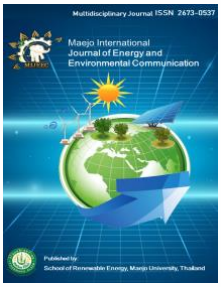
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ARTICLE

Valorization of spent mushroom substrate via anaerobic digestion: Process stability, co-digestion strategy, and energy recovery

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ABSTRACT

Spent mushroom substrate (SMS), an abundant lignocellulosic waste material from the mushroom production process, is an interesting feedstock for renewable biomethane production. This study cracks performance of anaerobic digestion (AD) of SMS under mesophilic conditions (35±1°C) during batch fermentation for 45 days. The impact of inoculum type and co-digestion with nitrogen-rich livestock manures (cow, pig, and chicken) was evaluated with regard to the optimization of the methane yield. Raw SMS had moderate methane potential because of fungal delignification; however, mono-digestion resulted in an unstable input because of a high C/N ratio and VFA accumulation. Methane yields from raw SMS only ranged between 140-180 mL CH_4 g^{-1} VS. Co-digestion showed a significant improvement in the performance, especially for chicken manure at a ratio of 1:1 in volatile solids. 300-350 mL of methane gas per g of volatile solids was obtained, representing more than a fourfold increase in comparison to SMS mono-digestion. Optimum mixtures have had balanced C/N ratios (25-30), stable pH (7.0-7.5), low VFA build-up, and improved volatile solids reduction (up to 65%). Manure digestate had superior buffering and a rapid onset of methane compared to using sewage sludge inoculum. Energy analysis revealed 55% of the energy content of SMS could be recovered in the form of biomethane as a result of co-digestion. The digestate contains mineral nutrients that are suited for biofertilizer circular agriculture. This study results in the confirmation of the potential of SMS co-digestion in improving biomethane production and stability, showing the feasibility for biogas production while supporting biomass vaporization.

1. Introduction

The fast growth of the global mushroom growing industry has resulted in the production of large amounts of lignocellulosic residues termed spent mushroom substrate (SMS) (Munir et al., 2021, 2024). It is estimated that about 4-6 kg of SMS is generated from a single kg of mushrooms based on the system of cultivation and the substrate formulation (Chuanchai & Xayyavong, 2025; Xayyavong et al., 2025). With an annual worldwide production of mushrooms of over 40 million tonnes, the volume of SMS is a substantial biomass production stream that must be managed well (Kousar et al., 2024; Ma et al., 2025). Conventional disposal

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practices - including landfilling, uncontrolled dumping, and incineration - not only have a logistical and economic cost, but they may also lead to greenhouse gas emissions, nutrient leaching and environmental degradation. Consequently, sustainable valorization of SMS has become an important research priority within the whole concept of circular bioeconomy and waste-to-energy strategies (Hu at al., 2022; Luo & Chen, 2024).

Spent mushroom substrate consists mainly of partially degraded agricultural residues such as straw, sawdust or other lignocellulosic resources, as well as other nutrients during cultivation, such as bran, manure, and mineral sources (Ravlikovsky et al., 2024). In the growth of a mushroom, filamentous fungi (typically species of Pleurotus, Agaricus and Lentinula) grow into and ECM the substrate matrix. These fungi release extracellular enzymes (laccases, peroxidases, and cellulases) capable of degrading lignin and hemicellulose that partially depolymerize the cellulose fibers and increase porosity and accessibility of cellulose fibers (Leong et al., 2022). As a result, the structure and chemical compositions of SMS are different from those of fresh lignocellulosic biomass (Ryden et al., 2017). Compared to untreated straw or sawdust, SMS generally has lower lignin content, a modified lignin structure, low crystallinity and an increased availability of accessible carbohydrates (Ma et al., 2025). This fungal preconditioning can be seen as a type of biological pretreatment, which is able to optimise the underlying microbial degradability.

Anaerobic digestion (AD) is a promising technology for the conversion of SMS to renewable energy in the form of methane-rich biogas (Dussadee et al., 2022; Nong et al., 2022; Van Tran et al., 2022). AD is a mature biotechnology that is typically used for the stabilization of organic wastes such as agricultural wastes, manure, and food waste, and the simultaneous production of biomethane, which can be used as a source of heat, electricity or upgraded to renewable natural gas (Ramaraj et al., 2015a; Sabarikirishwaran et al., 2023). The use of AD in SMS has a dual benefit of waste disposal problems mitigation and renewable energy recovery. Moreover, digestate produced from AD has a valuable stoichiometric composition of nutrients (nitrogen, phosphorus, potassium), for which recycling to agricultural soils is possible, and this will contribute to the concept of closed-loop nutrient management (Whangchai et al., 2022). Several studies have shown that fungal pretreatment can increase methane yields from lignocellulosic substrates to a significant extent (Pantawong et al., 2015; Ramaraj et al., 2015b; Unpaprom et al., 2021). The enzymatic degradation of lignin by white-rot fungi leads to a decrease in structural recalcitrance that can be followed by hydrolysis for AD. In this context, SMS may have advantages over untreated agricultural residues due to the fact that part of the most recalcitrant fraction has already been modified during the mushroom cultivation process. Indeed, integration of mushroom production with subsequent anaerobic digestion has been proposed as one solution that can form a sustainable strategy for biomass utilization that can enhance overall resource efficiency.

Despite the problems of mono-digestion of SMS, one of the biggest constraints is the carbon-to-nitrogen (C/N) ratio of 30-50. While the optimum C/N ratio for stable anaerobic digestion is 20-30, high carbon levels can lead to nutrient deficiency in methanogenic microorganisms and VFA accumulation with a decrease in pH (Qiu et al., 2023). Low formation of ammonia leads to low buffering capacity and acidification. Though fungi help in the breakdown of cellulose, easily degradable carbohydrates are consumed during the development of the mushroom. The residual SMS matrix of structural carbohydrate, fungal cell wall polymers and lignin has moderate methane yields and slower digestion kinetics. Nitrogen-rich manures from livestock fulfill a good C/N balance, microbial diversity, and buffering potential. Manures provide Nitrogen, alkalinity, trace elements, and an anaerobic microbial consortium. Co-digestion of lignocellulosic material and manure is synergistic with higher yields of methane. Optimized ratios reduce C/N ratios to 25-30, pH is stabilized and VFA production is reduced (Al-Zoubi et al., 2024). Poultry manure has a high synergistic effect due to its content of nitrogen and buffering capacity.

