Enhancing methane production by adding Fe^{3+} in mesophilic anaerobic digestion of cheese waste

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Abstract. The advancements in cheese production technology have resulted in increased waste generation, especially in the form of liquid byproducts left over after the milk clotting process. This research examines the possibility of using cheese waste to produce methane (CH_4) through mesophilic anaerobic digestion and investigates how adding iron (Fe) can improve CH_4. Field experiments were conducted to evaluate the effects of varying concentrations of cheese waste (0–33.33 g/L) and $FeCl_3$ (0–3.0 g/L) on CH_4 yield. Results revealed that the addition of 2 g/L $FeCl_3$ achieved the highest cumulative CH_4 yield and production rate, with increases of 68% and 65% over the control, respectively. The study also monitored pH levels and found that the best treatment maintained a near-neutral pH of 6.79 by day 50, which is important for sustaining effective microbial activity. This study highlights the potential of incorporating Fe supplementation to optimize CH_4 yields from cheese waste and other organic substrates, contributing to more sustainable and efficient renewable energy production.

1 Introduction

The cheese industry has undergone rapid technological advancements, leading to more efficient production methods. However, these advancements have also resulted in the generation of significant amounts of waste, particularly in the form of liquid byproducts left after the milk clotting process[1]. This waste, predominantly composed of carbohydrates (4.7 grams/100 ml) and proteins (0.9 grams/100 ml) with low-fat content (0.3 grams/100 ml), represents a significant environmental challenge due to its high biological oxygen demand (BOD) [2]. The cheese industry generates approximately 270 kg of high-BOD waste weekly, which can harm the environment if not properly managed [3]. Despite these challenges, cheese waste offers unique potential for valorisation as a substrate in methane (CH_4) production via anaerobic digestion[1], [4]. Cheese waste contains readily biodegradable components such as lactose and proteins, which can be rapidly converted into biogas, primarily composed of CH_4 and carbon dioxide (CO_2) [5]. Additionally, its consistent composition and abundance from dairy operations make it a reliable feedstock for biogas production by anaerobic digestion.

Anaerobic digestion is a technology recognised for its energy efficiency and environmental friendliness, focusing on processing organic waste through the biochemical activity of methanogenic microorganisms. Typically, CH_4 yields are conducted using a single-phase anaerobic fermentation technique, where both acidogenic (requiring an acidic environment) and methanogenic (requiring a neutral pH) stages occur within the same digester [5]. This setup often disrupts the pH balance necessary for optimal microbial activity, particularly during acetogenesis and methanogenesis, leading to reduced efficiency in breaking down complex organic compounds into simpler ones[6]. Recent studies suggest that a two-phase anaerobic digestion system could optimise CH_4 yields by improving the conditions for each stage and reducing the accumulation of volatile fatty acids (VFAs), which can inhibit the digestion process[5], [7].

In addition to optimising the digestion process, the introduction of mineral additives, such as iron (Fe), has shown potential in enhancing CH_4 yields. Fe plays a dual role by trapping hydrogen sulphide (H_2S), a common contaminant in biogas, and boosting the activity of methanogenic bacteria through pH modulation[8]. Different oxidation states of iron, including Fe^{2+}, Fe^{3+}, and Fe^0, have distinct impacts on anaerobic digestion[9], [10]. Fe^{2+} often facilitates electron transfer, enhancing methanogenesis by serving as a direct electron donor for hydrogenotrophic methanogens [10]. Fe^0 provides long-term electron transfer potential but may require specific conditions to become bioavailable[11]. In contrast, Fe^{3+}, particularly in the form of $FeCl_3$, is highly soluble and acts as an electron acceptor, thereby stabilizing microbial processes and enhancing methane yields[12]. Previous studies reported $FeCl_3$ increases CH_4 yields by 6.36% [12], while Fe increased it by approximately 90.3% [13]. Despite these promising results, Fe^{3+} application in the anaerobic digestion of cheese waste remains underexplored, presenting a unique opportunity for this study to address this knowledge gap.

