreover, the production of tofu and tempeh in Indonesia is often scattered throughout urban areas, making it difficult to implement efficient whey utilization practices.[20,21] Hence, to prevent the risk of harm to the environment and social aspects, it is vital for the soy processing industry to manage and recycle the wastewater by considering environmental, social, and economic factors, even though it will impact the production cost.[22,23] Those challenges can be achieved by implementing alternative waste management strategies that address the chemical and physical characteristics and pollution load in each step of the wastewater generation process.[5]

A highly promising solution is to utilize soy wastewater to produce volatile fatty acids (VFAs) as the acetic acid source through anaerobic digestion. Usually, micro- and small-scale tofu industries in Indonesia use tofu whey for coagulation owing to its economically friendly and easy-to-use properties.[24] Whey can serve as a coagulant in the food industry, similar to the tofu production process,

Table 1. Characteristics of tempeh and tofu wastewater.

Parameter	Tofu wastewater	Tempeh wastewater	Unit
COD	2290*	32 297.71**	mg L^{-1}
TSS	64*	0.75**	mg L^{-1}
pH	2.65*	4.8***	
Protein	1.11****	0.47	g L^{-1}
Total fat	8.2	0.04	g L^{-1}

Sources: *(Amalia *et al*. 2022)[9]; **(Pakpahan *et al*. 2021)[10]; ***(Nurhayati *et al*. 2011)[11]; ****(Shidik, 2025)[12]; *****(Nurandani *et al*. 2023)[13]; (Sari & Rahmawati, 2020)[14]; (Siska *et al*. 2025).[15]
COD, Chemical oxygen demand; TSS, total suspended solids.

offering an alternative to conventional coagulants.[25] Soy wastewater, especially tofu wastewater, is extensively used in the recovery of compounds or nutrients for microbial or enzymatic treatment to produce new beverages like nata de soya.[26,27] The addition of 30% *Acetobacter xylinum* bacteria to tofu wastewater in producing nata de soya resulted in the highest organoleptic tests (color, flavor, and elasticity), thickness (2.76 cm), yield (51.4%), fiber (54.025), and water content (85.8%).[28] Soy whey has been extensively studied for its potential applications in the production of soy protein isolates, soy cheese, and other soy-based products.

Volatile fatty acid production through an anaerobic digestion process is a traditional alternative that promises benefits from both economic and environmental aspects.[29] Volatile fatty acids are produced from the intermediate phase of the newly developed anaerobic digestion process.[30,31] The product of anaerobic digestion is biogas with H_2 and VFAs as intermediate products. Despite the challenges associated with VFA production, it has garnered significant attention owing to the superior value-added compared with biogas. The biogas market in 2024 is valued at $82.9 billion, with a compound annual growth rate (CAGR) of 9%. The global market for VFAs is $98.2 billion, also growing at a CAGR of 9.5%.[32] Volatile fatty acid production through an anaerobic digestion process is achieved at a shorter hydraulic retention time (HRT) than for biogas. Although VFAs are composed of two to six carbon organic acids, the primary acids are typically acetic acid and butyric acid. These acids can be converted into high-value chemicals such as bioplastics and biofuels, which confer a higher economic value compared with biomethane.[33]

To optimize VFA production during anaerobic digestion, pretreatment is of paramount importance. The key pretreatment techniques employed to achieve this include pH adjustment, temperature control, heat shock treatment for microorganisms, and methane inhibition.[34–36] The pH value is a very essential factor that impacts VFA yield and affects to the competition between acidogenesis and methanogenesis in the process of anaerobic digestion. At pH 5.5, the highest concentration of VFA composition is acetic acid, around 50%, however, at pH 7 obtaining a VFA yield of 36.6% g COD-VFA g^{-1} COD substrate is obtained with 20% of propionic and 30% butyric acid.[34] Additionally, heat shock or thermal treatment ranges from 140 to 170 °C, and the addition of 0.5–3% HCl and H_2SO_4 increases the solubilization of organic matter and can inhibit methanogens.[35] The effect of pretreatment influences microbial diversity; thermal treatment will reduce it and be more selective. The combination of thermal and acid pretreatments significantly affects the shift of the dominant microbial communities from non-dominant into more prominent such as *Cloacimonadota* and *Spirochaetota*.[37]

It is noteworthy that the low molecular weight proteins and carbohydrates, which serve as substrates for VFA production, accumulated as the pH increased.[38] Furthermore, bacteria compete with environmental microbes for nutrients and organic substrates, which can impact the growth and activity of VFA-producing bacteria, ultimately altering VFA yield and composition.[39] Pretreatment may have promoted metabolic pathways for butyrate formation, yet substrate availability and microbial rates probably limited overall VFA production.[40] The pretreatment used in this study was heat shock for the inoculum, and the temperature in the batch reactor in this study is thermophilic and mesophilic. Heat shock pretreatment of the inoculum was used to inhibit methanogenic activity during anaerobic digestion and systematically examine the combined effects of heat shock pretreatment, pH control, and temperature (mesophilic and thermophilic conditions) on VFA production from substrates. Optimizing heat shock pretreatment effectively suppressed methanogenic activity while enhancing VFA accumulation. The highest total VFA production was 14 883 mg L^{-1}, and the VFA yield was 496.1 mg g^{-1} VS, which was 26.98% higher than that for the untreated heat shock.[41] Furthermore, the impact of heat shock inoculum reduced the relative abundance of some microbial groups, including Porphyromonadaceae, the abundance of which dropped from 13 to 1% at pH 10 on day 1, but increased up to 12% by day 20. Then, at alkaline pH, Marinilabiacaea were completely inhibited, and Bacteroidaceae were strongly reduced, and with acidic heat treatment the abundance of Prevotellaceae was decreased.[42] Moreover, heat shock negatively affected bacterial diversity and archaeal richness; however, archaeal diversity remained largely unaffected as methanogenic activity during HA2 (mesopihlic temperature) was maintained.[43] Temperature influences biochemical processes and microbial growth, directly affecting hydrolytic bacteria and shaping the microbial community structure, which in turn impacts fermentation. Although mesophilic conditions are optimal for VFA production, the process remains energy intensive.[44] In addition, VFA accumulation can be caused by an imbalance between acid-producing and acid-consuming or by inhibition of the consumers.[45] The combination of pretreatment and pH was also reported by Logan *et al*.,[42] where the highest VFA yield of 0.516 g COD g^{-1} VS was achieved at alkaline pH, which was 45% higher than at acidic pH. The sequencing showed increased abundances of Clostridiaceae, Bacteroidaceae, and Prevotellaceae, which are involved in VFA production and selenium reduction.

This study aimed to obtain optimum total VFA production from tofu and tempeh wastewater through batch anaerobic digestion with adjusted substrate pH, heat shock inoculum pretreatment, and temperature treatment in the water bath.

Materials and methods

Sample preparation (tempeh and tofu wastewater)

The wastewater was generated through the simulated production of tofu and tempeh in a laboratory setting. The preparation of tempeh and tofu wastewater differed owing to the distinct production processes involved. In brief, soaking soybeans overnight increased the size of the soybeans and made them soft and thus easy to peel. Then, the soybeans were washed to remove all the dirt after peeling, and the soybeans were boiled until a white foam appeared. To obtain tempeh wastewater, the boiled soybeans were filtered and the boiled water was collected. The tempeh wastewater used in this study was collected from the soaking, washing, and boiling steps. The flowchart of tempeh and tofu production is provided in the Appendix, Fig. A1.