Integrated approaches of biorefinery are directed towards SMS utilization. In the case of cascading biomass systems, SMS is subject to enzymatic saccharification and fermentation processes for the production of bioethanol (Chuanchai & Xayyavong, 2025). The residue left after saccharification contains fewer carbohydrates but high amounts of lignin. Despite the recalcitrance of lignin in anaerobic conditions, partial degradation of lignin gives rise to the production of methane. Lignin-rich residues can be used in AD to enhance the efficiency of lignocellulosic biorefinery. However, the production of methane from post-processing SMS residues needs to be characterized better as compared to the raw SMS. Research on the biomethane production of both materials is required to optimize conversion, taking into account the yield of methane, stability, kinetics, and energy recovery. This study discusses the topic of anaerobic digestion of SMS under mesophilic conditions, including the biomethane potential of raw SMS, co-digestion with nitrogen-rich manures, the effect of inoculum sources and the biodegradability of lignin-enriched residues. It analyses the contribution of AD to bioenergy valorisation in the context of biomass utilisation in cascading approaches of sustainable waste management and sustainable renewable energy production.

2. Materials and Methods

2.1 Spent Mushroom Substrate (SMS) and Post-Fermentation Residue

Spent mushroom substrate was gathered, air-dried to around 10% moisture to obtain stable storage, and mechanically milled to a size smaller than 5 mm to obtain a homogenous size before anaerobic digestion trials. Two types of substrates were tested: (i) raw SMS, which is used without any other substrate treatment (except drying and size reduction), and (ii) post-fermentation residue (also called fermentation residue), which is obtained after respective enzymatic hydrolysis and yeast fermentation of SMS in parallel experiments. After the fermentation process, solids were separated by a filtration process and incubated at 60 °C in an oven and stored at 4 °C until the digestion tests. This storage condition was chosen in order to minimize possible microbial changes during

its storage period, with digestion tests starting within 2-4 weeks of drying to guarantee the stability of substrate quality. Compositional analysis (dry basis) showed that raw SMS contained around 15% lignin and around 40% cellulose, while fermentation residue was lignin-enriched (around 30%), containing residual cellulose (20-25%), with some added protein/chitin coming from fungal and yeast biomass and ash. Total solids (TS), volatile solids (VS), and elemental composition, i.e., C, H, and N, were assayed for each substrate before digestion.

2.1.2 Sources of Inoculum & Co-substrates

Two inoculum sources were used to study the importance of inoculum origin for the effect on methane yield and stabilization of digestion, as it allows the assessment of adaptation and buffering capacity of the microbial culture. The first inoculum was anaerobically digested sludge (collected at a mesophilic municipal wastewater treatment biogas plant). The second inoculum was digestate resources that come from an agricultural biogas facility that treats cattle manure. This comparative approach allows assessment of the differences in microbial community composition and buffering capacity of the municipal and the agricultural inocula when used for the application on lignocellulosic SMS. Both inocula have been sieved (1mm mesh) to degrade the large particle sizes and pre-incubated at 35 ±1°C for 7 days to minimize endogenous methane production before use. This pre-incubation step is important in order to correct for background methane production and ensure that only substrate degradation gas is measured and not residual inoculum metabolism.

Inoculum pH (usually between 7.5 and 7.8), volatile solids content, alkalinity, and concentrations of ammonium nitrogen were determined before biochemical methane potential (BMP) determinations to ensure consistency and reproducibility between trials. For co-digestion experiments, fresh manures from livestock production were collected from local town farms (chicken manure, dairy cow manure, and pig manure), then characterized for TS, VS, and total Kjeldahl nitrogen (TKN) and stored in -20 °C until needed to minimize uncontrolled biodegradation. Freezing at -20 °C will prevent spontaneous anaerobic fermentation during storage, and will therefore retain the original nutrient composition as well as microbial communities within manure. Chicken manure was chosen as a critical co-substrate because of its high nitrogen content and buffering potential, anticipated to complement the carbon-rich SMS and alter C/N balance to the optimum value of 25-30.

2.2. Batch Anaerobic Digestion Experimental Design

2.2.1 Reactor Configuration and Operating Conditions

Batch anaerobic digestion experiments were conducted to determine the biochemical methane potential (BMP) of individual substrates and co-digestion mixtures following standardized guidelines consistent with ISO 11734 and VDI 4630. Anaerobic reactors consisted of 500 mL borosilicate serum bottles with a working volume of 400 mL. The substrate-to-inoculum ratio (S/I) was maintained at 0.5 on a volatile solids (VS) basis to ensure inoculum excess and avoid overloading. In practice, each bottle contained approximately 10 g VS inoculum and approximately 5 g VS of the respective substrate (or substrate mixture) unless otherwise specified.

2.2.2 Treatment Groups

The following digestion conditions were evaluated: raw SMS mono-digestion, fermentation residue mono-digestion, and co-digestion of SMS with livestock manures (chicken, dairy cow, and pig manure). For each manure type, there were two VS mixing ratios (i.e., 50:50 and 25:75 SMS: manure) to assess the effect of blending proportions and nutrient balancing on the yield of methane and process stability. Co-digestion of fermentation residue with chicken manure was also performed at a 50:50 VS ratio to test if microbial synergy and dilution of the lignin fraction would increase methane production from the lignin-enriched residue.

2.2.3 Anaerobic Conditions and Incubation

Each reactor was flushed with high-purity nitrogen gas for 3-5 minutes to create anaerobic conditions, then capped with butyl rubber stoppers and aluminum crimps. Bottles were incubated at 35± °C for 45 days in a temperature-controlled orbital incubator and gently agitated (120 rpm) to prevent stratification and maintain contact of the substrate with the microorganisms. All treatments were done in triplicate, and blanks were exposed to inoculum only to correct for background methane production. Digestion was considered complete when daily methane production dropped below 1% of cumulative methane yield; however, all the experiments were run for the full 45-day period in order to obtain the complete kinetic profile.

Figure 1. Laboratory-Scale Anaerobic Fermentation Reactors



Anaerobic fermentation reactors (laboratory-scale) consisting of 500 mL borosilicate serum bottles with a 400 mL working volume (Quantity) (Figure 1). Bottles were sealed with butyl rubber stoppers and aluminum crimp caps, which incorporated airlock means, providing for the release of CO_2 and for stopping penetration of oxygen. Approximately 100 mL headspace has been maintained for biogas accumulation for incubation at 32 °C in static conditions.