This study aims to address this research gap by systematically evaluating the impact of Fe^{3+} addition on CH_4 yields during the mesophilic anaerobic digestion of cheese waste. The specific objectives of this research are to determine the optimal concentrations of both cheese waste and Fe^{3+} that maximise CH_4 yields. The study employs a series of controlled experiments to assess the influence of varying these concentrations on CH_4 yield, with the expectation that Fe^{3+} will act as a facilitate electron transfer to enhance microbial activity. By identifying these optimal conditions, the research seeks to contribute to more sustainable waste management and energy production practices, providing valuable insights into the effective use of cheese waste as a substrate for biogas production.

2 Experimental

The cheese waste utilized in this study served as the primary feedstock for anaerobic digestion (AD). To prepare the waste for digestion, the cheese was first grated into smaller, finer particles with an average size of approximately 2-3 mm in diameter. A thorough characterization of the cheese waste was conducted to understand its composition. This involved several key analyses, including proximate analysis, which measured total solids (TS), volatile solids (VS), moisture content (MC), and ash content. The results of these analyses provided insight into the chemical composition of the cheese waste, revealing that it contained 52.89% carbon (C), 7.89% hydrogen (H), 28.17% oxygen (O), and 1.08% nitrogen (N). Additionally, the carbon-to-nitrogen (C/N) ratio of the cheese waste was determined to be approximately 48.97%, which is a critical factor in the anaerobic digestion process as it influences microbial activity and CH_4 yields. For the inoculum, sludge was sourced from an anaerobic digester at a local industrial facility in Malang, Indonesia. The sludge was characterised to ensure its suitability for the study. It was found to have a total solids (TS) content of 1.09%, meaning that 1.09% of the sludge was made up of solid

particles, while the remaining 98.91% was water. The volatile solids (VS) content, which indicates the organic portion of the solids that microorganisms can break down, was exceptionally high at 99.45%.

2.1 Anaerobic digestion experimentation

The research used cheese waste effluent from the initial phase of two phases of anaerobic digestion (TPAD) as the substrate. A mix of 50 mL of this effluent and 10 mL of seed sludge was placed in a 100 mL serum bottle. The pH was initially adjusted to 7.8 using either hydrochloric acid (HCl) or sodium hydroxide (NaOH). To create an anaerobic environment, the bioreactors were flushed with high-purity nitrogen gas (>99.99%) at a flow rate of 20 L/min for 30 seconds. The bioreactors were then sealed with rubber stoppers and capped with aluminum lids before being placed in an incubator at 37°C for 50 days[14]. Experiments on CH_4 yields were split into two sets. The first set aimed to assess the overall effectiveness of cheese waste in methane generation by testing different cheese waste concentrations: 0 g/L, 8.33 g/L, 16.67 g/L, and 33.33 g/L [5]. After identifying the concentration yielding the highest gas production, a parallel study was conducted to examine the impact of Fe^{3+} addition, following the same procedure as the cheese waste experiments. The second experimental set focused on evaluating the influence of Fe^{3+} on CH_4 yields, with $FeCl_3$ added in concentrations of 0 g/L, 1.0 g/L, 2.0 g/L, and 3.0 g/L within a total mixture volume of 60 mL[5]. Each experiment was conducted in triplicate under consistent conditions.

2.2 Analytical methods

The pH of the samples was measured using a digital pH meter. Before each use, the pH meter was carefully calibrated using standard buffer solutions with known pH values of 7.0 and 9.2 to ensure accuracy. Total solids (TS) were determined to quantify the overall solid material present in the sample. In contrast, volatile solids (VS) were analysed to identify the portion of these solids that are organic and can be decomposed by microorganisms during the digestion process. Moisture content (MC) was measured to determine the water content in the samples, and ash content was assessed to evaluate the inorganic residue remaining after the complete combustion of the sample. All these analyses were conducted in accordance with Standard Method 2540 G, a widely recognised protocol for evaluating such parameters in environmental samples. The daily volume of CH_4 produced was recorded using the water displacement method. The recorded gas volumes were then converted to standard temperature and pressure (STP) conditions (273K and 1 atm) using the ideal gas law, which allows for consistent comparison of methane production data[14]. To estimate the theoretical methane yield from the organic biomass, the Buswell equation was applied. This equation assumes a complete breakdown of the organic material into methane and other byproducts. The necessary inputs for this calculation—namely, the levels of carbon (C), hydrogen (H), nitrogen (N), oxygen (O), and sulphur (S) in the substrate samples—were obtained through detailed elemental analysis. The elemental composition of the cheese waste was determined using a ThermoFisher Scientific FlashSmart™ CHNS/O elemental analyser (EA). This instrument provides precise measurements of carbon, hydrogen, nitrogen, and sulphur content in the samples. Additionally, the elemental composition in the leachate (liquid byproduct) was analysed using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), specifically with a Thermo Scientific iCAP 7400 Duo ICP-OES device. This technique allows for the accurate determination of various elemental concentrations within the samples. Finally, the potential methane yield in the anaerobic digestion process was theoretically calculated using the modified Buswell-Boyle equation[15]. This equation