The preparation methods for tofu and tempeh wastewater were identical, but the subsequent steps differed after the soybeans had been boiled. Following boiling, the soybeans were milled into soy porridge. Subsequently, the porridge was filtered to obtain soy milk. The soy milk was boiled until a white foam appeared. Furthermore, the soy milk was poured into a tofu mold and cooled to approximately 50°C. Subsequently, an acid solution was poured into the mold for the coagulation process. The acid solution utilized in this study was lemon juice. The top of the mold was pressed with a heavy weight to compact and separate the water. The tofu wastewater collected from soaking, washing, boiling, and tofu molding was used in this study. The wastewater resulting from boiling and molding is known as 'whey', which can generally be used as an acid solution for subsequent process production in small-scale industries.

Table 2. Characterization of substrate and inoculum before the experiment.

Parameter	Wastewater		Inoculum
	Tempeh	Tofu	
COD (mg L^{-1})	10 200	26 400	–
TS (%)	6.25	2.1	4.2
TSS (mg L^{-1})	9.6	10.8	3.01
VS (mg L^{-1})	5.0	2.0	3.33
VSS (mg L^{-1})	0.00065	0.0023	0.0033
pH	5.82	5.61	7.38

COD, Chemical oxygen demand; TS, total solid; TSS, total suspended solids; VS, volatile solid; VSS, volatile solid suspended.

Preparation of inoculum

The inoculum used in this study was granular sludge collected from an up-flow anaerobic sludge blanket reactor treating municipal sewage wastewater (Hammarby Sjöstad, Stockholm, Sweden). The inoculum was incubated for one week at 37°C (mesophilic temperature) and 57°C (thermophilic temperature) for thermophilic incubator. Before use, the inoculum was treated for heat shock (thermal pretreatment) to maximize the production as a result of the inhibited activity and growth of methanogens. The inoculum was added to a 100 mL experimental serum glass bottle, then put in the water bath, and heated at 80 °C for 15 min for heat shock treatment.[46] Furthermore, the mixture immediately cooled down in an ice chamber. The heat shock was applied for both inoculums, namely mesophilic and thermophilic. There are four types of inocula used in this study, namely mesophilic + heat shock, mesophilic + non-heat shock, thermophilic + heat shock, and thermophilic + non-heat shock. Initial measurements of substrate and inoculum were carried out as initial data for characterization before experiments on anaerobic digestion batches (Table 2).

Methods

Experimental setup for batch anaerobic digestion

The study was conducted in an anaerobic digestion batch experiment in the water bath. The assay was conducted in a 120 mL serum glass bottle, with an 80 mL working volume. The substrate and inoculum were mixed at a 1:1 ratio (40 mL each) to maintain balanced conditions. The serum glass bottle was tightly sealed, and immediately flushed with nitrogen gas for 2 min to replace the oxygen inside the reactor to obtain

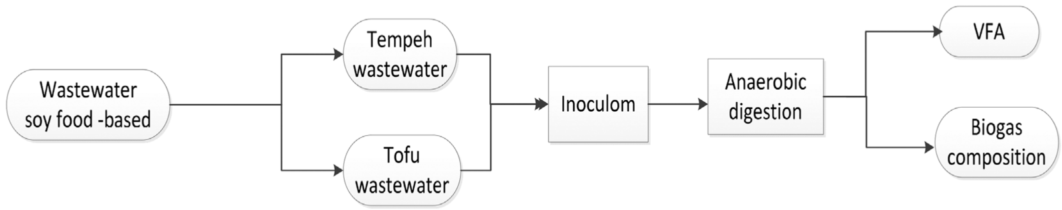

Figure 1. The experimental setup of the anaerobic digestion batch.

Table 3. Variable samples of anaerobic digestion batch.

Type of wastewater			
Tofu wastewater		Tempeh wastewater	
Name of sample	Details of sample	Name of sample	Details of sample
TF1	Tofu wastewater + unadjusted pH + thermophilic + heat shock	T1	Tempeh wastewater + unadjusted pH + thermophilic + heat shock
TF2	Tofu wastewater + unadjusted pH + thermophilic + non- heat shock	T2	Tempeh wastewater + unadjusted pH + thermophilic + non- heat shock
TF3	Tofu wastewater + pH 6 + thermophilic + heat shock	T3	Tempeh wastewater + pH 6 + thermophilic + heat shock
TF4	Tofu wastewater + pH 6 + thermophilic + non-heat shock	T4	Tempeh wastewater + pH 6 + thermophilic + non-heat shock
TF5	Blank + thermophilic + heat shock	T5	Blank + thermophilic + heat shock
TF6	Blank + thermophilic + non-heat shock	T6	Blank + thermophilic + non-heat shock
TF7	Tofu wastewater + unadjusted pH + mesophilic + heat shock	T7	Tempeh wastewater + unadjusted pH + mesophilic + heat shock
TF8	Tofu wastewater + unadjusted pH + mesophilic + non-heat shock	T8	Tempeh wastewater + unadjusted pH + mesophilic + non-heat shock
TF9	Tofu wastewater + pH 6 + mesophilic + heat shock	T9	Tempeh wastewater + pH 6 + mesophilic + heat shock
TF10	Tofu wastewater + pH 6 + mesophilic + non-heat shock	T10	Tempeh wastewater + pH 6 + mesophilic + non-heat shock
TF11	Blank + mesophilic + heat shock	T11	Blank + mesophilic + heat shock
TF12	Blank + mesophilic + non- heat shock	T12	Blank + mesophilic + non- heat shock

T, Tempeh wastewater; TF, tofu wastewater.

an anaerobic condition. Then, the reactor was incubated in a water bath shaker at 37 and 57 °C at 100 rpm (Fig. 1).

Three times a week, 250 µL of biogas was taken using a gas-tight syringe (VICI, Precision Sampling Inc., USA) to analyze biogas composition, and 1 mL of liquid was taken from the reactor using a syringe for VFA analysis. The experiment was carried out for 33 days with three replicates (Table 3).

Analytical method

The total solid (TS), volatile solid (VS), TSS, and volatile solid suspended (VSS) were measured using an oven and muffle furnace at 105 °C and 550 °C with the standard method American Public Health Association (APHA-AWWA-WEF-2005). pH value was analyzed by the pH meter (Mettler Toledo F20 FiveEasy, OH, USA). The COD was measured using a CSB 15000 test kit and the concentration of COD was analyzed with a Nanocolor 500D Photometer (MACHEREY-NAGEL GmbH & Co. KG, Germany).