2.2.4 Monitoring Bacterial Gas and Measuring Methane

Biogas accumulation was determined at 2-3 day intervals (more often at the times of rapid rate of gas evolution) using a digital pressure transducer with a needle port, and volumes were corrected to standard temperature and pressure (STP; 0°C, 1 atm). Methane concentration was measured by gas chromatography with a thermal conductivity detector (GC-TCD), and the sample collection was done in replicate 3 times for each time point to guarantee the analytical precision. Methane yield was expressed as cumulative methane production normalized to volatile solids added (mL CH_4 g^{-1} VS).

2.3 Analytical Methods

2.3.1 Analysis of Solid and Liquid Phases

Total solids (TS) and volatile solids (VS) were determined following the procedures of the Standard Methods by oven-drying to 105 °C (TS) and then igniting to 550 °C (ash), and VS was calculated by difference. Elemental composition (C, H, N) was determined with an elemental analyzer (Elementar Vario EL Cube or equivalent) and C/N ratios accordingly calculated. Liquid phase stability indicators were monitored throughout the digestion in selected sampling days (e.g. days 0, 3, 7, 14, 21, 35 and 45), pH was measured with a calibrated digital pH meter and volatile fatty acids (VFAs; acetic, propionic, butyric and valeric acids) determined with high performance liquid chromatography (HPLC) using a Rezex ROA-Organic Ammonium nitrogen (NH_4^+-N) was determined using the distillate-titration approach or using ion-selective electrode based on the availability of the instrumentation.

2.3.2 Degradation of Substrates Assessment

Selected solid samples before and after digestion were determined for lignin and cellulose content using the acid detergent fiber (ADF) method for cellulose and acid detergent lignin (ADL) method for lignin (ANKOM Technology Fiber Analyzer, or equivalent) to determine the patterns of degradation of substrate and confirm persistence of lignin-rich fractions. This approach gives quantitative data on structural carb loss and recalcitrant lignin persistence through the process of anaerobic digestion.

2.4 Analysis of Data and Statistical Methods

Cumulative methane yields were obtained as mL CH_4 g^{-1} VS added after subtraction of methane produced in inoculum-only blanks. Volatile Solids reduction (%) was calculated from the initial and final measurements of VS values. Synergistic effects were measured during the course of co-digestion by comparing measured methane yields of SMS-manure mixtures with calculated weighted theoretical yields on the basis of mono-digestion results. A synergistic enhancement was proven if measured methane production exceeded the predicted additive production [by 10%]. Statistical differences between treatments were examined with one-way analysis of variance (ANOVA) and post hoc test using Tukey. Differences were taken as significant when < 0.05. Before running the ANOVA, the data were tested to evaluate assumptions of normality using the Shapiro-Wilk test and homogeneity of variance using Levene's test; and in the case of violation of these assumptions, the non-parametric test Kruskal-Wallis test, was introduced as an alternative.

2.5 Kinetic Modeling of Methane Production

Methane production kinetics were described using the modified Gompertz model to fit cumulative methane production curves obtained during the 45-day incubation period. The model was expressed as:

$$M(t) = M_{max} \exp \{- \exp [\frac{R_{max}\, e}{M_{max}} (\lambda - t) + 1]\}$$

where M (t) is cumulative methane production at time t (mL CH_4 g^{-1} VS), Mmax is methane production potential (mL CH_4 g^{-1} VS), Rmax is maximum methane production rate (mL CH_4 g^{-1} VS d^{-1}), λ is lag phase duration (days), and e is Euler's constant (2.71828).

2.6 Energy Recovery and Efficiency Calculations

Energy recovery from anaerobic digestion was calculated using measured methane yields and the lower heating value (LHV) of methane (35.8 MJ m^{-3} at STP). Methane energy yield (MJ kg^{-1} VS) was determined as: Methane Energy Conversion Equation

$$E_CH_4 = Y_CH_4 \times 35.8$$

Where: E_CH_4 = Energy content of methane (MJ per kg VS or specified basis); Y_CH_4 = Methane yield (m^3 CH_4 per kg VS) and 35.8 = Lower heating value (LHV) of methane (MJ per m^3)

Methane/energy-based energy recovery was then compared between mono-digestion and co-digestion treatments to quantify energy enhancement due to the presence of synergic nutrient balancing/microbial. Volatile solids reduction participated in the interpretation of the conversion efficiency and the destruction of the organic matter. Overall energy recovery efficiency by comparing the methane energy production to the intrinsic energy of the substrate using a representative lower heating value: SMS was found to produce an overall energy recovery efficiency of 18.2 MJ/kg dry matter. 19.5 MJ/kg dry matter was observed from fermentation residue, assuming the typical lower HEV of lignocellulosic biomass composition. This energy, resulting from the assessment, helped to evaluate the feasibility of the production of BioMethane from SMS as a waste-to-energy pathway and also to provide a comparative discussion on process configurations.

3. Results and Discussion

3.1 Anaerobic Digestion Performance of Raw SMS

3.1.1 Methane Yield and Biogas Composition

SMS generated measurable biogas under mesophilic anaerobic digestion (35 ± 1 °C), confirming its suitability as a partially pretreated lignocellulosic feedstock for biomethane production. The cumulative methane yield was in the range of 150±6 mL CH_4/gVS after 40 days of incubation (n = 3), then methane yield stabilized at around 60+-2% (v/v), with the rest mostly consisting of CO_2 (38-40%) with minor amounts of H_2S (less than 0.2%). The concentration of methane rose quickly after day 3, and it stabilized from day 7 onwards (58-62%), which indicates that equilibrium of the gas phase and the establishment of methanogens occurred during the early digestion phase. Slight variation of days 0-5 reflected rapid acidogenesis and temporary CO_2 enrichment owing to the organic acid accumulation.