models the biochemical conversion of organic matter into methane, providing a theoretical estimate based on the elemental composition of the substrate.

$$C_cH_hO_oN_nS_s + \left(c - \frac{h}{4} - \frac{0}{2} + \frac{3n}{4} + \frac{s}{2}\right)H_2O \rightarrow \left(\frac{c}{4} + \frac{h}{8} - \frac{o}{4} - \frac{3n}{8} - \frac{s}{4}\right)CH_4 + \left(\frac{c}{2} - \frac{h}{8} + \frac{o}{4} + \frac{3n}{8} + \frac{s}{4}\right)CO_2 + nNH_3 + sH_2S$$

Where: c,h,o,n,s represent the moles of each mass fraction of the elements carbon (C), hydrogen (H), oxygen (O), nitrogen (N), and sulphur (S).

3 Results And Discussion

3.1 Effect of Cheese Waste Concentration on CH_4 yields

Variations in the concentration of cheese waste were identified as a key factor that significantly influenced the yield and level of CH_4 yields, both at the initial and advanced stages. Four different concentrations of cheese waste were used as substrates in this study: a control group with 0 g/L of cheese waste, as well as treatments with concentrations of 8.33 g/L, 16.67 g/L, and 33.33 g/L. Each concentration represents different experimental conditions, and these are detailed in Figure 1.

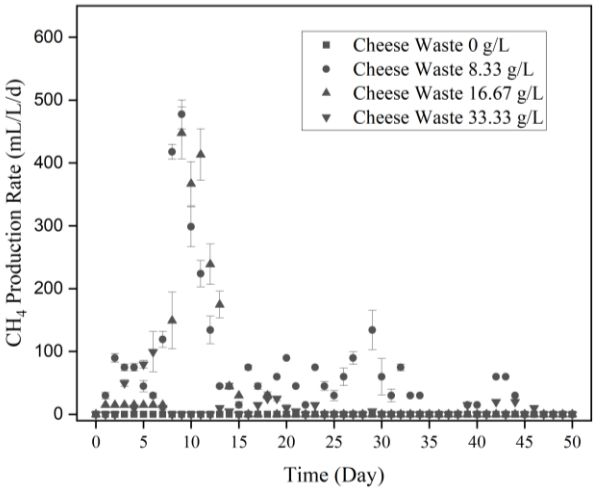

Fig.1. Effect of different concentrations of cheese waste regarding CH_4 production rates

Figure 1. shows the CH_4 production rate over time for various concentrations of cheese waste (0 g/L, 8.33 g/L, 16.67 g/L, and 33.33 g/L) during anaerobic digestion. The production rate offers insights into the rate at which methane is produced at each stage of the digestion process and how the concentration of cheese waste affects this rate. The control group with 0 g/L of cheese waste showed almost no methane production throughout the entire period, as anticipated. Since there was no organic substrate present, the microorganisms lacked the material to metabolise, leading to negligible methane production. This provides a baseline for comparing the other concentrations. The concentration of 8.33 g/L shows the highest peak