The analysis of biogas composition was using gas chromatography (Clarus 550; Perkin-Elmer, Norwalk, CT, USA) with a column (Carboxen™ 1000, 6 × 1.8 OD, 60/80 mesh, Supelco, Shelton, CT, USA). Furthermore, the VFA was analyzed by gas chromatography (Clarus 550; Perkin-Elmer, Norwalk, CT, USA) with a capillary column (Elite-WAX ETR, 30 m × 0.32 mm × 1.00 μm, Perkin-Elmer, Shelton, CT, USA) and a flame ionization detector. Prior to VFA analysis, the wastewater was mixed with an acid mix [25% (v/v) formic acid and 25% (v/v) ortho-phosphoric acid at a ratio of 1:3] and centrifuged at 10 000 rpm for 5 min. Then the supernatant was filtered through 0.2 μm of syringe filter to remove undissolved particles, and Butanol was added at a concentration of 1 g L^{-1} as an internal standard. It was put into the vial and milliQ water added to a total volume of 1 mL. The total production of VFA and the biogas composition from batch tests were compared to evaluate the effects of inoculum pretreatment, adjusted pH, and thermal pretreatment using statistical analysis. An analysis of variance (ANOVA) test followed by Duncan's multiple range test was used at the significance level of P-value < 0.05.

Results and discussion

Tofu and tempeh wastewater has a high potential to be used as a source of acetic acid. The research results in this study confirm this fact through an anaerobic digestion process. The result shows the dominant VFA compound to be acetic acid in both wastewaters (tofu and tempeh wastewater). In addition, the effect of heat shock treatment on the inoculum, pH value of the substrate, and temperature in the water bath resulted in variations in methane and VFA concentrations in each treatment which are discussed in detail in this section.

The effect of pH and heat-shock treatment on total VFA and VFA distribution of tempeh wastewater

The effect of pH and heat shock treatment on VFA production is crucial to the anaerobic digestion process. The results of the study show that unadjusted pH and heat shock treatment can improve VFA yield. Based on the results provided in Tables 4 and 5, the production of total VFA in the blank samples for both heat shock and non-heat shock treatment at fermentation day 14 was not high, and it only lasted for a few days. The total VFA for T5 and T6 at fermentation days 14, respectively, is 4.08 and 2.02 g L^{-1} (Table 4).

The high VFA production is influenced by several factors such as the types and dosage of substrates, the concentration of organic loading rate, temperature treatment, and reactor performance. A higher organic loading rate at 9 L^{-1} per day at a temperature of 40 °C results in the highest VFA.[30] Moreover, the total VFA production in mesophilic conditions at T11 and T12, respectively, is 3.73 and 2.36 g L^{-1} (Table 5).

Table 4. The results of blank samples T5 and T6 on fermentation day 14.

VFA composition	Blank samples	
	T5	T6
Acetic acid (%)	60.31	100
Propionic acid (%)	11.53	0
Isobutyric acid (%)	7.76	0
Butyric acid (%)	6.65	0
Isovaleric acid (%)	13.74	0
Valeric acid (%)	0	0
Caproic acid (%)	0	0
Total VFA (g L^{-1})	4.08	2.02
STDEV (±)	20.96	37.80

STDEV, standard deviation; VFA, Volatile fatty acid.

Table 5. The results of blank samples T11 and T6 on fermentation day 12.

VFA composition	Blank samples	
	T11	T12
Acetic acid (%)	56.56	100
Propionic acid (%)	11.52	0
Isobutyric acid (%)	8.31	0
Butyric acid (%)	7.77	0
Isovaleric acid (%)	15.81	0
Valeric acid (%)	0	0
Caproic acid (%)	0	0
Total VFA (g L^{-1})	3.73	2.36
Standard deviation (±)	19.51	37.80

VFA, Volatile fatty acid.

In addition, the highest concentration of acetic acid in the blank sample is reached in non-heat shock treatment for both temperature conditions, namely 100% at T6 and T12.

However, the blank sample with heat shock treatment at both temperatures produced low concentrations of acetic acid, namely 60.31% at T6 and 56.56% at T11. Although it produces high levels of acetic acid in the non-heat shock treatment, the total VFA in the heat shock treatment is higher. Hence, it can be concluded that the combination of heat shock and thermophilic treatment significantly affects in VFA production. The production of total VFA in thermophilic conditions is higher than in mesophilic conditions, however, the concentration of acetic acid is not much different and is still in the same range for all treatments in blank samples. Pretreatment of the inoculum is one of the effective methods to improve acidification in anaerobic digestion, reaching the optimum pretreatment method depends on the type of feedstock and inoculum.[47]

Furthermore, the highest total VFA production in thermophilic conditions was 10.08 g L^{-1}, achieved at T3, followed by 2.45 g L^{-1} at T7 for mesophilic conditions (Fig. 2). However, the acetic acid concentration from tempeh wastewater was the highest compared with butyric acid, propionic acid, and isobutyric acid for all treatments. Based on Fig. 2, the anaerobic digestion process on tempeh wastewater resulted in a concentration of acetic acid exceeding 50% compared with the other VFA compositions, such as propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, and caproic acid. The concentration of acetic acid for all treatments of tempeh wastewater was high. The high acetic acid content in anaerobic digestion inhibited the process of methanogens producing biogas. However, the acetic acid concentration decreased greatly at fermentation day 32 for all the treatments, with the lowest acetic acid concentration of 5.32% achieved in T1, followed by T4 (19.53%). Furthermore, the highest acetic acid concentration was attained at T7, T8, and T10 (Fig. 2) on day 1 of fermentation. The average acetic acid concentration in thermophilic conditions (T1, T2, T3, and T4) was 66.95, 53.79, 69.39, and 56.19%. Additionally, the average acetic acid concentrations in mesophilic conditions (T7, T8, and T9) were 75.84%, 77.32%, and 75.53%, which were within the same range. Consequently, a low acetic acid concentration at T9 (69.71%) was observed. The high acetic acid concentration on the first day of fermentation was probably due to the metabolic regulation of the microorganisms. The results from the addition of initial sugar from 320 to 450 g L^{-1} increased acetic acid rapidly by 1.06–1.62 g L^{-1} in the process of the final wine, which is due to the regulation of yeast metabolism which is driven by hyperosmotic stress.[48]

However, decreasing the concentration of acetic acid causes an increase in the number of carbons in the VFA molecular structure, particularly butyric acid, propionic acid, isobutyric acid, and isovaleric acid. The highest butyric acid content was 80.58% at T7 treatment, followed by 76.55% at T2 (Fig. 2). This high VFA content is based on the accumulation of acetate that produces high ammonia concentrations, which can inhibit acetogenesis and methanogenesis reactions.[49] The situation during the anaerobic digestion process can lead to high results of acetic acid and glycerol, as byproducts of fermentation. In general, the concentration of acetic acid in mesophilic conditions was higher than in thermophilic conditions (Fig. 2). However, this is not related to the total VFA produced. Overall, the total VFA was higher in thermophilic conditions and lower in mesophilic. The relation between temperature and VFA accumulation in anaerobic digestion is complex and influenced by several factors. Some studies suggested that thermophilic conditions can lead to increased VFA accumulation, while others indicate that mesophilic conditions may result in higher VFA yield. A study by David Fernández-Domínguez *et al.*[50] reported that the highest VFA yield was 0.49–0.59 $gCOD_{VFA}$ g^{-1} VS achieved at a temperature of 35°C (mesophilic conditions), but the VFA composition was not influenced by the fermentation temperature. On the other hand, the thermophilic temperature can increase the rate of hydrolysis and acidogenesis, resulting in high concentrations of acetic acid and isovaleric acid, and VFA accumulation. Thermophilic conditions can improve the activities and growth of bacteria, which can release the α-glucosidase and protease. Furthermore, thermophilic anaerobic digestion can enhance enzyme activities and metabolic pathways, enrich *Methanosarcina* and dominant bacteria such as thermodynamics, accelerating biological and chemical conversion, and providing advantages over mesophilic.[51–53]