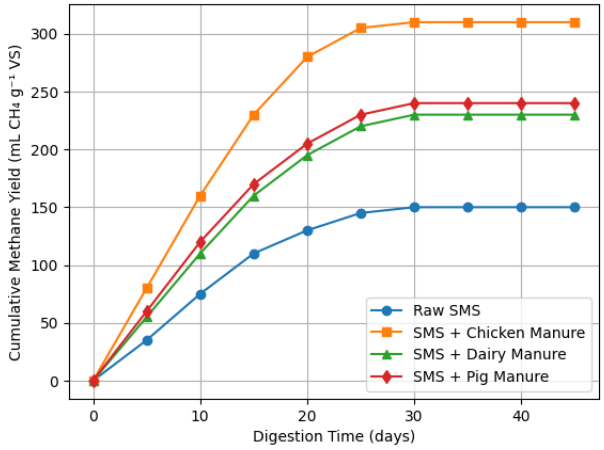


Figure 1. Cumulative Methane Production

As compared with untreated straw (original mushroom cultivation substrate), which has an average methane yield of about 100 mL CH_4 g^{-1} VS under similar conditions, the methane yield of SMS was about 50% enhancement (150 vs. 100 mL CH_4 g^{-1} VS). This improvement confirms that the cultivation of mushrooms is a biological pretreatment stage, in which the fungal enzymes working on ligninolysis (laccases, peroxidases, and manganese peroxidase) enable the partial depolymerization of lignin and hemicellulose (Poddar et al., 2022). Such structural disruption increases accessibility of cellulose as well as enhances the hydrolysis rate during anaerobic digestion. Previous literature reports a methane yield of 133-160 mL CH_4 g^{-1} VS for shiitake SMS, substantially higher than the results from untreated woody residues, giving support to the present results. A simplified carbon mass balance was done using elemental analysis and measured methane production. About 35-40% of the carbon in the total input was recovered as methane, which is a moderate conversion efficiency (Table 1). The presence of ~15% lignin (dry matter basis) and crystalline cellulose is responsible for incomplete biodegradation. This proves that hydrolysis of structural polymers is still the rate-limiting step.

Table 1. Carbon Recovery Distribution from Raw SMS Mono-Digestion

Carbon Fraction	Input Carbon (%)
Methane (CH_4)	35–40
Carbon dioxide (CO_2)	20–25
Residual solids	40–45
Microbial biomass	3–5

3.1.2 Digestion Kinetics and Stability of Raw SMS

The cumulative methane production profile of raw SMS showed a distinct pattern of successive phases in the degradation of discrete fractions of substrate of differing biodegradability. During the first 0-10 days, methane production rose rapidly as a result of the conversion of readily biodegradable material, consisting of residual soluble sugars, fungal biomass, and partially depolymerized hemicellulose produced during mushroom cultivation. This early phase is characterized by active hydrolysis and acidogenesis and quick methanogenic conversion of acetate and hydrogen (Induchoodan et al., 2022). After about day 10, methane production shifted to a slower phase that lasted until the end of day 40, which included gradual degradation of structural carbohydrates embedded inside the residual lignin matrix. This second phase shows hydrolysis of crystalline cellulose to become rate-limiting after the labile substrates are exhausted (Maurus et al., 2023). Such biphasic behaviour is characteristic of partially pretreated lignocellulosic residues and supports the fact that SMS still has a large degree of structural recalcitrance despite the biological change during mushroom growth.

Although two visible degradation phases were observed on the methane degradation curve, excellent overall fitting was achieved by the modified Gompertz model, with an R^2 value of 0.976±0.012 and a low RMSE value (3.2 ± 0.5 mL CH_4 g^{-1} VS), so that predicted accuracy was high. The lag phase of 5.2 ± 0.4 days is estimated to have been an efficient acclimatization of the inoculum to SMS. This relatively short period of adaptation is probably due to the presence of residual fungal biomass and partially degraded lignocellulose, which provided for greater accessibility of microbes than did untreated straw substrates, which typically have longer lag phases (8-12 days). The maximum methane potential (152±6 mL CH_4 g^{-1} vs) was close to the experimentally observed cumulative yield and therefore confirmed model reliability. However, based on the moderate maximum rate of methane production (8.5±0.7 mL CH_4 g^{-1} VS d^{-1}), hydrolysis-limited kinetics instead of methanogens inhibition appear to be the mechanism. If the methanogenesis step represented a limiting process, long-lasting VFA accumulation and delayed methane evolution would have been anticipated, whereas methane evolution gradually reached a plateau level as a result of lowering the availability of carbohydrate by provoking an increasing dominance of recalcitrant fractions (Maurus et al., 2023).

Volatile fatty acid dynamics are also in favor of this interpretation (Figure 3). During the first 5-7 days, total VFA concentrations peaked at half of about 3-4 g/L (as acetate equivalents) with a temporary decline of pH from 7.2 to 6.5. This transient acidification relates to the rapid activity of acid generation as opposed to the initial capacity of conversion of methane. Nevertheless, VFA concentrations did not exceed critical populations that were linked to instability of the process (>5-6 g L^{-1}), and pH slowly rebounded to 7.1 by day 40 when the methanogens used up the accumulated acids. The buffering capacity measured as alkalinity (1850-2050 mg $CaCO_3$/L) was sufficient to avoid system collapse, although rather modest due to

the rather moderate nitrogen content of SMS. Ammonium levels stabilized in the range of 800 milligram/L of NH_4^+-N; levels higher than that lead to inhibition. Ammonium levels stabilized at around 800 milligram/L of NH_4-N; levels higher than that lead to inhibition. When the lowest pH value (6.5) was encountered, most of the ammonia was in the protonated [NH_4^+] form and therefore, free ammonia [NH_3] toxicity was minimized. Therefore, inhibition in early digestion was mostly due to temporary acid stress and not to ammonia toxicity.

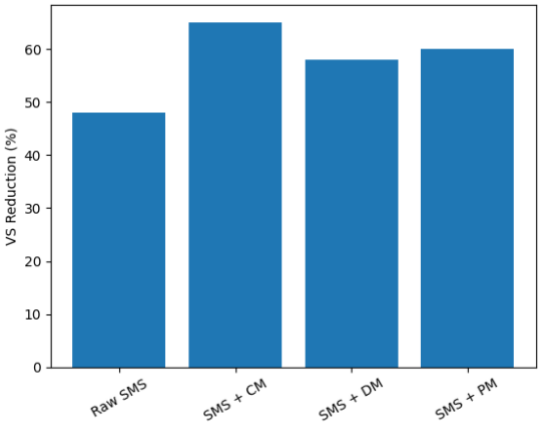


Figure 3. Volatile Solids Reduction

The volatile solids reduction of 28±4% is indicative of incomplete degradation of biomass. Residual solids included lignin (12-13% of original total solids), crystalline cellulose (8-10%), and microbial biomass (2-3%). Results show a recalcitrance of lignocellulose and not a limitation of microbes that is limiting methane production. Lignin inhibits the accessibility of enzymes to cellulose, thus inhibiting anaerobic degradation (Balakrishnan et al., 2023; Manmai et al., 2023; Nasution et al., 2024). Mushroom cultivation did disrupt lignocellulose but didn't do a whole lot of de-ligninlysis so less wanted hydrolyzing. SMS mono-digestion demonstrated moderate methane yield, low adaptation, and low buffering. The moderate C/N ratio caused acidogenesis to be at risk due to weakened alkalinity. Sodium bicarb or co-digestion with manure could increase the alkalinity and the methane. Additional pretreatment targeting of lignin would enable an improvement in hydrolysis. Raw SMS digestion is hydrolysis-limited and stable mesophilically; improving it is a question of improving the accessibility of the lignocellulosic (Tables 2 and 3).