in methane production rate, reaching 477.40±23 mL/L/day around day 10. This sharp peak indicates strong microbial activity as the microorganisms rapidly metabolise the available organic material. However, after this peak, the methane production rate decreases quickly. It stabilises at a much lower level, suggesting that the easily degradable portion of the substrate has been consumed, leaving behind more complex materials that degrade more slowly. At the concentration of 16.67 g/L, the methane production rate also exhibits a notable peak, although it is slightly lower than that of the 8.33 g/L concentration, with a maximum rate of around 447.57±41 mL/L/day. The decrease after the peak is more gradual, suggesting that the system may be nearing its metabolic limits and the remaining substrate is being broken down more slowly. This slower decline may also indicate the onset of inhibitory effects, which are taking longer to develop fully. At the highest concentration tested, 33.33 g/L, there is a noticeable decrease in methane production rate, barely exceeding 99.46±32 mL/L/day. The lower peak and the flatter curve indicate that the system may be experiencing inhibition at this concentration. This is likely due to the accumulation of inhibitory by-products such as volatile fatty acids (VFAs) or ammonia. These by-products can slow down or inhibit the activity of methanogenic bacteria, leading to a significant reduction in methane production efficiency. The data suggests that there is an optimal concentration of cheese waste for CH_4 yields, likely around 8.33 g/L. Higher concentrations, such as 16.67 g/L and particularly 33.33 g/L, appear to lead to substrate inhibition, reducing the overall efficiency of methane production. This result is consistent with findings in studies such as Zhuo et al. (2024), where high substrate concentrations led to the accumulation of VFAs and ammonia, which in turn inhibited methanogenic activity[16]. Substrate inhibition in anaerobic digestion occurs when the organic load surpasses the system's ability to degrade it efficiently. As reported by Wirasembada et al. (2021), an excessive organic load increases the production of intermediates like VFAs and ammonia, which lower the pH and create an inhibitory environment for methanogens[17].

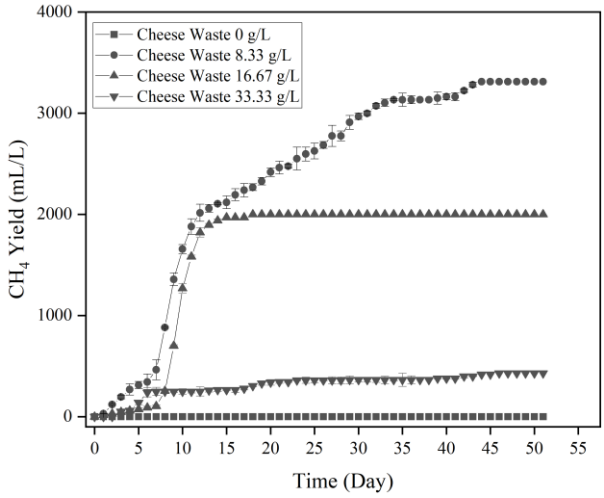

Fig 2. Effect of different concentrations of cheese waste regarding CH_4 yields

Figure 2 shows the cumulative CH_4 yield over time for different concentrations of cheese waste (0 g/L, 8.33 g/L, 16.67 g/L, and 33.33 g/L) during anaerobic digestion. The results

indicate that the control group (0 g/L cheese waste) did not produce significant methane during the 50-day period, as expected, due to the absence of organic substrate for microorganism metabolism. The 8.33 g/L concentration showed the highest methane yield, reaching 3311.99 mL/L by the end of the study period. Methane production began around day 5, increased sharply until day 25, and then plateaued, suggesting that this concentration is likely optimal for maximising methane production. The concentration of 16.67 g/L also led to methane production starting around day 5 but resulted in a lower final yield of 1999.13 mL/L. Although the initial production rate was similar to that of the 8.33 g/L concentration, it plateaued earlier, suggesting that the system was reaching its capacity to process the higher substrate level efficiently. The highest concentration tested, 33.33 g/L, showed the lowest methane yield among the non-zero concentrations, with production starting later and increasing more slowly, ultimately yielding 427.67 mL/L. This suggests that at 33.33 g/L, the system may be overloaded, leading to inhibition of microbial activity due to the potential accumulation of inhibitory by-products such as volatile fatty acids (VFAs) or ammonia. These findings indicate that optimising substrate concentration is essential for efficient methane production. While the concentration of 8.33 g/L seems to be the most effective, higher concentrations such as 16.67 g/L and particularly 33.33 g/L can decrease efficiency, likely due to substrate inhibition. Studies have similarly reported that higher substrate concentrations can lead to substrate inhibition, characterised by the accumulation of VFAs and ammonia, which disrupt the pH balance and hinder microbial metabolism[13], [14]. Higher organic loading rates led to significant drops in methane yield due to the inhibition of methanogenesis by excessive VFAs. This emphasises the importance of managing organic load in anaerobic digesters to maximise methane production while avoiding conditions that may hinder microbial activity[4].