The decrease in acetic acid content at the end of retention time (fermentation) owing to certain microorganisms like *Acetobacter* which oxidize ethanol to acetic acid during the advanced stage of fermentation.[54] However, it is not always the acetic acid that will decrease at the end of retention time several factors can decrease acetic acid during the anaerobic digestion process, such as strain yeast and the type of substrate with low acetic production are used.[55] Additionally, the environmental factors that slow down the anaerobic digestion process, such as low temperatures, can also lead to lower acetic acid production at a temperature of 20 °C, acetic acid decreases, moving slightly to the middle of the exponential growth phase.[56] The higher the concentration of acetic acid, the higher the inhibition of methanogens to produce methane, which affects the quantity and quality of biogas as the final result of the anaerobic digestion process, the increase of acetic acid up to 45 g L^{-1} can inhibit cell growth and ethanol oxidation.[57]

The types of substrates used in the anaerobic digestion process also affect the production of VFA. The premier sewage sludge is particularly effective in generating a substantial quantity of VFA and acetic acid, owing to its high content of readily biodegradable and soluble monomeric organic matter, including glucose, fructose, and amino acids.[58] The substrate utilized in this research was tempeh and tofu wastewater, which is high in protein content. Consequently, during the fermentation process associated with their production, a high concentration of acetic acid is produced. This occurs as microorganisms break down the organic compounds present in soybeans, resulting in acetic acid as a byproduct.[26,59] However, the fermentation process

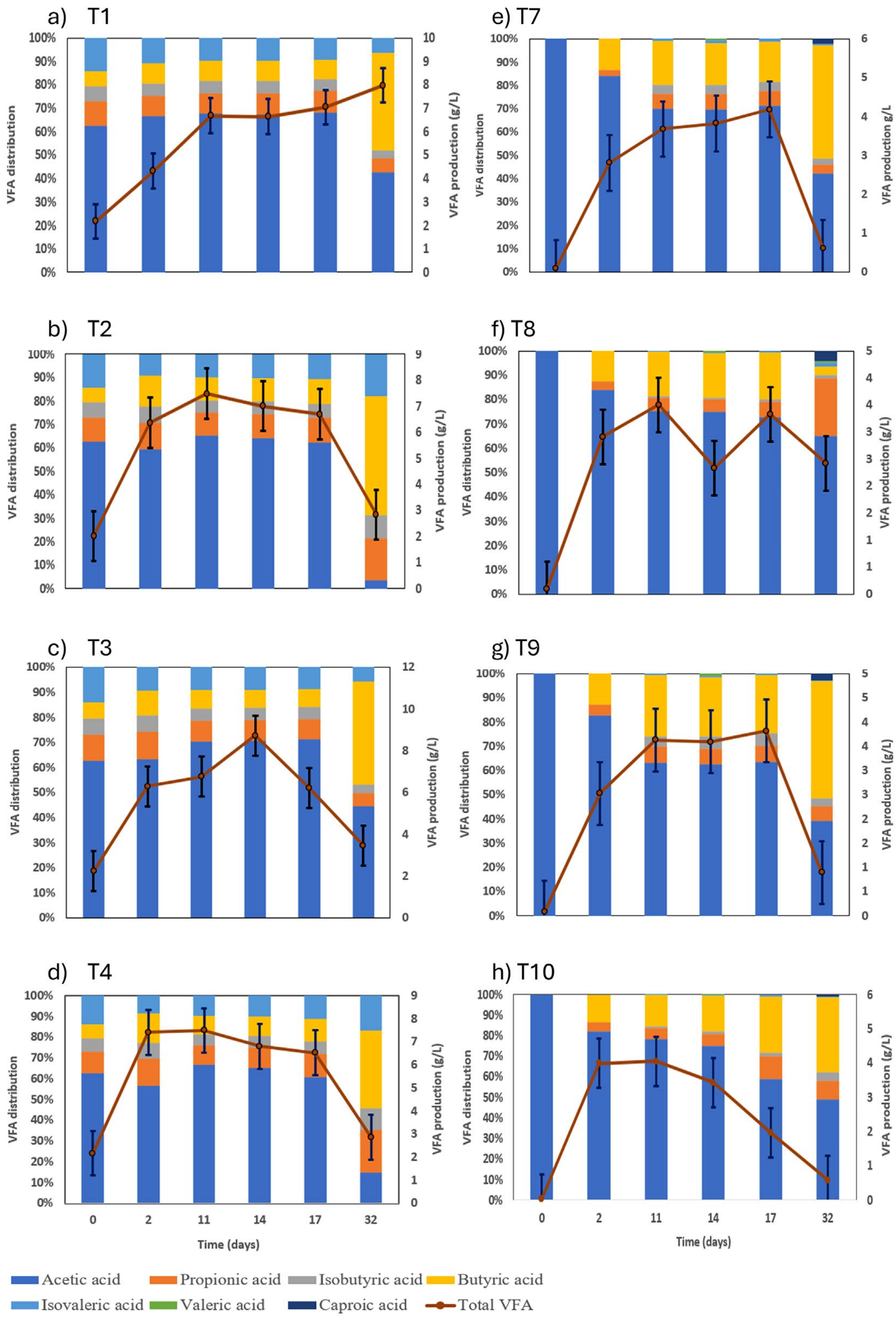

Figure 2. Volatile fatty acid concentration and distribution in tempeh wastewater of T1,T2, T3, T4, T7, T8, T9, and T10.

also leads to the production of other acidic compounds, such as propionic acid, which can further contribute to the acidity of the wastewater.[60]

Moreover, based on the result in Fig. 3. the lowest cumulative methane is 0.22 mL g^{-1} VS achieved at T9, and the highest is 44.98 mL g^{-1} VS at T2. The relation between

VFA production and the quality of biogas composition in this study is influenced by pretreatment heat shock in the inoculum. Heat shock treatment serves to select the microbial communities that can thrive under pressure conditions, including halt methanogenesis, thus the selected microbial communities can produce high VFA.[61] The heat shock treatment had a significantly positive impact on VFA production, contrary to the non-heat shock treatment. This study also employed adjustment pH as a parameter of observation (pH 6 and acidity pH) that influences the performance of reactors and the activities of microorganisms. This is because methanogenesis is highly sensitive to acidic conditions (pH changes) and is active within the range of pH 6.8–7.24.[62]

According to Fig. 3 in terms of methane production both treatments show a lazy S- profile with a slow rate until the first day, then increases in period retention time 14–21 days, that point the rate begins to decrease. The cumulative methane at T2 (44.98 mL g^{-1} VS) is higher than at T10 (24.78 mL g^{-1} VS). This is because the pH substrate has a substantial impact on biogas production. The pH range of 6.4–7.6 is considered ideal for the growth of bacteria and optimal for biogas production.[63] Both pH and temperature critically influence biogas production. The highest specific biogas yield reaches 161.09 mg L^{-1} of COD removal at 50 °C, demonstrating that thermophilic conditions (50–60 °C) optimize bacterial activity and enhance production efficiency.[64]