Table 2. Kinetic Parameters from Modified Gompertz Model (n = 3)

Parameter	Raw SMS	Interpretation
Lag phase (λ)	5.2 ± 0.4 days	Adaptation period
Maximum methane potential (M_{max})	152 ± 6 mL CH_4 g^{-1} VS	Ultimate yield
Maximum production rate (R_{max})	8.5 ± 0.7 mL CH_4 g^{-1} VS d^{-1}	Peak degradation rate
R^2	0.976 ± 0.012	Excellent model fit
RMSE	3.2 ± 0.5 mL CH_4 g^{-1} VS	Low prediction error

Table 3. Process Stability Indicators During Raw SMS Digestion

Parameter	Initial	Peak/Minimum	Final (Day 40)
pH	7.2	6.5 (Day 5)	7.1
Total VFA (g L^{-1})	0.6	3.8	0.9
NH_4^+–N (mg L^{-1})	620	820	780
Alkalinity (mg $CaCO_3$ L^{-1})	2100	1850	2050
VS reduction (%)	—	—	28 ± 4

3.2 Co-Digestion of SMS with Livestock Manure

Co-digestion of SMS with livestock manure significantly improved methane yield, digestion kinetics, and overall process stability as compared to SMS mono-digestion. Under all different types of manure assessed, a 50:50 VS mixing ratio produced the best methane yields, demonstrating that balanced carbon-nitrogen interaction and greater microbial synergy were key factors in enhanced performance. The improvements seen were not simply additive but indicated the existence of good synergistic interactions between the lignocellulosic SMS and nutrient-rich manure substrates.

3.2.1 SMS-Chicken Manure Co-Digestion

Co-digestion of SMS with chicken manure (CM) was found to have the most significant enhancement in methane production of the different combinations tested. At a 50:50 VS mixing ratio, the cumulative methane yield was increased to ca. 310 mL CH_4 g^{-1} VS, representing > 2-fold higher yields compared to SMS mono-digestion (150 mL CH_4 g^{-1} VS) and considerably higher compared to CH_4 yields from chicken manure alone (180-200 mL CH_4 g^{-1} VS). Overall, methane production was about 4.2 times higher than SMS mono-digestion, showing a high synergy of activity over simple substrate addition. Theoretical additive methane yield was calculated based on weighted substrate contributions:

Theoretical yield = (SMS: 150 × 0.5) + (CM: 190 × 0.5) = 75 + 95 = 170 mL CH_4 g^{-1} VS

Observed yield = 310 mL CH_4 g^{-1} VS

Synergy magnitude = [(310 − 170) / 170] × 100% = 82% enhancement above the theoretical average

This substantial positive deviation confirms that the interaction between SMS and CM generated metabolic acceleration rather than simple additive effects.

3.2.2 Mechanisms Driving Synergy

Several mechanisms led to improved performance of SMS-chicken manure co-digestion. Co-digestion improved the carbon to nitrogen (C/N) ratio to 24.8±1.2, which is in the optimal range (20-30) for anaerobic digestion. Raw SMS has a high C/N ratio (>30) from lignocellulosic composition, while chicken manure is the source of nitrogen from proteins. Their combination is balanced in nutrients for the growth of the microbes and conversion of methane, preventing the limitation of nitrogen and accumulation of ammonia. The degradation of protein increased alkalinity and buffering. NH_4^+-N increased from 1450mgL^{-1} to 1510mgL^{-1} at day

40, indicating mineralization of nitrogen. Ammonia levels remained below inhibitory levels so that the pH remained neutral. Chicken manure introduced anaerobic microbes such as hydrolytic bacteria, acidogens, acetogens, and methanogens, which accelerated the breakdown of polymers and the formation of methane (Johari et al., 2023). Pre-acclimated methanogens decreasedlag phase and enhanced rates of methane production.

Fourth, structural recalcitrance was decreased in the mixed substrate with lignin dilution. Raw SMS has 15% TC lignin content and chicken manure has 5% TC. A 1:1 mixture reduced lignin up to 10%, reducing the physical barrier around the cellulose fibres and increasing the hydrolytic accessibility. A lower ratio of lignin supports better interaction of the enzyme and substrate, as well as the degradability of carbohydrates. Cellulase assays supported the result of enhanced hydrolytic performance and the 1.2-1.5 times greater cellulase activity of SMS-CM digesters compared to SMS mono-digestion systems. This implies accelerated carbohydrate depolymerization with an increase in the amount of soluble intermediates available for methanogenesis. The increase in enzyme activity supports the idea that the increased methane yield was from synergistic effects between microfluidic, biochemical, and microbial components, rather than just the additive effect of substrates.

3.2.3 Kinetic Improvements and Process Acceleration

Modified Gompertz modelling revealed significant improvement for the kinetic parameters (Table 4). A 60% reduced lag phase corresponds to fast microbial adaptation, and a 114% higher rate of maximum production of methane corresponds to the enhanced metabolic efficiency. The range of doubling the methane potential confirms better carbon conversion with 4.2-fold yield enhancement by kinetic acceleration (Johari et al., 2023). The co-digestion process improved process stability compared to SMS mono-digestion, and VFA was kept below 1.5 g/L, and pH was kept at 7.2. SMS had chicken manure prevented pH content dropper and enabled fast methane production without acid stress. Increased buffering effect of stable ammonia and increased alkalinity are very effective in neutralizing organic acids and inhibiting organic acid intermediates, leading to continuous methane production (Wang & Wu, 2024).