3.2 Effect of Fe^{3+} addition on CH_4 yields from cheese waste

Figure 3 shows the data regarding the CH_4 production rate during mesophilic anaerobic digestion over time for different Fe^{3+}concentrations (0 g/L, 1 g/L, 2 g/L, and 3 g/L). All treatments exhibit an initial lag phase (0–5 days), followed by a rapid increase in production rates, peaking around days 10–15 and then declining to stabilize at low levels after day 30. The control treatment without Fe^{3+} addition (0 g/L) shows the lowest peak production rate at approximately 467.73 mL/L/day. In comparison, Fe^{3+} supplementation significantly enhances the production rate. The 1 g/L Fe^{3+} treatment peaks at 581.84 mL/L/day, reflecting a 24% increase over the control, while the 2 g/L Fe^{3+} treatment achieves the highest peak at 850.30 mL/L/day, representing a 65% increase. The 3 g/L Fe^{3+} treatment reaches a peak of 497.41 mL/L/day, corresponding to a 3% increase over the control. Furthermore, the Fe-supplemented treatments sustain higher production rates for a longer duration during the post-peak phase compared to the control, with 2 g/L demonstrating the most prolonged and elevated performance. However, the slight decline in performance at 3 g/L suggests potential inhibitory effects of excess Fe^{3+}.

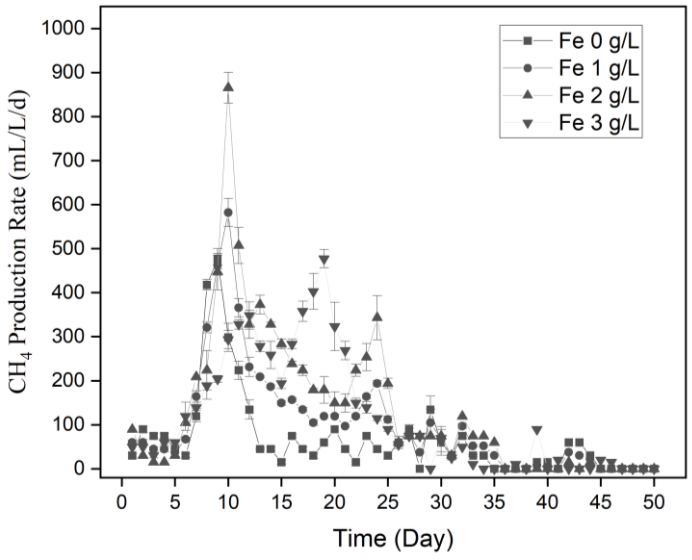

Fig.3 The Effect of Different Concentrations of Fe^{3+} on CH_4 production rates

Overall, Fe^{3+} addition significantly improves methane production rates, with 2 g/L identified as the optimal concentration, enhancing both peak production and sustained activity. These findings underscore the critical role of Fe^{3+} in optimizing anaerobic digestion processes. This decline suggests that while Fe^{3+} enhances methane production at lower concentrations, excessive Fe^{3+} can inhibit methanogenic activity. The observed peak in CH_4 production at 2 g/L Fe^{3+} aligns with findings from Zhang et al. (2020), who reported Fe enhances methane production by acting as a cofactor for key methanogenic enzymes and by reducing inhibitory compounds like hydrogen sulphide (H_2S). However, the decrease in CH_4 production rates at higher Fe^{3+} concentrations of 3 g/L highlights potential inhibitory effects. Excess Fe can lead to the formation of insoluble compounds such as FeS and $Fe(OH)_3$, which can precipitate out of the solution and become unavailable to microorganisms. This was observed by Almeida et al. (2023), where high Fe concentrations led to reduced methane production due to the precipitation of Fe compounds[4]. Additionally, high Fe^{3+} levels can disrupt the pH balance of the AD system, as Fe interactions with organic acids may lead to acidic conditions that inhibit methanogenic bacteria, consistent with Zhang et al. (2021), who noted that excessive Fe can negatively impact pH and microbial activity[18].