The kinetics of biogas production in mesophilic and thermophilic conditions are similar; however, the energy in thermophilic conditions is considerably higher compared with mesophilic conditions, thus, considerably required in optimizing the biogas production.[65]

The effect of pH and heat-shock treatment in total VFA and VFA distribution of tofu wastewater

The effect of pH and HS treatment on tofu wastewater differs slightly from tempeh wastewater. The total VFA of tofu wastewater is not significantly different from tempeh wastewater, because both wastewaters form a similar composition of organic matter and contain a high level of protein, carbohydrate, and other compounds that can contribute to VFA production during anaerobic.[13,66] Moreover, the high concentration of acetic acid in tofu wastewater is related to its fermentation process which contains eight types of amino acids, i.e. aspartic acid, glutamic acid, arginine, serine, glycine, leucine, lysine, and histidine that can be converted to produce VFA.[22]

The relationship between VFA and amino acid serves as the starting material for producing VFA. However, the acetic acid of tofu wastewater tends to stabilize or not much decrease at the end of fermentation unlike in tempeh wastewater. It is because tofu production involves coagulating soy milk with agents like acidic whey, acetic acid, gypsum (calcium sulfate dihydrate), and tofu seed solution (tofu wastewater that is left over one night), which could contribute acetic acid to the higher acetic acid in tofu wastewater.[8,67]

Moreover, tempeh production involves fermenting cooked soybeans with microorganisms, which might result in a different acid profile. Based on the results provided in Tables 6 and 7, the total VFA in the blank samples of tofu wastewater in thermophilic and mesophilic conditions with heat and non-heat shock treatment were in the same range. Total VFA in thermophilic conditions with heat shock treatment (TF5) is 7.92 g L^{-1} which is not different from non-heat shock treatment (TF6) 7.95 g L^{-1} (Table 6).

Similarly, total VFA values under mesophilic conditions with heat and non-heat treatment were significantly not different: 7.2 g L^{-1} at TF11 and 7.44 g L^{-1} at TF12 (Table 7). The highest acetic acid content in blank samples of tofu wastewater was 62.44% reached at TF6 (Table 6). However, the acetic acid contents in blank samples of tofu wastewater at TF5 (58.77%), TF11 (59.90%), and TF12 (55.62%) were not much different.

The main composition of VFA in tofu wastewater consists of acetic acid, butyric acid, propionic acid, isobutyric acid, and isovaleric acid. The highest average content of acetic acid from all treatments is achieved at TF10 and TF7, respectively, 92.40% and 91.68% (Fig. 4). The fermentation anaerobic digestion process of tofu wastewater was the same as that of tempeh wastewater. The highest acetic acid content based on the fermentation period is 100% achieved at TF7 (at day 1 HRT) (Fig. 4). However, the highest total VFA is 9.79 g L^{-1} achieved by TF4 at fermentation day 2 HRT. Hence, fermentation significantly affects the composition and total VFA production as a parameter on an anaerobic digester for performance and stability.[68]

The short retention time of 1.5 days produces a high VFA accumulation of 0.48 ± 0.01 g COD_{VFA} g^{-1} $TCOD_{fed}$ because the short retention time is inappropriate for methanogens and slow-growing in consumed VFA to biogas production.[69] The short fermentation at 8 h results in the highest main component of VFA, including acetic acid 1.1845 ± 0.0165 mM L^{-1}, propanoic acid 0.5160 ± 0.0141 mML^{-1}, and butyric acid 0.0148 ± 0.0009 mM L^{-1} with VFA yield 48.20 ± 1.21%.[70] This indicates that the short fermentation increases the production of the main VFA component which is more beneficial in the acidogenesis phase because it allows for a more efficient

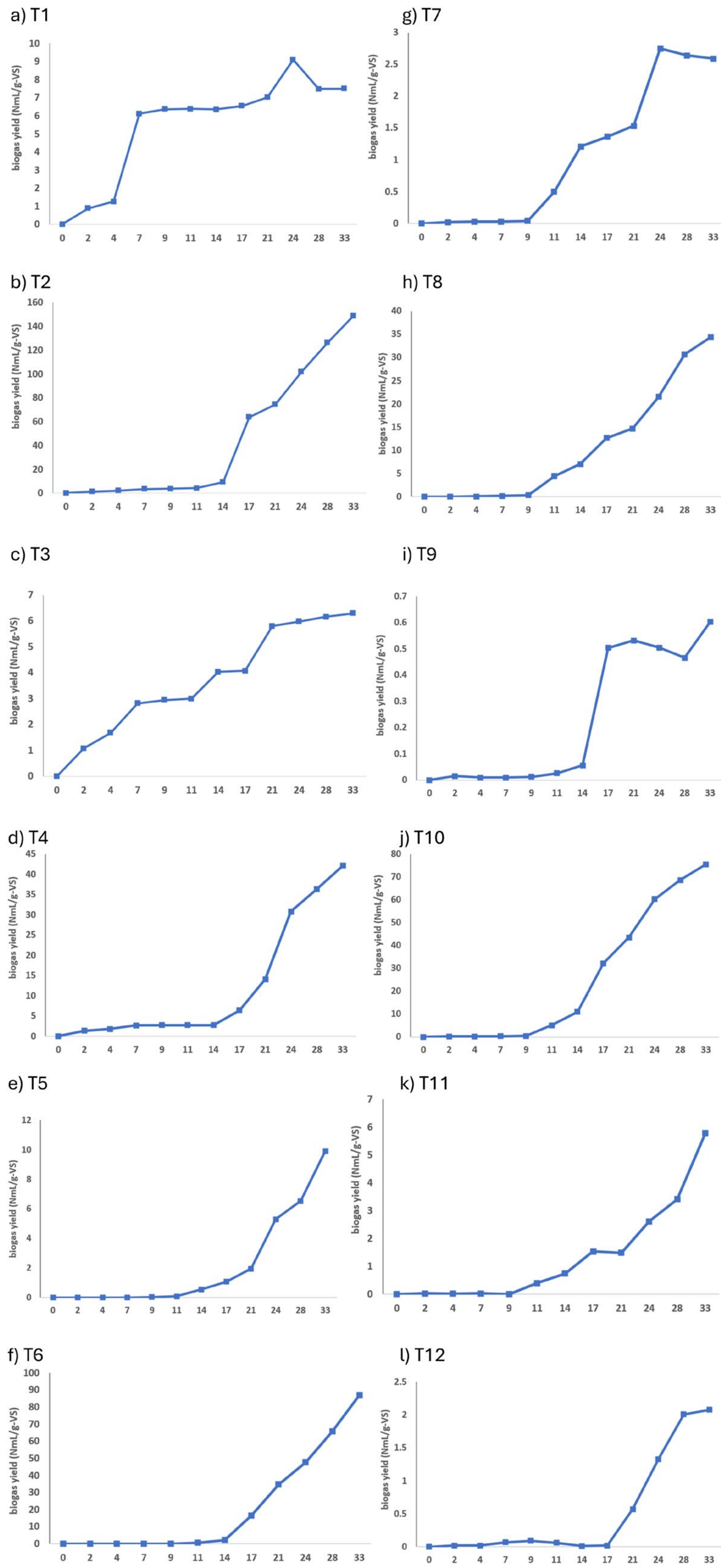

Figure 3. Methane content measured in tempeh wastewater across 12 experimental treatments (T1 - T12).