Table 4. Comparative Digestion Kinetics of Raw SMS and SMS–Chicken Manure Mixtures under Mesophilic Conditions

Parameter	Raw SMS	SMS–CM (50:50)	Improvement
Lag phase (λ), days	5.2 ± 0.4	2.1 ± 0.3	60% reduction
Max rate (R_{max}), mL CH_4 g^{-1} VS d^{-1}	8.5 ± 0.7	18.2 ± 1.5	114% increase
Methane potential (M_{max}), mL CH_4 g^{-1} VS	152 ± 6	315 ± 9	107% increase
R^2	0.976	0.982	Excellent fit

These results are consistent with earlier work, which has reported enhanced yields of methane in mixtures of SMS and chicken manure by virtue of nutrient complementation and improved alkalinity. However, the amount of improvement in the current system suggests stronger synergistic interaction, which may be caused by the optimized 50:50 mixing ratio and enrichment of active microbial populations (Induchoodan et al., 2022). The combination had both balanced substrate composition and microbial inoculation effects and achieved superior kinetic and stability performance. But when the SMS proportion was reduced to 25% (SMS:CM = 25:75), the cumulative methane yield was slightly reduced to around 280 mL CH_4 g^{-1} VS, which represents a slight reduction of 10% as compared with the 50:50 ratio.

The measured C/N ratio at 25:75 was determined as 19.2 ± 1.5, which falls slightly below the optimal C/N range of 20-30 for anaerobic digestion. This lower ratio represents a relatively higher availability of N to available C, which may lead to lower C conversion efficiency and higher exposure to ammonia, although levels were below those that caused inhibition. Importantly, the accumulation of VFA was not very high (<1.2 g L^{-1}) and pH stability was attained, which was taken as evidence that no process instability occurred. The slight reduction in yield is therefore attributed more to reduced structural carbon contribution from SMS and not inhibition or toxicity effects. Just at this reduced SMS fraction, methane production per unit SMS was still significantly higher than in mono-digestion, proving the long-lasting positive effect of manure as a co-substrate.

3.2.2 SMS–Dairy Manure Co-Digestion

Co-digestion of SMS with dairy manure (DM) also increased significantly more methane production compared to mono-digestion of the manure, but not as much as with chicken manure. At 50:50 VS mixing ratio, the production of methane was 230 mL CH4 g-1 VS, which is a 60% improvement compared to raw SMS digestion (150 mL CH_4 g^{-1} VS). Dairy manure contains moderate nitrogen content (C/N 16/20) and is full of microbes adapted to fiber degradation as a result of ruminant digestion. This microbial preconditioning helps in a better hydrolysis of lignocellulosic parts in SMS. The synergistic enhancement for SMS-DM was 35% above the theoretical weighted average [(170) / 170] x 100%, which was lower than that of SMS-chicken manure mixtures (82%). This is because dairy manure has lower nitrogen availability and lower buffering intensity.

The TKN content of dairy manure (2.5% dry basis) is less than that of chicken manure (4.2%), and thus less ammonia is released during digestion. SMS-DM digesters exhibited 1100 mg/L NH_4^+ - N as compared to 1500 mg/L in SMS-CM systems. The resulting C/N ratio of 22.5±1.8 approaches but does not reach the optimum of 25-30 for maximization of methanogenic efficiency, which limits microbial growth and buffering relative to SMS-CM systems. Kinetic modeling also showed further performance improvement. At a 50:50 ratio, the lag phase was shortened to 3.5 ±0.4 days, which is decreased by 33% as compared to uncooked raw SMS digestion. The maximal rate of methane production was elevated to 12.1±1.1 mL CH_4 g^{-1} VS d-1, which is 42% higher than mono-digestion, while ultimate methane potential was 235± 8 mL CH_4 g^{-1} VS, which is 55% higher than mono-digestion. These improvements, while much lower in magnitude than those with chicken manure, again confirmed the role of nitrogen concentration on the magnitude of synergy (Siyal et al., 2023).

SMS-DM exhibited enhanced production kinetics compared with mono-digestion, with negligible accumulation of VFA and stable pH. Better synchronisation between acidogenesis and

methanogenesis was indicated, probably as a result of fibre-adapted microbes present in dairy manure. The global buffering capacity was medium compared to SMS-CM systems, which was comparable to lower ammonia release. At a 25:75 SMS:DM ratio, the methane yield was decreased to 210mL CH_4 g^{-1} VS or ~9% lower than the 50:50 mixture, which is comparable to the behavior of SMS-CM systems. This gives optimization-type behavior of greater manure with diminishing returns past nutrient balance, with greater manure fractions with slightly reduced methane from lower structural carbon from SMS.

3.2.3 SMS-Pig Manure Co-Digestion

Co-digestion of SMS with pig manure (PM) at a 50:50 VS ratio resulted in a cumulative methane production of about 240 mL CH_4 g^{-1} VS, which was a significant enhancement of SMS mono-digestion. The synergistic enhancement was calculated to be 41% greater than a theoretical-weighted average of [(240 x 170) / 170] x 100%. This value puts SMS-PM performance between that of SMS-dairy manure (35% synergy) and SMS-chicken manure (82% synergy), which is a moderate but meaningful metabolic interaction. Pig manure has rather high fractions of soluble chemical oxygen demand (COD) coming from undigested feed residues, which may have been responsible for the somewhat higher methane production with respect to dairy manure. Measured soluble COD contents of pig manure were about 15-18 g/l. compared to 8-10 g/l. measured for dairy manure.

The presence of these readily biodegradable organics improved the formation of early stages of methane without transient accumulation of VFA, indicating efficient syntrophic cooperation between acidogens and methanogens. It is of note that no substantial vfa spike was noted and pH was stable during digestion (Siyal et al., 2023). Ammonia levels were stable around 1250 mg L^{-1} NH_4^+-N, which provided sufficient buffering capacity without inhibiting levels from being reached.

Performance improvement was also confirmed by kinetic modeling. The lag phase was reduced to 3.8±0.5 days, which represents a 27% reduction compared with raw SMS. The maximum methane production rate rose to 13.5±1.2 mL CH_4 g^{-1} VS d^{-1} or 59%, as shown by acquisition of 59%, whereas methane potential increased to 242±7 mL CH_4 g^{-1} VS or 59%, which is also shown by improvement of 59%. Although improvements observed in these studies were noted, they were still lower than levels observed for SMS-chicken manure systems, showing the importance of Nitrogen content and the strength of the buffer as dominant synergistic drivers. Differences between dairy and pig manure performance were fairly minor, which seems to show that the majority of livestock manures can be used effectively to boost SMS digestion if they are properly blended (Johari et al., 2023). However, poultry manure showed the greatest synergistic effect because it contains more nitrogen and has a better buffering capacity.