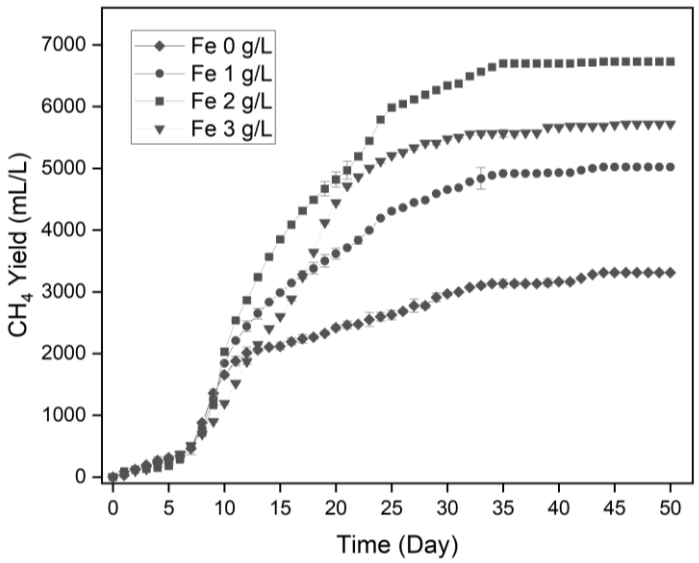

Fig.4. The Effect of Different Concentrations of Fe^{3+} on CH_4 yields

Figure 4 shows the effect of different concentrations of cheese waste with the addition of various Fe^{3+} concentrations (0 g/L, 1 g/L, 2 g/L, and 3 g/L). Across all treatments, methane production begins with a lag phase during the first 10 days, followed by an accelerated production phase and eventual stabilization. The treatment without iron addition (0 g/L) results in the lowest methane yield, which plateaus at 3311.99 mL/L after 40 days. In comparison, the addition of Fe^{3+} significantly enhances methane production. At 1 g/L Fe^{3+}, the final methane yield reaches approximately 5020.21 mL/L, representing a 51% increase compared to the control (0 g/L). The 2 g/L Fe^{3+} treatment achieves the highest methane yield, stabilizing at 6728.42 mL/L, which is a 68% increase over the control. However, the 3 g/L Fe^{3+} treatment shows a slight decline in yield, plateauing at around 5718.91 mL/L, which is a 35% increase compared to the control but lower than the yield at 2 g/L. These results indicate that Fe^{3+} addition significantly improves methane production, with the 2 g/L concentration being the most effective. The decline in performance at 3 g/L suggests that excessive Fe^{3+} may have inhibitory effects.

Overall, the findings highlight the critical role of Fe^{3+} in enhancing methane production and the importance of optimizing its concentration for maximum efficiency. One of the primary mechanisms is direct interspecies electron transfer (DIET). In methanogenic systems, DIET enables syntrophic relationships between fermentative bacteria and hydrogenotrophic methanogens, which rely on efficient electron transfer for methane production[10]. Fe^{3+} ions serve as effective electron acceptors, allowing microbes to bypass the less efficient interspecies hydrogen transfer (IHT) process, thereby enhancing the rate and yield of methane [19]. Additionally, Fe^{3+} ions participate in redox reactions that stabilize the anaerobic digestion environment. By cycling between Fe^{3+} and Fe^{2+} states, iron facilitates electron shuttling, which supports the metabolic activities of methanogenic archaea [8]. Moreover, Fe^{3+} enhances methanogenesis by influencing microbial community composition. Studies have shown that iron supplementation can increase the abundance of *Methanosaeta* and *Methanosarcina* species, which are critical for acetoclastic methanogenesis [4].

3.3 *pH profile*

Figure 5 illustrates the effect of varying concentrations of cheese waste and Fe^{3+} on pH levels over time, specifically on day 1 and day 50. The left graph shows that as the concentration of cheese waste increases (0, 8.33, 16.67, and 33.33 g/L), the pH remains relatively stable near neutral (around 7) on day 1 but significantly decreases by day 50, particularly at the highest concentration of 33.33 g/L. In contrast, the right graph reveals that the addition of Fe^{3+} (0, 1, 2, and 3 g/L) results in a stable pH of around 7 on both day 1 and day 50, with only a slight decrease in pH at higher Fe^{3+} concentrations over time.