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Table 6. The results of blank samples at TF5 and TF6 on fermentation day 2.

VFA composition	Blank samples	
	TF5	TF6
Acetic acid (%)	58.77	62.44
Propionic acid (%)	12.72	13.18
Isobutyric acid (%)	8.78	9.25
Butyric acid (%)	8.1	8.79
Isovaleric acid (%)	15.1	16.67
Valeric acid (%)	0	0
Caproic acid (%)	0	0
Total VFA (g L^{-1})	7.92	7.95
Standard deviation (±)	20.24	21.50

VFA, Volatile fatty acid.

Table 7. The results of blank samples at TF11 and TF12 on fermentation days 22.

VFA composition	Blank samples	
	TF11	TF12
Acetic acid (%)	59.90	55.62
Propionic acid (%)	11.52	27.5
Isobutyric acid (%)	7.83	0
Butyric acid (%)	6.91	0
Isovaleric acid (%)	13.82	16.8
Valeric acid (%)	0	0
Caproic acid (%)	0	0
Total VFA (g L^{-1})	7.2	7.44
Standard deviation (±)	20.79	21.24

VFA, Volatile fatty acid.

conversion of simple monomers into VFA.[71,72] In general, the total VFA production in both wastewater (tempeh and tofu synthetic wastewater) with heat shock pretreatment in thermophilic conditions is higher than non-heat shock pretreatment in mesophilic conditions.

The pretreatment heat shock in the inoculum is significantly effective in enhancing VFA production which is linked to the dynamics of the bacterial community.[73] Heat shock, also known as thermal treatment, as a pretreatment for inoculum, has a positive impact on inoculum to enhance the VFA production. The heat shock inoculum treatment is effective in increasing VFA yield 9315 ± 652 mg COD L^{-1} at alkaline pH and inhibits non-sporulating bacteria and methanogenic archaea (Methanobacteriaceae).[42] However, another study reports that a low pH value can improve the VFA and its composition. The highest VFA production yield from fermentation dairy milk was 0.92 gCOD g^{-1} VS, including the acid profile of VFA reached at pH 5.[74] This is because the adaptation of the microbial community in acidic pH increases VFA production, which also impacts in biogas composition.

Based on Fig. 5 the highest cumulative methane from synthetic tofu wastewater through an anaerobic digestion process was 33.42 mL g^{-1} VS reached at TF1, followed by TF3 (29.88 mL g^{-1} VS), and TF4 (25.23 mL g^{-1} VS). Furthermore, the lowest cumulative methane was 0.25 mL g^{-1} at TF7 and TF9. Hence, it can be indicated that the cumulative methane in thermophilic is higher than in mesophilic conditions, which is the same as the result from tempeh wastewater. According to Fig. 5 the cumulative methane in tofu wastewater also has a lazy S-profile, the same as in tempeh wastewater. The lazy S-profile refers to a slow rate of biogas production at the beginning, followed by a rapid increase and then a gradual decrease. The slow rate of biogas production indicated unstable operational performance and lower biogas production.[1]

Based on the results of this study, high cumulative methane in tempeh and tofu wastewater was found at thermophilic temperatures with heat shock pretreatment.

The same applies to the total VFA production in both wastewaters: the total VFA in the thermophilic temperature treatment was higher than that in the mesophilic. Acetic acid serves as an essential substrate for methanogenesis, directly influencing microbial metabolism, particularly in methane-producing bacteria. When fermentation temperatures decrease, both total VFA and acetic acid concentration progressively decline, negatively impacting methane production.

There is a relationship between total VFA and cumulative methane: if total VFA is high, then methane content is low.

The results show that the highest total VFA in tempeh wastewater was 10.08 g L^{-1} at T3 (Fig. 2), but the cumulative methane was low at 3.65 mL g^{-1} VS (Fig. 3). This indicates that methane production is related to VFA production, which is a decreasing methane content along with an increasing VFA.[75] However, in tofu wastewater, the highest total VFA was 9.79 g L^{-1} reached at TF3 (Fig. 4) with a high cumulative methane of 25.23 mL g^{-1} VS (Fig. 5). This is probably because tofu wastewater had a higher carbohydrate content than tempeh wastewater, thus achieving high total VFA and cumulative methane. The type of substrate with high carbohydrate content and biodegradability reached a high methane yield and VFA yield.[76] Therefore, further study is needed to optimize the production of VFA from tofu wastewater. There is a relation in VFA production that can decrease methane concentration. Increasing acidogenesis speeds up substrate breakdown, but excessive acid accumulation lowers pH, harming methanogenic bacteria. Propionic acid and its ions may also directly inhibit methanogenesis.[77] When VFA accumulation exceeds the tolerance of acidogenic bacteria, their activity is