Statistical analysis (one-way ANOVA followed by Tukey's post hoc test, $p < 0.05$) confirmed significant differences among co-digestion treatments. The ranking was:

- SMS–CM: 310 mL CH_4 g^{-1} VS (82% synergy; ~4.2% TKN)
- SMS–PM: 240 mL CH_4 g^{-1} VS (41% synergy; ~3.8% TKN)
- SMS–DM: 230 mL CH_4 g^{-1} VS (35% synergy; ~2.5% TKN)

Synergy magnitude correlated strongly with manure nitrogen content (Pearson $r = 0.98$, $p < 0.01$), confirming that nutrient balance and ammonia-derived buffering are primary determinants of co-digestion performance.

3.3 Digestion of Post-Fermentation Residue
3.3.1 Methane Potential

Mono-digestion of the post-fermentation residue (FR), which was extracted after ethanol extraction, produced a CH_4 production of about 120 mL CH4 g-1 VS (i.e., about 20% lower than the raw SMS digestion). The lower yield of methane reflects high carbohydrate depletion during ethanol fermentation, in which the readily hydrolysable sugars as well as amorphous fractions of cellulose were depleted. As a result, residual substrate became enriched for lignin (~35% of total solids) and crystallised cellulose (~20%), both of which can be very resistant to anaerobic biodegradation. Enzymatic hydrolysis in the process of fermentation has the effect of preferentially removing amorphous cellulose, leaving behind an ordered crystalline set that is less available to hydrolytic enzymes in anaerobic digestion.

Kinetic analysis with the modified Gompertz model was used to confirm the slower degradation of the dynamics for fermentation residue. The lag phase took a longer time, 7.1 ± 0.6 days, which is about 37% longer than raw SMS, which implicates prolonged microbial adaptation to the lignin-rich matrix. The maximum rate of methane production decreased to 6.2 ±0.8 mL CH4 g-1 VS d-1, which was 27% slower than raw SMS; while methane potential was 122±5 mL CH_4 g^{-1} VS, which was 20% lower overall. The increased time of the lag phase is probably a combination of structural recalcitrance and reduced accessibility of residual amounts of cellulose. Higher concentration of lignin (35% vs. 15% in raw SMS) creates a protective barrier around carbohydrate polymers, retarding the colonization and attack of microorganisms and enzymes. The model fit was quite good ($R^2 = 0.971$), obtaining the kinetic consistency of behaviour despite the lower digestibility.

Volatile Solids (VS) reduction further showed low efficiency of breakdown. After 50 days of incubation, VS reduction was as low as 19 +- 3%, which was significantly lower than that of raw SMS (28%). Residual solids were made up of mainly recalcitrant lignin (approximately 30% from the initial value TS), crystalline cellulose (approximately 18-20%), and small quantities of yeast biomass residues (approximately 1-2%). Given structural constraints, however, breaking down the digestion of remaining substrates for >50 days would not seem to yield much increase in the amount of methane (#5%), in which case degradation would be limited by substrate recalcitrance rather than by incomplete kinetic

progression.

3.3.2 Stability Considerations

In spite of decreased potential methane concentration, fermentation residue digestion processes showed process behavior stability. The C/N ratio was reduced to around 15-20 by incorporating the yeast biomass during the fermentation, which increased the nitrogen content. As a result, the hazard of acidification was lower than in raw SMS mono-digestion, whereas pH was near neutral (~7.2) throughout incubation. Higher nitrogen mineralization, represented by ammonia (NH_4^+) concentration, increased to about 2000 mg/L NH_4^+ - N. At neutral pH, only 3-5% of total ammonia is in the free ammonia (NH_3) form (~60-100 mg l-1 NH_3), far below classical inhibitory levels (~ 500 milligrams (mg) per litre (l) of NH_3). Therefore, acute ammonia toxicity was not a likely possibility.

While the increased concentration of NH_4^+ may have played a minor role in the osmotic stress and prolonged lag phase, structural recalcitrance was the major limiting factor. VFA concentrations were low-metabolically efficiently converted from the intermediate fermentation products (0.4 g/L). Thus, the main limitation in the fermentation residue digestion was substrate recalcitrance rather than process instability (Johari et al., 2023; Maurus et al., 2023). This observation is consistent with previous studies on the digestion of lignin-rich ethanol stillage, where stability is preserved, but a structural constraint for other potential by-product forms, namely methane, exists.

3.3.3 Co-Digestion of Residue of the Fermented

Co-digestion of fermentation residue and chicken manure (50:50 VS ratio) significantly enhanced the recovery of methane which was about 250 mL CH_4 g^{-1} VS. Calculated synergistic enhancement of 61% was based on [(250 x 155) / 155] x 100% 155 ml CH4 g-1 VS is the hypothetical weighted average of fermentation residue (120) and chicken manure (190). Although this synergy was slightly lower than that recorded for raw SMS-CM systems (82%), the significant improvement proves that co-digestion is a potent way to mitigate the limitations of lignin-rich residues.

Gompertz modeling was found to show strong kinetic enhancement in the FR-CM system. The lag phase decreased from 4.3±0.6 days by 46%, to 3.8±0.5 days, compared to FR mono-digestion. The maximum rate of methane production rose to 14.2 ±1.3 mL CH_4 g^{-1} VS d^{-1} (129% increase), and methane potential doubled to 252±8mL CH_4 g^{-1} VS (107% increase). These improvements have probably been caused by microbial enrichment, dilution of lignin concentration, increased buffering capacity, and improved degradation of residual cellulose (Induchoodan et al., 2022). The results of the study confirm that co-digestion plays an important role in the efficient recovery of biomethane from post-fermentation residues.

Advanced configurations, two-stage anaerobic digestion, may be able to even further improve the performance. Separating the hydrolytic/acidogenic and the methanogenic phases would, in turn, enable extended residence time for structural polymer degradation processes, optimized pH control (pH 5.5-6.5 for hydrolysis vs. 7 to 7.5 for methanogenesis), and also selective enrichment of cellulolytic consortia. However, such systems need to be validated at pilot-scale and were outside the scope of the present investigation.