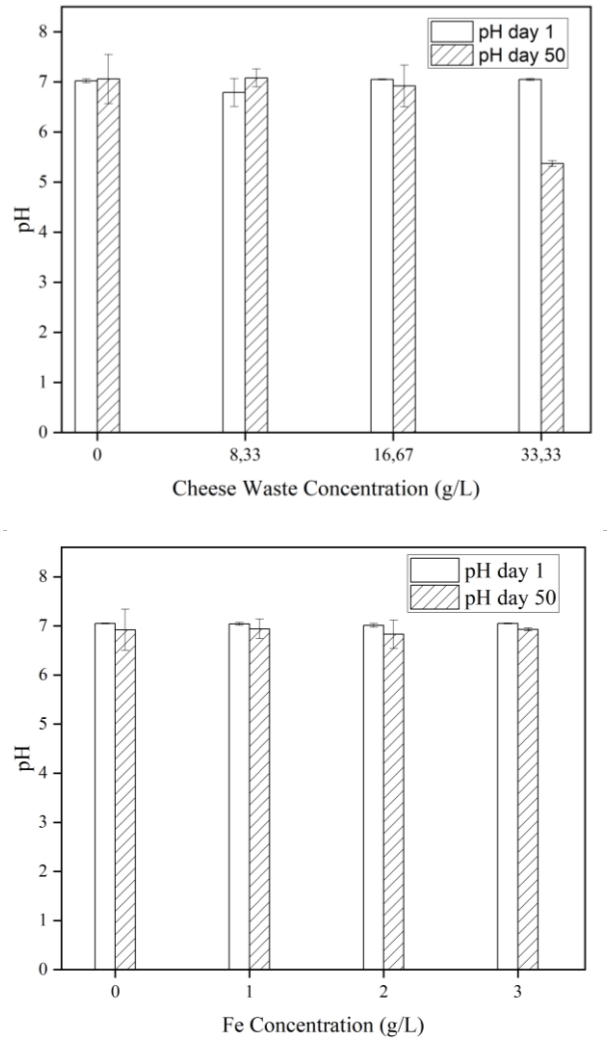

Fig.5. (a) pH of different cheese waste concentrations; (b) pH of different Fe^{3+} concentrations

The pH decreases as the concentration of cheese waste increases due to the organic matter in the waste, which acts as a food source for microbial activity. Microbes break down the

organic material over time, producing acidic byproducts such as lactic acid and fatty acids[18]. At first, microbial activity is not very strong, which is why the pH remains close to neutral on the first day, even at higher concentrations of cheese waste. However, by day 50, the combined effect of microbial metabolism causes a significant decrease in pH, especially at the highest concentration of 33.33 g/L, where the most organic material is available for breakdown.

The pH is not significantly affected by the addition of Fe^{3+} does not directly contribute to acid or base production. The pH remains stable around 7 regardless of the Fe^{3+} concentration on day 1, indicating that Fe^{3+} does not participate in reactions that would cause significant pH changes. Even by day 50, the slight decrease in pH at higher Fe^{3+} concentrations might be due to minor secondary interactions, such as Fe-catalyzed redox reactions or their influence on microbial dynamics [19]. Still, these effects are minimal compared to the impact of the organic load from cheese waste.

4. Conclusion

The research demonstrates that the addition of Fe^{3+} to the mesophilic anaerobic digestion of cheese waste significantly enhances CH_4 yields. The optimal CH_4 yield was achieved using a combination of 8.33 g/L of cheese waste and 2 g/L of $FeCl_3$, resulting in CH_4 yields of 6728.42 mL/L, which is a 68% increase over the control. The study also found that maintaining a close-to-neutral pH is critical for effective microbial activity, with the optimal treatment reaching a pH of 6.79 by day 50. These results suggest that the incorporation of Fe^{3+} into anaerobic digestion processes could be a promising strategy for optimising CH_4 yields from various organic substrates, contributing to more sustainable energy production.

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