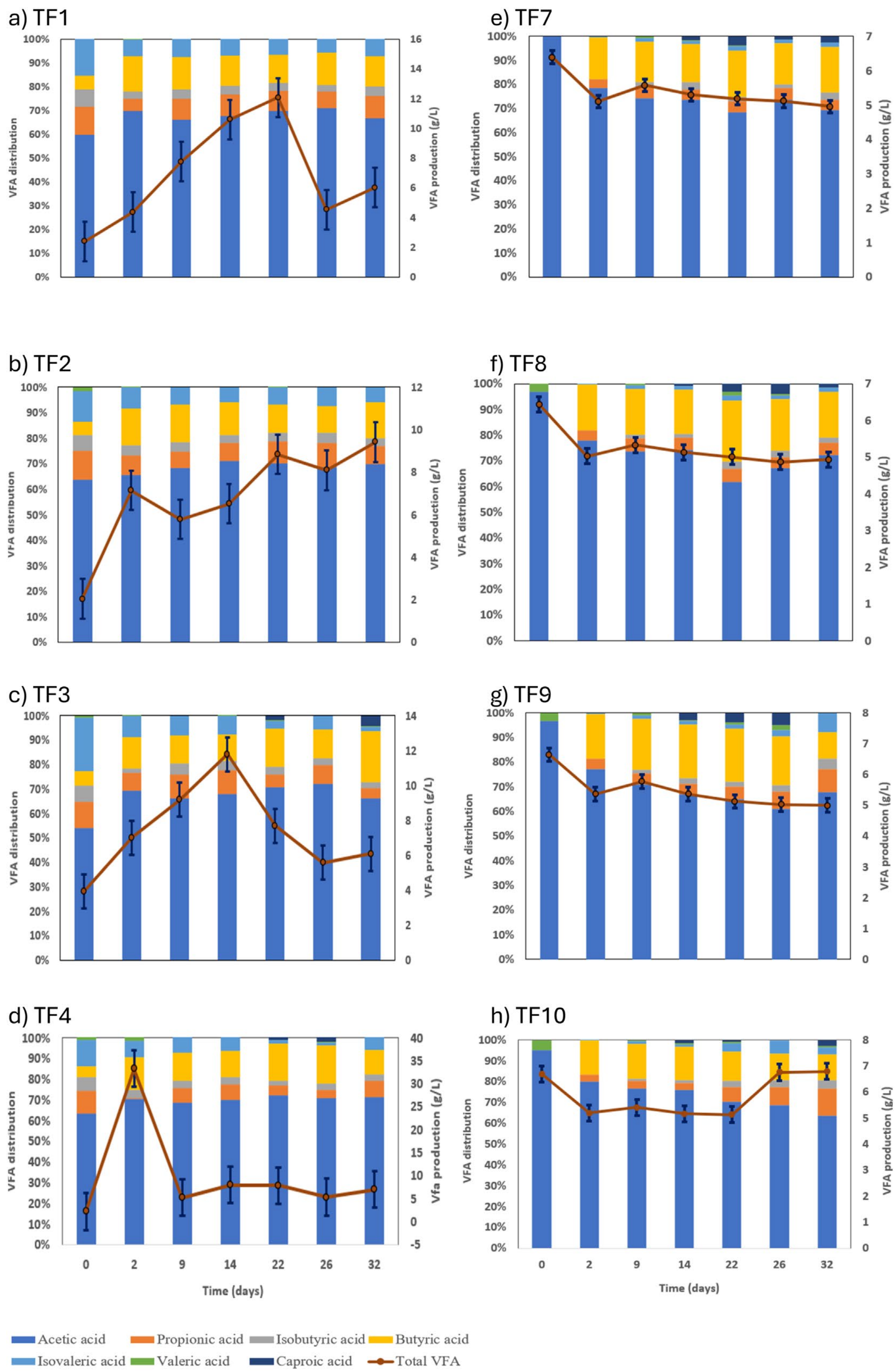

Figure 4. Volatile fatty acid concentration and distribution in tofu wastewater of T1, T2, T3, T4, TT7, T8, T9, and T10.

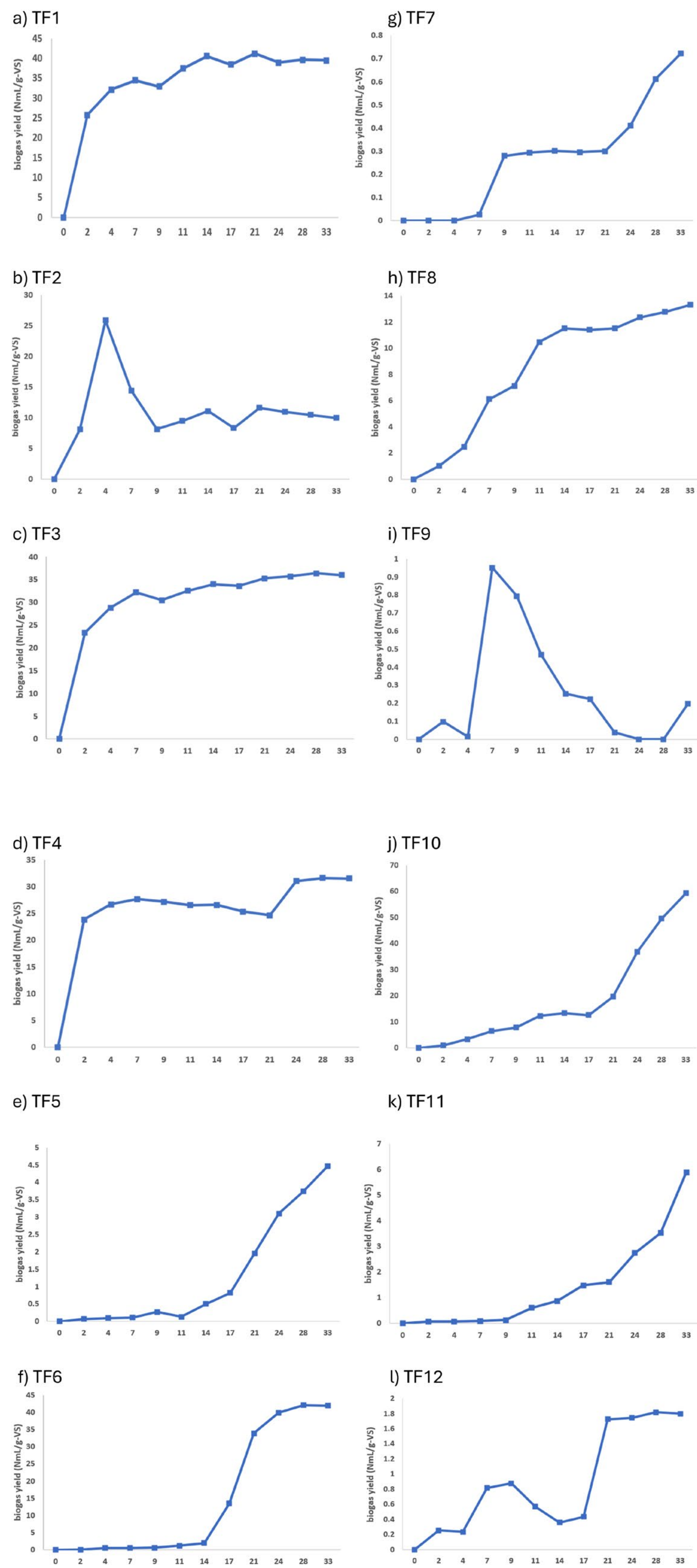

Figure 5. Methane content measured in tofu wastewater across 12 experimental treatments (T1 - T12).

further suppressed, causing a rapid decline in reactor pH and creating unfavorable conditions for methanogenic bacteria. Consequently, methanogenic activity and methane production decreased, leading to continued acetic acid accumulation and ultimately to the cessation of methane production.[78]

Mesophilic digestion is commonly applied but produces limited VFAs owing to slow hydrolysis of complex, cell-bound organics. However, thermophilic digestion enhances metabolic and hydrolytic rates and biomethane yields, yet requires more energy and is prone to VFA and ammonia inhibition. Thermophilic anaerobic digestion, which combines a short thermophilic phase to accelerate hydrolysis with a longer mesophilic phase for stable downstream digestion, addresses these limitations, although data on VFA yield and composition for waste-activated sludge remain limited.[79,80] Based on the results of this study, it can be concluded that total VFA production from tofu and tempeh wastewater was higher under thermophilic conditions than under mesophilic conditions, primarily owing to the influence of pH and oxidation–reduction potential. This is relevant to the study result by Sanjaya *et al.*[81] that anaerobic digestion under thermophilic conditions yielded almost threefold higher VFA than the mesophilic process, with maximum accumulation in both reactors observed on day seven (3311.57 ± 89 mg L^{-1} at -163 ± 16 mV oxidation–reduction potential in thermophilic reactor). Moreover, VFA production during anaerobic digestion is affected by both temperature and pH. In this study, tofu and tempeh wastewater produced the highest total VFA at pH 6, aligning with Chen *et al.*,[38] who observed that total VFA production increased from pH 7 to 8.9 (38.67–69.18% of total VFA) and declined at pH 9.9, indicating that hydrolysis continued at higher pH while acidogenesis slowed. Total VFA production increased at pH 8 and under uncontrolled conditions, probably owing to the higher soluble organic matter. Strong alkaline conditions (pH 12) further enhanced hydrolysis and VFA production; however, acidogenic bacteria were inhibited in thermophilic and extreme thermophilic fermentations, resulting in the lowest VFA accumulation under extreme thermophilic conditions.[82] Furthermore, heat-shock pretreatment enhances VFA production in both tofu and tempeh wastewater, probably owing to substrate changes that release substantial readily biodegradable organics during hydrothermal pretreatment, which accelerates fermentation, increases productivity, reduces fermenter heating energy, and enriches thermophilic microbial communities, resulting in higher amplicon sequence variant values compared with raw samples.[83] Heat shock pretreatment affect to inoculum shifted from being dominated by *Proteobacteria*, hydrogen-producing CO-oxidizers, to *Firmicutes*, which include hydrogen-producing bacteria like *Clostridium* spp. Heat shock inhibits methanogenesis as effectively as the chemical inhibitor, offering a safer alternative without harmful residues.[84] Moreover, acid, alkali, and heat shock effectively enhanced VFA production, the highest acidogenesis efficiency ($29.8 \pm 3.2\%$), followed by acid pretreatment ($18.2 \pm 1.5\%$), and the control ($11.7 \pm 0.4\%$).[47]