3.4 Integrated Energy Recovery

Energy recovery analysis showed that direct anaerobic digestion of 100 kg dry SMS produced about 482 Megajoules (MJ) of gross energy, while integrated 2-stage processing (fermentation of ethanol and subsequent anaerobic digestion of residue) produced about 556 MJ, which was 15% more gross energy capture (Zhang et al., 2023). However, when the energy inputs to the process were included, a substantially different net energy recovery was obtained. Direct AD required about 65-90 MJ for heating, feedstock handling, and digestate management, giving about 85% net energy recovery with respect to energy content of feedstock (~18.2 MJ/kg DM). In contrast, there were large energy inputs for enzyme production (80-120 MJ), fermentation heating (20-30 MJ), distillation (150-200 MJ), AD heating (about 50 MJ), and other ancillary operations, which totaled about 325-420 MJ. As a result, net recovery fell to a mere 39% despite the increased gross output. Thus, based on current assumptions of the technology, direct AD is superior in net energy performance because of its lower operational energy demand.

Methane conversion calculations are another example. Raw SMS had approximately 5.37 MJ per kg VS (9.6 MJ kg^{-1} DM) while SMS-CM co-digestion had approximately 11.1 MJ per kg VS (19.9 MJ kg^{-1} DM), which represents an approximately doubling of usable energy output. Final digestate contained 2-3 MJ/kgDM of recalcitrant organic matter, which could theoretically be recovered apart from digestion by combustion, although high moisture content (>60%) would require energy-intensive drying. Co-digestion of SMS with livestock manure has remarkable potential as an immediately implementable strategy for farm-scale digesters. A 1:1 VS ratio gives the maximum synergy. For medium-scale mushroom farms with an average production of 400-500 kg dry SMS per week (57-71 kg day-1), the co-digestion needs about 50-65 kg fresh manure/day (at about 50% moisture), which is feasible for integrated dairy or poultry operations. A 2 m3 digester with a 40-day HRT has a potential outline to produce up to ~55 kg DS5 / day under co-digestion conditions which yields approximately 10 MJ / day, about 100 kWh / day for electricity with 40% conversion efficiency.

Digestate from optimized systems had nutrient rich composition (N: 2.0-2.5%, 0.8-1.2% P, 1.5-2.0% DM K) and heavy metals below the regulatory limits. While a partial reduction in pathogen (level 2-3 log) was achieved with mesophilic digestion, enhanced sanitation could be performed with thermophilic operation, at added heating costs. Raw SMS mono-digestion produced ~150 mL CH4 g-1 VS, having moderate stability but being limited by the recalcitrance of lignin (15%) and by a high C/N ratio (ca. 60).

Co-digestion substantially improved performance, ranked as follows:

- **SMS–Chicken Manure (50:50):** 310 mL CH_4 g^{-1} VS, 82% synergy, 60% lag reduction (optimal).

- **SMS–Pig Manure (50:50):** 240 mL CH_4 g^{-1} VS, 41% synergy, 27% lag reduction.
- **SMS–Dairy Manure (50:50):** 230 mL CH_4 g^{-1} VS, 35% synergy, 33% lag reduction.
- **Fermentation Residue–CM (50:50):** 250 mL CH_4 g^{-1} VS, 61% synergy, 46% lag reduction.

Table 5. Comparative Methane Yield, Synergy, and Kinetic Enhancement in SMS Mono- and Co-Digestion Systems (50:50 VS Ratio)

Digestion System	Methane Yield (mL CH_4 g^{-1} VS)	Synergistic Enhancement (%)	Lag Phase Reduction (%)	Key Limiting / Enhancing Factors	Performance Ranking
Raw SMS (Mono-digestion)	150	—	—	High lignin (15%); High C/N (~60); Nitrogen deficiency	Baseline
SMS–Chicken Manure (50:50)	310	82	60	Optimal C/N (25–28); High N (~4.2% TKN); Strong buffering	1 (Optimal)
SMS–Pig Manure (50:50)	240	41	27	Moderate N (~3.8% TKN); High soluble COD	2
SMS–Dairy Manure (50:50)	230	35	33	Lower N (~2.5% TKN); Fiber-adapted microbiota	3
Fermentation Residue–CM (50:50)	250	61	46	Lignin-rich substrate (35%); Dilution & microbial enrichment	2–3

Synergy magnitude showed good correlation with the content of manure nitrogen, which confirms nutrient balance and buffering capacity as primary driving factors. Direct anaerobic digestion shows a higher net energy recovery (85%) compared with integrated two-stage processing (39%), applying current assumptions. SMS-chicken manure co-digestion is the most efficient and feasible route to optimise biomethane generation from the residues of mushroom cultivation.

4. Conclusion

Optimized anaerobic co-digestion of spent mushroom substrate (SMS) and nitrogen-rich livestock manure as a pathway for integrated waste management and renewable energy generation: A technically sound and immediately applicable pathway to integrated waste management. The results definitively prove that co-digestion at 50:50 volatile solids ratio opens the way for huge synergistic improvement with quite high methane production levels, around 310 mL CH_4 g^{-1} VS (4.2 fold higher than SMS mono-digestion) and up to 65% volatile solids reduction. The synergy between the SMS and chicken manure mixture was the highest (82%), whereas pig and dairy showed an improvement in performance, albeit lower than that of chicken. Balanced C/N ratios (25-30) increased significantly, buffering capacity, digestion kinetics, maintained constant pH (7.2-7.4), and reduced accumulation of volatile fatty acids (<1.4 g/L). These improvements support the critical role of nutrient complementarity to provide process stability and long-term methane production. Energy analysis undoubtedly shows that direct anaerobic co-digestion has an advantage of a much higher net energy recovery (~85%) than any integrated two-stage biorefinery pathway (~39%), where enzyme production and downstream processing have disfavored the process. Therefore, direct co-digestion is the technology of choice for farm-scale implementation. Feasibility assessment indicates that the implementation of medium-scale mushroom farms using mesophilic digestion (35°C, HRT = 40 days) by using locally available manure and produce renewable energy and high-quality digestate that can be used for soil amendment. Anything that is returned to the cultivated land (digestate) means that this system is closer to zero waste and supports the circular bioeconomy objective. Overall, SMS-manure co-digestion is an efficient, scalable, and sustainable solution for agricultural residues bioenergy without losing any nutrients in the process. Commercial-scale deployment will require pilot-scale continuous validation and agronomic field testing to validate long-term operational and environmental performance.

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