Overall, the findings of this study demonstrate the significant potential of producing high acetic acid content from two distinct types of wastewater for various industrial applications. Acetic acid holds immense value owing to its multifaceted utilization in industry, which is driven by its substantial economic worth and substantial global demand. The market size of acetic acid in 2023 is projected to reach US$23.23 billion. The period 2024–2032 is forecast to see the acetic acid market grow at a 5.10% CAGR, and the value will reach US$36.36 billion by 2032.[85] Further research is needed to utilize tofu and tempeh wastewater as a source of acetic acid, which is applied in the food manufacturing process or as other materials.

Conclusion

Anaerobic digestion of tofu and tempeh wastewater resulted in efficient production of high concentrations of VFA and acetic acids. The highest average total VFA in tempeh and tofu wastewater, respectively, was 10.08 and 9.79 g L^{-1}, achieved in T3 and TF3. Additionally, the highest concentration of VFA composition is acetic acid from both wastewaters. The highest average acetic acid concentration in tempeh wastewater is 77.32% in T7 and 92.40% in TF10 for tofu wastewater. High VFA production has an impact on the methane concentration. The highest total VFA in tempeh wastewater at T3 has a low cumulative methane of 3.65 mL g^{-1} VS. However, tofu wastewater has a high content of carbohydrates, thus resulting in a high VFA and a cumulative methane of 25.23 mL g^{-1} VS at TF3. This study concluded that tempeh and tofu wastewater has a high potential to produce VFA as a source of acetic acid. The combination of pretreatment heat shock, pH 6, and thermophilic conditions results in the highest total VFA and low cumulative methane. However, further research is needed to optimize the production of VFA and acetic acid, including their application as additives in the food industry.

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval

Not applicable.

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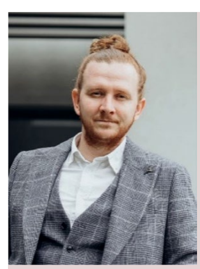

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Appendix A

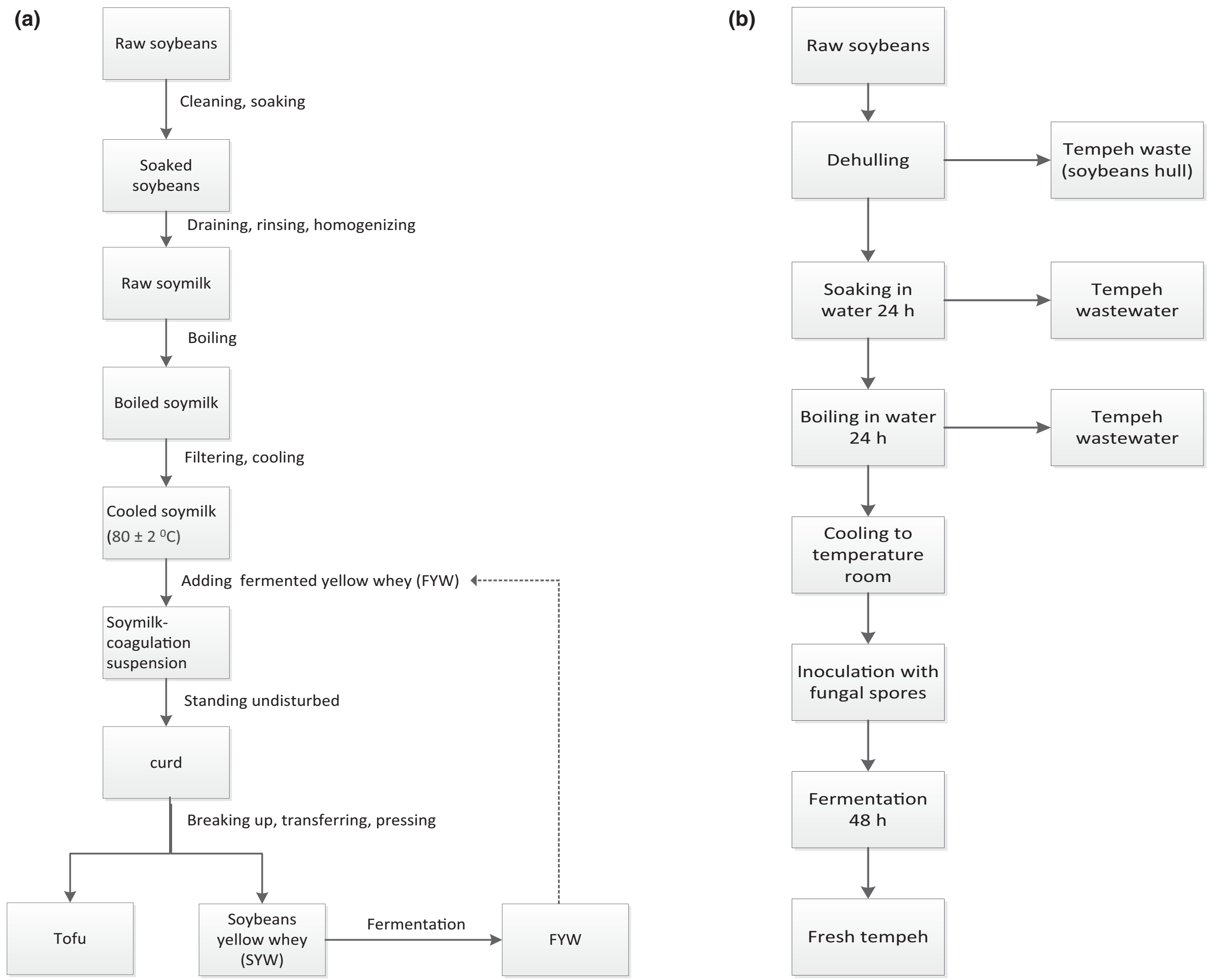

Figure A1. (a) Flow chart of tofu process production (Huang *et al*., 2021).[86] (b) Flow chart of tempeh process production. *Sources*: (Chaerun, 2009).[87]